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Study of the biological activities of *Physarum polycephalum* and *Physarella oblonga* plasmodial extracts

Nguyen TN Nguyen[1], Tuyen TM Huynh[1], Steven L Stephenson[2], Hanh TM Tran[*1]

[1] *School of Biotechnology, Ho Chi Minh International University, Thu Duc, Ho Chi Minh City, Vietnam*
[2]*Department of Biological Sciences, University of Arkansas, Fayetteville, AR 72701, United States*

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Abstract

Various species of myxomycetes have been found to produce bioactive compounds that appear to considerable potential for pharmaceutical applications. However, obtaining a sufficient amount of sample material for studying the bioactive compounds of myxomycetes has been a real challenge due to the small size of their fruiting bodies in the field and the slow growth rates of their plasmodia on synthetic media under laboratory conditions. The research reported herein was carried out first to study the effects of carbon source and carbon concentration on the growth of *Physarum polycephalum* and *Physarella oblonga* and then to evaluate antimicrobial and anticancer activities of their plasmodial extracts. *Physarella oblonga* was found to grow better on agar without nutrients, as it has ability to hydrolyze agar as a carbon source. The best agar concentration for the growth of this species was 19g/L, and at this concentration a remarkably high amount of plasmodia (92.05 g/L) was obtained. In contrast, *Physarum polycephalum* preferred glucose as a carbon source, and 20g/L of glucose was found to be the most suitable amount for this species. At this gulcose concentration, 65.02 g/L of plasmodia was produced. In term of antimicrobial activity, none of the *Phy. oblonga* extracts showed inhibitory activity on the pathogens tested. However, 90% MeOH and 90% MeOH:90% acetone extracts of *Ph. polycephalum* showed antimicrobial activity toward *Staphylococcus aureus*. Data on the minimum inhibitory concentration (MIC) showed that *S. aureus* was more susceptible to the extract of *Ph. polycephalum* 90% MeOH:90% acetone as the MIC of this extract was in the range of 1.56 to 0.78 mg/mL, whereas that of the 90% MeOH extract was between 6.25 and 3.12 mg/mL. However, the minimum bactericidal concentration values of these two extracts were found to be the same (at 12.5mg/mL). Initially, studies of the cytotoxicity of the extracts found that *Phy. oblonga* extracts could inhibit the tested cancer cell lines better when compared to those of *Ph. polycephalum*. Notably, MeOH:Chl *Phy. oblonga* had an inhibitory rate of 31.95 ± 6.37% on Jurkat blood cancer cells.

Key words – Anticancer activity - Antimicrobial activity - Myxomycete plasmodial extracts - Jurkat cells - *Staphylococcus aureus*

Introduction

The myxomycetes (plasmodial slime molds or myxogastrids) are a small group of fungus-like organisms (Lado 2001) with a life cycle that includes two trophic (or feeding) stages. The first stage consists of uninucleate amoebae and the other a multinucleate structure called a plasmodium. The latter is essentially a very large cell that contains numerous nuclei without having cell walls (Martin

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*Corresponding Author: Hanh TM Tran – e-mail – ttmhanh@hcmiu.edu.vn

et al. 1996). There are three distinct types of plasmodia produced by myxomycetes. These are the protoplasmodium, characteristic of members of the order *Echinosteliales*; the aphanoplasmodium, characteristic of members of the order *Stemonitales*; and the phaneroplasmodium, which is characteristic of members of the order *Physarales* (Gray & Alexopoulos 1968, Keller & Braun 1999). All three types of plasmodia are capable of movement and feed by migrating various distances within or on a given substrate. Under optimal environmental conditions, a plasmodium gives rise to one or more fruiting bodies containing spores. The phaneroplasmodium is the best-known and most easily studied type of plasmodium and has been the subject of numerous research projects. Phaneroplasmodia have rapid rates of growth and are relatively easy to culture under laboratory condition (Alexopoulos 1960).

