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# Stimulated Production of Triterpenoids of *Ganoderma lucidum* by an Ether Extract from the Medicinal Insect, *Catharsius molossus*, and Identification of the Key Stimulating Active Components

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**Abstract** The medicinal fungus *Ganoderma lucidum* was inoculated into the media with and without supplementation of medicinal insect extracts to screen stimulators from Chinese medicinal insects for mycelial growth and triterpenoids production in submerged fermentation. The methanol and ether extracts of the tested insects had no significant stimulatory effect on the mycelial biomass production ($P>0.05$), and those of *H. remigator* and *Mylabris phalerata* markedly inhibited the mycelial growth. However, the ether extract of *Catharsius molossus* at a concentration of 200 mg $l^{-1}$ led to a significant increase in triterpenoids concentration from 231.7±9.77 to 313.7±10.6 mg $l^{-1}$ ($P<0.01$). Analysis of fermentation kinetics of *G. lucidum* suggests that glucose concentration in the extract of *C. molossus*-added group decreased more quickly as compared to the control group from day 2 to day 7 of fermentation process, while the triterpenoids biosynthesis was promoted at the same culture period. However, the culture pH profile was not affected by the addition of the extract. Chemical study of the extract show that *cis*-9,10-methylenehexadecanoic acid (9,10-MEA) and hexadecanoic acid (especially 9,10-MEA) were the key active compounds of the extract responsible for the stimulatory effect on the triterpenoids production.

**Keywords** *Ganoderma lucidum* · Submerged fermentation · *Catharsius molossus* · Triterpenoids · Medicinal insects

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## Introduction

*Ganoderma lucidum* (Leyss. ex Fr.) Karst., a basidiomycete, lamellaless fungus, has been recognized as a traditional remedy used in Asia traditional medicine. The fruiting body of *G. lucidum* is called 'Lingzhi' in Chinese and 'Reishi' or 'Mannentake' in Japanese [1]. The polysaccharides from *G. lucidum* have been evidenced to activate immune effector cells and inhibit the growth of several cancer cells in vivo [1, 2]. More interestingly, this fungus also produces many oxygenated triterpenoids (especially ganoderic acids) with a wide range of biological activities. These include differential effects on the thromboxane A2-signaling pathways in human platelets [3], inhibition of eukaryotic DNA polymerases [4], cytotoxicity to several cancer cells in vitro [1, 5], inhibition of tumor invasion in vitro and in vivo [1, 6, 7], anti-human immunodeficiency virus-1 protease activity [8], and regulation of osteoclastogenesis [9].

*G. lucidum* usually takes several months to form its fruiting body under culture conditions. In addition, it is also difficult to control the quality of the produced fruiting body. Thus, submerged fermentation of *G. lucidum* is viewed as a promising alternative for efficient production of triterpenoids and polysaccharides [10–15]. Moreover, submerged fermentation of the fungus provides a practical method for screening the substances able to enhance the production of bioactive metabolites by *G. lucidum* [12, 16, 17].

Medicinal insects have proven to be very important sources of drugs for modern medicine [18]. Chemical screening applied to some insects has confirmed the presence of various types of bioactive substances [19]. In China, some insects are considered safe and used as traditional Chinese medicine. In our previous work, we found that the ethyl acetate extracts from *Eupolyphaga sinensis* and *Catharsius molossus* were useful to enhance the polysaccharides production of *G. lucidum* in submerged fermentation [12].

In this study, we examined the effects of the extracts from several Chinese medicinal insects on production of extracellular triterpenoids by *G. lucindum* in submerged fermentation, and traced the active compounds from the active extracts to find a solution to enhance the production of bioactive metabolites by the fungus.

## Materials and Methods

### Materials

Chinese medicinal insects, *E. sinensis* AMMF EW.02 (ES), *Tenodera aridifolia* (TA), *C. molossus* AMMF CM.01 (CM), *Aspongopus chinensis* AMMF AC.02 (AC), *Hydrotrechus remigator* AMMF HR.01 (HR), and *Mylabris phalerata* AMMF MP.02 (MP) were purchased from Anhui Medicinal Materials Factory (Hefei, China). After removal of shell, the insect samples were ground to powder (60 mesh), and then stored at 4°C.

