

MEDIA SELECTION FOR MYCELIA GROWTH, ANTIFUNGAL ACTIVITY AGAINST WOOD-DEGRADING FUNGI, AND GC-MS STUDY BY *PYCNOPORUS SANGUINEUS*

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Wood-decaying fungi present a serious threat to items made from rubberwood (*Hevea brasiliensis*). Though conventional chemical control has been a successful method for preserving wood against stain and decay fungi growth, the effects of these chemicals are of concern because they create problems for the environment and public health. *Pycnoporus sanguineus* (*P. sanguineus*), is a white-rot fungus that invades wood during its growth, storage, or use, causing decay or other property changes. It was considered in this work as a potential source of bioactive compounds and investigated for its natural antifungal activity using a minimum inhibitory concentration assay against wood-degrading fungi. It was found that media consisting of 10.0 g/L malt extract, yeast extract, dextrose, and maltose, respectively at pH 4.7±0.2 provided the highest biomass production by *P. sanguineus*. Results showed that the antifungal properties of methanol and water extract of *P. sanguineus* mycelia and supernatant ranged from MIC values of 0.1 to 5.0 µg/µL. 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP) was found to be the major component in the extract of this fungus, based on analysis using gas chromatography - mass spectrometry.

Keywords: Wood-degrading fungi; *Pycnoporus sanguineus*; Antifungal activity; 4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP)

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INTRODUCTION

Rubberwood is the wood from a para-wood species (*Hevea brasiliensis*). Its structural elements consist of 61.5% fiber, 9.5% vessels, and 29.0% parenchyma cells. The biodegradation problem is one of the main reasons why rubberwood has been less attractive for wood processing industries and has been almost neglected in the past, although it has been abundant in supply and readily available (Hong 1995; Killmann 2001). Most of the basidiomycetes, such as *Schizophyllum commune*, *Lentinus sajor-caju*, *Trametes versicolor*, *Microporus xanthopus*, *Gloeophyllum trabeum*, etc., are normally associated with the white-rot of living trees and on logs, dead twigs, and stumps; such fungi degrade wood, leading to economic losses in forestry (Quiroga et al. 2001; Zjawiony 2004). The high carbohydrate (sugar and starch) reserves deposited in the parenchyma and the lack of phenolic compounds in the wood are the major factors governing the high decay susceptibility of rubberwood (Salmiah 1997). Wood-decaying

fungi that cause severe breakdown of wood are classified either as brown-rot and white-rot (generally caused by basidiomycetes) fungi or as soft-rot (caused by ascomycetes and dueteromycetes as imperfect fungi) fungi (Hong et al. 1999). Although chemical agents are commonly employed to control wood degradation caused by stain and decay fungi, the application of chemical fungicides has become limited due to the requirement for low pesticide residues in products and to the increasing problems associated with resistance to the active principles (Mommaerts et al. 2008). According to Kinay et al. (2007), several synthetic fungicides have been removed from the market due to possible toxicological risks. Therefore, there is a significant requirement for the development of new and more environment-friendly formulations that reduce synthetic fungicide applications and minimize the pressure for resistance development.

Antifungal secondary metabolites isolated from the heartwood of plants have been considered to contribute to trees' resistance against wood-degrading fungi (Quiroga et al. 2001). There is a paucity of information on the use of the extracts of herbs for the control of wood degradation by wood-rot fungi. As reported by Ejechi (2001), the leaf extract from *Acalypha hispida* (Euphorbiaceae), a herbaceous ornamental plant, contains phenolic compounds that are capable of antagonizing wood-rot fungi. On the other hand, Verma et al. (2007) claim that *Trichoderma* spp. is currently the most extensively investigated biocontrol fungi for forest product preservation, particularly in protecting wood against basidiomycetes. As an example, Ejechi (1997) investigated the ability of *Trichoderma viride* to retard the decay of obeche (*Triplochiton scleroxylon*) wood in the field for 11 months covering dry and wet seasons in a tropical environment. The result obtained showed that the *T. viride* exhibited total inhibition of the decay by *Gloeophyllum sepiarium* and *Pleurotus ostreatus* through the means of mycoparasitism and competition for nutrients.

