Screening of anti-Aspergillus activities of edible wild and cultivated mushrooms from Northeast Thailand

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ABSTRACT: The antifungal activity of methanolic extracts from mushrooms was investigated. Ten edible wild mushroom species—*Amanita calyptroderma* Ark. et Bal., *Amanita princeps* Corner et Bas., *Astraeus odoratus*, *Boletus chrysenteron* Bull., *Boletus colossus* Heim., *Lentinus strigosus* (Schw.) Fr., *Russula alboareolata* Hongo, *Russula emetic* (Schaeff. ex Fr.) S.F. Gray., *Russula virescens* (Schaeff.) fr., *Termitomyces clypeatus* Heim and five cultivated mushroom species—*Auricularia auricula-judae* (Bull.), *Lentinus polychrous* Lev., *Lentinus squarrosulus* Mont., *Pleurotus sajor-caju* (Fr.) Sing, *Volvariella volvacea* (Bull. Ex.Fr.) Sing were selected to study. The antifungal activity from the methanol extracts of mushroom fruiting bodies were evaluated according to the agar well diffusion method on four *Aspergillus* spp.— *Aspergillus flavus, A. niger, A. ochraceus, A. paraciticus.* Agar well diffusion results were interpreted by measuring the zone of inhibition after incubation at 30°C for 48 hr on Potatoes dextrose agar. The inhibition fifteen mushroom tested, only *L. strigosus* and *B. colossus* show activities to inhibit the growth of *A. flavus* as clear zone was about 16.3 and 7.4 mm, respectively. However, none mushrooms tested showed inhibition against *A. niger, A. ochraceus* and *A. paraciticus*.

Keywords*:* mushroom extracts, antifungal activity, agar well diffusion method, *Aspergillus* spp.

Introduction

Aspergillus is a genera of fungi that distributes worldwide more than 180 species. Some *Aspergillus* species are human pathogens and some species can produce mycotoxin, a secondary metabolites contaminated in food that hazard to consumer (Hedayati et al., 2007). *Aspergillus flavus* and *A. paraciticus* can produce aflatoxins, a hepatocarcinegens in animal and humans (Guo et al., 1996). *A. niger* and *A. ochraceus* can produce ochratoxin A which causes various toxic effects, such as neurotoxicity, immunotoxicity, nephrotoxicity and genotoxicity (Sorrenti, et al., 2013; Soares et al, 2013). Prevention of food poisoning and spoilage mold is usually achieved by use of chemical preservatives which considered responsible for many carcinogenic and teratogenic attributes on human health (Mostafa et al., 2017). Moreover, growing problems with microbial resistance, the necessity to find a healthy safer, potentially effective and natural alternative preservatives is increased.

Among natural resources, mushrooms could be an alternative source of new antimicrobials. Kosanic et al. (2013) reported that methanolic extracts of the mushrooms *Amanita rubescens*, *Cantharellus cibarius*,

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Lactarius piperatus, *Russula cyanoxantha* showed to have antifungal activity against *A. flavus* and *A. fumigatus*. Gbolagade and Fasidi (2005) reported that the methanolic extract from the mushrooms *Corilopsis occidentalis* and *Daedalea elegans* showed weakly inhibited to *A. niger* and *A. flavus*. From our previous study, cultivated and wild mushroom showed to have phenol and flavonoid content (Srikram and Supapvanich, 2016) that involve antibacterial activity against foodborne pathogen; *E. coli*, *S. typhimurium*, *S. aureus* and *B. cereus* (Srikram et al., 2018). However antifungal activity were not investigated yet. Therefore, the objective of this work was to screen in vitro antifungal activity of fifteen mushrooms against *Aspergillus* spp.

Materials and Methods

Mushroom materials and preparation of mushroom crude extracts

The mushroom extracts were obtained from our previous study (Srikram et al., 2018) that reported on antibacterial activity of edible wild mushrooms and five commercial cultivated mushrooms. In brief, all mushrooms were purchased from a local market in Sakon Nakhon province, Thailand in 2016 (Table 1). Identification was done by comparing their morphological, anatomical and physiological characteristics and monographs with descriptions given in the manual (Royal academy, 2007), except Hed Phor Nung (*Astraeus odoratus*) was identified according to Phosri et al. (2004). Fruiting body of mushroom was cleaned of soil, washing with clean water and debris were removed using a sharp knife. Mushroom sample was cut and dry in hot air oven at 50 ^{o}C. Dried samples were ground to fine powder by blender. Twenty-five grams of mushroom powder were extracted by stirring with 200 ml of 95% (v/v) methanol in shaker at 150 rpm for 24 hr at room temperature (25±2 ^{o}C). The extracts were filtered through Whatman No. 4 paper. The extract was evaporated at 40 ^{o}C in vacuum dryer. After that, crude extract was dissolved in 5% dimethylsulfoxide (DMSO) to a final concentration of 500 mg/ml and filter-sterilization was done through a 0.45µm membrane filter. Then, the extracts were stored at -18 ^{o}C until used in the test.

