International Journal of Agricultural Technology 2016 Vol. 12(7.2):2155-2164
Available online http://www.ijat-aatsea.com
ISSN 1686-9141

In –Vitro and In-Vivo Antimicrobial Assay of *Pleurotus sajor caju, Volvariella volvacea* and *Auricularia auricula* Against *Streptococcus iniae* in Nile Tilapia (*Oreochromis niloticus L.*)

Viña Kristina D. Serrano[1*] and Cynthia C. Divina[2]

[1]College of Sciences Graduate Studies, De La Salle University-Dasmarinas,Dasmarinas City Cavite Philippines
[2]Department of Biological Sciences, College of Arts and Sciences, Central Luzon State University, Science City of Muñoz, Nueva Ecija 3120 Philippines

Serrano V.K.D., and C.C. Divina (2016). In-Vitro and In-Vivo Antimicrobial Assay of *Pluerotus sajor-caju, Volvariella volvacea* and *Auricularia auricula* Against *Streptococcus iniae* in Nile Tilapia (*Oreochromis niloticus* L.). International Journal of Agricultural Technology 12(7.2): 2155-2164.

The study aimed to evaluate the effect of selected mushrooms as antibacterial against *Streptococcus iniae* in Nile Tilapia (*Oreochromis niloticus L.*). Two hundred twenty five Nile Tilapia were equally distributed into five treatments with three replicates each. In vitro testing was first done before proceeding to in vivo testing. Mushroom extracts with the highest zones of inhibition were used for in vivo testing. Mushroom extracts were injected intraperitoneally, and the application of bacterial inocula was done by severing near the caudal fin and swabbing of the inoculumon it. Responses of the fishes against *S. iniae* were monitored every 24 hours for 96 hours after inoculation. Mortality and clinical signs of infection were observed. Results showed that percentage mortality of fish given with *Pleurotus* and *Volvariella* were lower than that of the negative control but higher than of the positive control. Analysis of Relative Percent Survival showed that fish treated with *Pleurotus* and *Volvariella* were significantly comparable to the positive control. There was significantly lower Relative Percent Survival for fishes given only with water. Results of the study showed that fish given with mushroom extracts (*Volvariella* and *Pleurotus*) had decreased mortality and clinical signs of infection. Results of the study also imply that mushrooms may have increased the resistance of Nile Tilapia against *Streptococcus iniae.*The overall results of the study showed that inhibition of Nile Tilapia against *S. iniae* using *P. sajor-caju* and *V. volvacea* increased the fish resistance to bacterial infection caused by *S. iniae.*

Keywords: intraperitoneal, Streptococcus iniae, Volvariella, Pluerotus, antimicrobials

[*] **Coressponding Author:** Viña Kristina D. Serrano, **E-mail:** vkdserrano@yahoo.com

Introduction

Mushrooms are well known to contain immunomodulationg bioactive compounds such as antioxidants, antitumor, and antimicrobial properties. The nutritive nutraceuticals present in mushrooms are dietary fibers, polyunsaturated fatty acids (PUFA), proteins, amino acids, keto acids, minerals, antioxidative vitamins, and other antioxidants (Sharma and Gautam , 2015) . Nile Tilapia (*Oreochromis niloticus L*.) has high nutritional value as it is a good source of proteins. The occurrence of bacterial diseases in tilapia causes a decline in production. One possible disease is caused by the bacterial pathogen *Streptococcus iniae,* a gram positive coccus. Researchers are now working on supplementing feeds with different bio compound to develop a better feed composition. Some mushrooms could be considered as feed supplement due to its high nutritional and medicinal value. As cited by Khatum et al. 2011,a nutraceuticals can be defined as a substance that may be considered a food or part of a food that provides medical or health benefits like the prevention and treatment of disease. Mushrooms have become attractive as a functional food and as a source for the development of drugs and nutraceuticals (Lakhanpal and Rana, 2005) responsible with their antioxidant, antitumor (Jones and Janardhanan, 2000) and antimicrobial properties. Besides their pharmacological features, mushrooms are becoming more important in our diet due to their nutritional value, related to high protein and low fat / energy contents (Agahar-Murugkar and Subbulakshmi, 2005). Mushrooms are well known source of bio-source of novel secondary metabolites. Molecular biology are now exploring the traditional medicines in regards to the wide biological spectrum spectrum which mushrooms posses. With this status a promise or potential for future nutraceutical designs could be harbored for human or animal health.