More than 100 secondary metabolites have been isolated from myxomycetes (Dembitsky et al. 2005). Many crude extracts obtained from myxomycete fruiting bodies and plasmodia have shown antimicrobial and antitumor properties. A number of fatty acids and their derivatives are known for their antimicrobial activity (Ishibashi et al. 2001, Misono et al. 2003a, Nakatani et al. 2005). A new glycerolipid (bahiensol), isolated from a 90% MeOH:90% acetone crude extract obtained from the plasmodium of *Didymium bahiense*, displayed an inhibition zone (12.5 mm) toward *Bacillus subtilis* (Misono et al. 2003b). Stigmasterol and fatty acids purified from 95% ethanol plasmodial extracts of *Phy. oblonga* showed positive activity against epymastigote forms of *Trypanosoma cruzi*. Moreover, a crude extract from *Physarum melleum* displayed growth inhibition of the phytopathogen *Fusarium oxysporum* (Herrera et al. 2011).

In addition, Herrera et al. (2011) indicated that an extract from an unidentified species of myxomycete showed strong antimicrobial and antifungal activities against isolated strains of *Bacillus cereus*, *Fusarium oxysporum* and *Rhizoctonia solani*, and Nakatani et al. (2005) reported that a 90% MeOH and 90% acetone crude extract from *Physarum melleum* exhibited antimicrobial activity against *Bacillus subtillis*. In terms of anticancer activity, a 95% ethanol extract of fresh *Phy. oblonga* plasmodial fractions was found to exhibit an inhibitory activity against approximately 22.7% of a Jurkat Lat GF cells population (Herrera et al. 2011). Arcyroxocin and a bisindole alkaloid isolated from a 90% MeOH:90% acetone extract obtained from the fruiting bodies of *Arcyria denudata*, together with dihydroarcyriacyanin A, obtained from 90% MeOH:90% acetone extract from *Arcyria obvelata* extract, showed antitumor activities against Jurkat cells (Kamata et al. 2006). Kehokorins A, a novel dibenzofuran, isolated from a 90% MeOH:90% acetone extract obtained from the fruiting bodies of *Trichia favoginea var. persimilis* displayed cytotoxic toward HeLa cells with an IC50 value of 1.5 µg/mL (Kaniwa et al. 2006). Moreover, cyclic phosphatidic acid (CPA), a novel bioactive lipid isolated from *Ph. polycephalum*, showed specific biological functions, including antimitogenic regulation of the cell cycle, regulation of actin stress fiber formation and rearrangement, inhibition of cancer cell invasion and metastasis, regulation of differentiation and viability of neuronal cells, and mobilization of intracellular calcium (Murakami-Murofushi et al. 2002).

In addition, two new bisindole alkaloids isolated from a 90% MeOH:90% crude acetone extract from the fruiting bodies of *Lycogala epidendrum* showed cytotoxicity against HeLa cells and Jurkat cells with IC50 values of 5.4 and 2.1 µg/mL, respectively. The alkaloids also had slightly weak cytotoxicity against vincristine resistant KB/VJ300 cells (Hosoya et al. 2005). Synthetic media with a sugar (e.g., glucose) or oat flakes as the carbon source has been commonly used for cultivation of several species of *Physarum*. However, from our observations, different species prefer different carbon sources and carbon concentrations. As such, the research described herein was carried out first to study the effects of carbon source and carbon concentration on the growth *Ph. polycephalum* and *Phy. oblonga* and then to evaluate antimicrobial and anticancer activities of their plasmodial extracts.

Materials & Methods

Myxomycete cultures

The strain of *Ph. polycephalum* used in the present study was obtained as a sclerotium from Carolina Biological Supply Company (Burlington, North Carolina, USA). *Phy. oblonga* was obtained

from the myxomycete culture collection of the Applied Microbiology Labratory (School of Biotechnology, Ho Chi Minh International University, Ho Chi Minh city, Vietnam).

Pathogenic microorganisms

The pathogenic microorganisms *Staphylococcus aureus* ATCC 25213, *Salmonella typhimurium* ATCC 14028, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 1413 were used for the antimicrobial tests.

Cancer cell lines

The human breast cancer MCF-7 cell line, the Cervical HeLa cancer cell line, the Jurkat blood cancer cell line and the human liver cancer cell line Hep-G2 were purchased from the American Type Culture Collection (Manassas, Rockville, Maryland, USA) by the Faculty of Biochemistry (Ho Chi Minh City University of Science, Ho Chi Minh City, Vietnam).