Antifungal activity

Antifungal activities of methanol extracts were in vitro tested against four *Aspergillus* spp.— *A. flavus* TISTR 3135*, A. niger* TISTR 5136*, A. ochraceus* TISTR 3120,and *A. paraciticus* TISTR 3557. All the microorganisms used were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). Antifungal activity of mushroom extract was carried out by the agar well diffusion method (Oyetayo et al., 2009) with some modifications. Briefly, suspensions of fungal spores were prepared from fresh mature cultures (5 day old) that grew at 30 °C on Potatoes dextrose agar (PDA). Spores were rinsed with 1 ml of sterile distilled water (Kosanic et al., 2013). Determine the number of spore by direct count on Hemacytometer under microscopy and adjusted to 10^5 conidia/ ml in distilled water. Then, 1 ml of spore suspension (10^5 conidia/ ml) were poured plate on PDA. The inoculated plates were left for 30 minutes at room temperature. Small wells (8 mm in diameters) were made in the agar plates by sterile cork borer. One hundred microliters (equivalent to 50 mg) of the extract of each isolate of the mushroom was loaded into the different well. DMSO was used as negative control for test fungal. The plates were incubated at 30 °C and the inhibition zones

were measured after 48 hr (2 days) of incubation (Arikan et al., 2003). In this study, antifungal activity of each mushroom variety from three samples were analyzed separately in triplicate and the results were recorded as mean ± SD. The means diameter of inhibition zone of each mushroom variety were examined for significance difference using ANOVA with Duncan's significant difference post-hoc test at $P < 0.05$.

Results and Discussion

The results of in vitro anti-*Aspergillus* activity of ten edible wild mushrooms and five commercial cultivated mushrooms extracts (50 µg) were investigated by agar well diffusion method as shown in the Table 1 and Figure 1. In a negative control, 5% DMSO had no inhibitory effect on the tested fungi. Although, from our previous study, cultivated and wild mushroom showed to have phenol and flavonoid content (Srikram and Supapvanich, 2016) that involve antibacterial activity against foodborne pathogen; *E. coli*, *S. typhimurium*, *S. aureus* and *B. cereus* (Srikram et al., 2018). However, in this study they showed low antifungal activity against *Apergillus* spp. From fifteen mushroom test, only *L. strigosus* and *B. colossus* showed inhibitory effect on the growth of *A. flavus* and the clear zone were 16.3 and 7.4 mm, respectively. However, none of tested mushroom showed inhibition to *A. niger*, *A. ochraceus* and *A. paraciticus*. Nedelkoska et al. (2013) have been reported that the mushroom extracts exhibiting more potent

Table 1 Inhibition zone diameters of methanolic extracts (50 mg) from cultivated and edible wild mushrooms from Northeast Thailand.

	Mushroom species		Inhibition zone diameter ± SD (mm)			
	No.	Scientific name	*A. flavus*	*A. niger*	*A. ochraceus*	*A. paraciticus*
Wild mushrooms	(1)	*Amanita calyptroderma* Ark. et Bal.	-	-	-	-
	(2)	*Amanita princeps* Corner et Bas	-	-	-	-
	(3)	*Astraeus odoratus*	-	-	-	-
	(4)	*Boletus chrysenteron* Bull.	-	-	-	-
	(5)	*Boletus colossus* Heim	7.4±2.1[b]	-	-	-
	(6)	*Lentinus strigosus* (Schw.) Fr.	16.3±0.4[a]	-	-	-
	(7)	*Russula alboareolata* Hongo	-	-	-	-
	(8)	*Russula emetic* (Schaeff. ex Fr.) S.F. Gray.	-	-	-	-
	(9)	*Russula virescens* (Schaeff.) fr.	-	-	-	-
	(10)	*Termitomyces clypeatus* Heim	-	-	-	-
Cultivated mushroom	(11)	*Auricularia auricula-judae* (Bull.)	-	-	-	-
	(12)	*Lentinus polychrous* Lev.	-	-	-	-
	(13)	*Lentinus squarrosulus* Mont.	-	-	-	-
	(14)	*Pleurotus sajor-caju* (Fr.) Sing	-	-	-	-
	(15)	*Volvariella volvacea* (Bull. Ex.Fr.) Sing	-	-	-	-
		5% DMSO (negative control)	-	-	-	-

- = indicate no antifungal effect. Each value is a mean of three replicates ± Standard Error. In each row, different lower case superscript letters in the same column indicate a statistical difference at $P<0.05$.

