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Comparative analysis of metabolites and in vitro hypoglycemic activity of *Taiwanofungus camphoratus* cultured using various methods

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Abstract

Taiwanofungus camphoratus has attracted much attention because it can abundantly produce various active substances that exhibit blood-sugar lowering, immunity improving, and antioxidant properties. Currently, *T. camphoratus* is cultured using four main methods: cutting wood culture, solid-state fermentation, submerged fermentation, and dish culture. *T. camphoratus* produces different metabolites under different culture methods. In this study, nontargeted metabolomics was used to compare the metabolites of *T. camphoratus* produced under these four culture methods. Principal component analysis and supervised partial least squares-discriminant analysis were used to analyze the differences in the metabolites. Moreover, in vitro hypoglycemic activity of *T. camphoratus* extracts produced under four culture methods was compared by assessing their ability to inhibit the activity of α-glucosidase, α-amylase, and sucrase. A total of 186 metabolites were identified. In total, 127 metabolites were common under the four culture methods. Under solid-state fermentation, submerged fermentation, and cutting wood culture, 12, 1, and 4 metabolites were unique, respectively. The differential metabolites produced by *T. camphoratus* under four culture methods were mainly triterpenoids, phenolic compounds, and fatty acid compounds. α-glucosidase, α-amylase, and sucrase activity inhibition was the best using *T. camphoratus* extract obtained under cutting wood culture; the inhibition rates were 55.97%, 51.96%, and 78.02%, respectively, which were comparable to those exhibited by 0.001, 3, and 12 mg/mL acarbose (positive control). The metabolites produced by *T. camphoratus* and α-glucosidase, α-amylase, and sucrase inhibitory activities were different under the four culture methods. Cutting wood culture exhibited the best enzyme inhibitory activity. This study provided a theoretical basis for further use and development of various culture methods for the rational production of active metabolites of *T. camphoratus*.

Keywords *Taiwanofungus camphoratus*, Metabolites, Nontargeted metabolomics, Enzyme inhibitory activity, Different culture methods

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Introduction

Taiwanofungus camphoratus (M. Zang & C.H. Su) Sheng H. Wu, Z.H. Yu, Y.C. Dai & C.H. Su, belongs to the division Basidiomycota, class Agaricomycetes, order Polyporales, family Incertae sedis, and genus *Taiwanofungus*. It is a famous, rare, edible, and medicinal fungus in China, mainly distributed in Taiwan Province of China. It is known as "forest ruby" [1, 2]. *T. camphoratus* is used since ancient times as a folk medicine. Taiwan aborigines used *T. camphoratus* as an effective cure for alcoholism and believed that regular consumption of *T. camphoratus* could maintain the vitality of body and prolong life [3]. Although *T. camphoratus* is being used since a long time, official literature reports on this fungus began to get published in the 1990s in China [4]. After years of research, it is currently confirmed that *T. camphoratus*, *Antrodia cinnamomea* T.T. Chang & W.N. Chou, and *Antrodia camphorata* Sheng H. Wu, Ryvarden & T.T. Chang can represent *T. camphoratus* [5–7]. *T. camphoratus* exhibits various therapeutic properties such as blood-sugar lowering, immunity improving, and antioxidant activities.

Cinnamomum kanehirae, an endemic plant species, in Taiwan Province of China is the only natural host of wild *T. camphoratus*. Therefore, obtaining the wild fruiting body of *T. camphoratus* is extremely rare. Moreover, in recent years, the resources of *C. kanehirae* are becoming increasingly scarce due to the wanton deforestation, making the wild fruiting bodies of *T. camphoratus* even harder to obtain. Therefore, the development and utilization of wild *T. camphoratus* resources is difficult. *T. camphoratus* resources can be obtained via artificial culture. At present, for the artificial culture of *T. camphoratus*, four methods are commonly used: cutting wood culture, solid-state fermentation, submerged fermentation, and dish culture.

For cutting wood culture, earlier, wood from *C. kanehirae* was used for the inoculation and culture of *T. camphoratus*. However, due to the scarcity and difficulty in obtaining this wood, researchers began to culture *T. camphoratus* on the wood of other trees. The composition and pharmacological activity of *T. camphoratus* cultured using cutting wood culture are the closest to those of wild *T. camphoratus*; however, it takes up to 2–3 years to obtain the fruiting body using this method. Therefore, it is difficult to meet the market demand for *T. camphoratus* only via cutting wood culture.

Solid-state fermentation does not exhibit the limitations of cutting wood culture to grow wild *T. camphoratus* (e.g., scarcity of *C. kanehirae* wood). In solid-state fermentation, metabolites of *T. camphoratus* with chemical characteristics and biological activities similar to those of wild *T. camphoratus* can be obtained [8]. In this method, mostly grains are used as raw materials. This method has the advantages such as high yield and less pollution. Antrodin C and Antroquinonol compounds are abundantly obtained in the solid-state fermentation of *T. camphoratus* [9]. Currently, solid-state fermentation is widely used to culture *T. camphoratus*; although it yields abundant active substances, it requires longer culture time than submerged fermentation [10].

In submerged fermentation, also known as deep fermentation, microorganisms are grown in a liquid medium containing the required nutrients [11]. This is the most commonly used method to culture *T. camphoratus* in factories, as well as laboratories [12]. Submerged fermentation method exhibits advantages such as short production cycle, high growth rate, and rapid maturation process; thus, more mycelia can be obtained in short time [13]. However, compared with other culture methods, the types of active substances from *T. camphoratus* obtained are few [14]. The mycelia obtained via conventional submerged fermentation mainly contain maleic acid and succinic acid derivatives, mainly Antrodin C, and some metabolites are differentially produced by different strains [15]. The content of Antroquinol in *T. camphoratus* is less when cultured using ordinary submerged fermentation. If the precursor of Antroquinol is added during the culture, submerged fermentation of *T. camphoratus* can be used to synthesize Antroquinol [16]. Many studies have reported the strategies to improve the biomass and content of active substances in the mycelium of *T. camphoratus* under submerged fermentation [17]. Liu et al. directly used the mycelial fermentation broth to study the pharmacological activity of *T. camphoratus* and reported that it could inhibit angiogenesis through HIF-1α, thus disrupting the characteristics of tumor stem cells and improving the radiosensitivity of esophageal cancer cells. This auxiliary effect can enhance the effect of radiotherapy in esophageal cancer. In addition, mycelial fermentation broth of *T. camphoratus* can inhibit the epithelial–mesenchymal transition in esophageal cancer cells [18–20].

Dish culture is a commonly used method to culture microorganisms in laboratory. Dish culture of *T. camphoratus* exhibits advantages such as convenience and moderate culture period and is now used in the production of *T. camphoratus* [3]. Under dish culture, *T. camphoratus* produces various metabolites including triterpenoids, which are similar to those found in wild *T. camphoratus* [21]. However, the limitations of this method include extensive use of material (dishes) and labor resources, increasing the cost, and the growth of *T. camphoratus* is limited because of small volume of the dish [3, 12].

Differences in the culture conditions under various culture methods result in differences in the composition of *T. camphoratus* [22]. Therefore, for the appropriate

application of *T. camphoratus* and to rationally obtain various active ingredients, it is urgent to study and optimize different culture methods. Though *T. camphoratus* is rich in various active substances, current studies mainly focus on the characteristic components such as triterpenoids and androquinol. Only few studies are available on other components of *T. camphoratus*, such as phenolic compounds. In addition, the metabolites produced by *T. camphoratus* under various culture methods are different. Therefore, analysis of metabolites produced by *T. camphoratus* under various culture methods is important for the rational use of *T. camphoratus*.

In this study, nontargeted metabolomic analysis was used to compare the metabolites produced by *T. camphoratus* under four culture methods. Principal component analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were used to analyze the differences in the metabolites under these culture methods. The ability of *T. camphoratus* to lower blood sugar level was assessed by studying inhibition of the activity of α-glucosidase, α-amylase, and sucrase by *T. camphoratus* extracts obtained under the four culture methods. This study laid a theoretical foundation for further development and use of various culture methods of *T. camphoratus* to obtain specific active ingredients.

Materials and methods

Material and reagent

Material

T. camphoratus was purchased from the Guangdong Microbial Strain Conservation Center (GIM No. 5.530) and preserved as a slant culture on PDA medium (composition: 200 g potato, 20 g glucose, 1 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, 3 g yeast extract powder, and 20 g agar in 1 L distilled water). Its molecular identification was performed via amplification of the ITS gene.

