Chapter 9

Natural Products from Plants and Fungi as Fungicides

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1. Introduction

In early fifties of the twentieth century the agrochemical industry provided agriculture with a vast array of chemicals for crop protection, including fungicides. Random synthesis, biological screening and empirical optimization yielded many effective compounds (Cremlyn, 1991). Whereas it is generally acknowledged that the use of pesticides has large benefits to farmers, the present use of pesticides in agriculture also causes negative environmental (and health-related) effects to society. For example, during and after application of pesticides a substantial amount of it could end up in soil, ground- and surface water or air. These negative effects demands for an effective policy. Such policies have been initiated, both at the level of the individual Member States of the European Union and at the level of the European Union itself (Oppenheimer & Donnelly, 1997). Their selectivity between target organisms and plants is mainly based on differences in uptake. The more recently developed protective chemicals are more potent in terms of dose required to control the pest or disease, and in distinguishing between target and non-target organisms. They usually have a specific mode of action. Since selective compounds are specific site inhibitors in the metabolism of target organisms, the risk of developing resistance is high. This has occurred for a number of fungal plant pathogens (Delp, 1988). Although pathogenic microorganisms are mainly controlled chemically, the use of synthetic compounds is limited due to several undesirable aspects, which include carcinogenicity, teratogenicity, acute toxicity and the requirement of an extended degradation period with consequent development of environmental pollution problems. The new awareness of modern consumers about these problems has created a "green" consumer profile that demands the absence of synthetic chemicals in food production and preservation together with extended shelf life of the majority of food products. Fungal infections remain a therapeutic problem in many fields despite the availability of a number of treatments. Such diseases in humans have markedly increased during the past ten years, especially in immunocompromised

patients. Consequently, up to 10% of hospital acquired systemic infections are caused by fungi. Altogether this forces the scientific community, agro–industry and pharmaceutical companies to search for natural compounds that will satisfy consumer requirements (Harvey, 2008). Furthermore, there is growing concern about chemicals for protection because of their undesirable side effects in humans, other target organisms and their behavior and fate in the environment (Jespers, 1994).

The total number of all known natural products is around one million, including both bioactive and inactive compounds, plants metabolites 600000, fungal metabolites 8600, microbial metabolites recognized until now is around 50000. It is an obvious question, where is the border in the diversity of natural products? The general needs of the human society are continuously increasing. We need every new compounds which may be useful for the human society. More food, new drugs, and other goods are highly necessary for the benefit of humankind. The only question is the existence of sufficient natural and technical resources to fulfill these demands. Fortunately, in the area of the research of bioactive microbial products it seems that the ever expanding scientific and technical possibilities are increasing together with the continuously widening needs of the human therapy, veterinary and agriculture. The problem really is not whether we would be able to discover further new useful microbial compounds, but rather how can we optimize and quickly and effectively apply the chances derived from the new discoveries. How can we pick up and use effectively the proverbial needle found in the haystack (Berdy, 2005). However, screening of more than a million substances in the last decade has resulted in the introduction of only a very limited number of compounds with novel modes of action and resistance. This explains the renewed interest of the chemical industry in natural compounds with a variety of unique characteristics, waiting to be exploited. Natural products derived from plants and fungi have traditionally been used in ethnomedicine. Throughout the development of both Western and Eastern civilizations, whole plants, fungi, their parts, derived compounds and extracts have functioned as sources of food and medicine, symbolic articles in religious and social ceremonies, and remedies to modify behavior. Plant and fungal extracts and compounds containing physiologically active biochemicals have immense potential for producing new agents of great benefit to mankind. In this context, systematic screening of secondary metabolites of folk herbs and fungi may result in the discovery of novel and effective antimicrobial compounds (Hussain et al., 2011). Recently, interest has been growing in natural products due to their availability, fewer side effects and less toxicity as well as better biodegradability when compared to other available antimicrobial agents and preservatives. Thus, plants and mushroom may offer great potential and hope. Consequently, natural products are attracting the attention of scientists because they are cheaper, safer, eco-friendly and within the reach of the current medical community. This paper gives an overview on the activity of plant and fungi derived extracts as well as their constituents against a wide variety of microfungi, methodology and potential uses (Figure 1.).

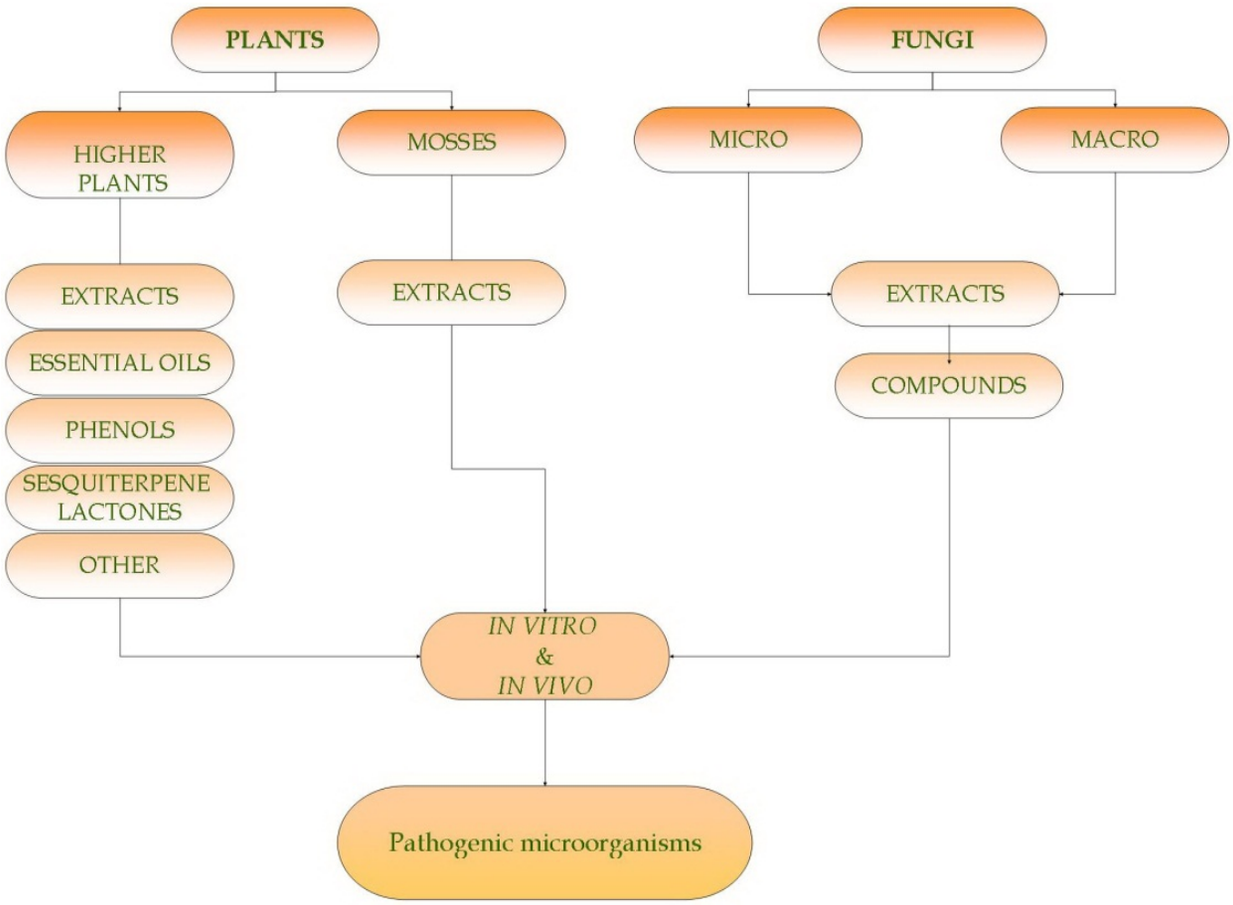

Figure 1. Our approach for testing of antifungal activity of natural products from fungi and plants.

2. Methodology

In order to test antifungal activities of natural products derived from plants and fungi few conventional and non-conventional methods were applied. Method is selected depending on the characteristics of extracts and compounds tested. A various number of plant, animal and human fungal pathogens were used. Growth cultures are conducted under optimal physical conditions for individual species. The growth of fungi was assessed visually or instrumentally. Two replicates were done for each compounds and the experiment was repeated two times.

Agar diffusion method is suitable for testing of antifungal activity of hydrophilic compounds which easily could be dispread trough to the agar medium. The compounds investigated were mixed with 0.01% Tween 20 surfactant and dissolved in molten MA medium. The fungal species were cultured for 7 days on Malt agar medium. Micromycetes were inoculated in the centre of Petri dishes and incubated for 21 days at 25° C. Mycelial growth was observed every 7 days and compared with the control. The commercial fungicide was used as a positive control (Ishii, 1995). The minimum inhibitory concentration (MIC) of compounds was determined when it achieved a complete stop in the growth of mycelium.

Microdilution method is suitable for testing small quantities of extracts, fractions or components, simultaneously in many different concentrations. In order to investigate the antifungal activity of the compounds, a modified microdilution technique was used (Hanel & Raether, 1988; Daouk et al., 1995). The fungal spores were washed from the surface of agar plates and adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μl per well. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in 5% DMSO solution containing 0.1% Tween 80 (v/v) (1 mg/ml) and added in broth Malt medium with inoculum. The microplates were incubated at Rotary shaker (160 rpm) for 72 h at 28 °C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of a 2 μl of tested compounds dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 μl of broth per well and further incubation 72 h at 28 °C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. DMSO was used as a negative control, commercial fungicides were used as positive controls.

Microatmosphere test, a slightly modified agar disc diffusion method, is perfectly suitable for the estimation of essential oil activity in vapor phase (Zollo et al., 1998). The assay was performed using mushroom pathogenic fungi. Petri dishes were filled with malt agar (MA), and then seeded with a 7 day-old mycelial culture of the tested fungi. The Petri dishes were then inverted and the determined amount of essential oils impregnated on sterile filter paper discs (4 mm) attached to the inverted lid (1 disc per lid). The Petri dishes were wrapped with parafilm along the rim, inverted and incubated for 21 days at 25 °C in an incubator. The results are presented as the diameter of the microorganism growth inhibition zone, or as the essential oil minimal inhibitory quantity (MIQ), which inhibits the total growth of microorganism. Commercial fungicides were tested as a control.

Bioautography is widely used for the examination of extracts. When the solvent evaporates, the broth and microorganisms are applied on the chromatographer paper or plates, and after the incubation time, growth is scrutinized. No growth is observed on the active spot components. Simultaneously, the components of the extract are eluted and identified. Different volumes of the mycelium extracts and pure compounds were dissolved in appropriate solutions. Ten micro liters of each sample were applied on TLC plates and sprayed either with freshly prepared fungal suspensions in nutrient broth (TSB). The plates were incubated for 18 h at 37°C and then sprayed with aqueous sol. 3% of p-iodonitrotetrazolium violet and stored for another 3 h. After this period plates were sprayed with 70% EtOH to stop fungal growth and were incubated for 36 h at 27 °C. White inhibition zones on a pinkish background were indicative of antimicrobial activity of tested extracts or compounds. The widths of these zones (mm) are the measure of efficiency and presented as minimal inhibitory concentration (MIC) (Pacher et al., 2001). As positive controls commercial fungicides have been used.

