RESULTS, DISCUSSION AND CONCLUSION

4.1 Plate Assay for Antagonistic Activity

The results for the plate assay of antagonistic activity are presented in Table 4.1. Most of the selected polypores did not inhibit test fungi except for *Microporus* sp. (POR 34), *Albatrellus* sp. (KUM 60500) and *Microporus* sp. (POR 18). *Microporus* sp. (POR 34) (Plate 4.1 a), *Albatrellus* sp. (Plate 4.1 b) and *Microporus* sp (POR 18) showed inhibition zones against *Fusarium* sp. Hazy zones could be observed as POR 36 and POR 34 partially inhibited *Candida parapsilopsis*.

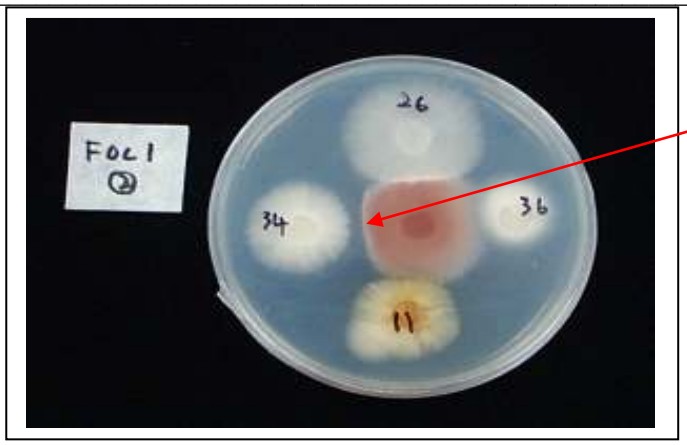
Table 4.1

Inhibition zones for antagonistic activity plate assay produced by selected polypores (PDA; 72 hours at 27 ± 2 ^{0}C).

POR [a]	**Inhibition zones of test fungi** [b]						
	SP	**CA**	**CP**	**Foc 1**	**Foc 2**	**Foc 4**	**GB**
KUM 60500	-	-	-	+	-	+	-
POR 34	-	-	hazy	+	+	-	-
POR 18	-	-	-	-	-	+	-
POR 36	-	-	hazy	-	-	-	-

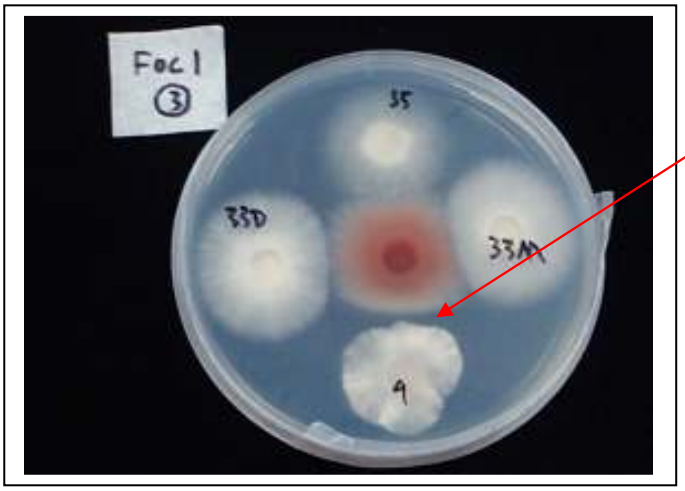
[a] KUM 60500: *Albatrellus.* sp.; POR 34: *Microporus* sp.; POR 18: *Microporus* sp.; POR 36: Unidentified 1.

[b] SP: *Saccharomycetes pombe*; CA: *Candida albicans*; CP: *Candida parapsilopsis*; Foc 1: *Fusarium oxysporum* f.sp. *cubense* race 1; Foc 2: *Fusarium oxysporum* f.sp. *cubense* race 2; Foc 4: *Fusarium oxysporum* f.sp. *cubense* race 4; GB: *Ganoderma boninense.*



Inhibition zone between *Microporus* sp. and *F. oxysporum*

Plate (a): *Microporus* sp. (POR34) against *F. oxysporum* after 72 hours.



Inhibition zone between *Albatrellus* sp. and *F. oxysporum*

Plate (b): *Albatrellus* sp. (KUM 60500) against *F. oxysporum* after 72 hours.

Plate 4.1

Inhibition zones of *Microporus* sp. (POR34) and *Albatrellus* sp. (KUM 60500) against *F. oxysporum* in antagonistic activity plate assay (PDA; 72 hours at 27 ± 2 ^{0}C).

This was the preliminary screening test for antifungal activity with cultured Polypores on solid artificial media. If polypores produced any antifungal substance, the substance would diffuse in the medium and an inhibition zone could be observed.

The report of Jonathan *et al*. (2007) saying that, the antifungal properties of the tested higher fungi were generally poor. There were four out of the eight screened mushrooms exhibited weak antifungal properties against pathogenic fungi (Jonathan *et al*., 2007). In addition, in a study by Suay *et al*. (2000), only 45 to 49% of methanolic extracts from Polyporales tested showed active antimicrobial activity. This is in agreement with the

results obtained in the present study because most of the fungi did not exhibit antifungal properties against pathogenic fungi.