Reagents

Anhydrous ethanol was purchased from Chengdu Cologne Chemicals Co., Ltd. (Chengdu, China). Oleanolic acid, vanillin, potassium sodium tartrate, 3,5-dinitrosalicylic acid, and 4-nitrophenyl-β-D-glucopyranoside (PNPG) were purchased from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China). Glacial acetic acid, perchloric acid, methanol, sodium hydroxide, phenol, sucrose, sodium carbonate, and soluble sucrose were purchased from Sinopharm Chemical Reagent Co., Ltd. (Tianjin, China). All reagents were of analytical grade. Three enzymes, namely, α-glucosidase (100 U/mg), α-amylase (50 U/mg), and invertase (sucrase) (≥200 U/mg), were purchased from Shanghai McLean Biochemical Technology Co., Ltd. (Shanghai, China).

Methods

Methods to culture T. camphoratus

T. camphoratus was cultured using cutting wood culture, solid-state fermentation, submerged fermentation, and dish culture at 26 °C. Raw materials were weighed using SQP electronic balance (Sartorius Scientific Instruments). The media for all culture methods were autoclaved prior to use.

For cutting wood culture, apple wood was used, inoculated with *T. camphoratus*, and cultivated for 2 years.

The medium for solid-state fermentation was oats with twice the amount of water. The *T. camphoratus* samples were collected at 30, 45, 60, 75, and 90 days of solid-state fermentation.

Submerged fermentation medium was prepared by mixing 200 g potato, 20 g glucose, 1 g potassium dihydrogen phosphate, 0.5 g magnesium sulfate, and 3 g yeast extract powder. Submerged fermentation was performed at 120 rpm. The *T. camphoratus* samples were collected at 10, 15, 20, 25, and 30 days of submerged fermentation.

For dish culture, the medium was the same as that used for submerged fermentation but added with 20 g agar. The *T. camphoratus* samples were collected at 20, 25, 30, 35, and 40 days of dish culture.

The obtained fruiting bodies and mycelia were separately dried in an oven (DHT-450A high-temperature blast drying oven, Shanghai Dao-han Industry Co., Ltd.) at 45 °C, crushed in a pulverizer (2500C pulverizer, Yongkang Red Sun Mechanical and Electrical Co., Ltd.), sieved (through a 0.425-mm sieve), and stored at 4 °C till further analysis.

Construction of standard curve of triterpenoids

The content of triterpenoids in the mycelia of *T. camphoratus* cultured using various methods was determined using vanillin–perchloric acid colorimetric method [23]. For constructing the standard curve for this experiment, 1 mg/mL oleanolic acid was used as the standard solution. In total, 25, 50, 75, 150, and 200 μL of the standard solution was taken in test tubes and evaporated to dryness in boiling water. To this, 0.1 mL of freshly prepared 5% vanillin–glacial acetic acid solution and 0.4 mg/mL perchloric acid solution was added, followed by heating in water bath (HHS-21-6 constant temperature water bath, Shanghai Boxun Industry Co., Ltd. Medical Equipment Factory) at 60 °C for 20 min and cooling to room temperature. To this, 5 mL glacial acetic acid solution was added and mixed well. The experiment included blank control and three replicates for each concentration. The absorbance was measured at 548 nm using T6 new Yue visible spectrophotometer, Beijing Persee General Instru-ment Co., Ltd.). The abscissa was concentration

of oleanolic acid, and the ordinate was the absorbance value. The standard curve was constructed, and the regression equation was obtained as $y = 6.9361x - 0.0815$ ($R^2 = 0.9999$).

Determination of triterpenoid content in T. camphoratus

To weigh 40 mg of *T. camphoratus* mycelium powder, 1 mL of 70% ethanol was added, and the mixture was placed in an ultrasonic extractor (KQ-500DE ultrasonic extractor, Kunshan Ultrasonic Instruments Co., Ltd.) for 40 min at 60 °C with power 400 W. The mixture was centrifuged (Neo15R high-speed refrigerated centrifuge, Shanghai Lishen Scientific Instruments Co., Ltd.) at 12,000 rpm for 5 min. The supernatant is hereafter referred to as the "crude extract." Using the supernatant, the triterpenoid content of *T. camphoratus* mycelia cultured using solid-state fermentation, dish culture, and submerged fermentation for various culture times was determined according to the method described in "Construction of standard curve of triterpenoids" section. The triterpenoid content of fruiting bodies of *T. camphoratus* cultured via cutting wood method served as the control. The duration (days) of culture was determined at which the highest triterpenoid content was obtained under each culture method.

Detection of metabolite profile of T. camphoratus using liquid chromatography–electrospray ionization–tandem mass spectrometry

The suspension of 40 mg/mL *T. camphoratus* in 70% ethanol was centrifuged. After centrifugation, the supernatant was collected, evaporated, redissolved in methanol, mixed, and centrifuged. The supernatant was collected and used for the analysis of metabolite profile as given below.

Liquid chromatography–electrospray ionization–tandem mass spectrometry (LC–ESI–MS/MS) was used for evaluating metabolite profile using the extracts of *T. camphoratus* obtained from four culture methods. The analysis was conducted using Thermo Fisher UPLC-Q Exactive Focus (Symefeishier Technology Company) equipped with an electrospray ionization source (ESI). The C18 column (100 × 2.1 mm, 3 μm) was used for the chromatographic separation. The solvent flow rate was 0.4 mL/min, and 2 μL of all samples and standards was injected. The mobile phase was solvent A (water with 0.1% formic acid) and solvent B (methanol). The mobile phase gradient was programmed as follows: 65% A (v/v) from 0 to 10.0 min, 30% A (v/v) from 10.0 to 30.0 min, 5% A (v/v) from 30.0 to 35.0 min, 65% A (v/v) from 35.0 to 35.1 min, and 65% A (v/v) from 35.1 to 40.0 min. The total run time was 40 min, and the column temperature was maintained at 40 °C.

Inhibition of α-glucosidase activity

α-Glucosidase activity inhibition rate was assessed using a method by Masao Hattori with slight modifications [24]. Acarbose was used as the positive control. In total, 20 μL of the crude extract of *T. camphoratus* or 20 μL of 0.001 mg/mL acarbose, 20 μL of 5 U/mL α-glucosidase solution, and phosphate buffer (to make up the total volume to 90 μL) were added to 96-well plates, mixed, and incubated at 37 °C for 30 min. To this, 20 μL PNPG solution (15 mM) was added, mixed, and incubated at 37 °C for 60 min. Finally, sodium carbonate solution was added to terminate the reaction. The absorbance of the released *p*-nitrophenol was measured at 405 nm using a microplate reader. The sample blank group contained the sample (or acarbose) but phosphate buffer was added instead of α-glucosidase solution. The control group contained phosphate buffer instead of sample (or acarbose). The control blank group contained phosphate buffer instead of sample (or acarbose) and PNPG. The inhibition rate of α-glucosidase can be calculated using formula (1).

$$\text{Inhibition rate of enzyme activity (\%)} = \frac{(A_0 - A) - (A_x - A_y)}{A_0 - A} \times 100\%, \quad (1)$$

where A_0, A, A_x, and A_y are the absorbance of control group, control blank group, sample group, and sample blank group, respectively.

Inhibition of α-amylase activity

The inhibition of α-amylase activity was assessed according to the method by Wang [25]. Acarbose was used as the positive control. First, DNS solution was prepared as follows. In total, 91 g of potassium sodium tartrate was dissolved in 250 mL distilled water. The mixture was heated at low temperature until potassium sodium tartrate was dissolved. Further, 10.5 g of sodium bicarbonate, 0.15 g of 3, 5-dinitrosalicylic acid, and 2.5 g of phenol were added and stirred to dissolve. The mixture was cooled, and the volume was adjusted to 500 mL using distilled water. The mixture was stored in dark for 1 week before using.

The crude extract of *T. camphoratus* (50 μL) or 50 μL of acarbose solution (3 mg/mL) were added to a centrifuge tube. To this, 50 μL of α-amylase solution (10 U/mL) was added and incubated at 37 °C for 10 min. To this, 100 μL of 1% soluble starch solution was added, mixed, and incubated at 37 °C for 10 min. Further, to terminate the reaction, 200 μL of DNS solution was added. The mixture was placed in boiling water bath for 5 min, taken out, cooled, and diluted with 1 mL distilled water. The absorbance was measured at 540 nm using a spectrophotometer. The

inhibition rate of α-amylase was calculated using formula (1).