Filamentous fungi are a major source of bioactive secondary metabolites. Through the study of ecological chemical interactions, researchers have established the existence of biochemical pathways with the sole purpose of producing mycotoxins and other natural products in fungi (Frisvad et al. 2008). In this approach, fungi have been widely applied in agriculture as biocontrol for pest management. According to Reddy and Mathew (2001), there has been much interest in the possible use of wood-rotting fungi as a biodegradation agent, particularly in the case of white-rot fungi. White-rot fungi are a physiological rather than a taxonomic grouping, belonging to the basidiomycetes group, and they exhibit the most efficient and extensive lignin degradation (Pointing 2001). These can be used as the active ingredients of bioherbicide, bioinsecticide, and biofungicide products (Bennett et al. 2001). Exploration, conservation, and utilization of the fungi belonging to the basidiomycetes group can be expected to prove beneficial for humankind, as well as the environment.

Pycnoporus sanguineus is a white-rot fungus, also of a slow-growing saprophytic nature, belonging to the basidiomycetes of the family of *Polyporaceae* (Smania et al. 1995; Siqueira et al. 1997). It is unique in its ability to completely degrade all components of lignocellulosic materials. The ability of this fungus to degrade lignin, as well as polysaccharides found in the cellulose and hemicelluloses, indicates that it has the potential not only to act as a biological pretreatment, but also to degrade all major components of lignocelluloses and yield valuable products (Park et al. 2006; Teoh and

Mat Don 2011). Recently, production of enzymatic activity has been shown (e.g., CMCase, FPase, and β -glucosidase) when *P. sanguineus* was grown in a suitable culture medium (Teoh and Mashitah 2010). In fact, it was reported that *P. sanguineus* had produced laccase as the sole lignolytic enzyme to degrade selected agro-residue in solid-state fermentation (Teoh and Mat Don 2011). On the other hand, this fungus is very common on dead trees and plays an important ecological role in degrading woody forest litter (Djarwanto and Tachibana 2009). Meanwhile, the antimicrobial activity of *P. sanguineus* had been known since 1946, when poliporin was isolated against gram-positive and gram-negative bacteria and shown to be non-toxic to experimental animals (Rosa et al. 2003). The basidiomes of *P. sanguineus* had already been in use for a long time by Brazilian indigenous people to prevent haemorrhages and by American and African indigenous tribes for treatment of several ailments (Smania et al. 1995; Rosa et al. 2003).

Thus, the present study is aimed to determine the nutritional requirement of *P. sanguineus* strains for mycelia growth and to provide information regarding its growth profile. The ability of a compound extracted from *P. sanguineus* culture to inhibit the growth of various wood-degrading fungi species will also be identified.

MATERIALS AND METHODS

Fungal Strain

The fungal strain, *P. sanguineus* was obtained from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. This stock culture was grown on agar slants comprised of 20 g/L glucose, 10 g/L yeast extract, and 10 g/L malt extract agar. The pH of the media was adjusted to 7.0 ± 0.2 prior to autoclaving at 121°C for 15 minutes. The culture was grown at 30°C and maintained on agar slants prior for subsequent studies.

Wood-degrading Fungi Used

Selected wood-degrading fungal strains of *Trametes versicolor*, *Trametes feei*, *Trametes menziesii*, *Lentinus* sp., *Lentinus sajor-caju*, *Lentinus strigosus*, *Microporus affinis*, *Microporus xanthopus*, and *Gloeophyllum trabeum* were obtained from the Biocomposite and Protection of Timber Forest Products Laboratory, Forest Research Institute Malaysia (FRIM), Kepong, Malaysia. The stock cultures were grown at 30°C and maintained on malt extract agar slants for subsequent studies.

Mycelial Suspension Preparation

A 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 of sterilized water were vortexed for 5 minutes in order to make the mycelial suspensions homogenous.