Cultivation of myxomycete plasmodia

Spore germination, sclerotial activation and inoculum preparation were carried out following Tran et al. (2012) and Tran et al. (2015). A piece of agar (about 2 cm^2) bearing a portion of the active plasmodium growing on oat flakes was transferred from the inoculum culture to either a water agar or nutrient agar medium by using sterile blade and forceps. The cultures were inoculated in the dark at room temperature (25^oC) for 5 days, when the plasmodia developed fully on the plate. The plasmodia were collected and placed in sterile falcon tubes and freeze-dried using a standard freeze-drier device (Tran et al. 2012).

One liter of nutrient medium containing 100 mL of a basal salt solution, 5.0 g of glucose [Difco], 2.5 g of yeast extract [Difco], 15 g of Bacto agar, and 900 mL of distilled water adjusted to pH 5.5 was used. The basal salt solution contained 29.78 g of citric acid, 33.10 g of K_2HPO_4, 2.50 g of NaCl, 1.00g of $MgSO_4.7H_2O$, 0.50 g of $CaCl_2.2H_2O$, and 1000 mL distilled water (Henney & Henney 1968).

Effects of carbon source and carbon concentration on myxomycete plasmodial growths

Preliminary studies showed that *Ph. polycephalum* preferred glucose and *Phy. oblonga* grew better in water agar without glucose (*Phy. oblonga* has agar hydrolytic activity). Thus, different carbon sources were used for these two species.

To study the effects of glucose concentration on *Ph. polycephalum* plasmodium culture, glucose with various concentrations (5 to 35 g/L) was used, whereas to study the effects of agar concentration on *Phy. oblonga* plasmodium culture, water agar was prepared with different amounts of agar (ranging from 10 to 25 g/L).

Myxomycete plasmodial extract preparation

A sample of freeze-dried plasmodial was ground to a fine powder for preparation of the extracts.

Methanol:Chloroform (MeOH:Chl) extract

The MeOH:Chl plasmodium extract was prepared following the procedure outlined by Bligh & Dyer (1959) with some minor modifications. One gram of dried plasmodium was homogenized with a methanol: chloroform (20:10, v/v) solution in a 35-mL sterile glass vial. After 15 minutes, 5 mL water was added to each vial, the vial was gently vortexed and set aside until the separation of three different phases was formed (5mL chloroform was added in case there was no obvious separation observed). The lower phase of the mixture was transferred to another glass bottle and evaporated until a constant weight was obtained (Tran et al. 2012).

90% MeOH extract

One gram of the dried plasmodium was added into a 35-mL sterile glass vial containing 20mL 90% methanol. This mixture was placed on a shaking incubator at room temperature for 12 hours. The

homogenized solution was then collected and transferred to another vial. The methanol mixture was evaporated until completely dried (Kang et al. 2011).

90% methanol:90% acetone extract

One gram of dried myxomycete plasmodium was homogenized with a 90% methanol:90% acetone (20:8, v/v) solution in 35 mL sterile glass vial. After 30 minutes, 2 mL of 90% MeOH and 0.8 mL hexane were added. The vial was set aside for another 30 minutes. The homogenized solution was then transferred to another glass vial. The extract was evaporated until completely dried (Misono et al. 2003b).

All of the dried extracts were dissolved in 2% Dimethyl sulfoxide (DMSO) (100mg/mL) for microbial tests and the cytotoxic assay.

Preparation of pathogenic microorganisms

Three pathogenic bacteria were cultured in LB broth until the cultures reached the concentration of approximately 1-1.5 x 10^8 CFU/mL. *Candida albicans* was cultured in Sabouraud Dextrose (SD) broth until the fungal culture had reached approximately 1-1.5 x 10^8 CFU/mL.