Inhibition of sucrase activity

The inhibition rate of sucrase was determined according to the method by Zhang with slight modifications [26]. Acarbose was used as the positive control. The crude extract of *T. camphoratus* (250 µL) or 250 µL of acarbose solution (12 mg/mL) was mixed with 250 µL of sucrase solution (200 U/mL) in a centrifuge tube and incubated at 37 °C for 10 min. To this, 500 µL of sucrose solution was added, mixed, and incubated at 37 °C for 20 min. Further, to terminate the reaction, 1 mL of DNS solution was added. The mixture was placed in boiling water bath for 5 min, taken out, and allowed to cool at room temperature. The absorbance was measured at 550 nm using a spectrophotometer. The inhibition rate of sucrase was calculated using formula (1).

Data analysis

The results of triterpenoid content and enzyme activity inhibition were analyzed using SPSS (IBM SPSS Statistics 26) software. Data were expressed as mean ± standard deviation (SD). One-way analysis of variance (ANOVA) and Tukey's post-hoc test ($P < 0.05$) was used to determine significant differences between the means. Ingredient testing data of the results were analyzed using Compound Discoverer software. Nontargeted analysis of the experimental results was performed using mz Cloud, mz Vault, and self-built databases [12]. Unsupervised PCA and supervised PLS-DA were conducted to explore the differences in the metabolites of *T. camphoratus* produced under four culture methods. The analysis of differential metabolites was performed using the free online data analysis platform OmicShare tool (https://www.omicshare.com/tools).

Results and analysis

Effect of different culture time on the triterpenoid content of *T. camphoratus*

Triterpenoids are the characteristic products of *T. camphoratus*. Our previous study reported that wild and artificially cultured *T. camphoratus* have some comparable characteristic triterpenoids with similar hepatoprotective activities [27].

The triterpenoid content of *T. camphoratus* mycelia cultured using solid-state fermentation, dish culture, and submerged fermentation for various culture times was determined. The triterpenoid content of *T. camphoratus* mycelia cultured via cutting wood method served as the control (Figs. 1, 2, 3). The result revealed that under solid-state fermentation, the triterpenoid content of *T. camphoratus* mycelia was the highest after 75 days of culture. Under submerged fermentation, it was the highest after 25 days of culture. Under dish culture, triterpenoid content increased with time and tended to be stable after 30 days. After comprehensive consideration including optimal time and high triterpenoid content, dish culture for 25 days was the best to obtain triterpenoids from *T.*

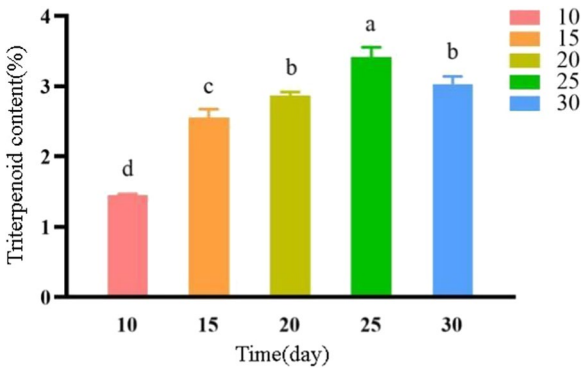

Fig. 2 Effect of different culture time on the content of triterpenoids in the mycelia of *T. camphoratus* under submerged fermentation

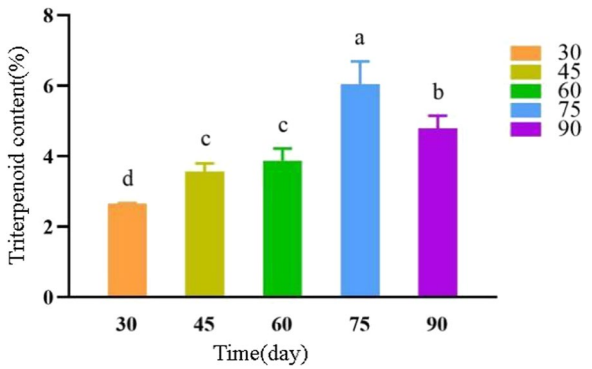

Fig. 1 Effect of different culture time on the content of triterpenoids in the mycelia of *Taiwanofungus camphoratus* under solid-state fermentation

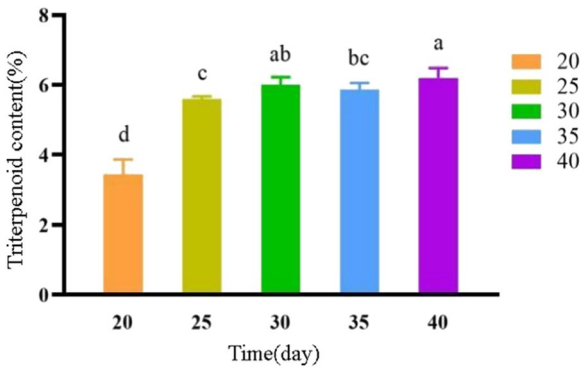

Fig. 3 Effect of different culture time on the content of triterpenoids in the mycelia of *T. camphoratus* under dish culture

camphoratus. For further analysis, the *T. camphoratus* samples collected at 75, 25, and 25 days under solid-state fermentation, submerged fermentation, and dish culture, respectively, were selected with three replicates in each group.

The effect of different culture time on the content of triterpenoids of *T. camphoratus* in the fruiting bodies was not included in this part, it is because the fruiting bodies grows very slowly. The fruiting bodies of *T. camphoratus* grow very slowly, and the triterpenoids in them are still accumulating after 5 years, but the content change of triterpenoids in them is very little in a few months. Therefore, triterpenoids content was not determined regularly as the other culture methods. The optimum culture time can be obtained by the determination of triterpenoids content in the other culture methods, and for the fruiting body, it seems that the longer the culture time, the higher the content of triterpenoids. In the following study, we selected the fruiting bodies of *T. camphoratus* that had grown on apple wood for 3 years.

Metabolite profile of *T. camphoratus* cultured using various methods

The metabolite profile of *T. camphoratus* cultured using various methods was determined using LC–ESI–MS/MS (Thermo Fisher UPLC-Q Exactive Focus, Thermo Fisher Scientific Inc.).

For this experiment, 10 μL of various samples was mixed, which was termed as quality control (QC) sample, and 100 μL of QC sample was injected for analysis.

The metabolites in the samples were analyzed according to their precise molecular weight, and fragment ions were obtained. A total of 186 metabolites were detected in the positive ion mode. The total ion current spectrum of the QC sample of the extract of artificially cultured *T. camphoratus* is shown in Additional file 1. Under solid-state fermentation, dish culture, submerged fermentation, and cutting wood culture, 158, 161, 157, and 160 metabolites of *T. camphoratus* were obtained, respectively. Therefore, *T. camphoratus* cultured using various methods contained abundant metabolites (Fig. 4). In total, 127 metabolites were common among the four culture methods. Under solid-state fermentation, submerged fermentation, and cutting wood culture, 12, 1, and 4 metabolites were unique, respectively. These metabolites were classified according to the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) and MeFSAT database (https://cb.imsc.res.in/mefsat/) (Table 1).

PCA of metabolites of *T. camphoratus* produced under four culture methods

Unsupervised PCA was conducted to explore the differences in the metabolites of *T. camphoratus* produced under four culture methods. PCA can show the rules of data through a few principal components while

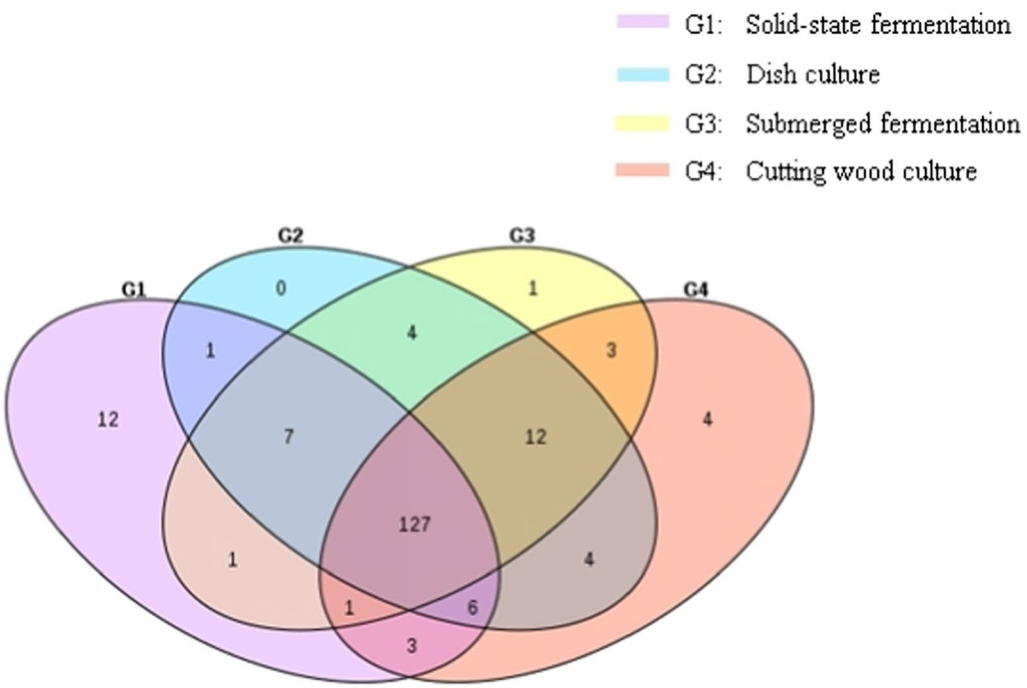

Fig. 4 Venn diagram of the metabolites of *T. camphoratus* produced under various culture methods

