Medicinal Polypores Indigenous to the Pacific Northwest Old Growth Forests of North America: Screening for Novel Antiviral Activity

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Abstract: Polypore mushrooms have been used medicinally for thousands of years. The Greek Physician Dioscorides first described the use of a wood conk, Agarikon now known as *Fomitopsis officinalis* as a treatment against consumption in 65 AD. Its use a topical anti-inflammatory agent also spans millennia. Other wood conks such as ling chi or reishi have had a similarly long history of use in Asia. In the past twenty years, wood conks are being carefully explored for their immunomodulating and anti-cancer properties. More recently, mushrooms, including polypores, have and are being explored for their antimicrobial properties. Upon submitting more than a hundred *in vitro* cultures of mushrooms to the U.S. Defense Departments' Bioshield BioDefense program, several tests show some of these polypore mushrooms have strong anti-viral activity. Within these verdant natural landscapes, trees hundreds of years old host ancestral strains of these elusive polypores. Species that are now rare, or in some cases thought to be extinct, still reside in the pristine old growth forests of the Pacific Northwest of North America. When clones from these mushrooms were grown *in vitro* and submitted for antiviral screening, several mycelial cultures produce antibiotics effective against Pox and other viruses. Notably strains vary in their antiviral properties. Our natural genomes hold within them great potentials for staving off disease and have not yet been fully explored. The fungal diversity within these genomes may prove critical for isolating the most active strains, similar to the lessons learned from the isolation of *Penicillium chrysogenum* strains that lead to the commercialization of penicillin, subsequently saving millions of lives. With deforestation, pollution, and industrialization, societies should re-evaluate the importance of their natural forests in the context that they hold within them novel medicines of enormous socio-economic importance. The old paradigm of viewing the forest as valuable only in terms of timber seems overly simplistic given this new knowledge.

Key words: Antiviral, antimicrobial polypores, *Fomitopsis officinalis*, pox, orthopox, smallpox, virus, Vaccinia, Variola

1 Introduction

Despite advances in modern medicine, microbes, especially viruses, continue to kill millions of people, stimulating the search for new anti-microbial agents, some of which have proven to be of significant commercial value. A major difficulty in the discovery of anti-microbial agents is their inherent toxicity to the affected host organism. For instance, a novel agent or treatment that kills the virus but also harms the human host is neither medically practicable nor commercially attractive. Hence, many new anti-viral drugs have never made it past preliminary screening studies as they have failed to prove non-toxicity and are unsafe to consume.
With the flow of airline passengers from remote regions of the world, concentrating in airports and being re-routed to their destinations, the contagiousness of foreign-borne viruses carried by passengers are likely to be exacerbated in these types of locations, especially within the closed compartments of passenger airplanes, cruise ships and auditoriums, increasing the likelihood of cross-infection. Virtually anywhere humans concentrate provide opportunities for contagions to spread, whether by air or by physical contact. With the increased

threat of bioterrorism from weaponized viruses, a new class of readily available broad-spectrum anti-viral agents serves the best interests of worldwide public health. A survey of the antimicrobial properties of mushrooms were published by Suay et al.,[1] while Brandt and Piraino[2] and Stamets[3, 4] discussed the published research on mushroom-derived anti-virals. Besides having a direct antiviral or antimicrobial effect, mushroom derivatives can also activate natural immune response, potentiating host defense, and in effect have an indirect but significant antimicrobial activity.[5]

As mushrooms share a more common evolutionary history with animals than with any other kingdom, mushrooms and humans often suffer from common pathogens in the microbial world, for instance, the bacteria, *Escherichia coli Staphylococcus aureus* and *Pseudomonas fluorescens*. Mushrooms have a vested evolutionary interest in not being rotted by bacteria, producing antibacterial agents to stave off infection. Work by Suay et al.[1] showed that various mushroom species have anti-bacterial specific properties. Viral infections, as in viral pneumonia, can precede, for instance infections from *Streptococcus pneumoniae* or *Staphylococcus aureus*, so the use of mushrooms having antibacterial properties can help forestall secondary infections from opportunistic pathogens. More particularly, mushrooms having both antibacterial and antiviral properties are especially useful for preventing infection. Mushrooms are likely to demonstrate anti-bacteriophagic properties, being dually antibacterial and antiviral, although no reports from the scientific literature are known to this author.

