Physarella oblonga-centered bioassays for testing the biological activity of myxomycetes

Herrera NA[1*], Rojas C[2], Franco-Molano AE[3], Stephenson SL[4] and Echeverri F[1]

[1]Laboratorio de Química Orgánica de Productos Naturales, SIU, Universidad de Antioquia, Medellín, Colombia
[2]Instituto de Investigaciones en Ingeniería, Universidad de Costa Rica, San Pedro de Montes de Oca 11501-2060, Costa Rica
[3] Laboratorio de Ecología y Taxonomía de Hongos, Universidad de Antioquia, Medellín, Colombia
[4]Department of Biological Sciences, University of Arkansas, Fayetteville AR 72701-1201, USA

To study the trypanocidal, antibacterial, antifungal activity and cytotoxicity of myxomycetes, a rapid assessment focused on the species *Physarella oblonga* was carried out. Optimum conditions for culturing were utilized to develop a protocol that was adequate for bioanalysis of chemical compounds. Nuclear magnetic resonance (NMR) and mass spectroscopy (MS) detected the presence of stigmasterol and fatty acids in plasmodial extracts of *Ph. oblonga* through H1 analysis. These plasmodial extracts showed low toxicity and positive activity against epymastigote forms of *Trypanosoma cruzi*. This activity was significantly higher than the activity shown by one of the controls used. Similarly, the extracts from an unidentified species of myxomycete showed strong antimicrobial and antifungal activities against isolated strains of *Bacillus cereus*, *Fusarium oxysporum* and *Rhizoctonia solani*, whereas the myxomycete *Physarum melleum* displayed growth inhibition of the phytopathogen *F. oxysporum*. These results showed that with the use of an appropriate methodology, bioprospective analysis can be carried out on myxomycetes. In addition, this is apparently the first report on the antifungal and antiparasitic potential of myxomycetes.

Introduction

The myxomycetes, also known as slime molds or myxogastrids, are a group of approximately 850 amoeboid protists (Lado 2005-2011) that occur in all terrestrial ecosystems but are poorly studied in some regions of the world. Myxomycetes are heterotrophic organisms that resemble certain microscopic fungi and produce spores as their primary dispersal mechanism (Stephenson & Stempen 1994). Their life cycle includes two different trophic stages, a uninucleate cell often referred to as a myxoamoeba or swarm cell, depending on the presence or absence of a flagellum, and a macroscopic, multinucleate structure known as a plasmodium (Martin & Alexopoulos 1969).

Myxomycetes are unique organisms from a biochemical point of view (Steglich 1989). This is partially due to the presence of a large group of secondary metabolites, some of which have been isolated from several of the more commonly cultured species. Among these metabolites, a number of fatty acid derivatives,

alkaloids, aromatic compounds, terpenoids and amides have been identified (Dembitsky et al. 2005). Interestingly, the derivates from fatty acids are known for their antimicrobial activity, which exhibits a reversal effect of multidrug resistance (Ishibashi et al. 2001, Misono et al. 2003, Nakatani et al. 2005). These facts cause myxomycetes to be a very interesting and innovative group to be analyzed from a biochemical perspective.

Similarly, their secondary metabolic pathways produce compounds with unique structures, and these compounds resemble a series of metabolites from both plants and fungi. However, a standard procedure for obtaining cultures of these microorganisms has not yet been standardized. Therefore, the availability of these chemical compounds is very low. In fact, most of the studies of spore germination, culturing and fruiting body formation in myxomycetes were carried out more than 40 years ago (Gray 1949) and few, if any, studies on secondary metabolites are still being carried out in the Western Hemisphere.

In spite of this, one group of myxomycetes that has been successfully cultured is the order Physarales (Gray & Alexopoulos 1968, Lado et al. 2007). Some species in this group, such as *Physarum polycephalum*, have even become model organisms. However, most of the species in this group are still understudied at the laboratory level. One of the species in the group, *Physarella oblonga,* has been cultured for a number of different purposes (e.g. Franke 1967, Bechtel 1975, Ribeiro et al. 2003) but apparently not yet examined for the presence of potentially useful active substances. The same can be said for the majority of myxomycetes.

