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Title: Polyphenolic composition and antioxidant, antiproliferative, and antimicrobial activities of mushroom *Inonotus sanghuang*

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Abstract

Inonotus sanghuang (IS) is a mushroom species with some bioactivity. In this study, we characterized in *Inonotus sanghuang* polyphenolic compounds and assessed antioxidant, antiproliferative, and antimicrobial activities. *Inonotus sanghuang* was first extracted using ethanol and then the resulting ethanol extract was sequentially partitioned into four fractions. Among these fractions, ethyl acetate fraction (EAF) appeared to contain constituents with the largest antioxidant capacity as demonstrated by DPPH, ABTS, FRAP, and lipid peroxidation inhibition assays ($p \leq 0.05$). This fraction also displayed the strongest antiproliferative effect against tumor cell PC3 (IC_{50} , 70.8 $\mu\text{g/mL}$) determined using a CCK-8 assay. Further, the EAF showed antimicrobial activities against 3 gram-positive bacteria evaluated using an agar well diffusion method. Using LC-MS, six polyphenolics, e.g., rutin, chlorogenic acid, quercitrin, isorhamnetin, quercetin and icarisid II, were identified in the EAF. Total phenol content of *Inonotus sanghuang* was correlated significantly with FRAP value ($r = 0.988$, $p \leq 0.001$). Thus, the EAF of wild *Inonotus sanghuang* contains constituents with potent antioxidant, antiproliferative and antimicrobial activities.

Keywords: *Inonotus sanghuang*, Antiproliferative activity, Antioxidant, Antimicrobial activity, polyphenolics

1. Introduction

Mushrooms are widely grown in nature, and many of them have been traditionally used as medicinal foods in Asian countries, particularly in China and Japan. Sanghuang mushroom (Sanghuang) is a polypore mushroom and well appreciated for its therapeutic use for stomachaches, inflammation, tumors, diabetes, and pneumonia (Jung et al., 2008; Wu et al., 2012). More importantly, no apparent adversities after its consumption have been reported. While 15 Sanghuang mushroom species have been found in the world, only some of them were found to display anti-inflammatory, antioxidant and anticarcinogenic activities (Dai & Cui, 2014 ; Hu, Zhang, Lei, Yang, & Sugiura, 2009; Park et al., 2005; Wu et al., 2012). For example, the methanol extract of *Inonotus obliquus* diminished acute paw edema in rats and had analgesic activity in mice (Park et al., 2005) and the ethanol extract of *Inonotus obliquus* displayed superoxide dismutase-like and antiproliferative activities in DLD-1 cells (Hu et al., 2009).

Inonotus sanghuang is one species of Sanghuang mushroom and a white-rot fungus in the family of Hymenochaetaceae. It has been known as a legendary medicinal herb in China since 2000 years ago (Wu et al., 2012). Tian (2014) reported that the ethanol extract of *I. sanghuang* mycelia produced from liquid fermentation scavenged DPPH and hydroxyl radicals, and the authors attributed the antioxidant activity to the existing polyphenolics, e.g., rutin, eriodictyol, naringenin and sakuranetin. However, the bioactions of wild *I. sanghuang* remains to be further characterized. Thus, in this study, we aimed to isolate and identify the bioactive fraction and

constituents of *I. sanghuang* by using an activity-guided fractionation approach. , The fractionation was achieved using four organic solvent, i.e., ethanol, petroleum ether, ethyl acetate, and *n*-butanol. The sequence of these administered solvents for the fractionation was employed according to the increasing polarity of the solvents (Balkan et al., 2017; Fu, Zhang, Guo, & Chen, 2015). These fractions, including ethanol extract (EE), petroleum ether fraction (PEF), ethyl acetate fraction (EAF), *n*-butanol fraction (NBF) and water fraction (WF) were then subject to the assessment of antioxidant, antiproliferative, and antimicrobial activities. Phenolics in the EAF were also characterized using LC/MS.

2. Materials and methods

2.1. Samples

I. sanghuang was collected from the Aershan Region of Inner Mongolia (Inner Mongolia, China) between July and September 2011 and stored at -80°C. After its authentication was confirmed by Professor Xiangmei Zhang, a voucher specimen (NoF121725) was deposited in the College of Life and Environmental Science of Minzu University of China for the future references.

