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Full Length Research Paper

Promotion of ganoderic acid production in *Ganoderma sinense* by the addtion of an ether extract from *Eupolyphaga sinensis*, a medicinal insect

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To screen stimulators from Chinese medicinal insects for mycelial growth and ganoderic acid (GA) production by *Ganoderma sinense*, the fungus was inoculated into the media with and without supplementation of a medicinal insect extract. The results show that all the water and ether extracts from the medicinal insects had no significant stimulatory effect on the biomass production ($P > 0.05$), and the extracts of *Hydrotrechus remigator* and *Mylabris phalerata* significantly inhibited the mycelial growth. However, the ether extract of *Eupolyphaga sinensis* at a concentration of 60 mgL^{-1} led to a significant increase in GA concentration from 187.6 ± 8.32 to 251.3 ± 11.27 mgL^{-1} ($P < 0.01$). Analysis of fermentation kinetics of *G. sinense* suggests that glucose concentration in the *E. sinensis* extract treatment group decreased more quickly as compared to the control group in the last 4 days of fermentation process, while the GA biosynthesis was promoted at the same period. However, the culture pH profile was not affected by the addition of the ether extract of *E. sinensis*.

Key words: Medicinal fungus, *Ganoderma sinense*, submerged fermentation, *Eupolyphaga sinensis*, ganoderic acid.

INTRODUCTION

Basidiomycets of various *Ganoderma* spp. have been found in the last decades as one of the most attractive groups of natural products in Asia and North America. *Ganoderma* has been used in traditional Asian medicines for the prevention and treatment of various types of diseases, such as cancer, hepatopathy, arthritis, hypertension, neurasthenia and chronic hepatitis (Liu and Zhang, 2005; Shiao, 2003; Weng and Yen, 2010). Among the genus, two species, *Ganoderma sinense* and *Ganoderma lucidum* are the key species for the production of medicinal materials. Chemistry studies on *G. sinense* and *G. lucidum* have shown that pharmaceutically active compounds from fruiting body and mycelia of *Ganoderma* include polysaccharides, triterpenoids (especially ganoderic acid, GA), steroids, alkaloids, nucleotides, lactones and fatty acids, among which, polysaccharides and GA are the major source for biological activity and therapeutic use of *G. sinense* and *G. lucidum* (Liu et al., 2010; Qiao et al., 2007; Sato et al., 2009a; Shiao, 2003). Recent studies show that the triterpenoids or GAs from *G. sinense* and *G. lucidum* have various biological functions such as cytotoxicity to several cancer cells *in vitro*, or inhibition of tumor invasion *in vitro* and *in vivo*, inhibition of human immunodeficiency virus (HIV)-1 protease, inhibition of eukaryotic DNA polymerases, inhibition of cholesterol synthesis and absorption, regulation of osteoclastogenesis and inhibition of U46619-

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Abbreviations: GA, Ganoderic acid; **HIV,** human immunodeficiency virus; **SCIM,** strain collection of industrial microorganisms.

induced platelet aggregation (Chen et al., 2010; Xu et al., 2010; Sato et al., 2009b; Chen et al., 1999; Liu and Zhang, 2005; Kimura et al., 2002; Min et al., 2000; Miyamoto et al., 2009; Mizushina et al., 1999).

Since it usually takes several months to complete a fruiting body culture of *G. sinense* or *G. lucidum* and it is also difficult to control the product quality, in recent years, a submerged cultivation of *G. sinense* and *G. lucidum* has received great interest as a promising alternative for efficient production of mycelial biomass, GA, and polysaccharides (Fang and Zhong, 2002a; Hsieh et al., 2005; Liu and Zhang, 2007; Tang et al., 2009; Xu et al., 2010; Zhang and Zhong, 2010). It has been a topic of concern to screen substances that stimulate mycelial growth and metabolites production of *G. lucidum* (Liu and Zhang, 2007; Yang et al., 2000, 2004). However, data on efficient submerged cultivation of *G. sinense* are scarce.

