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BACTERICIDAL ACTIVITES OF SELECTED MACROFUNGI EXTRACTS AGAINST *Staphylococcus aureus*

ABSTRACT: The increasing of the antibiotic resistance exhibited by pathogenic microorganisms has resulted in research directed toward evaluation of novel sources of antimicrobial compounds. Previous studies have indicated that macrofungi, as a specific response to the natural hostile environment, produce secondary metabolites with antimicrobial properties. In this study, antimicrobial activities of the extracts from six wild mushrooms: *Amanita echinocephala, Russula medulata, Cerena unicolor, Hericium erinaceus, Ishnoderma benzoinum* and *Laetiporus sulphureus* were evaluated against Gram-positive bacterium *Staphylococcus aureus*. The antimicrobial potential of the methanolic mushroom extracts was investigated by the microdilution method. Antimicrobial activity was observed in all species included in the study. All the extracts that demonstrated inhibitory activities were further tested for bactericidal activity and minimum bactericidal concentration (MBC) values were determined. The tested microorganism was most sensitive to the examined extracts from the polypore fungi *C. unicolor* and *H. erinaceus*. The highest bactericidal activity was obtained in the extracts from the species *C. unicolor* (MBC=1.563 mg/mL). The experimental results revealed that the methanolic extract of *C. unicolor* possessed significant bactericidal activity. The findings suggest the potential use of this wild mushroom as antimicrobial agent.

KEYWORDS: mushroom, antimicrobial activity, microdilution method, minimum bactericidal concentration (MBC)

INTRODUCTION

The emergence of multiple antibiotics resistance in bacterial pathogens is a common global problem posing enormous public health concerns in the past four decades. *Staphylococcus aureus* is the pathogen of great interest because of

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its virulence, ability to cause an array of life threating infections and capacity to adapt to environmental conditions (Lowy, 2003). The non-availability and high cost of new antibiotic generation with limited effectiveness has led to the search of more effective antimicrobial agents among resources of natural origin, with the aim to discover a source for production of new antimicrobial drugs.

Higher fungi or macrofungi are characterized by the production of macroscopic fruiting bodies, which have long been appreciated not only for the unique taste, but also for their nutritional value (Kalač, 2012). In addition, higher fungi synthesize a multitude of metabolites that have different roles in the fungal survival. Studies have displayed that some of these metabolites have antibacterial and antifungal properties (Rai *et al.* 2005; Stamets, 2002), while a higher antimicrobial activity of mushroom extracts was observed against Gram-positive bacteria (Yamac & Bilgili, 2006; Nikolovska Nedelkoska *et al.* 2013). Considering that fungi and humans share common microbial pathogens (e.g. *Escherichia coli*, *S. aureus*, and *Pseudomonas areuginosa*), antimicrobial compounds that are produced by fungi could be of benefit for humans. Several studies have shown that mushroom extracts and isolates demonstrate promising antimicrobial properties which can be employed to combat several diseases caused by pathogenic bacteria including drug-resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* (Ameri *et al.* 2011; Alves *et al.* 2012a; Karaman *et al.* 2012).

Therefore, considering the previous reports on the antimicrobial activity of macrofungi and the constant need for the development of new antimicrobial agents, this study aimed to examine the antimicrobial potential of the methanolic extracts from selected macromycetes: *Amanita echinocephala, Russula medulata, Cerena unicolor, Hericium erinaceus, Ishnoderma benzoinum* and *Laetiporus sulphureus* against Gram-positive bacterium *Staphylococcus aureus.*

MATERIAL AND METHODS

Fruiting body selection

Samples of the wild macromycetes *Amanita echinocephala, Russula medulata, Cerena unicolor, Hericium erinaceus, Ishnoderma benzoinum* and *Laetiporus sulphureus* were collected from different locations and habitats in the Republic of Macedonia. Geographical location and natural habitat of the mushroom specimens are shown in Table 1. Taxonomic identification was made in the Mycological Laboratory at the Institute of Biology, Faculty of Natural Sciences and Mathematics in Skopje, by implementing standard methods of microscopic and chemical techniques (coloring of fruit bodies and spores), as well as using appropriate literature. The representative voucher specimens were deposited at the Macedonian Collection of Fungi (MCF) of the Institute of Biology (Table 1).