2.2. Chemicals

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61 Chemicals used in this study were all analytical grade. RPMI1640 complete medium,
62 2,2'-azinobis(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS), 2,4,6-tri(2-pyridyl)-s-tri-azine
63 (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), quercitrin, chlorogenic acid, isorhamnetin,
64 icaridisid II, rutin, and quercetin were purchased from Sigma (St. Louis, MO, USA). Ascorbic
65 acid, hydrogen peroxide (H_2O_2), trichloroacetic acid (TCA), thiobarbituric acid (TBA),
66 Folin–Ciocalteu's reagent, sodium carbonate, ferric chloride ($FeCl_3$), dimethyl sulphoxide
67 (DMSO), and other reagents were obtained from Beijing Chemical Works (Beijing, China). All
68 solvents for HPLC analysis were HPLC grade.

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70 2.3. Extraction and fractionation

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72 Fresh fruiting bodies of *I. sanghuang* were obtained from the Aershan Region of Inner
73 Mongolia (Inner Mongolia, China). After air-dried for one week, the dehydrated fruiting bodies
74 were cut into small pieces and then pulverized to powder using a grinder (Tianjin Taisite
75 Instrument Co., Tianjin, China) set at the speed of 8000 rpm/min. The powder (0.15 kg) was
76 then extracted for 4 times with 4 L of 95% ethanol for 7 d each at room temperature (Liu,
77 Wang, Zhao, & Wang, 2013). The supernatants were filtered using a Whatman No. 1 filter
78 paper, combined, and then concentrated using a rotary evaporator (50°C) to yield 24.99 g EE
79 (paste). EE (16 g) was dissolved in distilled water, and then partitioned using a separating
80 funnel sequentially with 3 organic solvents, namely petroleum ether (3 × 300 mL), ethyl

acetate (3×300 mL) and then *n*-butanol (3×300 mL). The resulting four fractions were concentrated under vacuum and then lyophilized using a vacuum freeze dryer (Beijing Boyikang Instrument Co., Beijing, China) to generate PEF, EAF, NBF and water fraction (WF). These 4 fractions were stored at 4 °C in dark until further analyses. Yield of each extract was calculated as follows:

$$\text{Extraction yield (\%)} = (\text{weight of dry extract} / \text{weight of raw material}) \times 100$$

2.4. Total Phenol and Flavonoid Contents

Total phenol content (TPC) was determined according to a modified Folin–Ciocalteu method (Liu et al., 2013). One hundred twenty five microliters of properly diluted sample was mixed with 1 mL Folin–Ciocalteu's reagent. After 2-minute incubation at room temperature, 2 mL Na_2CO_3 (10%, w/v) was added and the resulting mixture was incubated for 15 min at 50 °C. At the end of the incubation, the absorbance was measured at 775 nm. TPC expressed as mg gallic acid equivalents/g dry-extract weight (mg GAE/g DW).

Total flavonoid content (TFC) was determined by a colorimetric assay, according to Tai et al. (2011). Briefly, 0.5 mL of the properly diluted sample was mixed with 9.5 mL methanol in a 25 mL volumetric flask, followed by the addition of 1 mL NaNO_2 (5%, w/v). After 6-minute incubation, 1 mL AlCl_3 (10%, w/v) was added. The mixture was incubated for 6 min at room temperature, followed by the addition 10 mL NaOH (4%, w/v) and 3 mL deionized water. The

solution was shaken vigorously for 1 min and then subjected to absorbance measurement at 510 nm. Results are expressed as mg rutin equivalents/g dry-extract weight (mg RE/g DW).

