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ANTIMICROBIAL ACTIVITY OF ETHANOLIC EXTRACTS MADE OF MUSHROOM MYCELIA DEVELOPED IN SUBMERGED CULTURE

Mihai Bogdan NICOLCIOIU, Gabriela POPA, Florentina MATEI

University of Agronomical Sciences and Veterinary Medicine of Bucharest,
Faculty of Biotechnologies, 59 Mărăşti Blvd., District 1, 011464, Bucharest, Romania

Corresponding author email: mihaibogdan_90@yahoo.com

Abstract

The ethanolic extracts of dried biomass made of mushroom mycelium produced in the submerged cultures of eight edible/medicinal macromycete species Ganoderma applanatum, Ganoderma lucidum, Laetiporus sulphureus, Flammulina velutipes, Trametes versicolor, Hericium coralloides, Pleurotus eryngii 2600 and Agaricus campestris were evaluated for their antimicrobial activities. Pathogenic tested microorganisms were represented by five bacteria and two yeasts B.subtilis subsp. spizizenii ATCC 6633, Staphilococcus aureus ATCC6538, Pseudomonas aeruginosa ATCC9027, Escherichia coli ATCC 8739, E.faecalis ATCC 29212, Candida albicans ATCC 10231 and Candida parapsilosis CBS604. The mushroom biomass was obtained from segments detached from the mycelium culture grown on solid culture medium (PDA or MEA) placed in Erlenmeyer flasks with a liquid culture medium containing 2% malt extract (ME). After inoculation, the probes were incubated at the temperature of 25°C for 21 days. The obtained biomass was filtered and dried at the temperature of 70 °C. The ethanolic extracts were prepared by adding 1 ml of 70% ethyl alcohol to 0.2 g of dried fungal biomass. Antimicrobial activities of the mushroom biomass extracts were evaluated by agar disk diffusion method. The results showed that G. applanatum, L. sulphureus, F. velutipes, T. versicolor, H.coralloides and A. campestris extracts had significant inhibitory activities especially against B. subtilis subsp. spizizenii ATCC 6633 bacterium while G. lucidum and Pleurotus eryngii 2600 extracts had no antimicrobial activity against any pathogenic microorganisms tested in this work. Further investigations will be conducted regarding the antimicrobial activity dependence on the fungal morphological part used in the extract(mycelium/fruiting body) and on the solvent type used for extracts' preparation.

Key words*: Antimicrobial activity, ethanolic extracts, mushroom biomass, pathogenic microorganisms.*

INTRODUCTION

The antibiotics represented a revolution in the field of medicinal sciences. The discovery and use of antibiotics during the 20th century have strongly decreased morbidity and mortality caused by bacterial infections (Chopra et al., 1997). Mathur and Singh, 2005 consider that the beginning of treating the bacterial infections using antimicrobial agents can be associated with the emerging of antimicrobial resistance in bacteria that cause diseases. This was a disadvantage because the antibiotics had to promise so much. Another study belonging to Lowy, F.D., 2003 show that in the early 1970's the idea that the large range of efficient antimicrobial agents can treat all bacterial infections faded among the physicians. This pessimistic attitude was adopted because the pathogens resistance to multiple antibiotics started to be present in the case of *S. aureus*, *P. aeruginosa* and other bacteria (Lowy, F.D., 2003). Fischbach and Walsh, 2009 show that *S. aureus* bacteria is a violent pathogen. These authors sustain that the methicillin-resistant *S.aureus* (MRSA) causes a big mortality rate in the United States and also this bacteria is capable of enormous health care costs per year (Fischbach and Walsh, 2009). In the opinion of these authors the probability that the same deadly as MRSA vancomycin-resistant *S.aureus* (VRSA) can become a new powerful pathogen in hospitals rises depending on the MRSA increasing prevalence (Fischbach and Walsh, 2009; Linda M. Weigel et al.,2003). Fischbach and Walsh, 2009 and Falagas et al.,2005 also state that there are other pathogenic bacteria having smaller prevalence compared to methicillin-resistant *S.aureus* but being also dangerous such as: *E. coli* and *P. aeruginosa* which are resistant to penicillins, cephalosporins, carbapenems, monobactams,

