

In vitro Antifungal Activities and Phytochemical Analysis of Filamentous White-rot Fungi, *Schizophyllum commune*

(Aktiviti Anti-kulat Pereput *In vitro* dan Analisis Fitokimia bagi Kulat Pereput Putih Berfilamen, *Schizophyllum commune*)

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ABSTRACT

In this study, the in-vitro antifungal activity and phytochemical analysis of Schizophyllum commune extracts have been investigated. The antifungal activity was tested against 11 species of selected wood degrading fungi of rubberwood. The results showed that water, methanol and ethanol extracts significantly inhibited the growth of wood degrading fungi with minimum inhibitory concentration (MIC) ranges 0.16-5.00 µg/µL. P. sanguineus was found as the strongest wood degrading fungus where it required the highest concentration of S. commune crude extracts (≥ 5.00 µg/µL) to inhibit its mycelia growth. Phytochemicals analysis revealed that the extracts contained flavonoid, phenol and saponin. The methanol extracts of S. commune was then applied on the rubberwood blocks and found that the growth of P. sanguineus was inhibited effectively at 5.00 µg/µL.

Keywords: Antifungal activity; minimum inhibitory concentration (MIC); rubberwood block; Schizophyllum commune; wood decaying fungi

ABSTRAK

Dalam kajian ini, aktiviti anti-kulat in vitro dan analisis fitokimia bagi kulat Schizophyllum commune telah dikaji. Aktiviti anti-kulat telah diuji ke atas 11 spesies kulat pereput kayu yang terpilih. Keputusan menunjukkan ekstrak air, metanol dan etanol boleh merencat pertumbuhan kulat pereput kayu dengan kepekatan rencatan minimum (KMM) di dalam julat 0.16-5.00 µg/µL. P. sanguineus telah dikenal pasti sebagai kulat pereput kayu yang terkuat kerana ia memerlukan kepekatan ekstrak kulat S. commune yang tinggi (≥5.00 µg/µL) untuk merencat pertumbuhan misilianya. Analisis fitokimia pula menunjukkan yang ekstrak ini mengandungi flavanoid, asid karbolik dan saponin. Ekstrak methanol daripada kulat S. commune ini juga telah diuji keberkesanannya ke atas blok kayu getah dan didapati kulat P. sanguineus telah terencat secara efektif pada 5.00 µg/µL.

Kata kunci: Aktiviti antikulat; blok kayu getah; kepekatan merencat minimum (KMM); kulat pereput kayu; Schizophyllum commune

INTRODUCTION

Natural resources are a gift for the synthesis of simple and effective antifungal which can provide new insights into potentially useful targets. Screening of such resources has had an impressive tool of determining bioactive agents, in which those results showed that the available biodiversity of natural sources and the isolation of bioactive compounds act as the potential ways for the development of clinical useful drugs (Orhan & Sener 2006; Shu 1998).

Plants have always been a common source for medicines, insecticides and pesticides, either in the form of traditional preparations or as pure active principles (Adisewojo et al. 1984). Previous report revealed that plants has limitless ability to synthesize aromatic secondary metabolites, most of which were phenols or their oxygen substituted derivatives, such as phenols, phenolic acids, flavones, flavonoids, flavonols, quinones, tannis and coumarins, in which these compounds

possessed antimicrobial effect and served as plant defense mechanisms against pathogenic microorganisms (Das et al. 2010; Ncube et al. 2008). Hence, it is reasonable for pharmaceutical company to spend a lot of time developing these natural products in developing more affordable and cost-effective remedies. However, some traditional used plants might disappear due to over exploitation and therefore the sustainable usage might concern the public (Farnsworth et al. 1985).

Filamentous fungi were major sources of bioactive secondary metabolites and researches have established the existence of biochemical pathways solely for the purpose of producing mycotoxins and other natural products in fungi through the study of ecological chemical interactions (Frisvad et al. 2008). In this approach, fungi have been widely applied in agriculture as bio-control for pest management. During the idiophase of fermentation (later stage of microbial growth), the most common secondary

metabolites occurred were antibiotics and others include mycotoxins and ergot-alkaloids, the widely used immune-suppressant cyclosporine and fumigillin as an inhibitor of angiogenesis and a suppresser of tumor growth (Nigam 2009). According to Reddy and Mathew (2001), there has been much interest in the possible use of wood-rotting fungi as biodegradation agent, particularly on white rot fungi.