2.5. LC/MS Analysis

EAF (6.3 mg) were dissolved in 20 mL methanol, followed by 5 min sonication. Polyphenols in the resulting solution were characterized using LC-MS-IT-TOF (Kyoto, Japan) equipped with a Shimadzu Shim-pack VP-ODS column (150 mm × 2.0 mm, 5 μm). The separation was performed using two mobile phases (A: 0.1% formic acid in water and B: acetonitrile) with the following gradient: 30 to 47% B (0-20 min), 47 to 80% B (20-30 min), 80-100% B (30-35), and 100% B (35-55 min). The injection volume was 5 μL, the flow rate was 0.2 mL/min, and polyphenols were monitored at 280 nm. The autosampler was maintained at 4 °C, and the LC column chamber was set at 40 °C. The ESI-MS was performed in the negative ion mode with interface voltage of -3.5 kV, nebulizing gas flow rate at 1.5 L/min, CDL temperature at 200 °C, heating block temperature at 200 °C, and detector voltage of 1.57 kV. MS scan ranged from 350 to 1300 m/z. Polyphenols were characterized and identified according to their MS spectra and retention times as compared to authenticated standards.

2.6. Quantification of selected polyphenols using HPLC-DAD

HPLC analysis of polyphenols was performed using a Shimadzu HPLC system (Kyoto,

Japan) equipped with a diode array detector (DAD). The chromatographic separation was achieved using a Supersil C18 column (150 × 2.0 mm, 5 µm) with the column temperature set at 40°C. Polyphenols were eluted using two mobile phases (A: water and B: acetonitrile) at a gradient mode: 20% B (0-10 min), 70% B (10-15 min), 20% B (15-20), and 100% B (20-30 min). Polyphenols were monitored at 280 nm. Detected polyphenols were quantified using a standard curve constructed using authentic standards.

2.7. *In vitro* antioxidant capacity

2.7.1. *DPPH* assay

The assay was performed according to the method of our previous study (Liu et al., 2013). The DPPH value is expressed as IC₅₀, which is defined as the concentration (µg DW/mL) scavenging 50% DPPH radicals. Ascorbic acid was used as the reference antioxidant. All samples were analyzed in triplicate.

2.7.2. *ABTS* assay

The ABTS assay was performed according to the method of Gursoy, Sarikurkcu, Cengiz, and Solak (2009) with minor modifications. ABTS solution was produced by mixing ABTS and K₂S₂O₈ and then incubating for 16 h in the dark at room temperature. Before use, the ABTS solution was diluted to yield the absorbance of 0.70 ± 0.02 at 734 nm. Samples (50 µL)

were mixed with 150 μ L of the working ABTS solution, and the absorbance at 734 nm was determined after 30 min incubation at room temperature.

2.7.3. FRAP assay

The FRAP assay was performed according to the method described by (Hayes, Allen, Brunton, O'Grady, & Kerry, 2011) with slight modifications. Briefly, 180 μ L of the FRAP working cocktail was added to 5 μ L sample. The tubes were covered in tinfoil and incubated at 37 °C for 5 min. At the end of the incubation, absorbance at 593 nm was measured. Trolox at the concentrations ranging from 0.15 to 1.5 mM were used to construct the standard curve. Results are expressed in mmol trolox/100 g DW. All samples were assessed in triplicate.

2.7.4. Inhibition of lipid peroxidation

A modified thiobarbituric acid-reactive species (TBARS) assay was performed to assess the capability of antioxidants in the samples to decrease ferrous ion-induced peroxidation of yolk lipids (Banerjee, Dasgupta, & De, 2005). Briefly, egg yolks were diluted with 9 volumes of ethanol to prepare a yolk suspension. After centrifugation at 5000 rpm for 10 min, the supernatant (200 μ L) of yolk suspension was mixed with 100 μ L sample or vehicle and 100 μ L of 250 μ M FeSO₄. The resulting mix was then incubated for 1 h at 37°C. At the end of the incubation, 1 mL trichloroacetic acid (15%, w/v) and 1 mL of thiobarbituric acid (0.67%, w/v) were added, followed by incubation for 40 min at 100 °C. After centrifugation for 10 min at 5000 rpm, absorbance was read at 532 nm. The inhibition of lipid peroxidation was calculated

according to the following equation:

$$\% \text{ inhibition of lipid peroxidation} = [(A_c - A_t) / A_c] \times 100$$

where A_c is absorbance of vehicle solution and A_t is absorbance of sample. All samples were analyzed in triplicate.