Many studies had utilized the agar plate assay method to determine biocontrol potential of some fungus but some studies showed that no correlation existed between inhibition of pathogen on agar plate in the lab and disease control in the field. It is agreed that the agar inhibition assay is not ideal and has limitation. However, many researchers are using a combination of methods to overcome the limitation of the agar assay (Inam-ul-Haq *et al.,* 2003).

Many reports showed that, most fungal metabolites were discovered from mycelia. But, compounds formed in the fruiting bodies also have attracted attention for several reasons. The fruit body has a distinct role in the life cycle of a fungus. It is normally short live and formes an entity that is separated from the rest of the organism. Therefore, the metabolites of the fruit body might be formed for other reasons than those found in the mycelium. One of the reasons is they protect the fruit body from parasites and predators (Marc and Olov, 1998).

Thus, to obtain bioactive polysaccharides from mushrooms, most investigators have spent their efforts to cultivate edible or medicinal mushrooms on solid artificial media (for fruit body production) rather than in submerged cultures (for mycelial extract production) (Bum *et al*., 2004). In this preliminary study, solid artificial media (PDA) was used to culture the mycelia of Polypores.

The results obtained from this assay were not conclusive. Firstly, the growth rate of test fungi and polypores were different. For example, *Trametes versicolor* (POR 33D) grew very fast but *Albatrellus* sp. (KUM 60500) grew very slow. The results could not be taken on the same day.

Secondly, the growth rate of polypores were very slow, the observation was done after 72 hours. In this long period of time, some bioactive compounds in the agar plate might be deactivated, degraded or not produced. So inhibition zone may not observed. The

result showed the potential of polypores to inhibit the test fungi was only in qualitative term but not in quantitative term. So, these selected polypores could be further studied by disc diffusion method to investigate the ability for antifungal activity.

4.2 Paper Disc Diffusion with Polypores Extracts from Agar Plate Cultures Against Test Fungi

The results of the paper disc diffusion assay with extracts from agar plate culture are given in Table 4.2. Most of the tested polypores did not inhibit test fungi except *Albatrellus* sp. (KUM 60500) and *Microporus* sp. (POR 18). Discs impregnated with 0.02 ml methanolic extracts of *Microporus* sp. gave an inhibition zone of 1 mm against *G. boninense* but did not inhibit the other test fungi. Discs impregnated with 0.02 ml methanolic extracts of *Albatrellus* sp. showed inhibition zones of approximately 10.5 mm against *S. pombe* and 2 mm inhibition zone against *F. oxysporum* (Plate 4.2 a).

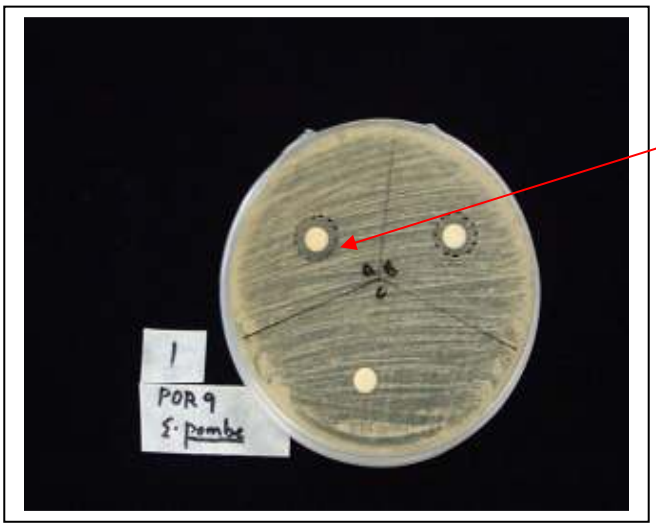
Table 4.2

Inhibition zones (mm) produced by methanolic extracts of *Albatrellus* sp. and *Microporus* sp from PDA plate culture (incubated at 27 ± 2 ^{0}C for 48 hours) at a concentration of 200 mg/ml against test fungi.

POR [a]	**Inhibition zones of test fungi** [b] **(mm)**						
	SP	**CA**	**CP**	**Foc 1**	**Foc 2**	**Foc 4**	**GB**
KUM 60500	+ (10.5)	-	-	-	-	+ (2)	-
POR 18	-	-	-	-	-	-	+ (1)
nystatin (100 ug / ml)	+ (12.5)	+ (12.5)	+ (6.5)	+ (2)	+ (2)	+ (2)	+ (2)

[a] KUM 60500: *Albatrellus* sp.; POR 18: *Microporus* sp..

[b] SP: *S. pombe*; CA: *C. albicans*; CP: *C. parapsilopsis*; Foc 1: *F. oxysporum* f.sp. *cubense* race 1; Foc 2: *F. oxysporum* f.sp. *cubense* race 2; Foc 4: *F. oxysporum* f.sp. *cubense* race 4; GB: *G. boninense.*



Inhibition zone produced by *Albatrellus* sp. against *S. pombe.*

Plate (a): Inhibition zones produced by *Albatrellus* sp. methanolic extracts against *S. pombe* after 48 hours.

