# Phenolic compounds from *Inonotus obliquus* and their immune-stimulating effects

ZHENG Wei-Fa[*]    ZHAO Yan-Xia    ZHANG Mei-Mei    YIN Zhao-Juan    CHEN Cai-Fa
WEI Zhi-Wen

*Key Laboratory for Biotechnology on Medicinal Plants of Jiangsu Province, Xuzhou Normal University, Xuzhou 221116,*
*China*

**Abstract:** Phenolic compounds from field-grown *Inonotus obliquus* sclerotia (Chaga) consist mainly of hispidin analogs and melanins, and are thought to be the active constituents to treat several human diseases. In submerged cultures of the fungus, however, no information is currently available on the production of phenolic compounds and their corresponding pharmacological functions. In this study, phenolic compounds from Chaga and submerged cultures of the fungus were assayed for their composition and immune-stimulating effects. Phenolic compounds produced by *I. obliquus* in submerged cultures mostly consist of flavonoids, together with small amounts of hispidin analogs and melanins. This is quite contrary to the situation in Chaga, where flavonoids are determined as trace elements. Furthermore, phenolic compounds from Chaga show capacity about two-fold higher than those produced in submerged cultures in inhibiting cyclophosphamide-induced reduction of bodyweight, spleen index and viability of peripheral lymphocytes in test mice. Thus less production of hispidin analogs and melanins is likely to be responsible for less immune-stimulating effects in phenolic compounds from submerged cultures, and additional factors should be imposed during submerged cultures of *I. obliquus* to regulate biosynthesis of phenolic compounds directed to the composition similar to Chaga.

**Key words:** sclerotium of *Inonotus obliquus*, Chaga, submerged cultures, flavonoids, melanins, hispidin, analogs

## 1 INTRODUCTION

The medicinal fungus *Inonotus obliquus* (Fr.) Pilát (Hymenochaetaceae) has been used as a folk remedy in Russia and Eastern Europe for more than four centuries (Zheng *et al.* 2007a), where its powerful effect on the treatment of several human diseases, in the absence of any unacceptable toxic side effects, has become established (Saar 1991; Huang 2002). In nature, this fungus is restricted to very cold habitats (45° N -50° N latitude) and grows very slowly on the trunks of *Betula* trees and forms sterile conk (Sclerotium) called "Chaga" (Campbell & Davidson 1938). Studies show that Chaga produces a diverse range of phenolic constituents including hispidin analogs (Lee *et al.* 2007) and melanins (Babitskaia *et al.* 2000), and these compounds are thought to be the active constituents for inhibiting replication of HIV-1 (Ichimura *et al.* 1998) and influenza virus H1NI and H3N2 (Ali *et al.* 2003). It has been established that antitumor and antiviral activities are mediated by cellular and humoral immunities, respectively (Griffith *et al.* 1999; Trkola *et al.* 2004). However, immune-stimulating effects of *I. obliquus* have not been documented except the description of immunity-mediated antitumor activity of an endo-polysaccharide (Kim *et al.* 2005; Kim *et al.* 2007). These effects by phenolic compounds from the fungus are still unclear.

Recently, owing to the increasing demand of healthy benefits from Chaga, natural reserves of this fungus have nearly been exhausted (Zheng *et al.* 2007b). Previous attempts to grow this fungus axenically focused mostly on the accumulation of mycelial biomass (Wang *et al.* 2006) and polysaccharides (Chen *et al.* 2007a), with little attention paid to the synthesis of phenolic compounds and their pharmacological activities. The differences if any in those synthesized by mycelia grown in its natural habitats and under laboratory conditions and in the pharmacological functions between them are still unknown. In this study, phenolic compounds from Chaga and submerged cultures of the fungus are assayed for the composition of phenolic compounds and their immune-stimulating effects. We aim to test the feasibility of substituting Chaga by submerged cultures of the fungus and provide some insights into understanding the accumulation of phenolic compounds in *I. obliquus*.