Objectives: This study aimed to evaluate in vitro and in vivo the antimicrobial properties of selected mushrooms against *Streptococcus iniae*. Specifically, this study aimed to to determine if *Pluerotus sajor-caju, Auricularia auricula, Volvariela volvacea* have antimicrobial properties against S. iniae by in vitro assay of disc plating method ; and to assay in vivo the effects of identified mushrooms with antimicrobial properties against *S. iniae* in *Oreochromis niloticus* by monitoring relative percentage survival and clinical signs of disease infestations.

Materials and methods

In-Vitro BioAssay of Anti-Microbial Properties of Selected Mushrooms

Basidiocarps of *P. sajor caju, V. volvacea, A. auricula* were used for this study. Three hundred grams of dried mushrooms were used in preparing the

crude extracts. Basidiocarps were washed with sodium hypochlorite (5.25%) and rinsed three times with sterile distilled water before placing in disinfected blender. The samples were blended until homogenous mixture was formed. The mixture was then filtered through a double layer filter paper and sterile 250ml Erlenmeyer flask (adapted from David, 2001).

Five ml of nutrient broth was transferred to screw capped test tube. The tubes were sterilized by autoclaving at 15psi, 121C for 15 minutes. A loopful of the bacterial culture *S. iniae* was dipped into the sterile broth. The tubes were incubated at 37C for 6 hours. This was used as inoculum swabbed in Todd Hewitt agar plate.

The affectivity of the extracts was evaluated. The capacity of assay discs was determined by using a 1ml syringe. The discs were seeded in lawn culture of the test bacterium. The plates were then incubated at 37C for 24 to 48 hours. Zone of inhibition or the clear zones around the disc were recorded. The treatments in the study were crude extracts of *A. auricula, V. volvacea, P. sajor caju*, water as negative control and antibiotic as positive control.

In-vivo BioAssay of Anti-Microbial Properties of Mushrooms

The study was conducted indoor and growing of Nile Tilapia was in polyethylene bags containing 25 liters of water, provided with sufficient aerator. Water parameters such as dissolved oxygen, temperature and pH were monitored daily using the YSIDO meter model.

Two hundred twenty five Nile Tilapia weighing 10-15 grams were used in this study. They were obtained from the Freshwater Aquaculture Center, Central Luzon State University (CLSU) , Science City of Munoz Nueva Ecija, Philippines. They were equally distributed in different bags with three replicates each, with fifteen fish per replicate.

Pure culture of *S. iniae* strain was obtained from Fish Pathology Laboratory, College of Fisheries, CLSU, Nueva Ecija. Mushrooms found with anti-microbial properties in the in-vitro testing were used for the in-vivo testing in Nile Tilapia. The fishes were distributed into different treatment, with three replication containing fifteen fish per bag. After a day of acclimatization the fishes were subjected to a challenge test against *S. iniae*. Fishes were scratched near their caudal fin and swabbed with the inoculum on the severed region. A number five MacFarland was prepared for the determination of concentration of the test organism. which corresponds to the bacterial density of 15x108 per ml. After introduction of the inoculum, mushroom extracts were injected intraperitoneally. Mortality was observed for 24, 48,72 and 96 hours. Clinical signs of infection of *S.iniae* were also noted.

Dead fish were removed and recorded. Dead and survived fishes were examined for diagnostic signs of *S.iniae* infection such as ulceration, darkening of the skin, fin rottening, exopthalmia and abdominal and vent swelling.

