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Antibacterial Agents from Lignicolous Macrofungi

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1. Introduction

Since ancient times, the mushrooms have been prized as food as well as source for drugs, giving rise to an increasing interest today ("functional food"). Number of macrofungi is of a medicinal importance and represents an unlimited source of secondary metabolites of high medicinal value while a large number of biologically active molecules are identified in many species of macrofungi throughout the world (Wasser & Weis, 1999; Kitzberger et al., 2007; Barros et al., 2007; Turkoglu et al., 2007; Kim et al., 2008; 2007; Wasser, 2011). In addition, of importance is the amount of produced substances namely, they must be simple for the manufacturing (industrial synthesis) or there must be enough raw material for extraction of active molecules. Such molecules, if chemical groups responsible for biological activity are known, should serve as basic compounds for the synthesis of new molecules.

Lignicolous macrofungi express significant biological effects, including antibacterial activity (Hur et al., 2004; Ishikawa et al., 2005; Kalyoncu et al., 2010) and their secondary metabolites can be easily extracted and identified. It has been found that secondary metabolites are very divergent in structure and play no essential role in their growth and reproduction, but probably have a function in biochemical evolution of a species ensuring its survival (Engler et al., 1998). The presence of these compounds in macrofungi is genetically determined, but also varies as a function of ecological factors and the growth stage of these organisms (Puttaraju et al., 2006). The fungal metabolites of fruiting bodies frequently differ from those of mycelia of submerged cultures or fermentation broth. Moreover, biogenetic pathways are rather dependent on their habitats or geographic origin. The chemical composition of fungal species significantly relies on the strains and sites (substrates) of the fruiting body production. The level of phenolic compounds seems to be very much dependent on the location and stress conditions (Kim et al., 2008). With regard to this, more geographical regions and more habitats should be analyzed in the future.

A great potential of these fungi is found in their use as dietary supplements, regardless active principle. A number of products derived from mushrooms that are sold in the market is untested and of suspicious quality. Since the natural style of life become more and more popular around the world, what means return to the organic, natural food and medicines, many people lack a critical attitude to the so-called ecological products. It would therefore be important to develop food supplements and medicines based on natural resources, but with the necessary scientific confirmation of values of such products.

1.1 Macrofungi

Macrofungi or mushrooms are not taxonomic categories, being most frequently used as terms for fungi with distinctive fruiting bodies, which are usually fleshy and edible, hypogeous or epigeous, large enough to be seen with the naked eye, and picked by hand (Chang and Miles, 2002, Karaman et al., 2012).

Lignicolous (wood-decaying) macrofungi, mostly belonging to the *Polyporaceae* family, are easily noticed, collected and recognized in the field. Taxonomically, these fungi mainly belong to the phyla Basidiomycota and Ascomycota, including about 20,000 known species, widely distributed on Earth. Recent estimations suggest that even more than 1.5 million species of fungi exist on our planet and about 140,000 species belong to macrofungi. However, only 10% of them are explored and 16% are cultured (Chang & Miles, 2004; Mueller, Bills & Foster, 2004).

1.2 Antibiotics and antimicrobial agents

From the beginning until now, the humankind has always been faced with a problem of spreading of infectious diseases. Today, more than 150 compounds make arsenal of antimicrobial substances used in the treatment of infectious diseases. Antibiotics are defined as low molecular weight organic natural products (secondary metabolites or idiolites) made by microorganisms, which are active at low concentrations against other microorganisms. There are estimations that among 12,000 antibiotics known, approximately 55% are produced by *Streptomyces*, 11% by other Actinomycetes, 12% from other bacteria and 22% from filamentous fungi (Inouye et al., 2004). In its broadest definition an antibacterial is an agent that interferes with the growth and reproduction of bacteria. Unlike antibiotics, antibacterials are not used as medicine for humans or animals, but are now most commonly described as agents used to disinfect surfaces and eliminate potentially harmful bacteria found in products such as soaps, detergents, health and skincare products and household cleaners.

Since Alexander Fleming's discovery, in 1928, of the first antibiotic, called penicillin, produced by the mold *Penicillium chrysogenum*, a real revolution in medicine with a new era of antibiotics have started. Later, the entire group of β -lactam antibiotics (penicillins and cephalosporins) was discovered, followed by the Waxman's discovery of streptomycin derived from *Streptomyces* bacteria, used in a treatment of tuberculosis), and then tetracyclines, quinolones, antifungal metabolites, antiparasitic substances and more recently antiviral drugs such as acyclovir. In 1971, the second significant antibiotic cyclosporin A and C were isolated from fungal organism *Hypocladium inflatum gams* (*Tolypocladium inflatum*) which is the asexual state of the pathogen of beetles *Elaphocordyceps subsessilis* (Petch) G.H. Sung, J.M. Sung & Spatafora). Its immunosuppressive activity was revealed in 1976 by J.F. Borel and was approved for use 1983 in order to reduce the risk of organ rejection in transplant surgery (Upton, 2001 as cited in Giovannini, 2006).

1.3 Antibiotic resistance and further perspectives

Today, antibiotic resistance is a serious problem and antibiotics are losing their effectiveness what is especially important and have serious threats for humans whose health is already compromised by stress in modern way of life or by illness (HIV patients, immunocompromised persons that are under chemotherapy). Along with the increasing use of antibiotics and antibiotic agents, the resistance of bacteria to common and more

frequently used antibiotics increased, resulting in low respond to the antibiotic treatment. The existence of multidrug-resistant diseases, once felt to be under control, increased as well, tuberculosis, penicillin-resistant pneumonia, resistant malaria (the cause of death of 1.1 million people in 1998), resistant strains of gonorrhea or dysentery caused by *Shigella* and *Salmonella* (2.2 million deaths in 1998).

Public concern about infection has been expanded, resulting in a greater public use of a variety of antibacterial agents designed to remove disease-causing organisms from external surfaces before they can enter the body. Today, antibacterials may also be impregnated into sponges, cutting boards, carpeting, and children's toys. However, if used too frequently and indiscriminately, certain antibacterial agents, those that leave trace chemical residues and that target particular processes in the life cycle of bacteria, may select for resistant strains (http://www.tufts.edu/med/apua/about_issue/agents.shtml).

Furthermore, no new class of antibacterial substances has been developed to combat infectious diseases since 1970 (WHO, 2000). It is therefore necessary to find some new compounds to fight against these resistant microorganisms. Then starts the parallel struggle against antibiotic resistance exhibited in the continuous screening of new natural resources of undiscovered antibiotics from the nature. In this manner, the potential of mushrooms have a great advantage, even in comparison to the bacteria. Nowadays it is much more complicated to find new pharmaceutical active substances by chemical synthesis than from the existing and unexplored natural resources. Screenings of biological activities have made great progress in exploring the rich unlimited and undiscovered natural products in order to use it for production of pharmaceutical and agrochemical products (Anke, 1989). Many organisms were studied as potentially new resources of undiscovered bioactive components, among which fungi from the phylum Basidiomycota gave the promising results. In the forties, the pioneers in such research were Anchel, Hervey, Wilkins et al. and Florey et al. 1949, who tested extracts derived from fruiting bodies and mycelia cultures of more than 2000 species, resulting in isolation of a tricyclic diterpene antibiotic (pleuromutilin from *Pleurotus mutilus*). During nineties of the last century many new structures and biological activities were detected (Anke, 1989). Since then, numerous studies have been performed. Today we are witnessing very important struggle not only against microorganisms but also against other human diseases such as cancer, viral and other diseases.

