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Evaluation of Therapeutic Activity of Hypogeous Ascomycetes and Basidiomycetes from North America

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ABSTRACT: This study is the first broad investigation of therapeutic activities of hypogeous truffles and truffle-like fungi (Ascomycetes and Basidiomycetes) from North America. Twenty-two species from 12 families were evaluated in several biological assays for antimicrobial, antimalarial, antiinflammatory, antioxidant, antituberculosis, and anticancer activities. Biological screening results indicate that 1 species showed antimalarial activity, 11 species were active in antioxidant assay, 9 species were active in antiinflammatory assay, 9 species showed antituberculosis activity, and 2 species showed anticancer activity. Among the screened species, *Elaphomyces granulatus, E. muricatus, Geopora clausa, Hymenogaster subalpinus, Melanogaster tuberiformis, Rhizopogon couchii, R. nigrescens, R. pedicellus, R. subaustralis, R. subgelatinosus,* and *Scleroderma laeve* expressed therapeutic activity in more than one assay. Our results indicate that this group of fungi has promising therapeutic activities that could lead to the development of new agents for the treatment and prevention of diseases.

KEY WORDS: Ascomycetes and Basidiomycetes fungi, hypogeous fungi, therapeutic activities, antimicrobial, antimalarial, antituberculosis, anticancer, antiinflammatory, antioxidant

INTRODUCTION

Fungi-producing hypogeous (underground) fruiting bodies occur over a wide range of orders in the Ascomycetes and Basidiomycetes (Castellano et al., 2004; Claridge and Trappe, 2005; Trappe and Claridge, 2005). This fruiting habit likely evolved from natural selection pressure to better withstand unfavorable environmental conditions, such as drought and frost, than their above-ground mushroom ancestors (Percudani et al., 1999; Trappe and Claridge, 2005). Ascomycetes, commonly termed

ABBREVIATIONS

COX-2: cyclooxygenase-2; **DCF:** 2′,7′-dichlorodihydrofluorescein; **DCFH-DA:** 2′,7′-dichlorodihydrofluorescein diacetate; **DMSO:** dimethyl sulfoxide; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl radical; **LPS:** lipopolysaccharide; **MIC:** minimum inhibitory concentration; **PMA:** phorbol 12-myristate 13 acetate; **SRB:** sulforhodamine B; **TCA:** trichloroacetic acid.

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true truffles, occur in orders Pezizales (including families Discinaceae, Helvellaceae, Pezizaceae, Pyronemataceae, and Tuberaceae) and Eurotiales (family Elaphomycetaceae) (Trappe, 1979; Castellano et al., 2004). Species belonging to the higher Basidiomycetes are usually referred to as *false truffles*, or *truffle-like fungi*, and occur in various orders and families, such as Cortinariaceae, Boletaceae, Leucogastraceae, Rhizopogonaceae, among others (Castellano et al., 1989, 2004).

Hypogeous fruiting bodies are produced by the mycelium of fungi that form ectomycorrhizae with a variety of gymnosperms and angiosperms (Trappe, 1971; Trappe and Claridge, 2005). These fruiting bodies are rounded to irregular in shape, mostly 1–10 cm in diameter. They produce spores, usually internally, that remain enclosed inside the fruiting body, which matures underground (Trappe and Claridge, 2005). As spores mature, the fruiting bodies emit aromatic substances that attract animals, which unearth and eat them (mycophagy). The animals digest the fruiting body tissues, but the spores pass through the digestive tract unharmed and are then dispersed through defecation. Each species produces its own combination of aromatics—humans may perceive these as pleasantly pungent, garlicky, cheesy, fruity, sweet, etc, which is the reason some species have high culinary values. However, the odors of many species are regarded as unpleasant to most human observers, although evidently not to other animals (Trappe and Claridge, 2005).