quinolones, aminoglycosides, tetracyclines and polymyxins. The fungal pathogens posessing resistance to antifungal agents are also important. Different authors indicate that *C. parapsilosis* is placed on the second place after *C. albicans* in the blood samples (Trofa et al., 2008; Almirante et al.,2006; Brito et al., 2006; Colombo et al.,2007; Colombo et al.,2006; Costa-de-Oliveira et al.,2008; Fridkin et al., 2006; Krčméry et al.,2006; Messer et al.,2006; Pfaller et al.,2001; Pfaller et al.,1998; Rodero et al.,2004). *C. albicans* is a pathogenic yeast with resistance to antifungals such as miconazole and ketoconazole according to some authors (Casalinuovo et al., 2004). According to some authors the thoughtlessly use of antibiotics led to development of resitant pathogenic microorganisms (Andrade et al.,2006; Alves et al., 2014). Considering all the above studies about pathogenic bacteria and yeasts much hope is put on new antimicrobial agents. The macroscopic fungi known also as basidiomycetes represent potent sources on the fight against various pathogens antibiotic resistance. Alves et al., 2014 specify the possibility that mushroom extracts can be used both to lower the therapeutic doses of standard antibiotics and reduce microorganism's resistance to these drugs. The studies of Deepalakshmi and Mirunalini (2014) and Iwalokun et al. (2007) showed that oil extracted from *P. ostreatus* using petroleum ether and acetone inhibited the growth of Gram-positive and Gram-negative bacteria such as *E. coli*, *P. aeruginosa*, *B. subtilis*, and *S. aureus*. Moreover, methanol and chloroform extracts of *P. ostreatus* were found to have antimicrobial activity against Gram-positive bacteria (Karaman et al., 2010; Deepalakshmi and Mirunalini, 2014). Other studies revealed the antimicrobial activity of *G. lucidum* extracts against *E. coli*, *S. aureus* and *P. aeruginosa* attributed to bacteriolytic enzyme, lyzozyme and acid protease. (Quereshi et al., 2010; Klaus and Miomir, 2007). Lindequist et al., 2005 and Smania et al., 1999 show that *G. applanatum* contains two steroids 5α-ergosta-7,22-dien-3β-ol and 5,8-epidoxy-5α,8α-ergosta-6,22-dien-3β-ol that have weak antimicrobial activity against both Gram-negative and Gram-positive pathogenic bacteria *E. coli*, *P. aeruginosa* and *S. aureus*. The study of Poucheret et al., 2006 and Wasser and Weis, 1999 also bring information about the antimicrobial activity of *G. lucidum* and *G. applanatum* mushrooms. On the other hand some authors consider that extracts of *Ganoderma* mushroom cannot be used as antibiotics because further research is needed (Gao et al., 2005). In the last years, more mushroom species are shown to have antimicrobial activity against pathogenic microorganisms. Mushrooms of *Trametes* genus contain coriolin which inhibit Gram-positive bacteria and *A. campestris* has a compound named campestrin which inhibit both Gram-positive and Gram-negative bacteria (Wasser and Weis, 1999). Other mushroom extracts, including *L. sulphureus* (Turkoglu et al., 2007) and *F. velutipes* (Poucheret et al., 2006) have already demonstrated their antimicrobial activity. Poucheret et al., 2006 states that *F. velutipes* possess antifungal activity. *L. sulphureus* was tested by Turkoglu et al., 2007 and it proved to have good antibacterial activity especially against Gram-positive bacteria such as *B. subtilis* and the ethanol extract had very good antifungal activity on *C. albicans*. In this context, the aim of our studies is to determine the antimicrobial activity of ethanolic extracts from the dry biomass (mycelia) of some mushroom species cultivated in submerged culture.

MATERIALS AND METHODS

Fungal material

The fungal material used in this experiment consisted of dry biomass of the mushroom species: *Ganoderma applanatum, Ganoderma lucidum, Laetiporus sulphureus, Flammulina velutipes, Trametes versicolor, Hericium coralloides, Pleurotus eryngii 2600* and *Agaricus campestris*. Fungal material was provided from the collection of Faculty of Biotechnology (UASVM, Bucharest).