2.8. Antiproliferative effect

Antiproliferative effect of test samples was assessed using Cell Counting Kit-8 (CCK-8) assay (Cao, Xia, Chen, Xiao, & Wang, 2013) with some modifications. Prostate cancer lines PC3 and DU145, liver cancer cell lines HepG2, and lung cancer cell lines A549) were purchased from Cell Resource Center, IBMS, and CAMS/PUMC (Beijing, China), respectively. Cells were grown in RPMI 1640 medium at 37 °C in a humidified atmosphere with 5% CO₂. Cells (2×10³ cells/well) were seeded in a 96-well tissue culture plate. The cells were allowed to adhere for 18 h and then treated with 20 µL samples, containing the appropriate solvent DMSO (0.1% in water), at concentration of 6.25, 12.5, 25, 50, 100, and 200 µg/mL for 24 h. Cell proliferation was measured using the CCK-8 kit (Dojindo Molecular Technologies, Inc. China), according to the manufacturer's instructions. The % inhibition was calculated using the following formula:

183 % inhibition = $[(A_c - A_t) / A_c] \times 100$

184
185 Where A_c is absorbance of control and A_t is absorbance of sample. Each concentration was
186 tested in triplicate. Results are expressed as IC_{50} , which is defined as the concentration (μ g
187 DW/mL) inhibiting cell growth by 50%.

188 189 2.9. Antimicrobial assay

190 191 2.9.1. Microbial strains

192 The bacterial strains, *Staphylococcus aureus* (ATCC25923-3), *Escherichia coli*
193 (ATCC25922-3), *Bacillus subtilis* (CMCC63507-3), *Pseudomonas aeruginosa*
194 (ATCC27853-3), *Proteus bacillus vulgaris* (CMCC49027), *Bacillus cereus* (ATCC11778-3)
195 were used for the studies (from the biology laboratory of the College of Life and
196 Environmental Sciences, Minzu University of China).

197 198 2.9.2. Inhibitory zone assay

199 Antimicrobial activity was determined using a modified agar well diffusion method (Liu et
200 al., 2013). Samples dissolved in methanol and control wells containing methanol as negative
201 control were also run in parallel. Antimicrobial activity was assessed by measuring diameter of
202 the zone of test samples as compared to that of control. Each sample was assessed in triplicate.

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204 2.9.3. Minimum inhibitory concentration

205 Minimum inhibitory concentration (MIC) of the test samples was evaluated by the two-fold
206 serial dilution method (Liu et al., 2013). Tested concentrations of samples ranged from
207 6.25-100 mg/mL. The MIC is defined as the lowest concentration inhibiting the visible growth
208 of tested microorganisms incubated for 24 h at 37°C.

209

210 2.10. Statistical Analysis

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212 The results are expressed mean $\pm$ standard deviation. All assays were performed in triplicate.
213 One-way ANOVA was performed to test statistical significance, followed by Tukey's HSD
214 multi-comparison test. P -value ≤ 0.05 was considered significant. Pearson's correlation test
215 was performed to test correlation between variables. All statistical analyses were performed
216 using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

217

218 3. Results and discussion

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220 3.1. Total phenols and total flavonoids

221

222 The yield of EE derived from *I. sanghuang* was 8.33% (w/w), a value larger than the yield of

methanol extract from *Inonotus hispidus* (4.55%, w/w) reported by (Smolskaitė, Venskutonis, & Talou, 2015). Of the 4 organic solvent-partitioned fractions generated from EE in the study, WF had the largest yield at 2.87%, followed by PEF at 2.33%, EAF at 2.01%, and NBF at 1.10%. The sum of the 4 fractions was 8.31%, indicating a full recovery from EE.

Phenolics and flavonoids have been considered the major contributors to antioxidant actions in mushrooms (Gan, Nurul Amira, & Asmah, 2013). In the *I. sanghuang* extracts, we found that TPC ranged from 0.79 to 43.60 mg GAE/g. EAF had the largest value, which was at least 19.2% larger than the other 3 fractions (Fig. 1A). Our results appeared in line with the results of Smolskaitė et al. (2015), in which TPC in cyclohexane, dichloromethane, methanol and water extracts of 8 mushroom species (including *I. hispidus*) ranged from 4.21 to 31.88 mg GAE/g. The ranking order of TFC values of *I. sanghuang* extracts was the same as TPC. Thus, ethyl acetate appeared to possess a better extraction potency in extraction of phenolics from mushrooms than other organic solvents. Consistently, Jeon et al. (2009) reported that TFC and TPC of EAF from *Phellinus linteus* were larger than other fractions.