Truffles have been used as a culinary delicacy for thousands of years and were known to the ancient Egypt, Greek, and Roman civilizations. In Europe, *Tuber melanosporum* (Perigord black truffle) and *Tuber magnatum* (Italian white truffle) are the most highly valued, both gastronomically and economically. Truffles have been used fresh and unprocessed as a condiment in cooking, in making liqueurs, for scenting tobacco, and in the perfume industry (Gao et al., 2001). They are rich in proteins, amino acids, vitamins, and minerals, such as phosphorus and potassium (Ashour-Ahmed et al., 1981; Claridge and Trappe, 2005). Research on the chemical composition of truffles has revealed alcohols, aldehydes, ethers, ketones, and sulphur compounds forming truffle aromatic constituents (Talou et al., 1987; Diaz et al., 2002). Research on the chemistry and medicinal properties of truffles has been limited to a few species of *Terfezia*, *Tirmania*, and *Tuber* from Europe, North America, and Asia (Marin et al., 1984; Marin and McDaniel, 1987; Chellal and Lukasova, 1995; Lanzotti and Iorizzi, 2000; Gao et al., 2004; Janakat et al., 2005; Shaker, 2005).

The goal of this study was to investigate the therapeutic activity of hypogeous fungi from North America as a potential source of bioactive compounds that could contribute to the development of new drugs. Twenty-two species were screened in biological assays for antimicrobial, antimalarial, antioxidant, antiinflammatory, antituberculosis, and anticancer activities.

MATERIALS AND METHODS

Collection

Fruiting bodies of hypogeous fungi from Ascomycetes and Basidiomycetes were collected by members of the North American Truffling Society and identified by Dr. James M. Trappe. Voucher specimens have been deposited in the Mycological Herbarium, Department of Botany and Plant Pathology, Oregon State University, USA (Table 1). *Astraeus pteridis* is not a truffle-like species in the strict sense, but its early stages develop underground much of the same as truffles, and it emerges only at maturity.

Extract Preparation

The fruiting bodies of fungal specimens were dried for 24 hours in a forced air dehydrator at 35°C. Five grams of the dried, powdered material of each fungal species were separately extracted with an ASE 200 Accelerated Solvent Extractor (Dionex, Sunnyvale, CA, USA) by the following procedure: extracted 3 times with 95% EtOH at 40°C, then extracted 2 times with 70% EtOH at 40°C. The 95% EtOH extract was concentrated under reduced pressure to yield residue A; the 70% EtOH extract was concentrated under reduced pressure to yield residue B.

Table 1. Screened Fungus Species

Class	Family	Species	OSC accession no.	Collection location
Ascomycetes	Elaphomycetaceae	*Elaphomyces granulatus* Fr.	20881	Bonner County, Idaho
		Elaphomyces muricatus Fr.	12300	Benton County, Oregon
	Helvellaceae	*Barssia oregonensis* Gilkey	27997	Clackamas County, Oregon
	Pyronemataceae	*Geopora clausa* Tul. et C. Tul.	5715	Inyo County, California
Basidiomycetes	Boletaceae	*Gastroboletus subalpinus* Trappe et Thiers	27882	Douglas County, Oregon
		Truncocolumella citrina Zeller	22967	Clackamas County, Oregon
	Cortinariaceae	*Hymenogaster subalpinus* A. H. Sm.	27992	Benton County, Oregon
		Thaxterogaster pingue (Zeller) Singer et A. H. Sm.	23395	Linn County, Oregon
	Gautieriaceae	*Gautieria monticola* Harkn.	6004	Benton County, Oregon
	Leucogastraceae	*Leucogaster rubescens* Zeller et C. W. Dodge	23589	Pend Oreille County, Idaho
	Melanogastraceae	*Melanogaster tuberiformis* Corda	27998	Lane County, Oregon
	Rhizopogonaceae	*Rhizopogon couchii* A. H. Sm.	27933	Lebanon State Forest, New Jersey
		Rhizopogon idahoensis A. H. Sm.	25707	Clearwater County, Idaho
		Rhizopogon nigrescens Coker et Couch	27932	Lebanon State Forest, New Jersey
		Rhizopogon pedicellus A. H. Sm.	25562	Pend Oreille County, Idaho
		Rhizopogon subareolatus A. H. Sm.	23132	Lewis County, Washington
		Rhizopogon subaustralis A. H. Sm.	27931	Lebanon State Forest, New Jersey
		Rhizopogon subgelatinosus A. H. Sm.	7108	Jackson County, Oregon
	Sedeculaceae	*Sedecula pulvinata* Zeller	19197	Valley County, Idaho
	Sclerodermataceae	*Astraeus pteridis* (Shear) Zeller	27893	Linn County, Oregon
		Scleroderma laeve Lloyd	27936	Lebanon State Forest, New Jersey
	Tricholomataceae	*Hydnangium carneum* Wallr.	8842	Humboldt County, California