That medicinal mushrooms have been ingested for hundreds, and in some cases, thousands of years, is strong support for their non-toxicity, making them appealing candidates in the search for new antimicrobial and antiviral agents. The cell surface of mycelium secretes antibiotics within extracellular exudates, often referred to as secondary metabolites. These indigenous antibiotics and enzymes target distinct sets of microbes. Useful antibiotics isolated from mushrooms include calvacin from the giant puffball (*Calvatia gigantea*), armilliaric acid from honey mushrooms (*Armillaria mellea*), campestrin from *Agaricus campestris*, the meadow mushroom, coprinol from inky caps (Coprinus species) corolin from turkey tail mushrooms (*Trametes versicolor* = *Coriolus versicolor*), cortinellin from shiitake (*Lentinula edodes*), ganomycin from reishi (*Ganoderma lucidum*) and sparassol from cauliflower mushrooms (*Sparassis crispa*).

Suzuki et al.[6] characterized an antiviral water-soluble lignin in an extract of the mycelium of shiitake mushrooms (*Lentinula edodes*) isolated from cultures grown on rice bran and sugar cane bagasse which limited HIV replication *in vitro* and stimulated the proliferation of bone-marrow cells. Clinical trials with lentinan in the treatment of HIV patients showed inhibitory activity.[7] However, Abrams[8] found no significant advantage in using lentinan in treating AIDS patients. Another mushroom recognized for its antiviral activity is *Fomes fomentarius*, a hoof-shaped wood conk growing trees, which inhibited the tobacco mosaic virus.[9] Collins & Ng[10] identified a polysaccharopeptide inhibiting HIV type 1 infection from Turkey Tail (*Trametes versicolor*) mushrooms while Sarkar et al.[11] identified an antiviral substance resident in an extract of Shiitake (*Lentinula edodes*) mushrooms. Yan[12] reported that polysaccharides from Zhu Ling (*Polyporus umbellatus*) effectively limited the reproduction of chronic hepatitis.

More recently, derivatives of the Gypsy mushroom, *Rozites caperata*, were found by Piraino & Brandt[13] to have significant inhibition against the replication and spread of varicella zoster (the 'shingles' and 'chickenpox' virus), influenza A, herpes simplex I and II and the respiratory syncytial virus but not against HIV and other viruses. Eo et al.[14, 15] found antiviral activity from the methanol-soluble fractions of reishi mushrooms (*Ganoderma lucidum*), selectively inhibiting Herpes simplex and the vesicular stomatitus virus (VSV). Wang & Ng[16] isolated a novel ubiquitin-like glycoprotein from oyster mushrooms (*Pleurotus ostreatus*) that demonstrated inhibitory activity toward the HIV-1 reverse transcriptase. Maitake (*Grifola frondosa*) is currently the subject of research in the treatment of HIV. Kahlos et al.[17] noted that crude fractions from chaga (*Inonotus obliquus*) showed anti-viral activity.

Arabinoxylane inhibits HIV indirectly through the enhancement of NK cells that target the virus. Arabinoxylanes are created from mushroom mycelia's enzymatic conversion of rice bran.[18] In response to hot water extracts of reishi mushrooms, preserved in ethanol, versus saline controls, NK cell activity was significantly augmented

when cancer cells were co-cultured with human spleen cells.[19] A mycelial combination of seven species grown on rice ("Stamets-7™") achieved a similar result, greater than any one species at the same dosage. As the water extract of the fruitbodies is high in beta glucans while the mycelium-on-rice is low in beta glucans, but is high in arabinoxylanes, two causal agents are identified as NK effectors. Both the extract and the heat treated, freeze dried, powdered mycelium from seven species share common activity levels of enhancing NK activity by 300+%. These compounds may be synergistic. This same combination of seven species fermented on rice had a strong effect against HIV, inhibiting replication by 99% while the water extract of Reishi fruitbodies was 70%, respectively. These results underscore that water extractions of fruitbodies and oral administration of myceliated rice positively influence the immune system, activating different subsets of immunological receptor sites.