3. Antifungal activity against plant pathogens

3.1. Fungal extracts and metabolites

The fungi constitute a very large group of organisms that are found everywhere and are of great importance to life on earth and to human society. This is mainly due to the many interactions among fungi and other organisms. Most fungi produce a wide variety of secondary metabolites with biological activity. A wealth of literature exist on the discovery and potential use of mycotics in agriculture (Berdy, 1980; Guterson, 1990). However, their exploitation in agriculture remained limited, because widespread application in crops might simultaneously select for resistance to these mycotics in human pathogens (Isono, 1990). The concept that substances derived from one living organism may affect another organsm is old. Some of the secondary metabolites that occur in fungi are fairly widespread, but many are confined to a few species. Hence, screening of further fungi species usually leads to the discovery of new bioactive secondary metabolites. The broad diversity of the fungi, as well as their easy acquisition makes them especially interesting for natural products screening program. Among fungal species, the various microscopic (filamentous) fungi (ascomycetes, fungi imperfecti, *etc.*) are the most frequent producers with about 6400 produced compounds. From the most common ascomycetes, namely from *Aspergillus*, *Penicillium* and *Fusarium* species 950, 900 and 350 compounds have been isolated, respectively. Besides them several other filamentous and endophytic species (*Trichoderma*, *Phoma*, *Alternaria*, *Acremonium* and *Stachybotrys*), are also good producers, each produces several hundreds of bioactive compounds. From higher fungal species – basidiomycetes or mushrooms – exemplified by *Ganoderma*, *Lactarius* or *Agaricus* species, altogether about 2000 active compounds have been derived. From yeasts, only 140 and from Myxomycetes (slime moulds) species 60 bioactive metabolites have been isolated. The chemically relatively simple fungal compounds, over the antibiotic activities frequently exhibit diverse biological effects, mainly phytotoxic and pharmacological activities. We should not forget, the great practical and historical importance of beta-lactams (penicillins, cephalosporins), the cyclosporin, and various statins (mevinolin, compactin, lovastatin, pravastatin, atrovastatin), which are all fungus derived compounds. Recently it is unquestionable that the interest to all types of fungal species, but mainly to endophytic and the so called marine fungi as possible sources of new bioactive compounds is highly increasing. The expansion of the very quick new screening methods led to the appearance of the increasing number of "unidentified" fungus as bioactive metabolite producers (over 250 new metabolites in the last two years), especially in the patent literature. It indicates the high speed of isolation, and identification/patenting process of new fungal products, and the long time need for taxonomical identification of new fungal species (Berdy, 2005). Particularly desirable is the discovery of novel prototype antimicrobial agents representing new chemical classes that operate by differet modes of action from existing agents and, consequently, lack cross-resistance to chemicals currently used. Kurobane et al., (1981) reported that *Penicillium brefeldianum* produces fulvic acid which possesses antiviral, antifungal, antioxidant and antibiotic activities. Maskey et al., (2003) isolated two active substances,

8-O-methylaverufin and 1,8-O-dimethylaverantin as new antifungal agents from *Penicillium chrysogenum*. Nam et al., (2000) found that 8-O-methylsclererotiorinamine isolated from *Penicillium multicolor* showed antimicrobial activity. Funiculosin, found in *Penicillium funiculosum* possesses antibiotic activity (Ando et al., 1969), as well as substance SQ 30,957, a new antibiotic produced by *P. funiculosum* (Singh et al., 1986). We recently started an investigation on the antifungal activity of fungal extracts and metabolites from both micro- and macrofungi. The extracts of 17 microfungi (*Alternaria alternata, Cladosporium cladosporioides, C. fulvum, Fusarium sporotrichioides*, *F. trincintum, Paecilomyces variotii, Penicillium ochrochloron, P. funiculosum, Phoma magdonaldii, Phomopsis helianthi, Stachybotrys chartarum, Trichoderma viride,* and five dermatomycetes, *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes*, *T. rubrum* and *T. tonsurans* were tested against the yeast *Candida albicans* using the bioautographic assay test on TLC plates (Rančić, 2004).

While herbs are rather commonly used in the Western hemisphere, medicinal use of mushrooms, which has a long tradition in Asian countries, has also slightly increased in Europe during the last few decades. Although there has been extensive research on properties of medicinal mushrooms, their true potential is yet to be revealed. A number of compounds possessing significant antimicrobial activity have been isolated from polypore fungi. They provide a rich variety of active secondary metabolites and polysaccharides. Medicinal mushrooms such as *Agaricus brasiliensis, Coprinus comatus, Coriolus versicolor*, *Ganoderma lucidum, Lentinula edodes, Phellinus linteus*, and many others have traditionally been used as health foods or supplements for the prevention and cure of a range of diseases, including atherosclerosis, cancer, chronic hepatitis, and diabetes. The preventive and therapeutic effects of these mushrooms and their components have been well documented in mouse and rat model systems and in cancer cell lines. This has led to a considerable amount of knowledge about the effects of mushroom extracts and of their modes of action. It is generally accepted that mushroom extracts contain a variety of components, such as polysaccharides (*i.e.* glucans), small proteins, lectins and polyphenols, each of which may have its own biological or medicinal effect. The most common immunomodulatory action of mushroom are attributed to β-(1→3)-(1→6)-glucans, which have been studied in some detail (Smiderle et al., 2010). Vaz et al., (2011) described and compared the chemical constituents (phenol compounds, macronutrients, sugars, fatty acids, tocopherols and ascorbic acid) of four wild edible mushrooms widely appreciated in gastronomy: *Armillaria mellea, Calocybe gambosa*, *Clitocybe odora*, *Coprinus comatus*. Polysaccharides have emerged as an important class of bioactive substances, and many medicinal and therapeutic properties are attributed to them (Alquini & Carbonero, 2004). *Trametes versicolor*, *Laetiporus sulphureus* and *Ganoderma lucidum* are just some of the known mushrooms with this potential. This alone has made them suitable candidates for critically needed new antibiotics and antimycotics (Zjawiony, 2004). *Laetiporus sulphureus* is a wood-rotting basidiomycete, growing on several tree species and producing shelf-shaped fruit bodies with a bright yellow fleshy margin. This recognizable pigmentation along with the fruit body form is

responsible for the trivial name under which this fungus is known, and that is sulfur shelf (Weber et al., 2003). Even though it is recognized as a source of active compounds, and is widely used as a food among human, reports on the antimicrobial activity of *L. sulphureus* extracts are scarce (Turkoglu et al., 2007; Zjawiony, 2004). The potential barrier to everyday use of medicinal mushrooms as therapy is the manner in which the mushroom is consumed. Most research on fungi as potential antimicrobial agents is based on ethanol and methanol extracts of the fungal fruit body (Barros et al., 2007; Turkoglu et al., 2007). Consumption of products on this basis is of no practical use. To test the antifungal activity of extracts and metabolites of macrofungi we chose the wood-rotting basidiomycete, *L. sulphureus*, also named chicken of the woods. It is known for its nutritional value. An aqueous extract obtained from *L. sulphureus* was investigated for antimicrobial properties using a microdilution assay *in vitro* against seven fungi (four *Aspergillus,* two *Penicillium* species and *Trichoderma viride*). This extract showed strong activity against the tested microorganisms in a dose dependent manner (Šiljegović et al., 2011a). The presence and growth of microfungi in food may cause spoilage and result in reduction in quality and quantity. The presence of toxigenic fungi in foods stored for long periods of time is a potential hazard to human and animal health. Consumption of tomato products has been associated with a lower risk of developing digestive tract and prostate cancer. Therefore, preservation of tomato paste seems to be of great importance, both for the food industry, and for human well-being. We have used a methanol extract of *L. sulphureus* as an *in vivo* inhibitor of *Aspergillus flavus* growth in tomato paste. The results indicated complete inhibition of *A. flavus* growth in tomato paste for 15 days. An inhibition rate of 99.83% was achieved with 0.15 mg/ml of extract. Complete fungicide activity (100%) and no spore survival in the tomato product was recorded using 0.25 mg/ml of *L. sulphureus* extract in tomato medium. Since *L. sulphureus* is widely consumed as an edible macrofungus, its use as a natural preservative in tomato products can be considered as safe (Stojković et al., 2011a).

3.2. Plant extracts and metabolites

With increasing acceptance of traditional plants as an alternative form of health care the search for active compounds in plants becomes very important. Medicinal and aromatic plants have been employed for many centuries and they are mentioned in folklore from ancient times. After the advent of antibiotics in the 1950s, the use of plant derivatives as antimicrobials become virtually nonexistent to be rediscovered, as well as other alternative forms of medical treatments in the late 1990s (Cowan, 1999). There are several approaches to choosing sources of natural products for the discovery of potential antifungal compounds. One of approach is to investigate whole extracts of potential antifungal plants. Other approaches are to obtain biological material, which has not previously been studied for fractionation and testing, or some other sources. One strategy is to use ethnobotanical and/or chemical ecology clues to select which plants to sample (Duke et al., 2000). Here at first we will discuss the antifungal activities of plants extracts and after that some secondary metabolites derived from plants.

3.2.1. Plant extracts

There are many reports concerning the antifungal activity of plant extracts, but we will mention only a few. Ushiki et al., (1996) found that root extracts from twelve medicinal plants displayed antimicrobial activity against certain pathogens of soil-borne plant diseases. Among these plants, *Geranium pratense* (Bigroot geranium) strongly inhibited the growth of *Streptomyces scabies* which causes common scab of potato. It was shown that geranin, isolated from Bigroot geranium roots possessed a 1.25% higher antimicrobial effect than streptomycin (Ushiki et al., 1997). Previous studies indicated that certain crops and vegetables contain antimicrobial substances in their roots, and that these substances directly suppress growth of the pathogen and development of the disease (Clarke, 1966; Masaoka et al., 1993; Naqvi & Chauhan 1980; Yoshihara et al., 1988). In a program to screen extracts from medicinal plants for fungicidal activity, it was found that aqueous extracts of *Reynoutria suchalinensis* (Polygonaceae) showed favorable protecting control of powdery mildew (Herger et al., 1988).

An ethanol extract of *Phlomis fruticosa* (Jerusalem sage) (Labiatae) tested by diffusion method inhibited *Aspergillus niger, Penicillium ochrochloron, Trichoderma viride, Fusarium tricinctum* and *Phomopsis helianthi.* Moreover, this extract had fungicidal activity against *Cladosporium cladosporioides* and *Aspergillus ochraceus* at a very low concentration (10-20 μg/ml), (Ristić et al., 2000). Further investigation showed that when this extract was hydrolyzed with HCL and β-glucosidases, which remove sugars from flavonoids, it possessed greater antifungal activities than the original ethanol extract. Lower antifungal activity of the whole ethanol extract may be due to the presence of some aglycones, unstable flavonoid glycosides (Soković et al., 2000). It is generally known that flavonoid glycosides show lower activity against the microorganisms than aglycones (Raoha et al., 2000). Strong antifungal activity of a dealcoholized extract of leaves of *Cassia tora* was obtained against *Aspergillus niger* (Mukherje et al., 1996). Sato et al., (2000) analyzed 29 plants extracts against *Arthrinium sacchari* and *Chaetonium funicola*. The ethanol extracts of fifteen plants showed antifungal activity, but *Acer nikoense* (Nikko maple), *Glycyrrhiza glabra* and *Thea sinensis* (Tsa) were the most effective plants in very low amounts. Whole, fresh involucral bracts of cardoon, *Cynara cardunculus* L. (Compositae), were extracted with EtOH and an aqueous suspension of this extract was partitioned successively with $CHCl_3$, EtOAc and n-BuOH, leaving a residual water extract. Each extract was evaluated for antifungal properties. Antimicrobial activity was estimated using a microdilution technique against *Aspergillus niger, A. ochraceus, A. flavus, Penicillium ochrochloron, P. funiculosum, Trichoderma viride, Fusarium tricinctum* and *Alternaria alternata*. All cardoon extracts were found to possess antifungal activity comparable with standard mycotics (Kukić et al., 2008). Antifungal assays of branched centaury *Centaurium pulchellum* (Gentianaceae) extracts and secoiridoid glycosides isolated from this extracts have been studied as potent bioactive compounds against five fungal species. Methanol extracts from both aerial parts and roots exhibited excellent antifungal (0.1-2 mg/ml) activity. Pure secoiridoid glycosides isolated from these extracts demonstrated very strong antifungal (0.001-0.1 mg/ml) activity (Šiler et al., 2010). The antifungal activity of methanol extracts of three different Labiatae species (Catmint),

Nepeta rtanjensis, N. sibirica and *N. nervosa* (grown *in vitro*) against eight fungal species, was evaluated. All tested extracts showed significant antifungal activity, with that from, *N. rtanjensis* being the strongest (Nestorović et al., 2010).

Genuine mosses constitute a large group of nonvascular higher plants, consisting of about 14 000 species. Generally, bryophytes are not damaged by microorganisms, insects, snails, slugs, and other small mammals. Up to date, over several hundred new compounds have been isolated from bryophytes and their structures elucidated (Veljić et al., 2009). In spite of a number of secondary metabolites identified from various mosses, the chemical profiles of most species are insufficiently known or even unknown. Secondary metabolites from mosses, identified so far, include terpenoids, flavonoids and bibenzyls, and also derivatives of fatty acids. Mosses rich in flavonoids has been found to possess strong antimicrobial activity (Veljić et al., 2009). An ethanol extract of bryophyte, *Bryum argenteum* (silver moss), showed antifungal activity against two fungi (*A. niger* and *P. ochrochloron*) (Sabovljević et al., 2006). Our investigations also demonstrated that methanol extracts of selected genuine mosses (*Pleurozium schreberi*, *Palustriella commutata*, *Homalothecium philippeanum*, *Anomodon attenuatus, Rhytidium rugosum*, *Hylocomium splendens*, *Dicranum scoparium* and *Leucobryum glaucum*) possess antimicrobial activity when tested by the microdilution method (Veljić et al., 2008). When the antifungal activity of methanol extracts of the mosses *Fontinalis antipyretica* var. *antipyretica*, *Hypnum cupressiforme* and *Ctenidium molluscum* were analyzed, that of the first species showed strongest activity against the following micromycetes: *Trichoderma viride*, *Penicillium funiculosum*, *P. ochrochloron*, *Aspergillus fumigatus, A. flavus* and *A. niger* (Veljić et al., 2009). The antifungal activity of extracts of three bryophyte species, two mosses (*Atrichum undulatum, Physcomitrella patens*) and a liverwort (*Marchantia polymorpha* ssp. *ruderalis*), grown under natural conditions and in axenic culture, was evaluated by the microdilution method against five fungal species. Each bryophyte extracts was active against all fungi tested. In general, extracts made from material grown in laboratory (*in vitro*) conditions express stronger antifungal activity than those made from material from under natural conditions. Some of the fungi tested reacted similarly to both extracts (Sabovljević et al., 2011). The antimicrobial activity of a dimethyl sulfoxide extract of the moss *Rhodobryum ontariense* was evaluated by microdilution method against *Aspergillus versicolor*, *A. fumigatus*, *Penicillium funiculosum*, *P. ochrochloron* and *Trichoderma viride*. The extract was active against all the fungi tested but to varying degrees. This finding implies that *R. ontariense* could be considered as a promising material for natural antifungal products (Pejin et al., 2012 in press).