1.4 Antimicrobial substances - Antibiotics from fungi and macrofungi

Microbial metabolites and their derivatives play an important role in the development of medicines. The use of these metabolites has grown extensively over the past century, starting with the Fleming's discovery of penicillin (1924), originally from *Penicillium notatum* filamentous micro-fungus, via Brotzu's discovery of cephalosporins from another fungus, mold *Cephalosporium acremonium* (*Acremonium chrysogenum* now), until today when the Japanese clinics use 30 penicillin derivatives and about 49 derivatives of cephalosporin. Although the metabolites originating from fungi were the main targets of antimicrobial screening, these studies were interrupted for a short time by Waksman's discovery of streptomycin (1945) originating from Actinomycetes. It is believed that the cause of the break helped by the fact that fungi often produce mycotoxins with pronounced cytotoxicity in humans and animals, and one example is the aflatoxin from the mold *Aspergillus flavus*, the most prominent cause of chronic hepatitis that leads to tumor malignancy.

However, in recent years the trend has changed and fungal metabolites have again attracted the attention of pharmacological research. This can be seen from the statistics presenting fungal metabolites increasingly important as bioactive agents and showing that the percentage of medicines versus the metabolites originating from actinomycetes are as it follows (according to the Journal of Antibiotics (I), Tokyo): 13 versus 66% (1983), 16 versus 74% (1990), 38 versus 53% (1994) and 47 versus 44% (2000), while the percentage of metabolites originating from the bacteria remained at about 8%, except 1983 when it was 21%. A similar tendency was observed for metabolites that are registered as patents in Japan, showing that the products from the fungi grew intensively: 11% (1983), over 21% (1990) to 36% (2000), and for the products from actinomycetes decreased sharply from 74% (1983), over 66% (1990) to 48% (2000). According to Tanaka and Omura (1993), 43% of more than 8000 new microbial metabolites were discovered thanks to Japanese scientists. It is possible that the abundance of secondary metabolites of fungi and actinomycetes, compared with bacteria and yeasts, is associated with the characteristics of the environment poor in nutrients. Nutritional limitation further induces secondary metabolism and production of various compounds, in order to exploit scarce nutrients in the best extent possible (Alderred et al., 1999). Taking into account the antibiotic screening, review of Inouye et al., 2004 showed that the number of antifungal metabolites increased significantly, anticancer metabolites - moderately, while the number of antibacterial metabolites decreased in the last ten years. However, the most significant increase was observed in bioactive metabolites of non-antibiotic mode of action, especially regarding the screening of inhibitors of cholesterol synthesis, of which 93% originated from fungi (Yagisawa, 2000).

In this sense it is considered that the eukaryotic fungal metabolites in action in mammalian cells could have far fewer side-effects compared with prokaryotic metabolites. Cultures of micro-organisms usually contain complex mixtures of different compounds, small and large molecular weight, what makes a direct pharmacological screening more difficult, considering the fact that can easily be masked by the activity of other compounds in the mixture. Being sessile organisms, which are in their natural environment constantly exposed to the influence of different competitors (parasitic organisms), it is not surprising that many antibiotics are isolated from fungi (Lindequist et al., 2005). Although today, still only compounds originating from micro-fungi or synthetic medicines have been used, literature data pointing to higher fungi, macro-fungi, primarily Basidiomycetes as natural sources rich in new antimicrobial substances are infrequently found (Suay et al., 2000).

As potential new sources of natural antibiotics, lignicolous mushrooms again become the subject of study (Smania et al., 2001). The fact that humans and animals share common microbial pathogens with fungi (*E. coli*, *S. aureus* and *P. aeruginosa*) has prompted the thought that they produce compounds that may have similar effects in humans (Zjawioni, 2004). In Western Europe, the interest for this group of fungi start with the discovery of antibiotics (penicillin), when a group of scientists with their pioneering research of new antibiotics originating from macrofungi Basidiomycota, led by M. Anchel, A. Hervey, WH Wilkins and Kavanagh, started research of extracts and culture mycelia and fruit body of about 2000 species (Florey et al., 1949). This research has resulted in isolation of antibiotics three-cyclic diterpene pleuromutilin (Kavanagh et al., 1951) from *Pleurotus mutilus* species. Pleuromutilin has demonstrated its antibacterial activity by inhibiting bacterial protein synthesis by interacting with RNA (Lorenzen & Anke, 1998). After that, the first semisynthetic antibiotic tiamulin was produced together with valnemuline, used in veterinary medicine (Egger & Reinshagen, 1976) for the treatment of *Mycoplasma* infections in animals (Lorenzen & Anke, 1998).

Many studies have shown that macrofungi produce many interesting pharmacological substances. By comparing the number of studied fungi with those whose chemical and pharmacological effects are completely unknown, we realized that only a very small, even insignificant fraction of potentially active fungal substances are known. For instance, the illustrative example is the species *Ganoderma lucidum*, witnessing that each species contains many different active components. In addition, production of certain secondary metabolites may depend on the characteristics of the strains (isolates) or culture conditions. Therefore, many scientists coping with this problem are actually trying to find new active compounds to be used in the future. It is clear that only a small number of active compounds studied *in vitro* or *in vivo* on animals as biological models suits the needs of allopathic medicine, defined by chemical composition, precise dosing, toxicology, pharmacodynamics and clinical studies.

Macrofungi need antibacterial and antifungal compounds to survive in their natural environment. Since fungi and humans share common microbial pathogens (e.g. *E. coli*, *S. aureus* and *P. aeruginosa*), antimicrobial compounds that are produced by fungi against microorganisms, can benefit to humans (animals). Compounds of special interest are those that exhibit antibacterial activities against multiresistant bacterial strains (methicillin resistant *S. aureus* – MRSA or vancomycine resistant *Enterococcus* – VRE).

According to a recent biological evaluation, more than 75% of screened polypores showed strong antimicrobial activity inhibiting mostly Gram-positive bacterial strains (*B. subtilis*, *S. aureus* and *M. flavus*). It was reported that new sesquiterpenoid hydroquinones produced by some species of the European *Ganoderma* genus, named ganomycins, inhibit the growth of methicillin-resistant *S. aureus* and other bacteria (Mothana et al., 2000).

Based on our results of antibacterial screening, 60% methanol and 55% chloroform extracts reached a significant antibacterial activity, giving the diameter of inhibitory zone (>15mmØ) against one or more target bacteria. Gram –negative bacteria were less sensitive to the applied extracts than Gram-positive ones, except *G. lucidum* ethanolic extract (25mg/ml) against *P. aeruginosa* (h) and *E. coli* (ATCC 25922). Three extracts of lignicolous macrofungi *P. betulinus*, *C. versicolor* and *G. lucidum* showed a wide range of activities against all tested Gram-positive and some of Gram-negative bacteria, reaching MIC values mainly at a concentration of 17.5 mg/ml. Unlike methanol, chloroform extracts did not show concentration dependence while the concept of a dose response phenomenon- hormesis (low dose stimulation and high dose inhibition) may be used for explanation of this phenomenon. The precise composition of examined extracts of fungi is unknown and can only be assumed that the effect of crude extracts, which are concentration dependent, is a consequence of complex interactions between cells and mixtures of compounds in the extracts (Karaman et al., 2009a).

In a recent screening of antibacterial activity of water and methanol crude extracts of the species *Meripilus giganteus* against nine species of Gram-positive and four species of Gram-negative bacteria, the most active extract was methanolic extract, inhibiting all the Gram-positive bacteria (mostly *S. aureus*sm, *Rh. equi*, *Bacillus*) and only two Gram-negative ones, *C. perfringens* and *P. aeruginosa*, ATCC strains (Karaman et al., 2009b) The animal strains showed to be the most susceptible analyzed strains, indicating a possible application of this fungus against Gram-positive bacterial infections in animals. Since water extract exhibited only a narrow antibacterial effect, we assumed that the obtained results could not be attributed to the compounds like proteins or polysaccharides. These results are in agreement with the literature data for similar polypore fungi (Lindequist et al., 2005; Zjawioni, 2004), demonstrated sterols and lanostanoid

terpenoids as well as phenolic compounds as the main active components responsible for the obtained activity (Turkoglu et al., 2007; Barros et al., 2007; Elmastas et al., 2007).