## 2 MATERIALS AND METHODS

### 2.1 Organism, inoculum preparation and conditions in submerged cultures

*Inonotus obliquus* (Fr.) Pilát (KLBMP04005) was provided by Key Lab for Biotechnology on Medicinal Plants of Jiangsu Province, and the seed culture was prepared as previously detailed (Zheng *et al.* 2007a). Submerged cultures were conducted in a 10L fermenter (EastBio CSTR fermentation system, Zhengjiang, China). Fermenter configuration and parameters used were all the same as described in Zheng *et al.* (2008). The culture period lasted 14 days.

### 2.2 Preparation of phenolic compounds from Chaga and submerged cultures

A total of 0.5kg well-chopped Chaga (collected from Changbai Mountains, Mudanjiang, Northeast China) was extracted with 4L of 50% ethanol at room temperature for 24 hours. The extract of Chaga was concentrated under vacuum and applied to Sephadex LH-20 (8cm ×120cm) and eluted

with methanol. The fractions with yellow and dark color were collected and combined respectively after excluding possible contaminating triterpenoids using vanillin reagent. These were evaporated to dryness under $N_2$ stream, and produced 6.8g total phenols and 0.84g melanins, which were stored at -20℃ until assayed. For preparing phenolic compounds from submerged cultures, a total of 20L mycelium-free culture broth was concentrated under vacuum and subject to mixing with three-fold volume of ethanol to remove any exocellular polysaccharides, followed by column chromatography on Sephadex LH-20 as described above, which yielded 3.78g total phenols and 85mg melanins.

**2.3 HPLC analysis of phenolic compounds**

Identification and quantification of phenolic compounds were conducted by HPLC on a Shimadzu Class-VP HPLC with computer-controlled upgraded Class-VP 5.03 software and a SCL-10A VP System controller with accessories identical to those described previously (Yao *et al.* 2005). Column used and gradient eluting program, as well as standards for quantification and identification were all the same as detailed by Zheng *et al.* (2008).

**2.4 Animals**

ICR mice (male, 3-weeks old, bodyweight at 20.79 ± 1.56g) were purchased from National Rodent Laboratory Resources, Shanghai, China (Licensed ID: SCXK2003-0003), and housed at 23 ± 1°C, with relative humidity of 55 ± 10% and light/dark cycle of 12h/12h, and were freely accessible to standard pellet diet and drinking water. The animals were cared humanly according to the standards for Laboratory Animals of China (GB 14923-94, GB 14922-94 and GB/T 14925-94).

**2.5 *In vivo* assays for immune-stimulation**

*In vivo* assays for immune-stimulating effects were conducted using ICR mice, where cyclophosphamide (CYP) (Hengrui, China, product ID: 07020821) was used as immune-suppressing agent. The test mice were grouped randomly into different sets including normal set (intraperitoneally injected with physiological saline), immune suppressed set (intraperitoneally injected with CYP at a dose of 40mg/kg), phenolic compounds combined with melanins from Chaga set (PCG) (CYP-treated mice were orally administered with PCG at a dose of 50mg/kg per day), phenolic compounds combined with melanins from culture broth set1 ($PCF_1$) (CYP-treated mice orally received PCF at a dose of 50mg/kg per day) and PCF set2 ($PCF_2$) (CYP-treated mice orally administered with PCF at a dose of 100mg/kg per day). The bodyweight variation of test mice was monitored daily. On day 30, after scaling the bodyweight, the mice were sacrificed for determining the index of spleen and thymus and viability of peripheral lymphocytes as protocols described previously (Chen *et al.* 2007b).

**2.6 Data analysis**

Experiments for submerged cultures were conducted in triplicates, and for immune-stimulating bioassay, ten replicates. Results from representative experiments are shown as means ± standard deviation. Data of all experiments were analyzed using SPSS 11.0 software. The assumptions of analysis of variance were considered to be statistically significant at $p < 0.05$.