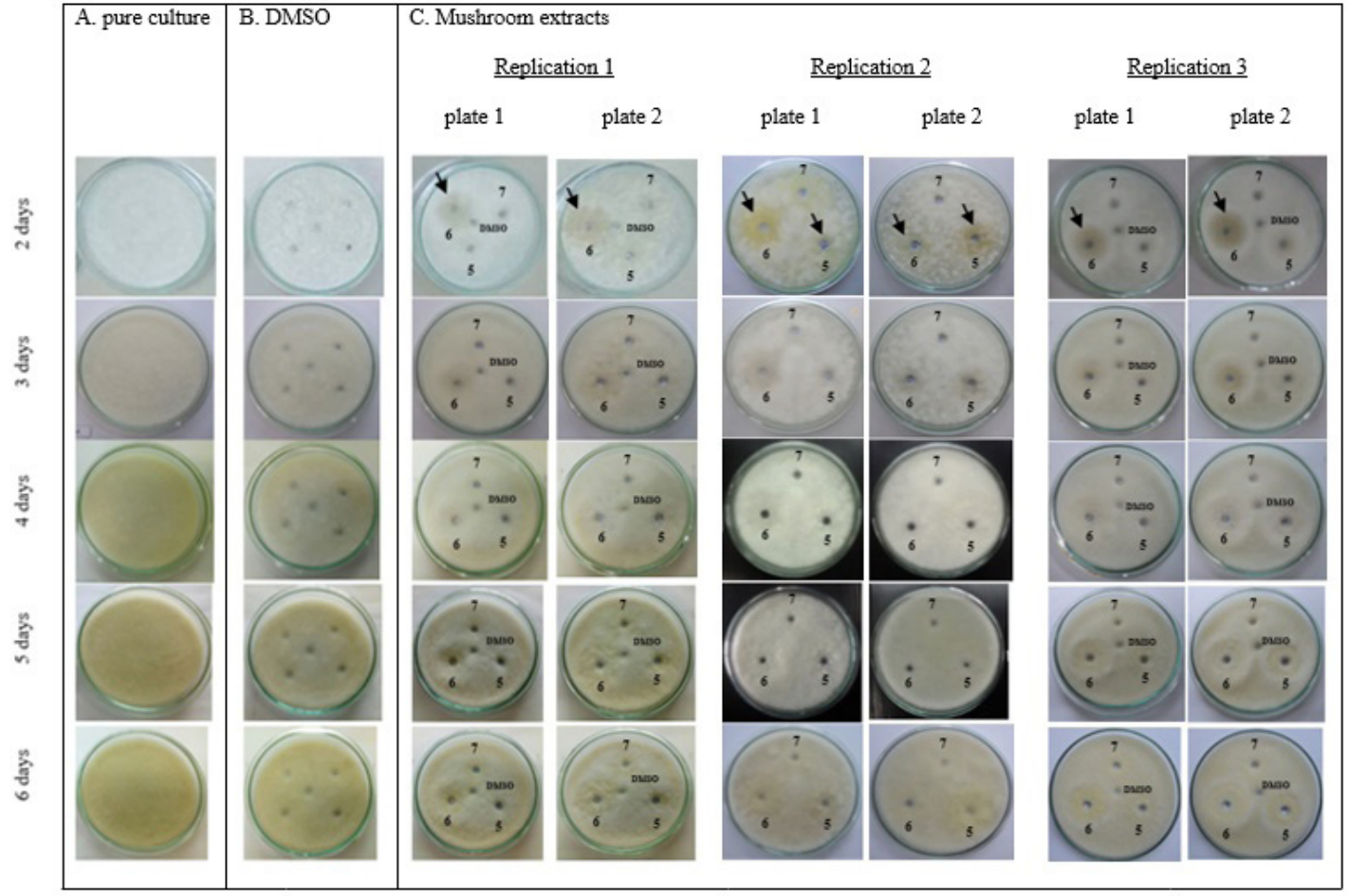

Figure 1 Inhibition zone of mushroom extracts against A. flavus by agar-well diffusion method. The growth of A. flavus on PDA at 30 ˚C for 2 days to 6 days (A). The well were loaded with 100 µl of 5% DMSO (B) and mushroom extracts (C): B. colossus (5); L. strigosus (6); R. alboareolata (7). The inhibition zone (↓) diameter were measure at 2 days after incubation. All the tests were carried out in triplicates (n=3) with two plate.