Inhibition zone produced by nystatin (100 µg/ml) against *S. pombe*.

Plate (b): Inhibition zones produced by nystatin against *S. pombe* after 48 hours.

Plate 4.2

Inhibition zones produced by methanolic extracts of *Albatrellus* sp. and nystatin as control against S. *pombe* after 48 hours at 27 ± 2 ^{0}C in paper disc diffusion test.

Plate 4.3

The growth morphology of ten days old culture of *Albatrellus* sp. (KUM 60500) grown on different agar media.

A: PDA

B: PDA + 1% yeast plate culture

C: glucose – yeast – malt – peptone agar medium (GYMP)

D: malt extract (2% w/w malt extract + 1.8% w/w agar)

E: corn meal agar

The methanolic extract of *Albatrellus* sp. showed an exciting result because the inhibition zone against *S. pombe* was closed to the inhibition zone generated by positive control, nystatin (10 µg / ml) (Plate 4.2 b).

Subsequently, *Albatrellus* sp. (KUM 60500) was cultured on different agar media to determine the production of antifungal bioactive compound. The selected agar media were commonly used in polypores culture, such as PDA or PDA with 1% w/w yeast or glucose–yeast–malt–peptone agar medium (GYMP) or malt extract (2% w/w malt extract with 1.8% w/w agar) or corn meal agar (Plate 4.3).

However, the results of this experiment (Table 4.3) showed that *Albatrellus* sp. only expressed the ability to inhibit *S. pombe* when grown on the PDA.

Table 4.3

Inhibition zones (mm) produced by methanolic extracts (200 mg/ml) of *Albatrellus* sp. (KUM 60500) from different media agar plate against *S. pombe,* which incubated at 27 ± 2 ^{0}C for 48 hours in discs diffusion test.

Methanolic extract of *Albatrellus* sp. (KUM 60500) grown in different agar medium	**Inhibition zones against *S. pombe* (mm)**
Potato dextrose agar (PDA)	8
Potato dextrose agar (PDA) + 1% yeast	8
Glucose–yeast–malt–peptone agar medium (GYMP)	-
Malt extract (2% malt extract + 1.8% agar)	-
Corn meal agar (CMA)	-
10% DMSO – negative control	-
nystatin (100 µg/ml) – positive control	10

Disc diffusion method also known as agar diffusion method. It is a popular and commonly used method for studying antifungal action (Anupama *et al.,* 2005). With the absence of growth, it is called inhibition zone. The diameter of the zone was proportional to the amount of sample added to the disc, the solubility of the sample, the diffusion coefficient and the overall effectiveness of the sample used.

According to Bauer *et al.*, (1966), the diameters exhibited by the crude extracts in the paper disc diffusion method cannot be taken as the exact antifungal activity. This was because the diffusion and solubility properties of the active substances in the crude extracts into agar media were different. Thus, crude extracts of polypores in the present's study which did not show activity against the test fungi might be caused by the low diffusion and solubility of the active substances from the disc to the agar media.

Information about the nutritional requirement for antifungal compound production by *Albatrellus* sp. is either very scanty or not available in the literatures. In the present study, various growth morphologies of *Albatrellus* sp. could be found on different agar media (Plate 4.3). As an example, *Albatrellus* sp. (KUM 60500) produced a thick mycelium layer but grew very slowly when grown on PDA, meanwhile *Albatrellus* sp. produced a thin mycelium but growth was very fast on malt extract and corn meal agar.

The growth rate and pattern of mycelium would affect the production of bioactive compounds. Stamets and Chilton (1983) noted that slower growing cottony mycelium of *Agaricus* cultivators was inferior as compared to the faster growing rhizomorphic mycelium. Therefore, faster growing mycelium with higher weight of dried mycelia biomass might contain higher amount of bioactive compounds. Beside, David *et al.,* (2006) demonstrated that micronutrient supplementation is a potential opportunity for mushroom growers to improve efficiency and quality of freshly harvested mushrooms.

Indeed, different media would support the growth of an organism but not all would lead to the production of a desired compound. As an example, in one screening programme, two different media, Raulin-Thom and Czapek-Dox, which differ primarily in the nitrogen source (ammonia in the former and nitrate in the latter) were used. It was frequently observed that the biological activity occurred only on one medium but not the other. A striking example of the effect of medium on the production of secondary metabolites was provided by *Penicillium baarnense* which produced mainly orsellinic acid on Czapek-Dox medium but barnol on Raulin-Thom medium. The nitrogen source was the key factor in determining which bioactive compound produced by mycelium (Tan, 2001).

On the other hand, according to Stamets and Chilton (1983), the characteristics of fruiting mycelium were often species specific. The growth of mycelium on nutrient agars could display a remarkable diversity of forms. The use of various media might help the mycelium to adapt better for survival. Besides, different species vary greatly in their preferences to certain agar nutrient.