3.2.2. Plant secondary metabolites

Plants produce a diverse array of secondary metabolites, many of which have antifungal activity. Some of these compounds exist in healthy plants in biologically active forms. Others, such as cyanogenic glycosides and glucosinolates, occur as inactive precursors and are activated in response to tissue damage or pathogen attack. This activation often involves plant enzymes, which are released as a result of breakdown in cell integrity. Compounds

belonging to the latter category are still regarded as constitutive because they are immediately derived from preexisting constituents (Mansfield, 1983). A large number of plant compounds have been reported to have antifungal activity. Well known examples include; flavonoids (Ćirić et al., 2011; Karioti et al., 2011; Weidenbörner & Jha 1997), lactones (Djeddi et al., 2007; Janaćković et al., 2002; Skaltsa et al., 2000a, 2000b; Vajs et al., 1999, 2004), proteins (Giudici et al., 2000), sulfur compounds (Ilić et al., 2012), cyanogenic glycosides and glucosinolates (Osbourn, 1996) and essential oils (Daouk et al., 1995; Džamić et al., 2010; Garg & Siddiqui, 1992; Glamočlija et al., 2006a, 2006b, 2009; Marinković et al., 2002; Mishra & Dubey, 1994; Müller-Riedau et al., 1995; Rančić et al., 2005; Shimoni et al, 1993; Soković, 2001, 2002, 2008a, 2008b, 2009a, 2009b, 2009c; Stojković et al., 2011b, 2011b; Thompson, 1989).

3.2.2.1. Essential oils

Essential oils from aromatic and medicinal plants have been known since antiquity to possess biological activity and constitute one of the most investigated groups of secondary metabolites. With growing interest in their use in the pharmaceutical and agrochemical industries, systematic examination of oils for these properties has become increasingly important. Over the last hundred years antimicrobial properties of common spice oils have been demonstrated (Bullerman et al., 1977) and many studies have been made on antifungal activities of essential oils (Daouk et al., 1995; Garg & Siddiqui, 1992; Glamočlija, 2006b, 2009; Kalemba & Kunicka, 2003; Mishra & Dubey, 1994; Müller-Riedau et al., 1995; Shimoni et al, 1993; Soković, 2001; Thompson, 1989). Thus, Maruzzela & Balter (1959) found that, out of 119 spice oils tested, 100 essential oils possessed an antagonistic effect to at least one of twelve phytopathogenic fungi and 50 of these compounds showed wide spectrum activity against all fungi tested. The essential oil of *Origanum majorana* exerted considerable inhibitory powers against *Aspergillus flavus, A. niger, A. ochraceus, A. parasitcus* and *Trichoderma viride* (Deans & Svoboda, 1990). A comparative study of the antifungal activity of essential oils extracted from thyme, rosemary, eucalyptus and mugwort was carried out by a group of investigators against 39 mold strains. The essential oil of thyme was found to be the most effective (Conner & Beuchat, 1984). Essential oils of allspice and cloves totally inhibited *Trichoderma viride, Alternaria alternata, Fusarium oxysporum, Mucor circinelloides, Rhizopus stolonifer, Cladosporium cladosporioides, Aspergillus versicolor* and *Penicillium citrinum* in a concentration of 2% (Schmitz et al., 1993). Essential oils from other plants such as Wormwood *Artemisia afra,* Lavender tree, *Heteropyxis natalensis* and Sweet gale *Myrica gale,* were found to have strong inhibitory effects against a broad spectrum of fungal species. The essential oil of *Origanum syriacum* showed very strong antifungal activity against *Penicillium, Aspergillus* and *Fusarium* species (Daouk et al., 1995 and references cited therein). The essential oil of Soldier's herb *Piper angustifolium* was very effective effect against *Aspergillus niger* and *A. flavus* (Trillini et al., 1996). The antifungal activities of the essential oils from Lemon mint *Monarda citriodora* and Tea tree *Melaleuca alternifolia* were evaluated *in vitro* on fifteen common post-harvested pathogens of a variety of crops. Both essential oils exhibited a high level of antifungal activity, by direct contact and in the vapor phase. Oil from Lemon mint was generally more active than that from Tea tree, particularly against rapidly growing fungal species (Bishop & Thornton, 1997). Baratta et al., (1998) examined essential oil from

eight commercial plants (*Cinnamomum zeylanicu* (Cinnamon), *Cananga odorata* (Ylang ylang), *Ocimum basilicum* (Sweet basil), *Citrus limon* (Lemon), *Cymbopogon citratus* (Lemon grass), *Baswellia thurifera (*Boswellia), *Majorana hortensis* (Marjoram) and *Rosmarinus officinalis* (Rosemary) and showed that all the oils tested were able to inhibit the growth of the common spoilage fungus, *A. niger*, even at a concentration of 1 μl/ml broth, with the exception of lemon and rosemary oils which exhibited inhibitory effects on higher concentrations. Essential oils extracted from different parts of some angiosperms (Cedarwood *Cedrus deodara* and Ajwain *Trachysremum ammi*) were analyzed for fungitoxicity against the mycelial growth of *Aspergillus niger* and *Curvularia ovoides,* two fungi found in *Vigna mungo.* Since the essential oils from both plants exhibited fungitoxic properties, it may be possible to use them to control various fungi and exploit them as fungicides (Singh & Tripathi, 1999). The antifungal activities of four essential oils from spice (sage, thyme, oregano and savory) were analyzed against *Fusarium oxysporum, Macrophomina phaseoli, Botrytis cinerea, Rhizoctonia solani, Alternaria solani* and *Aspergillus parasiticus.* Earlier results showed weak activity for sage, while thyme, oregano and savory were active against all moulds tested (Ozcan & Boyraz, 2000). Different essential oils have antifungal activity against a wide range of fungi. Thus, considering the importance of these oils, 75 different essential oils were tested against *A. niger*. All the oils possessed antifungal activity (Pawer & Tacker, 2006). We examined a variety of essential oils from several plant families (Compositae, Labiatae, Lauraceae, Apiaceae, Cupressaceae, Poaceae, Illiaceae, Myrtaceae, Verbenaceae). Essential oils of Wild marjoram *Origanum onites,* Thyme-leaved savory *Satureja thymbra,* Greek sage *Salvia fruticosa* and *S. pomifera* subsp. *calycina* plants growing wild in Greece and their components; carvacrol, camphor, and 1,8-cineole, were assayed for antifungal activity against thirteen fungal species. The oils inhibited all fungi investigated. The highest and broadest activity was shown by oils containing the carvacrol (Wild marjoram and Thyme-leaved savory, while Greek sage was the least effective (Soković et al., 2002). The antifungal activity of essential oils from three *Micromeria* species: *M. dalmatica*, *M. albanica* and *M. thymifolia* was investigated against seven fungal species. The oils from all three *Micromeria*: (*M. dalmatica*-0.2-0.4 μl/ml, *M. thymifolia*-0.4-2 μl/ml) and particularly *M. albanica* (0.2-0.4 μl/ml) showed strong antifungal effects against all fungi tested: *Aspergillus niger*, *A. ochraceus*, *Penicillium ochrochloron, Cladosporium cladosporioides, Fusarium tricinctum*, *Phomopsis helianthi* and *Trichoderma viride* (Marinković et al., 2002). The essential oil of *Foeniculum vulgare* was tested for antifungal activity against nine different plant pathogenic fungi. This oil was most effective against *C. cladopsorioides* and *P. helianthi* (0.8-4.0 μl/ml), more effective than bifonazole (Mimica-Dukić et al., 2003). The antifungal activity of oils from eight *Stachys* species were tested against fungal pathogens (*A. niger* and *Penicillium ochrochloron*). The greatest activity was obtained for *S. scardica* due to the high content of sesquiterpene hydrocarbons (69.3%) in this oil (Skaltsa et al., 2003). Essential oil from *Juniperus excelsa* (Cupressaceae) was evaluated for antifungal activity against twelve micromycetes from the following genera (*Alternaria, Aspergillus, Cladosporium, Fusarium, Penicillium, Phomopsis* and *Trichoderma*). This essential oil showed moderate-high antifungal characteristics with MIC of 8.0–40.0 μl/ml and MFCs of 10.0–50.0 μl/ml. The essential oil was the most effective against the phytopathogenic species *Phomopsis helianthi* while *Trichoderma*

viride was the most resistant species treated with this essential oil. This essential oil exhibited higher antifungal activity than the commercial fungicide bifonazole (Soković et al., 2004). The antifungal activity of essential oils from different plant species of Lauracaea family (*Aniba rosaeodora* Rosewood, *Laurus nobilis* Bay tree, *Sassafras albidum* Sassafras and *Cinnamomum zeylanicum* Cinnamon) was investigated against seventeen micromycetes. In order to determine fungistatic and fungicidal concentrations (MIC and MFC) both diffusion and microdilution tests were employed. Essential oil from cinnamon was the most effective as an antifungal agent, followed by rosewood and sassafras oils. Laurel oil possessed the lowest antifungal activity (Simić et al., 2004). The antimicrobial activities of essential oils isolated from Sweet cicely *Myrrhis odorata*, St. John's wort *Hypericum perforatum* and *Helichrysum arenarium* were determinated by microdilution test. The greatest antifungal activity was observed for sweet cicely oil, while *H. arenarium* showed the lowest antifungal potential. Minimal inhibitory and fungicidal concentrations were 0.5-60 μg/ml. The oil of sweet cicely showed had higher activity than commercial product and was very effective against all fungi tested (Rančić et al., 2005).

Essential oils and their components from three spices of *Salvia* (Labiatae) were tested for antifungal activity in our laboratory. The antifungal activity of the corolla, calyx and leaf oils of *Salvia brachyodon* were analyzed. Antifungal activities of the essential oils were determined by the microdilution method against *Aspergillus niger*. The oil from the calyx possessed the strongest antifungal activity probably due to synergistic activity of all the components present. This oil contains high concentrations of sesquiterpenes and diterpenes, which were not found in the other samples investigated (Soković et al., 2005). Another species of *Salvia* genus, Clary sage (*Salvia sclarea*) was also tested as an antifungal agent. A concentration of 25 μl/ml showed fungicidal activity against *Aspergillus*, *Penicillium*, *Trichoderma viride* and *Fusarium* species. For *Mucor mucedo* the MFC was 15 μl/ml. Fungistatic and fungicidal activities of the oil against *Cladosporium cladosporioides* was recorded at concentrations of 2.5 μl/ml and 5 μl/ml. The most sensitive micromycetes were *Cladosporium fulvum*, *Alternaria alternata*, *Phomopsis helianthi*, and *Phoma macdonaldii*, where a concentration of 2.5 μl/ml was lethal (Džamić et al., 2008). The essential oil of Sardinian sage *S. desoleana* and its main components were investigated for antifungal activity against eleven micromycetes by macro- and micro-dilution methods. The essential oil and components investigated were diluted in ethanol and Tween 80 in both methods. We analyzed whole essential oil from Sardinian sage and its main components; linalyl acetate, 1,8-cineole and linalool, which together represent 52.71% of the total oil. Whole essential oil of Sardinian sage and linalool possessed strong antifungal activity, while 1,8-cineole exhibited only moderate potential. Linalyl acetate had the lowest antifungal potential. Values for MIC were lower with microdilution method (Soković et al., 2009a). The antifungal activity of *Nepeta rtanjensis* essential oil on mycelia growth has been determined by the diffusion method. It acted most efficiently against *Alternaria* species (0.6 μl/ml). *Bipolaris spicifera* and *Cladosporium cladosporoioides* had MIC values of 1.0 μl/ml, whereas *Trichoderma viride* with an MIC value of 1.6 μl/ml against this essential oil (Ljaljević Grbić et al., 2007). Antimicrobial activity from Citronella *Cymbopogon winterianus* (Poaceae) and Karawya *Carum carvi*

(Apiaceae) essential oils was investigated against seventeen fungal species. Oil of Karawya at concentration of 0.25–2.5 μl/ml stopped the growth of all tested micromycetes except for *T. viride*. For this resistant fungus, the concentration of Karawya oil had to be increased to 10 μl/ml. The essential oil from Citronella was less effective but still showed stronger antifungal activity than the commercial drug bifonazole. The MIC and MFC values of this oil were 1–20 μl/ml (Simić et al., 2008).