Mycelial Extract Preparation

Ten milliliters (10% v/v) of the mycelia suspension was added to 90 mL of medium containing different typical compositions in 250 mL Erlenmeyer flasks. The medium was autoclaved at 121°C for 15 minutes before transferring the mycelia suspension into the culture media. The culture was shaken at 200 rpm in an incubator shaker at 30±2°C for 5 days. The culture broth was then harvested and centrifuged at 4000 x g for 15 minutes. The residue (also known as mycelia biomass) was then dried and homogenized prior to 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.

Effect of Different Media on *P. sanguineus* Growth in Shake Flask Culture

The effect of different growth media on growth of *P. sanguineus* was studied under shake flask culture conditions. The medium compositions are listed in Table 1. The medium (90 mL) was adjusted to pH 4.7±0.2 using 1M NaOH and 1M HCl and incubated with 10 mL fungal mycelia suspension for 5 days at 30±2°C and 200 rpm. The fungal biomass was vacuum-filtered and dried at 60°C in an oven for 24 hours until constant weight was achieved. The biomass concentration was determined as shown in Eqn. (1).

$$\text{Mycelia dry weight (g/L)} = \frac{\text{g(dried mycelia filter paper)} - \text{g(filter paper)}}{\text{Volume measure (L)}} \quad (1)$$

A control experiment was also carried out using only distilled water as the medium.

Table 1. Composition of Different Media for Growth of *P. sanguineus* in Shake Flask Culture

Composition	Medium 1 (g/L)	Medium 2 (g/L)	Medium 3 (g/L)	Medium 4 (g/L)	Medium 5 (g/L)
Malt extract	-	-	10.0	10.0	10.0
Yeast extract	10.0	10.0		10.0	10.0
Peptone	3.0	3.0	3.0	-	-
Dextrose	-	-	10.0	-	10.0
Glucose	20.0	20.0	-	20.0	-
Maltose	-	-	10.0	-	10.0
KH ₂ PO ₄	-	0.5	0.5	-	-
MgSO ₄ ·7H ₂ O	-	0.5	0.5	-	-

Batch Solvent Extraction Process

A hydrodistillation process was carried out. Dry residues (100 g) either obtained from the mycelia or supernatant were boiled in water and methanol (ratio 1 g: 20 mL) for 48 hours. The percentage yield for water and methanol was about 1.21% and 2.95%, respectively. Then, the crude extract obtained was dried and kept at 4°C for further analysis.

Minimum Inhibitory Concentration (MIC) Assay

A broth dilution method using a 96-well microtitre plate was employed for minimum inhibitory concentration (MIC) determination. Six different concentrations of each of the extracts were prepared ranging from 0.1 to 5 µg/µL. Briefly, the wood-degrading fungi mycelia suspension of each strain was prepared and standardized according to 0.5 McFarland standard turbidity. Then, the crude extract obtained was dissolved into 5% dimethyl sulfoxide (DMSO). Each well contained malt extract broth (Media 5, Table 1) with 0.09 mL volume of serially diluted crude extracts. Each well was inoculated with the mycelial suspension of wood-degrading fungi in a final volume of 0.1 mL. The control contained only malt extract in the medium. Incubations were performed at 30°C for 48 hours. The effectiveness of inhibition was quantified by adding 0.02 mL of the yellow tetrazolium MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) reagent into each well. The results were obtained after an incubation period of 2 hours. Formation of a blue colour indicated fungal growth, while clear colour of the well indicated growth inhibition.

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. DMSO at the concentrations used did not interfere with the reaction conditions (Slanc et al. 2004).

Analytical method

The crude extract was dissolved with 70% (v/v) ethanol prior to the gas chromatography mass spectrometry (GC-MS) analysis. In this study, a GC-MS was used to analyze the sample qualitatively by referring to the molecular weight of the compounds in a library (Model: NIST), which was incorporated into it. The gas chromatography analyses were performed using a Perkin Elmer Clarus 600 gas chromatograph equipped with an ELITE-5 column. The gas chromatograph was coupled to the Perkin Elmer 600T mass spectrometer. The oven temperature was programmed at 65°C for 4 minutes and then increased to 280°C at a rate of 8°C/min.