From Fasola and his team's study (2006), all the media used supported mycelium growth of *V. speciosa*, although PDA stimulated the best mycelium growth for this fungus. The enhancement of growth on PDA was not a surprise, because this medium has been widely reported to support growth of mushroom mycelia (Alofe, 1985; Fasidi, 1996; Huang, 1993). Jonathan and Fasidi (2001) also obtained better mycelial yields of *Psathyerella atroumbonata, Lentinus subnudus* and *Schizophyllum commune* when PDA was supplemented with 0.5% (w/w) yeast extract.

This indicated that culture media was important to the yield of any bioactive compounds. Carbon and nitrogen sources generally play a significant role because these nutrients are directly linked with cell proliferation and metabolite biosynthesis (Tang and Zhong, 2002). Beside that, it was possible that different carbon sources might have different catabolic repression effect on the cellular secondary metabolism (Mao *et al.*, 2005).

So, the medium used to grow the polypores could affect greatly in the accuracy of the test results. In this study, *Albatrellus* sp. only excreted bioactive compounds that inhibit *S. pombe* when cultured on PDA plate agar. The carbon and nitrogen sources might be the key factor in determining the production of antifungal compounds (Mao *et al.*, 2005).

Beside, Tan and Moore (1994), Irinoda *et al.* (1992) and Tochikura *et al.* (1988), showed that independently purified extracts of edible mushrooms are more effective against microorganisms than crude extracts. In a recent report, Jonathan *et al.* (2007) also observed that purified extract of the tested macrofungi exhibited more potent antimicrobial activities than crude extracts. In agar well diffusion method, the inhibition values obtained for crude extracts and purified extracts for *Marasmius jodocodo* against *Bacillus cereus* were 4.0 and 8.0 mm respectively. Similar result was obtained for this mushroom against *A. niger* and *A. flavus* (Jonathan *et al.*, 2007). Therefore, antifungal activity of polypores by using purified extract should be carried out in the future.

In addition, the bioactive compounds might be present in too low concentration to manifest the antifungal activities at the concentration used. Farnsworth and Bingel (1977), showed that there may be other components present which may have a suppressive effect towards the bioactive compounds. Based on this present study, there is a possibility of having antagonistic substances in the crude extract since it was prepared in single solvent, methanol.

Based on the above result, nutrient components of potato dextrose could be selected as a growing medium for further study. A purified extract should be used for assays.

4.3 Paper Disc Diffusion with Polypores Extracts from Static Liquid Culture Against Test Fungi.

The results of the paper disc diffusion test with extracts from static liquid cultures are given in Table 4.4. Most of the polypores tested did not inhibit test fungi except for *Albatrellus* sp. (KUM 60500) and POR 58.

POR 58 showed the ability to inhibit *F. oxysporum* (Plate 4.4 c). However, *Albatrellus* sp. (KUM 60500), which showed remarkable antifungal activity when grown on solid agar in the previous test, did not inhibit *S. pombe* but inhibit *Fusarium* sp. when cultured in liquid media (Table 4.4). *Albatrellus* sp. (KUM 60500) exhibited one mm inhibition zone against *F. oxysporum* (Plate 4.4 a and b) when cultured on potato dextrose and malt culture extracts.

Table 4.4

Inhibition zones (mm) produced by *Albatrellus* sp. and POR 58 methanolic extracts from static liquid culture at a concentration of 200 mg/ml against test fungi.

POR [a]	**Inhibition zones against test fungi** [b] **(mm)**				
	SP	**CA**	**CP**	**Foc 4**	***GB***
PDB KUM 60500	-	-	-	+ (1)	-
ME KUM 60500	-	-	-	+ (1)	-
PDB POR 58	-	-	-	+ (1)	-
nystatin (100ug / ml)	+ (11.5)	+ (12.5)	+ (6.5)	+ (2)	+ (2)

[a] KUM 60500: *Albatrellus* sp.; POR 58: unidentified 7; PDB: potato dextrose broth; ME: malt extracts.

[b] SP: *S. pombe*; CA: *C. albicans*; CP: *C. parapsilopsis*; Foc 4: *F. oxysporum* f.sp. *cubense* race 4; GB: *G. boninense.*

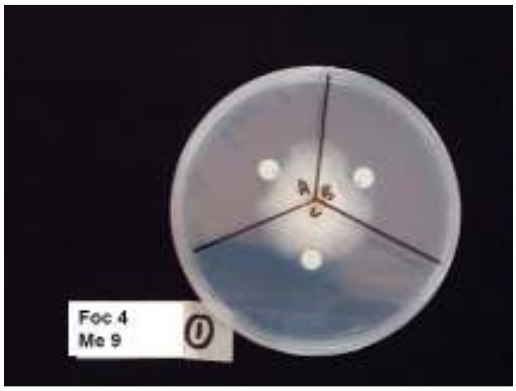


Plate (a): Inhibition zones produced by *Albatrellus* sp. from malt extract culture against *F. oxysporum.*

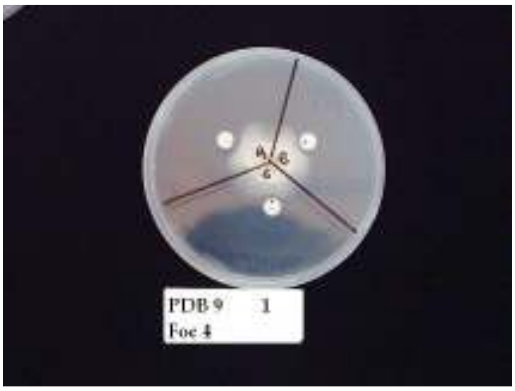


Plate (b): Inhibition zones produced by *Albatrellus* sp. from PDB culture against *F. oxysporum*.

