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ANTIMICROBIAL ACTIVITY OF CRUDE ETHANOLIC EXTRACTS FROM SOME MEDICINAL MUSHROOMS

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ABSTRACT

In this paper the antimicrobial activity of 1 year old crude ethanolic extracts obtained from *Cordyceps sinesis*, *Laricifomes officinalis*, *Oudemansiella mucida* and *Coprinus comatus* were investigated. The antimicrobial activities of extracts against two Gram-positive bacteria (*Bacillus thuringiensis*, *Staphylococcus aureus*) and two Gram-negative bacteria (*Klebsiella pneumoniae*, *Enterobacter aerogenes*) were determined by disk diffusion and microbroth dilution method according by EUCAST in 96-well microplates. Microorganisms were obtained from Czech Collection of Microorganisms. Absorbance after and before the experiment were substracted, converted to binary system and obtained values to Probit analysis were used. Not all macromycetes ethanolic extracts showed antimicrobial activity against tested bacteria. Antimicrobial activity determined by MIC methodology showed extracts from *Oudemansiella mucida*, *Cordyceps sinesis*, *Coprinus comatus* in the tested range. Conversely, the best antimicrobial activity tested by disc diffusion methods showed extract from *Laricifomes officinalis*. Equally, more better studying of antimicrobial activity in these mushrooms will needed.

Keywords: Antimicrobial activity, macromycetes ethanolic extracts, MIC, edible mushrooms

INTRODUCTION

Nature is a very good source of many medical compounds for thousands of years. In the last decades problem with antibiotic resistant bacteria has emerged. Bacterial pathogens have evolved numerous defense mechanisms against antimicrobial agents, and nowadays, the need to discover new and more potent of these agents as accessories or alternatives to antibiotic therapy is stronger (**Butler *et al*., 2004; Lam *et al*., 2007**). Macromycetes as higher fungi are rich sources of biologically active compounds with an enormous variety of chemical structures. Therefore, mushrooms could be useful in the search of new potent antimicrobial agents (**Alves *et al*., 2012**). Mushrooms need antibacterial compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activities could be isolated from many mushrooms and that they could be of benefit for human (**Lindequist *et al*., 1990**). There is many different studies about antimicrobial activity of different types of fungal extracts from India (**Seena *et al*., 2003; Quereshi *et al*., 2010**) and China (**Gao *et al*., 2005**). In this country fungi medicine has tradition for many years ago. For example, *Ganoderma lucidum* is a one of the most famous traditional medicinal fungi, being used as functional food and in preventive medicines, mostly in the form of extracts with an annual global market (**Sullivan *et al*., 2005; Pala *et al*., 2011**). But only compounds from microscopic fungi are on the market as antibiotics till now (**Lindequist *et al*., 2005**).
The present work is focus to antimicrobial activity of 1 year old medicinal mushrooms extracts isolated from *Cordyceps sinesis*, *Laricifomes officinalis*, *Oudemansiella mucida* and *Coprinus comatus* against some selected Gram-positive and Gram-negative bacteria.

MATERIALS AND METHODS

Fungi materials

The fungi materials used in this experiment consist from SSF (Solid State Fermentation) of *Cordyceps sinesis* and fruiting bodies (basidiocarps) of *Laricifomes officinalis*, *Oudemansiella mucida* and *Coprinus comatus*. Dried fungi SSF and fruiting bodies were obtained from Mykoforest company, Slovakia. Selected fungi were identified by Martin Rajtar (Mykoforest, Slovakia). Fungi were dried at the room temperature in the dark. More detailed information are showed in Table 1.

Table 1 Additional information about tested fungi

Orig. Latin title	Fungal part	Yield*	Origin	Dissolving time	Extracted by
Cordyceps sinesis	SSF	0.1006	Mykoforest	1 year at room temperature	Vacuum evaporator from methanol at room temperature at -800 mbar
Laricifomes officinalis	fruiting body	1.7269	Mykoforest		
Oudemansiella mucida	fruiting body	1.0893	Mykoforest		
Coprinus comatus	fruiting body	0.3379	Mykoforest		

* Obtained yield of evaporated ethanolic extracts in g, SSF – Solid state fermentation

Test microorganisms

Four strains of microorganisms were tested in this research. Two Gram-negative bacteria include *Klebsiella pneumoniae* CCM 2318, *Enterobacter aerogenes* CCM 2531, two Gram-positive bacteria include *Staphylococcus aureus* subsp. *aureus* ser. a5 CCM 2461, *Bacillus thiringiensis* CCM 19. All tested strains were collected from the Czech Collection of Microorganisms. The bacterial suspensions were cultured in the nutrient broth (Imuna, Slovakia) at 37 °C, expect *Bacillus thiringiensis* which was cultivated at 30°C.

