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Influence of Olive Oil Press Cakes on Shiitake Culinary-Medicinal Mushroom, *Lentinus edodes* (Berk.) Singer (Higher Basidiomycetes) Fruiting Bodies Production and Effect of their Crude Polysaccharides on CCRF-CEM Cell Proliferation

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ABSTRACT: *Lentinus edodes* (Berk.) Singer fruiting bodies were cultivated on substrates composed of beech sawdust, wheat bran, and calcium sulfate hemihydrate (gypsum), containing different proportions of olive oil press cakes (OOPC). We determined the influence of OOPC on fruiting bodies production and proliferation of CCRF-CEM leukemia cells. A negative influence of OOPC on mycelia growth and maturation was noticed. When growth medium contained 80% OOPC, fruiting bodies ceased forming. To investigate the cytotoxicity on CCRF-CEM cells *in vitro*, cells were treated with crude polysaccharides extracted from *L. edodes* fruiting bodies. Also in this case a negative correlation between OOPC content and cytotoxicity was found.

KEY WORDS: medicinal mushrooms, *Lentinus edodes*, Shiitake mushroom, polysaccharides, olive oil press cakes, cytotoxicity, CCRF-CEM leukemia cells

ABBREVIATIONS: CCRF-CEM: acute lymphoblastic leukemia cells; **BE:** biological efficiency; **BS:** beech sawdust; **DMSO:** dimethyl sulfoxide; **HPLC:** high pressure liquid chromatography; **OOPC:** olive oil press cakes; **SEM:** standard error of the mean; **TLC:** thin layer chromatography; **WB:** wheat bran

I. INTRODUCTION

Shiitake culinary-medicinal mushroom, *Lentinus edodes* (Berk.) Singer (Marasmiaceae, Agaricales s.l., higher Basidiomycetes), is a popular species especially used in Asia. It is known for its nutritional value as well as medicinal potential, which is primarily linked to polysaccharides (beta glucans). One of these polysaccharides is lentinan, which is reported to possess antitumor, antibacterial, antiviral, anticoagulatory, as well as wound-healing activities.[1] It is also registered as a medicine in some parts of the world.[2,3] Beta glucans possess the ability to link themselves to immunoglobulins in the blood serum and to stimulate the immune system.[4] *In vitro* and clinical investigations have revealed that the consumption of fruiting bodies of higher Basidiomycetes can prevent oncogenesis, increase the level of activated macrophages, and lead to shrinkage and disappearance of tumors.[5]

Lentinus edodes have been cultivated on logs since 700 years ago.[6] Today, they are cultivated worldwide, most often using sawdust[7,8,9] and straw-based substrates. Some authors report successful cultivation on wood chips of *Quercus*, *Lithocarpus*, and *Acer species,*[10] different grass species,[11] sunflower seed husks, hazelnut husks,[12] wheat straw, corn, flax, cacao, coffee, waste products of wine production,[2,13–15] and other organic materials. It has been shown that lipids, such as oleic and palmitic acids contained in olive oil press cakes (OOPC),

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TABLE 1. Substrate Mixtures Used for *Lentinus edodes* Cultivation

Wheat bran (%)	Olive oil press cakes (%)	Calcium sulfate hemihydrate—gypsum (%)	Beech sawdust (%)
18	80	2	0
18	60	2	20
18	40	2	40
18	20	2	60
18	0	2	80

stimulate the growth of *L. edodes* mycelium.[16] They also appear to be a suitable source of nutrients for fruiting bodies cultivation. OOPC, being the major waste product in olive oil production, have been successfully used as a cultivating substrate component for different mushroom species, such as *Grifola frondosa*,[17] *Pleurotus pulmonarius*,[18] *P. ostreatus*,[19] and *Ganoderma lucidum*. In our study, we have cultivated *L. edodes* on substrates supplemented with different proportions of OOPC. We studied the effect of OOPC on fruiting bodies production and on proliferation of acute lymphoblastic leukemia cells (CCRF-CEM).

II. MATERIALS AND METHODS

A. *Lentinus edodes* Fruiting Bodies Cultivation

L. edodes strain No. 4080 was obtained from the culture collection of Zavod za naravoslovje, Ljubljana, Slovenia. Cultures were cultivated on potato dextrose agar (Difco, Franklin Lakes, NJ, USA) at 24°C.

Substrates were composed of different portions of olive oil press cakes (OOPC), wheat bran (WB), beech sawdust (BS), and calcium sulfate hemihydrates (gypsum). The components were mixed and their water content adjusted to 65% (Table 1). Subsequently, the substrate (3.5 kg) was filled into polypropylene bags (Combiness, Nazareth, Belgium) and sterilized for 5 h at 121°C. Four replicates were prepared for each substrate mixture.