Fatty acids, *cis*-9-octadecenoic acid, hexadecanoic acid, *cis*-octadecanoic acid, *cis*-9,12-octadecadienoic acid, *cis*-9,10-methylenehexadecanoic acid and *cis*-pentadecanoic acid were purchased from Larodan Fine Chemicals (Malmo, Sweden). Ganoderic acid A was purchased from Shanghai Winherb Medical Co. Ltd (Shanghai, China).

### Preparation of Methanol and Ether Extracts of the Medicinal Insects

For the preparation of the methanol extracts, a total of 100 g insect samples was separately extracted by circumfluence with 1 l methanol or ether for 3 h, and the extracts were obtained by removing the solvents under reduced pressure.

## Microorganism

The strain of *G. lucidum* CGMCC 5.616 was purchased from China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The stock culture was maintained on potato–dextrose–agar slants, stored at 4 °C and transferred monthly.

## Shake Flask Cultures

*G. lucidum* CGMCC 5.616 was grown in a 250-ml flask containing 80 ml medium (see below) at 28 °C for 8 days with shaking at 160 rpm. This was then inoculated at 12 % (v/v) into the medium containing different insect extracts and incubated at 28 °C for 7 days. The culture medium composed of glucose (38 g $l^{-1}$), peptone (4.5 g $l^{-1}$), $KH_2PO_4$ (0.75 g $l^{-1}$), $MgSO_4 \cdot 7H_2O$ (0.45 g $l^{-1}$) and vitamin $B_1$ (0.01 g $l^{-1}$).

## Determination of Mycelial Biomass

Samples collected at various intervals from the culture were filtered using a 40-mesh stainless sieve to harvest mycelia, the harvested mycelia were then washed with distilled water for three times followed by centrifugation at 8,000 rpm for 15 min, and desiccation at 60°C to a constant weight. [20].

## Measurements of Extracellular Triterpenoids

The extracellular triterpenoid was determined as the method described by Fang and Zhong [10] with a modification. After removal of mycelia by centrifugation, the fermented supernatants were collected, and the culture broths were mixed with ethanol in a ratio of 1:4 (v/v) followed by centrifugation. The supernatants were extracted with chloroform thrice. The extracts containing triterpenoids were prepared by removal of chloroform under reduced pressure, and dissolved in absolute ethanol, and its absorbance was measured at 245 nm by using the triterpenoid, ganoderic acid A as standard.

## Glucose Analysis

The glucose content of the medium during fermentation was monitored by HPLC (Waters Sugar-Pak 6.5×300 mm column and with an RI detector). The column was maintained at 85°C. The solvent, water, was delivered at 0.5 ml $min^{-1}$. Glucose was quantified by relating its peak area to a standard curve [17].

## Analysis and Identification of the Compounds from Ether Extract of *C. molossus* by GC-MS

The ether extract was dissolved in absolute ether, and analyzed by a Hewlett-Packard gas chromatograph (5890 Series II) equipped with a flame ionization detector. The sample was injected at 250°C (split 1:12) onto a fused-silica capillary column (Omegawax; 30 m× 0.32 mm i.d.; Supleco, Bellefonte, PA). The oven temperature was kept at 180°C during the injection (1 min), then the temperature was increased at 6°C $min^{-1}$ up to 215°C (maintained for 3 min) and then to 230°C at a rate of 3°C $min^{-1}$ and kept at 230°C for 20 min. Helium was used as the carrier gas at the constant flow rate of 0.8 ml $min^{-1}$. The mass detector was operating in the electron impact ($EI^+$) mode. Compounds from ether extract were tentatively

identified by matching mass spectral data of sample components with those of known compounds in a database (NIST database and Wiley database).

### Statistical Analysis

Incubations were performed in a triplicate and data were analyzed by using Statistics Analysis System (SAS) 8.1 version (SAS Institute Inc., Cary, NC, USA). The results were expressed as the mean±SD. The significance of the mean difference between the control and each treatment groups was determined by Student's *t*-test.