## 3 RESULTS

### 3.1 Phenolic compounds from Chaga and the broth of submerged cultures

A total of 15 phenolic compounds were identified from Chaga and 12 from the filtrates of submerged cultures of *Inonotus obliquus*, reaching 82.99％ and 79.73％ of total yield, respectively. Phenolic compounds occurred in Chaga mainly consists of hispidin analogs including phelligridins A and D, inoscavins A and B, and melanins, together with small amounts of gallic acid, ferulic acid and flavonoids (fortuneletin, naringenin, kaempferol, EGC and narirutin); whereas those identified in culture filtrate are characterized by predominant presence of flavonoids including kempferol, naringin and narirutin, EGC, ECG, naringenin and fortuneletin, together with small amounts of melanins. Hispidin analogs was only determined as trace element. Gallic acid and ferulic acid were hardly detectable. In addition, phenylalanine (Phe) and tyrosine (Tyr) were also detected both in Chaga and culture filtrates of the fungus (Fig. 1).
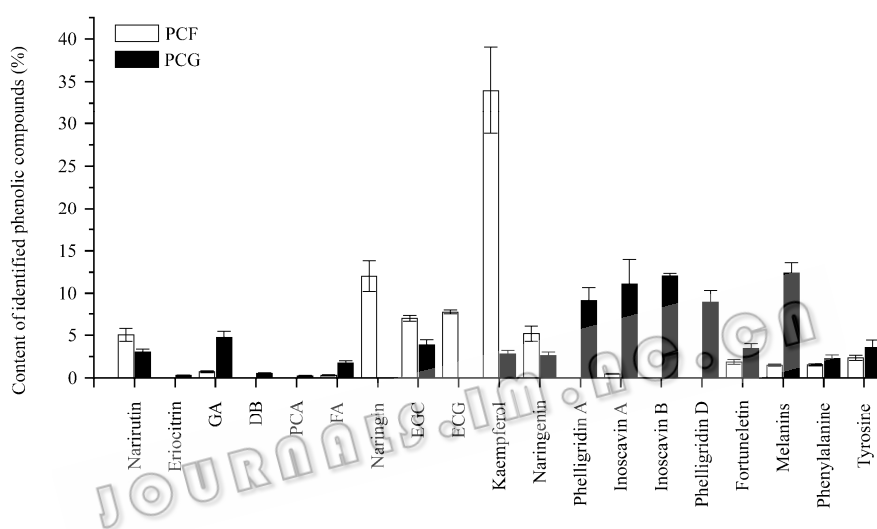


Fig. 1 Phenolic compounds identified from Chaga (PCG) and culture filtrates (PCF) of *Inonotus obliquus.* GA, gallic acid; DB, 2,3-dihydroxybenzaladehyde; PCA, protocatechuic acid; FA, ferulic acid; EGC, epigallocatechin; ECG, epicatechin gallate.

### 3.2 Effects on bodyweight of immune-suppressed mice

Bodyweight of test mice received CYP at a dose of 40mg/kg per day was reduced with time and reached more than 10% reduction by the end of experiment. This reduction can be reversed in the mice orally treated with PCG at a dose of 50mg/kg per day. Furthermore, the bodyweight of the test mice with PCG treatment increased from day 12 onwards, and remained unreduced throughout the experiment. In comparison, oral administration of PCF at a dose of 50mg/kg did not inhibit CYP-induced reduction of bodyweight from day 24 onwards, with a reduction pattern similar to that of control. A marked inhibition in bodyweight reduction occurred on day 25, and recovered on day 26, where it sustained up to the end of experiment. Oral administration of PCF at a dose of 100mg/kg did