Table 1 Types of metabolites in *T. camphoratus*

Classification	Quantity
Terpene	61
Nitrogenous compounds	22
Phenol compound	42
Glycoside	2
Amino acid	3
Vitamins	1
Fatty acids	26
Miscellaneous	29
Aggregate	186

retaining as much original data as possible. The results of PCA indicated that the data in the four groups were well duplicated, and differences existed among the four groups (Fig. 5). The four culture methods of *T. camphoratus* could be distinguished to a certain extent. In addition, the metabolites of *T. camphoratus* cultured using dish culture and submerged fermentation were more similar under principal component 1 and were clearly distinguishable from those of *T. camphoratus* cultured using solid-state fermentation and cutting wood culture. The mycelia of *T. camphoratus* cultured using solid-state fermentation and fruiting bodies of *T. camphoratus* cultured using cutting wood culture could not be completely distinguished under principal component 2, and the difference was small.

PLS-DA of the metabolites of *T. camphoratus* produced under four culture methods

Supervised PLS-DA was performed to explore the differences among the metabolites produced using four methods. PLS-DA more focuses on the analysis of differences among groups. The results revealed that the data from four groups did not overlap; the distinction was obvious, and the fitting accuracy of the model was good (Fig. 6). Compared with the metabolites of *T. camphoratus* cultured using cutting wood culture, those in the other three groups were significantly different. The metabolites of *T. camphoratus* cultured using solid-state fermentation and submerged fermentation were more similar.

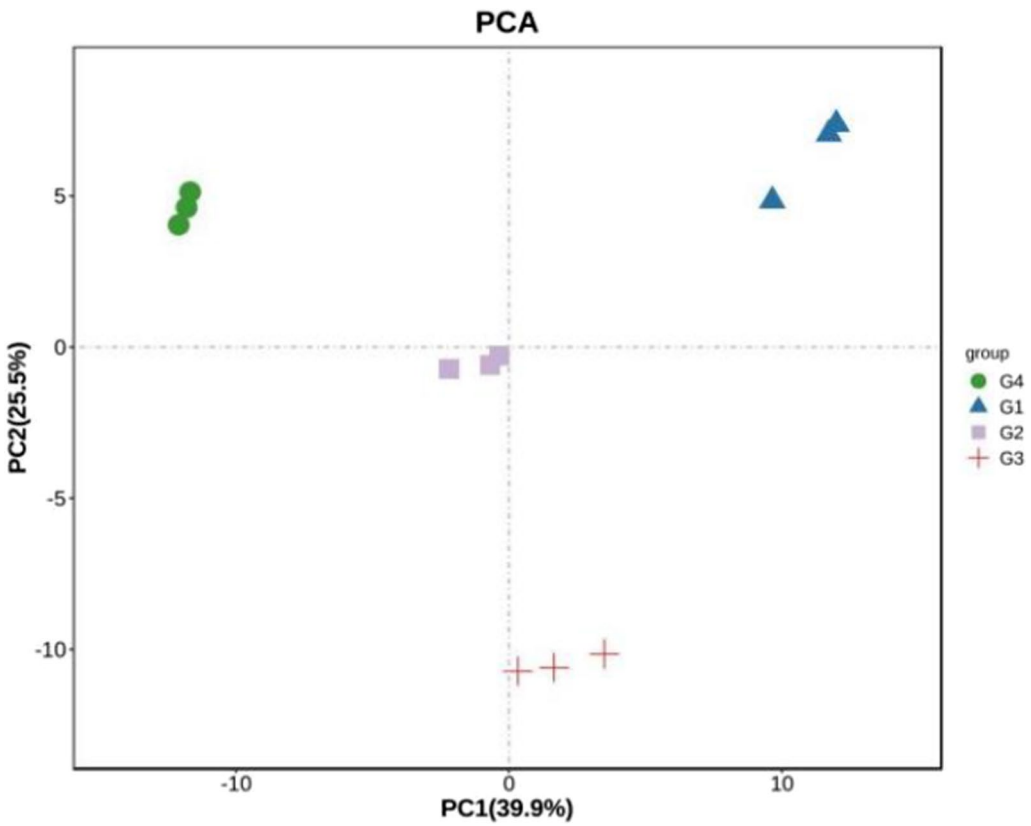

Fig. 5 Principal component analysis of metabolites of *T. camphoratus* produced under four culture methods (G1: solid-state fermentation; G2: dish culture; G3: submerged fermentation; G4: cutting wood culture)

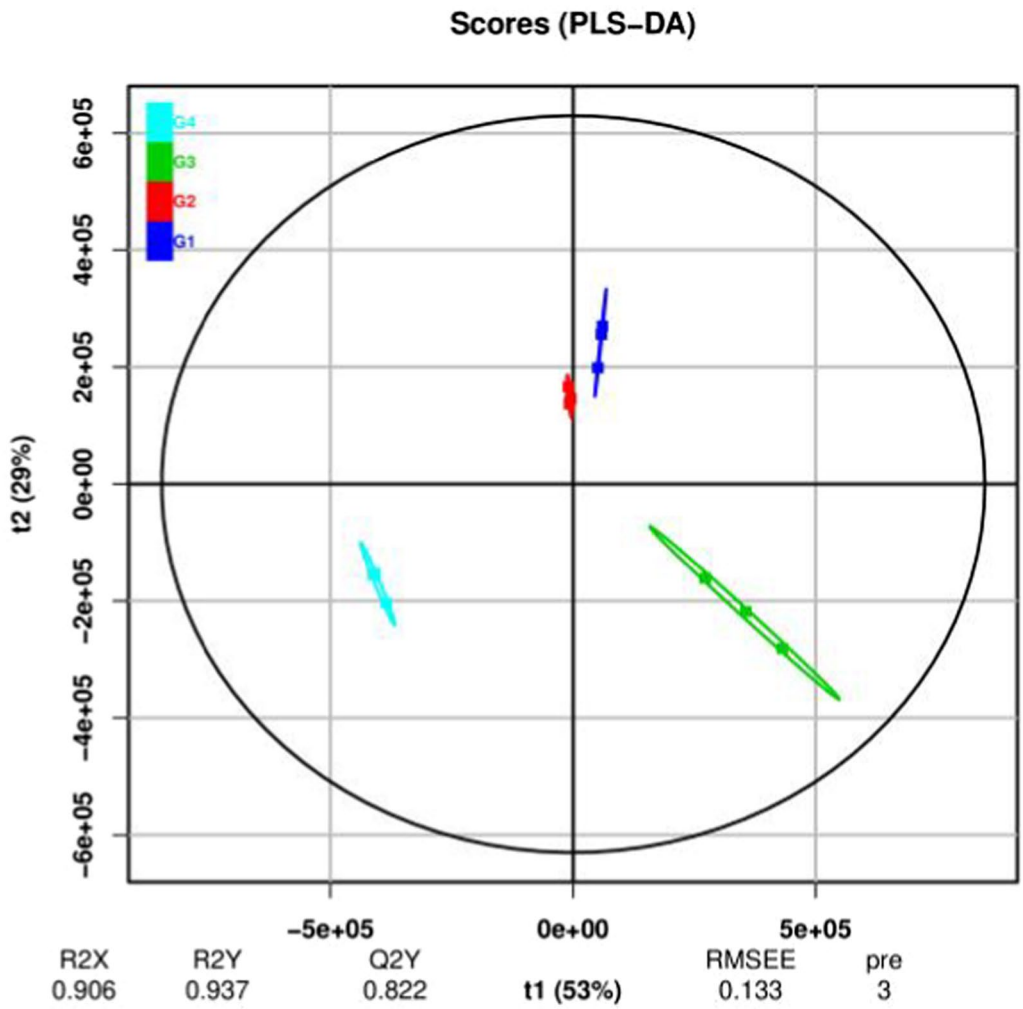

Fig. 6 Partial least squares-discriminant analysis of metabolites of *T. camphoratus* cultured using four different methods (G1: solid-state fermentation; G2: dish culture; G3: submerged fermentation; G4: cutting wood culture)

Analysis of significantly differential metabolites of *T. camphoratus* cultured using four different methods

Based on the PLS-DA model, 20 metabolites with significant differences were screened under the condition of $P < 0.05$ and $VIP > 1$. Through the cluster heat map (Fig. 7), the differences among the metabolites could be clearly seen. Under cutting wood culture and solid-state fermentation, the metabolites with significant differences were mostly triterpenoids and fatty acid compounds, respectively. The top-20 differential metabolites were analyzed in Table 2. These metabolites were observed to be important for production of food products, medicine.