1.4.1 Antiviral substances

Presented antiviral activity of fungi is related to their whole, complex extracts, but also to the isolated compounds. Agents isolated from fungi can directly cause the inhibition of viral enzymes, the synthesis of viral nucleic acid, or adsorption and absorption of virus in mammalian cells. The most often small molecules are active in the direct antiviral effect, while the indirect effects are mediated by antiviral activity immunostimulative polysaccharides and other complex molecules (Zjawioni, 2004).

1.4.1.1 Low molecular weight compounds with antiviral activity

Several triterpenes from *G. lucidum* (ganoderiol F, ganodermanontriol and ganoderic acid and B) are active antiviral agents against HIV-1 virus. *In vitro* antiviral activity of influenza viruses type A and B was noticed in extracts of mycelium of mushroom *Kuehneromyces mutabilis* (Schaeff.: Fr.) (Singer & AH Sm.), while the extract and two isolated phenolic components from the mushroom *Inonotus hispidus* (Bull.; Fr.) P. Karst, as well as ergosterol peroxide, are present in many different fungal species.

1.4.1.2 High molecular weight compounds with antiviral activity

Water-soluble lignins isolated from *Inonotus obliquus* (Pers.: Fr.) Pilate, inhibit HIV protease with IC 50 value of 2.5 mg/ml. Anti-HIV activity is recorded for the submerged culture media of *L. edodes* and water-soluble lignin isolated from the same fungus. Protein-polysaccharide complex PSK and PSP from *Coriolus versicolor*, also shows antiviral activity on HIV and cytomegalovirus *in vitro*. Inhibition of HIV-1 reverse transcriptase is caused by velutin, protein from *Flammulina velutipes*, which inactivates ribosomes. MD fractions of mushroom *Grifola frondosa* showed general improvement of condition of the patients (85%) who had various symptoms of HIV and other secondary diseases (Zjawioni, 2004).

1.4.2 Antifungal substances

Compounds with antibacterial and antifungal activity of mushrooms assists in their survival in their environment. These substances can be very useful in the treatment of human infections, but the official antibiotic therapeutics in the world market can be only found originating from microfungi so far. Opportunistic fungal infections are always a big problem, especially in immunocompromised patients receiving chemotherapy or in cases of transplantation of organs or bone marrow, as well as in HIV infection. During the last ten years, the interest in compounds that show antifungal activity has been increased. Among them the sordarin (tricyclic diterpene glycoside) was for the first time isolated in 1971 (Hauer and Sigg as cited in Inouye et al., 2004), and slightly more potent zofimarin was isolated for the first time in 1987 (Ogita et al. 1987 as cited in Inouye et al., 2004). In addition, suggestive is xylarin (compound SCH57404) isolated from the lignicolous fungus *Xylaria* sp. (Schneider, 1995). Many derivatisations of sordarin antibiotics have been performed in research groups of the GlaxoSmith Kline company by biotransformation with *Streptomyces avermitilis*, what resulted in the synthesis of GM237354 (Herreros et al., 1998), with the MIC of 90% that was 0.015 mg/ml for isolates of *C. albicans* and 0.12 for *C. tropicalis*. Further development of these compounds has led to the azasordarin group in which the sugar component is replaced by N-substituted morpholine (Herreros et al., 2001 as cited in Inouye et al., 2004).

Several antifungal metabolites with steroid structure have been also isolated from fungi A25822 A and B from *Geotrichum* (Gordee and Butler, 1975 as cited in Inouye et al., 2004) and from *Wallemia sebi*; Mer-NF8054 A and X from the genus *Aspergillus*. The most famous triterpene, favonol isolated from basidiomycetous *Favolashia* sp. (Anke et al., 1995 as cited in Inouye et al., 2004) is a metabolite that exhibited antifungal activity against Ascomycetes, Basidiomycetes, Zygomycetes and Oomycetes, but did not show antibacterial activity. Researchers of Merck Group have discovered four acidic terpenoids from filamentous fungi: ergokonin A (from *Trichoderma koningii*), ascosteroid (from *Ascotricha amphitricha*) arundifungin (steroid from *Arthrinium arundinis*) and enfumafungin (pentacyclic terpenoid from from mould *Trichoderma koningii*), ascosteroid (from ascomycetous *Ascotricha amphitricha*), arundifungin (steroid from mould *Arthrinium arundinis*) and enfumafungin (pentacyclic terpenoid from *Aureobasidium*), which were found to affect the biosynthesis of β -D-glucan but not the biosynthesis of steroids. Among them the best antifungal activity on *Candida* species and species of *Aspergillus* genera showed enfumafungin.

1.5 Chemical nature of antibacterial agents

A large number of pharmacologically active substances like sesquiterpenes (Abraham, 2001), hydroquinones (Mothana et al., 2000), polysaccharides and complexes of polysaccharide-peptide (Liu, 1999), lanostanoide triterpenoids (Shiao, 1992, Leon et al., 2004) steroids (Smania, 2003), nucleosides, alkaloids and vitamins (Paterson, 2006) from fruitbodies of polypore fungi have been detected. Recent studies pronounced phenolic compounds (Turkoglu et al., 2007, Paterson, 2006, Ribeiro et al., 2007) as the main active antioxidative components in fungal extracts (Kityberger et al., 2007; Barros et al., 2007). It is assumed that antibacterial effects exhibited by fungal extracts of different polarities could be related to an overall effect of phenolic compounds (e.g. phenolic acids: caffeic acid, ellagic acid; flavonoids, hydroquinones) detected in similar extracts of the species *G.lucidum*, *F.velutipes*, *P.ostreatus* or organic acids (oxalic, malic) previously detected in *L. sulphureus* and *F. hepatica*, as well as terpenoids.

1.5.1 Products of primary metabolism

Polysaccharides. Polysaccharide molecules that form an integral part of the fungal cell wall also exhibit antimicrobial properties (Stamets, 2002). Polysaccharides are the most important components of fungal bioactive substances, proven to provide many medical and therapeutic possibilities (Fan et al., 2006) while their antibiotic effect is often specific to certain microorganisms (Stamets, 2002). Most of these compounds belong to glucans or heteroglycans (Fan et al., 2006). It is believed that the antibacterial and antifungal effects of β -glucan is based on the activation and strengthening of the immune response, and their use is recommended in combination with other antibiotics and immunostimulators in prevention and treatment of infectious diseases, especially immunocompromised individuals (Chen & Seviour, 2007).

Proteins and polypeptides. Proteins that act inhibitory on microorganisms are found frequently in organisms of plant and animal species, whereas their presence is rare in fungi (Wang & Ng, 2006). It is believed that these proteins are often positively charged, and that the mechanism of their action is realized by forming ion channels in cell membranes of microorganisms as well as by competitive binding to host cell polysaccharide receptors (Cowan, 1999). Proteins and peptides are isolated from macrofungi whose antimicrobial effect is limited to a small number of mostly phytopathogenic species (Table 1).