Table 1. Geographical location and natural habitat of the mushroom species studied for antimicrobial potential

Species	Habitat	Geographical location	Collection number
Amanita echinocephala	mycorrhizal (*on ground in park*)	Botanical garden, Skopje	MAK 10/13309
Russula medulata	mycorrhizal (*on ground in park*)	Gazi Baba, Skopje	MAK 10/13305
Cerena unicolor	saprotrophic (*on living beech trunks in conifer forest*)	Suva Gora Mt.	MAK 11/13368
Hericium erinaceus	saprotrophic (*on living oak trunks in deciduous forest*)	Sk. Crna Gora Mt.	MAK 11/13360
Ishnoderma benzoinum	saprotrophic (*on stump of pine trees*)	Suva Gora Mt.	MAK 11/13252
Laetiporus sulphureus	parasitic (*on living black locust trunks*)	Kozle, Skopje	MAK 11/13361

Preparation of methanolic extracts of mushrooms

The fruiting bodies were cleaned to remove any residual compost/soil and subsequently air-dried in the oven at 40 ºC. Dried specimens were ground to fine powder and extracted by stirring with 80% (v/v) methanol in ultrasonic bath for 30 min at 4 ºC, and then centrifuged at 12,000 rpm for 15 min. Supernatants were used for the evaluation of antimicrobial potential of the samples. The organic solvent in the extracts was evaporated to dryness under vacuum. The yields of methanolic extracts of the fruiting bodies are presented in Table 2.

Table 2. Yield of mushroom methanolic extracts

sample	mushroom species	yield of extracts[a] (g/100 g of dry mushroom)
1	*Amanita echinocephala*	33.082 ± 3.356
2	*Russula medulata*	4.167 ± 0.577
3	*Cerena unicolor*	20.800 ± 1.131
4	*Hericium erinaceus*	17.333 ± 0.764
5	*Ishnoderma benzoinum*	15.000 ± 1.323
6	*Laetiporus sulphureus*	33.833 ± 4.254

[a] Each value is the mean of three replicate determinations ± standard deviation.

The tested extracts were dissolved in 10% (v/v) DMSO in sterile water. A solvent control test was performed to study the effect of DMSO on the growth of microorganisms. The test approved that DMSO had no inhibitory effect on the tested organisms.

In vitro antimicrobial assay

Test microorganism

Antimicrobial activities of methanol extracts were tested against Gram-positive bacterium *Staphylococcus aureus* ATCC 6538.

The microorganism was provided from the collection of the Microbiology Laboratory at the Faculty of Natural Sciences and Mathematics in Skopje.

Suspension preparation

Microbial suspension was prepared by the direct colony method. The turbidity of initial suspension was adjusted by comparison with 0.5 McFarland's standard (Andrews, 2005). The initial suspension contained about 10^8 colony forming units (CFU)/mL. Additionally, 1:100 dilutions of initial suspension were prepared into sterile 0.9% saline.

Microdilution method

The antibacterial activities of the mushroom extracts were assessed using the microdilution method with resazurin as an indicator of microbial growth (Sarker *et al.* 2007). The antimicrobial assay was performed by using a sterile 96-well plate, and the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values were determined. The test plates were prepared by dispensing 50 µL of Mueller-Hinton broth into each well. A volume of 50 µL from the stock solution of tested mushroom extracts was added into the first row of the plate and then two-fold serial dilutions of extracts were performed. Each test plate included growth control and sterility control. MIC was defined as the lowest concentration of tested extracts that prevented a resazurin color change from blue to pink. All tests were performed in triplicate and MIC values were constant.

The extracts that demonstrated inhibitory activities were further tested for bactericidal activity. A sample from each well that tested positive for inhibitory activity was inoculated on fresh sterile Mueller-Hinton agar (MHA) plates and incubated additionally for 24 h at 37 °C. Absence of colonies was regarded as positive for bactericidal activity, while growth of colonies was regarded as negative. MBC was defined as the lowest concentration of the mushroom extract that results in microbial death. All tests were performed in triplicate and MBC values were constant.

RESULTS AND DISCUSSION

The antimicrobial activity of the tested mushroom extracts was quantitatively assessed and minimum bactericidal concentration (MBC) values were determined. The bactericidal activity of the mushroom extracts is shown in Table 3. Antimicrobial activity was observed in all species included in the study, with MBC values ranging from 1.563 to 31.250 mg/mL.

Table 3. Minimum bactericidal concentration (MBC) of methanolic extracts from mushroom samples

	Samples					
	A. echinocephala	*C. unicolor*	*H. erinaceus*	*I. benzoinum*	*L. sulphureus*	*R. medulata*
MBC	25	1.563	2.344	31.250	15.625	25

[a] Minimum bactericidal concentration (MBC) values given as mg/mL

The results showed that *Cerena unicolor* fruiting body extract exhibited the strongest bactericidal activity against the tested bacterium with the lowest MBC value (1.563 mg/mL), followed by the extracts of *Hericium erinaceus* and *Laetiporus sulphureus* (2.344 mg/mL and 15.625 mg/mL, respectively). The methanolic extracts of *Amanita echinocephala* and *Russula medulata* demonstrated antimicrobial potential with the same MBC value of 25 mg/mL. In this study the highest MBC value (31.250 mg/mL), which corresponded to the lowest bactericidal potential against tested *S. aureus*, was observed in methanolic extract of polypore fungus *Ishnoderma benzoinum*.