This fact does not really represent a problem in relation to the search of alternative cures for diseases, but it does represent a gap in a line of research with a potentially high social impact. For instance, diseases such as the American Trypanosomiasis, also known as Chagas disease, have been traditionally treated with potent chemicals, but it has been poorly investigated with respect to the use of more natural medicines (e.g. Ferraz et al. 2009). Since more than 40 million people are at risk of being infected with this disease (Schofield 2006) and its eradication has been difficult, there is a need and responsibility to try new alternatives for its treatment. In this sense, an answer to the question as to whether or not myxomycetes can be potential candidates for research against Chagas-like diseases is still unknown, since these organisms have not yet been studied in this way. However, as a relatively new biological group for bioprospecting research, myxomycetes provide a wide spectrum of possibilities due their unique biochemistry.

For those reasons, the project described herein was designed with two main objectives. The first was to rapidly assess the biological activity and cytotoxicity of some myxomycete plasmodia and their secondary metabolites, and the second was to develop a suitable protocol for the production of large amounts of myxomycete plasmodial mass. The ultimate goal of this project is to make available, at some point in the future, the material required to evaluate active chemical compounds from myxomycetes that might act as bioactive agents against microorganisms.

Methods

This study was carried out in the Laboratory of Organic Chemistry of Natural Products at the University of Antioquia in Medellin, Colombia, during 2007 and 2008. The morphological concept of species was used to identify myxomycetes, following the taxonomic treatment of Lado (2005-2011). Plasmodia were first cultivated in moist chamber cultures following a modification of the protocol provided by Stephenson & Stempen (1994).

In all cases, plasmodia were placed on filter paper (Whatman #1, 90 mm) in a standard Petri dish (150 x 15 mm), and a small amount of distilled water was added to the dish. After approximately 2 weeks, small sections of the plasmodia were cut off and placed on 1.6% water agar plates. The first fruiting bodies obtained in this study were deposited in the herbarium of the University of Antioquia (HUA).

For all bioassays, the myxomycete *Physarella oblonga* (Berk. & M.A. Curtis) Morgan was used. However, to test the antimicrobial activity, two more taxa, *Physarum melleum* (Berk. & Broome) Massee and an

unidentified species also were utilized. Culturing conditions were established for the first species and then applied to the other two myxomycetes as a way of testing the viability of the obtained protocol for generating an adequate amount of plasmodial extracts for bioassay purposes.

Culturing

Seven days after the initial growth on water agar, an evaluation of the best type of media and amount of food for the plasmodial cultivation of *Ph. oblonga* was carried out. For the first part, three sections of plasmodia approximately 1 cm^2 in extent were transferred onto sets of three plates prepared with eight different types of media, for a total of 24 different cultures. The media types evaluated were 1.6% water agar, oatmeal agar, malt extract agar, potato dextrose agar (PDA), oxytetracycline glucose yeast agar (OGY), sabouraud agar, mixed-bark infusion agar and a mushroom (*Flammulina velutipes*) infusion agar.

To evaluate the amount of food necessary to obtain the largest plasmodia, four quantities (0.1, 0.2, 0.5 and 1 g) of oatmeal flakes previously autoclaved at 121ºC and 15 lb/atmospheric pressure for 20 minutes, were used in bark infusion agar plates. A set of three replicates was prepared for each of the four quantities, for a total of 12 different cultures. The inoculated plasmodia on bark infusion agar were subjected to temperature of 26°C. For extraction of secondary metabolites, an additional experiment was carried out to determine the kinetic plasmodium growth. During six consecutive days, the weight of the plasmodial mass was determined on three cultures per day after an initial inoculation of eighteen plates of bark infusion agar with plasmodia, following the best combination of parameters as determined from the previous experiments.