Essential oils isolated from the aerial parts during the flowering and vegetative phases, roots and seeds of the plant *Portenschlagiella ramosissima* were tested for antimicrobial activity against micromycetes (*Aspergillus flavus, A. niger, A. versicolor, Penicillium ochrachloron, P. funiculosum* and *Trichoderma viride*). All of the oils tested showed activity. The most effective was that isolated from the aerial parts of the plant during flowering, followed by the oil from the vegetative phase, seeds and roots. The most resistant fungal species were *Aspergillus flavus* and *A. versicolor* (Soković et al., 2008b). Essential oils of Star anise *Illicium verum* (Illiaceae) and Clavos *Eugenia caryophyllata* (Myrtaceae) were investigated as a potential antifungal agents. Star Anise oil exhibited fungicidal characteristics with MIC and MFC values of 2.5–25 μl/ml. Clavos oil showed strong antifungal activity at 0.1–2.5 μl/ml. The most resistant fungi were *Trichoderma viride*, *Penicillium* and *Aspergillus* species. The antimicrobial activity of star anise is mainly due to anethole while eugenol is responsible for antifungal effect of cloves oil. The authors raised the possibility that interactive effects of other compounds present in smaller quantities may also contribute. Thuse, both oils, but especially clove, showed powerful antifungal activity (Džamić et al., 2009). The antifungal activity from essential oils of wild carrot *Daucus carota* L. (Apiaceae) collected in Serbia were tested. The antifungal activity of oils from the ripe fruits, unripe fruits, flowers, root, leaves, and stem, were examined against eight fungal strains (*Fusarium sporotrichoides, Fulvia fulvum, Trichoderma viride, Penicillium ochrochloron, P. funiculosum, Aspergillus ochraceus, A. flavus* and *A.fumigatus*) by the microdilution technique. The essential oil of unripe fruits manifested the strongest antifungal potential followed by oils from ripe fruits, roots, stems, leaves and flowers. These oils were more efficient than the commercial drug bifonazole and much more active than ketoconazole. The most prominent biological activity was exhibited by the essential oils from ripe and unripe fruits of wild carrot oil (Soković et al., 2009c). The chemical composition and effectiveness of the essential oil obtained from *Echinophora spinosa* (Apiaceae) was tested on different fungi. The most resistant fungal species were *Penicillium ochrochloron* and *P. funiculosum* while *Trichoderma viride* was the most sensitive. This essential oil tested showed higher antifungal potency against *T. viride* than the commercial drugs bifonazole and ketoconazole (Glamočlija et al., 2011a). The essential oil of *Lippia alba* Bushy lippia (Verbenaceae) is reported as an antifungal agent against human pathogenic microorganisms but few articles concern its use for green mould control. We determined the antifungal activity of Bushy lippia essential oil against green molds (*Aspergillus ochraceus, A. niger, A. versicolor. A. fumigatus, Penicillium ochrochloron, P. funiculosum* and *Trichoderma viride*) as an alternative to synthetic fungicides. Microdilution assays evaluated the essential oil MIC and MFC. Bushy lippia essential oil has MIC of 0.3-1.25 mg/ml and MFC of 0.6-1.25 mg/ml. Bushy lippia essential oil is classificated as citral type and the results indicate that it

is a potential alternative to synthetic fungicides (Glamočlija et al., 2011b). The chemical composition and antimicrobial activities of the essential oils isolated from Pink savory *Satureja thymbra* and Black thyme *Thymbra spicata* (Labiatae) were compared. The oil of Black thyme possessed higher antifungal potential than Pink savory *S. thymbra* oil. *A. versicolor* and *A. fumigatus* were the most sensitive species, while *P. ochrochloron* was most resistant to these oils. Both oils showed much greater antifungal activity than a commercial antifungal agent (Marković et al., 2011). The essential oil of *Seseli montanum* subsp. *tommasinii* was tested for antifungal activity on four fungal species (*Aspergillus ochraceus*, *A. fumigatus*, *Penicillium ochrochloron* and *Trichoderma viride*). It showed moderate activity against all the tested fungi, but activity against *A. fumigatus* and *T. viride* was stronger than that of bifonazole. In the case of *A. fumigatus*, which is a very common and invasive pathogen, this is important due to the rising problem of fungal resistance to antifungal agents (Šiljegović et al., 2011b). We have studied the antimicrobial activity of *Seseli* species, examining essential oils obtained from the aerial parts of *S. anuum* (Milosavljević et al., 2007), *S. globiferum* fruits (Stojković et al., 2008a), *S. globiferum* aerial parts (Janaćković et al., 2011) and flowers of *S. rigidum* (Stojković et al., 2009). Differences in their activity were found. The essential oil from aerial parts of *S. anuum* showed activity against twelve fungi in the range of 12.5 to 50 μl/ml. That from fruits of *S. globiferum* had the strongest antifungal activity with the MICs and MFCs in the range of 0.5-50 μl/ml. Oil from the aerial part of *S. globiferum* showed significant activity against micromycetes (2.5-10 μl/ml). The essential oil from the aerial parts of *S. montanum* subsp. *tommasinii* was more active than that from the aerial parts of *S. anuum*. In the case of *P. ochrochloron* and *T. viride* this activity was twice that of *S. anuum* oil. On the other hand, the essential oil from *S. rigidum* had greater antifungal activity than that of *S. montanum* subsp. *tommasinii* against *A. fumigatus* and *P. ochrochloron* (Šiljegović et al., 2011b). The results of our investigation of the antifungal activities of essential oils from sixteen aromatic and medical plants and their components (Bitter orange *Citrus aurantium,* Lemon *C. limon, Hyssopus officinalis,* Lavender *Lavandula angustifolia,* Wild lavender *L. stoechas,* Chamomile *Matricaria chamomilla,* Melissa *Melissa officinalis,* Peppermint *Mentha piperita*, Spearmint *M. spicata,* Sweet basil, Rusmary *Rosmarinus officinalis*, Sage *Salvia officinalis,* Sardinian sage *S. desoleana,* Clary *S. sclarea,* Thyme *Thymus vulgaris* and *T. tosevii*) by the microdilution method showed that *Thymus* essential oils were the most effective in tests *in vitro*, while those from *Citrus* species and *S. officinalis* showed the lowest antifungal activities (Soković, 2001, 2009b). The same essential oils plus Oregano *Origanum vulgare* oil were assayed for inhibitory activity against major pathogens of the button mushroom, *Agaricus bisporus*, i.e. the fungi *Verticillium fungicola* and *Trichoderma harzianum*. The highest and broadest activity was shown by oregano and thyme oils with very low active concentrations (0.05-5.0 μg/ml) (Soković & van Griensven, 2006a). All the essential oils mentioned previously were also tested by microatmospheric method *in vitro* against 5 different isolates of *Mycogone perniciosa* (causal agent of wet bubble disease) from *Agaricus bisporus*. Essential oils which contained phenol components (thymol and carvacrol), (oregano, Greek oregano *O. heracleoticum,* Pink savory *S. thymbra,* thyme and *T. tosevii* with MIC values of 0.001 to 0.7 μl/disc and MFC of 0.1 to 1.0 μl/disc) showed significantly

stronger antifungal potential than those with high alcohol contents (Spearmint and Peppermint with MIC 1.0 μl/disc and MFC 2.5 μl/disc), and those oils with high keton contents (*S. pomifera* with MIC 1.0 μl/disc and MFC 15.0 μl/disc and *H. officinalis* with MIC 5.0 μl/disc and MFC 25.0 μl/disc), and especially than oils with monoterpenic hydrocarbons as dominant components (Lemon with MIC 4.0 μl/disc and MFC 5.0 μl/disc Bitter orange with MIC 0.7 μl/disc and MFC 1.0 μl/disc and Lavender MIC 1.5 μl/disc and MFC 2.5 μl/disc). The fungicide prochloraz showed much lower antifungal potential than all the oils tested, with MIC 5.0 μl/disc and MFC 50.0 μl/disc (Glamočlija, 2006a, 2006b, 2009). Also, there is only limited information in the literature on the antifungal activity of essential oils *in vivo.* The experiments *in vivo* are in relation with several problems of application of essential oils. Those related to the volatility of the oils and their poor solubility in water must be resolved before trials are performed *in vivo*. The persistence of a volatile oil on the treated plant and consequently its protection against pathogens is of short duration. The solubility problem means that organic diluents may have to be used, with the risk of environmental pollution and even phytoxicity. In an attempt to overcome these problems, some experimental formulations of essential oil and camphor, the most active components of Sage against *Botrytis cinerea* were prepared using as excipient a polymeric matrix obtained by the graft polymerization of acyclic monomers on gelled starch. Besides being absolutely harmless to plants, these polymers showed the capacity to form aqueous dispersions of the oil that were homogenous and adhered well to the surface of the leaves in some preliminary tests. Once the water had evaporated, the dispersions left a solid film which could be reduced to a hydrogel on the laminae of the leaves, from which the oil was gradually released in concentration related to the humidity of the microenvironment. This guarantees an extended duration of effective concentrations of the oil on treated plants (Moretti et al., 1998). The same researchers analyzed the effects of essential oil from Sage and camphor on tomato plants infected with *Botrytis cinerea*. A significant reduction of infection was found, but they not able to eliminate entirely the appearance of spots on the leaves. However, this encouraged further research in this field, with other essential oils which possessed greater antifungal activitiy *in vitro.* The effects of eleven plants essential oils for protecting maize kernel against *Aspergillus flavus* were studied. The optimal doses for maize protection, the influence of combinations of oils, residual effects and toxicity of the essential oils to maize plants were determinated. The principal constituents of eight essential oils were tested for their ability to protect maize kernels. Essential oils of Cinnamon, Peppermint, Sweet basil, Oregano, *Telaxys ambrosioides, Syzygium aromaticum* and thyme totality inhibited fungal development on maize kernel. Thymol and methoxycinnamaldexyde significantly reduced maize grain contamination. No phytotoxic effect on germination and corn growth was detected with any of these oils (Montes-Belmont & Carvajal, 1998). Reddy et al., (1998) showed *in vivo* that essential oils of thyme exhibited antifungal activity against *Botrytis cinerea* and *Rhizopus stolonifer,* two common pathogens of *Fragaria anamassa.*

Among a variety of oils tested *in vitro* against the pathogenic fungi *Mycogone perniciosa,* Oregano oil was singled out as the best. Although disease caused by *M. perniciosa* is routinely controlled with different fungicides, it remains a constant threat. In order to find

alternative preventive methods we evaluated the antifungal activity of Oregano oil *in vivo* in a mushroom growing unit. To treat experimentally induced mushroom disease in the growing house we tested the antifungal activity of oregano oil when applied in casing soil. The most favorable results were achieved with 2% of oregano oil and simultaneous application of the spores suspension when oregano oil completely inhibited the growth of *M. perniciosa*. As essential oils are largely nontoxic and easily biodegradable we advise on disinfection of commercial casing soil with 2 % oregano oil before applying the casing to the compost (Glamočlija, 2006a, 2007). The chemical composition and antimicrobial activity of essential oils of *Vitex agnus-castus* L. and their main constituents *in vitro* and *in vivo* showed that these oils could be used equally *in vitro* and *in vivo* (Figure 2.). The oils from all plant parts possessed great antifungal potential against eight fungal plant pathogens. Using the same technique 1,8-cineole and α-pinene (dominant compounds) showed very high antifungal potency as well. As 1,8-cineole was the predominant constituent of the oils, we tested it *in vivo*. Randomly chosen apples were treated with 1,8-cineol solution and infected with *Aspergillus niger* in order to provoke Aspergillus rot in apples. As apple fruits were treated with an acceptable amount of 1,8-cineole, we suggest it as a potent agent for preventing apple rot caused by *A. niger* and its application as a bioactive compound to control *A. niger* infection during apple storage (Stojković et al., 2011b).