Insects have proven to be very important sources of drugs for modern medicine (Ahn et al., 2002). Chemical screening applied to some insects has confirmed the presence of various types of bioactive substances (Costa-Neto, 2002). In China, some insects are considered safe and used as traditional Chinese medicines, including *Eupolyphaga sinensis*, *Catharsius molossus*, *Aspongopus chinensis*, etc. In our previous work, we found that the ethyl acetate extracts of *E. sinensis* and *C. molossus* were useful in enhancing the polysaccharides production of *G. lucidum* (Liu and Zhang, 2007). The aim of this research is to examine whether Chinese medicinal insect extracts stimulate mycelial growth and GA production in *G. sinense* in submerged fermentation. A strain, *G. sinense* SCIM 0701 was used and inoculated into the media with and without supplementation of a medicinal insect extract, and the effects of extracts from some typical medicinal insects on the cell growth and GA production of the fungus were studied.

MATERIALS AND METHODS

Insect materials and extracts preparation

Chinese medicinal insects, *C. molossus* (CM), *E. sinensis* Walker (ES), *A. chinensis* Dallas (AC), *Hydrotrechus remigator* (HR) and *Mylabris phalerata* Pallas (MP) were purchased from Anhui Medicinal Materials Factory (Hefei, P.R. China). After removal of shell, the insect samples were ground to powder (60 mesh), and then stored at 4°C.

For the preparation of the water extracts, 100 g insect samples was separately extracted by circumfluence with 1 L deionized water for 3 h, the obtained extracts were filtered, and then water was removed under reduced pressure to obtain dry extracts. The ether extracts were prepared in the same manner as the water extracts.

Microorganism and culture conditions

G. sinense SCIM 0701 was screened and collected by strain collection of industrial microorganisms (SCIM), Central South University of Forestry and Technology (Changsha, China). It was grown in a 250-ml flask containing 80 ml medium at 30°C for 6 days with shaking at 160 rpm. This was then inoculated at 10% (v/v) into the same medium, but containing various insect extracts. The culture time was 6 days. The culture medium contained (gL^{-1}): glucose, 40; peptone, 3.0; yeast extract 1; KH_2PO_4, 0.8; $MgSO_4 \cdot 7H_2O$, 0.6; vitamin B_1, 0.01.

Determination of biomass

Samples collected at various intervals from flasks were filtered using a 40-mesh stainless sieve and the mycelium was harvested. Biomass was collected by centrifuging the mycelium at 8000 rpm for 15 min, washing the precipitated cells for three times with distilled water and drying at 60°C until it reached a constant weight (Liu and Wang, 2007).

Measurements of GA

The determination of GA was made by a similar method described by Fang and Zhong (2002b). The dried mycelia (2 g) were extracted by circumfluence with 50% (v/v) ethanol (100 ml) for 2 h (twice). After removal of mycelia by centrifugation, the supernatant was dried at 50°C under vacuum. The residues were suspended in water, and later extracted with chloroform. The GA in chloroform was extracted by 5% (w/v) $NaHCO_3$. After adding 2 M HCl to adjust the pH of the $NaHCO_3$ layer to be lower than 3, the GA in the $NaHCO_3$ layer was extracted with chloroform. After removal of chloroform by evaporation at 40°C, GA was dissolved in absolute ethanol, and its absorbance was measured at 245 nm.

Glucose analysis

The glucose content of the medium during fermentation was monitored by high performance liquid chromatography (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. Glucose was quantified by relating its peak area to a standard curve.

Statistical analysis

Incubations were performed in triplicate and data were analyzed by using SAS 8.1 version. The results were expressed as the mean ± SD. The significance of the mean difference between the control group and each treatment group was determined by the student's t test.

RESULTS

Effect of various insect extracts on the production of biomass and GA of *G. sinense*

The water and ether extracts from various insects at 50 mgL^{-1} were added to the medium of *G. sinense* to investigate their effects on the production of biomass and GA. The results in Table 1 reveal that not all the insect extracts stimulated the production of biomass, and the extracts of *H. remigator* and *M. phalerala* significantly inhibited the mycelial growth. However, ether extract of *E. sinensis* at 50 mgL^{-1} showed stimulatory effects on the production of GA ($P < 0.05$), and as compared to the con-

Table 1. Effect of extracts of various insects at 50 mgL^{-1} on the mycelial growth and GA production.