not inhibit CYP-induced reduction of bodyweight in test mice from day 6 onwards, but indicated substantial inhibition against reduction in bodyweight of test mice, and recovered to initial level of bodyweight between day 9 and 17, and increased with treatment time up to the end of experiment (Fig. 2).
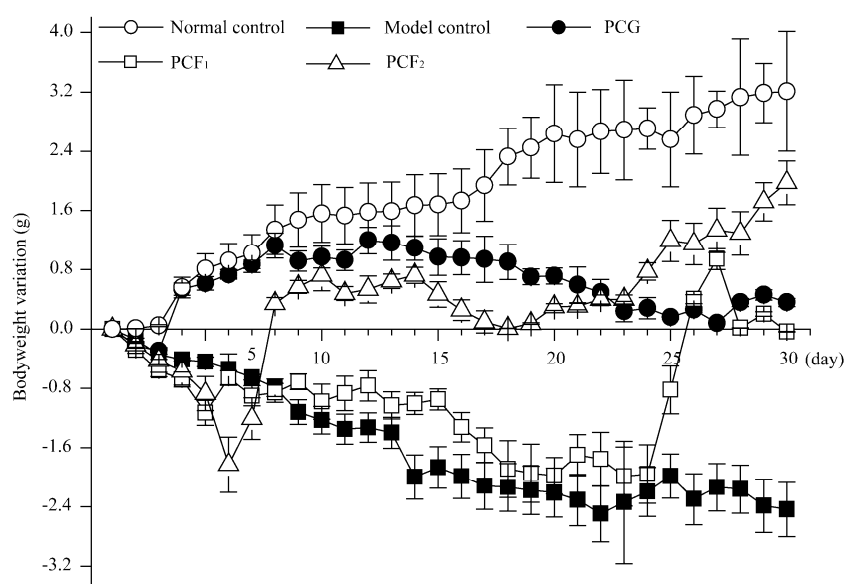


Fig. 2 Inhibitory effects of PCG and PCF on CYP-induced reduction of bodyweight of ICR mice. PCF, phenolic compounds from culture filtrates; PCG, phenolic compounds from Chaga; CYP (cyclophosphamide) was administered intraperitoneally at a dose of 40mg/kg per day; PCG, $PCF_1$ orally treated at a dose of 50mg/kg per day, and $PCF_2$, at a dose of 100mg/kg per day. Results are expressed as the mean ± S.D (n = 10).

### 3.3 Effects on spleen and thymus index in immuno-suppressed mice

Peritoneal treatment of CYP led to more than 50% decrease in spleen index, and this decrease was inhibited by oral administration of PCG at a dose of 50mg/kg per day or PCF at doses of 50 and 100mg/kg per day. Thymus index was not reduced in CYP-treated mice, and oral treatment of PCG and PCF at a dose of 50mg/kg per day did not affect it either, but increased markedly by oral treatment of PCF at a dose of 100mg/kg per day (Fig. 3-A). The viability of peripheral lymphocytes markedly decreased following CYP treatment compared to those in normal mice and reached to about $1.1 \times 10^6$/mL. This reduction, however, is inhibited following oral treatment of PCG, where the viable number of lymphocytes increased to nearly $5 \times 10^6$/mL by the end of experiment. While oral treatment of PCF at a dose of 50mg/kg per day did not enhance the viability of lymphocytes in CYP-treated mice, but increased to $3.2 \times 10^6$/mL following the treatment by PCF at a dose of 100mg/kg per day (Fig. 3-B).
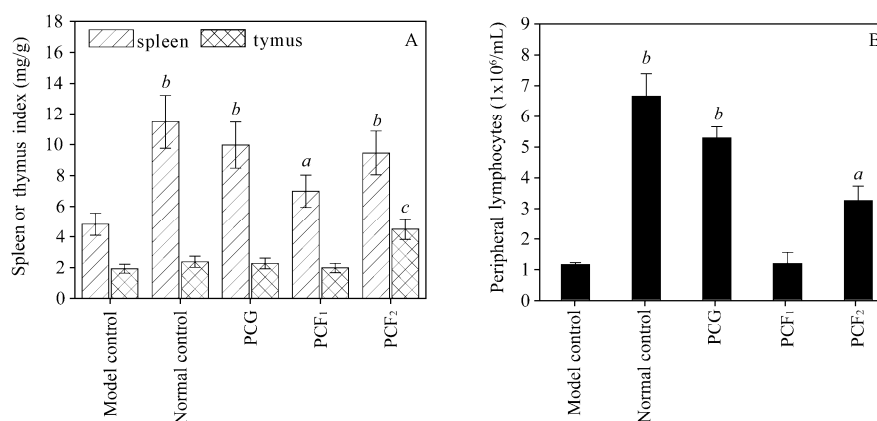
Fig. 3 Inhibitory effects of PCG and PCF on CYP-induced reduction of spleen or thymus index (A) and viability of peripheral lymphocytes (B). Other details are the same as in Fig. 2. [a]$P<0.05$, [b]$P<0.01$ versus mold control; [c]$P<0.05$ versus model control.