2 Surveying Polypores for Antiviral Activities

The author submitted more than ethanol/water extracts of more than 150 strains and species of medicinal mushrooms, primarily polypores, over the course of two years to the U.S. Defense Departments' Bioshield BioDefense program coordinated by the United States Army Medical Institute for Infectious Diseases (USAMRIID) and the National Institutes of Health (NIH). The screening program tested the mushroom extracts against viruses which could be potentially weaponized, including viruses responsible for causing Yellow Fever, Dengue, SARS, respiratory viruses, and pox. Of the samples submitted, several showed potent activity preventing and reducing infection from Orthopoxes. One species, in particular, *Fomitopsis officinalis*, proved to be particularly potent in anti-pox activity. Subsequently, the author filed several U.S. and international patents on the antiviral properties of mushrooms within this taxon.[20] Further research using extracts from this mushroom for in vivo testing against mousepox and monkey pox are planed in the near future at the Center for Disease Control in Atlanta, Georgia.

Fomitopsis officinalis (Villars) Bondarzew & Singer (= *Agaricum officinalis, Fomes officinalis, Fomes laricis* and *Laricifomes officinalis*) has the common names agarikon, quinine conk, larch bracket mushroom, brown trunk rot, eburiko, adagan ('ghost bread') and tak'a di ('tree biscuit'). Once widespread throughout the temperate regions of the world, this perennial wood conk saprophytizes larch, Douglas fir, hemlock, preferring mature woodlands. Classically a brown rot fungus, this mushroom causes a top-rot in mature trees, and in doing so provides essential habitat for cavity dwellers, including birds, squirrels and insects. Not an aggressive parasite, this slow-acting fungus can often be found in trees hundreds of years in age. Once the tree dies, the conk can persist for several years as a saprophyte. It is hyphothesized by the author, although not yet proven, that this mushroom may behave as an endophyte, forestalling the invasion of more pernicious parasitic fungi.

Now nearly extinct in Europe and Asia, this mushroom is a resident of the old growth forests of Oregon, Washington and British Columbia. Although reported from China, this mushroom is now rare due to deforestation. Known constituents include beta glucans, triterpenoids, agaricin and extracellular antibiotics. *F. officinalis* has traditionally been used for centuries for the treatment of tuberculosis and/or pneumonia, the primary causal organisms being *Mycobacterium tuberculosis, Bacillus pneumoniae* and/or other microorganisms. Mizuno et al.[21] include this mushroom in a group of polypores, the hot water extracts of which provide a strong host mediated response.

Traditionally this mushroom has been boiled, dried and/or burned to create a topical poultice as an anti-inflammatory medicine for treating muscle/skeletal pain. Described by the first century Greek physician Dioscorides in Materia Medica, the first encyclopedic pharmacopoeia on the medicinal use of plants, in approximately 65 C. E., as a treatment for a wide range of illnesses, most notably consumption, later known as tuberculosis. A resident on the old growth conifers, especially spruce, hemlock, Douglas fir, and on larch, this amazing mushroom produces a chalky cylindrical fruitbody that adds layers of spore-producing pores with each growth season, allowing for a rough calculation of age. Conks up to 50 years have been collected, and often times they re-

semble a woman's body shape, reminiscent of the so-called Venus of Willendorf form.
The Haida Indians of the Haida Gwaii (also known as the Queen Charlotte Islands) and elsewhere on the coast of British Colombia, associated this mushroom with the powerful creator spirit Raven, and as a protector of women's sexuality. [4, 22] This mushroom was carved into animalistic forms and placed on shaman's graves to protect them from evil spirits. Grzywnowicz[23] described the traditional use of this mushroom by Polish peoples, as a treatment against coughing illnesses, asthma, rheumatoid arthritis, bleeding, infected wounds, and was known for centuries as a "elixirium ad longam vitam": elixir of long life. The North Coast First Peoples of Northwestern North America also discovered the use of this mushroom as a poultice to relieve swellings and in teas for treating feverish illnesses. Called the quinine fungus in many forestry manuals because of its bitter taste, this mushroom is not the source of quinine, an alkaloid from the bark of the Amazonian *Cinchona ledgeriana* tree which was widely used since the late 19th century to treat malaria, caused by *Plasmodium falciparum*. Despite the long history of use, few modern studies have been published on its medicinally active compounds. *F. officinalis* merits further research as the number of strains is in rapid decline, especially in Europe, where it is on the verge of extinction.[24] The author has cloned six strains from the Old Growth forests of the Pacific Northwest of North America, three of which have thus far shown to have significant anti-pox activity.