Insect extract	Mycelium (g·L^{-1})	GA (mg·L^{-1})
Control	9.21 ± 0.41	187.6 ± 8.32
WCM[a]	9.19 ± 0.72	179.9 ± 6.23
WES	9.38 ± 0.02	191.5 ± 11.41
WAC	8.10 ± 0.62	196.3 ± 9.65
WHR	6.13 ± 0.46**	91.7 ± 4.22**
WMH	1.23 ± 0.41**	4.7 ± 0.52**
ECM	9.44 ± 0.74	192.3 ± 7.48
EES	9.11 ± 0.47	226.7 ± 12.76*
EAC	8.78± 0.27	194.9 ± 7.22
EHR	6.83 ± 0.23*	123.6 ± 8.61**
EMH	2.19 ± 0.47**	6.8 ± 0.71**

[a]WCM, WES, WAC, WHR and WMP are water extracts of medicinal insects CM, ES, AC, DH and MP, respectively; ECM, EES, EAC, EHR and EMP are ether extracts of CM, ES, AC, DH and MP, respectively. Cells were cultivated at 30°C for 6 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 $P < 0.01$.

trol, GA level increased from 187.6 ± 8.32 to 226.7 ± 12.76 mgL^{-1}.

Effect of insect extract concentration on the production of biomass and GA

Table 1 shows that the insect extracts did not promote the production of biomass at 50 mgL^{-1}. This may be due to the improper extract concentration, for a new material (insect extract), lower concentrations may have no effect on the production of biomass or GA, and higher concentrations may have inhibitory effect on biomass or GA formation. Therefore, to select the proper extract concentration for efficient biomass and GA production, the effects of different levels of the extracts of three types of insects on the production of biomass and GA were further compared (Figures 1 and 2). The results show that all the water and ether extracts from the three insects at the tested concentrations had no significant stimulatory effect on the biomass production ($P > 0.05$), and the cell growth was inhibited as all the extract concentrations increased in the range of 100 - 200 mgL^{-1}.

For the GA production, all water extracts from the three insects had no positive effects. However, ether extract from *E. sinensis* at 60 mgL^{-1} showed significant stimulatory effect on the production of GA ($P < 0.05$). The addition of the extract led to a maximal increase in GA concentration from 187.6 ± 8.32 to 251.3 ± 11.27 mgL^{-1}. These results clearly show that the optimization of insect extract concentration enhances GA production in *G. sinense*.

Effect of ether extract of *E. sinensis* on fermentation kinetics of *G. sinense*

Mycelial growth, GA production, glucose consumption and change of culture pH in samples containing 60 mgL^{-1} ether extract of *E. sinensis* in the medium and the control culture were monitored for 8 days (Figure 3). As shown in Figure 3A, glucose concentration in the treatment group decreased more quickly as compared with the control group in the last 4 days, and its concentration decreased to 5.62 gL^{-1} in treatment group and 8.33 mL^{-1} in the control group on the 6th day. Mycelial growth was affected alittle, but GA production was stimulated by 60 mgL^{-1} ether extract of *E. sinensis* in the last 4 days (Figures 3B and C). Figure 3D shows the course profile of culture pH, and the culture pH profile in the treatment group was almost the same as that of the control group, indicating that the culture pH was not affected by the addition of ether extract of *E. sinensis*.

DISCUSSION

As *Ganoderma* is very rare in nature, the amount of wild mushroom is not sufficient for commercial exploitation. Its cultivation on solid substrates, stationary liquid medium, or in a submerged cultivation has become essential to meet the increasing demands in the international markets (Xu et al., 2010; Hsieh et al., 2005; Tang et al., 2009).

Currently, most of the researches has been related to *G. lucidum*, and to accelerate mycelial growth and metabolite production in *G. lucidum*, the effects of environmental conditions, two-stage culture process, etc. have been studied (Fang and Zhong, 2002a; Zhang and Zhong, 2010; Tang et al., 2009). In addition, some inducers to increase the mycelial growth and polysaccharide production have been reported (Yang et al., 2000, 2004). However, data on efficient submerged cultivation of *G. sinense* are scarce.

In this work, the water and ether extracts of various Chinese medicinal insects were added into the media to investigate their effects on the mycelial growth and production of GA by *G. sinense* in submerged fermentation. The results show that the addition of the ether extract of *E. sinensis* at 60 mg/l significantly enhanced the production of GA, though the biomass yield was not increased.

Glucose concentration in the extract treatment group decreased more quickly when compared to the control group in the late 4 days, while GA biosynthesis was promoted at the same period, suggesting that the utilization efficiency of glucose by *G. sinense* was improved after addition of the ether extract of *E. sinensis*, thus the GA biosynthesis metabolism was enhanced.