In addition, 166 metabolites produced by *T. camphoratus* were common under all four culture methods (see it in Additional file 1). These metabolites include some characteristic components of *T. camphoratus*, such as Antcin E and AntcinF. Further studies should assess whether these compounds can be obtained from *T. camphoratus* via culture methods (other than cutting wood culture) with short culture time and easy bulk production.

Analysis of metabolites with significant differences among the four groups

The differences in the metabolites of *T. camphoratus* produced under different culture methods were further analyzed using PLS-DA model (Fig. 8). The results revealed that the metabolites of *T. camphoratus* produced under different culture methods could be distinguished by pairwise comparison, which once again proved that there were differences in the metabolites of *T. camphoratus* under different culture methods.

Based on the PLS-DA model of pairwise comparison, with the threshold of $P < 0.05$ and $VIP > 1$, the metabolites with significant differences were screened to plot the heat map (Fig. 9). Compared with solid-state fermentation,

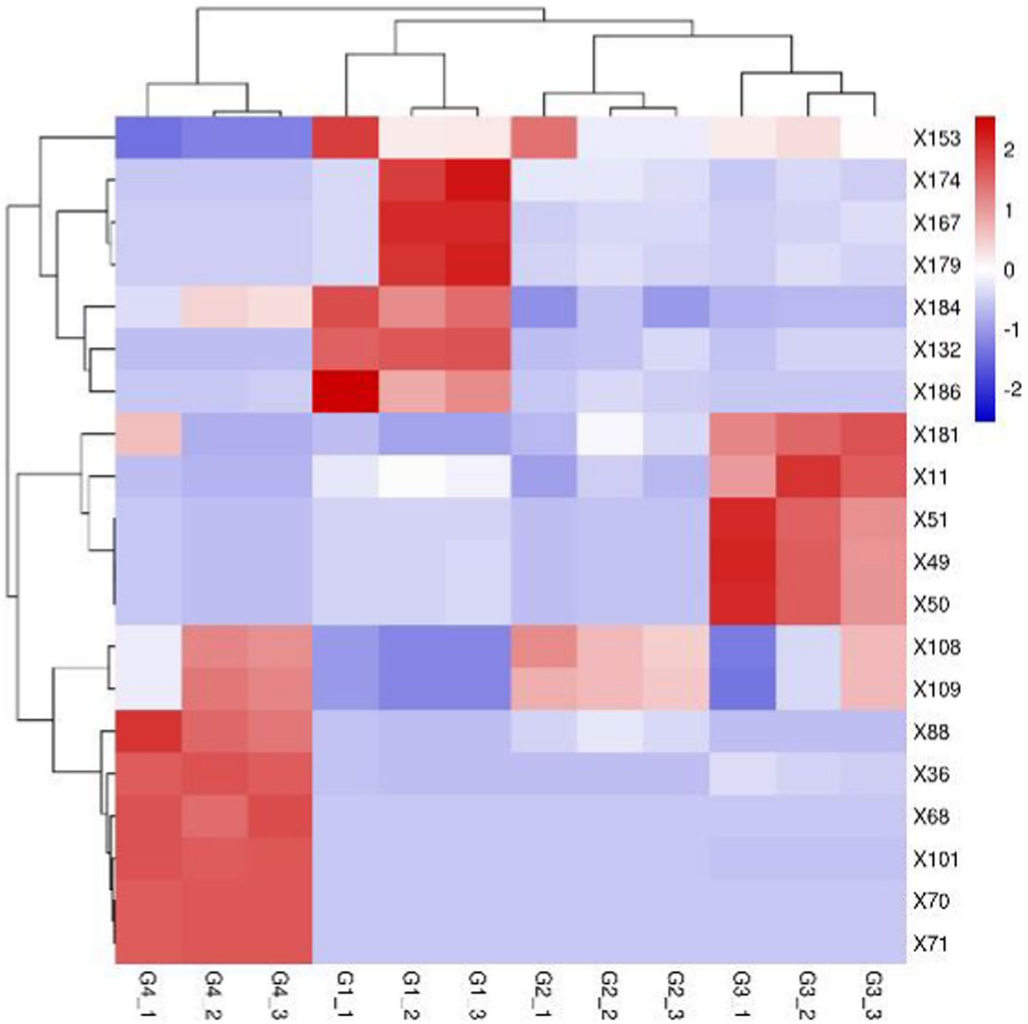

Fig. 7 Heat map analysis of 20 differential metabolites of *T. camphoratus* produced under four culture methods (G1: solid-state fermentation; G2: dish culture; G3: submerged fermentation; G4: cutting wood culture)

the triterpenoid expression under dish culture was higher, and the main fatty acids and phenolic compounds produced by *T. camphoratus* were significantly different under these two methods. Compared with solid-state fermentation, the upregulated metabolites under submerged fermentation were mostly phenolic compounds. Compared with dish culture, the upregulated metabolites under submerged fermentation were mostly phenolic compounds. Compared with solid-state fermentation, dish culture, and submerged fermentation, the upregulated metabolites under cutting wood culture were mostly terpenes and mainly triterpenoids, which are the characteristic metabolites of *T. camphoratus* (Table 3).

Inhibitory activity of α-glucosidase, α-amylase and sucrase

α-Glucosidase is one of the key components affecting postprandial blood glucose and is considered an important target for the treatment of diabetes [60]. The inhibition of α-glucosidase activity by the crude extracts of *T. camphoratus* cultured using various methods was assessed (Fig. 10A). Acarbose was used as the positive control. Under solid-state fermentation, submerged fermentation, dish culture, and cutting wood culture, the inhibition rates of α-glucosidase activity by *T. camphoratus* crude extracts were 23.86%, 17.37%, 26.56%, and 55.97%, respectively. The inhibition rate of α-glucosidase activity by 0.001 mg/mL acarbose was 59.87%. The rates of α-glucosidase activity inhibition by acarbose and *T. camphoratus* extract under cutting wood culture were not significantly different.

The inhibition rates of α-amylase activity by *T. camphoratus* crude extract obtained under solid-state fermentation, submerged fermentation, dish culture, and cutting wood culture were 39.43%, 28.98%, 47.52%, and 51.96%,

Table 2 TOP 20 significantly different metabolites

Serial number	Compound	Molecular formula	*P*-value	VIP	Biological activity and mechanism of action	References
X153	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$	2.65E−02	1.304089	Anti-inflammatory: can reduce inflammation caused by apolipoprotein CIII (a small glycoprotein that binds to the surface of certain lipoproteins)	[28]
X174	Ethyl linoleate	$C_{20}H_{36}O_2$	4.58E−02	4.386933	1. Treatment of atherosclerotic disease: by inhibiting the progression of plaque in ApoE gene deficient mice, the development of atherosclerosis was slowed down 2. Anti-inflammatory, antibacterial: (1) can be used to treat inflammatory and non-inflammatory acne and seborrheic disease; (2) it can down-regulate the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), thereby reducing the production of nitric oxide (NO) and prostaglandin E2, and achieving anti-inflammatory effects 3. Inhibition of melanin formation: the formation of melanin was inhibited by Akt/GSK3β/β-catenin signaling pathway	[29–32]
X167	Linolenic acid ethyl ester	$C_{20}H_{34}O_2$	4.69E−02	1.903281	Pro-fibrosis	[33]
X179	Ethyl oleate	$C_{20}H_{38}O_2$	4.78E−02	1.65851	Can be used as excellent solvent for steroids and other lipid drugs	[34]
X184	β-Sitostenone	$C_{29}H_{48}O$	3.12E−05	1.618023	Inhibition of tyrosinase activity	[35]
X132	Eleostearic acid	$C_{18}H_{30}O_2$	2.96E−09	1.105423	Treatment of breast cancer: it can block the proliferation of breast cancer cells and induce apoptosis through an oxidation-dependent mechanism	[36]
X186	Linolenic acid	$C_{18}H_{30}O_2$	1.18E−03	1.124406	–	–
X181	(22E)-Ergosta-4, 6, 8(14), 22-tetraen-3-one	$C_{28}H_{40}O$	1.26E−03	2.300434	Against cancer: neuro-2a, Saos-2, MCF7 and LNCaP-C42	[37]
X11	Syringaldehyde	$C_9H_{10}O_4$	3.35E−05	1.229216	Anti-inflammatory, anti-oxidation, anti-diabetes	[38]
X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$	1.27E−05	3.710584	Inhibition of cyclooxygenase 2 (COX-2)	[39]
X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$	2.72E−05	5.483619	Attract bees and other insects to complete plant pollination	[40]
X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$	2.03E−05	3.133247	–	–
X108	Gamabufotalin	$C_{24}H_{34}O_5$	2.21E−02	3.260203999	1. Anti-cancer: (1) non-small cell lung cancer can significantly inhibit the expression of COX-2 in non-small cell lung cancer cells, and its mechanism is to inhibit IKKβ/NF-κB signaling pathway. (2) Human multiple myeloma: inhibit the growth of human multiple myeloma cells and induce apoptosis, IC50 < 50 nM; (3) osteosarcoma: inhibition of the viability and tumorigenicity of osteosarcoma cells by blocking the TGF-β/periostin/PI3K/AKT signaling pathway 2. Treatment of angiogenesis-related diseases: inhibition of angiogenesis by inhibiting the activation of VEGFR-2 signaling pathway	[41, 42]
X109	Methyl 2-methoxyphenylacetate	$C_{10}H_{12}O_3$	2.33E−02	1.146733678	One of the compounds for the synthesis of trans-pterocarpin compounds	[43]
X88	Camphoratin C	$C_{29}H_{42}O_6$	7.26E−07	2.385113	–	–

