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Mycelial Growth, Antioxidant and Antibacterial Properties of *Boletus griseipurpureus* from South Thailand

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Boletus griseipurpureus Corner is a popular edible ectomycorrhizal mushroom in the south of Thailand. The objective of this study was to identify optimal media for mycelial formation, antioxidant and antimicrobial activities. Of the four media tested, PDA, Boletus medium, MMN, and MMN with 10 % fresh basidiocarp hot water extract, the latter medium was superior for mycelial dry weight increment. Methanolic extraction of dried basidiocarps showed that the DPPH radical scavenging activity (200.3$\pm$4.4 µg vitamin C/g sample) was stronger than crude ethyl acetate extract (77.9$\pm$0.4 µg vitamin C/g sample). Ferric reducing antioxidant power (FRAP) gave similar results. Crude basidiocarp extracts inhibited *Pseudomonas aerunginosa* TISTR 2370 (MIC = 0.39 mg/ml) and *Enterococcus faecalis* TISTR 379 (MIC = 0.78 mg/ml) in agar well diffusion tests.

Keywords: *Boletus griseipurpureus*, mycelial formation, antioxidation and antibacterial activity

Introduction

Utilization of edible wild mushrooms in indigenous communities is a common practice in Malaysia, Thailand, Philippines, India and Indonesia (Lee and Chang, 2004; Cai *et al.*, 2010; Tibuhwa, 2013; Lau, 2014; Venkatachalapathi and Paulsamy, 2016). Mushrooms are an important source of food, being high in both carbohydrate and protein contents but low in fat (Sanmee *et al.*, 2003; Cheung, 2010). Some are high priced because they not only have nutritional values but also contain bioactive compounds such as antioxidants, anti-inflammation and antibacterial substances (Hamza *et al.*, 2016). Many edible and medicinal mushrooms are ectomycorrhizal fungi that

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fruit in association with host plants, for example *Tricholoma matsutake* (Islam and Ohga, 2013) with Pinaceae, *Tuber melanosporum* with Fagaceae (Roy, 2003) and *Terferzia boudieri* with Cistaceae (Dogon and Aydim, 2013). In the south of Thailand, *Boletus griseipupureus* is a popular edible ectomycorrhizal fungus associated with indigenous and exotic trees (Seehanan and Petcharat, 2008). The mushroom has a high protein but low fat content (Aung-aud-chariya *et al*., 2012). Recently a group of Malaysian researchers have reported that hot water extracts of *B. griseipurpureus* contained therapeutic compounds including antioxidants (Muniandy *et al*., 2016). Preliminary studies in Thailand indicated that crude extracts of this species inhibited growth of *Staphyloccous aureus* and *Escherichia coli* (Aung-aud-chariya *et al*., 2015). This study aimed to identify a suitable culture medium for studying antioxidant and antibacterial properties of the fungus.

Materials and methods

Microbial strain

Mycelial culture of *B. griseipurpureus* was obtained in 2016 from a basidiocarp collected from a mixed *Melaleuca leucadendron* and *Acacia mangium* forest in Trang province.

Culture media

Mycelium from a single isolate was produced on sterilized cellophane discs placed on the surface of different media in 90 mm diameter petri dishes (De Araujo and Roussos, 2002). The media tested were: Modified Melin Norkran medium (MMN), MMN with 10% fresh basidiocarp hot water extract (Xu *et al.*, 2008), Boletus medium (Ohta and Fujiwara, 2003), and Potato Dextrose Agar (PDA). Plugs of inoculum (6 mm in diameter) were cut from 21 days old colony grown on MMN medium and placed centrally on each medium. There were three replicate plates per medium. The petri dishes were wrapped with Parafilm and incubated at 27 ℃ in darkness.

Preparation and percentage yield of crude extract

Field-collected basidiocarps were dried at 45 ℃ for 16 hours, chopped in a Moulinex blender and 100 g soaked in 1300 mL of ethyl acetate or methanol at room temperature for one week, and then extracted using a rotary evaporator (IKA RV 10 digital). Yield was determined as follows:

$$\text{Yield (\%)} = \frac{\text{Weight of extract after drying x100}}{\text{Weight of milled mushroom soaked}}$$

DPPH assay

The DPPH assay was performed according to Tepe *et al*. (2005) with some modifications. Fifty microlitres of various concentrations of the extracts were added to 5 ml of a 0.004% methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition of free radicals by DPPH in percent (I%) was calculated as follows:

$I\% = (A_{blank} - A_{sample} / A_{blank})$ x10

Where A_{blank} = the absorbance of the control reaction

A_{sample} = the absorbance of the test compound.

Tests were carried out in triplicate. Absorbance decreases were calculated as DPPH values by comparing with standard curves created by vitamin C (5 - 50 μg/ml), and the results were reported as μg vitamin C equivalent per gram of fresh weight.