White-rot fungi that belongs to the basidiomycetes were the most efficient and had extensive lignin degradation capabilities (Howard et al. 2003; Pointing 2001). These fungi can be used as active ingredients for bio-herbicides, bio-insecticides and biofungicides products (Bennett et al. 2001). The bioactive metabolites that were present in most fungi includes phenolic compounds, flavonoids and polysaccharides. It is necessary to explore, identify, conserve and utilize the vast use of fungal diversities for the benefit of mankind, in particular and the mycobiota as well as environmental.

Schizophyllum commune is a white rot fungus belonging to the Schizophyllaceae of Agaricales. It is one of the most commonly found fungus and can be isolated on all continents except for Antarctica. It was also known as a potential producer for different metabolites during submerged cultivation (Teoh et al. 2012). But, its exact mode of action has not yet been elucidated. Thus, the present work was carried out with the aim of determining the *in-vitro* antifungal properties as well as the phytochemicals analysis from the extracts of *S. commune*. The effects of its extractant on commonly found wood degrading fungi in Malaysia were also looked at.

MATERIALS AND METHODS

FUNGAL STRAIN

The wild strain of white rot fungus, *Schizophyllum commune* (*S. commune*) was obtained from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute of Malaysia (FRIM), Kepong, Malaysia. The stock culture was grown on malt extract agar (MEA) (containing 30 g/L malt extract, 3 g/L peptone, 15 g/L agar-agar, pH5.6) at 30°C and maintained on agar slants before subsequent studies.

PREPARATION OF MYCELIA SUSPENSION

Mycelia suspension was prepared by suspending mycelia discs from 7 days old culture plates in sampling bottles containing sterilized distilled water and 0.1% (v/v) Tween 80. The disc of 5 mm diameter was cut on the mycelia mats of the agar plate using a sterilized cork borer. A total of 10 discs for every 100 mL sterilized water were vortexed for 5 min in order to homogenize the mycelia suspensions.

PREPARATION OF MYCELIA EXTRACT

A total of 10 mL (10% v/v) mycelia suspension was added to 90 mL of nutrient medium (containing 26.9 g/L yeast

extract, 10.0 g/L malt extract, 49.2 g/L glucose, 1.0 g/L KH_2PO_4 , 1.0 g/L K_2HPO_4 , 0.93 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.0 g/L $(\text{NH}_4)_2\text{SO}_4$, pH 6.5) in 250 mL Erlenmeyer flasks. The medium was sterilized at 121°C for 15 min before transferring the mycelia suspension into the culture media. The culture was incubated at 30±2°C in an incubator shaker at 200 rpm for 5 days. The culture broth was then harvested and centrifuged at 4000 × g for 15 min. The residues were then dried and homogenized before the extraction process. Meanwhile, the supernatant was evaporated using a rotary evaporator and the residues were maintained in vacuum until the extraction process was carried out.

PREPARATION OF WATER EXTRACT

Dry residues (100 g) obtained from the mycelia (biomass) were boiled in distilled water for 48 h in a ratio of 1 g: 20 mL. The crude extract was then dried and kept at 4°C for further analysis. A similar procedure was also carried out for the extraction using methanol and ethanol.

WOOD DEGRADING FUNGI USED

The selected wild species of wood degrading fungi: *Earliella scabrosa*, *Gloeophyllum trabeum*, *Lentinus* sp., *Lentinus sajor-caju*, *Lentinus strigosus*, *Microporus affinis*, *Microporus xanthopus*, *Pycnoporus sanguineus*, *Trametes versicolor*, *Trametes feei* and (11) *Trametes menziesii* were collected from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. All stock cultures were grown at 30°C and maintained on malt extract agar slant for subsequent studies.

SCREENING OF ANTIFUNGAL ACTIVITY

Screening of antifungal activity of *S. commune* extract (tested concentration of 5 µg/µL) was carried out using two methods, which were minimum inhibitory concentration (MIC) assay (Teoh et al. 2012) and poison food technique (Das et al. 2010). All experiments were carried out in triplicate.