Microbial material

The tested microbial material was represented by: *B. subtilis subsp. spizizenii* ATCC 6633, *Staphylococcus aureus* ATCC6538, *Pseudomonas aeruginosa* ATCC9027, *Escherichia coli* ATCC 8739, *Enterococcus faecalis* ATCC 29212, *Candida albicans* ATCC 10231 and *Candida parapsilosis* CBS604. The

pathogenic microorganisms were provided from the Institute of Biology, Bucharest.

Mushroom biomass

Segments of 10 x 10 mm were detached from the mycelium culture grown on solid culture medium (PDA or MEA) and placed in Erlenmeyer flasks with a liquid culture medium containing 2% malt extract (ME). After inoculation, the probes were incubated at the temperature of 25^{o}C under stirring conditions at 110 rpm for 21 days. After the incubation period, the obtained biomass was filtered and dried at the temperature of 70 ^{o}C for 3 hours.

Preparation of ethanolic extracts

Extracts preparation was performed by using 1 ml of 70 % ethyl alcohol added to 0.2 g of dried fungal biomass. The alcoholic solutions were kept for 24 hours at the room temperature (±25^{0}C) until use.

Determination of antimicrobial activity

Antimicrobial activities of the extracts were screened by the agar disk diffusion method. A volume of 1 ml from each bacterial and yeasts suspensions were inoculated in Petri dishes on Luria Broth and YPG media respectively. After removing the excess suspension, sterile filter paper discs (5 mm diameter) soaked in ethyl alcohol extracts were placed on the surface of the inoculated medium. At 24 hours after the incubation at 37^{o}C for bacteria and 30^{o}C for yeasts, occurrence of inhibition halos around each disk was observed. Ethanol (70%) was used as negative control.

RESULTS AND DISCUSSIONS

Biomass extracts obtained from mycelia developed in submerged culture were tested against the mentioned bacterial and fungal pathogenic strains. The results showed a microbial activity in the case of *L. sulphureus* (P1), *A. campestris* (P3), *F. velutipes* (P4), *G. applanatum* (P6), *T. versicolor* (P7) and *H. coralloides* (P8) extracts (Table 1). The data relating to the antimicrobial activities of extract samples is summarized in Table 1.

Table 1. The antimicrobial activity of fungal dry biomass extracts against pathogenic microorganisms

Variant	Pathogen microorganism						
	B. subtilis subsp. spizizenii ATCC 6633	*S. aureus* ATCC6538	*P. aeruginosa* ATCC9027	*E.coli* ATCC 8739	*E. faecalis* ATCC 29212	*C. albicans* ATCC 10231	*C. parapsilosis* CBS604
P1	++	+	-	++	+	-	-
P2	-	-	-	-	-	-	-
P3	+	+	+	-	-	-	-
P4	++++	+++	+	-	-	-	-
P5	-	-	-	-	-	-	-
P6	++	-	-	-	-	-	-
P7	+	-	+	+	-	-	-
P8	+	-	+	-	-	-	-
M	-	-	-	-	-	+	+

P1.L. sulphureus; P2. P. eryngii; P3. A. campestris; P4. F. velutipes; P5. G. lucidum; P6. G. applanatum; P7. Trametes versicolor; P8. H. coralloides; M=control (70% ethyl alcohol).

Activities were classified according to the diameter of the inhibition zones around the disks containing 10µl/disk extract or control: +, <10 mm; ++, 10–15 mm; +++, 15–20 mm, ++++, >20 mm; -, without activity.

Data included in Table 1 shows that most tested mushroom extracts have antimicrobial activity against *B. subtilis subsp. spizizenii*. It can be noted that in the case of *L. sulphureus* the extract has medium to low inhibition on *B. subtilis subsp. spizizenii*, *S. aureus*, *E. coli* and *E. faecalis*. In the case of *A. campestris* extract small inhibition zone was visible in the case of *P. aeruginosa*, *B. subtilis subsp. spizizenii* and *S. aureus*. *F.velutipes* extract had strongly inhibited the *B. subtilis* and *S. aureus* bacteria developing the largest inhibition halos. *G. applanatum* extract had moderate inhibitory effect against *B. subtilis subsp. spizizenii,* while *T. versicolor* and *H. coralloides* extracts had low inhibitory effect against *P. aeruginosa*. The extracts–pathogens inhibition effect is shown in Figure 1.