Evaluation of Biological Activity

Antimicrobial assay. All organisms were obtained from the American Type Culture Collection (ATCC) and included the fungi *Candida albicans* ATCC 90028, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 90906 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 43300 (MRS), *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed by a modified version of the NCCLS methods (NCCLS. 1997, 1998, 2000a,b). *M. intracellulare* was tested by a modified method of Franzblau et al. (1998). Briefly, samples (dissolved in DMSO) were serially diluted with 0.9% saline and transferred in duplicate to 96-well microplates. Inocula were prepared by diluting microbe suspensions with assay media (Sabouraud Dextrose [Difco, www.fishersci.com] for *C. albicans* and *C. neoformans*, cation-adjusted Mueller-Hinton [Difco] for *Staphylococcus*, YM broth [Difco, buffered with 0.165M MOPS at pH 7.3] for *A. fumigatus*, and 5% Alamar Blue [BioSource International, Camarillo, CA, USA] in Middlebrook 7H9 broth with OADC enrichment, pH = 7.3 for *M. intracellulare*) to afford the desired colony forming units/mL at turbidimetric readings of 630 nm. The microbial inocula were added to the samples to achieve a final volume of 200 µL and final sample concentration of 200 µg/mL. Growth (saline only), solvent, and blank (media only) controls were included on each test plate. Drug controls (Ciprofloxacin [ICN Biomedicals, Warrendale, PA, USA] for bacteria and Amphotericin B [ICN Biomedicals] for fungi) were included in each assay. Except for *A. fumigatus*, which was

inspected visually, all other organisms were read at either 630 nm with the EL-340 Biokinetics Reader (Bio-Tek Instruments, Winooski, VT, USA) or 544ex/590em, gain = 25 (*M. intracellulare*) with the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Bottco, Germany) prior to and after incubation: *C. albicans*, *S. aureus*, and *P. aeruginosa* at 37°C for 18–24 hours. *C. neoformans* and *A. fumigatus* at 30°C for 72 hours, and *M. intracellulare* at 37°C and 10% CO_2 for 72 hours.

Antimalarial assay. The assay is based on the determination of plasmodial LDH activity. For the assay, a suspension of red blood cells infected with D6 or W2 strains of *Plasmodium falciparum* (200 µL, with 2% parasitemia and 2% hematocrit in RPMI 1640 medium supplemented with 10% human serum and 60 µg/mL amikacin) was added to the wells of a 96-well plate containing 10 µL test samples diluted in medium at various concentrations. The plate was placed in a modular incubation chamber (Billups-Rothenberg, Del Mar, CA, USA) and flushed with a gas mixture of 90% N_2, 5% O_2, and 5% CO_2 and incubated at 37°C for 72 hours. Parasitic LDH activity was determined by use of Malstat™ reagent (Flow Inc., Portland, OR, USA) according to the procedure of Makler and Hinrichs (1993). Briefly, 20 µL of the incubation mixture was mixed with 100 µL of the Malstat™ reagent and incubated at room temperature for 30 minutes. Twenty microliters of a 1:1 mixture of NBT/PES (Sigma Aldrich, www.Sigma-Aldrich.com) was then added, and the plate was further stopped by the addition of 100 µL of a 5% acetic acid solution. The plate was read at 650 nm with the EL-340 Biokinetics Reader (Bio-Tek Instruments). IC_{50} values were computed from the dose response curves. Artemisinin and chloroquine were included in each assay as the drug controls. DMSO (0.25%) was used as vehicle control.