Figure 2. Treatment of Aspergillus rot in apples with 1,8-cineol

3.2.2.1.1. Essential oils components

We will also discuss here the antifungal activities of essential oil components. Eugenol has exhibited very strong antifungal activity against *Absida glauca, Aspergillus nidulans, A. niger, Colletotrichum capsici, Fusarium monoliformae, Pestolotia psidi* and *Rhizopus nadssus*, while caryophyllene inhibited *A. glauca*. Cineole showed favorable activity against *Fusarium monoliformae* and *Alternaria alternata* and good to moderate activity against the remaining fungi tested. The chillie crop in India suffers from rips tot and die Back diseases caused by *C. capsici*. The growth of this fungus has very successfully been inhibited by cumaldehyde and eugenol. Cineole, cumalaldehyde and eugenol also very satisfactorily inhibited growth of *F. monoliformae, P. psidi* and *R. nadssus. F. monoliformae* causes localized "Softrot" of apical tissue, head blight, scab and root rot of wheat and other cereals, beke disease of rice and twisted top of sugarcane. These compounds could be used for inhibition of *A. alternata*, which attacks crucifers such as mustard, cauliflower, knol-knol and radish. Cumalaldehyde may also find application as an inhibitory agent against the growth of *P. expansum* which

causes infection if it gains a foothold in injured tissues. It also causes soft rot in apple fruit (Garg & Siddiqui, 1992). The essential oil of *Foeniculum vulgare* and components (anethole, fenhone and camphor) were screened for antifungal activity against nine plant pathogens. Anethole possessed the greatest activity (1.3-2.8 μl/ml), then fenchone (3.7-6.0 μl/ml), while camphor had the lowest antifungal effect (2.8-9.7 μl/ml) (Mimica-Dukić et al., 2003).

Essential oil components (β-caryophyllene, β-caryophyllene oxide, α-pinene, cadinene, linalool) from eight *Stachys* species were analyzed for antifungal activity. Linalool exhibited the greatest activity (0.03 mg/ml), while the lowest effect was observed for β-caryophyllene (0.3 mg/ml) (Skaltsa et al., 2003). We investigated the antifungal activity of limonene against fourteen fungal species using micro- and diffusion tests. Limonene showed antimicrobial activity against all fungi tested, in concentrations of 8.0-13.0 μl/ml in the diffusion and 6.0-10.0 μl/ml in the microdilution method. Differences in MICs obtained by these two methods could be attributed to low solubility of limonene in the agar medium used in the diffusion method. Limonene showed stronger antifungal potential than bifonazole, especially in the microdilution assay when the component was dissolved in Tween (Rančić et al., 2003). Phenol compounds were the most active among the components investigated. Limonene and linalyl acetate were less effective against micromycetes. All essential oils and components were investigated against twelve fungi: *Aspergillus niger, A. ochraceus, A. versicolor, A. flavus, A. terreus, Alternaria alternata, Penicillium ochrochloron, P. funiculosum, Cladosporium cladosporioides, Trichoderma viride, Fusarium tricinctum* and *Phomopsis helianthi* (Soković, 2001, 2009b). The components linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol and menthol were assayed for inhibitory activity against three major pathogens of the button mushroom, *Agaricus bisporus*, the fungi *Verticillium fungicola* and *Trichoderma harzianum*. The highest and broadest activity was shown by carvacrol and thymol with very low MIC and MFC values (0.02-1.5 μl/ml), while linalyl acetate and limonene possessed the lowest activity (MIC/MFC 5.0-11.0 μl/ml) (Soković & van Griensven, 2006a). The essential oil of *Critmum maritimum* and its components (α-pinene and limonene) possessed antifungal activity against the mycopathogen *M. perniciosa* tested by the microatmospheric method. MIC for α-pinene was 5 μl/disc, and MFC 10 μl/disc, while limonene showed higher antifungal activity with MIC 1 μl/disc, and MFC 5 μl/disc (Glamočlija et al., 2009) (Table 1.).

It can be seen that growth of the tested fungi responded diversely to the essential oils and their components, which indicates that different components may have different modes of action or that the metabolism of some fungi is able to overcome the effect of the oil or adapt to it. Terepenic compounds inhibit electron transport, proton translocation, phosphorylation steps and other enzyme-dependent reactions or act on the cell membrane. Their antifungal activity will depend on the chemical composition of the cell wall and on the structure of the terpenoid molecules. Terpenic hydrocarbons are water insoluble and revealed poor activity, while among the water soluble compounds vanilin, piperonal and camphor, were not remarkably active, whereas the non-aromatic ester borneol acetate showed antiseptic effects. Aliphatic alcohols, such as linalool or citronellol and ketones like pipertone or carvone exhibited antifungal properties. Phenol compounds

showed very strong antifungal activity in spite of their relative low capacity to dissolve in water (Knobloch et al., 1988). The most active terpenoids were found among phenols, followed by aldehydes and ketones, alcohols and hydrocarbons. Thymol and carvacrol were the most effective compounds which causing total inhibition of oxidative phosphorylation. The ability of terpenoids to inhibit the reactions described above arises both from lipophilic properties, which enables them to dissolve in the cytoplasmic membrane, and from their functional groups, which interfere with enzyme structure (Griffin et al., 1999; Knobloch et al., 1988; Shelef, 1983; Soković, 2001). Studies of antimicrobial activity of essential oils and their components showed that, terpene acetates and hydrocarbons tended to be relatively inactive, regardless of their structural type, and that this inactivity appears to be closely related to their limited hydrogen bonding capacity and water solubility. Ketones, aldehydes and alcohols showed activity but with differing specificity that was not always defined by the functional group present but was associated with hydrogen-bonding parameters in all cases (Griffin et al., 1999). Our results concerning the antifungal activity of many essential oils and their components indicate different efficacy. Also, the modes of action of essential oils differ among fungal species. The strong antifungal activity of some oils (*Mentha* species, Thyme, Oregano) can be explained by the high percentage by their high percentage of active components such as menthol, thymol, carvacrol. For the remaining oils, no significant correlation between antifungal activity and relative amounts of the major components has been found. This suggests that the components present in large proportions are not necessarily responsible for a great share of the total activity. Different antifungal activity exhibited by an oil, compared with those of it major components, can be explained by either synergistic effect of diverse components in the oil and/or by the presence of other components that may be active even in small concentrations (Soković & van Griensven, 2006a). To examine the problem of a lack of unified criteria in greater depth, we can look particularly at studies of the antimicrobial activity of essential oils. Janssen et al., (1987) reviewed the characteristics of complex mixtures as well as the techniques used to evaluate them and concluded that many results are difficult to compare as the test methods differed so widely. They proposed that in future the strain number of the tested microorganism, the composition of the essential oil and the conditions under which it was obtained be included as an integral part of the report.

Recently, Kalemba & Kunicka (2003) reviewed the classical methods commonly used to evaluate the antibacterial and antifungal activities of essential oils, including the agar diffusion method (paper disc and well), the dilution method (agar and liquid broth) and turbidimetric and impedimetric monitoring of microorganism growth in the presence of these oils. Besides drawing conclusions about factors that influence the antimicrobial activity of essential oils *in vitro* and their mechanisms of action, they included an overview of the susceptibility of human and foodborne fungi towards different essential oils and their constituents. The most relevant ones, which included oils from Thyme, Oregao, Mint, Cinnamon, Sage and Clove, have antimicrobial properties. Other criteria were the study of plants used as preservatives, as well as examination of the use of spices

as antimicrobial agents. The cited criteria seem sufficient to justify the studies, but Ríos & Recio, (2005 and references cited therein) believe that research should be focused on achieving definitive knowledge about the plant and its properties. With increasing acceptance that the chemical diversity of natural products is well suited to provide core scaffolds for future antimicrobial agents, there will be more developments in the use of novel natural products and chemical libraries based on natural products (Harvey, 2008). The methodology employed is another point that needs to be considered in depth. For non-polar extracts, the use of diffusion techniques is probably inadequate, although many reports employing these techniques have been published. Solid dilution techniques are suitable for studying plant extracts or nonpolar compounds. Only when the amount of sample available is small is the use a diffusion techniques possibly more appropriate (Rios & Recio, 2005).

Our results showed that the MICs for essential oils and their components are generally higher, in disc-diffusion assays and with diffusion methods than with the microdilution method. Poor activity can be explained by low water solubility of the oil and its components, which limits diffusion through the agar medium in the disc diffusion and agar diffusion methods. Only more water-soluble compounds, such as 1,8-cineole, diffuse into the agar. The hydrocarbon components either remain on the surface of the medium or evaporate. This could be the reason for better results obtained using the microdilution method. Also, essential oil and their components showed greater antifungal activity when diluted in Tween 80. Both MICs and MFCs were lower in the microdilution than in the diffusion method, especially when Tween 80 was employed. Non-ionic emulsifiers, such as Tween 20 or 80, are relatively inactive when tested alone and have been widely reported as useful emulsifying agents (Soković, 2001; Soković et al., 2002; Soković & van Griensven, 2006a). We observed that some oils and compounds acted not only as fungicidal agents but also inhibited sporulation of different fungi (Glamočlija et al., 2006a, 2006b; 2007; Soković & van Griensven, 2006a). Treatment was not only effective in solution or by contact, but even in a vapor treatment their were very effective enabling fungal growth to be inhibited by a smaller amount of essential oil while also acting as a potent inhibitor of sporulation. Vapor concentration and the duration of exposure are important. The gaseous contact activity was demonstrated primarily by the maximum vapor concentration at an early stage of incubation. Maintaining a high vapor concentration for long periods of time appeared to be unnecessary. Essential oil vapors might serve to control proliferation of moulds that are now treated with other sanitizing agents. Oils and their components have high vapor pressures and are relatively volatile. Solutions and emulsions used in the form of sprays with or without a carrier therefore represent the preferred form in which these agents should be applied to large areas of casing soil surface with minimal effort. Also evaporation by heating could be considered. An additional advantage of the volatile of essential oils is that no or only little residue will be left on the product after treatment. Our own experience leads us to propose the use of the microdilution method, carried out in microtiter trays, which involves a low

workloads for a larger number of replicates and the use of small volumes of test substance and growth medium (Soković 2001, Soković et al., 2006a, 2009b). On other hand, for investigation of some plants and fungal extracts and separation of fractions and highly pure compounds bioautographic methods on TLC plates were recommended. This qualitative techniques will only give an idea of the presence or absence of substances with antimicrobial activity in very small amounts. The method was useful for screening plants and fungi for antimicrobial activity and for the bioassay-guided isolation of natural antimicrobial compounds. Bioautography allows easy localization of activity even a matrix as complex as that derived from natural products. Comparison chromatograms developed under identical conditions and visualized using suitable chromogen reagents can provide useful information about the nature of active compounds (Figure 3.) (Rančić et al., 2006, Ćirić, 2010).

Considerable changes in legislation have been made and there are increasing consumer trends for more natural alternatives to chemical fungicides (Brul & Coote, 1999). The use of essential oils is particularly advisable because herbs and spices are common plant additives. Among the natural antimicrobials that were tested in our laboratory the essential oils of Oregano and Thyme, as well as their components, carvacrol and thymol were the most promising. Addition of various plant derived antimicrobials in combination should improve both the spectrum of activity and the extent of inhibition due to synergistic effects. Thus, combination of these compounds might have even higher potential. The use of essential oils is limited, and possible reasons for this may be their strong smell and taste when used at effective doses (Skandamis & Nychas, 2000). Although the majority of essential oils are classified as Generally Recognized As Safe (GRAS) (Kabara, 1991), their use in foods as preservatives is often limited due to flavor considerations, as effective antimicrobial doses may exceed organoleptically acceptable levels. Therefore, there is an increasing demand for accurate knowledge of the minimum inhibitory (effective) concentrations (MIC) of essential oils to enable a balance between sensory acceptability and antimicrobial efficacy (Lambert et al., 2001).