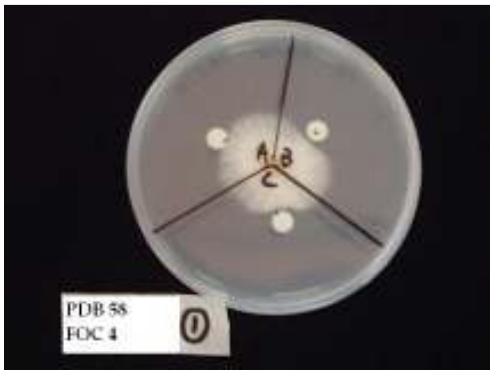


Plate (c): Inhibition zones produced by POR 58 from PDB culture against *F. oxysporum*.

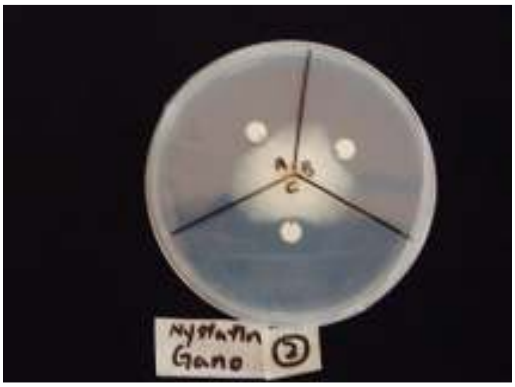


Plate (d): Inhibition zones produced by nystatin against *G. boninense*.

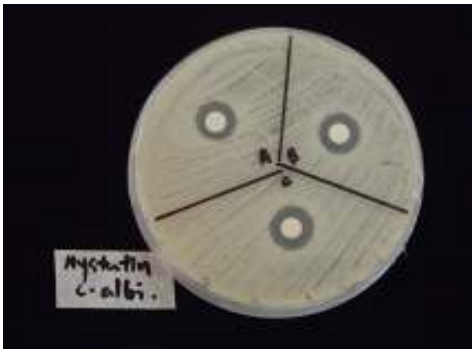


Plate (e): Inhibition zones produced by nystatin against *C. albicans*.

Plate 4.4

Inhibition zones in paper disc diffusion for methanolic extracts from static liquid culture (PDA plate; 72 hours at 27 ± 2 ^{0}C).

The culture conditions are very important factors that affect the production of bioactive compounds. In nature, mushrooms grown on solid surface but not in liquid media. These mushrooms might produce different compounds when cultured in different conditions (David, 1996). *Albatrellus* sp. only produced antifungal compounds that inhibited *S. pombe* when grown on solid surface but not in liquid culture. When in static liquid culture, *Albatrellus* sp. did not produce the same bioactive substances but excreted the other bioactive compounds, which could inhibit *Fusarium* sp. Further, the effect of medium may need to be investigated.

4.4 Effect of Submerged Agitated Culture Medium of *Albatrellus* sp. Against Test Fungi.

The effect of submerged culture of *Albatrellus* sp. (KUM 60500) in various liquid media such as PD broth, ME broth and GYMP broth for three, six and nine days incubation were demonstrated. The methanolic extracts were used in disc diffusion test. However, no antifungal bioactive compound was detected in this study.

4.5 Effect of Extracts from Solid Substrate Fermentation Against *Saccharomyces pombe*.

Since *Albatrellus* sp. did not produce the anti *S. pombe* bioactive compound in liquid culture fermentation, it was assumed that *Albatrellus* sp. actively produced bioactive compounds when cultured on solid substrate fermentation. Thus, solid substrate fermentation was carried out to culture the *Albatrellus* sp. in variety substrates, such as wheat, corn, green bean and rice. However, the results of paper disc diffusion for *Albatrellus* sp. (KUM 60500) extracts from solid substrate fermentation against *S. pombe* were negative.

In the course of screening for novel naturally occurring fungicides from mushrooms in Yunnan province, China, the ethanol extract of the fruiting bodies of *Albatrellus dispansus* was shown antifungal activity against plant pathogenic fungi. The

bioactive compound was isolated from the fruiting bodies of *A. dispansus* by bioassay guided fractionation of the extract and identified as glifolin by mass spectral analysis. Its antifungal activities were evaluated *in vitro* against nine plant pathogenic fungi and *in vivo* against the plant disease of *Erysiphe graminis*. *In vitro, Sclerotinina sclerotiorum* and *Fusarium graminearum* were the most sensitive fungi to glifolin. Spore germination of *F. graminearum, Gloeosporium fructigena* and *Pyricularia oryzae* was almost completely inhibited by grifolin (Luo *et al*., 2005).