PHYTOCHEMICAL SCREENING ANALYSIS

An analysis of phytochemicals from the solvent free extract of mycelium was carried out using various qualitative tests for alkaloids (Mayor's test), flavonoids (Alkaline reagent test), phenols (Ferric chloride test) and saponins (Froth test) using the method as described by Tiwari et al. (2011).

EFFECTIVENESS OF ANTIFUNGAL ACTIVITY ON RUBBERWOOD BLOCK

The rubberwood block treating procedure was carried out according to ASTM D4445 (2003). Each fungicide was evaluated using at least five concentrations. The prepared solutions were then poured into beakers containing the specimens. Similarly, the control specimen was treated with distilled water. After 5 min, the solution was poured

out and the beakers were tightly covered and stored overnight. This would allow the draining of excess solution and time for the fungicides to be deposited onto the wood before inoculation. After overnight storage, the specimens were placed into prepared petri dishes and inoculated with selected wood decaying fungi. They then were incubated at room temperature for 4 weeks.

RESULTS AND DISCUSSION

ANTIFUNGAL ACTIVITIES OF *S. COMMUNE* EXTRACTS AGAINST SELECTED WOOD DEGRADING FUNGUS

The filamentous fungus, *S. commune* produced metabolites that possessed antifungal activity (Teoh et al. 2012). In this study, the tested *S. commune* extract concentration was set at 5 µg/µL, in which was similar to the commercial level. Minimum inhibitory concentration (MIC) assay was used as an antifungal activity method in this study. The MIC was defined as the lowest concentration of fungal mycelia extract to which no growth of wood degrading fungi was observed after the incubation period (Das et al. 2010). The value of the MIC for antifungal agents represents the ability of the active compounds of *S. commune* in the minimum quantity that can suppressed the growth of wood decaying fungi as shown in Table 1. On the other hand, food poison technique has been also used generally for antifungal activity determination. The percentage inhibition of mycelia growth were recorded and also reported in Table 1.

In this present study, the antifungal activities of the crude extract of *S. commune* against the selected wood degrading fungi were found to be in the range of 0.16 to 5.00 µg/µL based on the MIC values (Table 1). This was in agreement with the data obtained by Jayakumar et al. (2010), who found that the antibacterial activity was in the range of 3.00 and 8.00 µg/µL using various analytical methods such as disc diffusion assay, MIC and a minimum bactericidal concentration assay. Among the wood degrading fungi tested, *P. sanguineus* showed the lowest growth inhibition susceptibility to both water and ethanol extracts of *S. commune*, in which the MIC values were >5.00 µg/µL, respectively. In fact, the methanolic extract from *S. commune* effectively reduced the growth of *P. sanguineus* with MIC value of 5.00 µg/µL (Table 1). Meaning that, the methanol extracts provided better antifungal activities as compared to water and ethanol extract against the growth of *P. sanguineus*. According to Margaritis and Jajuee (2007), methanol is a strong solvent and exhibited as the smallest alcoholic molecule, that was able to undergo more complete extraction reaction as compared to water to obtain the less polar compound. In addition, a group of saponins might be produced during the methanol extraction process (Masoko & Eloff 2006; Teoh et al. 2012), which might exhibit higher toxicity against fungi, thus possessing better antifungal activities.

PHYTOCHEMICAL ANALYSIS OF *S. COMMUNE* MYCELIUM EXTRACT

Phytochemicals were bioactive compounds found in plants that work with nutrients and dietary fibre to protect against diseases (Agbafor & Nwachukwu 2011). Plants had limitless ability to synthesize aromatic secondary metabolites, most of which were phenols or their oxygen substituted derivatives, such as phenols, phenolic acids, flavones, flavonoids, flavonols, quinones, tannis and coumarins, in which these compounds possessed antimicrobial effect and served as plant defense mechanisms against pathogenic microorganisms (Das et al. 2010; Ncube et al. 2008). Javale and Sabnis (2010) stated that the flavonoids and saponins extracts from plants exhibited better inhibitory as compared to that of phenol which also showed inhibition, but to a lesser extent. This phenomenon can be explained by the fact that flavonoid and saponins were synthesized by plants in response to microbial infection; therefore their *in-vitro* antimicrobial activities are justified (Cowan 1999; Das et al. 2010; Ncube et al. 2008).