## Results

### Effect of the Insect Extracts on Biomass and Triterpenoids Production in *G. lucidum*

In order to investigate the effects of insect extracts on the production of biomass and triterpenoids, the methanol and ether extracts from the tested insects at 100 mg $l^{-1}$ were added to the medium of *G. lucidum*. The results showed that all the tested insect extracts did not stimulate the production of biomass, and those of *M. phalerala* and *H. remigator* significantly inhibited the mycelial growth ($P<0.01$). However, addition of the extracts of *C. molossus* at 100 mg $l^{-1}$ was best with increasing production of triterpenoids to 258.6± 8.61 mg $l^{-1}$ compared to 231.7±9.77 mg $l^{-1}$ in the controls (Table 1).

### Effect of Insect Extract Concentration on Biomass and Triterpenoids Production in *G. lucidum*

Table 1 shows that the insect extracts did not promote the production of biomass at 100 mg $l^{-1}$. This may be due to the improper extract concentration. Therefore, to select the proper extract concentration for the efficient biomass and triterpenoids production, the effects of different levels of the extracts of the tested insects on the production of biomass and triterpenoids were further compared (Fig. 1). The results showed that all the methanol and ether extracts from the four insects at the tested concentrations had no significant stimulatory effect on the mycelial biomass ($P>0.05$), and the cell growth was inhibited by the tested extracts with the final concentrations more than 200 mg $l^{-1}$ (Fig. 1a and b).

For the triterpenoids production, all the tested methanol extracts did not show any primitive effects on production of triterpenoids (Fig. 1c). However, ether extract from *C. molossus* at 200 mg $l^{-1}$ showed significant stimulatory effect on the production of triterpenoids ($P<0.01$). Additions of the extract increased the production of triterpenoids to 313.7±10.6 against 231.7±9.77 mg $l^{-1}$ (Fig. 1d).

### Effect of Ether Extract of *C. molossus* on Fermentaion Kinetics of *G. lucidum*

Figure 2 describes the changes in time-course profiles of glucose consumption, cell growth, triterpenoids production and culture pH in the cultures containing 200 mg $l^{-1}$ ether extract of *C. molossus*. As shown in Fig. 2a, substrate concentration in the extract-added cultures decreased more quickly as compared with the control group from day 2 to day 7, and its concentration decreased to 5.08 g $l^{-1}$ on day 7 against 6.31 g $l^{-1}$ in the controls. Mycelial growth was not affected in the presence of the extract (Fig. 2b). But production of

**Table 1** Effect of extracts of the tested insects at 100 mg $l^{-1}$ on production of mycelial biomass and triterpenoids by *G. lucidum*

| Insect extract | Mycelial biomass (g $l^{-1}$) | Triterpenoids (mg $l^{-1}$) |
|---|---|---|
| Control | 14.73±0.62 | 231.7±9.77 |
| MES[a] | 14.51±0.49 | 233.4±10.47 |
| MTA | 13.99±0.39 | 225.1±8.21 |
| MCM | 14.17±0.56 | 223.6±11.03 |
| MAC | 13.06±0.73 | 239.3±7.43 |
| MMP | 1.77±0.32** | 6.2±0.51** |
| MHR | 6.92±0.57** | 96.1±3.73** |
| EES | 15.64±0.63 | 239.2±9.11 |
| ETA | 14.71±0.49 | 236.9±10.32 |
| ECM | 14.63±0.75 | 258.6±8.61* |
| EAC | 14.01±0.19 | 231.6±7.69 |
| EMP | 4.62±0.31* | 101.5±5.39** |
| EHR | 2.33±0.67** | 11.7±0.57** |

*G. lucidum* was cultivated at 28 °C for 7 days on a rotary shaker at 160 rpm

*Values are significantly different from that of the control group by Student's *t*-test at $P<0.05$

**Values are significantly different from that of the control group by Student's *t*-test at $P<0.01$

[a] MES, MTA, MCM, MAC, MMP and MHR were methanol extracts of medicinal insects ES, TA, CM, AC, MP, and HR, respectively; EES, ETA, ECM, EAC, EMP and EHR were ether extracts of ES, TA, CM, AC, MP, and HR, respectively

triterpenoids was evidently promoted on day 7 with the maximal level reaching 310.1 mg $l^{-1}$ compared to 226.2 mg $l^{-1}$ in the controls (Fig. 2c).