Antiinflammatory assay. The assay is based on the determination of COX-2 activity. Mouse macrophages (RAW 264.7, ATCC) were cultured in 75 cm^2 culture flask in RPMI-1640 medium (Gibco™, Carlsbad, CA, USA; Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% bovine calf serum (Hyclone, Logan, UT, USA) and 60 mg/L amikacin (Sigma) at 37°C in an environment of 95% humidity and 5% CO_2. For the assay, cells were seeded in the wells of 96-well plates (50,000 cells/well) and incubated at 37°C for 24 hours. After washing with RPMI-1640 medium supplemented with 3% bovine calf serum, they were then incubated with 5 µg/mL lipopolysaccharide (LPS) (*Escherichia coli* 055:B5, Sigma) for 16 hours to induce the production of COX-2. Induced cells were washed thoroughly with medium to remove LPS completely and treated with different concentrations of test samples (extracts or pure samples) for 2 hours. Arachidonic acid (300 µM, Sigma) was added, and the cells were further incubated for 30 minutes. The amount of PGE_2 released in the medium was determined with the PGE_2 Enzyme Immunoassay kit (Cayman Chemical Co., Ann Arbor, MI, USA). COX-2 activity was determined by the conversion of exogenous arachidonic acid to PGE_2, expressed as percentage of the vehicle control. The concentration that caused 50% inhibition of enzyme activity (IC_{50}) was calculated from the dose curves generated by plotting % COX-2 activity against the test concentrations. NS-0398 (Cayman), a specific inhibitor of COX-2, was included as a positive control in each assay.

Assay for antioxidant activity. Myelomonocytic HL-60 cells (ATCC) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (Hyclone) and 60 mg/mL amikacin at 37°C in an environment of 95% humidity and 5% CO_2. For the assay, 125 µL of the cell suspension (1×10^6 cells/mL) was added to the wells of a 96-well plate. After treating with different concentrations of the test samples for 30 minutes, the cells were stimulated with 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) for 30 minutes. DCFH-DA (Molecular Probe, Carlsbad, CA, USA; 5 µg/mL) is added, and the cells were incubated for 15 minutes. The levels of DCF produced were measured on a PolarStar plate reader with an excitation wavelength at 485 nm and emission at 530 nm, as described previously (Takamatsu et al., 2003; Choi, et al., 2006). The ability of the test materials to inhibit exogenous cytoplasmic ROS-catalysed oxidation of DCFH to fluorescent DCF in HL-60 cells was measured in comparison to PMA-treated controls without the test materials. IC_{50} values were calculated from dose curves of % DCF production versus test concentrations. Vitamin C (Sigma) was included as a positive control.

Antituberculosis assay. The antituberculosis activity was determined against *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) in the Microplate Alamar Blue assay (Collins and Franzblau, 1997). The minimum inhibitory concentration (MIC) was defined as the lowest concentration affecting a reduction in fluorescence of 90% relative to controls. Rifampin was included as a positive quality control compound for each test with expected MIC ranges of 0.06–0.125 µg/mL.