As in this study, all the eleven species of wood degrading fungi were found to be inhibited by the methanolic extract from *S. commune* mycelium (Table 1). So further attempt was made to determine phytochemicals such as alkaloids, flavonoids, phenols and saponins that were not only found in plants but also could be present in the fungal extract. The preliminary analysis of the extracts in Table 2 showed that it contained flavonoids, phenols and saponins but not alkaloids. Thus showing that fungi also produced secondary metabolites during their growth in shake flask culture in this study; and this was related to the antifungal activities that were explained previously. Due to that, further effectiveness study of *S. commune* methanol extract on rubberwood was carried out in subsequent studies.

EFFECTIVENESS OF *S. COMMUNE* METHANOL EXTRACT ON RUBBERWOOD BLOCK

Based on the MIC result (Table 1), *P. sanguineus* served as the strongest wood degrading fungus where it required the highest concentration of *S. commune* methanol extract in order to inhibit its mycelia growth, thus it was chosen in the effectiveness study. This tested species was also unique in its ability to completely degrade all components of lignocellulosic materials (Teoh et al. 2011).

Figure 1 shows the growth of *P. sanguineus* on rubberwood blocks treated with 5 sets of mycelium extract of *S. commune* at concentration ranges 0 – 5.00 µg/µL and incubated for 0, 7, 21 and 30 days. A significant growth of *P. sanguineus* mycelia on the rubberwood blocks surface were observed, especially for the untreated block (Control) (Figure 1(b)-1(d)). After 30 days of incubation, it was found that no growth was seen on the rubberwood block treated with 5.00 µg/µL extractant, as shown in Figure 1(d). This result was in good agreement with the data obtained in Table 1, which was explained previously. Thus showing that *P. sanguineus*, one of the wood degrading fungi, very common on dead trees and played an important ecological

TABLE 1. Antifungal activity from crude extract of *Schizophyllum commune* toward selected wood-degrading fungi

Wood-degrading fungi	Minimum Inhibitory Concentration, MIC ($\mu\text{g}/\mu\text{L}$)			Mycelia Inhibition (%)		
	Water Extract	Methanol Extract	Ethanol Extract	Water Extract	Methanol Extract	Ethanol Extract
(1) <i>Earitella scabrosa</i>	5.00	1.25	2.50	99.4%	100.0%	100.0%
(2) <i>Gloeophyllum trabeum</i>	5.00	2.50	2.50	99.5%	100.0%	100.0%
(3) <i>Leninus</i> sp.	0.31	0.16	0.16	100.0%	100.0%	100.0%
(4) <i>Leninus sajor-caju</i>	2.50	1.25	1.25	100.0%	100.0%	100.0%
(5) <i>Leninus strigosus</i>	5.00	2.50	2.50	99.5%	100.0%	100.0%
(6) <i>Microporus affinis</i>	0.61	0.31	0.31	100.0%	100.0%	100.0%
(7) <i>Microporus xanthopus</i>	0.61	0.31	0.31	100.0%	100.0%	100.0%
(8) <i>Pycnoporus sanguineus</i>	>5.00	5.00	>5.00	0.2%	100.0%	1.5%
(9) <i>Trametes versicolor</i>	5.00	1.25	2.50	99.8%	100.0%	100.0%
(10) <i>Trametes feei</i>	5.00	1.25	2.50	99.7%	100.0%	100.0%
(11) <i>Trametes menziesii</i>	5.00	0.31	2.50	99.7%	100.0%	100.0%

*Tested concentration was at 5.0 $\mu\text{g}/\mu\text{L}$. Activity concentration: weak activity: MIC > 5.0 $\mu\text{g}/\mu\text{L}$; moderate activity: 1.0 $\mu\text{g}/\mu\text{L}$ < MIC \leq 5.0 $\mu\text{g}/\mu\text{L}$; strong activity: MIC \leq 1.0 $\mu\text{g}/\mu\text{L}$.