Preparation of fungal extracts

After drying, the fungal materials were crushed, weighed out to 10g and soaked separately in 100 mL of ethanol p.a. (99,5 %, Sigma, Germany) during 1 year at room temperature in the dark. Why one year? The main reason was determining of antimicrobial activity after the long time of storage. Exposure to sunlight was avoided to prevent the degradation of active components. Then, ethanolic fungal extracts were subjected to evaporation under reduced pressure at 40 °C in order to remove the ethanol (Stuart RE300DB rotary evaporator, Bibby scientific limited, UK, and vacuum pump KNF N838.1.2KT.45.18, KNF, Germany). For the antimicrobial assay, the crude fungal extracts were dissolved in dimethyl sulfoxid (DMSO) (Penta, Czech Republic) to equal 102.4 mg/mL as stock solution. Stock solutions of fungal extracts were stored at -16 °C in refrigerator until use.

Preparation of discs and disc diffusion method

Synchronously with evaporation of ethanol from mushroom extract blank discs (Oxoid, UK) were added to extracts and impregnated with extracts. Discs stayed in extracts until evaporated completely. Obtained impregnated discs served as pre-determination experiment for detection of antimicrobial activity. Concentration of extracts in discs were unknown. Impregnated discs were used for disc diffusion methodology, which was done on Mueller-Hinton agar (Biolife, Italy) at 37 °C for three bacteria, expect *Bacillus thuringiensis* (30°C) during 16-20 hours. Bacterial inoculum in physiological solution at the final density of 0,5 McF° was spread out on the agar surface evenly. Impregnated discs were stacked on to the agar surface evenly with adequate spacing. Inhibition zones were read in millimeter.

Antimicrobial assay

The minimum inhibitory concentration (MIC) is the lowest concentration of the sample that will inhibit the visible growth of microorganisms. Fungal extracts dissolved in DMSO were prepared to a final concentration of 4096 µg/mL. Minimum inhibitory concentrations (MICs) were determined by the microbroth dilution method according to the Clinical and Laboratory Standards Institute recommendation (**CLSI, 2009**) in Mueller Hinton broth (Biolife, Italy) for bacteria. Briefly, the DMSO fungal extracts solutions were prepared as serial two-fold dilutions, in order to obtain a final concentration ranging between 2 – 4096 µg/mL. Each well was then inoculated with microbial suspension at the final density of 0.5 McF°. After incubation at 37 °C for three bacteria and 30 °C for *Bacillus thuringirnsis* during 16-20 hours. The inhibition of microbial growth was evaluated by measuring the well absorbance at 590 nm in an absorbance microplate reader Biotek EL808 with shaker (Biotek Instruments, USA). The 96 micro-well plates were measured before and after experiment. Differences between both measurements were evaluated as growth. Measurement error was established for 0.05 values from absorbance. Wells without fungal extracts were used as positive controls of growth. Pure DMSO was used as negative control. This experiment was done in eight-replicates for a higher accuracy of the minimum inhibitory concentrations of used fungal extracts.

Statistical analysis

Using obtained absorbance before and after the analysis, we were able to express the differences in absorbance between the measurements as a set of binary values. These values were assigned to exact concentrations. The following formula was created for this specific experiment: value 1 (inhibitory effect) was assigned to absorbance values lower than 0.05, while value 0 (no effect or stimulant effect) was assigned to absorbance values higher than 0.05. For this statistical evaluation Probit analysis in Statgraphic software was used.

RESULTS AND DISCUSSION

Disk diffusion method

Results from disc diffusion tests showed antimicrobial activity in the case of extracts from *Laricifomes officilnalis* and *Oudemansiella mucida*. Some authors (**Anke *et al*., 1979; Anke *et al*., 1990; Florianowicz *et al*., 1999**) presented results that extract from *Oudemansiella mucida* inhibited only fungal cells. In their study, scientists determined that main compounds strobilurins and oudemansins inhibited growth of yeasts like *Candida albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis*. In our study we determined inhibition effect against *Staphylococcus aureus* too, which is showed in Figure 1 D, sample 9. Inhibition zone (12 mm) around *Oudemansiella* mucida extract was formed in the case of *Staphylococcus aureus* only. Extracts from *Laricifomes officinalis* had inhibitory activity against the all used bacterial strains in this experiment. Inhibition zones around the *Laricifomes officinalis* extract were formed in the case of *Staphylococcus aureus* (27 mm), *Bacillus thuringiensis* (13 mm), *Klebsiella pneumoniae* (20 mm) and *Enterobacter aerogenes* (20 mm). Other used macromycetes extracts in this study didn't showed antimicrobial activity. In the other side authors like **Demir and Yamac, 2008** tested *Coprinus comatus* basidiocarp extract dissolved in different solutions and they determined its antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecium*, *Proteus vulgaris* and *Candida glabrata*. They tested submerged mycelium and some exopolysacharides from *Coprinus comatus*, but they didn't determined so extensive activity like in previous test with basidiocarps. Inhibition zones are showed on the Figure 1.