Anticancer assay. A colorimetric assay that uses sulforhodamine B (SRB) reaction has been adapted for a quantitative measurement of cell growth and viability (Skehan et al., 1990). This form of the assay employs 96-well cell culture microplates of 9 mm diameter (Mosmann, 1983; Faircloth et al., 1988). Most of the cell lines were obtained from ATCC derived from different human cancer types. Tested cell lines included HT-29 (colon carcinoma), A549 (lung carcinoma), LOVO-DOX (colon carcinoma), and SK-MEL-28 (malignant melanoma). Cells were maintained in RPMI 1640 10% FBS, supplemented with 0.1 g/L penicillin and 0.1 g/L streptomycin sulfate, and then incubated at 37°C, 5% CO_2, and 98% humidity. For the experiments, cells were harvested from subconfluent cultures with trypsin and resuspended in fresh medium before plating. Cells were seeded in 96-well microtiter plates at 5×10^3 cells per well in aliquots of 195 µL medium, and they were allowed to attach to the plate surface by growing in drug-free medium for 18 hours. Afterward, samples were in aliquots of 5 µL in a range of 10 to 10^{-8} µg/mL dissolved in DMSO/EtOH (0.2% in PS buffer). After 48 hours' exposure, the antitumor effect was measured by the SRB methodology: cells were fixed by adding 50 µL of cold 50% (wt/vol) trichloroacetic acid (TCA) and incubating for 60 minutes at 4°C. Plates were washed with deionized water and dried. One hundred µL of SRB solution (0.4% wt/vol in 1% acetic acid) was added to each microtiter well and incubated for 10 minutes at room temperature. Unbound SRB was removed by washing with 1% acetic acid. Plates were air-dried, and bound stain was solubilized with Tris buffer. Optical densities were read on an automated spectrophotometric plate reader at a single wavelength of 490 nm.

RESULTS AND DISCUSSION

Twenty two species from 12 families, representing both the Ascomycetes and Basidiomycetes, were evaluated in this study (Table 1).

Crude mushroom extracts were evaluated in several biological assays for antimicrobial, antimalarial, antiinflammatory, antioxidant, antituberculosis, and anticancer activities. Biological screening results indicate that 1 species showed weak antimalarial activity, 11 species exhibited moderate to weak antioxidant activity, 9 species showed significant antiinflammatory activity, 9 species were active in antituberculosis assay, and 2 species showed weak anticancer activity in the in vitro cell-based assays (Table 2). *Elaphomyces granulatus, E. muricatus, Geopora clausa, Hymenogaster subalpinus, Melanogaster tuberiformis, Rhizopogon couchii, R. nigrescens, R. pedicellus, R. subaustralis, R. subgelatinosus,* and *Scleroderma laeve* expressed biological activity in more than 1 assay.

This study is the first broad investigation of therapeutic activities of hypogeous Ascomycetes and Basidiomycetes fungi from North America. Our results indicate that this group of fungi has promising biological activities that could lead to the development of new agents for the treatment and prevention of diseases.

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Table 2. Therapeutic Activity of Screened Species

Species	Extract[a]	Therapeutic activity					
		AMI	AMA[b]	AO[c]	AI[d]	AT[e]	AC[f]
Astraeus pteridis	A	–	–	–	–	SA	–
	B	–	–	–	–	MA	–
Barssia oregonensis	A	–	–	–	–	MA	–
	B	–	–	–	–	MA	–
Elaphomyces granulatus	A	–	–	WA	SA	SA	–
	B	–	–	WA	SA	–	–
Elaphomyces muricatus	A	–	–	–	A	WA	–
	B	–	–	WA	–	–	–
Gastroboletus subalpinus	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
Gautieria monticola	A	–	–	–	–	–	–
	B	–	–	WA	–	–	–
Geopora clausa	A	–	–	–	–	–	WA
	B	–	–	MA	–	–	WA
Hydnangium carneum	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
Hymenogaster subalpinus	A	–	–	–	A	SA	–
	B	–	–	–	A	–	–
Leucogaster rubescens	A	–	–	MA	–	–	–
	B	–	–	–	–	–	–
Melanogaster tuberiformis	A	–	–	WA	A	SA	–
	B	–	–	WA	A	–	–
Rhizopogon couchii	A	–	–	MA	A	MA	–
	B	–	–	WA	A	–	–
Rhizopogon idahoensis	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
Rhizopogon nigrescens	A	–	–	WA	SA	–	–
	B	–	–	–	SA	–	–
Rhizopogon pedicellus	A	–	–	–	–	WA	–
	B	–	–	MA	–	–	–
Rhizopogon subareolatus	A	–	WA	–	–	–	–
	B	–	WA	–	–	–	–
Rhizopogon subaustralis	A	–	–	WA	A	–	–
	B	–	–	–	A	–	–
Rhizopogon subgelatinosus	A	–	–	–	A	–	WA
	B	–	–	–	A	–	–
Scleroderma laeve	A	–	–	SA	SA	SA	–
	B	–	–	WA	A	WA	–
Sedecula pulvinata	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
Thaxterogaster pingue	A	–	–	–	–	–	–
	B	–	–	–	–	–	–
Truncocolumella citrina	A	–	–	–	–	–	–
	B	–	–	–	–	–	–