RESULTS AND DISCUSSION

Effect of Different Growth Media on *P. sanguineus* Growth in Shake Flask Culture

Figure 1 shows the effect of different media on the growth of *P. sanguineus* in shake flask culture. The highest biomass was recorded in Medium 5 and therefore used throughout the study.

Of all the mineral nutrients in the culture media, nitrogen is usually required in the largest amounts and can be the limiting factor for fungal growth (Deacon 2006). In addition, it serves as a mean of physiological control and regulation of microorganism metabolites (Thomas et al. 1996). In this study, malt and yeast extract served as the key component in the media for *P. sanguineus* growth, which is similar to results reported by Yahaya (2008). According to Cheng et al. (1991), yeast extract was the best supplement for a nitrogen source and provided convenient growth factors for most microorganisms. The results are inconsistent with those of Fasidi and Olorunmaiye (1994), who found that

yeast extract, a complex nitrogen source, sustained the highest growth of *Pleurotus tuberregium*. On the other hand, the simulating effect of malt extract may be attributed to its carbon and protein components in the media (Yahaya 2008) that could have improved the mycelia growth of fungi, particularly of *Agaricus bisporus* (Friel and McLoughlin 2000) and *Antrodia cinnamomea* (Lin and Chen 2007).

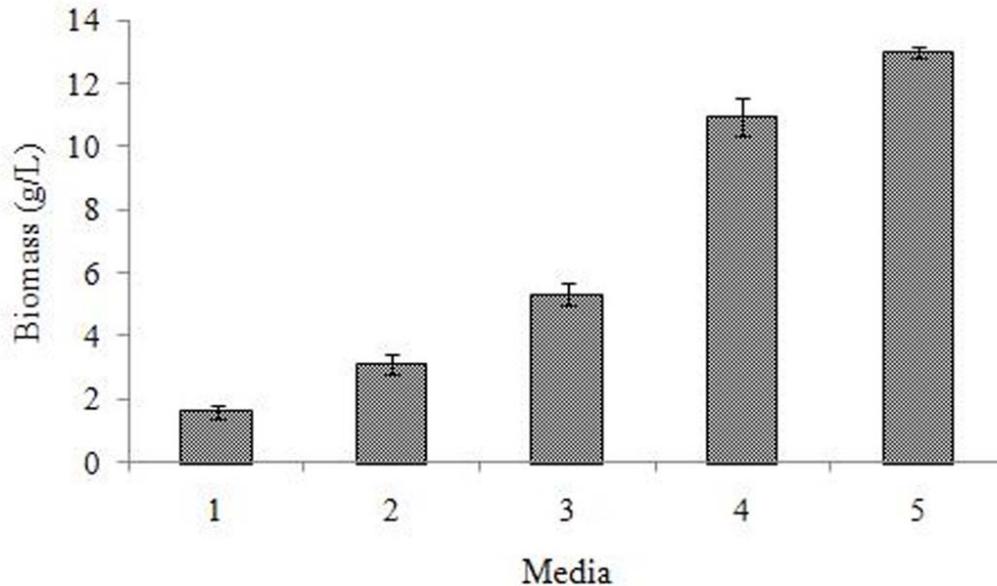


Fig. 1. Biomass of *P. sanguineus* in different production media

Glucose is another nutrient that has been used as an excellent carbon source for growth, and it interferes with the biosynthesis of many secondary metabolites (Demain 1986). Medium 5 yielded the higher biomass as compared to Medium 4 (Fig. 1). Herewith, the result contrasted with the studies undertaken by Martin et al. (1999), Jonathan and Fasidi (2001), and Lin and Chen (2007). Martin et al. (1999) reported that carbon catabolite regulation of penicillin biosynthesis and cephalosporin production is exerted by glucose. Jonathan and Fasidi (2001) indicated that the preference of glucose over other carbon compounds on the growth of *Psathyrella atroumbonata* may be due to the ease with which this sugar was metabolized to produce cellular energy. Besides, Lin and Chen (2007) concluded that glucose was utilized preferentially for mycelial growth in submerged cultivation of *A. cinnamomea*. However, several researchers had found that dextrose and maltose can serve as a better carbon source during the mycelial incubation process. Pandey et al. (2005) observed that dextrose was an excellent carbon source for antibiotic production by *Streptomyces kanamyceticus*. In addition, Manjunathan and Kaviyaran (2010) reported that dextrose proved to be the best source of carbon for the growth of *Lentinus tuberregium*, and this was followed by maltose.