After sterilization and cooling, substrates were inoculated with mycelium, mixed, and incubated at 24 ± 1°C in a dark environment. When the surface of the overgrown substrate became dark brown in color, bags were completely removed from the substrate and moved into cultivation rooms at 17 ± 2°C, 10 h of light daily, and 80% relative humidity. Fruiting bodies were harvested after they were fully matured, cut into small pieces, and dried at 60°C. Biological efficiency [BE = (fresh fruiting bodies weight/weight of fresh substrate) × 100] was calculated. Five replicates were prepared for each substrate mixture.

B. Crude Polysaccharides Extraction

Approximately 7 g of dry, ground fruiting bodies were used for extractions, which were performed in an accelerated solvent extractor. ASE 100 (Dionex), at 121°C and 100 bar, using water as a solvent with extraction parameters as follows: static time 5 min, flush volume 40%, purge time 180 sec, number of static cycles 3. Extracts were dried under reduced pressure with a rotary evaporator at 60°C, re-dissolved in water, precipitated with 90% ethanol at 4°C overnight, and centrifuged for 5 min at 3000 rpm. Precipitated crude polysaccharides were dialyzed against distilled water and further lyophilized.

C. Cell Culture

CCRF-CEM were cultured in RPMI 1640 medium (Sigma, St. Louis, MO, USA), 2 mM L-glutamine (Sigma), 10% heat-inactivated fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria) and 1% Pen/Strep (PAA Laboratories). Cells were kept in a humidified 5% CO_2 atmosphere at 37°C. At 90% confluence cells were passaged. Cells were counted using a Casy Cell Counter (Innovatis, Reutlingen, Germany). Only cell suspensions with viability above 90% were used for the XTT viability assay.

D. XTT Viability Assay

Cell proliferation kit II (XTT) (Cat. No. 11 465 015 001) was obtained from Roche Diagnostics (Mannheim, Germany). Aliquots (100 µL) of 1×10^5 cells/mL were seeded in 96-well plates (flat bottom) and extracts were added immediately. All cells were incubated with the extracts for 72 h before

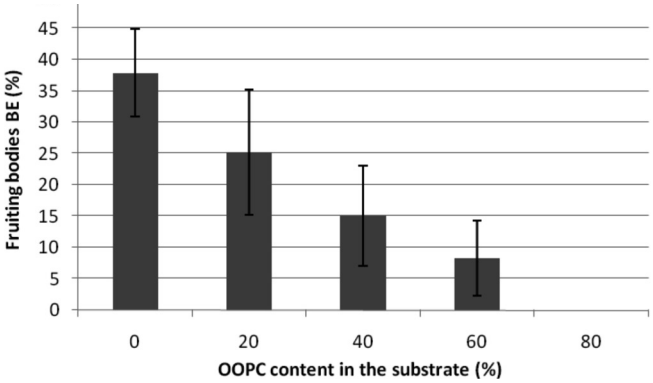

FIGURE 1. Biological efficiency (BE) of *Lentinus edodes* fruiting bodies cultivation on substrates with different olive oil press cakes (OOPC) content. *L. edodes* fruiting bodies were cultivated on substrates containing different portions of OOPC and harvested when mature. Their BE was expressed as a percentage.

XTT solution was added. XTT solution consisted of a XTT labeling reagent (5 mL) and an electron-coupling reagent (100 µL). XTT is a yellow tetrazolium salt (sodium 3′-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro) benzene sulfonic acid hydrate) and cleaved by metabolic active cells into an orange formazan dye. This color change occurs only in viable cells and can be directly quantified using a scanning multiwell spectrophotometer.[20] Numbers of viable cells were determined with the following formula and expressed as percentage of control: (absorbance of treated cells/absorbance of untreated cells) × 100.[21]

As control, cells treated with 0.5% DMSO (vehicle) were used. Six replicates were conducted for each sample and mean ± SEM calculated. Crude polysaccarides were dissolved in DMSO at a concentration of 2 mg/mL and diluted with sterile water.

III. RESULTS

A. Biological Efficiency (BE) of *Lentinus edodes* Fruiting Bodies Cultivation

Negative influence of OOPC on the growth and maturation of *L. edodes* mycelia was noticed. Mycelium tends to grow slower and in some cases does not grow at all on substrates containing 80% OOPC. Overgrown substrates containing higher proportions of OOPC tend to mature (change color) later than substrates with lower OOPC content. Highest biological efficiency (BE) (38%) of *L. edodes* fruiting bodies occurred on substrates composed of 80% BS, 2% calcium sulfate hemihydrate, and 18% WB. BE of fruiting bodies decreased in correlation to increasing portions of OOPC in the growing substrates, indicating that presence of OOPC inside the substrate inhibits fruiting bodies formation. When substrate contained 80% OOPC, fruiting bodies ceased formation completely (Fig. 1).

Substrates containing OOPC absorbed less water, were of softer consistency, and tended to fall apart after soaking. They became infected more rapidly with unfavorable molds.