Well diffusion method

One hundred µL of the microorganism suspension was spread on either a LB or SD agar plate using sterile cotton swab. Four 8-millimeter diameter wells were made on each plate, and 100 µL of the extract was added to two wells. Two remaining wells were used for positive and negative control. Gentamycine (1 mg/mL) and Ketoconazole (0.4 mg/mL) were used as positive antimicrobial controls for bacteria and fungus, respectively, whereas 2% DMSO was used as the negative control (Kang et al. 2011; Herrera et al. 2011). The plates were incubated at 37ºC in 12-18 hours for bacteria and 24-36 hours for the fungal cultures. Diameters of the inhibition zone in millimeters (minus the well diameter) were recorded (Anesini et al. 1993, Azizkhani et al. 2013)

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) was carried out using the micro-titer broth dilution method (Hassan et al. 2011). The assay was performed in a sterile 96-well micro-titer plate. Two positive controls (medium and inoculum; 2% DMSO and inoculum) and two negative controls (extract and medium; 2% DMSO and medium) were included to obtain more accurate evaluations of the results.

Each treatment/well contained 100µL of broth medium and 100µL of standard bacteria inoculum (10^6cfu/mL) and the plasmodial extract with a particular concentration (within the range of 25 to 0.39 mg/mL). The micro-titer plates were incubated at 37ºC for 12-18 hours. MICs were then recorded (Chanthaphon et al. 2008). As the extracts exhibited colors, MBC was investigated to confirm the MIC values. The MBC was the plate with the lowest concentration of extract which showed no evidence of microbial growth (Betts et al. 2012).

Cytotoxic assay by Sulforhodamine B (SRB) assay

The cell lines were cultured at 37°C and 5 % CO_2 in Eagle's Minimal Essential Medium (EMEM) supplemented with 10 % (v/v) FBS (Sigma), 2 mM L-glutamine (Sigma), 20 mM HEPES (Sigma), 0.025µg/mL amphotericin B (Sigma), 100 IU/mL penicillin G (Sigma) and 100 µg/mL of streptomycin (Sigma) (Nguyen et al. 2016).

SRB assay

Cells seeded at a density of 10,000 cells/well in 96-well plates were cultured for 24 h before being incubated with the tested plasmodial extract for 48 h. Treated cells were fixed with cold 50 % (w/v) trichloroacetic acid (Merck) solution for 1-3 h, washed, and stained with 0.2 % (w/v) SRB (Sigma) for 20 min. After five washes with 1% acetic acid (Merck), protein-bound dye was solubilized in 10 mM Tris base solution (Promega). Optical density values were determined with a 96-well micro-

titer plate reader (Synergy HT, Biotek Instruments) at the wavelengths of 492 nm and 620 nm. The percentage of growth inhibition (Inh %) was calculated according to the formula

Inh (%) = (1-[ODt/ODc] x 100) %

in which ODt and ODc are the optical density values of the test sample and the control sample, respectively. Camptothecin (Calbiochem) was used as the positive control (Nguyen et al. 2016).

Experimental statistics

All the experiments were carried out in triplicate. The results were reported as mean ± standard deviation (SD). Statistical analysis of the data was conducted by analysis of variance (One-way ANOVA) using the Statistical Package for the Social Sciences (SPSS) software version 16.0. A probability value of $p \leq 0.05$ indicated a significant difference in the result of the particular test being carried out.

Results and Discussion

Effects of carbon concentration on plasmodial growth of *Phy. oblonga* and *Ph. polycephalum*

Phy. oblonga was observed to have agar-hydrolytic activity and could grow better on a water agar medium, which is different from other myxomycetes, including *Ph. polycephalum*. Therefore, agar and glucose were chosen as the carbon source for cultivation of *Phy. oblonga* and *Ph. polycephalum*, respectively. Effects of the carbon concentrations on the plasmodial cultures are shown in Fig. 1 and 2.