COMPOUNDS	ORIGIN/SOURCE	BIOLOGICAL ACTIVITY/REFERENCE
EXTRACELULAR POLYSACCHARIDES (noncellulose β-glucans)		
LENTINAN	<i>Lentinus edodes</i> mycelial extract of <i>Lentinus edodes</i>	antifungal: <i>Candida albicans</i> , antibacterial: <i>Mycobacterium tuberculosis</i> , <i>Listeria monocytogenes</i> , <i>S. aureus</i> , <i>M. luteus</i> i <i>B. cereus</i> (Stamets, 2002; Kitzberger et al., 2007; Chen & Seviour, 2007) antiviral: Herpes simplex-a type 1 (Stamets, 2002)
SCHYZOPHYLLAN (SPG)	<i>Schyzophyllum commune</i>	antifungal: <i>Candida albicans</i> , antibacterial: <i>S. aureus</i> . (Stamets, 2002)
KRESTIN (PSK), proteoglycan	<i>Trametes versicolor</i>	antifungal effect: <i>C. albicans</i> (Stamets, 2002; Kitzberger et al., 2007)
GRIFOLAN (GRN)	<i>Grifola frondosa</i> <i>Lepista nuda</i>	
INTRACELLULAR POLYSACCHARIDES - containing 1,6-α-D-galactopyranosyl units, substituted on O-2 position with α-L-fucopyranosyl or 3-O-α-D-manopyranosyl-α-L-fucopyranosyl units. Found only in fungi and concerned as a type of reserve materials (Fan et al., 2006)		
FUCOGALACTAN CMP3 (hydrosoluble heteroglucan)	from the mycelium of <i>Coprinus comatus</i>	not yet investigated (<i>C. comatus</i> showing antibacterial activity) (Fan et al., 2006).
FUCOGALACTAN	<i>G. applanatum</i>	
MANOFUCOGALACTANES	<i>F. velutipes</i> , <i>Polyporus pinicola</i> , <i>P. fomentarius</i> and <i>P. igniarius</i>	not yet investigated, concerned as a reserve material (Fan et al., 2006)
FUCOMANOGLACTANS	<i>Laetiporus sulphureus</i>	
GANODERMIN	<i>Ganoderma lucidum</i>	antifungal to phytopathogens <i>Botrytis cinerea</i> , <i>Fusarium oxysporum</i> and <i>Physalospora piricola</i> (Wang & Ng, 2006)
PLEUROSTRIN	<i>Pleurotus ostreatus</i>	antifungal effect : <i>Fusarium oxysporum</i> , <i>Mycosphaerella arachidicola</i> and <i>Physalospora piricola</i> (Chu et al., 2005)
LYOPHYLLIN	aqueous solution of <i>Lyophyllum shimeiji</i>	antifungal effect : <i>Mycosphaerella arachidicola</i> and <i>Physalospora piricola</i> (Takakura et al., 2001; Wang & Ng, 2006)
TRICHOGIN	<i>Tricholoma giganteum</i>	antifungal activity against <i>Fusarium oxysporum</i> , <i>Mycosphaerella arachidicola</i> and <i>Physalospora piricola</i> , as well as inhibitory effect on HIV-1 reverse transcriptase (Guo et al., 2005)
ERYNGIN	<i>Pleurotus eryngii</i>	inhibition of <i>Fusarium oxysporum</i> and <i>Mycosphaerella arachidicola</i> , its N-terminal end shows certain similarity with antifungal protein liophyllin (Wang & Ng, 2004)
AGROCYBIN	<i>Agrocybe dura</i>	antibacterial effect against Gram + and Gram - bacteria: <i>B. mycoides</i> , <i>B. subtilis</i> , <i>E. coli</i> , <i>Klebsiella pneumoniae</i> , <i>Mycobacterium pheli</i> , <i>M. smegmatis</i> , <i>Photobacterium fischeri</i> , <i>P. aeruginosa</i> , <i>S. aureus</i> (Kavanagh et al., 1950)
	<i>A. cylindracea</i>	antifungal effect: <i>Aspergillus niger</i> , <i>Gliomastix convoluta</i> , <i>Memnoinella echinata</i> , <i>Myrothecium verrucaria</i> , <i>Penicillium notatum</i> , <i>Phycomyces blackesleeanus</i> , <i>Stemphylium consortiale</i> and <i>Trichomonas mentagrophytes</i> (Ngai et al., 2005)

Table 1. Polysaccharides, proteins and peptides from macrofungi with antimicrobial effect

Dietary fibers. High molecular weight substances that are excreted without digestion and absorption from the human body are called dietary fibers (Mizuno, 1999). Mushrooms contain these substances, which are composed of β -glucan, chitin and heteropolysaccharide (pectin substances, hemicellulose, polyuronidase, etc..) in the range of 10-50% in dry weight of the substance. Since they absorb harmful substances, hindering their intestinal absorption, dietary fibers are effective in preventing colon and rectal cancers (Mizuno, 1999).

Lectins. Lectins (Latin *legere* = to take, to choose) are defined as carbohydrate-binding proteins of non-immune origin which agglutinate cells or precipitate polysaccharides or glycoconjugates (Kawagishi, 1995). Many species of plants, animals and microorganisms contain lectins, but the fungal lectin is still not explicitly defined. So far, several lectins were isolated from mushrooms of the genus Polyporales: *Grifola frondosa* (GFL), *Fomes fomentarius* (FFL), *Ganoderma lucidum* (GLLs). Some are isolated from the fruit bodies and some from the mushroom mycelium. GFL is cytotoxic to HeLa cells, and its activity is explained by binding of lectins to carbohydrate parts of the cell by preventing aggregation of cells (Wasser & Weis, 1999).

1.5.2 Products of secondary metabolism

Secondary metabolites produced by a large number of macrofungi have great therapeutic significance. These compounds occur as intermediate products of primary metabolism, but most of them are classified according to the five major metabolic sources (Table 2,3,4). The most productive pathways of synthesis of secondary metabolites are polyketide and mevalonate pathways (Zeidman et al., 2005, from Giovaninni, 2006).

1.5.2.1 Phenolic compounds

Phenols are one of the largest classes of secondary biomolecules, which are characterized by the presence of aromatic rings with hydroxyl group bonded directly to an aromatic hydrocarbon group. Although they are firstly identified in plants (Cowan, 1999), their presence was also observed in fungi (Barros et al., 2008, Mattila et al., 2001, Karaman, 2002, Karaman et al., 2012a). In recent years, there was a causal relationship between the total content of these compounds with biological activities recorded in a large number of macrofungi (Barros et al., 2007), which include anti-inflammatory, antiallergic, anticancer, antihypertensive, antirheumatic and antibacterial activity. Antimicrobial properties of phenolics are explained by the presence of phenol hydroxyl groups, which number is in correlation with their toxicity toward microorganisms (Cowan, 1999). The possible mechanisms of their action include inhibition of extracellular microbial enzymes, deprivation of the substrates required for microbial growth or direct action on microbial metabolism through inhibition of oxidative phosphorylation, by sulfhydryl groups and some non-specific interactions (Cowan, 1999).

It has been shown that the antimicrobial effects of extracts of mushroom *Lactarius deliciosus*, *Sarcodon imbricatus* and *Tricholoma portentosum* directly correlated with total content of phenols and flavonoids in them (Barros et al., 2007). Extracts of all three fungi showed antibacterial effects on *Bacillus cereus* and antifungal to *Candida neoformans*, while the extract of mushrooms *Lactarius deliciosus* and efficiency demonstrated against *P. aeruginosa* and *Candida albicans*. High content of phenols has been recorded in lignicolous fungi *Meripilus giganteus*, *G. lucidum* and *Flammulina velutipes* in the form of coumarins and tannins, as well

as in *Ganoderma applanatum*, where they were detected in the form of coumarins, flavonoids and tannins (Karaman, 2002, Karaman et al., 2005). Data on the antimicrobial action of these fungi also exist (Karaman et al., 2010). Analyses of extracts of the genus *Ganoderma* species shown the presence of polyphenolic compounds, and antimicrobial properties of these mushrooms explains the activity of compounds of hydrohynon composition - ganomycin A and B (Ofodile et al., 2005 as cited in Mothana et al., 2000).

High concentrations of phenolic acids (> 1.0 mg / g), mainly a high concentration of gallic acid and protocatechuic, could be interpreted as anti-microbial activity of the following species: *L. sulphureus*, *F. hepatica*, *P. ostreatus*, *F. velutipes* and partially *M. giganteus*, which in antimicrobial screening showed moderate activity (Karaman, 2009b). Further studies of mechanisms of antimicrobial components originating from mushrooms could be suggested, including the influence of the protein compounds and organic acids such as oxalic acid, which accumulates in the fruit bodies of brown rot mushrooms, but also malic acid, ellagic acid, or some other compounds.