Antifungal Activity from *P. sanguineus*

The results obtained from this study concerning the biological activity of the anti-fungal agents produced by *P. sanguineus* against different species of wood-degrading

fungi are recorded in Table 2. It is clear that the minimum inhibitory concentration (MIC) depended mainly on the types of solvent used and the tested fungal strains. Results showed MIC values ranging from 0.1 to more than 5.0 $\mu\text{g}/\mu\text{L}$. The methanolic extract of *P. sanguineus* showed better inhibition on the tested wood-degrading fungi, particularly against *Lentinus* sp. with MIC 0.1 $\mu\text{g}/\mu\text{L}$.

As reported by Anke (1989), most basidiomycetes produce a great variety of antimicrobial activities. As shown in Table 2, the extract of *P. sanguineus* exhibited considerable *in-vitro* activity against all filamentous fungi tested. This observation was in agreement with that reported by Smania et al. (1995), who found that the fraction obtained from the culture fluid of *P. sanguineus* fungus contained a compound with biological activity against strains of *Escherichia coli*, *Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Staphylococcus aureus*, and members of the genus *Streptococcus*. These results show that *P. sanguineus* can be used as an alternative to treat various types of wood-degrading fungi.

Concerning the anti-fungal effect, the result showed that the effectiveness of anti-fungal activity using mycelia extract was higher in comparison to that from the supernatant. In the light of the experimental results concerning the anti-fungal activity against the tested fungal organisms, the results recorded in Table 2 clearly showed that the methanol extracts of *P. sanguineus* gave better biological activity as compared to the water extract. This observation is in slight agreement with the study carried out by Parthasarathy et al. (2009), who indicated that the relatively wider spectrum of activity of the methanol extracts over the positive control was significant from the disc diffusion assay, while the water extract was devoid of any antimicrobial activity. Oppositely, Ali-Shtayeh and Abu Ghdeib (1999) observed success with water extracts against dermatophytes. According to Webster et al. (2008), alcohol extracts provide a more complete extraction, including less polar compounds, and many of these extracts have been found to possess anti-fungal properties.

Table 2. Composition of the Extract from *P. sanguineus* Mycelia

Wood-degrading fungi species	Minimum inhibitory concentration (MIC) obtained by extract of <i>P. sanguineus</i> ($\mu\text{g}/\mu\text{L}$)			
	My ^m	My ^w	Sus ^m	Sus ^w
<i>Schizophyllum commune</i>	5.0	>5	>5	>5
<i>Trametes versicolor</i>	1.3	>5	>5	>5
<i>Trametes elegans</i>	2.5	5	5	5
<i>Trametes feei</i>	1.3	5	>5	>5
<i>Trametes menziezi</i>	0.3	5	5	5
<i>Lentinus</i> sp.	0.1	2.5	5	5
<i>Lentinus sajor-caju</i>	1.3	5	>5	>5
<i>Lentinus strigosus</i>	2.5	>5	>5	>5
<i>Microporus affinis</i>	0.3	2.5	2.5	5
<i>Microporus xanthopus</i>	0.3	2.5	2.5	5
<i>Gloeophyllum trabeum</i>	2.5	5	>5	>5
<i>Eariella scabrous</i>	2.5	5	>5	>5

*The symbols My and Sus denote the type of residues used by mycelia and supernatant, respectively after incubation process. The superscripts m and w denote the extraction carried out by methanol and water, respectively. In this study, the tested concentration was 5 $\mu\text{g}/\mu\text{L}$. Activity

concentration: weak activity: MIC > 5 µg/µL; moderate activity: 1 µg/µL < MIC ≤ 5 µg/µL; strong activity: MIC ≤ 1 µg/µL.