Ferric reducing antioxidant power (FRAP)

The FRAP assay was done according to Benzie and Strain (1996) with some modifications. The stock solutions included 300 mM acetate buffer (3.1 g $C_2H_3NaO_2$ $3H_2O$ and 16 mL $C_2H_4O_2$), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3$ $6H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3$ $6H_2O$ solution. Crude basidiocarp extracts (0.2 mL) were allowed to react with 6 mL of the FRAP solution for 8 min in the dark. Readings of the colored product [ferrous tripyridyltriazine complex] were then taken at 593 nm. The standard curve was linear between 5 and 45 μg/ml for gallic acid. Results are expressed in μg gallic acid equivalent per g dry sample. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.

Agar well diffusion

Two types of basidiocarp extract were tested by the agar well diffusion method. Two bacterial strains were used: *Enterococcus faecalis* TISTR 379 and *Pseudomonas aerunginosa* TISTR 2370. TISTR 379 was inoculated in Tryptic soy broth (TSB) while TISTR 2370 was inoculated in Nutrient broth, and both

were incubated at 37 ℃ for 18 - 24 hrs. They were diluted in 0.85% NaCl as compared with the McFarland No.0.5 standard, and evaluated by agar well diffusion using 6 mm diameter wells in Mueller Hinton Agar (MHA) and 80 µl of crude extract per well. The basidiocarp crude extracts were replicated. The positive control was Ampicillin. Data were analysed by ANOVA.

Minimal inhibitory concentration method

The resazurin solution was prepared using a 270 mg tablet in 40 mL of sterile distilled water which was vortexed to produce a homogenous solution. Then two sterile 96 well plates were prepared (Sarker *et al*., 2007). A volume of 100 µl test material in 10 % (v/v) DMSO (200 mg/ml) was used. The methanolic extract from dried basidiocarps was pipetted in the first row of the microtitre plate and 50 µl of normal saline was applied to all the other wells. For serial dilution, 50 µl of the crude methanolic extract was performed serially in descending concentration in each well. Then, 10 µl of resazurin indicator solution was added to each well followed by 30 µl of 3.3 x strength MHB broth, and finally 10 µl of bacterial suspensions (diluted in 0.85% NaCl as compared with McFarland standard No.0.5) was added to each well. The positive control was 0.0045 mg/ml of ampicillin. Plates were wrapped with shrink film and incubated at 37 ℃ for 18 - 24 hrs. The crude ethyl acetate extract assay was made using the same method.

Results and Discussion

Optimal medium for Boletus griseipurpureus

The dry weight of mycelium on four media are given in Table 1. Biomass was highest ($P<0.05$) on MMN with 10 % fresh basidiocarp extract from hot water, followed by MMN and Boletes medium. No growth occurred on PDA. Some ectomycorrhizal fungi produce vegetative mycelium on synthetic media containing high nutrients from natural components or the addition of micronutrients and vitamins. For example, *Lactarius insulus*, *Lactarius delicious* and *Boletus edulis* formed mycelia on media which contained 20% pine juice, potato juice media, and iron-magnesium agar (Xu *et al*., 2008). Mycelial formation of *Tricholoma matsutake* and *Rhizopogon roseolus* were enhanced on both MMN and potato dextrose agar with increased micronutrients and vitamins (Islam and Ohga, 2013). In the case of *Phlebopus portentosus*, the best media were modified Murashige and Skoog or MS and a fungal-host medium (Kumla *et al*., 2011). However, the presence of high

concentration of vitamins and hormones may stimulate enzymes that inhibit mycelial growth (Hatakeyama and Ohmasa, 2004), and this can vary with mushroom species. More work needs to be done to define suitable economic substrates for commercial inoculum production of *B. griseipupureus*.

Table 1. Dry weight of mycelial growth of *Boletus griseipurpureus* on four media after 21 days.

Type of medium	Dry weight (g)
MMN	0.012 $\pm$ 0.0015^{bc}
MMN+ 10% extract fresh Bg	0.0169 $\pm$ 0.0017^{c}
PDA	0^{a}
Boletes medium	0.008 $\pm$ 0.0054^{b}

Values are means (n = 3) with standard deviations. Subscripts with different common letters are significant by Duncan 's multiple range test (P<0.05).

Antioxidant activity

Percentage yield of crude methanolic extraction from dried basdiocarps was higher than the yield of crude ethyl acetate extraction. Antioxidant property of *B. griseipupureus* was determined by DPPH assay and Ferric Reducing Antioxidant power (FRAP) (Table 2). DPPH has been used extensively as a free radical to evaluate reducing substances (Cotelle *et al*., 1996). In the same solvent, the results varied according to the assay used. When the same assay was compared, the antioxidant activity of dried basidiocarp powder extracted with methanol showed the best activity. It has been reported that the antioxidant activity of plant materials was well correlated with the content of their phenolic compounds (Velioglu *et al*., 1998). Similarly, a mushroom phenolic compound has been found to be an excellent antioxidant and synergist that is not mutagenic (Ishikawa *et al*., 1984). This study confirmed antioxidant activity of *Boletus* spp. as reported by Yuswan *et al*. (2015) indicating that this mushroom species may be a good nutritional food for consumption. In Malaysia, *B. griseipurpureus* (Gelam mushroom) in Tok Bali, Kelantan, is considered to have medicinal properties useful for treating diabetes, cervical cancer and breast cancer (Muniandy *et al*., 2016) but scientific evidence is lacking. Basidiocarp extracts contained alkaloids, anthraquinones, flavonoids, reducing sugar, saponins, steroids and tannins. (Muniandy *et al*., 2016).