3.2. Antioxidant activity

Four assays were employed to assess antioxidant activity of the fractions. Of the 4 assays, DPPH and ABTS have been commonly used to determine antioxidant activity of constituents in plant foods (Alam, Bristi, & Rafiquzzaman, 2013). We noted that all fractions displayed potent scavenging activities toward DPPH and ABTS radicals in a dose-dependent manner

with concentrations ranging from 6.25 to 200 $\mu\text{g/mL}$. Consistent to the ranking order of the TPC and TFC contents, EAF exhibited the largest scavenging activity than other fractions ($p \leq 0.05$) (Fig. 1A). We noted that the DPPH radical scavenging activity of EAF with the IC_{50} value of $3.79 \pm 0.18 \mu\text{g/mL}$ was comparable to that of the reference antioxidant ascorbic acid with the IC_{50} value of $3.61 \pm 0.11 \mu\text{g/mL}$. Further, the ABTS radical scavenging activity of EAF was slightly larger than that of ascorbic acid (19.31 ± 1.49 vs. $16.46 \pm 1.24 \mu\text{g/mL}$, $p \leq 0.05$). These results were in agreement with the study of Lin, Ching, Chen, and Cheung (2015) in which they found that EAF of *Pleurotus tuber-regium* displayed larger DPPH and ABTS radical scavenging activities than other fractions.

FRAP assay assesses the reducing capability of antioxidant (Katalinić, Milos, Modun, Musić, & Boban, 2004). We found that FRAP activity was significantly different between *I. sanghuang* fractions ($p \leq 0.05$). EAF had the highest FRAP value, followed by EE, NBF, WF, and PEF in a descending order. Smolskaitė et al. (2015) reported that the reducing power of cyclohexane, dichloromethane, methanol and water extracts of 8 mushroom species (including *I. hispidus*) ranged from 0.043-30.1 mmol Trolox/100 g DW. FRAP value of EAF noted in the present study was at least 10-fold higher. While there are many factors contributing to the observed higher FRAP value, we speculate that phenolics existing in *I. sanghuang* may carry more hydroxyl functional moieties in the phenol ring(s), which act as an electron donor in the reduction reaction (Orak, 2007). There is a consensus that The TPC and TFC of mushrooms were correlated to their antioxidant activities (Gan et al., 2013). Our results were consistent with this consensus. Particularly, TPC and TFC were strongly correlated with FRAP ($r = 0.988$ and 0.999 , respectively, $p \leq 0.001$). These strong correlations implicate that polyphenolics in *I.*

sanghuang possess potent reducing antioxidant capacity. Interesting, we did not have significant correlations between TPC and TFC and DPPH and ABTS values, implicating compounds other polyphenols may be more potent to scavenge DPPH and ABTS radicals. Our results were in agreement with the study of Kumari and Chang (2016), in which they found that the correlation between TFC and DPPH and between TFC and ORAC (Oxygen Radical Absorbance Capacity) was -0.37 and 0.95, respectively.

Lipid peroxidation is an oxidative reaction of polyunsaturated fatty acids induced by free radicals (Su, Wang, & Liu, 2009). The end product of lipid peroxidation, malondialdehyde (MDA), has been widely used to assess *in vivo* oxidative stress status in humans. This same measurement is also employed to assess antioxidant capability of compounds against lipid peroxidation. In this study, we evaluated the magnitude of protection of *I. sanghuang* antioxidants against ferrous ion induced yolk lipid peroxidation (Fig. 1D). At 200 µg/mL, we found all fractions inhibited ferrous ion induced MDA production in different degrees. The most protective fraction was EAF, and it was approximately 5-fold more effective than the least fraction, PEF. We also found that EAF was as efficacious as ascorbic acid at 100 µg/mL. As compared to EE of *Pleurotus ostreatus* (oyster mushroom), EAF of *I. sanghuang* was at least 50-fold more efficacious in the protection against lipid peroxidation (Jayakumar, Thomas, & Geraldine, 2009).