Note: – = not active, A = active, WA = weakly active, MA = moderately active, SA = strongly active; AMI = antimicrobial assay, AMA = antimalarial assay, AO = antioxidant assay, AI = antiinflammatory assay, AT= antituberculosis assay, AC = anticancer assay.

[a] A: 95% EtOH extract. B: 70% EtOH extract.

[b] WA: samples showing % inhibition > 50 at 15.9 μg/mL in primary assay and $IC_{50} \geq 40.000$ ng/mL in secondary assay.

[c] SA: samples showing $IC_{50} < 20$ μg/mL; MA: IC_{50} 20–50 μg/mL; WA: $IC_{50} > 50$ μg/mL.

[d] SA: samples showing % inhibition of COX-2 > 60% at 50 μg/mL; A: 45–60% at 50 μg/mL.

[e] SA: samples showing $IC_{50} < 20$ μg/mL; MA: IC_{50} 20–50 μg/mL; WA: $IC_{50} > 50$ μg/mL.

[f] WA: based on the proposed scale by PharmaMar.

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Neurotropic and Trophic Action of Lion's Mane Mushroom *Hericium erinaceus* (Bull.: Fr.) Pers. (Aphyllophoromycetideae) Extracts on Nerve Cells *in Vitro*

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ABSTRACT: The neurotropic and trophic effect of extract obtained from the edible and medicinal mushroom *Hericium erinaceus* on nerve cells was studied. Spike reactions of hippocampal neurons during application of *H. erinaceus* fruiting bodies extract were studied on rat brain slices *in vitro*, using whole-cell patch clamp recording. Spike activity was inhibited in a concentration-dependent, reversible manner in 34%–90% of studied neurons by extracts obtained with ethanol, ether, or broth. Extract suppressed the excitation of neurons caused by L-glutamic acid application. The assumption is made that the *H. erinaceus* extract contains substances that may activate receptors that cause an inhibition of spike activity. The inhibitory effect of extract was not induced by GABA and serotonin receptor activation or activation of M- and N-cholinoreceptors. Inhibition of spike activity was caused by hyperpolarization of the neuronal membrane during extract application. The hyperpolarization was accompanied by an increase of apamin-sensitive Ca-activated K^+ current (I_{AHP}) and apamin-insensitive, slow Ca-activated K^+ current (sI_{AHP}), but it was not caused by an increase of inward rectifier K^+ current (I(ir)) or by changes of hyperpolarization-activated cationic currents (I(h)). The effect of the extract was observed in the presence of tetrodotoxin, suggesting that the extract acts postsynaptically. Extract application did not suppress biochemical processes in cell respiratory circuits. The extract did not affect the regenerating abilities of the neurons and glial cells of the cerebellum and hippocampus. It was demonstrated that the *H. erinaceus* extract concentrate exerted neurotropic action and improved the myelination process in the mature myelinating fibers, did not affect nerve cell growth *in vitro*, and did not evoke a toxic effect or nerve cell damage.

KEY WORDS: *Hericium erinaceus*, edible and medicinal mushrooms, extracts, neurons, brain slices, spike activity, cell cultures

INTRODUCTION

Recently, much attention has been given to the mechanisms of neurotropic action of medicinal mushroom components (Moldavan et al., 1999a–c, 2000a–c). One of the most promising mushroom species for use in medicine is *Hericium erinaceus* (Bull.: Fr.) Pers. It is a good edible cultivated mushroom and is widely used in Eastern traditional medicine (Dudka and Wasser, 1987). In Japan it is known by the name

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