Studies have shown the significance of culture pH on the production of GA (Fang and Zhong 2002a). We investigated the course profile of culture pH after the addition

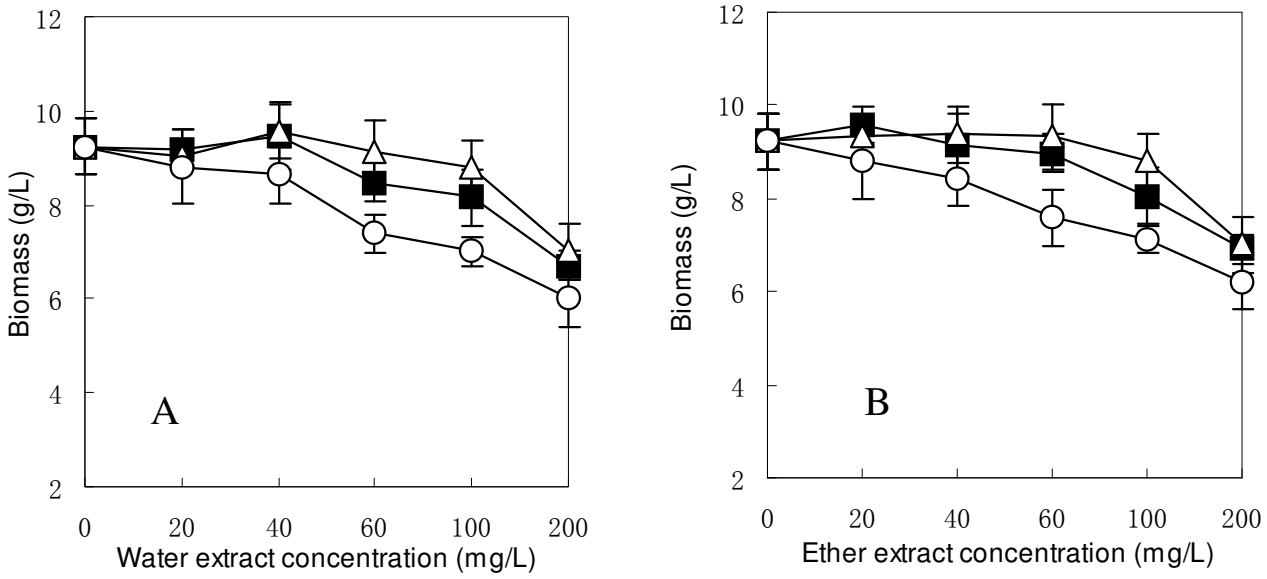

Figure 1. Effects of water extract (A) and ether extract (B) of medicinal insects on biomass production. △, *E. sinensis*; ■, *C. molossus*; ○, *A. chinensis*. Cells were cultivated at 30℃ for 6 days on a rotary shaker at 150 rpm.