EAF showed the highest antioxidant activities among all fractions assessed using a battery of tests. Thus, we characterized polyphenolics in this fraction using LC-DAD and LC-MS-IT-TOF. We identified 6 compounds, namely chlorogenic acid, rutin, quercitrin, icaridin II, quercetin and isorhamnetin, by comparing their retention times monitored at 280 nm (LC-DAD) and in

total ion chromatogram mode (LC-MS-IT-TOF) to authenticated standards (Fig. 2). The content of 6 polyphenols in EAF, quantified using the HPLC-DAD method, is presented in Table 1. Of 6 compounds, chlorogenic acid is the most dominant, followed by rutin, quercitrin, and quercetin with their concentrations being 93.06%, 93.89% and 95.32% smaller. As compared to the study of Kaewnarin, Suwannarach, Kumla, and Lumyong (2016), EAF contained a higher amount of rutin than methanol extract of *Rugiboletus extremiorientalis* (168.9 vs. 9.5 mg/kg) and a higher amount of quercetin than methanol extract of *Russula emetica* (113.9 vs. 17.6 mg/kg).

It is noteworthy that these compounds are reported in the EAF of *I. sanghuang* for the first time. Though we expect the observed antioxidant activity of EAF is a product of additive/synergistic/antagonistic interactions between all constituents, these 6 identified polyphenols have been reported to be potent antioxidants. For example, rutin at 0.05 mg/mL scavenged 90.4% DPPH radicals and inhibited 68.8% lipid peroxidation (Yang, Guo, & Yuan, 2008). Both isorhamnetin and quercetin have been shown to scavenge DPPH radicals (IC₅₀ at 24.61 and 3.07 µmol/L, respectively) and ABTS radicals (IC₅₀ at 14.54 and 3.64 µmol/L) and attenuate lipid peroxidation (IC₅₀ at 6.67 and 6.67 µmol/L) (Zuo et al., 2011). Quercitrin displayed DPPH, superoxide, and hydroxyl radical scavenging activity (Ham et al., 2012). Chlorogenic acid has also been shown to be one of major contributors to DPPH radical scavenging activity (Kumari & Chang, 2016). Further, chlorogenic acid at the same concentration (10 µmol/L) was more potent than dl- α -tocopherol or ascorbic acid to scavenge DPPH radicals (Ohnishi et al., 1994). Thus, the noted antioxidant property of EAF of *I. sanghuang* is most likely attributed to polyphenols. However, it should be noted NBF also

contained constituents with antioxidant activity scavenging DPPH and ABTS radicals in a potency closer to those in EAF.

3.3. Antiproliferative activity

Cancers are one of the main causes for morbidity and mortality worldwide (Costantini, Colonna, & Castello, 2014). Wu et al. (2012) reported that Sanghuang mushrooms displayed potent anti-carcinogenic activity. To assess anti-carcinogenic potential of EE, PEF, EAF, NBF, and WF of *I. sanghuang* in this study, we determined proliferation of human cancer cell lines (PC3, DU145, HepG2 and A549) treated with the fractions at various concentrations. We found that EAF was the most efficacious to inhibit the proliferation of prostate (PC3 and DU145), liver (HepG2), and lung (A549) cancers than the other fractions (Table 2). Further, we noted that among the 4 cancer cell lines, the IC₅₀ value toward PC3 was at least 1-fold smaller than the other 3 cell lines, suggesting that the anti-proliferative effect of EAF constituents was cell type dependent. This is also applicable to the anti-proliferative effect of the other 3 fractions. It shall be noted that the results of the *in vitro* anti-proliferation tests is readily translatable to *in vivo* bioefficacy because the *in vitro* experiments do not take absorption and metabolism of bioactive constituents into account. Nevertheless, these results inform design of future preclinical studies targeting anti-carcinogenic effect of *I. sanghuang* on prostate cancer.