3 The Need for Novel Antivirals and the Threat of a Smallpox Resurgence

Smallpox is a serious acute, contagious and infectious disease marked by fever and a distinctive progressive skin rash. The majority of patients with smallpox recover, but death may occur in up to 30% of cases. Many smallpox survivors have permanent scars over large areas of their body, especially their face, and some are left blind. Occasional outbreaks of smallpox have occurred for thousands of years in India, western Asia and China. European colonization in both the Americas and Africa was associated with extensive epidemics of smallpox among native populations in the 1500s and 1600s, including use as a biological weapon in the United States against indigenous peoples. Smallpox was produced as a weapon by several nations well past the 1972 Bioweapons convention that prohibited such actions. That reservoirs of this virus exist, and the potential for these strains to be utilized by bioterrorists, has caused great concern within the U.S. Defense Department.
There is no specific treatment for smallpox and the only prevention is vaccination. In 1980, the disease was declared eradicated following worldwide vaccination programs. However, in the aftermath of the terrorist and anthrax attacks of 2001, the deliberate release of the smallpox virus is now regarded as a possibility and the United States is taking precautions to deal with this possibility.
Smallpox is classified as a Category A agent by the Centers for Disease Control and Prevention. Category A agents are believed to pose the greatest potential threat for adverse public health impact and have a moderate to high potential for large-scale dissemination. Other Category A agents are anthrax, plague, botulism, tularemia, and viral hemorrhagic fevers. Even the remote potential for release of a deadly communicable disease in an essentially non-immune population is truly frightening.
Orthopox (orthopoxviruses or poxviruses) includes the virus that causes smallpox (variola). Smallpox infects only humans in nature, although other primates have been infected in the laboratory. Other members of the *Orthopox* genus of viruses capable of infecting humans include monkeypox, camelpox, cowpox, pseudocowpox, *Molluscum contagiosum* and Orf. Monkeypox is a rare smallpox-like disease, usually encountered in villages in central and west Africa. It is transmitted by monkeys and rodents. Camelpox is a serious disease of camels. The genetic sequence of the camelpox virus genome is most closely related to that of the variola (smallpox) virus. Cowpox is usually contracted by milking infected cows and causes ulcerating "milker's nodules" on the hands of dairy workers. Cowpox protects against smallpox and was first used for vaccination against smallpox. Pseudocowpox is primarily a disease of cattle. In humans it causes non-ulcerating "milker's nodes." *Molluscum contagiosum* causes minor warty bumps on the skin with a central indentation. It is transferred by direct contact, sometimes as a venereal disease. Orf virus occurs worldwide and is associated with handling sheep

and goats afflicted with "scabby mouth." In humans it causes a single painless lesion on the hand, forearm or face. Vaccinia, a related *Orthopox* of uncertain origin, has replaced cowpox for vaccination. Other viruses of the Poxviridae family include buffalopox virus, rabbitpox virus, avipox virus, sheep-pox virus, goatpox virus, lumpy skin disease (Neethling) virus, swinepox virus and Yaba monkey virus.
Poxviruses are very large rectangular viruses the size of small bacteria. They have a complex internal structure with a large double-stranded DNA genome enclosed within a "core" that is flanked by two "lateral bodies." The surface of the virus particle is covered with filamentous protein components, giving the particles the appearance of a ball of knitting wool. The entire virus particle is encapsulated in an envelope derived from the host cell membranes, thereby "disguising" the virus immunologically. Most poxviruses are host-species specific, but Vaccinia is a remarkable exception. True pox viruses are antigenically rather similar, so that infection by one elicits immune protection against the others.