Table 2 (continued)

Serial number	Compound	Molecular formula	*P*-value	VIP	Biological activity and mechanism of action	References
X36	Fraxetin	$C_{10}H_8O_5$	1.28E−11	1.286482	1. Anticancer: ovarian cancer, through the inhibition of TLR4/STAT3 signaling pathway to play a therapeutic role in ovarian cancer 2. Antioxidation 3. Anti-inflammatory 4. Antibacterial	[44, 45]
X68	Antcin H	$C_{29}H_{42}O_6$	1.07E−09	1.025206	1. Protective liver: (1) by disrupting the binding of p-JNK to the mitochondrial outer membrane scaffold protein (Sab), the self-sustaining activation of the ROS-dependent MAPK cascade is interfered, thereby protecting the liver from damage 2. Anti-cancer: (1) renal cell carcinoma inhibits the invasion of renal cancer cells by inhibiting the FAK-ERK-C/EBP-β/c-Fos-MMP-7 pathway; (2) lymphoma induces apoptosis of lymphoma cell lines by inhibiting LMP1-induced JAK/STAT pathway-related signals	[45–48]
X101	Zhankuic acid B/Antcin I	$C_{29}H_{42}O_5$	7.35E−14	3.571542	1. Anti-inflammatory 2. Cancer: it can effectively regulate the drug efflux transporter P-gp through non-competitive inhibition and reverse the multidrug resistance (MDR) of tumors	[49–51]
X70	Antcin C	$C_{29}H_{42}O_5$	7.42E−15	1.296406	1. Anti-cancer, liver cancer: (1) promote the apoptosis of liver cancer cells by regulating the phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin pathway; (2) protect hepatocytes from oxidative stress and cell death by activating Nrf2/ARE pathway; (2) (s)-antcin C was cytotoxic to Hep G2 and MCF-7 cells with IC50 values of 14.5 and 12.8 μg/mL, respectively 2. Protect cerebral hemorrhage injury: control microglial inflammation through the TLR-4 pathway, thereby protecting cerebral hemorrhage injury	[52–54]

Table 2 (continued)

Serial number	Compound	Molecular formula	*P*-value	VIP	Biological activity and mechanism of action	References
X71	Antcin K	$C_{29}H_{44}O_6$	1.96E−15	5.708815	1. Treatment of liver injury, liver cancer, hepatitis: (1) it has a good potential to reduce the risk of liver cancer metastasis, and can inhibit the metastasis of human liver cancer cells by inhibiting integrin-mediated adhesion, migration and invasion; (2) induce apoptosis mediated by mitochondria and endoplasmic reticulum stress in human hepatocellular carcinoma cells; 3. Protective and therapeutic mechanisms against *N*-nitrosodiethylamine-induced liver injury and inflammation by directly scavenging ROS activity and up-regulating antioxidant defense mechanisms 2. Treatment of diabetes and hyperlipidemia: (1) it can dose-dependently reduce blood glucose and lipid levels in high-fat diet (HFD) mice and improve glucose tolerance; (2) it can significantly increase the expression level of glucose transporter 4 (GLUT4) in skeletal muscle cell membrane and reduce the level of glucose-6-phosphatase (G6Pase) mRNA in liver, thus reducing blood glucose level. (3) The expression of peroxisome proliferator-activated receptor α (PPARα) was increased, and the mRNA level of hepatic sterol response element binding protein-1c (SREBP-1c) was decreased, which contributed to the decrease of plasma triglyceride, hepatic steatosis and total cholesterol levels 3. Treatment of rheumatoid arthritis (RA): by down-regulating the phospholipase C-γ/protein kinase C-α pathway, the expression of VEGF in rheumatoid arthritis synovial fibroblasts (RASFs) was significantly inhibited and the migration and lumen formation of endothelial progenitor cells (EPC) were improved	[55–59]

respectively (Fig. 10B). The rate of α-amylase activity inhibition by 3 mg/mL acarbose was 51.17%. The rates of α-amylase activity inhibition by acarbose and *T. camphoratus* extract under dish culture and cutting wood culture were not significantly different.

The inhibition rates of sucrase activity by *T. camphoratus* crude extracts obtained under solid-state fermentation, submerged fermentation, dish culture, and cutting wood culture were 49.96%, 41.61%, 62.23%, and 78.02%, respectively (Fig. 10C). The inhibition rate of sucrase activity by 12 mg/mL acarbose was 76.92%. The rates of sucrase activity inhibition by acarbose and *T. camphoratus* extract under cutting wood culture were not significantly different.

Although the above experiments have confirmed that the extract of *T. camphoratus* has the inhibitory activity to the target proteins related to diabetes, the specific substances that play the main role still need to be further verified. According to the current research results, triterpenoids are mainly involved, and the content and types of triterpenoids directly affect the enzyme inhibitary activity. In the future, we will continue to conduct network pharmacology studies, and to isolate and purify the active substances of *T. camphoratus* to further verify their enzyme inhibition efficacy.

Discussion

In recent years, studies on *T. camphoratus* focused on its characteristic metabolites such as triterpenoids and androquinol and their activities. However, *T. camphoratus* produces abundant active metabolites and produces different metabolites under different culture methods. Most studies have only used liquid chromatography–tandem mass spectrometry or high-pressure liquid