Previous chemical investigations of *Albatrellus* sp. belonging to the *Scutigeraceae* family had revealed that they were abundant sources of grifolin and its isomer neogrifolin (Dang *et al.*, 2005). Two new farnesyl phenols named grifolinones A and B, together with known grifolin and neogrifolin were also isolated from methanolic extract of the inedible mushroom *Albatrellus caeruleoporus* (Dang *et al*., 2005). These compounds and their derivatives possess many interesting biological activities besides antifungal activity, such as anti-oxidative, antimicrobial, plant growth inhibitory, tyrosinase inhibitory, controlling the anti-cholesteremic activity level in blood and liver and promoting melanin synthesis by B16 melanoma cells (Dang *et al*., 2005). For example, in 1950, grifolin was defined as an antibiotic (Hirata and Nahanishi, 1950). Research had shown that grifolin added to the high cholesterol diet could significantly lower plasma cholesterol level with limited or no toxicity (Sugiyama *et al*., 1992). Additionally, grifolin possesses antioxidative activity and significantly inhibit histamine release from rat peritoneal mast cells. However, antitumor activity of grifolin is yet to be investigated (Mao Ye *et al*., 2005).

From the study of Makiko, *et al.* (2002), three neogrifolin derivatives namely 3-hydroxyneogrifolin, 1-formylneogrifolin and 1-formyl-3-hydroxyneogrifolin were isolated along with grifolin and neogrifolin from the Japanese mushroom *Albatrellus ovinus.* 3-hydroxyneogrifolin and 1-formyl-3-hydroxyneogrifolin showed more potent antioxidative activity properties compared to the synthetic antioxidant compound, α-tocopherol or BHA.

According to Lau (2006), the methanolic extracts of *Albatrellus* sp. (KUM 60500) from GYMP liquid medium tested for the scavenging effect on 1, 1–diphenyl–2-picrylhydrazyl (DPPH) radicals, showed that the highest EC_{50} value was 41.67 mg/ml.

Brand, *et al* (1995) showed that there are three types of reaction kinetics that depend on the nature of the antioxidant being tested. Compounds that reacted rapidly with the DPPH radicals and reach a steady state in less than 1 minute are assumed to have a rapid kinetic behaviour. The second type of behaviour was intermediate where the compounds reacted a steady state after approximately 5 to 30 minutes. For slower kinetics, the compounds took about 1 to 6 hour to reach a steady state. So, *Albatrellus* sp. (KUM 60500) exhibited slow kinetic reaction with the DPPH radical as it required 90 minutes to reach a steady state.

Lau (2006) also reported that dichloromethane extracts of *Albatrellus* sp. (KUM 60500) from GYMP liquid medium showed the most potent cytotoxic activity against CaSki cells. About 40% of the cells lysed at concentration of 10µg/ml and about 80% cells were lysed at concentration of 100 µg/ml compared to the other nine Polypores that showed percentages of lysed cells lower than 30% at concentration of 10µg/ml. *Albatrellus* sp. produced the highest cytotoxic activity with the EC_{50} value of 37 µg/ml.

This result provided initial evidence of antitumor activity of *Albatrellus* sp. (KUM 60500). Thus encouraging further molecular study of the mechanism involved in the lyses of the cancer cells.

The ability to inhibit *S. pombe* might indicate the anti tumor activity of *Albatrellus* sp. (KUM 60500). So, although *Albatrellus* sp. (KUM 60500) only gave positive in anti *S. pombe* ability in PDA agar culture, but not in liquid culture or solid surface fermentation, the potential to inhibit *S. pombe* should not be under looked. The *Albatrellus* sp. may have a potential in anti tumor bioactive compound production.

4.6 Agar Spot Test for Enzymatic Activity

In spot test for enzymatic activity, laccase reacted with 0.1 M syringaldazine dissolved in 50% ethanol to form a pink colour; tyrosinase, reacted with 0.1 M P. cresol to form a yellow to red colour, and lignin peroxidase, reacted with 1% pyrogallic acid in H_2O_2 to form brown colour.

The results of spot test for enzymatic activity are shown in Table 4.5. In the agar spot test for lignin peroxidase, 12 of polypores gave positive results except *Trametes* sp. (POR 33M), *Microporus* sp. (POR 34) and *Polyporus* sp. (POR26). 10 of the 15 polyporales showed positive results in agar spot test for laccase except *Bjerkandera* sp. (POR 31), *Trametes* sp. (POR 33M), *Microporus* sp. (POR 34), *Polyporus* sp. (POR 26) and *Albatrellus* sp. (KUM 60500).

All polypores showed negative results in agar spot test for tyrosinase activity except *Albatrellus* sp. (KUM 60500), *M. xanthopus* (POR 57) and POR 48. Among 15 polypores, only *T. versicolor* (POR 33D) and *Polypore* sp. (POR 35) showed remarkable enzymatic activities in spot test for both laccase and lignin peroxidase.

Recently, extensive research on basidiomycetes fungi has been conducted with aim to isolate new organisms for ligninolytic enzymes as well as enzymes with properties important for their industrial application (Kiiskinen *et al*., 2004).