Figure 2d shows that both the initial pHs in the extract added cultures and the controls decreased to 3.9 during the first 3 days of cultivation. After that, they remained relatively constant for about 3 days, and then increased to about 4.3 on day 8. The culture pH profile in the extract added cultures was almost the same as that of the controls, suggesting the addition of the extract did not change pH profiles compared to those in the controls.

## Chemical Components in the Ether Extract of *C. molossus*

The constituents of the ether extract from *C. molossus* were analyzed by GC-MS. The summarized GC-MS data and the percentage of each individual compound with respect to the all contents were shown in Table 2.

As demonstrated in Table 2, 14 compounds whose match index was more than 800 (999= 100%) were identified; the main constituents among these were *cis*-9-octadecenoic acid (39.43%), hexadecanoic acid (21.06 %), *cis*-octadecanoic acid (14.09%), *cis*-9,12-octadecadienoic acid (8.07%), *cis*-9,10-methylenehexadecanoic acid (2-hexyl-cyclopropaneoctanoic acid, 2.56%), and *cis*-pentadecanoic acid (2.31%).

## Active Compounds for Stimulating Production of Triterpenoids in Ether Extract of *C. molossus*

Based on the identification of compounds of the ether extract, the effects of the main compounds on production of triterpenoids and biomass by *G. lucidum* were studied. It

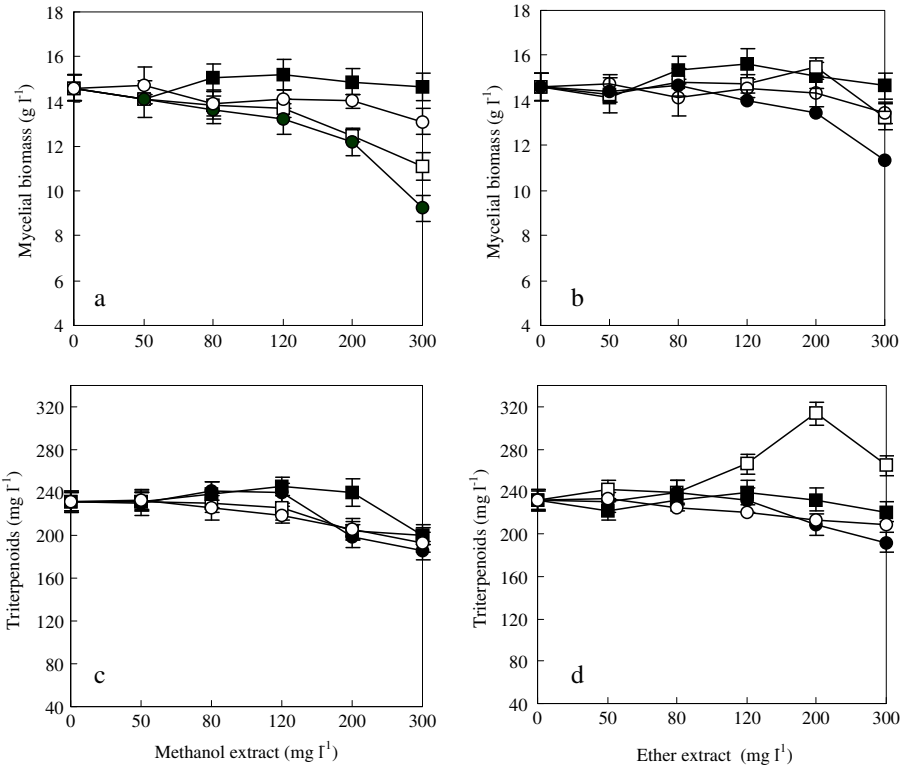


**Fig. 1** Effects of methanol and ether extracts of the medicinal insects on production of mycelial biomass (**a**, **b**) and triterpenoids (**c**, **d**) production by *G. lucidum*. *Filled square*, *E. Sinensis*; *open square*, *C. molossus*; *filled circle*, *A. chinensis*; *open circle*, *T. aridifolia*. *G. lucidum* was cultivated at 28 °C for 7 days on a rotary shaker at 160 rpm