Table 2. Antioxidant activities of *Boletus griseipurpureus* extracts obtained using two solvents.

Solvent	% yield	Radical scavenging activity DPPH (µg vitamin C/g sample)	FRAP (µg gallic acid/g sample)	Sig.
Bg ETOAc	2.8719	77.959 $\pm$ 0.457	19.072 $\pm$ 0.047	*
Bg MeOH	6.2304	200.356 $\pm$ 4.490	65.351 $\pm$ 0.247	*
	Sig.	*	*	

*Statistical significance (P<0.05) by Independent-samples t test.

Antibacterial test

Antibacterial properties of *B. griseipupureus* were examined by agar well diffusion and minimal inhibition concentration or MIC method. The pathogenic *Enterococcus faecalis* and *Pseudomonas aeruginosa* display high susceptibility to antimicrobial agents (Alves *et al.*, 2012). The crude ethyl acetate extract from dried B. *griseipurpureus* revealed inhibition zones against *P. aerunginosa* TISTR 2370 (17.33$\pm$1.155 mm) and *E. faecalis* TISTR 379 (13.33$\pm$2.082 mm) (Table 3). By contrast, the crude methanolic extract only inhibited *P. aerunginosa* TISTR 2370 (15.33$\pm$0.577). Raw and fermented ethyl acetate extracts of *Lenzites quercina* inhibited *Pseudomonas aerunignosa* ATCC 27853 but the latter extract did not inhibit *Enterococcus faecalis* (Ogidi *et al.*, 2015). In a preliminary study, crude methanol extracts from dried B. *griseipupurues* inhibited *Staphyloccoccus aureus* ATCC 29523 and *Esherichia coli* ATCC 25922 (Aung-aud-chariya *et al.*, 2015).

Table 3. Inhibition of two basidiome extracts on two species of pathogenic bacteria

Bacterial strain	Inhibition zone of BgETOAC (200mg/ml) (mm) X$\pm$ SD	Inhibition zone of BgMeOH (200mg/ml) (mm) X$\pm$ SD
Pseudomonas aerunginosa TISTR 379	17.33$\pm$1.155^{b}	15.33$\pm$0.577^{ab}
Enterococcus faecalis TISTR 2370	13.33$\pm$2.082^{a}	0*

Values are means (n = 3) with standard deviations. Subscripts with different common letters are significant by Duncan's multiple range test (P<0.05). *No inhibition zone was observed. Hence, this treatment was excluded from one-way ANOVA (Gomez and Gomez, 1984).

Based on primary screening, the crude extract with the best activity was determined for minimum inhibitory concentration (MIC) value. The metanolic

extract of basidiocarp against *Pseudomonas aerunginosa* TISTR 379 with MIC had a value of 6.25 mg/ml by microtitre plate-based antibacterial assay but *Enterococcus faecalis* TISTR 2370 was not inhibited by the methanolic extract. The crude ethyl acetate extract had a MIC value of 0.78 mg/ml against *P. aerunginosa* and a MIC value 0.39 mg/ml against *E. faecalis* TISTR 2370. In another study, an aqueous methanolic extracts of wild *Fistulina hepatica, Ramaria botrytis* and *Russula delica* with MIC values of 20 mg/ml were capable of inhibiting *E. faecalis* (Alves *et al*, 2012). Against *E. faecalis*, the MIC values of methanolic and ethyl acetate extracts from *Terfezia boudieri* were 0.625 and 2.5 mg/ml, respectively, while against *P. aerunginosa* the two extracts had identical MIC values of 2 mg/ml. (Hamza *et al*., 2016). Some species of boletes such as *Boletus pseudocalopus* are inedible but have potential medical value due to the presence of chemicals that attack some human tumor cell lines (Kim *et al*., 2015).

Conclusion

Edible ectomycorrrhizal fungi have been widely studied as a source of food, but knowledge on other health benefits remains incomplete. *Boletus griseipurpureus* is a seasonally edible ectomycorrhizal mushroom that is popular in the diet of some people in the south of Thailand. The fungus can be grown on MMN agar medium containing 10 % hot water extract of fresh basidiocarps. This species was other usefulness further nutrition value. Laboratory tests have confirmed both antioxidant and antimicrobial properties of basidiocarp extracts. Further in depth studies are necessary to ascertain whether this species has potential for pharmacology.

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