According to Stuardo *et al*. (2005), not all white rot fungi produce laccase. It is true as in this study, of the 15 polyporales tested for laccase, only *T. versicolor* (POR 33D) and POR 58 (Table 4.5) gave good enzymatic activities.

Table 4.5

Laccase, tyrosinase and lignin peroxidase activities of polyporales on agar spot test (Polyporales were cultured on PDA plate; then incubated at 27 ± 2 ^{0}C for 7 – 8 days).

Polypores	Laccase	Tyrosinase	Lignin Peroxidase
Albatrellus sp. (KUM 60500)	-	+	+
Bjerkandera sp. (POR 31)	-	-	++
Microporus sp.(POR 18)	+	-	++
Microporus sp.(POR 34)	-	-	-
Microporus xanthopus(POR 57)	++	+++	+
Polypore sp. (POR 35)	++	-	++++
Polyporus sp. (POR 26)	-	-	-
Pycnoporus sp.(POR 11)	+	-	+++
Trametes sp. (POR 33M)	-	-	-
Trametes versicolor (POR 33D)	+++	-	+++
Unidentified 1 (POR 36)	+	-	+++
Unidentified 2 (POR 38)	+	-	++
Unidentified 3 (POR 48)	+	++++	+
Unidentified 4 (POR 52)	++	-	+
Unidentified 5 (POR 58)	+++	-	++

(-), Negative reaction; (+), weak activity; (++), moderate activity; (+++), strong activity; (++++), very strong activity.

The formation of oxidase and peroxidase by these 15 fungi were examined with agar spot tests for tyrosinase, laccase and lignin peroxidase. Based on report from Grams *et al.* (1998), extracellular peroxidase was released by virtually all isolated fungi. Tyrosinase was presumably intracellular and occurred in the majority of isolates. The strong laccase reaction was predominantly extracellular, although in some genera the intracellular laccase seemed to be dominant.

In this present study, the enzymes were assumed to be extracellularly produced and diffused widely into the agar that would give strong enzymatic activities in spot test for laccase and lignin peroxidase. For the tyrosinase spot test, only a few polypores (*Albatrellus* sp., *M. xanthopus* and POR 48) had shown positive results.

According to Marx and Peterson (1969), macrofungi were recovered by plating pieces of fruit body on several culture media of which modified Melin–Norkrans medium (MMN) and 2.5% malt extract medium (ME) were most appropriate compared to the PDA. For the present study, only PDA culture plate was used to culture polypores for agar spot test. In the future study, MMN and ME should be used to culture these polypores.

4.7 Lignin Peroxidase (LiP) Enzyme Activity

T. versicolor (POR 33D) and *Polypore* sp. (POR 35) showed the presence of lignin peroxidase, 6.146 U / mL and 8.148 U / mL respectively when cultured in potato dextrose broth.

From the previous result, *Polypore* sp. (POR 35) and *T. versicolor* (POR 33D) which had LiP activity did not exhibit antifungal properties. On the other hand, *Albatrellus* sp. (KUM 60500), which had shown the antifungal properties, did not possess strong enzymatic activity. Thus, the polypores which could excrete bioactive compounds were not necessarily excreted enzymes at the same time. Enzymes are primary metabolites but many lignin modifying enzymes are secreted in stationary phase of growth.

T. versicolor and *Polypore* sp. were chosen to test the LiP enzymatic activity because these two polypores had good enzymatic activity in agar spot test for lignin peroxidase. *T. versicolor* and *Polypore* sp. were cultured in malt extract and potato dextrose broth separately. After incubation, the liquid cultures were used for the LiP enzyme activity assay. However, the results of LiP enzyme activities for *T. versicolor* and *Polypore* sp. were negative when they were cultured in malt extract. *T. versicolor* and *Polypore* sp. were shown to have positive results for LiP enzyme activities when cultured in potato dextrose broth.

From the study of Ng (2004), many examined fungal species secreted more than one isoenzyme. Different conditions of growth and culture media may produce different isoenzymes. For example, Elisashvili (2006) reported that the presence of lignocellulosic substrate is mandatory for MnP production by *Pleurotus dryinus* IBB 903 since there was no enzyme production when the fungus was cultivated in the synthetic medium with different carbon sources.

This was proved by another study by Celia *et al.* (2005), in which two *Ganoderma* strains showed differences in enzymatic activity depending on the culture condition. In spite of having similar protein secretion patterns and biomass yields, two *Ganoderma* sp. strains showed interesting differences in enzymatic activity depending on the culturing condition. The production of LiP by strain GASI3.4 in the culture medium without supplementation was only detected after 10^{th} day of incubation and this activity increased until the end of the evaluation period. In the case of the medium supplemented with bran, stimulated the production of this enzyme and activity was detected in the first three days of evaluation and the activity measured at the end of the experimental period was 10-fold higher when compared with the control treatment. In general, the presence of wheat bran induced a high production of the enzymes, while the presence of herbicide inhibited the activities of LiP only in the strain GASI3.4 (Celia *et al*., 2005).