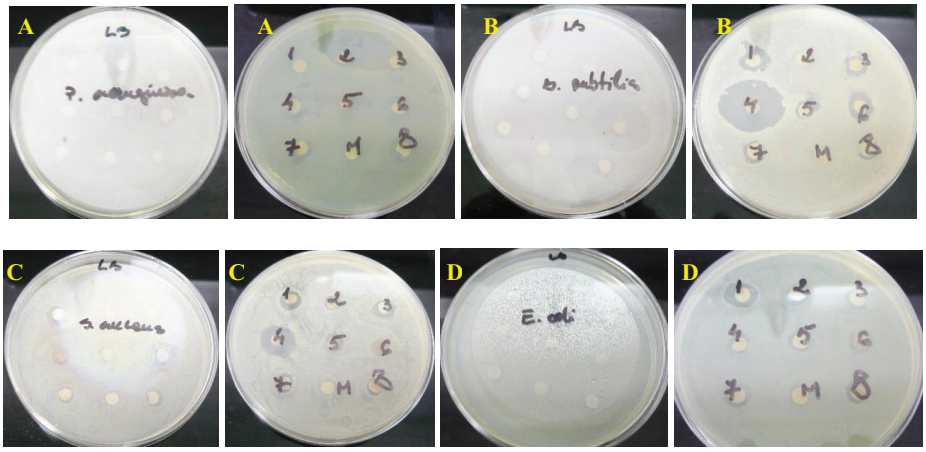

Figure 1. Antimicrobial activity of mushroom ethanolic extracts
A. P.aeruginosa; B. B.subtilis; C. S.aureus; D. E.coli
1. L. sulphureus; 2. P. eryngii; 3. A. campestris; 4. F. velutipes; 5. G. lucidum; 6. G. applanatum; 7. Trametes versicolor; 8. H. coralloides; M-martor (ethanol 70%)

The etahnolic extract of the *G. lucidum* and *P. eryngii* strains used in this study haven't shown any antimicrobial activity against the tested pathogen.

However, there are positive reports regarding the antimicrobial activity of *G. lucidum* mycelium tested by agar-well diffusion method.

For the preparation of dry biomass extracts methanol, acetone, chloroform and distilled water were used as solvents (Dijde et al., 2014; Kamble et al, 2011). Kamble et al, 2011 show that the extracts had good inhibitory effect at a concentration of 100 mg extract/ml distilled water on some pathogenic bacteria such as: *S. aureus*, *B. subtilis*, *E. coli*.

In the case of *P.eryngii* species the antimicrobial activity was tested on some pathogens such as: *S. aureus* COWAN 1, *E. coli ATCC 25922*, *C. albicans* FMC 17 by disk diffusion method with methanol extracts (Akyüz and Kirbag, 2009). Akyüz and Kirbag, 2009 show that the extracts of *P. eryngii* inhibited the growth of test microorganisms in various proportions. Meanwhile, in our study, none of the tested macromycete extracts showed any antimicrobial activity in interaction with pathogenic yeast species *C. albicans ATCC 10231* and *C. parapsilosis CBS604.* In these cases, the results of extracts–pathogens interactions were not conclusive because the ethanol used as control had inhibitory effects.

CONCLUSIONS

The results showed that *G. applanatum, L. sulphureus, F. velutipes, T. versicolor, H. coralloides and A. campestris* extracts had medium to high inhibitory activities especially against *B. subtilis subsp. spizizenii* bacterium. In our study, none of the tested mushroom extracts had any antimicrobial activity in interaction with pathogenic yeast species *C. albicans ATCC 10231* and *C. parapsilosis CBS604.* In this case, for better information about the mushroom antimicrobial activities more studies and experiments concerning the type of the fungal material (mycelium/fruiting body) and the solvents used for extracts preparation are needed.

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