For In-vitro Assay, data gathered were percent mortality relative percent survival and clinical signs of infection such as darkening of the body, reddish color of the body, erratic swinnig, bulging of the abdomen, ulceration, exopthalmia, tail rottening, fin rottening and vent swelling. Experiment was set up following the complete random design and data were analyzed using analysis of variance. Means were compared using the Duncan Multiple Range Test with level of significance set at 0.05.

Results

In Vitro Analysis

In vitro analysis for antimicrobial properties of the mushroom extracts was done through disc-plating method. The formation of zone of inhibition around the discs indicates an antimicrobial activity of the mushroom extracts. Table 1 shows the zone of inhibition of different mushroom extracts against *Streptococcus iniae* at 12, 24 and 48 hours. Results revealed that the zones of inhibition were formed in discs with antibiotic, *Pleurotus* and *Volvariella* extracts. No zone of inhibition was formed in discs with *Auricular*ia and water. This was observed to be true from 12 to 48 hours after inoculation. Analysis of variance revealed that there was a significant difference in the sizes of zone of inhibition of the mushroom extracts. At 24^{th} and 48^{th} hours, zones of inhibitionof *Volvariella, Pleurotus* and antibioticsshowed highly signicant differences among each other. *Pleurotus* had significantly bigger zone of inhibition than Volvariella but smaller than the positive control. Comparison among means at 12^{th} , 24^{th} and 48^{th} hours revealed that the zone of inhibition of *Volvariella* extract was significantly bigger than the negative control, comparable to Pleurotus but smaller than the antibiotic discs.

Table 1 Zone of inhibition (mm) of different mushroom extracts against *S.iniae*

Treatment	Zone of Inhibition (mm)		
	12^{th} hours	24^{th} hours	48^{th} hours
Water	0.00^{a}	0.00^{a}	0.00^{a}
A. auricula	0.00^{a}	0.00^{a}	0.00^{a}
V. volvacea	8.71^{b}	9.51^{b}	9.51^{b}
P. sajor caju	9.83^{bc}	11.07^{c}	11.07^{c}
Antibiotic	10.86^{c}	12.70^{d}	12.70^{d}

Means in a column with different letter superscripts are significantly different at .05 level of significance

In Vivo BioAssay

Pleurotus and Volvariella crude extracts that showed positive antimicrobial activities in the in-vitro assay were further tested in in-vivo bioassay by challenge test against *S. iniae* in nile tilapia. Table 2 shows the percent mortality of the nile tilapia inoculated with S. iniae and injected with extracts and antibiotics. The highest mortality of the tilapia subjected to test organism died after 96 hours was observed in negative control, 80 percent and only 8.89 percent both for Pleurotus amd Volvariella extracts and 4.44 percent in antibiotics of fish died. Analysis of variance revealed highly significant differences among the percentages of mortality means among the treatments

Table 2. Percent mortality of nile tilapia exposedto S. iniae and injected with different mushrooms extracts at 24, 48,72 and 96 hours

Treatment	Percent Mortality (%)			
	24^{th} hour	48^{th} hour	72^{nd} hour	96^{th} hour
Antibiotic (Positive Control)	2.22^{a}	4.44^{a}	4.44^{a}	4.44^{a}
P. sajor caju	2.22^{a}	4.44^{a}	6.67^{a}	8.89^{a}
V. volvacea	4.44^{a}	6.67^{a}	8.89^{a}	8.89^{a}
Water (Negative Control	48.89^{b}	66.67^{b}	73.33^{b}	80.00^{b}

Means in a column with different letter superscripts are significantly different at .05 level of significance

Comparison among means showed that the percent mortality of fish injected with *Pluerotus* and *Volvariella* were comparable to those injected with antibioticsc at all times during the 96 hours post inoculation observations. This may indicate that Pleurotus and Volvariella have antibacterial activities as they

significantly decreased the mortality rate of infected fishes. This result concurs with the earlier results in the in vitro testing for antibacterial activities.