Extraction of chemical compounds

For *Ph. oblonga*, the chemical extraction of secondary metabolites was carried out by starting with a mixture consisting of 1.0 L of 95% ethanol and approximately 50 g of fresh plasmodial mass. This step was followed by filtration and evaporation of the solvent and fractioning of the crude extract (5 g) by column

chromatography using Merck silica gel 60, eluted with 1000 ml of a mixture of dichloromethane:methanol (4:1 v/v). Fractions were obtained and monitored using thin layer chromatography (TLC) on silica gel 60 chromatoplates using dichloromethane:methanol 4:1 v/v. From the latter plates, candidate fractions were recovered and purified using subsequent TLC plates (dichloromethane:methanol 4:1 v/v) and column separation, using Merck silica gel 60, eluted with 500 ml of a mixture of hexane:ethyl acetate (3:1 v/v).

Isolated compounds were analyzed by means of nuclear magnetic resonance (NMR) analyses, using a Bruker AMX at 300 MHz for 1H and 75 MHz for 13C. In addition, mass spectroscopy (MS) analyses were performed on isolated compounds in order to evaluate the chemical composition and possible identity of the metabolites.

Cytotoxicity

For this essay, Jurkat Lat GFP cells were used. Approximately 500,000 cells were deposited in individual 1 ml wells on a 24-well plate. Samples were activated with a concentration of 100 μg/ml from the fractions of plasmodial extracts of *Ph. oblonga*, a polyunsaturated fatty acid and a yellow compound from *Ph. oblonga* plasmodia recovered after chromatrography. A negative control was also used. Samples were incubated for 12 hours at 37 ºC in an atmosphere of 5% CO_2. The liquid in each well was transferred to a microcentrifuge tube in which propidium iodide at a concentration of 1 μl/100 μl was added. Immediately after this, samples were analyzed using a flow cytometer by collecting ten thousand events per sample (see Zaritskaya et al. 2010).

Trypanocidal activity

The activity against *Trypanosoma cruzi* was evaluated by first cultivating a series of samples corresponding to the epimastigote forms of the Tulahen strain of the parasite at 26˚C in a liver infusion-tryptose medium supplemented with 5% of heat-inactivated fetal bovine serum. The parasites in logarithmic growing phase were distributed by triplicate in 96-flat bottom-well microplates at a concentration of 5 x 10^4 cells/ml. Each well was exposed

to different concentrations of crude plasmodial extract and two fractions isolated from crude extract of *Ph. oblonga* at a concentration of 100, 50 and 25 μg/ml, respectively, for a period of 72 hours.

The anti-trypanosome activity was determined by optical count, using an inverted microscope. For this, the reference drugs utilized correspond to Benznidazole and Amphotericin B at a concentration of 10 μg/ml. The results of the antiparasite activity are expressed in terms of IC50, the drug concentration that inhibits 50% of the development in the parasite. These values were obtained by creating a dose-response curve which corresponds to the logarithm of the drug concentration versus the percentage of inhibition.

Determination of antimicrobial activity

For these series of assays, three species of myxomycetes—*Ph. oblonga*, *P. melleum* and one unidentified species—were used to test biological activity of the group on pathogenic microorganisms (see Table 1). The cultures of these organisms were obtained from the Microbiology laboratory at the Universidad de Antioquia. The inhibitory effects of the extracts produced were tested by performing a disc diffusion method (Thitilertdecha et al. 2008).

For this, 50 μl of sample solutions at a concentration of 0.5 mg/ml (dry residue/volume of dimethyl sulfoxide, DMSO) were used. Three repeats were tested in every inhibition experiment. The magnitude of the antimicrobial action was assessed by measuring the diameter (in mm) of the inhibition zones, in relation to those of positive and negative controls. The latter were co-assayed by using 10 μg of Gentamicine and 1g/L of Benomil as positive antimicrobial references and plain DMSO as negative control.