Flavonoids are hydroxylated phenolic compounds (C6-C3 units associated with the aromatic core) and antimicrobial activity can be explained by their ability to create complexes with extracellular soluble proteins and polypeptides that builds cell wall of microorganisms, as well as disruption of the function of cell membrane (Cowan, 1999). There are only few data dealing with detection of flavonoids (rutin, chrysin, naringin, myrcetin and quercetin) in tericolous (Turkoglu, 2007; Baros et al., 2007) lignicolous fungal species (Kim et al., 2008, Jayakumar et al., 2009). Since flavonoids are phenolics that generally occur in plants acting as antioxidants, antimicrobials, photoreceptors, feeding repellants or UV protectors (Pietta, 2000) we assume that the presence of these metabolites in TP of fungi that generally are in tight connection with wood, could have impact on the expressed bioactivity. Recent studies conducted with mushrooms showed a positive correlation between the TP and antioxidant capacity (Turkoglu, 2007, Ribeiro, 2007), possibly due to their ability to chelate metals, inhibit lipoxygenase and scavenge free radicals. Plotting TP content versus antibacterial activity (Karaman et al., 2010), revealed a good positive correlation between these two parameters, showing higher values for MeOH than CHCl₃ extracts against most of the bacteria. By comparing different strains of the same bacteria (*S. aureus*) it was concluded that the effect of TP upon the antibacterial activity may be strain specific.

Worthy of note is the antibacterial activity of fungi against the multidrug-resistant strains of bacteria. New sesquiterpenoid hydroquinone from *Ganoderma pfeifferi* Bres., called ganomycin (Mothana, et al., 2000) inhibit methicillin-resistant strains of *Staphylococcus aureus* and the growth of other, mainly Gram-positive bacteria. In addition, sterol-type compounds, isolated from the species *G. applanatum* such as 5 α -ergost-7-en-3 β -ol, 5 α -ergost-7, 22-dien-3 β -ol, 5.8-epidioxy-5 α , 8 α -ergost-6,22-dien-3 β -ol and another new lanostanoid showed weak activity against many Gram + and Gram - bacteria. Oxalic acid is one of the substances responsible for the antimicrobial effects of mushroom *Lentinula edodes* (Berk.). Chloroform extract of mycelium *L. edodes* has bactericidal properties (Hirasawa et al., 1999).

Tannins are complex polyphenolic compounds that are divided into the two groups: the hydrolized (esters of phenolic acids and sugars), and condensed (constructed from flavonoid monomers). Antimicrobial activity of tannins is expressed due to their ability to link amino acids in proteins, inactivating adhesions, enzymes and transport proteins of cell

membranes of microorganisms (Cowan, 1999), as well as the formation of complexes with metal ions (Biradar et al., 2007). In addition, tannins could form complexes with polysaccharides, affecting microorganisms.

The equivalent of tannic acid was detected in extracts of shiitake mushrooms (*Lentinus edodes*), which show the antibiotic effect against bacteria *M. luteus* and *B. cereus* and the fungus *Candida albicans*, while against the strains of *E. coli* and *S. aureus* did not show the same activity (Kitzberger et al., 2007). While the focus of previous mycochemical (gr. myces=fungi) analysis of *Pleurotus ostreatus* was mainly put on the vitamins and minerals content, indicating a high nutritional value of mushrooms (Mattila et al., 2005), recent research revealed its exceptional antimicrobial and antioxidant effects that are associated with the presence of terpene and phenolic compounds (Iwalokun et al., 2007). The presence of phenols in the form of pyrocatechols, and flavonoids in the form of quercetin, was noted in extracts of fungus *Laetiporus sulphureus*, which explains its strong antioxidant properties (Turkoglu et al., 2006). This study also shows that ethanol extract of *L. sulphureus* exhibits strong antibiotic effect against Gram-positive bacteria (*B. subtilis*, *B. cereus*, *M. luteus* and *M. flavus*) and the yeast *Candida albicans*, while its activity against Gram-negative bacteria is much lower.

Coumarins are phenolic compounds of characteristic odor, and, according to the chemical structure, they are lactones built from the benzene and pyrone ring (Cowan, 1999). Despite the antiviral activity of some coumarins and the evidence of their inhibitory effect on the fungus *Candida albicans* *in vitro* conditions (Cowan, 1999), data on antimicrobial activity of these compounds are scarce. The presence of coumarin in fungi has been established in most genera of Xylariaceae family (Ascomycetes) (Whalley et al., 1999), as well as in certain fungi belonging to lignicolous basidiomycetes based on preliminary TLC profiling (Karaman, 2002).

Other agents with weak antibacterial effects found in macrofungi are steroids like 5 α -ergosta-7,22-dien-3 β -ol or 5,8-epidioxy-5 α ,8 α -ergosta-6,22-dien-3 β -ol, isolated from *Ganoderma applanatum* (Pers.) Pat., proved to be weakly active against a number of Gram-positive and Gram-negative bacteria and organic acids like oxalic acid proved to be responsible for the antibacterial effect of *Lentinula edodes* (Berk.) Pegler against *S. aureus* and other bacteria.

Other, non-phenolic, compounds including terpenoids (Leon et al., 2004) and polysaccharides (Tseng et al., 2008) have also been designated as mushroom antioxidants or antimicrobials.

1.5.2.2 Terpenoid compounds

Terpenes are a broad class of lipophilic secondary metabolites whose general chemical structure is C₁₀H₁₆. In nature they appear as diterpenes, triterpenes and tetraterpens (carotenoids) - C₂₀, C₃₀, C₄₀, as well as the hemiterpens and sesquiterpens - C₅, C₁₅. If include additional elements (mostly oxygen within the hydroxyl and carbonyl groups), they are called terpenoids (Cowan, 1999). Terpenoids originate from simple acyclic compounds, isoprene and mevalonic acid, and their structure may be acyclic, monocyclic or bicyclic. Basically, their structure is isopentenyl-pyrophosphate (IPP), whose synthesis is realized in two ways, and pathways of synthesis of higher isoprenoids continue on after the

isomerization of IPP in DMAPP. For all animal and fungal cells characteristic is the mevalonic pathway of isopentenyl-pyrophosphate synthesis, while most plants, bacteria, actinomycetes and protozoa have non-mevalonic mode of its synthesis (Inouye et al., 2004).

One of the many functions of these compounds is their antimicrobial activity, but the mechanism of action of terpenoids on microorganisms is not fully understood (Cowan, 1999). According to their lipophilic nature, it is assumed to act by disrupting membrane functions of microbial cells (Cowan, 1999), and some authors believe that they may cause increasing of non-specific cell membrane permeability for the antibiotic molecule (Byron et al., 2003). Though plant organisms are thought to be the largest source of triterpenoids, in recent years more and more data indicate the presence of these compounds in some representatives of macrofungi (He et al., 2003, Akihisa et al., 2005; de Silva et al., 2006; Abraham, 2001, Deyrup et al., 2007).

Sesquiterpenes. One of the many strategies that representatives of the higher fungi use to protect themselves against a number of parasites that feed on their fruit bodies is the production of toxins. It is interesting that many of these toxic chemical suits sesquiterpens (Abraham, 2001). For most basidiomycota fungi the presence of sesquiterpens of protoiludane type is characteristic, which originate from humulene, compounds present in a rare fungus, formed by cyclization of farnesyl-pyrophosphate. Of the few ways of humulene transformation, the most important pathway of synthesis of protoiludane, tricyclic compound which, due to the high reactivity caused by the presence of cyclobutane, is further transformed into a series of compounds. Some of these sesquiterpenes show interesting biological activity, and are considered to be a very interesting object of study in terms of medical chemistry. Several groups of sesquiterpenes originating from higher fungi show a greater or lesser antimicrobial effect (Tables 2, 3). It is interesting to note that some representatives of the genera *Russula* and *Lactarius* synthesize sesquiterpene alcohols that are esterified with fatty acids. These esters do not show strong antibiotic activity, but in the case of mushroom fruit body injury, leads to cleavage of ester bonds and release of alcohols that are highly reactive and therefore very toxic to microorganisms. Therefore, the mentioned esters may be considered as pro-medicines or precursors of compounds that in metabolic processes are transformed into an active form.