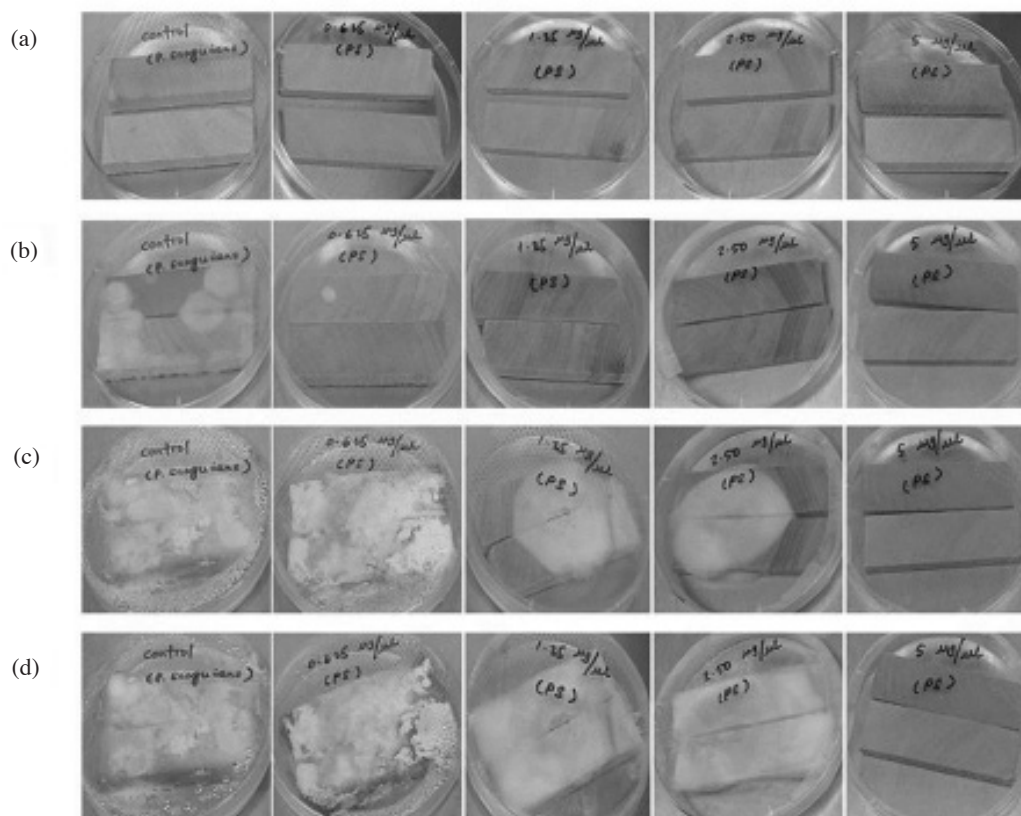


FIGURE 1. Growth of *Pycnoporus sanguineus* on rubberwood treatment using five set concentration of *Schizophyllum commune* mycelia extract for (a) 0 day, (b) 7 days, (c) 21 days and (d) 30 days

TABLE 2. Qualitative analysis of phytochemicals from methanol extract of *Schizophyllum commune* mycelia

Alkaloids	Flavonoids	Phenols	Saponins
-	+	+	+

role in degrading woody forest litter, failed to grow with the concentration of *S. commune* methanol extract at 5.00 µg/µL of the treated rubberwood blocks.

CONCLUSION

The methanolic extract of *Schizophyllum commune* mycelium provide the most efficient antifungal activity towards the selected wood decaying fungi used in this study with the range of minimum inhibitory concentration values from 0.16 to 5.00 µg/µL. The phytochemicals study indicated the presence of flavonoids, phenols and saponins in the extractant, but none for alkaloids. The effectiveness of antifungal activity produced by this fungus was also tested using rubberwood blocks with *P. sanguineus*, incubated from 0 to 30 days. The results were significantly observed and agreed well with the experimental data of the *in-vitro* antifungal properties. Thus showing that the presence of the secondary metabolites at higher concentration might inhibit the growth of the wood degrading fungus, *P. sanguineus*.

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