Statistical analysis

In order to evaluate the performance of plasmodial growth under the different culturing conditions tested, a series of Kruskal-Wallis tests was carried out on the respective data. This non-parametric test uses ranks to evaluate the independence of three or more groups that do not necessarily meet the normality requirements of similar tests for heterogeneous

data structure. On the other hand, the cytotoxicity and trypanocidal activity were tested using analysis of variance due the more normally structured nature of the datasets. In the latter instances, effect-size correlation values were also calculated to test variability due to biological variation (population-based differences). In all cases, the probability value associated with the rejection of the null hypothesis was 0.05 and all tests were performed using the software JPM, version 7.0.2. (SAS 2007).

Results

As result of the experiments described above, a successful protocol for the laboratory cultivation of myxomycete vegetative (plasmodial) mass was obtained. This was based on the performance of *Ph. oblonga* plasmodial growth, which was observed to take place only on water agar, mushroom infusion agar and bark infusion agar. Five days after inoculation the amounts of fresh plasmodial mass for the three positive media types were 0.041, 0.312 and 0.980 g, respectively. The ranks of plasmodial growth were significantly different on both water agar and mushroom infusion agar than on bark infusion agar ($H=7.20$, $d.f.=2$, $P<0.05$). Based on these results, it was determined that bark infusion agar was a suitable medium for the cultivation of *Ph. oblonga*. In a similar way, the largest plasmodial fresh weight rank differences were obtained by using the smallest amount of food ($H=8.33$, $d.f.=3$, $P<0.05$).

The average fresh weight obtained for each day was determined to be 0.039, 0.186, 0.528, 0.932, 0.856 and 0.715 g from the first to the sixth day. There was a progressive increase in plasmodial mass until the fourth day, after which the plasmodia seemed to decrease in mass. Interestingly, the fresh weight of the last three days was not significantly different ($H=5.68$, $d.f.=2$, $P>0.05$) in spite of obvious differences for the entire period of time ($H=15.9$, $d.f.=5$, $P<0.05$).

Chromatographic fractioning, NMR and MS analyses allowed the isolation of a large number of triglycerides and several polyunsaturated fatty acids. However, only one compound (stigmasterol) could be identified successfully using these techniques. The cytotoxicity test showed that both of the plasmodial

Table 1 Antimicrobial activity of crude extracts and fractions from myxomycetes and positive and negative controls.

Sample type	Organism used					
	Bs	Sa	Ec	Fo	Rs	Bc
Crude extract *Physarella oblonga*	-	-	-	-	-	-
Crude extract *Physarum melleum*	-	-	-	++	+++	-
Crude extract Unidentified	+++	-	-	+++	+++	-
Fraction 1	-	-	-	-	-	-
Fraction 4	++	-	-	-	-	-
Gentamicine (0.1 mg/ml)	+++	+++	+++	-	-	-
Benomil (1 mg/ml)	-	-	-	+++	-	+++
DMSO (50 μl)	-	-	-	-	-	-

Symbols: − no activity, + inhibition zone less than 10 mm, ++ inhibition zone 10-20 mm, +++ inhibition zone greater than 20 mm.
Abbreviations: Bs = *Bacillus subtilis*, Sa = *Staphylococcus aureus*, Ec = *Escherichia coli*, Fo = *Fusarium oxysporum*, Rs = *Rhizoctonia solani*, Bc = *Botrytis cinerea*.

fractions isolated from the crude extract from plasmodia of *Ph. oblonga* have low toxicity when compared to the negative control (Table 2) at a concentration of 100 μg/ml. The highest percentage of inhibition was observed for one of the plasmodial fractions, designated as number two, at 22.7%. In spite of the latter, the cytotoxicity observed for fractions and extracts from *Ph. oblonga* plasmodia is not significantly different from the control ($F(1,5)=3.07$, $P=0.14$, $R^2=0.38$).

The trypanocidal activity of *Ph. oblonga* is shown in Table 3. Both the crude extract and the two fractions evaluated showed strong differences in the activity against the parasite when compared to the Amphotericin B control group (for experimental versus control only, $F(1,2)=1406$, $P=0.0007$, $R^2= 0.99$) but no differences when Benznidazole was considered ($F(1,3)=0.50$, $P=0.52$, $R^2=0.14$).