4 Materials and Methods

F. officinalis mushrooms were cloned from tissue directly behind the youngest hymenial, tube-producing layer by excising tissue or using a cork-borer. Once purified by selectively culturing, the mycelium was grown via liquid fermentation and the antiviral products harvested subsequent to colonization. The methods for cultivation of mycelium used are described by Stamets[25] in Growing Gourmet and Medicinal Mushrooms. *F. officinalis* I and *F. officinalis* IV are strains respectively collected from Morton and Elwha river valley, Washington State, USA while *F. officinalis* V was cloned from a dead Douglas Fir tree on Cortes Island, British Columbia, Canada. Mycelial cultures were grown in sterile Petri dishes containing sterilized malt yeast rice agar. After three weeks of colonization in a clean room laboratory, the cultures were aseptically transferred into a 1000 ml. EBERBACH™ stirrer containing 800 ml. of sterilized water. The EBERBACH™ container was activated using a WARING™ blender base, chopping the mycelium into thousands of fragments. This myceliated broth was then transferred, under sterile conditions, into a sterilized glass 2000 ml. fermentation vessel containing a 3% concentration of malt sugar, .3% yeast and .3% powdered rice, stir bar and 800 ml. of sterilized water. Once transferred, the fermentation flask was placed on a magnetic stir plate, and stirred at 300-400 rpm for a period of 3-4 days in front of a laminar flow hood at a temperature of 70-75 F. During that time, three-dimensional colonies of mycelium appeared, increasing in numbers and in density. The fermentation was stopped prior to the coalescing of the mycelium into a contiguous mycelial mat. The dissociated fragmented mycelial mass allows for a multiple loci inoculation, resulting in accelerated colonization and allowing for the ease of further dilutions and inoculations. The fermented broth was then diluted 1:10 into sterilized water, and transferred, under sterile conditions, into polypropylene incubation bags containing approximately 6.6 lbs or 3 kg. moistened sterilized rice, adjusted to approximately 45-50% moisture content. Approximately 50-100 ml. of diluted fermented fluid was transferred into each of the 10 rice bags under sterile conditions. The fresh mycelial cultures were then incubated for 60-180 days in class 100 clean room.
Once colonization was determined to be sufficient, the mycelium-colonized rice was transferred to glass containers for extraction. The mycelium being delicate in nature, was handled with utmost gentle care so as to not to cause cell damage in transfer and immediately covered with an approximately equal weight of 50% ethanol-water (prepared by mixing equal weights of 95% (190 proof) organic ethyl alcohol and spring water), agitated, and then allowed to rest for room temperature infusion-extraction for a total of 14 days. Cultures of *Fomitopsis officinalis* and various other species were treated separately in a similar fashion to the methods described herein. The clear fluid, the supernatant, was drawn off and decanted into 2 ounce amber bottles.
The general approach for determining antiviral activity and toxicity for orthopoxviruses as described by E. Kern (http://www.niaidaacf.org/protocols/orthopox.htm) was utilized. An inexpensive, rapid assay such as a CPE-inhibition assay that is semi-automated was used initially to screen out the negatives. Screening assays were conducted in low-passaged human cells. Each assay system contained a positive control (CDV) and a

negative control (ACV). Toxicity was determined using both resting and proliferating human fibroblast cells. Compounds were screened for activity against Vaccinia virus (VV) and cowpox virus (CV) using the CPE assay *in vitro* with cultured human follicle foreskins (HFF) cells. The screening assay systems utilized were selected to show specific inhibition of a biologic function, i.e., cytopathic effect (CPE) in susceptible human cells. In the CPE-inhibition assay, drug is added 1 hr prior to infection so the assay system will have maximum sensitivity and detect inhibitors of early replicative steps such as adsorption or penetration as well as later events. To rule out non-specific inhibition of virus binding to cells all compounds that show reasonable activity in the CPE assay can be confirmed using a classical plaque reduction assay in which the drug is added 1 hr after infection. These assay systems also can be manipulated by increasing the pre-treatment time in order to demonstrate antiviral activity with oligodeoxynucleotides and/or peptides. By delaying the time of addition of drug after infection, information regarding which step in the virus life cycle is inhibited (i.e., early vs. late functions) can be gained.

In all the assays used for primary screening, a minimum of six drug concentrations was used covering a range of 100µg/ml to 0.03µg/ml, in 5-fold increments. These data allowed good dose response curves. From these data, the dose that inhibited viral replication by 50% (effective concentration 50; EC_{50}) was calculated using the computer software program MacSynergy II by M.N. Prichard, K. R. Asaltine, and C. Shipman, Jr., University of Michigan, Ann Arbor, Michigan.

To determine if each compound has sufficient antiviral activity that exceeds its level of toxicity, a selectivity index (SI) was calculated according to CC_{50}/EC_{50}. This index, also referred to as a therapeutic index, was used to determine if a compound warrants further study. Compounds that had an SI of 2 or more (~1.5-2.5) are considered active, 10 or greater is considered very active.