This indicated that the culture media and culture condition play important roles in enzyme production. In the present study, *T. versicolor* and *Polypore* sp. only showed positive result in LiP enzymatic activity test when cultured in potato dextrose broth. On the contract, *T. versicolor* and *Polypore* sp. may secrete other enzymes but not the lignin peroxidase when cultured in malt extract. Therefore, in the future study, different types of culture media and culture condition should be carried out to optimize the production of desired enzymes.

From Rothschild *et al.* (2002), enzymes activities (laccase and lignin peroxidase) were detected and their formation followed a temporal sequence peaking at 4, 7 and 11 days respectively. Besides, Celia *et al*. (2005) also reported that the specific enzymes

activities of LiP and laccase continued to increase after the 10th day. However, the liquid cultures that were used for the LiP enzyme activity assay were only incubated for 7 – 8 days in this present study and this was a short period for enzyme secretion. Therefore polypores should be incubated for longer time to obtain higher yield of enzymes because some polypores need longer time to secret the desired enzymes.

4.8 Conclusion

15 Malaysian polypores were selected for screening of antifungal activity against five fungi (*S. pombe*, *C. albicans*, *C. parapsilopsis*, *F. oxysporum* f.sp. *cubense* race 1, *F. oxysporum* f.sp. *cubense* race 2, *F. oxysporum* f.sp. *cubense* race 4 and *G. boninense*). The 15 polypores were cultured on various agar media such as potato dextrose, yeast – peptone – glucose, sabouraud dextrose, glucose – yeast – malt – peptone, malt extract and corn meal. In the plate assay for antagonistic activity, only 3 (20%) polypores (*Microporus* sp. POR 34, *Albatrellus* sp. KUM 60500 and *Microporus* sp. POR 18) showed inhibition activity against at least vone of the test fungi. The methanolic extracts from PDA plate cultures of *Albatrellus* sp. (KUM 60500) displayed high activity in the paper disc assay against *S. pombe* and *F. oxysporum.* The inhibitory zone (10.5 mm) of *Albatrellus* sp. against *S. pombe* was closer to inhibitory zone (12.5 mm) produced by standard positive control (Nystatin).

The effect of cultivation systems and cultivation media were studied in order to obtain a suitable fermentation medium for production of bioactive compounds. From the result, it was shown that *Albatrellus* sp. (KUM 60500) produce antifungal compound against *S. pombe* when cultured on PDA plate. On the other hand, the methanolic extracts of POR 58 from the static liquid culture showed the ability to inhibit *F. oxysporum.* Besides that, methanolic extracts of *Albatrellus* sp. grown in potato dextrose and malt extract gave 1 mm inhibition zone against *F. oxysporum*.

Fungi are rich in proteins. A multitude of proteins has now been purified and characterized and many more remain to be isolated. Some of these proteins have proven applicable value e.g. laccases, lignin peroxidases and tyrosinase.

15 selected polypores were also screened for enzymes activity by agar spot tests. It was found that polypores which could excrete antifungal bioactive compound did not necessary be good for enzyme excretion. *Albatrellus* sp. (KUM 60500), *Microporus* sp. (POR 34), *Microporus* sp. (POR 18) and POR 58, showed ability to inhibit *S. pombe*, *F. oxysporum* and *G. boninense* but they did not display the enzymatic activity. On the other hand, polypores which were strong in enzymatic activity did not play a role in antifungal activity.

It was found that lignin peroxidase was released by 12 of 15 polypores but was strong activity for *Trametes versicolor* (POR 33D), *Polypore* sp. (POR 35), *Pycnoporus* sp. (POR11) and *Microporus* sp. (POR 36). The strong laccase reaction was predominantly by POR 33D and POR 58. On the contrary, *Microporus xanthopus* (POR 57) and POR 48 actively released the tyrosinase.

Since *Trametes versicolor* (POR 33D) and *Polypore* sp. (POR 35) had potential in enzymatic activity, both were cultured in two different media, malt extract and potato dextrose broth. The results showed that no LiP enzyme activities for *Trametes* sp. and *Polypore* sp. was detected when they were cultured in malt extract. The results only showed positively when they were cultured in potato dextrose broth. This indicated that some substances in potato dextrose broth induced the production of LiP, but not malt extract. The culture filtrate of *Trametes* sp. and *Polypore* sp. showed the presence of lignin peroxidase with 6.146 U / mL and 8.148 U / mL respectively.

For the results obtained in the present study, there were some suggestions for future study:

a. 15 Polypores showed weak antifungal activity against five tested fungi. Thus, methanol was probably not a good solvent to extract antifungal properties. Therefore, antifungal activity of Polypores extract using other solvents such as ethyl acetate and dichloromethane should be carried out in the future.

b. polysaccharide structure formed in cultured mycelium may depend on the composition of the nutrient medium used for cultivation. Antifungal properties of crude extracts of Polypores grown on various media can be investigated because different species vary greatly in their preferences to certain agar nutrient. Thus, antifungal properties of the 15 Polypores can be improved through the optimization of the growth media and especially the carbon and nitrogen sources for the production of mycelia.