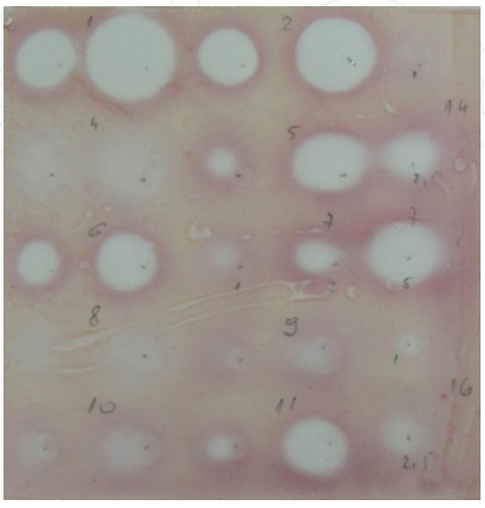

Figure 3. Antifungal activity of sesquiterpene lactones using a bioautographic method on TLC plate

Group of compounds	Species	Methods	Activity	References
Fungal extracts	*Cladosporium fulvum* *Fusarium sporotrichioides* *Penicillium ochrochloron* *P. funiculosum* *Phomopsis helianthi* *Stachybotrys chartarum Trichoderma viride* *Microsporum canis* *Trichophyton rubrum* *T. mentagrophytes* *T. tontonsurans*	bioautographic assay on TLC plates bioautographic	50-100μg/ml 50-100 μg/ml 50-100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 100 μg/ml 50-100 μg/ml	Rančić et al., 2006
	Laetiporus sulphureus	microdilution	0.30 mg/ml	Šiljegović et al., 2011a
plant extracts	*Phlomis fruticosa*	diffusion	10-20 μg /ml	Ristić et al., 2000
	Cynara cardunculus	microdilution	1.0–1.5 mg/ml	Kukić et al., 2008
	Centaurium pulchellum	microdilution	0.1-2.0 mg/ml	Šiler et al., 2010
	Nepeta rtanjensis, N.sibirica, N. nervosa	microdilution	25-100 μg/ml	Nestorović et al., 2010
mosses	*Bryium argenteum*	microdilution	0.29-0.52 μg/ml	Sabovljević et al., 2006
	Pleurozium schreberi *Palustriella commutata* *Homalothecium philippeanum* *Anomodon attenuatus* *Rhytidium rugosum* *Hylocomium splendens* *Dicranum scoparium* *Leucobryum glaucum*	 microdilution diffusion	0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 0.5-10 mg/ml 0.5-10 mg/ml 0.5-10 mg/ml 1.0 mg/disc 0.5-10 mg/ml 0.5-10 mg/ml	Veljić et al., 2008
	Fontinalis antipyretica var. *antipyretica* *Hypnum cupressiforme Ctenidium molluscum*	microdilution	2.5-5 mg/ml 5 mg/ml 5 mg/ml	Veljić et al., 2009
	Atrichum undulatum, *Physcomitrella patens, Marchantia polymorpha* ssp. *ruderalis*	microdilution	0.1-2 mg/ml 0.1-2 mg/ml 0.25-1 mg/ml	Sabovljević et al., 2011
	Rhodobryum ontariense	microdilution	0.25-1 mg/ml	Pejin et al., 2012
essential oils	*Origanum onites, Satureja thymbra, Salvia fruticosa,* *S. pomifera* subsp. *calycina*	microdilution	0.05-25 μl/ml	Soković et al., 2002
	Micromeria dalmatica, M. albanica, M. thymifolia	diffusion	0.2-2.0 μl/ml	Marinković et al., 2002
	Foeniculum vulgare	microdilution	0.8-4.0 μl/ml	Mimica-Dukić et al., 2003
	Stachys sp.	microdilution	0.01-1.0 mg/ml	Skaltsa et al., 2003
	Aniba rosaeodora *Laurus nobilis* *Sassafras albidum* *Cinnamomum zeylanicum*	 diffusion microdilution 	0.5-7.5 μl/ml 1.0-20 μl/ml 10-40 μl/ml 10-50 μl/ml 5-15 μl/ml 5-30 μl/ml 0.1-1 μl/ml 0.1-2.5 μl/ml	Simić et al., 2004
	Juniperus excelsa	microdilution	8–50 μl/ml	Soković et al., 2004
	Myrrhis odorata *Hypericum perforatum* *Helichrysum arenarium*	microdilution	0.5-2.5 μg/ml 15-30 μg/ml 10-60μg/ml	Rančić et al., 2005
	Salvia brachyodon	microdilution	15-40 μl/ml	Soković et al., 2005
	Salvia sclarea	microdilution	2.5-25 μl/ml	Dzamić et al., 2008
	Salvia desoleana	diffusion microdilution	1.5-6 μl/ml 1.5-6 μl/ml	Soković et al., 2009a
	Nepeta rtanjensis	diffusion	0.6-1.8 μl/ml	Ljaljević Grbić et al., 2007
	Cymbopogon winterianus *Carum carvi*	microdilution	0.5-20 μl/ml 0.25-20 μl/ml	Simić et al., 2008
	Portenschlagiella ramosissima	microdilution	50-200 μl/ml	Soković et al., 2008b
	Eugenia caryophyllata *Illicium verum*	microdilution	0.1-2.5 μl/ml 2.5–25 μl/ml	Dzamić et al., 2009
	Daucus carota	microdilution	2-150 μl/ml	Soković et al., 2009c

Group of compounds	Species	Methods	Activity	References
	Echinophora spinosa	microdilution	0.0625-1 mg/ml	Glamočlija et al., 2011a
	Lippia alba	microdilution	0.3-1.25 mg/ml	Glamočlija et al., 2011b
	Satureja thymbra *Thymbra spicata*	microdilution	1.25-5 μg/ml 0.3-2.5 μg/ml	Marković et al., 2011
	Seseli anuum aerial	diffusion	12.5-50 μl/ml	Milosavljević et al., 2007
	Seseli globiferum fruits	microdilution	0.5-50 μl/ml	Stojković et al., 2008a
	Seseli globiferum aerial	microdilution	2.5-10 μl/ml	Janaćković et al., 2011
	Seseli rigidum flowers	microdilution	10-50 μl/ml	Stojković et al., 2009
	Seseli montanum subsp. *tommasinii*	microdilution	25-100 μl/ml	Šiljegović et al., 2011b
	Matricaria chamommilla, *Mentha piperita*, *M. spicata* *Lavandula angustifolia* *Ocimum basilicum*, *Origanum vulgare*, *Salvia officinalis*,*Citrus limon*, *C. aurantium*, *Thymus vulgaris*	microatmosphere diffusion microdilution microatmosphere	0.5-15 μl/ml 0.5-35 μl/ml 0.125-20 μl/ml 0.001-25 μl/disc	Soković & van Griensven, 2006[a] Glamočlija, 2006a; 2006b, 2009
	Vitex agnus-castus	microdilution	44.5-267 μg/ml	Stojković et al., 2011
components of essential oils	carvacrol, camphor 1,8-cineole	microdilution	0.1-0.5 μg/ml 3.0-10 μg/ml 4.0-15 μg/ml	Soković et al., 2002
	anethole fenhone camphor	microdilution	1.3-2.8 μl/ml 3.7-6.3 μl/ml 2.8-9.7 μl/ml	Mimica-Dukić et al., 2003
	β-caryophillene, β-caryophillene oxide, α-pinene, cadinene, linalool	microdilution	0.03-0.3 mg/ml	Skaltsa et al., 2003
	limonene	diffusion microdilution bioautographic	8-13 μl/ml 6-10 μl/ml 5 μl/ml	Rančić et al., 2003
	linalyl acetate, linalool, limonene, α-pinene, β-pinene, 1,8-cineole, camphor, carvacrol, thymol, menthol	diffusion microdilution	0.05-13 μl/ml 0.02-11 μl/ml	Soković & van Griensven, 2006a
	linalyl acetate 1,8-cineole linalool	diffusion microdilution	7.0-11.5 μl/ml 7.5-11 μl/ml 2-8 μl/ml 3-8 μl/ml 2-7 μl/ml 2-7 μl/ml	Soković et al., 2009a
	α-pinene limonene	microatmospheric	5-10 μl/disc 1-5 μl/disc	Glamočlija, 2009
	1,8-cineole α-pinene	microdilution	3.5-7 μg/ml 4-8 μg/ml	Stojković et al., 2011
Flavonoids	*Quercus ilex*	microdilution	0.056-2.95 μmol/ml	Karioti et al., 2011
	Centaurea spruneri	microdilution	0.694-1.3 μmol/ml	Ćirić, et al., 2011
Sesquiterpene lactones	*Centaurea nicolai*	diffusion	0.8-25 μg/ml	Vajs et al., 1999
	Centaurea achaia, C. thessala, C. attica	microdilution	0.03-4 μg/ml	Skaltsa et al., 2000a;b
	Centaurea pullata	microdilution	0.0001-0.0007 μmol/ml	Djeddi et al., 2007, 2008
	Anthemis melanolepis	microdilution	25 μg/ml	Saroglou et al., 2010
sulphur products	*Allium sativum*	microdilution	0.001-0.03 mg/ml	Ilić et al., 2012

Table 1. An overview of antifungal activities of natural products from fungi and plants

3.2.2.1.2. Morphophysiological changes in fungi due to inhibition activity by essential oils

Dematiceous fungi are characterized by the presence of the dark brown pigment – melanin within their cell wall structure. Melanins are negatively charged, hydrophobic biopolymers of high molecular weights. They are typically brown or black and formed by the oxidative polymerization of phenol or indolic compounds in organisms from all biological kingdoms, including fungi. Fungal melanins are usually found in the cell walls of spores, sclerotia,

mycelia or fruiting bodies. They enable fungi to survive adverse environmental conditions by protecting them from oxygen free radicals, UV radiation and wall-degrading enzymes produced by antagonist microbes (Butler et al., 2001).

Many human pathogenic fungi contain melanin within their cell wall structure (e.g. *Aspergillus fumigatus, A. nidulans, A. niger, Alternaria alternata, Cladosporium carionii, Cryptococcus neoformans, Exophiala jeanselmei, Fonsecaea compacta, F. pedrosoi, Hendersonula toruloidii, Histoplasma capsulatum, Paracoccidioides brasiliensis, Penicillium marneffei, Phaeoannellomyces wernickii, Phialophora richardsiae, P. verrucosu, Sporothrix schenckii, Wangiella dermatitidis*). For several of these fungi, melanin has been described as a virulence factor due to its ability to reduce a pathogen's susceptibility for killing by host antimicrobial mechanisms and by influencing the host immune response. Due to the protective role of fungal melanin, dematiceous fungi are extremely difficult to treat with antifungal drugs (Nosanchuk & Casadevall, 2006). Plant secondary metabolites could be a suitable alternative for the treatment of fungal infections in the light of increasing fungal resistance to commercial antifungal agents (Vivek et al., 2009). Essential oils are known to cause morphophysiological changes in fungi through a lack of sporulation, depigmentation and aberrant development of conidiophores (e.g. Sharma & Tripathi, 2008; Moreira et al., 2010). We tested several essential oils for this purpose. Using the diffusion method it was observed that essential oils of *Hyssopus officinalis*, Thyme, *T. tosevii*, Spearmint and Peppermint induced changes in some morphophysiological characteristics of the fungi *Trichoderma viride*, *Penicillium ochrochloron* and *Aspergillus niger*. Depigmentation of the colonies of *A. niger* was noted. Untreated control colonies were black, blue, green and the mycelium was well developed, while the colonies treated with oils were white with sparse mycelium. In the treated *A. niger, P. ochrochloron* and *T. viride* cultures, mycelium is rare, the conidiogenic apparatus was atypical, the vesicles deformed, phialides were abnormal and other variations included lack of sporulation, visible loss of pigmentation and aberrant development of conidiophores (Soković, 2001). *Nepeta rtanjensis* an essential oil showed the ability to interfere with all stages in the reproduction cycle of the human pathogenic fungus *Bipolaris spicifera*: conidia germination, mycelial growth and intensity of sporulation which is demonstrated with radial mycelial growth inhibition. Thus, inhibition of conidia germination and low conidia production were demonstrated in the treated samples. The most significant documented orphophysiological changes in *B. spicifera* included demelanization (bleaching) and an aberrant conidial apparatus (Ljaljević Grbić et al., 2011).

3.2.2.2. Flavonoids

Flavonoids are another group of secondary metabolites with great antifungal potential. Besides other biological activities, have been shown to be active against microorganisms. At least in some cases their presence might serve as a chemical barrier to invading microorganisms. Since they are natural compounds and possess highly specific antimicrobial activity, flavonoids may be an alternative to conventional fungicides in the control of plant diseases caused by fungi. Twenty-five flavonoids were examined for their effect on the mycelial growth of a crop pathogen, *Verticillim albo-atrum*. The minimum

inhibitory concentrations (MIC) for the two most active compounds, flavone and flavanone. Other flavonoids inhibited hyphal growth and some compounds were ineffective at the highest concentration used. Active compounds did not share a common pattern of substitutions. The unsubstituted flavonoids were stronger growth inhibitors and, in most cases, increasing the number of substittions (hydroxylation, methoxylation and glycosylation) resulted in the loss of antifungal activity (Picman et al., 1995). The fungicidal activity of two isoflavones, one isoflavanone and seven isoflavans was tested against *Aspergillus repens, A. amstelodami, A. chevalieri, A. flavus* and *A. petrakii* (Weidenbörner et al., 1989). While the isoflavones showed low activity, the two isoflavans were highly inhibitory (Weidenbörner & Jha, 1997). Two naturally occurring isoflavones, genistein and biochanin A, and their dihydroderivates (isoflavanones) as well as nine perhydrogenated isoflavones (isoflavans) were tested for their effects against *Rhizoctonia solani* and *Sclerotium rolfsii* (Weidenbörner et al., 1990). All the isoflavonoids of the biochanin A series showed high antifungal activity. Genistein isoflavan and the other isoflavans with two hydroxyl groups and one methoxy group were fungitoxic, while isoflavans with two or three methoxy groups were almost inactive (Weidenbörner & Jha, 1997), although earlier results demonstrated that isoflavans, generally possess higher activity than the corresponding isoflavones and isoflavanones.