B. Proliferation of CCRF-CEM Cells Treated with *Lentinus edodes* Polysaccharides

Investigation of the cytotoxicity of the crude polysaccharides against CCRF-CEM leukemia cells revealed that the OOPC content is negatively correlated with the cytotoxicity of the crude polysaccharides (Fig. 2). While the cytotoxicity was very high (below 10% of control cells) when *L. edodes* was cultivated without OOPC in the substrate, it was lost in the presence of OOPC.

IV. DISCUSSION

This study revealed that OOPC have a negative influence on mycelia growth and fruiting bodies production. The reason could be polyphenolic compounds in OOPC.[22] Polyphenols could also be the reason for decreased fruiting bodies yields with OOPC-containing substrates. Normally, *L. edodes* maturing mycelium turns brown in color. This process is hindered when higher proportions of OOPC are used in the growth substrate.

Besides polyphenolic compounds inside OOPC, the low porosity of OOPC and consequently lower substrate aeration could also be the reason for lowered yields of fruiting bodies. It has been

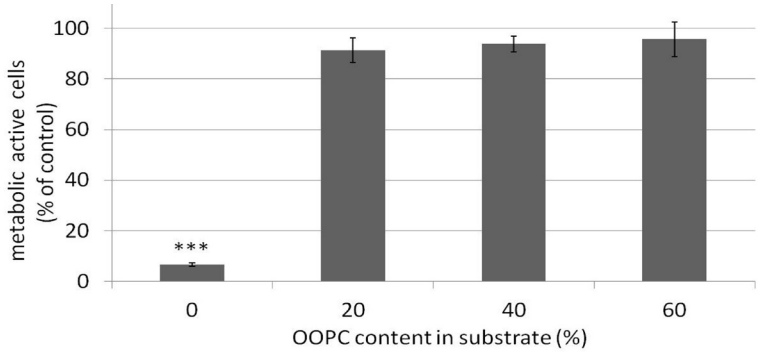

FIGURE 2. Influence of olive oil press cakes content in cultivation substrate on CCRF-CEM cells treated with crude polysaccharides extracted (final concentration: 10 µg/mL) from *Lentinus edodes* fruiting bodies. XTT viability assay, incubation time 72 h, n = 6, mean ± SEM, ****P* < 0.001 (compared to vehicle treated cells). Aliquots (100 µL) of 1 × 10^5 acute lymphoblastic leukemia (CCRF-CEM) cells/mL were seeded in 96-well plates (flat bottom) and extracts were added immediately. All cells were incubated with the extracts for 72 h before XTT solution was added. Numbers of viable cells were determined with the following formula and expressed as percentage of control: (absorbance of treated cells/absorbance of untreated cells) × 100.

reported previously that aeration greatly influences mycelial overgrowth and *L. edodes* fruiting bodies production.[23–25] On the other hand, specific strain characteristics and inability to utilize OOPC and its nutrients, or to withstand higher contents of polyphenolic compounds in the substrate could be the reason for lowered yields in the presence of OOPC. Strain characteristics tend to strongly influence mycelial growth as well as quantity and quality of produced fruiting bodies.[26,27] Reduction of fruiting bodies yields on substrates containing higher proportions of OOPC is in accordance with the findings of other authors, who tested OOPC as a substrate component for cultivation of other mushroom species, such as *Grifola frondosa*,[17] *Pleurotus pulmonarius*,[18] *P. ostreatus*,[19] and *Ganoderma lucidum*. For a better understanding of the influence the substrate has on fruiting bodies yield and mycelium growth rate, different fungal strains should be used. Also strain adaptation techniques could be used, because they could have a strong influence on fruiting bodies yield.

The second part of the study investigated the effect of the crude polysaccharides on growth and viability of CCRF-CEM leukemia cells. It has been reported that *L. edodes* possesses antitumor activity.[1] Although there is evidence that this effect is mediated via an immunomodulating action,[28,29] it has also been shown that polymers derived from *L. edodes* have a direct cytotoxic effect on several cancer cell lines.[30,31] Therefore, we studied *in vitro* cytotoxicity in CCRF-CEM leukemia cells. It could be shown that OOPC had not only a negative effect on mycelia growth and fruiting bodies production but also on the cytotoxicity of the polysaccharide fraction. Cytotoxic effects were observed only in crude polysaccharides obtained from fruiting bodies that were grown without OOPC. Even with 20% OOPC in the substrate, no cytotoxicity could be detected. It was demonstrated by other authors that the structure of *L. edodes* polysaccharide is the same on substrates containing olive oil waste water in comparison to control substrates.[32] From this we can speculate that pharmacological activity may be the same with both types of polysaccharides and further that crude polysaccharides used in our experiments were not purified enough and could also contain other substances responsible for characteristic activity against CCRF-CEM cells. For identification of the active principle, further investigations are necessary. Each active crude polysaccharide has to be activity-guided fractionated to yield the active compounds. In addition, the development of chemical analysis for characterization (TLC, HPLC) is very important for finding more information on the chemical structures in the mixture.

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