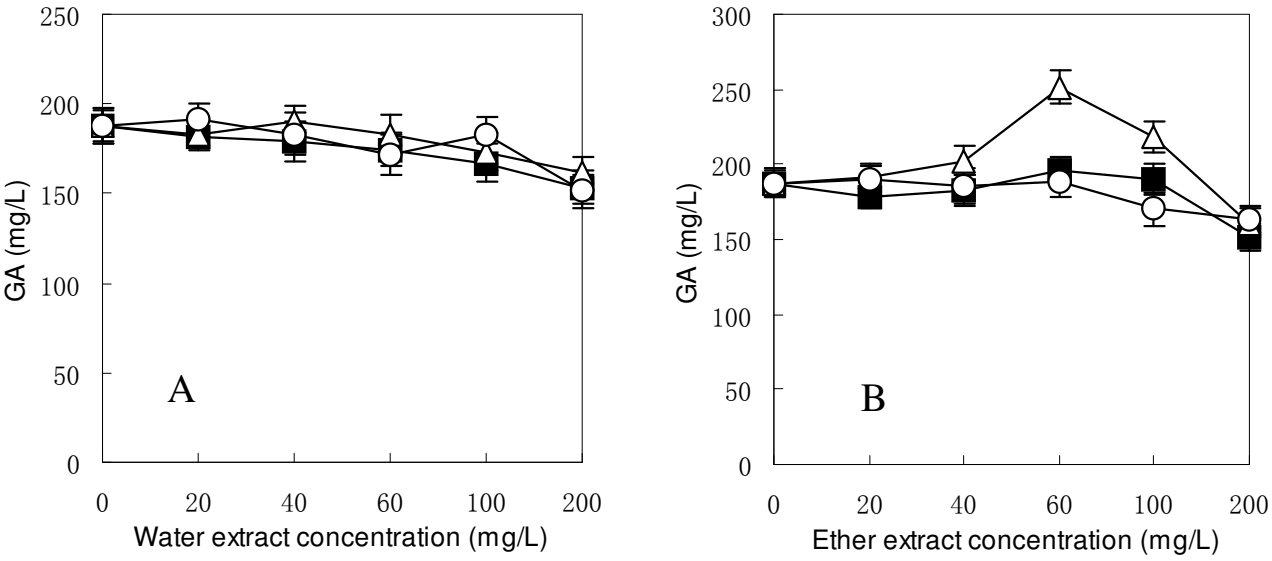

Figure 2. Effects of water extract (A) and ether extract (B) of medicinal insects on GA production. △, *E. sinensis*; ■, *C. molossus*; ○, *A. chinensis*. Cells were cultivated at 30℃ for 6 days on a rotary shaker at 150 rpm.

of the ether extract of *E. sinensis*. However, as shown in Figure 3D, the culture pH profile in sample containing 60 mg/l ether extract of *E. sinensis* in the medium was almost the same as that of the control, indicating that the stimulatory effect by *E. sinensis* was not linked with the changes of culture pH.

Previous studies (Fukushima et al., 1991; Yang et al., 2000, 2004) have shown that oils, surfactants, fatty acids and ethanol promoted the production of fungal metabolites like protease, extracellular enzymes and polysaccharides. The function was mainly explained in terms of membrane structure and its permeability. The mechanism of stimulatory effect by the ether extract of *E. sinensis* might be proposed as the extract worked by modifying membrane composition and increase permeability, or by directly affecting the level of synthesis of the enzymes involved in GA production. However, further studies are required to elucidate the real mechanism.

In conclusion, the ether extract of *E. sinensis* is advantageous to enhance the GA production of *G. sinense* in