In the assays carried out on the antimicrobial activity (Table 3), the bacterium *B. cereus* was inhibited by the extract of the unidentified myxomycete. The diameter of the inhibition zone was about 20 mm at 500 μg/ml. None of the other combinations of myxomycetes and bacteria tested yielded positive results. On the other hand, when the antifungal activity was tested, both crude extracts from *P. melleum* and the unidentified species showed growth inhibition of *F. oxysporum*, although the inhibition zone of the latter was larger than that of *P. melleum*. In the

case of both antibacterial and antifungal activity, the positive controls showed a strong effect on the target organisms. Interestingly, the strain of *R. solani* used in this study seemed to be very resistant to the different antifungal agents used, except in the case of plasmodial extracts from *P. melleum* and the unidentified species.

Discussion

The production of fruiting bodies by *Ph. oblonga* only in water agar, mushroom infusion agar and bark infusion agar is not surprising. It is well known that myxomycete spores germinate when water is available (Stephenson & Stempen 1994). Also, bark represents one of the best substrates on which myxomycetes can grow (see Ing 1994) under natural conditions. This is probably related to the chemical composition of bark from different tree species and also to the capacity of different types of bark to retain air-borne myxomycete spores. As such, it is very likely that some myxomycetes are well adapted to this corticolous environment and exploit its microresources efficiently. If this is the case, the Physarales, the order that contains *Ph. oblonga*, is likely to be one example of this type of situation, as can be observed in its high degree of specificity for bark (see Everhart & Keller 2008). Even though mushrooms are also known as a substrate for myxomycetes, the relationship does not seem to be that intricate

Table 2 Percentage of inhibition of Jurkat Lat GF cells shown by different fractions extracted from the myxomycete *Physarella oblonga*.

Fraction	Inhibition (%)
Polyunsaturated fatty acid	22.7
Unidentified compound	12.4
Fraction 1	15.2
Fraction 2	27.0
Fraction 3	11.6
Fraction 4	16.7
Negative control	6.1

(Ing 1994). The fact that fatty acids and stigmasterol were successfully isolated from myxomycete plasmodia provides some evidence that these organisms can be used for biosprospecting. Even though these types of metabolites already have been reported from myxomycete plasmodia (Dembitsky et al. 2005), this is the first time that stigmasterol has been reported for *Ph. oblonga*. This compound has been found in other members of the Physarales such as *Physarum polycephalum* and *Physarum flavicomum* (Bullock & Dawson 1976). The problem is that these sterols are very common in plants as well (e.g. Shibuya 2001), which makes the evaluation of the nature of this compound difficult in the case of the present study. Whether this compound is being directly produced by the myxomycete or actively taken up from the woody substrates is a question that warrants additional study. For example, stigmasterol is known to occur in oat plants, but it also has been observed in examples of the Physarales cultured without this as a food source (Bullock 1976). It is likely that the metabolic pathway used by myxomycetes to produce this sterol is very similar to the one found in plants (e.g. Schaller 2004), but the true nature of the compound in the cultures we studied remains uncertain for now.

On the other hand, even though polyunsaturated fatty acids are known to possess antibacterial activity (e.g. McGaw et al. 2002), it is currently impossible to determine whether or not some of the compounds extracted from *Ph. oblonga* have such properties. However, it seems possible that this is indeed the case. For example, Chiappeta et al. (1999) reported antibacterial activity in *Fuligo septica*, a species that is phylogenetically related to *Ph. oblonga*.

Similarly, *Lycogala epidendrum* has been reported to produce fatty acids that showed inhibitory activities toward the growth of gram-positive bacteria (Rezanka & Dvorakova 2003). What makes bioprospecting in myxomycetes more interesting is that it has been suggested that fatty acids can be used for other important applications. For example, it seems that these compounds have a high potential for use against malaria, tuberculosis and fungal diseases (Carballeira 2008). Interestingly, even though there are no studies of trypanocidal activity in any of these compounds, the results obtained in the present investigation provide an alternative future approach to study Chagas disease as well.