revealed that hexadecanoic acid, particularly *cis*-9,10-methylenethxadecanoic acid had significant positive effects on the triterpenoids production of *G. lucidum*. As compared with the control, the triterpenoids concentration significantly increased in the cultures supplemented by 9,10-MEA with the concentrations ranging from 10 to 160 mg $l^{-1}$, and hexadecanoic acid ranging from 30 to 160 mg $l^{-1}$. However, other tested compounds had no stimulatory effects on production of triterpenoids, and 9,12-octadecadienoic acid inhibited production of triterpenoids at concentrations raging from 80 to 160 mg $l^{-1}$. In addition, the production of mycelial biomass was not affected in the presence of 9,10-MEA and hexadecanoic acid, but was enhanced in the cultures containing *cis*-9-octadecenoic acid with concentrations ranging from 30 to 160 mg $l^{-1}$ (Table 3). These suggest that 9,10-MEA and hexadecanoic acid were the key active compounds able to increase the production of triterpenoids in submerged cultures of *G. lucidum*.

## Discussion

In recent years, submerged cultivation of *G. lucidum* has received great attention and perceived as a promising alternative for efficient production of mycelial biomass,

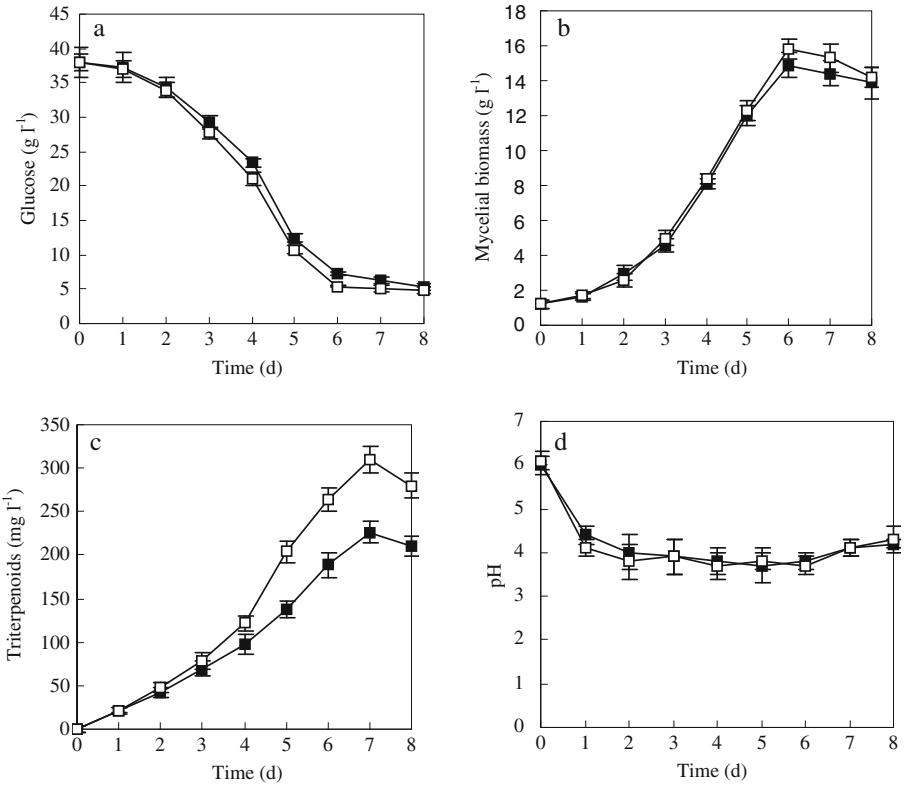


**Fig. 2** Time courses of glucose consumption (**a**), cell growth (**b**), triterpenoids production (**c**), and the change in culture pH (**d**) during the cultivation of *G. lucidum* in the medium containing 200 mg $l^{-1}$ of ether extract of *C. molossus* (*open square*) and the control (*filled square*). *G. lucidum* was cultivated at 28 °C for 8 days on a rotary shaker at 160 rpm

triterpenoids, and polysaccharides [10, 14, 15]. To accelerate mycelial growth and production of metabolites by *G. lucidum*, the effects of environmental conditions [10, 11], two-stage culture process [21], fed-batch fermentation [22], pH-shift and DOT-shift integrated fed-batch fermentation [13], etc. have been studied.