## 4 DISCUSSION

Data presented here suggested that phenolic compounds produced by *Inonotus obliquus* in submerged cultures mainly consisted of flavonoids, together with small amounts of melanins and trace element of hispidin analogs, while those produced by Chaga included predominantly hispidin analogs and melanins. Furthermore, phenolic compounds from Chaga were more effective in inhibiting CYP-induced reduction of bodyweight, spleen index and viable number of peripheral lymphocytes in mice than those determined in submerged cultures.

In nature, phenolic compounds produced by *Inonotus obliquus* are reported to consist of hispidin analogs including inoscavins A, B and C (Lee & Yun 2007), inobilins A, B and C, phelligrinds D, E and G (Lee *et al.* 2007). This class of compounds are thought to be derivatized in fungi from hispidin, synthesized from phenylalanine via the polyketide pathway (Lee & Yun 2007). Lower levels of hispidin analogs observed in culture filtrates indicates that biosynthesis of these compounds is largely inhibited. It is well established that Phe is the precursor for flavonoid production (Jiang *et al.* 2005), and Tyr can be transformed in the presence of tyrosine ammonia-lyase, to precursors for flavone (Yan *et al.* 2005) and flavan-3-ol (Chemler *et al.* 2007) synthesis. In this study, presence of Phe and Tyr, epigallocatechin and a number of flavones in culture filtrates of *I. obliquus* would implicate existence of pathways for flavone and flavan-3-ol synthesis, where chalcone is the common substrate for chalcone isomerase, flavone synthase and cytochrome 450 flavone synthase (Chemler *et al.* 2006; Chemler *et al.* 2007). This is opposite to the situation in Chaga, where flavonoids are detected only as minor components (Fig. 1).

It has also been established that long-term peritoneal treatment of CYP results in breakdown of DNA in lymphocytes and thereby causes lymphocyte apoptosis (Wang *et al.* 1999). Consequently, CYP treatment leads to suppressing both cellular and humoral immunity (Zhang *et al.* 2003). It has been evidenced that benzoic derivatives, phenylpropanoid acids, flavonoids and hispidin analogs are

all able to scavenge free radicals (Graf 1992; Lee & Yun 2007; Nakajima *et al.* 2007), and thus inhibit oxidative DNA damage in lymphocytes (Park *et al.* 2004). In our study, inhibiting reduction of bodyweight, spleen index and peripheral lymphocytes reinforced the protective effect of PCF and PCG on immune system in immune-suppressed mice.

It is noted that PCG show capacity about two-fold higher in inhibiting CYP-induced reduction of bodyweight, spleen index and viability of peripheral lymphocytes than those found in PCF. This possibly implicates that hispidin analogs and melanins are more capable for protecting oxidative DNA damage of lymphocytes in immune-suppressed mice than flavonoids, and underlie potential for inhibiting replication of HIV-1 (Ichimura *et al.* 1998) and influenza virus H1NI and H3N2 (Ali *et al.* 2003).

It is generally believed that polyphenols like hispidin analogs and melanins are secondary metabolites, and not required for growth or development of the producing organisms under laboratory conditions, but are thought to aid the fungus in competing successfully with factors in its natural habitats (Shwab & Keller 2008). Thus preferred production of hispidin analogs and melanins in Chaga can be attributed to the interaction with harsh environmental factors, and flavonoids are the metabolites accumulated preferably under laboratory conditions. For these reasons, suitable oxidative stress should be imposed during submerged cultures of *Inonotus obliquus* to regulate biosynthesis of phenolic compounds directed to the composition similar to Chaga.

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