This alternative is of course highly dependent on the properties of the system analyzed. For example, whether or not a system shows toxicity against human cells is something critical to be determined. In the present study, all the cytotoxicity tests showed that plasmodial extracts from *Ph. oblonga* are highly innocuous. This is an important aspect to consider in biochemical systems when potential medical uses are studied. It has been known for a number of years that myxomycetes do not have an adverse effect on humans (see Stephenson & Stempen 1994), but their properties at the cellular level have not been studied completely. The low levels of cytotoxicity observed in this investigation are important since they support the idea that this group of non-toxic organisms can be used for bioprospecting research.

The results from the trypanocidal activity tests support the latter as well. Even though they do not show differences when the experimental treatments are compared to Benznidazole, the significant differences with Amphotericin B provide evidence for the

Table 3 Trypanocide activity of the crude extract and fractions from *Physarella oblonga* plasmodium on epimastigotes of *Trypanosoma cruzi*.

Sample type	Compound	IC_{50}- µg/ml
Crude extract	NH-MX-A	7.5 ± 0.14
Fraction 1	NH-MX-1A	7.8 ± 0.14
Fraction 2	NH-MX-2A	7.8 ± 0.07
Reference	Amphotericin B	0.2
Reference	Benznidazole	10

potential use of myxomycete plasmodia against *T. cruzi*. Of course, developing a more comprehensive research protocol is necessary to evaluate this effect more carefully. However, the indication of a possible positive effect against the parasite as shown herein is important, since myxomycetes have not been reported previously to be active against any type of human parasite. Also, in the fight against this disease, any new empirical data supporting potential new lines of research are helpful for setting the stage for future investigations.

The unidentified myxomycete showed a great inhibitory activity against some of the organisms tested. Interestingly, in a manner similar to one of the other species, it seems that the crude extract is more bioactive than the fractioned one. The low level of biological activity against bacteria and fungi in the case of *Ph. oblonga* contrasts dramatically with the high level of activity against *T. cruzi*. However, in the present study, it was not possible to evaluate this aspect completely, since a more detailed approached would have to have been followed. In any case, what seems likely is that distinct species of myxomycetes have different types and levels of biological activity, which may account for their individual strategies to cope with different biotic elements of their environment.

It is evident that future studies are necessary to provide the evidence required to fill in the information gaps that exist today. In spite of this, the present investigation represents a first step in the study of one additional group of non-traditional organisms with potential applications. The successful evaluation of culturing conditions and the isolation, using NMR, of some secondary metabolites in *Ph. oblonga* represents a step forward in the bioprospecting study of myxomycetes with the use of modern research tools. At the same time, the evaluation of antimicrobial activity displayed by two other species of myxomycetes provides an indication of the potential use of the group.

References

Bechtel DB, Horner HT. 1975 – Calcium excretion and deposition during sporogenesis in *Physarella oblongga*. Calcified Tissue Research 18, 195-213.

Bullock E, Dawson CJ. 1976 – Sterol content of the Myxomycetes *Physarum polycephalum* and *P. flavicomum*. Journal of Lipid Research 17, 565-571.

Carballeira NM 2008 – New advances in fatty acids as antimalarial, antimycobacterial and antifungal agents. Progress in Lipid Research 47, 50-61.

Chiappeta A, de Sena KXFR, Nascimento SC, Rocha CS, Cavalcanti L. 1999 – Influence of pH on the extraction of bioactive substances of *Fuligo septica*. Phyton (Buenos Aires) 65, 7-11.

Dembitsky VM, Řezanka T, Spížek J, Hanus LO. 2005 – Secondary metabolites of slime molds (myxomycetes). Phytochemistry 66, 747-769.

Everhart SE, Keller HW. 2008 – Life history strategies of corticolous myxomycetes: the life cycle, plasmodial types, fruiting bodies, and taxonomic orders. Fungal Diversity 29, 1-16.

Ferraz M, Gazzinelli R, Alvez R, Urbina J, Romanha A. 2009 – Absence of CD4 T lymphocytes, CD8 T lymphocytes, or B lymphocytes has different effects on

the efficacy of Posaconazole and Benznidazole in treatment of experimental acute *Trypanosoma cruzi* infection. Antimicrobial Agents and Chemotherapy 53, 174-179.