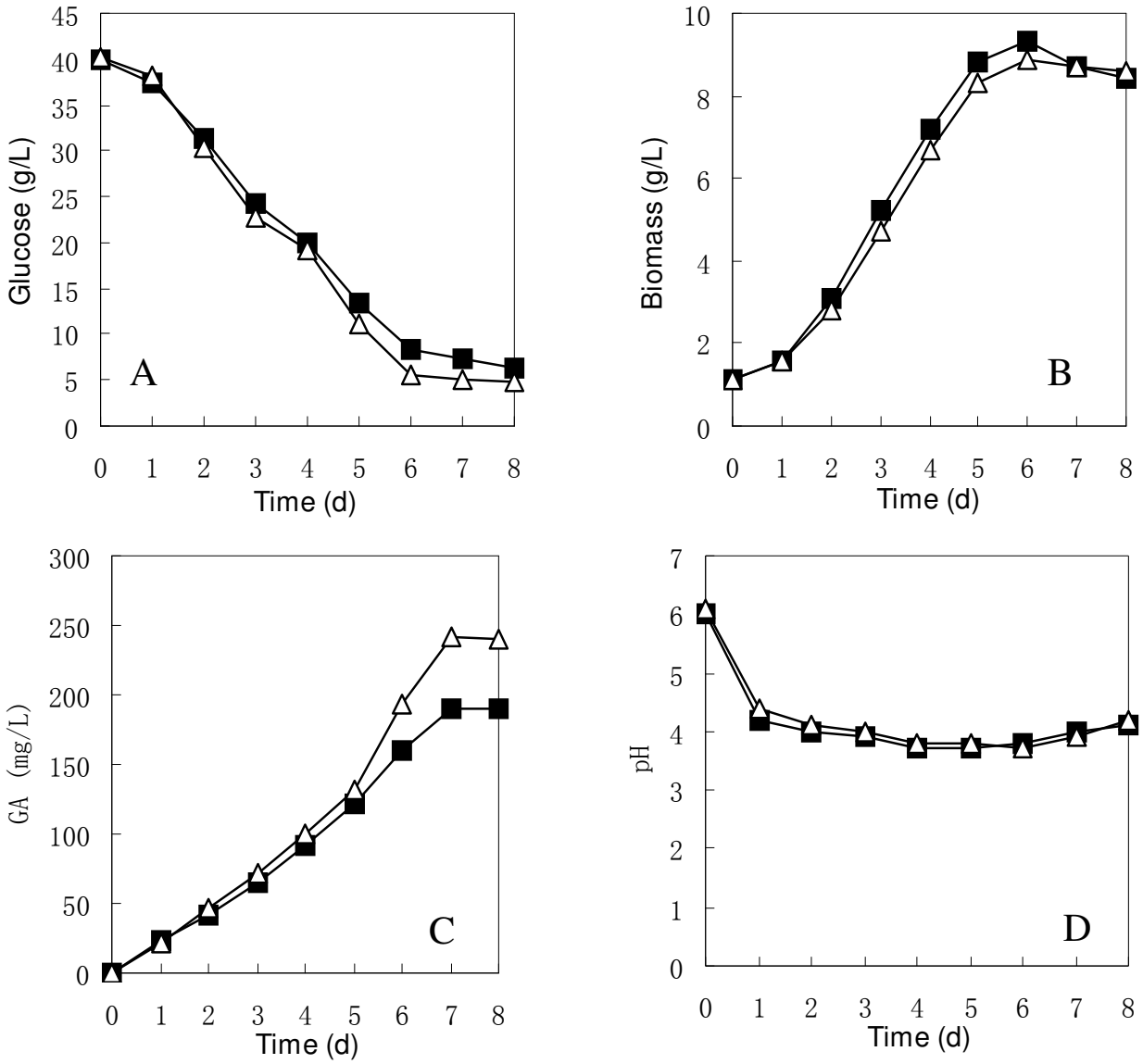

Figure 3. Time-courses of glucose consumption (A), cell growth (B), GA production (C) and the change in culture pH (D) during the cultivation of *G. sinense* in samples containing 60 mgL^{-1} of ether extract of *E. sinensis* in medium (△) and the control (■). Cells were cultivated at 30°C for 8 days on a rotary shaker at 150 rpm.

submerged fermentation. Considering the insect, *E. sinensis* is cheap because it can be propagated on a large scale in factory, it will be attractive to use the ether extract of the insect as a stimulator for GA production by submerged fermentation of *G. sinense*. Further studies are required to separate the detailed constituents of the ether extract from *E. sinensis* that are responsible for the stimulatory effect on GA production and to elucidate the detailed mechanisms of stimulation.

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REFERENCES

Ahn MY, Hahn BS, Ryu KS, Cho SI (2002). Effects of insect crude drugs on blood coagulation and fibrinolysis system. Nat. Prod. Sci. 8: 66-70.

Chen NH, Liu JW, Zhong JJ (2010). Ganoderic acid T inhibits tumor invasion *in vitro* and *in vivo* through inhibition of MMP expression. Pharmacol. Rep. 62: 150-163.

Chen YS, Ming SS, Cheng TW (1999). Differential effects of ganodermic acid S on the thromboxane A2-signaling pathways in human platelets. Biochem. Pharmacol. 58: 587-595.