Polyphenols are the secondary metabolites of mushrooms in the genus *Inonotus*. These

compounds have been found to possess anti-carcinogenic activities (Hao et al., 2008; Hu et al., 2009). As we discussed above, EAF of *I. sanghuang* contained 6 polyphenols including quercetin and chlorogenic acid. The literature has shown that quercetin could inhibit migration and invasion of PC3 cell (Senthilkumar et al., 2011) and chlorogenic acid protected against cancer cell A549 and HepG2 (Barahuie, Hussein, Arulselvan, Fakurazi, & Zainal, 2014). Thus, we speculate that quercetin and chlorogenic acid in EAF may account partially for the observed anti-proliferative effect of EAF on PC3, A549 and HepG2 cells. Further, constituents in NBF inhibited proliferation of DU145 and HepG2 cells in the potency close to EAF as compared to the other 3 fractions. However, the bioactive constituents in NBF remains to be identified.

3.4. Antimicrobial activity

The antimicrobial potential of *I. sanghuang* fractions against gram-negative (*E. coli*, *P. aeruginosa* and *P. vulgaris*) and gram-positive bacteria (*S. aureus*, *B. cereus* and *B. subtilis*) was evaluated by assessing the size of the inhibition zone and minimum inhibitory concentration. We found that constituents in *I. sanghuang* were ineffective toward 3 gram-negative bacteria species but somewhat effective against gram-positive ones (Table 3). We speculate the differences in the structure of their cell walls may explain the dissimilarity (Nikaido, 2003; Siddiqi, Naz, Ahmad, & Sayeed, 2011). The zone of inhibition (ZOI) values indicated that among the tested gram-positive bacteria, the most susceptible bacterium to *I. sanghuang* fractions was *S. aureus*, followed by *B. cereus* and *B. subtilis*. Our results were consistent with the study of (Barros, Baptista, Estevinho, & Ferreira, 2007), in which extracts

of *Leucopaxillus giganteus* only inhibited the growth of gram-positive bacteria, i.e., *S. aureus*.

The exact mechanism by which the study extracts were more potent in the inhibition of gram positive bacteria was not examined in the current study, we speculated that due to the differences in the structure of cell walls between, gram-positive bacteria are more sensitive than gram-negative bacteria to anti-microbial compounds (Siddiqi et al., 2011). EAF displayed the highest antimicrobial potential, followed by EE and NBF. PEF and WF were ineffective. Similar to the inhibition zone results, the same trend in MIC values was noted. While it is unknown what constituents in *I. sanghuang* exerted antimicrobial actions, we speculate that rutin and chlorogenic acid in EAF may contribute partially to the observed antimicrobial activity of EAF (Rajoria, Mehta, Mehta, Ahirwal, & Shukla, 2015).

4. Conclusions

Bioassay-guided preparative isolation was used to characterize antioxidant, antiproliferative and antimicrobial activities of constituents in *I. sanghuang*. Six polyphenols, including rutin, chlorogenic acid, quercitrin, isorhamnetin, quercetin and icarisisid II, were identified and quantified in the ethyl acetate fraction for the first time. As compared to ethanolic extract, petroleum ether, *n*-butanol, and water fractions, and ethyl acetate fraction was found to exhibit higher antioxidant, antiproliferative, and antimicrobial activities, most likely being attributed to polyphenolics in the fractions. It shall be noted that the antimicrobial activity was only observed toward gram-positive bacteria *S. aureus*, *B. cereus* and *B. subtilis*. Our *in vitro* results

suggest that *I. sanghuang* mushrooms contain polyphenols and other substances with antioxidant, antiproliferative, and antimicrobial activities. *In vivo* studies are warranted to examine these bioactions of *I. sanghuang* constituents that are soluble in ethyl acetate.

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Figure captions

Fig. 1. Antioxidant activity of different solvent fractions derived from *I. sanghuang* are assessed using Total phenolic and flavonoid content assays (A), DPPH and ABTS scavenging activity assays (B), FRAP assay (C), and lipid peroxidation inhibition assay at a dose of 200 µg/mL (D). Values are expressed as mean ± SD (n = 3). Values with the same letter (a, b, c, etc.) are not significantly different ($p \leq 0.05$), tested using Tukey's HSD Test. Abbreviations: total phenolic content (TPC), total flavonoid content (TFC), gallic acid equivalence (GAE), quercetin equivalence (QE), ethanolic extract (EE), petroleum ether fraction (PEF), ethyl acetate fraction (EAF), n-butanol fraction (NBF), water fraction (WF), lipid peroxidation (LP), inhibition activity (IA)

Fig. 2. Chromatogram trace of HPLC-DAD analysis monitored at 280 nm and total ion chromatogram of LC-MS-TI-TOF analysis, polyphenol standards (A, C) and EAF fraction (B, D). Peak identification: chlorogenic acid (1), rutin (2), quercitrin (3), icarisid II (4), quercetin (5), isorhamnetin (6).