Since the individual unsubstituted flavonoids showed strong antifungal activity, various mixtures have been tested against fungi occurring on grain to enhance the fungicidal potential of each substances by anticipating synergy. In general a combination of flavones and flavanones in different proportions was most effective. However, it was interesting that a mixture containing flavonoid molecules with one methoxy group and several hydroxy groups in general exhibited higher activity than a mixture containing only hydroxylated flavonoids. It becomes obvious that combinations of several suitable flavonoids (depending on the number, kind and location of the substitutents) may result in even greater increase in antifungal potential. Consequently, lower active concentrations may make flavonoids more attractive as natural protectants (Silva et al., 1998; Weidenbörner & Jha, 1997). Concerning the antifungal activity of flavonoid glycosides, it should be noted that no substantial effect could be detected (Weidenbörner & Jha, 1997). Krauze-Baranowska et al., (1999) found that cupressuflavone and 4'-O-methylcupressuflavone, isolated from leaves of *Cupressocyparis leylandrii*, possessed antifungal activity against *Alternaria alternata, Cladosporium oxysporum, Fusarium culmorum* and *F. avenaceum.* Matshumoto & Tahara, (2001) separated ampelosin, a flavonol, from *Salix sachalinensis* leaves and reported antifungal activity against *Cladosporium herbarum*.

The antifungal activities of many phenol compounds isolated from Holm Oak *Quercus ilex* leaves, belonging to the classes of flavonoids, proanthocyanidins, and phenol acids, have been examined against fourteen fungal species (Karioti et al., 2011). Two coumarins, scopoletin and isoscopoletin, two simple phenol acids, protocatechuic acid and isovanillic acid and one flavonoid, eriodictyol separated from the aerial parts of *Centaurea spruneri*, showed fungistatic activity at 0.259–2.38 μmol/ml and fungicidal at 0.69–2.6 μmol/ml against all fungi tested. The flavonoid, eriodictyol, possessed the greatest antifungal activity in the

range of 0.694–1.388 μmol/ml for MIC and MFC, while the activity of protocatechuic acid was lower (0.65–1.3 μmol/ml for MIC and 1.3–2.6 μmol/ml for MFC). The simple phenol acids, protocatechuic acid expressed the lowest antifungal activity (Ćirić et al., 2011). The inhibitory activity of flavonoids generally decreased with the increasing number of substitutions on the molecule, and the strongest inhibitors were unsubstituted compounds. The most active flavonoids, flavone and flavanone, have excellent potential as new natural antifungal agents (Picman et al., 1995).

3.2.2.3. Sesquiterpene lactones

Sesquiterpene lactones are natural products present in many families of plants, but mostly distributed in the family Compositae. They display a wide spectrum of biological activity, one of the most important of which is antifungal activity. The general mechanism of action is considered to be alkylation of biological nucleophiles such as cysteine (cys) and glutathione or sulfhydryl-containing systems, phosphofructoki-nase and glycogen synthetase by a,b-unsaturated carbonyl structures in a Michael-type addition (Koukoulitsa et al., 2005). This group of compounds was analyzed for potential antifungal activity, *in vitro* against *Aspergillus niger, A. ochraceus, Penicillium ochrochloron, Trichoderma viride, Fusarium tricinctum, Phomopsis helianthi* and *Cladosporium cladosporioides.* Guaianolides from *Centaurea nicolai* were found to be highly active (0.8-25 μg/ml) (Vajs et al., 1999). Sesquiterpene lactones isolated from *Centaurea achaia, C. thessala* and *C. attica* also exhibited excellent antifungal activity against *Aspergillus niger, A. ochraceus, A. versicolor, A. flavus, Penicillium ochrochloron, P. funiculosum, Trichoderma viride, Fusarium tricinctum, Phomopsis helianthi, Alternaria alternata* and *Cladosporium cladosporioides* with germacranolides providing the greatest antifungal activity (Skaltsa et al., 2000a; 2000b).

The fungicidal activities of 36 natural and synthetic sesquiterpene lactones with guaianolide, trans-germacranolide, cis-germacranolide, medampolide, and eudesmanolide carbon skeletons were evaluated against the phytopathogenic fungi *Colletotrichum acutatum, C. fragarie, C. gloeosporioides, Fusarium oxysporum, Botrytis cinerea* and *Phomopsis* sp. by Wedge et al., (2000). Dehydrozaluzanin showed the highest antifungal activity due the presence of an α,β-unsaturated carbonyl group in the cyclopentananone ring. In addition to the previously isolated sesquiterpene lactones, 11,13-dihydrocnicin and 11,13-dihydro-19-desoxycnicin, the aerial parts of *Centaurea pullata* afforded three minor sesquiterpene lactones, namely, a new germacranolide, 8R-*O*-(4-acetoxy-5-hydroxyangeloyl)-11,13-dihydrocnicin, and two new eudesmanolides, 8R-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11,13-dihydrosonchucarpolide and 8R-*O*-(4-hydroxy-2-methylenebutanoyloxy)-11,13-dihydro-4-*epi*-sonchucarpolide. The antimicrobial activity of all previously mentioned compounds and some newly isolated sesquiterpene lactones (a novel elemanolide with an α-methyl-γ-lactone moiety, 8α-O-(4-hydroxy-2-methylenebutanoyloxy)melitensine, in addition to other four known sesquiterpene lactones with the same ring, melitensine, 11β-dihydrosalonitenolide, 8α-hydroxy-11β-13-dixidroxy-4-epi-sonchucarpolide and 8α-hydroxy-11β-13-dihydroxy-onopordaldehyde) from *Centaurea pullata* was tested against eight fungal species, using a

microdilution method. All compounds evaluated showed greater antifungal activity than the positive controls used. Moreover, the pharmacokinetic profile of these compounds was investigated using computational methods and was in agreement with our *in vitro* data (Djeddi et al., 2007, 2008). Three linear sesquiterpene lactones, anthecotulide, hydroxyanthecotulide and acetoxyanthecotulide were isolated from the aerial parts of *Anthemis auriculata* together with five known flavonoids, taraxa-20(30)en-3â-ol and methyl vanillate. Comparing these results with previously published data (Konstantinopoulou et al., 2003) concerning the antimicrobial potential of sesquiterpene lactones of *A. altissima*, it was concluded that linear lactones are more active. This differentiation in antimicrobial activity could be explained in terms of solubility, as linear lactones are more lipophilic than the oxygenated eudesmanolides and germacranolides of *A. altissima* (Theodori et al., 2006). Nine sesquiterpene lactones, anthemin A, 1α-hydroxydeacetylirinol-4 α,5β-epoxide, anthemin C, tatridin A, 1-epi-tatridin B, anthemin B, 6-deacetyl-β-cyclopyrethrosin, elegalactone A and 1β,4 α,6trihydroxyeudesm-11-en-8 -12-olide were isolated from the aerial parts of *Anthemis melanolepis*. All sesquiterpene lactones showed an inhibitory effect against almost all fungi tested (Saroglou et al., 2010), which adds to evidence from previous studies concerning related compounds isolated from other *Anthemis* species (Konstantinopoulou et al., 2003).

The biological activity of sesquiterpene lactones is generally attributed to the alkylating property of the α-methylene-γ-lactone moiety. Moreover, the presence of other alkylating sites (epoxides and conjugated carbonyl groups) may enhance their biological activities. Lipophilicity seems to play an important role in antifungal activity. Since the chemical composition of fungal cells walls is highly lipophilic, they generally provide a strong barriers against penetration of hydrophobic compounds and transport of polar compounds through the outer lipid layer. According to Skaltsa et al., (2000a) an inverse relationship exists between polarity and antifungal activity for sesquiterpene lactones in general. Their polarity decreases in the order eudesmanolides > elemanolides > germacranolides. Some of the differences between the responses of *Verticillium albo-atrum* to flavonoids and sesquiterpene lactones suggest these two groups of plant metabolites have different modes of action. Thus, inhibition of mycelial growth by sesquiterpene lactones remained relatively constant during incubation times of 24, 48 and 72h. This suggests that these lactones affected the metabolism of the pathogen, slowing its growth, but that the fungus evidently did not produce specific enzymes to degrade the lactones. In contrast, flavonoids and especially flavanone, significantly reduced hyphal growth during the first 24h by up to 100% but growth was much less inhibited during the next 48h of incubation which indicated that the flavonoids were subject to degradation by enzymes produced by the pathogen (Picman et al., 1995).

3.2.2.4. Other compounds tested

Inhibition of certain thiol-containing enzymes in microorganisms by the rapid reaction of thiosulfinates with thiol groups was assumed to be the main mechanism involved in the antimicrobial effect of allicin. The mode of action of allicin on the fungal cell has not yet been elucidated but it is assumed to act on thiol enzymes as in other microorganisms. Other

requirements such as molecular accessibility and lipophilicity seem to play an important role for in their antifungal activity (Yamada & Azuma, 1997). Antifungal activities of allicin and related organo-sulfur products obtained by microwave-assisted transformation of allicin in ethanol were studied in our laboratory against eight fungi: *Aspergillus flavus, A. niger, A. fumigatus, Penicillium funiculosum, P. ochrochloron, Trichoderma viride, Candida albicans* and *C. kruzei.* The mixture of transformation products of allicin was analyzed using liquid chromatography-mass spectrometry (LC-MS) and consisted of ajoenes, vinyldithiins and diallyl disulfide. Allicin showed very powerful antifungal activity with minimum inhibitory concentration (MIC) at 0.001 to 0.008 mg/ml and MFC at 0.004 to 0.03 mg/ml. The transformation products of allicin also possessed very strong antifungal activity, but less than allicin (Ilić et al., 2012).

3.2.2.5. Synergistic effect of different natural compounds and fungicides

Fungi that are pathogenic to plants which produce antimicrobial compounds often have greater tolerance to these natural compounds *in vitro* than do nonpathogens of these plants, suggesting that resistance may be a prerequisite for infection. Nevertheless, many other factors will be required for fungal pathogenicity in addition to resistance to host antimicrobial compounds. Although *in vitro* tests of antifungal activity usually involve individual purified compounds, phytopathogenic fungi may often be exposed to more than one antifungal compound simultaneously during infection of plants. The combination of different compounds may be synergistic. The combined therapy is used with the aim of expanding the antimicrobial spectrum, minimizing toxicity, preventing the emergence of resistant mutants during therapy, and obtaining synergistic antimicrobial activity. Some steroidal glycoalkaloids have low antifungal activity when tested separately, but exhibit pronounced synergistic activity when mixed in plants (Osbourn, 1996). Synergism between ketoconazole and *Agastache rugosa* oil against *Blastichizomyces capitatus* was reported, as well as between *Pelargonium graveolens* oil and amphotericin B plus ketoconazole on strains of *Aspergillus* sp. (Silva & Fernades, 2010). Thus, studies on the interactions between natural products and antifungal drugs have also multiplied in recent years, indicating the importance of elucidating types of interactions, which can be favorable, such as in synergism, or harmful, as in antagonism. Our results showed that proanthocyanidins (procyanidin and prodelphinidin) isolated from Holm oak when combined with bifonazole and ketoconazole, increased the activity of both conventional fungicides (Karioti et al., 2011). Compounds tested showed higher activity than bifonazole and ketoconazole, against *A. fumigatus* and *A. niger*. Almost all compounds tested exhibited good ability to inhibit fungi, even much better than commercial antifungal agents used as reference drugs. Even more, compounds showed few times higher activity than bifonazole and especially than ketoconazole. Also, we tested the synergistic effect of mixture of several essential oils and found that some combinations could possess great antifungal potential. The following mixture of essential oils: Thyme, Peppermint, Sage, *Hyssopus officinalis*, Sweet basil and Lavander showed greater antifungal potential *in vitro* than each oil separately (Soković, 2001). Structurally unrelated compounds are also likely to have combined and possibly synergistic antifungal activity. Therefore, this study supports the potential use of a weak

antifungal products together with another compound to increase its activity. This type of finding could further boost the use of medicinal plants, extracts or natural products, either alone, combined or together with mycotics.

4. Antifungal activity against animal and human pathogens

Among animal and human pathogens, dermatomycetes are the main cause of dermatomycoses (infections of the hair, skin, and nails), superficial infections that are not life threatening but are chronic and cause considerable morbidity. The unpleasant side effects of therapy including nausea, abdominal pain and itching, and its toxicity, can limits its therapeutic use in many cases (Shin & Lim, 2004).