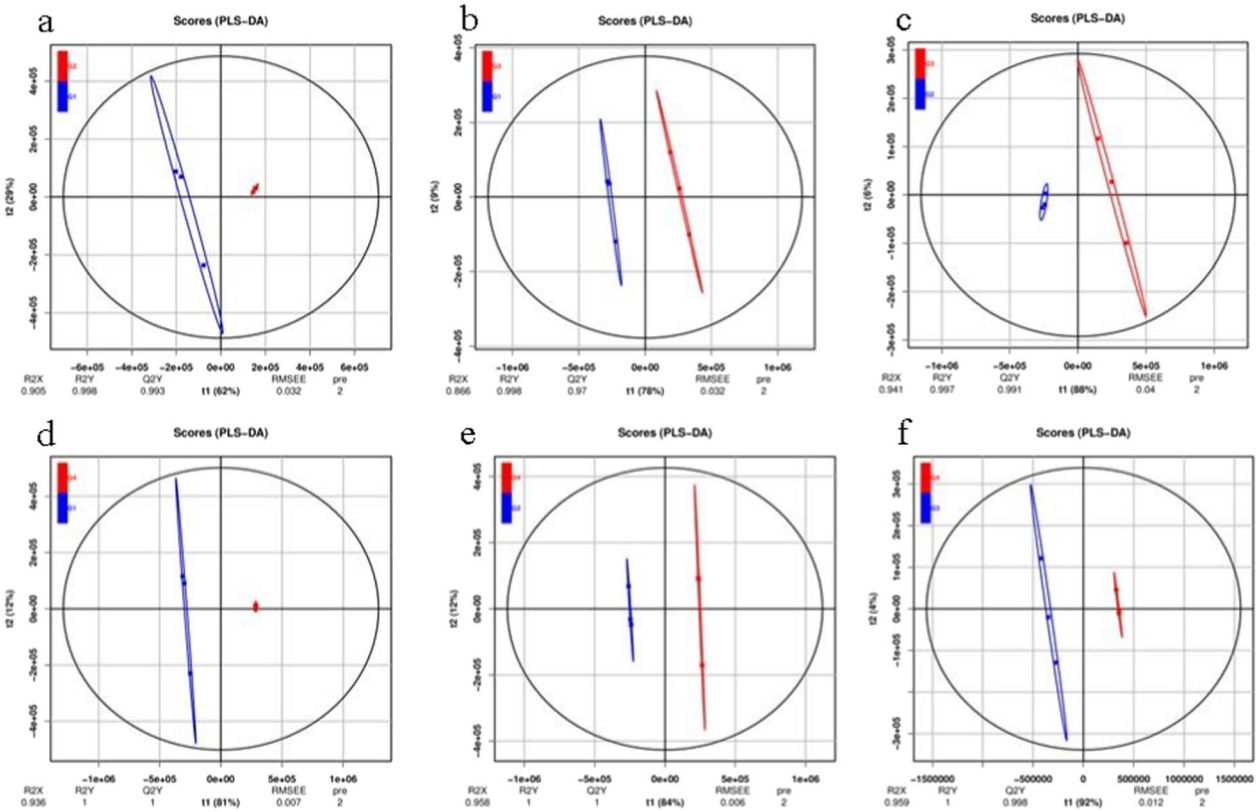

Fig. 8 Score plot of PLS-DA model for two groups (**a** G1 vs G2; **b** G1 vs G3; **c** G2 vs G3; **d** G1 vs G4; **e** G2 vs G4; **f** G3 vs G4; G1: solid-state fermentation; G2: dish culture; G3: submerged fermentation; G4: cutting wood culture)

chromatography to compare the characteristic components of *T. camphoratus* under different culture methods. However, no study has compared the metabolome of *T. camphoratus* under different culture methods, and this is the first study to perform nontargeted metabolome analysis of *T. camphoratus* cultured using cutting wood culture, dish culture, solid-state fermentation, and submerged fermentation.

In this study, the metabolites of *T. camphoratus* produced under four culture methods were statistically analyzed in detail. First, the content of total triterpenoids in *T. camphoratus* under solid-state fermentation, dish culture, and submerged fermentation was measured and compared with that in the fruiting bodies of *T. camphoratus* cultured using cutting wood culture. The metabolites produced under four methods were analyzed using LC–ESI–MS/MS. A total of 186 compounds were detected including 61 terpenoids, 22 nitrogen-containing compounds, 42 phenolic compounds, 2 glycoside compounds, 3 amino acid compounds, 1 vitamin compound, 26 fatty acid compounds, and 29 other compounds. These compounds were classified according to the PubChem database and the MeFSAT database. *T. camphoratus* produced abundant metabolites under four culture methods.

Multivariate statistical analysis can simplify and reduce the dimension of complex data while retaining the original information to the greatest extent. PCA and PLS-DA were used to explore the similarities and differences in the metabolites produced via four culture methods. Multivariate statistical analysis revealed that the differences in the metabolites of *T. camphoratus* under four culture methods were mainly in terms of some terpenes, phenolic compounds, and fatty acid compounds. The metabolites without significant differences were mainly nitrogen-containing compounds, glycosides, some terpenes, and some phenolic compounds.

The significant upregulation of fatty acid compounds under solid-state fermentation may be related to the rich active substances such as oleic acid, linolenic acid, and palmitic acid in oat medium [61]. The significant upregulation of phenolic compounds in solid-state fermentation of *T. camphoratus* may also be related to the use of oats as a medium, which is consistent with the results of Drzymaa et al. [62]. Therefore, culturing *T. camphoratus* with oats as a medium can provide more abundant fatty acids and phenolic compounds.

The significant upregulation of phenolic compounds under submerged fermentation is related to the culture

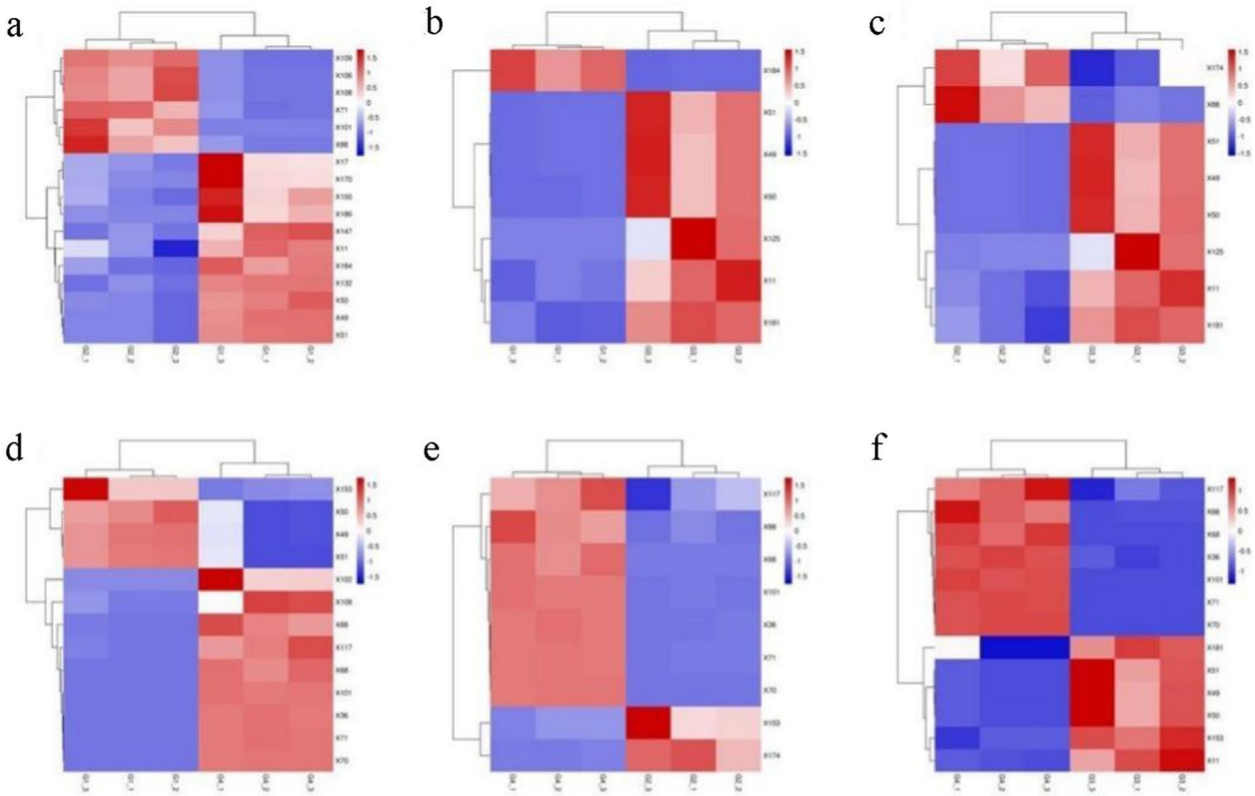

Fig. 9 Heat map analysis of differential metabolites between two groups (**a** G1 vs G2; **b** G1 vs G3; **c** G2 vs G3; **d** G1 vs G4; **e** G2 vs G4; **f** G3 vs G4; G1: solid-state fermentation; G2: dish culture; G3: submerged fermentation; G4: cutting wood culture)

cycle and liquid medium. Under submerged fermentation, the accumulation of phenolic substances is more obvious, and because the culture cycle is relatively short, the generated phenolic substances are degraded less. At present, the process of obtaining phenolic substances in edible and medicinal fungi by submerged fermentation is well developed, and several phenolic acids can be obtained by submerged fermentation [63–65]. The significant upregulation of triterpenoids under cutting wood culture in this study was consistent with the results of other studies [66, 67]. This may be because the culture conditions of *T. camphoratus* under cutting wood culture are closest to those in natural habitat, providing suitable culture conditions and substrates for the accumulation of triterpenoids.

The inhibition of α-glucosidase, α-amylase, and sucrase by *T. camphoratus* extract was studied. The α-glucosidase and α-amylase inhibitory abilities of the crude extracts were different under the four culture methods, and they were the best under cutting wood culture. This could be because of the differences in the active substances produced by *T. camphoratus* under different culture methods, and the unique triterpenoids produced by *T. camphoratus* under cutting wood culture may play an important role in this. The α-amylase inhibitory activity of the same concentration of *T. camphoratus* crude extracts under dish culture and cutting wood culture was better than that of the extracts under other two culture methods. This could be because the content of active ingredients with α-amylase inhibitory activity was higher under dish culture and cutting wood culture. PCA and PLS-DA analysis revealed that these active substances with good α-amylase inhibitory ability were mainly triterpene compounds. Therefore, the extract of *T. camphoratus* obtained under cutting wood culture exhibited superior enzyme inhibitory ability than that obtained under other culture methods, which may be related to rich triterpenoid content produced under cutting wood culture.