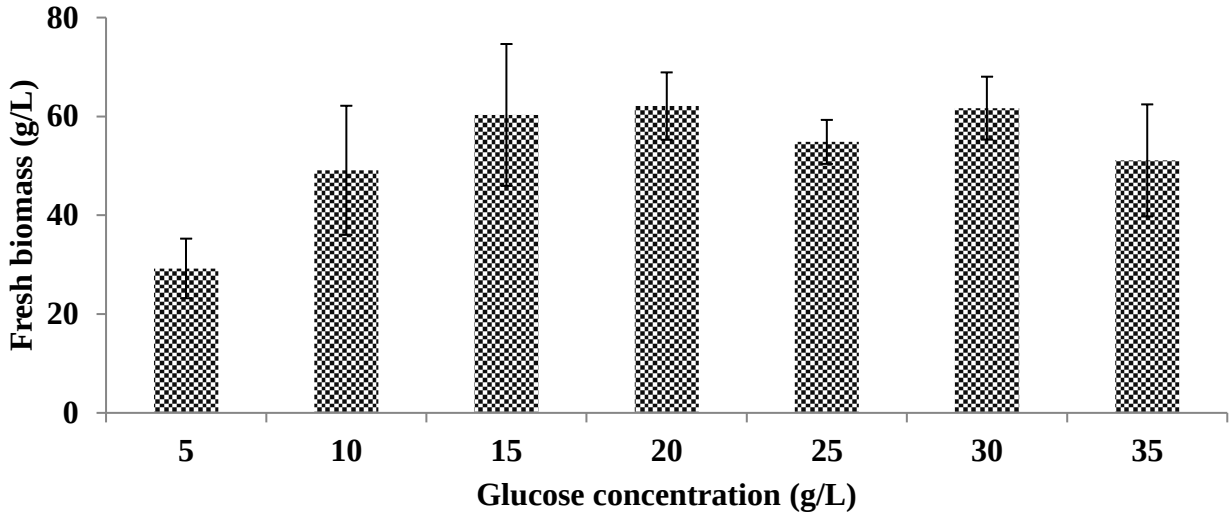

Fig. 1 - Effect of glucose concentrations on biomass production by *Ph. polycephalum*

The fresh biomass production of *Ph. polycephalum* was found to increase with increasing glucose concentrations from 15 to 35 (g/L). Notably, the highest amount of biomass (62.09 g/L) was recorded for a 20g/L glucose culture, which is significantly different when compared with the cultures with lower glucose concentrations. Since glucose is the main carbon source in the medium, higher glucose concentration could enhance plasmodial production. However, there was no significant difference in biomass production when glucose fell in the range from 20 to 30 g/L, and the plasmodial concentration decreased when the glucose was 35 g/L. It seems likely that the high concentrations of glucose would cause high osmotic pressure, and this would negatively affect plasmodial growth. For *Ph. polycephalum* this effect began to become evident at 35 g/L.