Newborn human foreskins are obtained as soon as possible after circumcision and placed in minimal essential medium (MEM) containing vancomycin, fungizone, penicillin, and gentamicin at the usual concentrations, for 4 hr. The medium is then removed, the foreskin minced into small pieces and washed repeatedly with phosphate buffered saline (PBS) deficient in calcium and magnesium (PD) until red cells are no longer present. The tissue is then trypsinized using trypsin at 0.25% with continuous stirring for 15 min at 37°C in a CO_2 incubator. At the end of each 15-min. period the tissue is allowed to settle to the bottom of the flask. The supernatant containing cells is poured through sterile cheesecloth into a flask containing MEM and 10% fetal bovine serum. The flask containing the medium is kept on ice throughout the trypsinizing procedure. After each addition of cells, the cheesecloth is washed with a small amount of MEM containing serum. Fresh trypsin is added each time to the foreskin pieces and the procedure repeated until all the tissue is digested. The cell-containing medium is then centrifuged at 1000 RPM at 4° C for 10 min. The supernatant liquid is discarded and the cells resuspended in a small amount of MEM with 10% FBS. The cells are then placed in an appropriate number of 25 cm^2 tissue culture flasks. As cells become confluent and need trypsinization, they are expanded into larger flasks. The cells are kept on vancomycin and fungizone to passage four, and maintained on penicillin and gentamicin. Cells are used only through passage 10.

Low passage HFF cells are seeded into 96 well tissue culture plates 24 hr prior to use at a cell concentration of 2.5 x 10^5 cells per ml in 0.1 ml of MEM supplemented with 10% FBS. The cells are then incubated for 24 hr at 37°C in a CO_2 incubator. After incubation, the medium is removed and 125µl of experimental drug is added to the first row in triplicate wells, all other wells having 100 µl of MEM containing 2% FBS. The drug in the first row of wells is then diluted serially 1:5 throughout the remaining wells by transferring 25 µl using the BioMek 2000 Laboratory Automation Workstation. After dilution of drug, 100µl of the appropriate virus concentration is added to each well, excluding cell control wells, which received 100 µl of MEM. The virus concentration utilized is 1000 PFU's per well. The plates are then incubated at 37°C in a CO_2 incubator for 7 days. After the incubation period, media is aspirated and the cells stained with a 0.1% crystal violet in 3% formalin solution for 4 hr. The stain is removed and the plates rinsed using tap water until all excess stain is removed. The plates are allowed to dry for 24 hr and then read on a BioTek Multiplate Autoreader at 620 nm. The EC_{50} values are

determined by comparing drug treated and untreated cells using a computer program.

Two days prior to use, HFF cells are plated into 6 well plates and incubated at 37°C with 5% CO_2 and 90% humidity. On the date of assay, the drug is made up at twice the desired concentration in 2× MEM and then serially diluted 1:5 in 2× MEM using 6 concentrations of drug. The initial starting concentration is usually 200 µg/ml down to 0.06µg/ml. The virus to be used is diluted in MEM containing 10% FBS to a desired concentration which will give 20-30 plaques per well. The media is then aspirated from the wells and 0.2 ml of virus is added to each well in duplicate with 0.2 ml of media being added to drug toxicity wells. The plates are then incubated for 1 hr with shaking every 15 min. After the incubation period, an equal amount of 1% agarose will be added to an equal volume of each drug dilution. This gives final drug concentrations beginning with 100µg/ml and ending with 0.03µg/ml and a final agarose overlay concentration of 0.5%. The drug/agarose mixture is applied to each well in 2 ml volume and the plates are incubated for 3 days, after which the cells are stained with a 0.01% solution of neutral red in phosphate buffered saline. After a 5-6 hr incubation period, the stain is aspirated, and plaques counted using a stereomicroscope at 10× magnification.

Twenty-four h prior to assay, HFF cells are plated into 96 well plates at a concentration of 2.5 × 10^4 cells per well. After 24 hr, the media is aspirated and 125µl of drug is added to the first row of wells and then diluted serially 1:5 using the BioMek 2000 Laboratory Automation Workstation in a manner similar to that used in the CPE assay. After drug addition, the plates are incubated for 7 days in a CO_2 incubator at 37°C. At this time the media/drug is aspirated and 200µl/well of 0.01% neutral red in PBS is added. This is incubated in the CO_2 incubator for 1 hr. The dye is aspirated and the cells are washed using a Nunc Plate Washer. After removing the PBS, 200 (g/well of 50% ETOH/1% glacial acetic acid (in H_2O) is added. The plates are rotated for 15 min and the optical densities read at 540 nm on a plate reader. The EC_{50} values are determined by comparing drug treated and untreated cells using a computer program.