According to these results, all extracts included in the study showed antimicrobial properties, while the variation in the antimicrobial activity among the species could be attributed to the fungal genomics and environmental factors (Keller *et al.* 2005), that resulted in the presence of different components with antimicrobial properties within species. Based on the evidence reported in the literature, various taxonomic mushroom groups have been investigated for their antimicrobial activities and many low- and high-molecular-weight compounds with antimicrobial properties were identified. Numerous secondary metabolites, such as sesquiterpenes and other terpenes, steroids, anthraquinones, benzoic acid derivatives, and quinolines, primary metabolites such as oxalic acid, and high-molecular-weight compounds, mainly peptides and proteins, are among the identified antimicrobial compounds in mushrooms (Alves *et al.* 2012b).

In this study the examined extracts from the polypore fungi *Cerena unicolor, Hericium erinaceus*, and *Laetiporus sulphureus* exhibited the most potent bactericidal activity against tested microorganism. These results are in accordance with earlier reported data that confirm the antimicrobial activity of these macrofungi species (Jaszek *et al.* 2013; Demiri and Yamaç, 2008; Zjawiony, 2004; Wong *et al.* 2009). Jaszek *et al.* (2013) evaluated the antibacterial activity of the isolated bioactive fractions from *C. unicolor* and showed that the low molecular subfraction of secondary metabolites possesses potent inhibitory effect against *Staphylococcus aureus*. Demiri and Yamaç (2008) reported relatively high antimicrobial activity for the crude exopolysaccharides of *C. unicolor* and *L. sulphureus*, while the highest antimicrobial activity against the tested cultures in their study they observed for mycelial extracts from *C. unicolor.* Several studies have shown the antimicrobial potential of mushroom species *H. erinaceus* and some bioactive molecules with antimicrobial activity against pathogenic microorganisms have been identified (Okamoto *et al*. 1993; Kim *et al*. 2000; Wong *et al*. 2009). All these observations were in accordance with the results of bactericidal activity reported here. Taking into account all these observations,

further studies could be focused on *in vitro* biological activity of extracts from *Cerena unicolor, Hericium erinaceus* and *Laetiporus sulphureus*.

CONCLUSIONS

Results from the present study showed that *Amanita echinocephala, Russula medulata, Cerena unicolor, Hericium erinaceus, Ishnoderma benzoinum* and *Laetiporus sulphureus* extracts could be considered a potential sources of various compounds with bactericidal properties against Gram-positive bacterium *Staphylococcus aureus*. Particularly, extracts from the species *C. unicolor* and *H. erinaceus* exhibited most promising antimicrobial activity against *S. aureus* that may serve as potential candidates for the development of novel antibiotics. Further studies are needed toward the chemical characterization of specific classes of antimicrobial compounds of these selected mushrooms and understanding the possible mechanisms for their utilization in food and pharmaceutical industries.

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БАКТЕРИЦИДНА АКТИВНОСТ ОДАБРАНИХ ЕКСТРАКАТА МАКРОГЉИВА ПРЕМА *Staphylococcus aureus*

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РЕЗИМЕ: Пораст резистентности патогених врста микроорганизама резултирала је усмеравањем истраживање ка изналажењу нових извора антимикробних једињења. Предходне студије указују да макрогљиве као специфичан одговор на услове средине продукују секундарне метаболите с антимикробним особинама. У оквиру ове студије, испитивана је антимикробна активност екстраката пореклом из шест врста дивљих гљива: *Amanita echinocephala, Russula medulata, Cerena unicolor, Hericium erinaceus, Ishnoderma benzoinum* и *Laetiporus sulphureus* на *Gram* позитивни микроорганизам *Staphylococcus aureus*. Антимикробни потенцијал

метанолних екстраката гљива је испитиван применом микродилуционе методе. Антимикробну активност су показале све врсте укључене у студију. Сви испитивани екстракти су показали антимикробну активност, а након испитивања бактерицидне активности одређена је минимална бактерицидна концентрација (MBC). Тестирани микроорганизам је највећу осетљивост показао на екстракте пореклом од полипорних гљива *C. unicolor* и *H. erinaceus*. Највећа антимикробна активност постигнута је с екстрактом врсте *C. unicolor* (MBC=1.563 mg/mL). Резултати истраживања указују да метанолни екстракт *C. unicolor* поседује значајну бактерицидну активност. Резултати ових истраживања сугеришу на потенцијалну употребу ове дивље гљиве као извора антимикробних једињења.

КЉУЧНЕ РЕЧИ: гљиве, антимикробна активност, микродилуциони метод, минимална бактерицидна концентрација (MBC)