The relative percent survival at 96 hours was found highest in antibiotics (78.78%) followed by *Volvariella* and *Pluerotus* extracts (72.11%). Analysis of variance revealed no significant differences between relative percent survival means of fish injected antibiotics and the two mushroom extracts agains S. iniae infection. Results imply the efficacy of the two mushroom extracts injected in the infected fishes was comparable to the each other. It was also further revealed that their efficacy was comparable with that of the antibiotics in promoting survival of infected fishes. This was found to be true in all observations from 24, 48, 72 and 96 hours of post-inoculation observations.

Fish affected with *S. iniae* may exhibit one or more of the following clinical signs, erratic swimming,lethargy,darkening, uni or bilateral exopthalmia, haemorrhages in or around the eye, the gill plate, base of fins or elsewhere the body and ulcerations. Clinical signs and manifestation of infection were observed in the study. These were erratic swimming, darkening ofthe body, reddish color of the body, abdominal swelling, ulceration tail rotting ,fin rottening exopthalmia, sloughing off of scales and vent swelling . Table 3 shows the clinical signs observed in fishes in the different treatments. It could be observed that there were more in number and intensity observed in the negative control compared to the other treatments. Erratic swimming, reddening and blackish color of the body was noted in fishes after 24 hours of post inoculation of bacteria especially in treatment with distilled water .The darkening of the body is a manifestation that the bacterium penetrates the subcutaneous connective tissue and muscles. After 24 hours, less erratic swimming was observed in the fishes. And the dark color turns to original or normal gray color. Other clinical signs of infection were observed in negative control such as exopthalmia, swelling of the abdominal region, fin and tail rottening, sloughing off scales and acute ulceration. Vent swelling was noted in all treatments except to the control which is untreated.

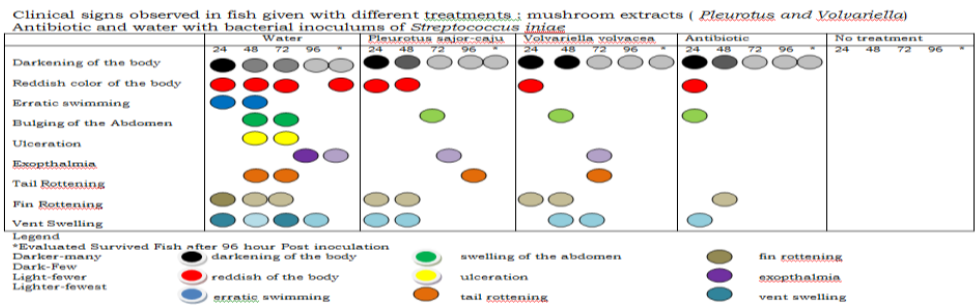

Figure 1. Darkening of the body, clinical sign of *Streptococcus iniae* infection
Figure 2. Reddish color of the body, clinical sign of *Streptococcus iniae* infection
Figure 3. Swelling of the abdomen, clinical sign of *Streptococcus iniae* infection
Figure 4. Ulceration, clinical sign of *Streptococcus iniae* infection
Figure 5. Tail rottening, clinical sign of *Streptococcus iniae* infection
Figure 6. Fin Rottening, clinical sign of *Streptococcus iniae* infection
Figure 7. Exopthalmia (puff eyes), clinical sign of *Streptococcus iniae* infection
Figure 8. Swelling of the vent, clinical sign of *Streptococcus iniae* infection

Fish given *Pleurotus* and *Volvariella* extracts showed other symptoms aside from erratic swimming and darkening of the body. There were red patches on their body. Fishes that were neither subjected to *S. iniae* nor injected with anything did not show any clinical signs of infection. Results showed that fish treated with mushrooms had lesser signs of infection compared to those infected fishes given with water but more symptoms werer observed compared to those treated with antibiotics. This indicates that mushrooms might help to strengthen the immune system or provide health benefits to tilapia from bacterial infection.