Triterpenes. Compounds of triterpene composition are found in many mushroom extracts which showed some antibiotic properties. Genus *Ganoderma* contains about 200 species known for the production of triterpene compounds. Many of these species have found wide application in the prevention and treatment of various diseases due to the numerous biological activities based on the presence of triterpene components (Ofodile et al., 2005). Although thought to be active against bacteria just due to the presence of triterpenes in these fungi, there are data that disagree with such opinions, giving the example of seven different triterpenes isolated from a Vietnamese species *G. collosum*, which showed no antimicrobial effect, but exhibit strong anti-inflammatory activity (Ofodile et al., 2005). Most triterpenes synthesized by species of the genus *Ganoderma* belong to the lanostane type (de Silva et al., 2006). Over 100 compounds from this group have been identified, among them a few newly discovered (Akihisa et al., 2005; de Silva et al., 2006, Jian et al., 2003, Kamo et al., 2003). The review of triterpene compounds isolated from macrofungi is given in Table 3.

Overview of other compounds isolated from macrofungi, which exhibit antimicrobial activity is shown in Tab. 4

COMPOUND	NAME OR CHEMICAL STRUCTURE	ORIGIN	EFFECT (ACTIVITY)
CARYOPHYLLENE	Naematolin	<i>Hypholoma fasciculare</i>	weak antibacterial
COLLYBIAL	α,β -unasturated aldehyde	<i>Collybia confluens</i>	low antifungal, high antibacterial (<i>Bacillus</i> sp.), high antiviral, cytotoxic, nonselective antibiotic
PROTOILLUDANES (esters of protoilludanol)	Armillyl orselinate Arnamiol (chlorinated derivatives) Melleolide B, C, D, E, F, G, H (everniate-armillarin) Melleolide I, J Radulon A Lentinellic acid methyl-estars of lentinellic acid	<i>Armillaria mellea</i> (similar to <i>A. tabescens</i>) <i>Clitocybe elegans</i> <i>A. novae-zelandiae</i> <i>Radulomyces confluens</i> <i>Lentinellus</i>	prevent trombocyte aggregation, cytotoxic, antimicrobial low antifungal, high antibacterial, cytotoxic high antifungal , low antibacterial
MARASMANES	Marasmic acid hydroxy derivative of marasmic acid Pilatin Velutinal and fatty acid esters	<i>Marasmius conigenus</i> culture – <i>Flagelloscypha pilatii</i> contain many Basidiomycota by damage of fruiting-bodies, converting to Isovelleral	antibacterial less antifungal, cytotoxic and phytotoxic lower antibiotic and cytotoxic high antibacterial, antifungal & cytotoxic
HYDROGRAMMANE (modified marasmic sesquiterpenes)	10-hydroxy-isovelleral Hydrogrammic acid	<i>Clitocybe hydrogramma</i>	antibacterial against <i>Bacillus</i> sp., non against <i>E. coli</i> and fungi
CUCUMANES		culture – <i>Macrocystidia cucumis</i>	antimicrobial and cytotoxic
FOMANNOSANES	Fomanosin Illudosin	<i>Fomes annosus</i> <i>Omphalotus olearius</i> <i>Omphalotus nidiformis</i> .	bactericidal, phytotoxic antibacterial against Gram + (<i>Sarcina lutea</i> and <i>Bacillus</i> spp.), non against <i>E. coli</i> antifungal
ILLUDANES	Illudin S (lampterol) Illudin M Hydroxydihydroilludin M Illudin A, B, C, D i E Illudalenol, Illudin F, G i H Illudin C ₂ i C ₃ Illudinic acid	<i>Omphalotus olearius</i> <i>Lampteromyces japonicus</i> , <i>Omphalotus olivascens</i> <i>Clitocybe subilludens</i> <i>Pleurotus japonicus</i> <i>Omphalotus olearius</i> <i>Omphalotus nidiformis</i> <i>Coprinus atramentarius</i> <i>Agrocybe aegerita</i>	anticancerogenic properties weak antibiotic activity on <i>B. subtilis</i> cytotoxic, antibiotic (<i>S. aureus</i>), antifungal
ILLUDALANES (dicoumaric sesquiterpenes)	Fomajorin D & S Illudalic acid illudinine Candicansol Clavicornonic acid	<i>Fomes annosus</i> <i>Omphalotus olearius</i> <i>Clitocybe candicans</i> <i>Clavicornona pyxidata</i> <i>Mycena leaiana</i>	antiviral (inhibits reverse transcriptase of viruses causing leukemia in rats -weak antibiotic activity (<i>Acinetobacter</i>), high
ISOILLUDANES	Leaianafulven		cytotoxic, mutagenic

Table 2. Antimicrobial effects of sesquiterpenoids orginated from macrofungi (according to Abraham, 2001)

Compound	Name or chemical structure	ORIGIN	EFFECT (ACTIVITY)
HIRSUTANES		Hirsutic acid C Complicatic acid	<i>Stereum hirsutum</i> <i>Stereum complicatum</i> - culture <i>Pleurotus hypnophilus</i>
PLEUROTELANES	pleurotelic skeleton, created by modification of hypnophillin	Hypnophillin Pleurotelic acid Pleurotellol Coriolin A, B, C Hypnophillin 1-desoxyhypnophylin	<i>Coriolus consors</i> <i>Lentinus crinitus</i> - <i>Gloeostereum incarnatum</i> - culture
		Gloeosteretriol Incarnal (dehydro-hirsutanol A)	<i>Macrocystidia cucumis</i>
CUCUMANES		Cucumins A-H	<i>Merulius tremellosus</i> - culture
MERULANES		Merulidial	
ISOLACTARANES			
TRITERPENOIDES		Meruliolactone Stereopolide Dihydrostereopolide	<i>Stereum purpureum</i> – culture, <i>Merulius tremellosus</i>
TRITERPENES	Lanostane-type	<i>G. applanatum</i> <i>G lucidum</i>	(de Silva et al., 2006)
		Lanostane-type, fatty acids	<i>Fomes</i>
		lanostane and ergostane derivatives	
Triterpenoid lactons		Fomlactons A, B, C	<i>Fomes cajanderi</i>
Triterpenic glycosides		Kolocosides A, B, C, D	common in plants and lichens, so far only three representatives found: <i>Xylaria</i> from Hawaiian Islands
		Fuscoatroside Enfumafungin - WF11605	
Triterpenoid saponins		glycosides with <i>betulin</i> as a aglyconic component	<i>P. ostreatus</i>
		Favolon (with variable cyclic structure and method of substitution)	<i>Favolaschia</i>

Table 3. Antimicrobial effects of sesquiterpenoids and triterpenoids from macrofungi (according to Abraham, 2001)

1.6 Extraction methods

Extraction procedures are important in assessing good antibacterial activities of extracts. Macrofungi are commonly collected either randomly or by locals in geographical areas or forest habitats where the fruiting bodies are found. Initial screenings of fungi for possible antibacterial activities usually begin by using crude aqueous or alcohol extractions. Since the majority of the identified components of mushrooms are active against microorganisms, they are mostly obtained through initial ethanol or methanol extraction.

Water-soluble compounds, such as polysaccharides and polypeptides, including lectins, are commonly more effective as inhibitors of virus adsorption and cannot be identified in the screening techniques commonly used. Tannins and terpenoids are occasionally obtained by treatment with less polar solvents.

For alcoholic extraction, the intact mature fruiting bodies or their segments are brush cleaned, air-dried to constant mass and pulverized, and then soaked in methanol or ethanol for extended periods (24-72h). The resultant filtrated extracts are then filtered and washed, concentrated under reduced pressure at low temperature to avoid destroying of any thermo-labile antimicrobial agents present in the extract and redissolved in the alcohol (or 5% DMSO) to a determined concentration. Water extractions, generally used distilled water, blending of slurry, filtration and centrifugation (approximately 15,000 for 30 min) multiple times for clarification.