Table 1
Quantitative analysis of phenolic content (mg/kg dry weight) of EAF using HPLC.

Standards	Retention time (min)	Regression equation	*r ² Value	Content (mg/kg)
Chlorogenic acid	1.428	y = 40444x + 1679.9	0.9994	2435.25 ± 26.54
Rutin	4.423	y = 16007x - 2813.2	0.9993	168.93 ± 13.85
Quercitrin	8.269	y = 27087x - 11426	0.9992	148.82 ± 0.45
Icarisid II	16.86	y = 23335x + 2552.5	0.9998	73.02 ± 3.07
Quercetin	17.783	y = 11200x - 1175.7	0.9991	113.94 ± 2.61
Isorhamnetin	18.687	y = 54610x + 9235.1	0.9995	78.56 ± 7.6

Values are mean ± standard error mean (SEM) of mean of triplicate analyses.
*r² value is the R-squared value obtained from the regression line, using different concentrations of the various reference standards

Table 2
The antiproliferative activities of the ethanol extract and four fractions of *I. sanghuang*, tested using four cell lines*

Extract	PC3	DU145	HepG2	A549
	IC ₅₀ (µg/mL)			
EE	592.8 ± 8.1(3) ^c	453.1 ± 6.4(4) ^b	346.9 ± 5.2(3) ^c	376.7 ± 3.8(2) ^d
PEF	1000.1 ± 22.1(5) ^a	475.98 ± 5.5(5) ^a	3665.7 ± 30.6(4) ^b	3836.8 ± 32.8(5) ^a
EAF	70.8 ± 3.4(1) ^e	145.1 ± 2.7(1) ^e	308.5 ± 4.7(1) ^e	216.3 ± 3.5(1) ^e
NBF	236.8 ± 5.5(2) ^d	193.1 ± 2.9(2) ^d	327.4 ± 5.7(2) ^d	602.6 ± 7.8(3) ^c
WF	682.7 ± 11.8(4) ^b	402.2 ± 8.0(3) ^c	5046.3 ± 42.3(5) ^a	829.5 ± 21.1(4) ^b

*Values were expressed as mean ± SD (n = 3). The number in the parenthesis is the ranking order of the fractions within the cell line.

^{abcd}Values without sharing the same superscript letter within the same column significantly differ, tested by one-way ANOVA followed by Tukey’s HSD test ($p \leq 0.05$).

Table 3
Antimicrobial activity of the ethanol extract and four fractions of *I. sanghuang**

Bacteria species	EE	PEF	EAF	NBF	WF	EE ^b	PEF	EAF	NBF	WF
	Inhibition zone (cm) ¹					Minimum inhibitory concentration (mg/mL)				
Gram negative										
<i>E. coli</i>	—	—	—	—	—	—	—	—	—	—
<i>P. aeruginosabs</i>	—	—	—	—	—	—	—	—	—	—
<i>P. vulgari</i>	—	—	—	—	—	—	—	—	—	—
Gram positive										
<i>S. aureus</i>	2.22±0.13 ^a	—	2.31±0.14 ^a	0.87±0.05	—	25 ^c	—	25 ^c	100	—
<i>B. subtilis</i>	1.01±0.09 ^c	—	1.07±0.08 ^c	—	—	100 ^a	—	100 ^a	—	—
<i>B. cereus</i>	1.68±0.11 ^b	—	1.71±0.07 ^b	0.80±0.06	—	50 ^b	—	50 ^b	100	—

*Values were expressed as mean ± SD (n = 3).
^{abcd}Values without sharing the same superscript letter within the same column significantly differ, tested by one-way ANOVA followed by Tukey’s HSD test ($p \leq 0.05$).
¹Treatment dosage was 5 mg/well.

Fig. 1.

Fig. 2.