Discussions

Results of in-vivo bioassay wherein the extracts of *Pleurotus* and *Volvariella* prevented the growth of *S. iniae* may imply that they may have substances that can inhibit activities of *S. iniae*. Studies of Pardeshi and Pardeshi (2009) stated that active constituents found in mushrooms are polysaccharides, dietary fibres, oligosaccharides, triterpenoids, peptides and proteins, alcohols and phenols, and mineral elements such as zinc, copper, iodine, selenium and iron, vitamins, amino acids etc. These have been found to boost the immune system, have anti-cancerous properties, act as anti-hypercholesterolemia and hepato-protective agents, and anti-viral activity.

Oxidation is essential for all living organisms for the production of energy to fuel biological processes. However, oxygen-centeredd free radicals and other reactive oxygen species that are continuously produced *in vivo*, result in cell death and tissue damage. Oxidative damage caused by these free radicals may be related to ageing and diseases, such as atherosclerosis, diabetes, cancer and cirrhosis. Studies of Breen (1990) stated that the mushrooms prove to contain polysaccharides (e.g. B-glucans), nucleic acid derivatives (the hypercholesterolemic eritadenine), lipids, peptides, proteins, and glocoproteins have been isolated and identified. Some of the mechanisms of activity have been elucidated, e.g., antiviral and antibacterial activity via stimulation of interferon production in the host.

Results imply that Volvariella and Pluerotus may have substances that can inhibit activities of S. iniae. As cited by Khatun *et al.* (2011) *Pleurotus* species have high medicinal value. Compounds extracted from these mushrooms exhibit activity against various chronic diseases including hypertension, hypercholesterolemia. Oyster mushrooms (*Pleurotus* species) are excellently edible and nutritious, rank among one of the most widely cultivated mushrooms in the world (Chang, 1999). Species of *Pleurotus* are found to possess significant antioxidant, anti-inflammatory and antitumor activities (Jose and Janardhanan, 2000; Jose *et al.*, 2002). The methanol extract of fruiting bodies of *Pleurotus florida* was found to possess OH radical scavenging and lipid peroxidation inhibiting activities (Jose and Janardhanan, 2000). The extract also showed significant reducing power and radical scavenging property as evident from FRAP and DPPH radical scavenging. Studies of Jakapovic (1990) stated that mushrooms are sources of active ingredients that can be antibiotics with antibacterial properties and immunostimulants. Adams et al (2002) as cited by Duro (2005) described the Relative Percent Survival (RPS) as the index of efficacy if treatments. According to Morales (1995), the higher the RPS obtained the higher the level of fish resistance. Results showed that fish given with antibiotics were the most resistant followed by Pluerotus and Volvariella. In the absence of further studies on the mode of actionof the extracts in inhibiting the growth of S. iniae, it could only be implied that the mushroom extracts may have acted as directly inhibiting growth of S. iniae as shown in in-vitro assay or may stimulate the immune system as they were the extracts were injected before the onset of the disease. Several studies have pointed out those medicinal values of mushrooms.

Acknowledgement

The author expresses her deepest thanks to her family and friends who gave time and support to the conduct of this study.

References

Agahar-Murugkar, D., Subbulakshmi, G. (2005). Nutritional value of edible wild mushroomscollected from the Khasi hills of Meghalaya. Food Chem, 89, 599-603.

Breen W. 1990. Nutriceutical and Medicinal Values of Mushrooms. Journal of Food Production 53, p.883-894

Castro-Alves VC, Gomes D., Menoli N. Sforca ML and Nascimento JR. (2016). Characterization and immunomodulatory effects of glucans from Pleurotus albidus, a promising species of mushroom for farming and biomass production. Int J Biol Macromol. 2016 Nov 19;95:215-223. doi: 10.1016/j.ijbiomac.2016.11.059.

Chang, S.T. (1999). Global impact edible and medicinal mushrooms on human welfare in the 21st century: Non green evolution. Int. J. Med. Mushr., 1, 1-7.