In summary, all four methods, namely, solid-state fermentation, submerged fermentation, dish culture, and cutting wood culture of *T. camphoratus* provided valuable metabolites. The differences in the metabolites under different culture methods lead to differences in enzyme inhibitory activity. Further studies are needed to explore whether this difference in enzyme inhibitory activity is related to the content of characteristic triterpenoids or other compounds of *T. camphoratus*.

Table 3 Pairwise comparison of four groups of samples significantly different metabolites summary

Group	Serial number	Compound	Molecular formula
G1-vs-G2	X109	Methyl 2-methoxyphenylacetate	$C_{10}H_{12}O_3$
	X106	4-Methylbenzoic acid	$C_8H_8O_2$
	X108	Gamabufotalin	$C_{24}H_{34}O_5$
	X71	Antcin K	$C_{29}H_{44}O_6$
	X101	Zhankuic acid B/Antcin I	$C_{29}H_{42}O_5$
	X88	Camphoratin C	$C_{29}H_{42}O_6$
	X17	6-Methylquinoline	$C_{10}H_9N$
	X170	Nuatigenin	$C_{27}H_{42}O_4$
	X155	Neotame	$C_{20}H_{30}N_2O_5$
	X186	Linolenic acid	$C_{18}H_{30}O_2$
	X147	Linoleoyl ethanolamide	$C_{20}H_{37}NO_2$
	X11	Syringaldehyde	$C_9H_{10}O_4$
	X184	β-Sitostenone	$C_{29}H_{48}O$
	X132	Eleostearic acid	$C_{18}H_{30}O_2$
	X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$
	X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$
	X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$
G1-vs-G3	X184	β-Sitostenone	$C_{29}H_{48}O$
	X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$
	X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$
	X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$
	X125	Cortisone	$C_{21}H_{28}O_5$
	X11	Syringaldehyde	$C_9H_{10}O_4$
	X181	(22E)-Ergosta-4,6,8(14),22-tetraen-3-one	$C_{28}H_{40}O$
G2-vs-G3	X174	Ethyl linoleate	$C_{20}H_{36}O_2$
	X88	Camphoratin C	$C_{29}H_{42}O_6$
	X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$
	X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$
	X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$
	X125	Cortisone	$C_{21}H_{28}O_5$
	X11	Syringaldehyde	$C_9H_{10}O_4$
	X181	(22E)-Ergosta-4,6,8(14),22-tetraen-3-one	$C_{28}H_{40}O$
G4-vs-G1	X153	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$
	X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$
	X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$
	X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$
	X102	Antcin F	$C_{29}H_{40}O_5$
	X108	Gamabufotalin	$C_{24}H_{34}O_5$
	X88	Camphoratin C	$C_{29}H_{42}O_6$
	X117	Dexamethasone	$C_{22}H_{29}FO_5$
	X68	Antcin H	$C_{29}H_{42}O_6$
	X101	Zhankuic acid B/Antcin I	$C_{29}H_{42}O_5$
	X36	Fraxetin	$C_{10}H_8O_5$
	X71	Antcin K	$C_{29}H_{44}O_6$
	X70	Antcin C	$C_{29}H_{42}O_5$

Table 3 (continued)

Group	Serial number	Compound	Molecular formula
G4-vs-G2	X117	Dexamethasone	$C_{22}H_{29}FO_5$
	X88	Camphoratin C	$C_{29}H_{42}O_6$
	X68	Antcin H	$C_{29}H_{42}O_6$
	X101	Zhankuic acid B/Antcin I	$C_{29}H_{42}O_5$
	X36	Fraxetin	$C_{10}H_8O_5$
	X71	Antcin K	$C_{29}H_{44}O_6$
	X70	Antcin C	$C_{29}H_{42}O_5$
	X153	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$
	X174	Ethyl linoleate	$C_{20}H_{36}O_2$
G4-vs-G3	X117	Dexamethasone	$C_{22}H_{29}FO_5$
	X88	Camphoratin C	$C_{29}H_{42}O_6$
	X68	Antcin H	$C_{29}H_{42}O_6$
	X36	Fraxetin	$C_{10}H_8O_5$
	X101	Zhankuic acid B/Antcin I	$C_{29}H_{42}O_5$
	X71	Antcin K	$C_{29}H_{44}O_6$
	X70	Antcin C	$C_{29}H_{42}O_5$
	X181	(22E)-Ergosta-4,6,8(14),22-tetraen-3-one	$C_{28}H_{40}O$
	X51	2,4,5-Trimethoxybenzaldehyde	$C_{10}H_{12}O_4$
	X49	1,4-Dimethoxybenzene	$C_8H_{10}O_2$
	X50	3-(2-Hydroxyphenyl)propanoic acid	$C_9H_{10}O_3$
	X153	1-Linoleoyl glycerol	$C_{21}H_{38}O_4$
	X11	Syringaldehyde	$C_9H_{10}O_4$

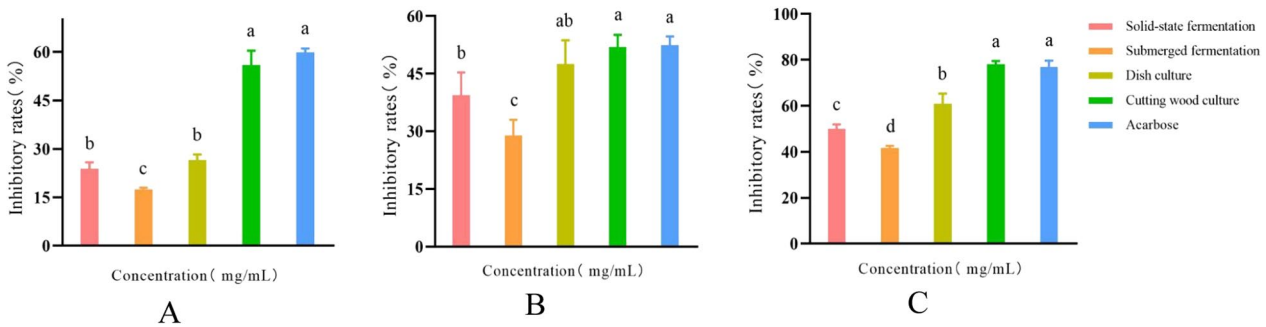

Fig. 10 **A** Inhibition rate of α-glucosidase activity by *T. camphoratus* extracts; **B** inhibition rate of α-amylase activity by *T. camphoratus* extracts; **C** inhibition rate of α-amylase activity by *T. camphoratus* extracts. (*T. camphoratus* crude extract: 40 mg/mL; acarbose: 0.001 mg/mL)

In addition, for the metabolites with no significant difference among various culture methods, it is feasible to use the other three culture methods instead of cutting wood culture to obtain these compounds more quickly and in abundance considering the limitations of cutting wood culture. Therefore, In addition to the compounds with significant differences in *T. camphoratus* cultured in cutting wood culture, further studies should verify pay more attention to the activity of these non-significant differences metabolites.

The mycelium of *T. camphoratus* is reported to contain rich active substances. However, studies are needed to further explore the active ingredients in the mycelium of *T. camphoratus*, especially small molecular active substances. As a "treasure" of China, *T. camphoratus* is rich in various active substances. Although *T. camphoratus* is being studied since several years,

further studies are needed to develop strategies to obtain quick and extensive yield of active metabolites of *T. camphoratus*. This study provided a theoretical basis for further rational use of different culture methods for *T. camphoratus*.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13765-024-00890-x.

Additional file 1: Figure S1. Total ion current spectrum for the extract of artificially cultured *T. camphoratus*. **Table S1.** No significantly different metabolites.

Author contributions

R. Z. and X. C. designed this study and led the project. Y. M. (Yongfei Ming), Y. L., J. C., X. Z. and Y. H. performed the experiments, analyzed the data, and wrote the manuscript. S. Y., Y. M. (YueJun Mu) and L.W. edited and revised the manuscript. All authors have read and approved the final version of this manuscript.

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Data availability

The authors declare that the data supporting the findings of this study are available within the paper. Should any raw data files be needed in another format they are available from the corresponding author upon reasonable request. Source data are provided with this paper.

Declarations

Competing interests

All authors declare no conflict of interest.

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