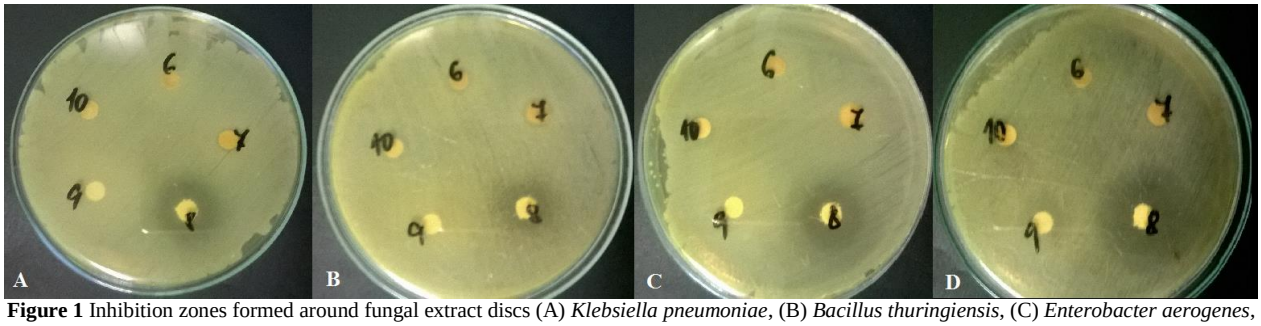

Figure 1 Inhibition zones formed around fungal extract discs (A) *Klebsiella pneumoniae*, (B) *Bacillus thuringiensis*, (C) *Enterobacter aerogenes*, (D) *Staphylococcus aureus*, (6) *Coprinus comatus*, (7) *Cordyceps sinensis*, (8) *Laricifomes officinalis*, (9) *Oudemansiella mucida* and (10) not presented extract in this study

Minimal inhibition concentration

The antimicrobial activity (expressed as µg/mL) of four ethanolic fungal extracts from *Cordyceps sinesis*, *Laricifomes officinalis*, *Oudemansiella mucida* and *Coprinus comatus* against four strains of bacteria are summarized in Table 2. The most effective was tested fungal extract from *Coprinus comatus* against *Enterobacter aerogenes* with a MIC 50 value 2048 ug/mL. Also, *Oudemansiella mucida* extract showed inhibitory activity against tested bacteria. Extract from *Laricifomes officinalis* inhibited the growth of all bacterial strains tested by disk diffusion method, but minimal inhibition concentration method didn't showed any inhibition activity in tested concentration range. Some studies about antimicrobial activity of *Cordyceps sinensis* determined that main compounds cordycepin had effects against *Clostridium perfringens*, *C. paraputrificum*, *Bifidobacterium* spp. and *Lactobacillus* spp. (**Kniefel *et al*., 1977; Ahn *et al*., 2000**). There are exist many studies about antimicrobial activity of higher fungi (**Yoon *et al*., 1994; Rosa *et al*., 2003; Poucheret *et al*., 2006; Molitoris 1994; Lindequist *et al*., 2005**) because is known that mushrooms need antibacterial and antifungal compounds to survive in their natural environment. It is therefore not surprising that antimicrobial compounds with more or less strong activity could be isolated from many mushrooms and that they could be of benefit for human (**Lindequist *et al*., 1990**). But only compounds from microscopic fungi are on the market and antibiotics till now (**Lindequist *et al*., 2005**).

Table 2 The minimum inhibitory concentration (MIC) of ethanolic fungal extracts on four test bacteria

Tested bacteria	Antimicrobial activity of macromycetes fungal extract (ug/mL)							
	Cordyceps sinensis		***Laricifomes officinalis***		***Oudemensiella mucida***		***Coprinus comatus***	
	MIC 50	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**	**MIC 50**	**MIC 90**
Gram-positive bacteria								
Bacillus thuringiensis CCM	> 4096	> 4096	> 4096	> 4096	3064,03	3257,04	> 4096	> 4096
Staphylococcus aureus CCM	4096	> 4096	> 4096	> 4096	4096	> 4096	3064,03	3257,04
Gram-negative bacteria								
Klebsiella pneumoniae CCM	4096	> 4096	> 4096	> 4096	3064,03	3257,04	3064,03	3257,04
Enterobacter aerogenes CCM	3064,03	3257,04	> 4096	> 4096	3064,03	3257,04	2048	2285,99

Legend: Abr. – abbreviations,

CONCLUSIONS

In conclusion, we can state that the ethanolic fungal extracts of all fungi (*Cordyceps sinesis*, *Laricifomes officinalis*, *Oudemansiella mucida* and *Coprinus comatus*) showed antimicrobial activity in high concentration. Very interesting in this experiment was that extract from *Laricifomes officinalis* showed strongest antimicrobial activity detected by disk diffusion methodology and not by MIC methodology. We think that more studies and experiments and more range of concentration of fungal extracts are needed for better information about antimicrobial activity of macro fungal extracts.

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