Costa-Neto EM (2002). The use of insects in folk medicine in the state

of Bahia, northeastern Brazil, with notes on insects reported elsewhere in Brazilian folk medicine. Hum. Ecol. 30: 245-263.
Fang QH, Zhong JJ (2002a). Effect of initial pH on production of ganoderic acid and polysaccharide by submerged fermentation of *Ganoderma lucidum*. Process Biochem. 37: 769-774.
Fang QH, Zhong JJ (2002b). Two-stage culture process for improved production of ganoderic acid by liquid fermentation of higher fungus *Ganoderman lucidum*. Biotechnol. Progr. 18: 51-54.
Fukushima Y, Itoh H, Fukase T, Motai H (1991). Stimulation of protease production by *Aspergillus oryzae* with oils in continuous culture. Appl. Microbiol. Biotechnol. 34: 586-90.
Hsieh CY, Hsu TH, Yang FC (2005). Production of polysaccharides of *Ganoderma lucidum* (CCRC36021) by reusing thin stillage. Process Biochem. 40: 909-916.
Kimura Y, Taniguchi M, Baba K (2002). Antitumor and antimetastatic effects on liver of triterpenoid fractions of *Ganoderma lucidum*: Mechanism of action and isolation of an active substance. Anticancer Res. 22: 3309-3318.
Liu C, Zhao F, Chen RY (2010). A novel alkaloid from the fruiting bodies of *Ganoderma sinense* Zhao, Xu et Zhang, Chin. Chem. Lett. 21: 197-199.
Liu GQ, Wang XL (2007). Optimization of critical medium components using response surface methodology for biomass and extracellular polysaccharide production by *Agaricus blazei*. Appl. Microbiol. Biotechnol. 74: 78-83.
Liu GQ, Zhang KC (2005). Mechanisms of the anticancer action of *Ganoderma lucidum* (Leyss. ex. Fr.) Karst: A new understanding. J. Integr. Plant Biol. 47: 129-135.
Liu GQ, Zhang KC (2007). Enhancement of polysaccharides production in *Ganoderma lucidum* by the addition of ethyl acetate extracts from *Eupolyphaga sinensis* and *Catharsius molossus*. Appl. Microbiol. Biotechnol. 74: 572-577.
Min BS, Gao JJ, Nakamura N, Hattori M (2000). Triterpenes from the spores of *Ganoderma lucidum* and their cytotoxicity against meth-A and LLC tumor cells. Chem. Pharm. Bull. 48: 1026-1033.
Miyamoto I, Liu J, Shimizu K, Sato M, Kukita A, Kukita T, Kondo R (2009). Regulation of osteoclastogenesis by ganoderic acid DM isolated from *Ganoderma lucidum*. Eur. J. Pharmacol. 602: 1-7.
Mizushina Y, Takahashi N, Hanashima L, Koshino H, Esumi Y, Uzawa J, Sugawara F, Sakaguchi K (1999). Lucidenic acid O and lactone, new terpene inhibitors of eukaryotic DNA polymerases from a basidiomycete, *Ganoderma lucidum*. Bioorg. Med. Chem. 7: 2047-2052.
Qiao Y, Zhang XM, Qiu MH (2007). Two novel lanostane triterpenoids from *Ganoderma sinense*. Molecules, 12: 2038-2046.
Sato N, Ma CM, Komatsu K, Hattori M (2009a). Triterpene-Farnesyl Hydroquinone Conjugates from *Ganoderma sinense*. J. Nat. Prod. 72: 958-961.
Sato N, Zhang Q, Ma CM, Hattori M (2009b). Anti-human immunodeficiency virus-1 protease activity of new lanostane-type triterpenoids from *Ganoderma sinense*. Chem. Pharm. Bull. (Tokyo) 57: 1076-1080.
Shiao MS (2003). Natural products of the medicinal fungus *Ganoderma lucidum*: Occurrence, biological activities, and pharmacological functions. Chem. Rec. 3: 172-180.
Tang YJ, Zhang W, Zhong JJ (2009). Performance analyses of a pH-shift and DOT-shift integrated fed-batch fermentation process for the production of ganoderic acid and *Ganoderma* polysaccharides by medicinal mushroom *Ganoderma lucidum*. Bioresour. Technol. 100: 1852-1859.
Weng CJ, Yen GC (2010). The *in vitro* and i*n vivo* experimental evidences disclose the chemopreventive effects of *Ganoderma lucidum* on cancer invasion and metastasis. Clin. Exp. Metastasis, 27: 361-369.
Xu JW, Zhao W, Zhong JJ (2010). Biotechnological production and application of ganoderic acids. Appl. Microbiol. Biotechnol., 87: 457-66.
Yang FC, Ke YF, Kuo SS (2000). Effect of fatty acids on the mycelial growth and polysaccharide formation by *Ganoderma lucidum* in shake flask cultures. Enzyme Microb. Technol. 27: 295-301.
Yang HL, Wu TX, Zhang KC (2004). Enhancement of mycelial growth and polysaccharide production in *Ganoderma lucidum* by the addition of ethanol. Biotechnol. Lett. 26: 841-844.
Zhang WX, Zhong JJ (2010). Effect of oxygen concentration in gas phase on sporulation and individual ganoderic acids accumulation in liquid static culture of *Ganoderma lucidum*. Biosci. Bioeng. 109: 37-40.