Franke RG. 1967 – Preliminary investigation of the double-diffusion technique as a tool in determining relationships among some myxomycetes, order Physarales. American Journal of Botany 54, 1189-1197.

Gray WD 1949 – The laboratory cultivation and development of the myxomycetes *Physarella oblonga* and *Physarum didermoides*. Ohio Journal of Science 49, 105-108.

Gray WD, Alexopoulos CJ. 1968 – Biology of the myxomycetes. The Ronald Press Company, New York.

Ing B 1994 – The phytosociology of myxomycetes. New Phytologist 126, 175-201.

Ishibashi M, Iwasaki T, Imai S, Sakamoto S, Yamaguchi K, Itos A. 2001– Laboratory culture of the myxomycetes: formation of fruiting bodies of *Didymium bahiense* and its plasmodial production of makaluvamine A. Journal of Natural Products 64, 108-110.

Lado C 2005-2011 – An on line nomenclatural information system of Eumycetozoa. Real Jardín Botánico de Madrid, Spain. www.nomen.eumycetozoa.com

Lado C, Mosquera J, Estrada-Torres A, Beltrán-Tejera E, Wrigley de Basanta D. 2007 – Description and culture of a new succulenticolous *Didymium* (Myxomycetes). Mycologia 99, 602-611.

Martin GW, Alexopoulos CJ. 1969 – The Myxomycetes. University of Iowa Press, Iowa City.

McGaw LJ, Jager AK, van Staden J 2002 – Antibacterial effects of fatty acids and related compounds from plants. South African Journal of Botany 68, 417-423.

Misono Y, Ishikawa Y, Yamamoto Y, Hayashi M, Komiyama K, Ishibashi M. 2003 – Dihydrolindbladiones, three new naphthoquinone pigments from a myxomycete *Lindbladia tubulina*.

Journal of Natural Products 66, 999-1001.

Nakatani S, Kamata K, Sato M, Onuki H, Hirota H, Matsumoto J, Ishibashi M. 2005 – Melleumin A, a novel peptide lactone isolated from the cultured myxomycete *Physarum melleum*. Tetrahedron Letters 46, 267-271.

Rezanka T, Dvorakova R. 2003 – Polypropionate lactones of deoxysugars glycosides from slime mold *Lycogala epidendrum*. Phytochemistry 63, 945-952.

Ribeiro SM, Cavalcanti LH, Pereira EC, Silva NH. 2003 – Plasmodium immobilization of *Physarella oblonga* (Berk. & Curt.) Morgan (Myxomycetes) using kaolinite as a matrix of entrapment. Research in Microbiology 154, 55-58.

SAS 2007 – JMP Statistical Discovery Software. Statistical Analysis System (SAS) Institute, Cary, North Carolina.

Schaller H 2004 – New aspects of sterol biosynthesis in growth and development of higher plants. Plant Physiology and Biochemistry 42, 465-476.

Schofield CJ, Jannin J, Salvatella R. 2006 – The future of Chagas disease control. Trends in Parasitology 22, 583-588.

Shibuya M 2001 – Biosynthesis of sterols and triterpenes in higher plants. Natural Medicines 55, 1-6.

Steglich W 1989 – Slime moulds (Myxomycetes) as a source of new biologically active metabolites. Pure and Applied Chemistry 61, 281-288.

Stephenson SL, Stempen H. 1994– Myxomycetes: a handbook of slime molds. Timber Press, Oregon.

Thitilertdecha N, Teerawutgulrag A, Rakariyatham N. 2008 – Antioxidant and antibacterial activities of *Nephelium lappaceum* L. extracts. LWT Food Science and Technology 41, 2029-2035.

Zaritskaya L, Shurin MR, Sayers TJ, Malyguine AM. 2010 – New flow cytometric assays for monitoring cell-mediated cytotoxicity. Expert reviews of vaccines 9, 601-616.