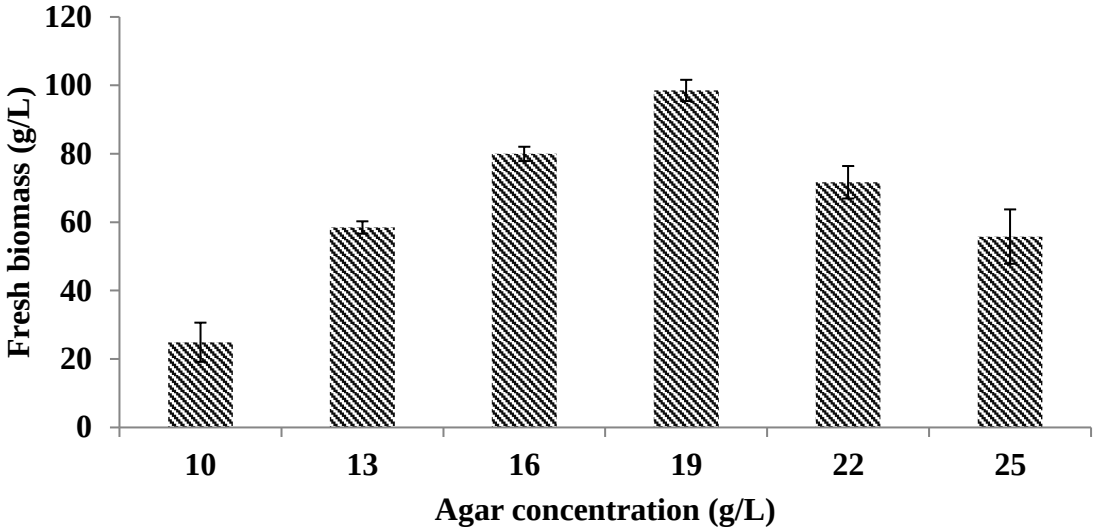

Fig. 2 - Effect of agar concentrations on biomass production of *Phy. oblonga*

As noted in Fig. 2, the biomass production of *Phy. oblonga* was found to increase when agar concentration was increased in increments from 16 to 19 g/L. The highest amount of fresh biomass (98.5 g/L) was observed at 19 g/L. When the agar concentration exceeded 22 g/L, plasmodial production decreased. The lowest biomass production (24.9 g/L) was obtained from the 10g/L agar culture. Because *Phy. oblonga* can utilize agar for as a carbon source, the higher agar concentration would be expected. In addition, higher agar concentration also increased the firmness of agar surface, which would help *Phy. oblonga* plasmodia easily to expand. The negative effects recorded from cultures with 25 g/L of agar would be explained by the fact that when the concentration of agar is too high, the availability of water in the culture decreased. This made the agar crack; moreover, the lack of water would negatively affect the metabolic activities of plasmodia. However, a low concentration of agar, which would result in high water content in the culture, also would make it difficult for a plasmodium to obtain oxygen because the water could cover the plasmodium, resulting in poor biomass production (Tran et al. 2015)

Primary study on antimicrobial activities of *Phy. oblonga* and *Ph. polycephalum* extracts

Antimicrobial activity of *Phy. oblonga* and *Ph. polycephalum* plasmodial extracts was primarily determined by the well diffusion method. The antimicrobial activity was assessed by determining the diameter (in mm) of the growth inhibition zone and showed in Table 1.

In general, only the 90% MeOH:90% acetone and 90% MeOH extracts of *Ph. polycephalum* were found to have antimicrobial activities. The extracts showed inhibitory activities toward *S. aureus*, for which the inhibition zone diameters were 15 ± 1.5 mm and 5 ± 1 mm, respectively.

Thus, far, the ability of extracts obtained from *Ph. polycephalum* has not been reported in the literature. In the present study, although *S. aureus* was susceptible to the *Ph. polycephalum* extracts, it should be noted that after 36 hours, *S. aureus* grew back into the inhibition zones, causing the zones to become smaller. However, since the extracts were crude, the amount of the actual antimicrobial compounds present would be expected to be rather low.

Phy. oblonga in our research did not exhibit any antimicrobial activity. It seems likely that different species of myxomycetes species display different types and levels of biological activity, which may be the result of their individual strategies to cope with the different biotic components of their environment (Herrera et al. 2011).

Since *Ph. polycephalum* extracts (90% MeOH:90% acetone and 90% MeOH) displayed antibacterial activities toward *S. aureus*, MICs of the extracts against this gram-positive bacterium were determined and the results are shown in Table 2.

MIC results showed that *S. aureus* was more susceptible to the *Ph. polycephalum* 90% MeOH: 90% acetone extract in the concentration range of 1.56 to 0.78mg/mL, whereas with the 90% MeOH

extract, the MIC value was between 6.25 to 3.12 mg/mL. The MBC values of the two types of *Ph. polycephalum* extracts were found to be the same (12.5mg/mL).

Table 1 Antimicrobial activities of myxomycete plasmodial extracts

Test pathogen	*Ph. polycephalum*			*Phy. oblonga*			Control		
	ME[a]	ME-C[a]	ME-A[a]	ME[a]	ME-C[a]	ME-A[a]	G[b]	K[c]	2% DMSO
S. aureus	+	-	+++	-	-	-	+++ +	-	-
E. coli	-	-	-	-	-	-	+++ +	-	-
S. typhi	-	-	-	-	-	-	+++ +	-	-
C. albican	-	-	-	-	-	-	-	+++ +	-

Notes: ME: 90% MeOH extract, ME-C: MeOH:Chl extract, ME-A: 90% MeOH: 90% Acetone extract, G: Gentamycine, K: Ketoconaole. [a,b,c] concentration of 100mg/mL, 1mg/mL, 0.4mg/mL, respectively. [-] no inhibition zone, [+] inhibition zone less than 10 mm, [++] inhibition zone from 10 to less than 15 mm, [+++] inhibition zone from 15 to less than 20 mm, [++++] inhibition zone greater than 20 mm.