All strains below were incubated for approximately two months prior to extractions except for those designated "4 months," which were incubated for approximately four months prior to extraction. Those strains designated "Hot" were incubated for the final 48 hours at approximately 35°C (95°F). With those strains designated as "shaken," the mycelium and ethanol/water were shaken and allowed to settle prior to decanting the extract.

5 Results

The antiviral tests (Table 1 and 2) show that several strains of *F. officinalis* generate strong anti-pox activity, with the highest activity from the strain designated as *F. officinalis* I showing a SI rating of 14.7. (See Table 2). That several strains varied in their antiviral activities underscores the importance of mycodiversity. As researchers search the fungal genome for antiviral strains, it is anticipated that more and less potent strains of *F. officinalis* and allies will be discovered.

Since the ecological niche for these unique mushrooms is increasingly jeopardized as humans destroy old growth forest habitats, the pool of available strains of *F. officinalis* will be further impaired. Acquiring as many strains as possible should be an international priority so that preventive or curative medicines against pox and related viruses can be developed.

Table 1. Antiviral Therapeutic Index of *F. officinalis* extracts inhibiting vaccinia pox virus co-cultured with human cells

Drug Name	Vaccinia-HFF Cells					
	CPE EC50	CPE EC90	CPE CC50	CPE SI	CPE CDV EC50	CPE CDV EC90
Fomitopsis officinalis I	3.4	4.8	>10	2.9	2.1	3.4
Fomitopsis officinalis I Hot	5.7	8.7	>10	>1.8	2.1	3.4
Fomitopsis officinalis I 4 months	1.9	3.3	>10	>5.3	2.5	5.4
Fomitopsis officinalis I 4 months	3	6.5	>10	>3.3	2.1	3.4
Fomitopsis officinalis I	1.1	1.8	>10	>9.1	2.5	5.4
Fomitopsis officinalis IV	6.5	>10	>10	>1.5	2.1	3.4
Fomitopsis officinalis IV Hot	6.7	>10	>10	>1.5	2.1	3.4
Fomitopsis officinalis V	7.4	>10	>10	>1.4	2.1	3.4
Fomitopsis officinalis V Hot	5	8.9	>10	>2	2.5	5.4
Fomitopsis officinalis V Hot	2.8	4.7	>10	>3.6	2.1	3.4

Table 2. Antiviral Therapeutic Index of *F. officinalis* extracts inhibiting cowpox virus when co-cultured with human cells

Drug Name	Vaccinia-HFF Cells					
	CPE EC50	CPE EC90	CPE CC50	CPE SI	CPE CDV EC50	CPE CDV EC90
Fomitopsis officinalis I	2.3	3.9	>10	4.3	2.7	76
Fomitopsis officinalis I Hot	7.1	>10	>10	1.4	2.7	76
Fomitopsis officinalis I 4 months	1.3	2.2	>10	>7.7	3.1	
Fomitopsis officinalis I 4 months	4.1	6.5	>10	>2.4	2.7	76
Fomitopsis officinalis I	0.68	1.1	>10	>14.7	3.1	
Fomitopsis officinalis IV	>10	>10	>10	0	2.7	76
Fomitopsis officinalis IV Hot	6	>10	>10	2.7	7.6	
Fomitopsis officinalis V	7.1	>10	>10	>1.4	2.7	76
Fomitopsis officinalis V Hot	1.7	2.5	>10	>5.9	3.1	
Fomitopsis officinalis V Hot	3.8	6.6	>10	>2.6	2.7	76