GC-MS Analysis

The interpretation of the mass spectra obtained by the GCMS method was conducted using the database of the National Institute of Standards and Technology (NIST). The spectrum of unknown components from the sample was compared with the spectrum of the known components stored in the NIST library version 2.0. On this basis the name, molecular weight, and structure of the components of the tested materials were ascertained. Concerning the effect of anti-fungal activity towards fungi tested, this study involved only the extract from mycelia of *P. sanguineus*, which performed much better compared to those from culture suspension. Figures 2 and 3 show the GCMS chromatogram of the extracts from *P. sanguineus* mycelia. The corresponding major components present in the extract of this fungus based on the GCMS spectrum are recorded in Table 3.

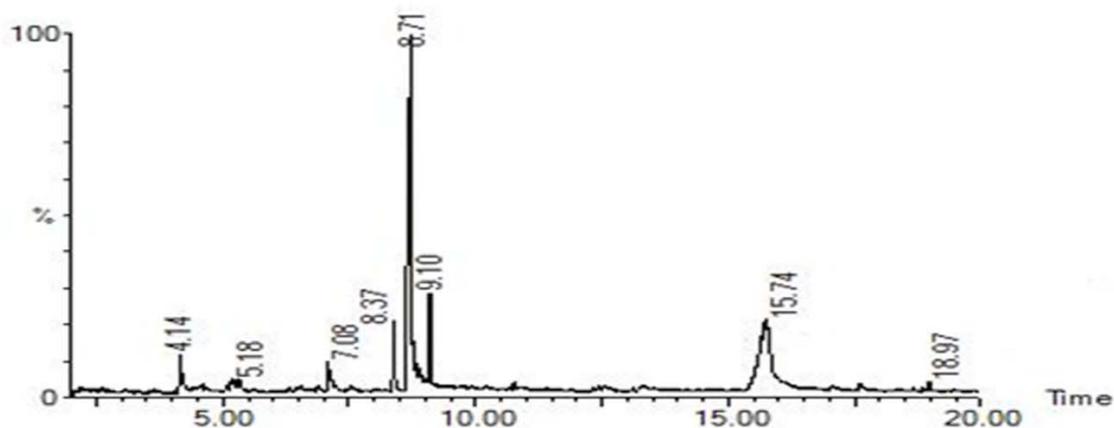


Fig. 2. Chromatogram for the methanol extract from *P. sanguineus*

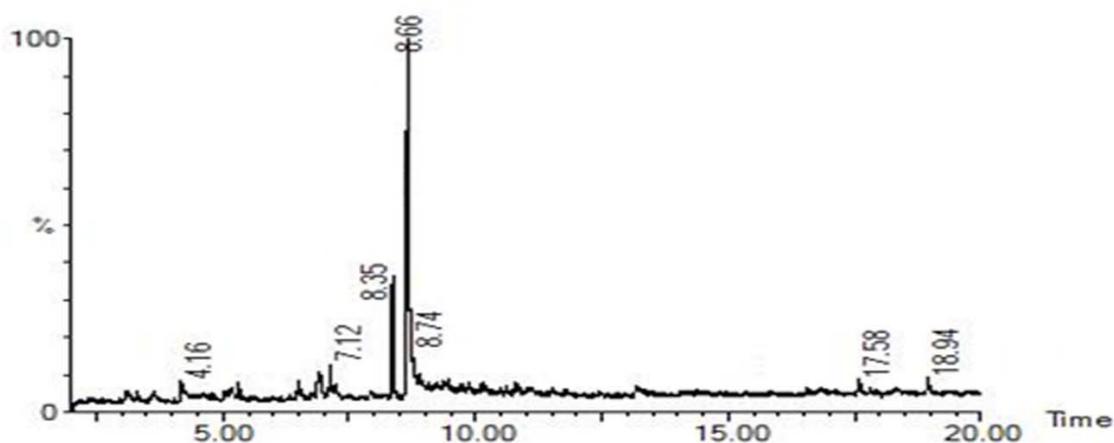


Fig. 3. Chromatogram for the water extract from *P. sanguineus*

As shown in Table 3, the major component that was present in the methanol and water extracts of *P. sanguineus* was 4H-pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl- (DDMP). It was suggested that the formation of DDMP might be the intermediate product during the aqueous Maillard reaction of D-glucose, hexoses, dextrose, and maltose (Preininger et al. 2009). For the effect of anti-fungal activities of these extracts, the results showed that the methanol extract had a higher percentage of DDMP as compared to that in water extract. Thus, the methanol extract provided a better result with lower MIC value to inhibit the growth of fungi tested. Based on previous studies, DDMP was first identified from *Phaseolus coccineus* by Yoshiki et al. (1994). In addition, several researchers had investigated several important biological activities of DDMP, such as anti-alpha-glucosidase activity in patients with diabetes mellitus (Quan et al. 2003), reactive oxygen-scavenging activity (Takara et al. 2007), anti-mutagenic activity against arylamine and 2-acetoxyacetylaminofluorene (2AAAF)-induced DNA damage in Chinese hamster ovary cells (Berhow et al. 2000), and also anti-tumour activity (Ban et al. 2007). Meanwhile, it was reported that the flavones, flavonoids, and flavonols had a phenolic structure with one carbonyl