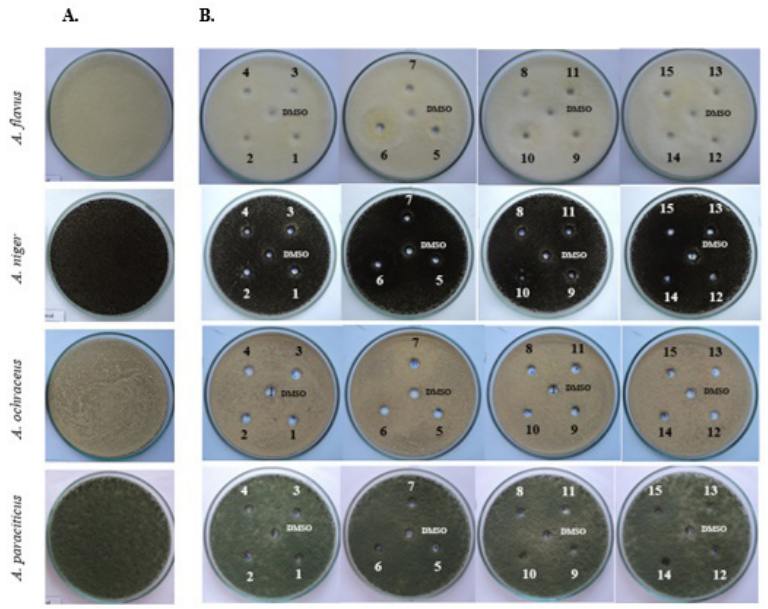

Figure 2 In vitro antifungal activity of mushroom extracts by agar well diffusion method against *Aspergillus* spp. on PDA after incubated 30 °C for 6 days. The growth of *Aspergillus* spp. on PDA after incubated 30 °C for 6 days (A). The well (8 mm in diameter) were loaded with 100 µl (equivalent to 50 mg) of mushroom extracts: (1) *A. calyptroderma*; (2) *A. princeps*; (3) *A. odoratus*; (4) *B. chrysenteron*; (5) *B. colossus*; (6) *L. strigosus*; (7) *R. alboareolata*; (8) *R. emetic*; (9) *R. virescens*; (10) *T. clypeatus*; (11) *A. auricula-judae*; (12) *L. polychrous*; (13) *L. squarrosulus*; (14) *P. sajo-cajur*; (15) *V. volvacea* (B). One hundred microliters of 5% dimethyl sulfoxide (DMSO) were used for a negative control in each plate.

inhibitory effects on the growth of bacteria than on fungi. This observation may be explained by the differences in cell wall of component between bacteria and fungi (Van, 2001; Farkas, 2003).

There were many in vitro methods to evaluating antimicrobial activity such as diffusion methods—agar disk diffusion method, agar well diffusion method, agar plug diffusion method, antimicrobial gradients method, cross streak method, poisoned food method or dilution method —broth dilution methods, agar dilution method (Balouiri et al., 2016). In this study we used agar well diffusion method to evaluating the anti-*Aspergillus* activity of mushroom.From our finding suggests that, the inoculum size of 10^5 conidia/ml in to 20 ml of PDA after incubation at 30 °C for 48 hr, *Aspergillus* showed the sufficient growth (Figure 1) for interpreted by measuring the zone of inhibition. And notice that after 72 hr (days 3 to day 6) of incubation, the inhibition zones were hardly observed as overgrowth of the fungal (Figure1) and after 6 day of incubation all *Aspergillus* spp. tested were overgrowth cover the clear zone as showed in figure 2. Arikan et al. (2003) and Qiao et al. (2007) were study in vitro anti-*Aspergillus* activity of medicine by disk diffusion methods were also evaluated the inhibition zone after incubate at 35 °C for 24 and 48 hr. Therefore, we suggest that, the agar well diffusion method for investigate the inhibitory effect of *Aspergillus* spp. should measure the zone of inhibition within 48 hr after incubation at 30 °C

Conclusions

Our results showed that from fifteen mushrooms test, only *Lentinus strigosus* (Schw.) Fr. and *Boletus colossus* Heim has antifungal activity against *A. flavus* in vitro.

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