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References

[1] Suay IF, Arenal F, Asenio A, *et al.* Screening of basidiomycetes for antimicrobial activities. Antonie van Leeuwenhoek, 2000, 78: 129-139.
[2] Brandt CR, Piraino F. Mushroom antivirals. Recent Research Developments for Antimicrobial Agents and Chemotherapy, 2000, 4:11-26.
[3] Stamets P. Novel anti-virals from mushrooms. Herbalgram, 2001, 51:24-27.
[4] Stamets P. Novel antimicrobials from mushrooms. Herbalgram, 2002, 54:29-33.
[5] Stamets P. Potentiation of cell-mediated host defense using fruitbodies and mycelia of medicinal mushrooms. Int. J. Med. Mush. 2003, 5:179-192.
[6] Suzuki H, Iiyama K, Yoshida O, *et al.* Structural characterization of immunoactive and antiviral water-solubilized lignin in an extract of the culture medium of Lentinus edodes mycelia (LEM). Agr. Biol.Chem.1990, 54:479-487.
[7] Gordon M, Bihari B, Goosby E, *et al.* A placebo-controlled trial of the immune modulator, lentinan, in HIV- positive patients: a phase I/II trial. J. Med. 1998, 29:305-330.
[8] Abrams D. Personal communication, 2002.
[9] Aoki M, Motomu T, Fukushima A, *et al.* Antiviral substances with systemic effects produced by Basidi omycetes such as *Fomes fomentarius.* Biosci. Biotechnol. Biochem. 1993, 57:278-293.
[10] Collins RA, Ng TB. Polysaccharopeptide from *Coriolus versicolor* has potential for use against human immunodeficiency virus type 1 infection. Life Sciences, 1997, 60:PL383-387.
[11] Sarkar S, Koga J, Whitley RJ, *et al.* Antiviral effect of the extract of culture medium of *Lentinus edodes* mycelia on the replication of herpes simplex virus 1. Antiviral Research, 1993, 20:293-303.
[12] Yan SC. Clinical and experimental research on *Polyporus umbellatus* polysaccharide in the treatment of chronic viral hepatitis. Chung Kuo Chung Hsi I Chieh Ho Tsa Chih. 1988, 8:141-143.
[13] Piraino F, Brandt C. Isolation and partial characterization of an antiviral, RC-183, from the edible mushroom, *Rozites caperata.* Antiviral Res. 1999, 43:67-68.
[14] Eo SK, Kim YS, Lee CK, *et al.* Antiviral activities of various water and methanol soluble substances isolated from *Ganoderma lucidum.* J. Ethnopharmacol. 1999, 68:129-136.
[15] Eo SK, Kim YS, Lee CK, *et al.* Possible mode of antiviral activity of acidic protein bound polysaccharide isolated from *Ganoderma lucidum* on herpes simplex viruses. J. Ethnopharmacol. 2000, 72:475-481.
[16] Wang HX, Ng TB. Isolation of a novel ubiquitin-like protein from *Pleurotus ostreatus* mushroom with anti- human immunodeficiency virus, Translation-inhibitory and ribonuclease activities. Biochem. Biophys. Res. Commun. 2000, 276:587-593.
[17] Kahlos K, Lesnau A, Lange W, *et al.* Preliminary tests of antiviral activity of two *Inonotus obliquus* strains. Fitopterapia, 1996, 6: 344-347.
[18] Ghoneum M. Enhancement of human natural killer cell activity by modified arabinoxylane from rice bran (MGN-3). Int. J. Immunother. 1998, XIV:89-99.
[19] Ohtomo M. In vivo and in vitro test study: physiological activity in immune response system of representative basidiomycetes. Unpublished research report provided to Fungi Perfecti from Tamagawa University, Japan, 2001.
[20] Stamets P. U.S Patent Application serial no. 11/029,861 for Antimicrobial activity from medicinal mushrooms (*Fomitopsis*), filed 1/04/2005. PCT International Patent application. Antimicrobial activity from medicinal mushrooms, PCT/US05/00266. 2005.
[21] Mizuno TC, Zhuang K, Abe H, *et al.* Studies on the host-mediated antitumor polysaccharides, Part XXVII. Mush. Sci. Biotechnol. 1996, 3:53-60.
[22] Blanchette RA, Compton BD, Turner NJ, *et al.* Nineteenth century grave guardians are carved *Fomitopsis officinalis* sporophores. Mycologia, 1992, 84:119-124.
[23] Grzywnowicz K. Medicinal mushrooms in Polish folk medicine. Int. J. Med. Mush. 2001, 3:154.
[24] Leck C. Mass extinction of European fungi. Trends Ecol. Evol. 1991, 6.
[25] Stamets P. Growing Gourmet & Medicinal Mushrooms. Berkeley, California:Ten Speed Press, 2000, 574 pp.