Duro GJ. (2004). Immunostimulation of Nile Tilapia (oreochromis niloticus) Against Aeromonas hydrophila Using Selected Mushrooms

Jakapovic I and Jakapovic M., (1990). DR MYKO SAN_HEALTH FROM MUSHROOMS. Website : http://www.zdravljeizgljiva.html

Jones, S., Janardhanan, K.K. (2000). Antioxidant and antitumor activity of Ganoderma lucidum (Cart. Fr.) P.Karst.-Reishi (Aphyllophoromycetidae) from South India. Int. J. Med. Mushroom, 2, 195-200.

Jose, N., Janardhanan, K.K. (2000). Antioxidant and antitumor activity of Pleurotus florida. Curr. Sci., 79, 941-943.

Jose, N., Ajith, T.A., Janardhanan, K.K. (2002). Antioxidant, anti-inflammatory and antitumor activities of culinary medicinal mushroom Pleurotus pulmonarius (Fr.) Quel. (Agaricomycetideae). Int. J. Med. Mushr., 4, 329-335.

Khatun S, Islam A, Cakilcioglu U and Chahatterjee C. (2011).Research on Mushroom as a Potential Source of Nutraceuticals: A Review on Indian Perspective 2011 American Journal of Experimental Agriculture 2(1): 47-73, 2012

KozarskiM, Klaus A., Jakavlievic D., Todorovic N., Vunduk J, Petrovic P, Niksic M, Vrvic MM, Van Griensven LJ. (2015). Antioxidants of Edible Mushrooms. Molecules. 2015 Oct 27;20(10):19489-525. doi: 10.3390/molecules20101948

KozarskiM, Klaus A., Jakavlievic D., Todorovic N., Vunduk J, Petrovic P, Niksic M, Vrvic MM and Van Griensven LJ. (2015). Nutraceutical properties of the methanolic extract of edible mushroom Cantharellus cibarius (Fries): primary mechanisms. Food Funct. 2015 Jun;6(6):1875-86. doi: 10.1039/c5fo00312a.Int J Med Mushrooms. 2015;17(2):187-97.

Khaund, P. and Joshi, S.R. (2014). Functional nutraceutical profiling of wild edible and medicinal mushrooms consumed by ethnic tribes in India. Food Funct. 2014 Dec;5(12):3170-8. doi: 10.1039/c4fo00703d.

Lakhanpal, T.N. and Rana, M. (2005). Medicinal and nutraceutical genetic resources of mushrooms. Plant Genetic Resources: Characterization and Utilization. 3, 288-303.

Pardeshi, B.M. and Pardeshi, P.M. (2009). The edible medicinal mushrooms as supportive natural nutrients: Study of nonvolatile mineral contents of some edible medicinal

mushrooms from India; eastern remedies for modern western maladies. Proc. 5th Int. Medicinal Mushroom Conference, Mycological Society of China, Nantong, China. pp.514-18.

Sharma S.K., Gautam N. and Atri N.S., (2015). Optimization ,Composition, and Antioxidant activities of Exa-and Intracellular Polysaccharides in Submerged Culture of Cordyoceps gracilis (Grev.) Durieu & Mont. Evidence Baased Complementary and Alternative Medicine Volume 2015 (2015)

Thankthaeng N, Miller MG, Gomes SM and Shukitt-Hale B. (2015). Daily supplementation with mushroom (Agaricus bisporus) improves balance and working memory in aged rats. Nutr Res. 2015 Dec;35(12):1079-84. doi: 10.1016/j.nutres.2015.09.012. Epub 2015 Oct 5.

Xu N, Rem Z, Zhang J, Song X, Gao Z, Jing H, Li S, Wang S, Jia L. (2016) Antioxidant and anti-hyperlipidemic effects of mycelia zinc polysaccharides by Pleurotus eryngii var. tuoliensis. Int J Biol Macromol. 2016 Nov 18;95:204- . doi: 10.1016/j.ijbiomac.2016.11.06

Xu X, Yang J, Ning Z, Zhng X. (2015). Lentinula edodes-derived Polysaccharide rejuvenates mice in terms of immune responses and gut microbiota. Food Funct. 2015 Aug;6(8):2653-63. doi: 10.1039/c5fo00689a. Epub 2015 Jul 2.