Compounds	Origin/Source	Biological activity	Reference
<u>β-methoxyacrylates</u> strobilurins and oudemansins	cultures of <i>Oudemansiella mucida</i> , <i>Xerula malanotricha</i> and <i>Xerula longipes</i>	- antifungal activity against a large number of saprotrophic and phytopatogenic fungi, inhibiting the process of respiration	Anke et al., 1979; Anke et al., 1983
<u>Polyenes</u> xerulin, dihydroxerulin and xerulinic acid	<i>Xerula malanotricha</i>	- antimicrobial, anticancer, antiviral and anti-inflammatory activity - inhibition of cholesterol biosynthesis and cytotoxic effect	Negishi et al., 2000; Kuhnt et al., 1990
Agrocybolacton	cultures of representatives of the genus <i>Agrocybe</i>	- moderate antibacterial activity against Gram-positive bacteria <i>B. subtilis</i> and <i>M. smegmatus</i>	Rosa et al., 2003
Lentionine (1,2,3,5,6-enthathiocycloheptane) and its disulfide derivate	<i>Lentinus edodes</i>	antibacterial antifungal effect	Hirasawa et al., 1999)
Cinnabarine	<i>Pycnoporus cinnabarinus</i>	antibacterial (<i>B. subtilis</i> <i>S. aureus</i>) antifungal effect - <i>in vitro</i> antifungal activity	Shitu et al., 2006
Laschiatrion new antibiotic with steroid skeleton	submerged cultures of the genus <i>Favolaschia</i>	against some human pathogens - antibacterial and citotoxic effects not detected	Anke et al., 2004

Table 4. Other compounds from macrofungi with antimicrobial activity

1.7 Evaluation of antibacterial activity

1.7.1 Techniques used in research of new substances

Basidiomycota and fungi in general, represent an inexhaustible source of new substances, even though each species contains hundreds of active metabolites. Therefore, test systems for research of new substances must be fully simplified, fast, efficient and as cheap as possible (Hostettmann et al., 1997, as cited in Giovaninni, 2006). In addition, biotests (bioassays) must be sufficiently sensitive to detect the activity of substances in low concentrations, in the so-called solid (crude) extracts.

Crude products can be used in antimicrobial testing disc-diffusion and broth-dilution assays to test for antibacterial properties including bioautography according to standard procedures (NCCLS or CLSI procedures). The use of standard cultures of familiar characteristics is recommended though several precautions have to be taken into account. In a recent study the differences between two screening methods applied were not statistically significant (t-test at level $p < 0.05$). Both *Meripilus* extracts analyzed (water and methanol) showed wider inhibition zones in disc-diffusion method, indicating that it is more appropriate for the testing of polar extracts (Karaman et al., 2009b). Similar results were confirmed for extracts of the genus *Fomes* although showed broader inhibitory zones using the method of "wells", compared with inhibitory zones obtained by disc-diffusion method. For other extracts, however, the disk-diffusion method could be recommended, indicating that polarity of active substances in extract influence on results obtained in particular method applied.

MIC and MBC determination is used to quantify antimicrobial activity using the two-fold dilution method according to CLSI guidelines. The MIC is defined as the lowest concentration preventing visible growth while complete absence of growth is considered as the MBC. The lower MIC or MBC values with respect to the extract concentration indicate a higher activity, implying better quality of the extract. To confirm MBCs, aliquots of the experimental suspensions (100µl) could be sub-cultured on Müller Hinton agar plates incubated overnight.

Potent source of antibacterial agents is the species *M. giganteus* (50mg/ml), showing high activity against both groups of bacteria reaching MIC values in a wide range of concentrations (<17.5 -1125µg/ml). Various activities have been detected among different strains of *S. aureus*, indicating that fungal extracts are target specific on intraspecific level (strain specific).

Antibacterial assay may be performed in 96-well micro-plates instead of tubes. If 5% DMSO is applied for dissolving a negative control with 0.5% DMSO must be used to ensure that DMSO did not affect bacterial growth. Results are recorded after incubation at 35-37°C for 18-24h and all the samples should be tested in triplicate.

Bioautography is one of the most effective tests for detection of antimicrobial metabolites, considering the fact that it localizes the place of the active component, therefore enabling the isolation of the active component precisely. Bioautography may be the direct, when microorganisms grow directly on the TLC plate, then contact, when the active compound is transferred from the TLC plates to inoculated agar and agar-spill-over (so-called immersion bioautography), when the inoculated agar medium is spilled over the TLC plate (Rahalison

et al., 1991). In the bioautography agar overlay method, the drug to be evaluated is adsorbed onto the TLC plate and the inoculum is laid onto the plate as a very thin layer (1 mm). The advantage of this method is that the amount of sample being used is very small and that the fractionalisation of the crude extracts on its different components simplifies the identification of active compound.²⁶

In our recent work, the TLC chemical profile of the analyzed species of lignicolous macrofungi showed that they are rich in phenols, although the differences in the number and quality of the extracted compounds have been noticed. Comparing the TLC profiles, fungi can be classified into three groups according to the obtained retention factor e.g. R_f values representing the distance traveled by the compound divided by the distance traveled by the solvent: 1) three species: *C. versicolor*, *G. lucidum* and *G. applanatum* contain compounds with similar ($R_f = 0.68$, $R_f = 0.69$, $R_f = 0.70$, respectively), 2) five species *M. giganteus*, *L. sulphureus*, *F. velutipes*, *F. hepatica* and *P. ostreatus* showed a small amount of eluted compounds and intense fluorescence at the start line after the spraying, 3) the species *P. betulinus* expressed with three spots in the MeOH extracts ($R_f = 0.62$, $R_f = 0.65$, $R_f = 0.68$), which extinguished fluorescence in the UV 254th (Karaman, 2009c).

Furthermore we made slight modifications of the standard procedure of bioautography in the same study using the following: soft (top) agar (0.7% Nutrient agar) which was mixed with freshly prepared inoculum of bacteria (0.5Mac Farland optical density) and with the aqueous solution of tetrazolium red dye 0.1% w/v (1mg/ml)- 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) (3:1:0.1). The strain *S. aureus*sm was used as the indicator organism. Amoxicillin (64µg/ml) was used as positive control. Approximately 10µl of the solution of each extract was applied on a TLC plates (silica gel 60, F 254, DC-Plastikfolien, 0.2 mm thick, Merck, Germany) for about 2h, equally prepared as a reference plate for chemical analysis. Bioautography test plate was developed in the same tank using the pre-determined mobile phase which was removed from the plate by drying with a stream of cool air from a heating gun. Separated spots were visualised under UV light and marked by pencil (Figure 2A). Developed plates were placed upside-down in the petri dishes containing bottom agar (nutrient agar, Torlak, Belgrade). Soft agar (0.7% Nutrient agar) was melted and poured into sterile tubes (100 ml) in which the dye and bacteria were added quickly. That mixture was flowed over the chromatograms in the petri dishes. After the agar has solidified, the plates were inverted and incubated at 35°C for 24h. The clear zones on the chromatogram indicate areas of inhibition zones on the red background where bacteria are present. Comparing clearing zones with reference TLC plate according to R_f values the most active components of crude fungal extracts could be approximately detected (Fig. 1B).

Bioautography results showed many antibacterial compounds against animal strain of *S. aureus* that were mostly present in the polar region of the bioautogram. According to detected clearing zones, chloroform extracts were more active corresponding to more detected UV absorptive substances along the chromatogram. However, these substances were not active in methanolic extracts on bioautogram for *C. versicolor* and *P. betulinus*.