Chinese medicinal insects or herbs are sources of modern drugs. Chemical screening applied to some herbs and insects has confirmed the presence of various types of bioactive substances [19, 23]. Studies have reported that many types of extracts from herbs displayed various pharmacological effects [24–26]. Our previous work showed that ethyl acetate extract from the medicinal insect, *E. sinensis* at 55 mg $l^{-1}$ lead to significant increase in the production of biomass and intracellular polysaccharides [12].

In the present study, extracts of the tested Chinese medicinal insects were added into the media to investigate their effects on the mycelial growth and production of triterpenoids by *G. lucidum* in submerged fermentation. The results show that the addition of the ether extract of *C. molossus* at 200 mg $l^{-1}$ significantly enhanced the production of triterpenoids. This is the first report that the ether extract of Chinese medicinal insect stimulated the triterpenoids production of *G. lucidum* in submerged fermentation.

Studies have shown that culture pH had significant effect on the production of triterpenoids (ganoderic acids) of *G. lucidum* [10]. However, in this study, the culture pH

**Table 2** Compounds from the ether extract from *C. molossus*

| Retention time | Area % | Compound name[a] |
|---|---|---|
| 3.18 | 0.36 | Benzenepropanoic acid |
| 3.47 | 0.76 | Tetradecanoic acid |
| 3.95 | 1.67 | 12-Methyl-tetradecanoic acid |
| 4.17 | 2.31 | *cis*- Pentadecanoic acid |
| 5.07 | 21.06 | Hexadecanoic acid |
| 5.40 | 0.39 | |
| 5.50 | 1.90 | 9-Hexodecanoic acid |
| 5.58 | 0.69 | *cis*-Heptadecanoic acid |
| 5.80 | 0.76 | 14-methyl-hexadecanoic acid |
| 5.97 | 0.29 | Heptodecanoic acid |
| 6.13 | 2.56 | *cis*-9,10-Methylenehexadecanoic acid |
| 6.58 | 0.47 | |
| 7.40 | 14.09 | *cis*-Octadecanoic acid |
| 7.74 | 4.81 | *cis*-9-Octadecenoic acid |
| 7.87 | 34.62 | *cis*-9-Octadecenoic acid |
| 7.96 | 0.66 | |
| 8.04 | 0.53 | |
| 8.32 | 0.32 | |
| 8.71 | 8.07 | *cis*-9,12-Octadecadienoic acid |
| 8.78 | 0.51 | |
| 9.28 | 0.36 | |
| 10.27 | 1.02 | *cis*-6,9-Octadecadienoic acid |
| 10.33 | 0.59 | *cis*-Eicosanoic acid |
| 10.97 | 0.43 | |
| 13.66 | 0.22 | |

The constituents of the ether extract from *C. molossus* were analyzed by GC-MS. The sample was injected at 250°C (split 1:12) onto a fused-silica capillary column (Omegawax; 30 m×0.32 mm i.d.; Supleco, Bellefonte, PA). The oven temperature was maintained at 180°C during the injection (1 min) then the temperature was increased at 6°C $min^{-1}$ up to 215°C (maintained for 3 min) and then to 230°C at a rate of 3° C $min^{-1}$ and maintained at 230°C for 20 min. Compounds from the ether extract were tentatively identified by matching mass spectral data of sample components with those of known compounds in a database (NIST database and Wiley database)

[a] Data were not given in the table for the compounds whose match index was less than 800

profile was not changed in treatment group, indicating the stimulatory effect on the triterpenoids production by the medicinal extract was not contributed by the changes of pH value.