Table 2 Minimum inhibitory concentration (mg/mL) of *Ph. polycephalum* extracts

Extract	MIC (mg/mL)	MBC (mg/mL)
90%MeOH:90%Acetone	0.78	12.5
90% MeOH	3.12	12.5

Cytotoxicity of *Phy. oblonga* and *Ph. polycephalum* extracts

The extracts of *Phy. oblonga* and *Ph. polycephalum* were dissolved in absolute DMSO to reach the concentration of 100µg/mL and tested with different cancer cell lines. The results obtained are displayed in Table 3.

As a general observation, MeOH:Chl and 90% MeOH:90% acetone extracts of *Phy. oblonga* were observed to have inhibitory activities against the cancer cell lines tested in the present study, especially toward Jurkat cells. At a concentration of 100 µg/mL, the MeOH:Chl extract from *Phy. oblonga* could inhibit Jurkat, MCF-7, Hep-G2 cancer cell lines with the rates of 31.95 %, 12.02% and 11.33%, respectively. The 90% MeOH:90% acetone extract from *Phy. oblonga* also showed cytotoxic city against Jurkat cancer cell lines, with a rate of 26.15%. The *Ph. polycephalum* MeOH:Chl extract was toxic only on the Jurkat cell line with a rate of 13.26 ± 3.43.

Percentage inhibition of Jurkat Lat GF cells by the 90% ethanol *Phy. oblonga* extract as reported from previous studies was 22.7% (Herrera et al. 2011). In term of cytotoxicity of the 90% MeOH:90% acetone extract involving other species of myxomycetes, we are aware only of research on *Trichia favoginea var. persimilis*, in which the crude extract obtained from fruiting bodies was reported to inhibit 50% of the HeLa cells at 1.5µg/mL (Kaniwa et al. 2006). This extract concentration is quite low compared with the concentration used in the present study.

Conclusions

Different species of myxomycetes require different optimized medium compositions for their growth. Glucose with a concentration of 20 g/L was optimal for *Ph. polycephalum*, whereas 19 g/L of agar was optimal for *Phy. oblonga*. In term of microbial activity, 90% MeOH:90% acetone and 90% MeOH extracts of *Ph. polycephalum* displayed inhibitory activities toward *S. aureus*. Moreover, a

preliminary investigation of the cytotoxicity of these extracts found that the MeOH:Chl extract from *Phy. oblonga* displayed the potential to inhibit the Jurkat cell line.

Table 3 Cytotoxicity of *Phy. oblonga* and *Ph. polycephalum* extracts on various cancer cell lines (%)

Types of extracts (100µg/mL)	**Cytotoxicity on cancer cell lines (%)**			
	MCF-7	**HeLa**	**Jurkat**	**Hep-G2**
Phy. oblonga MeOH: Chl	12.02 ± 0.69	-0.09± 3.16	**31.95 ± 6.37**	11.33 ±1.11
Ph. polycephalum MeOH:Chl	0.60 ± 0.95	-6.57 ± 4.61	13.26 ± 3.43	-0.43 ± 2.67
Ph. polycephalum 90% MeOH: 90% acetone	-11.31 ± 3.29	3.84 ± 3.56	4.94 ± 4.23	-13.89 ±1.65
Phy. oblonga 90% MeOH: 90% acetone	-6.86 ± 4.07	2.85 ± 4.97	**26.15 ± 3.10**	10.41 ±3.29
Ph. polycephalum 90% MeOH	-11.67 ± 0.96	4.80 ± 5.51	-1.02 ± 6.57	-31.22 ±2.48
Phy. oblonga 90% MeOH	5.82 ± 5.23	6.83 ± 3.67	9.88 ± 0.17	-4.93 ± 3.90

Note: the minus figures indicate that the extracts stimulated the growth of the cells.

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