group, which was often found to be effective *in-vitro* as an antimicrobial substance against the wild array of microorganisms (Das et al. 2010). In fact, DDMP belongs to the flavanoid fraction, a group of complex natural products of current medicinal interest, in which its anti-microbial, anti-oxidant, and anti-inflammatory properties have been recognized (Kumar and Maneemegalai 2008; Taylor et al. 2009; Chaturvedi et al. 2010; Kumar et al. 2010).

Table 3. Composition of the Extract from *P. sanguineus* Mycelia

My ^m			My ^w		
Retention time (minutes)	Area (%)	Chemical composition	Retention time (minutes)	Area (%)	Chemical composition
4.14	2.33	2-Propanone,1,3-dihydroxy-	4.16	1.48	2-Propanone,1,3-dihydroxy-
7.08	0.74	4,5-Diamino-6-hydroxy pyrimidine	7.12	0.56	4,5-Diamino-6-hydroxy pyrimidine
8.38	2.04	N-Aminomorpholine	8.35	1.64	N-Aminomorpholine
8.71	20.58	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-	8.66	5.43	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl-
9.10	1.92	2(3H)-Furanone, dihydro-4-hydroxy	8.74	0.39	2(3H)-Furanone, dihydro-4-hydroxy
15.75	16.01	Sucrose	17.58	0.42	3-Deoxy-d-mannonic lactone
18.98	0.44	Xylitol	18.94	0.39	Xylitol

*The symbol My denotes the type of residues used by mycelia after incubation process. The superscripts m and w denote the extraction carried out by methanol and water, respectively.

These results indicate that the presence of chemical compounds in *P. sanguineus* might be the reason for it to have anti-fungal activities against the wood-degrading fungi.

CONCLUSIONS

It was found that *P. sanguineus* yielded the highest growth in Medium 5, comprising of 10.0 g/L yeast extract, 10.0 g/L malt extract, 10.0 g/L dextrose, and 10.0 g/L maltose at pH 4.7±0.2. *P. sanguineus* is a potential source of bioactive compounds, and the results revealed that the methanol extract gave better antifungal activity compared to that of water extract with a lower MIC value. In addition, quantitative analysis using a GC-MS showed the presence of DDMP, a flavanoid compound, which due to the reaction of glucose with complex nitrogen may be formed during the fermentation process. This phenomenon thus could explain the antifungal properties of the *P. sanguineus*. These results can help to justify the use of *P. sanguineus* for the treatment against various species of wood-degrading fungi.

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