Studies have also shown that oils, surfactants, fatty acids and ethanol promoted the production of fungal metabolites like protease [27], exocellular enzymes and polysaccharides [16, 17]. The possible mechanism for this promotion is the interaction with the cell membrane, leading to its changes in structure and its permeability. Thus, the promotion in production of triterpenoids might also be contributed by these fatty acid derivatives in the way similar to those seen in the production of metabolites by other fungi. In nature, fungi including *G. lucidum* are exposed to a wide range of environmental factors including UV irradiation, attack from pathogenic microorganisms and insects [28, 29]. Among these,

**Table 3** Effects of the main components of the ether extract from *C. molossus* on mycylial biomass and triterpenoids production of *G. lucidum*

| Concentration (mg $l^{-1}$) | Mycylial biomass (g $l^{-1}$) and triterpenoids concentration (mg $l^{-1}$) in each treatment group | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | *cis*-9-Octadecenoic acid | | Hexadecanoic acid | | *cis*-Octadecanoic acid | | *cis*-9,12-Octadecadienoic acid | | *cis*-9,10-Methylenehexadecanoic acid | | *cis*-Pentadecanoic acid | |
| | Biomass | Triterpenoids | Biomass | Triterpenoids | Biomass | Triterpenoids | Biomass | Triterpenoids | Biomass | Triterpenoids | Biomass | Triterpenoids |
| 0 | 15.23±0.51 | 226.7±11.2 | 15.23±0.51 | 226.7±11.2 | 15.23±0.51 | 226.7±11.2 | 15.23±0.51 | 226.7±11.2 | 15.23±0.51 | 226.7±11.2 | 15.23±0.51 | 226.7±11.2 |
| 10 | 15.27±0.33 | 221.8±9.7 | 15.41±0.57 | 221.2±10.8 | 15.32±0.47 | 209.3±10.1 | 15.06±0.64 | 219.5±9.6 | 15.22±0.56 | 292.7±11.8* | 15.17±0.43 | 209.6±10.1 |
| 30 | 16.33±0.27 | 214.2±7.8 | 15.29±0.53 | 281.9±9.7* | 15.27±0.57 | 230.5±8.9 | 13.64±0.42 | 221.3±10.4 | 15.61±0.43 | 317.9±9.7* | 15.21±0.57 | 230.9±10.7 |
| 80 | 17.67±0.48* | 231.5±11.6 | 16.01±0.52 | 287.7±10.8* | 15.21±0.42 | 221.2±10.6 | 12.99±0.27* | 183.6±7.9* | 15.93±0.68 | 326.6±11.7** | 15.46±0.36 | 221.7±11.2 |
| 160 | 17.04±0.32 | 239.2±1.2 | 15.54±0.29 | 261.3±12.6* | 15.02±0.63 | 210.7±7.9 | 10.34±0.34* | 150.8±9.3* | 15.32±0.43 | 291.3±10.4* | 15.29±0.72 | 226.3±10.4 |

* Values are significantly different from that of the control group by Student's *t*-test at $P<0.05$

** Values are significantly different from that of the control group by Student's *t*-test at $P<0.01$. *G. lucidum* was cultivated at 28 °C for 7 days on a rotary shaker at 160 rpm

attack to basidiomycetes fungi (mushroom) from insects is widespread in natural habitats, invariably leading to the interactions between the insects and mushrooms [29]. The insects feed the mushrooms in one hand, and in the other hand the mushrooms defend insect feeding by producing antifeedant metabolites like triterpenoids. In this context, the fatty acid derivatives might act as signaling molecules to trigger the enhanced biosynthesis of antifeedant metabolites. Studies have shown that methyl jasmonate (MeJA) and phenobarbital enhanced biosynthetic gene expressions and production of ganoderic acids of *G. lucidum* [30–32]. In addition, hormone-like substances like jasmonic acid, oxylipins are the signaling molecules responsible for growth and development of *Inonotus obliquus* [33]. In our study, 9,10-MEA and hexadecanoic acid are structurally similar to oxylipins, and thus might act as the signaling molecules to mediate interactions between the insects and *G. lucidum*.

In summary, this study has examined the promotion effects on production of mycelial biomass and triterpenoids by *G. lucidum*. Further experiments are needed to elucidate the receptors of these signaling molecules in the fungus and the mechanisms of interaction. However, this study uncovered for the first time that the active compounds from *C. molosus* can increase the production of triterpenoids, and gained insights into a possible solution for industrial production of triterpenoids by *G. lucidum*.

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