Developing system: toluene-ethyl acetate – 90% formic acid (5:4:1 v/v/v). **Detection:** 366 nm UV light without spraying. **Extracts:** lane 1- *M. giganteus* (MeOH), lane 2- *L. sulphureus* (MeOH), lane 3- *C. versicolor* (MeOH), lane 4- *F. velutipes* (MeOH), lane 5- *G. lucidum* (EtOH), lane 6- *G. applanatum* (MeOH), lane 7- *P. tigrinus* (MeOH), lane 8- *P. betulinus* (MeOH), lane 9- *P. ostreatus* (MeOH), lane 10- *F. hepatica* (MeOH), lane 2'- *L. sulphureus* (CHCl₃),

lane 3'- *C. versicolor* (CHCl₃), lane 4'- *F. velutipes* (CHCl₃), lane 6'- *G. applanatum* (CHCl₃), lane 7'- *P. tigrinus* (CHCl₃), lane 8'- *P. betulinus* (CHCl₃) **B: Bioautogram of extracts for *S. aureus*^a. Extracts:** lane 1- *M. giganteus* (MeOH), lane 2- *L. sulphureus* (MeOH), lane 4- *F. velutipes* (MeOH), lane 3- *C. versicolor* (MeOH), lane 6- *G. applanatum* (MeOH), lane 5- *G. lucidum* (EtOH), lane 7- *P. tigrinus* (MeOH), lane 8- *P. betulinus* (MeOH), lane 9- *P. ostreatus* (MeOH), lane 10- *F. hepatica* (MeOH), lane 2'- *L. sulphureus* (CHCl₃), lane 3'- *C. versicolor* (CHCl₃), lane 4'- *F. velutipes* (CHCl₃), lane 6'- *G. applanatum* (CHCl₃), lane 7'- *P. tigrinus* (CHCl₃), lane 8'- *P. betulinus* (CHCl₃).

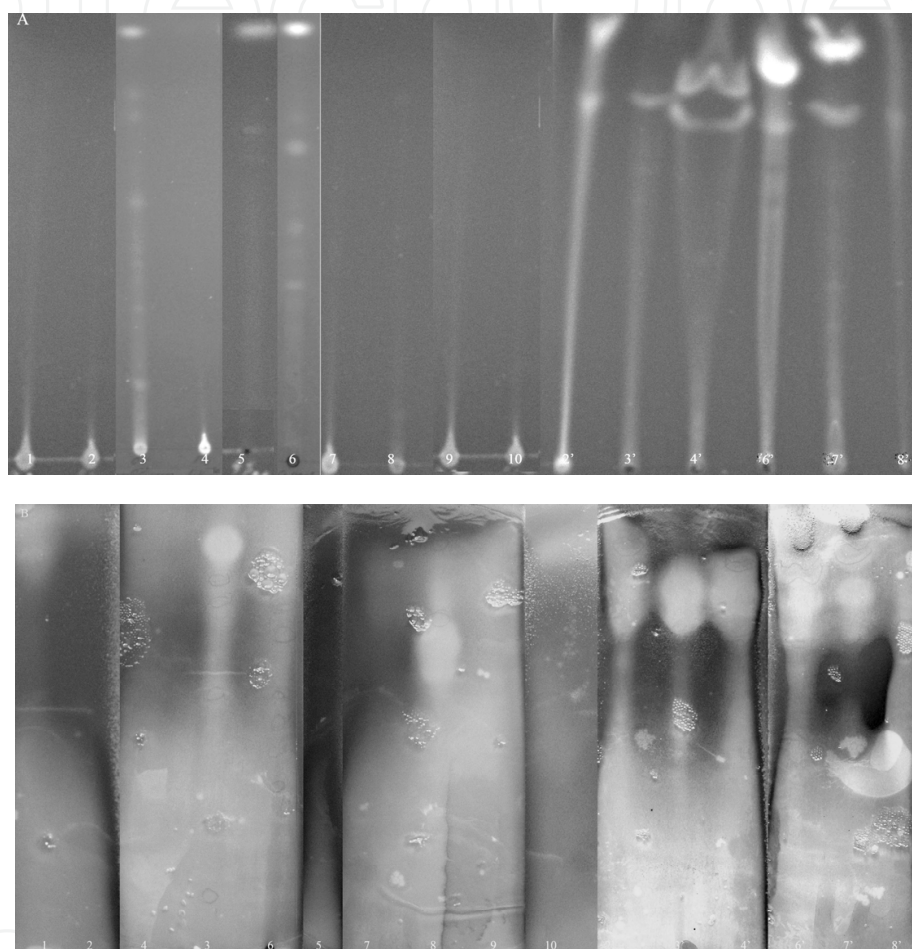


Fig. 1. **A:** TLC separation of crude extracts (methanol - MeOH and chloroform - CHCl₃) of selected lignicolous species prepared for bioautography assay and **B:** bioautogram of extracts for Gram- positive bacteria *S. aureus*, animal strain

1.8 Target organisms

Bacillus subtilis is a Gram + bacteria, non-pathogenic to humans and can be used as a model organism in similar tests, since the representative of the same genus, bacteria *B. anthracis* is responsible for the disease anthrax, which is characterized by the appearance of edema, hemorrhage and tissue necrosis. It is common in some animals, often used as a biological weapon in bioterrorism. If an extract shows activity against *B. subtilis*, it is possible to be active against *B. anthracis* and possibly against other pathogenic Gram + bacteria such as specieses of the genera *Staphylococcus* and *Streptococcus*.

Escherichia coli. *E. coli*, Gram - bacteria, inhabits the gastro-intestinal tract of humans and warm-blooded animals, making their normal indigenous microflora. In immuno-suppressed patients, however, it can cause infections, sometimes fatal (Giovannini, 2006). Gram - bacteria cause more problems than Gram +, as a result of their different cell wall structure. Since penicillin and cephalosporin antibiotics belong to the group that act at the level of cell wall synthesis, the exploration of new types of antibiotics is very important for group of Gram - organisms.

C. albicans belongs to Deuteromycota, representing yeasts forming pseudo-mycelia. It lives as a part of the normal human microflora, especially in the mucosa of the mouth and vagina. In immuno-suppressed individuals (AIDS, chemotherapy, inadequate nutrition and poor hygiene), or after prolonged use of antibiotics, it can cause disease called candidiasis, which is the most common caused by *C. albicans* as the most widespread species. It may affect almost any tissue, starting with simple children's thrush, and ending as the systemic infections. Most commonly it is manifested in the form of slimy mucus. *C. albicans*, is very convenient target organism in the detection of new antifungal drugs.

2. Determination of active substances

In the last decades of the 20th century, the study of macrofungi was intensified, including the research of structurally different metabolites (polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins, lectins, etc..) originating from the primary or secondary metabolism of fungi, as well as different biological activities that they express. Metabolites from fungal fruit bodies or spores themselves are substantially different from those that come from extracellular liquid of the medium in which submerged mycelium was grown or from cells of the culture. Since the phenomenon of multidrug-resistance of microorganisms is on the rise, the studies of macrofungi increased in range, in spite of the fact that they are very slow growing organisms. The value of macrofungi and the dietary supplements, originating from these organisms, grows each year on the world market. They are very safe and considered as the factors useful in the daily diet, especially for people suffering from various diseases.

Natural-products chemists further purify active chemicals from crude extracts by a variety of methods. The chemical structures of the purified material can then be analyzed. Techniques for further chemical analysis include chromatography, bioautography, radioimmunoassay, various methods of structure identification, or modern techniques such as atom bombardment mass spectrometry, Gas chromatography-mass spectrometry, high-performance liquid chromatography, capillary zone electrophoresis, nuclear magnetic resonance spectroscopy, and X-ray crystallography.

3. Conclusion

The presented results indicate that extracts from lignicolous macrofungi could be used in the prevention and treatment of Gram-positive bacterial infections resistant to antibiotics in animals (humans), although further toxicity assays (*in vivo*) must be performed before its application. The fact that fungi can have bactericidal properties with low cytotoxicity to the animal host underscores their usefulness as natural sources of human or veterinary medicines.

Also, the results obtained should stimulate further studies of other, so far unexplored, species such as *M. giganteus* and *P. tigrinus*, since current knowledge of the antibacterial activities or chemical composition of their active agents is not capable of fulfilling the expectations.

4. Acknowledgment

This work is fully supported by the project No 172058 of Ministry of Education and Science of Republic of Serbia

5. References

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