Despite the advancements of science and technology, surprisingly the development of novel and efficient antifungal drugs is still lagging behind due to the very fact that fungi are also eukaryotic and have mechanisms similar to human beings. Hence it becomes very difficult to develop an antifungal agent that is more specific in targeting the fungi alone without any damage to human beings. For successful treatment of the disease, proper diagnosis of the disease is always essential. The treatment is chosen based on the infection site, etiological agent and penetration ability of the drug. The penetration ability and retention in the site of infection of the agent determines its efficacy and frequency of utility. Since the dermatomycetes reside in the stratum corneum especially within the keratinocytes, the antifungal agents should have a good penetrating ability (Lakshmipathy & Kannabiran, 2010). Therefore, the development of more effective and less toxic antifungal agents is required for the treatment of dermatomycosis. Essential oils play a great role in these investigations, the majority have good penetration possibilities the lipophilic properties of oil components might have also aided in the ability of the oil to penetrate the plasma membrane, strong antifungal activity and if they are used in active (MIC and MFC) concentration they are not harmful for animals and humans.

Previous studies *in vitro* and *in vivo* on investigations of the antifungal activity of essential oils from some medicinal and aromatic plants, *Origanum vulgare* subsp. *hirtum*, Spearmint, Lavander, and *Salvia fruticosa*, indicated that they could be employed as effective antifungal agents (Adam et al., 1998). Our selection of plants for evaluation in our study was based on traditional usage for treatment of infectious diseases (Sokmen, 1999). The *in vivo* evaluation of antifungal activity of several essential oils and their components was tested for the therapeutic potency against experimentally induced dermatomycoses in rats (2-month old male Wistar rats), using the most frequent dermatomycetes, *Trichophyton mentagrophytes, T. rubrum* and *T. tonsurans.* Essential oil of Lavander exhibited therapeutic activity after 13 days of treatment. The group of rats treated with Sweet basil oil were cured after 25 days of treatment. The shortest period of currency was observed at animals treated with Sage-12 days. The longest period of treatment was observed at rats treated with oils of Bitter orange and Lemon, 45 days. The main essential oil components were also used as a potential antifungal agents. Linalool showed antifungal activity after 32 days of treatment, while limonene needs 50 days for this activity. Rats treated with 1,8-cineole were cured after 40

days. Camphor exhibited therapeutic and antifungal activity after 14 days of treatment. The best antifungal activity was observed for menthol, which showed therapeutic potential after 10 days of treatment (Soković, 2001). We examined the antifungal activity of essential oil from *Mentha* x *piperita* and menthol. The oil completely cured the animals infected with *T. mentagrophytes* within 15 days, with *T. rubrum* within 30 days and with *T. tonsurans* within 29 days. Menthol possessed higher therapeutic and antifungal activities than the essential oil, as it cured the animals within 10 days. Also, menthol showed stronger activity than bifonazole (Soković et al., 2006b). The antifungal activity of essential oil from Lavander showed therapeutic and antifungal potential during the 13-day observation period and cured the animals completely (Soković et al., 2007). The essential oil of thyme and its main component thymol was also tested for therapeutic potency *in vivo.* This oil completely cured animals infected with *T. mentagrophytes* within 24 days, with *T. rubrum* within 37 days, and with *T. tonsurans* within 32 days of treatment. The animals treated with the commercial drug bifonazole were cured after 14–15 days of treatment. Moreover thymol possessed higher therapeutic and antifungal activity than essential oil and cured the animals within 14 days (Figure 4.) (Soković et al., 2008c).

In vitro susceptibility of the turpentine oil obtained from Cluster pine *Pinus pinaster* oleoresin was evaluated against three Sudanese clinical isolates of *Actinomadura madurae*, which is the main causative agent of actinomycetoma in man and animals. The minimum inhibitory concentrations (MICs) of the oil ranged from 100.3-124.8 μl/ml, and the minimum microbicidal concentrations (MMCs) were between 100.3 μl/ml and 150.0 μl/ml. The main component of oil, α-pinene, exhibited prominent bioactivity with MICs ranging between 3.3 and 5.0 μl/ml, while the MMC was 10.0 μl/ml against the same clinical isolates. Cluster pine turpentine oil and α-pinene might be useful agents in the treatment of mycetoma caused by *A. madurae* (Figure 4.) (Stojković et al., 2008b). From the above results it can be concluded, that all essential oils tested showed beneficial antifungal activity both *in vitro* and *in vivo.*

After reviewing of the results of the antifungal activity of essential oils and individual components *in vivo* experiment, knowing that the composition of essential oils and the proportion of the tested individual components may be, to some extent, explain the differences between their activities. Menthol, camphor and thymol showed better antifungal activity than essential oils tested individually. Since the individual essential oils showed lower antifungal activity than the tested components, it is evident that the active principles can be explained by individual components. Although it is possible that interactions between the constituents of essential oils block the active principles of individual components when the treatment is the total essential oil. Added to that are antagonistic effect (Davidson & Parish, 1989) which does not mean that it can be completely neglected the role of individual components of essential oils on the expression of antifungal potential. Menthol, camphor and thymol, which showed the best antifungal activity *in vivo* among all tested components, are the dominant components of the essential oils of Spearmint and Thyme, and therefore can be justified by the high antifungal potential of these oils *in vivo*. The essential oil of Sweet basil, which is known for the beneficial activity of the skin, healing wounds, etc. is used to treat fungal infections showed good antifungal activity, but only

better than lemon and orange. Dominant component of this oils was linalool, which proved to be good, but the tested components as one of the weaker fungicides, in front of limonene. Similarly, Lemon and Bitter orange oil showed the lowest antifungal potential, as well as among individual components of limonene, which is present in these oils with a high proportion, which is certainly influenced the decrease in the efficiency of oil. Essential oils of *S. officinalis* and *L. angustifolia* have proved to be most effective in the treatment of experimental induced dermatomycoses. If we compare the results obtained during investigation of antifungal activity of essential oils *in vitro*, and this generated *in vivo*, it is obvious that the essential oil of Sage and Lavander reacted with lower potential *in vitro*. *In vivo* experiments, these oils, in contrast, have proved to be most effective. Obviously, they have better therapeutic activity than other essential oils. In addition, it is known that Sage, Lavender and above all, always been used to treat various skin diseases and cosmetic products for skin care (Bremnes, 1994). Lavender essential oil possessed as the dominant components linalool, linalyl acetate, limonene, cineole and camphor. Good efficacy of essential oil it can be explained by interactions of individual components, but given the importance of some of the components, especially interactions linalyl acetate and linalool (Lis-Balchin et al., 1998). These essential oils and components could represent possible alternatives for the treatment of animals and humans infected by dermatomycetes. However, there are still only limited data available on the antifungal activity of essential oils towards human fungal pathogens *in vivo* (Soković, 2001).

Extracts of seventeen microfungi (Alternaria alternata, Cladosporium cladosporioides, *C. fulvum*, Fusarium sporotrichioides, F. trincintum, Paecilomyces variotii, *Penicillium ochrochloron*, *P. funiculosum*, *P*homa magdonaldii, Phomopsis helianthi, *Stachybotrys chartarum*, *Trichoderma viride*, and five dermatomycetes, *Epidermophyton floccosum*, *Microsporum canis*, *Trichophyton mentagrophytes*, *T. rubrum and T. tonsurans* were tested against the yeast *Candida albicans* using a bioautographic assay on TLC plates. The extracts weer active against *C. albicans* at concrentations of 50-100 μg/mL. The extact of *P. ochrochloron* was most active. Further bioguided chemical analysis of *P. ochrochloron* afforded two components with antimicrobial activity identified as (−)2,3,4-trihydroxybutanamid and (−)-erythritol. (−)-Erythritol showed moderate antifungal activity, while (−)2,3,4-trihydroxybutanamide was highly active against the fungi tested (Rančić et al., 2006). The antifungal activity of limonene was tested against *Candida albicans* and five dermatomycetes. The antifungal potential of limonene was evaluated against *C. albicans* using a bioautographic method on TLC plates. It showed better potential than bifonazole. When the activity of limonene towards five dermatomycetes was determined by the micro- and diffusion methods, it was more effective against these human and animal pathogens than bifonazole (Rančić et al., 2003). The antifungal activity of garlic bulb powder, allicin and the lozenge with 15% of garlic powder was tested using broth microdilution method against *C. albicans*. The tested garlic powder, as well the lozenge, have shown activity with MIC 1.25–7.50 mg/ml. The major compound, allicin, was highly active at a very low concentration with MIC 0.4 μg/ml. Those concentrations are lower then concentrations of commercially available fungicides (Kundaković et al., 2011). These antifungal agents in development offer

extended half-lives, possibly reduced drug interaction profiles and good tolerance. In addition to activity against animal and human pathogens, they have a broad spectrum of activity including activity against resistant and emerging fungal species. According to these results microfungi may be the source of new biologically active substances, so special attention should be given to research on the biological activity of fungal metabolites and their application.

5. Susceptibility of fungal species to tested compounds

In our investigation, the extracts of different plants exhibited inhibitory effect on the growth of micromycetes. Among the tested extracts one of *Phlomis fruticosa* showed the best activity (10-20 μg/ml) (Table 1.). All investigated mosses extracts have been proved to be active against all fungi tested, where the ethanol extracts of silver moss showed the best potential (0.29-0.52 μg/ml) (Table 1.). The results of the tested essential oils are summarized in Table 1. All tested essential oils exhibited antifungal activity ranging from 0.1-1250 μl/ml using different methods. Among all oils analyzed the essential oil of cinnamon was the most effective as an antifungal agent in concentration 0.1-1 μl/ml using microdilution method. Also, the components of essential oils such as carvacrol, thymol, menthol, showed very strong antifungal activity (0.02-300 μl/ml), where carvacol showed the best potential (0.1-0.5 μg/ml). Among all flavonoides, sesquiterpene lactones and other compounds analyzed in our investigation (Table 1), lactones from *Centaurea pullata* showed the best antifungal activity (0.0001-0.0007 μmol/ml). Thus, comparing the activity of our investigation (extracts, essential oils and components, pure compounds) we can conclude that the sesquiterpene lactones was the most effective as an antifungal agent, followed by essential oils and their components.

The human and food-borne pathogens are most frequently chosen for testing essential oil antimicrobial activity. Many laboratories deal with plant pathogens but fewer with animal pathogens. The essential oils which are the most tested compounds in our investigation of antifungal activity showed different effect on plant and human pathogens species. In our earlier investigation (Soković, 2001) essential oils in general exhibited higher antifungal activity against plant pathogen species (*Phomopsis helianthi, Cladospoirium cladosporioides, Alternaria alternata, Fusarium* species) than human (*Trichophyton* species, *Microsporum cannis, Epidermophyton flocosum*). The essential oils tested possessed different range of minimal fungicidal concentration where human pathogens were more resistant than plant pathogens: *Mentha spicata* 1-2 μl/ml for plant pathogens (pps) and 2 μl/ml for human pathogens (hps), *M. piiperita* 1.5-2.5 μl/ml pps and 2.5 μl/ml for hps, Thyme 0.125-0.25 μl/ml pps and 0.25 μl/ml for hps., *Salvia* species for pps 2-15 μl/ml and 3-20 μl/ml for hps, *Lavandula* species 0.5-9 μl/ml for pps and 0.5-10 μl/ml hps, *Citrus aurantium* 10 μl/ml for pps and 7-15 μl/ml for hps. Some oils had the same antifungal ability against both, pps and hps: *Melissa officinalis* (5-6 μl/ml), *Rosmarinus officinalis* (6-8 μl/ml), *Citrus lemon* (7-10 μl/ml), while *Matricaria chamommilla* exhibited better antifungal capacity against hps (6-9 μl/ml) than for pps (7-9 μl/ml) and Sweet basil (4-5 μl/ml for pps) and 3-5 μl/ml for hps. These results are confirmed

by our investigation of antifungal activity with some other essential oils that we tested latter (Soković et al., 2002; Ristić et al., 2004; Simić et al., 2008; Džamić et al., 2009). Other researchers also obtained the similar results and confirmed that dermatomycetes are the most resistant, although numerous essential oils demonstrate high effectiveness against them. Among 22 samples of essential oils from 11 species of *Cinnamomum*, the oil of *C. suvabenium* was the most active against *Microsporum canis*, *Trichophyton mentagrophytes* and *T. rubrum* as well as some candidiasis (*C. albicans* and *C. glabrata*), (Kalemba & Kunicka, 2003). The results indicate that different essential oils have different efficacy. Also, the modes of action of essential oils are not the same against different fungal species. The mode of action of antimicrobial agents also depends on the type of fungal species and is mainly related to their cell wall structure and the outer membrane arrangement (Villar et al., 1986).

6. A novel approach to solve the problem

Fungal disease is responsible for significant losses of global crop production every year, and thus has a major impact on the world's agricultural productivity. Numerous strategies have been developed in attempts to minimize the losses caused by plant pathogens. Traditional approaches are based on the avoidance of sources of infection, vector management, modification of cultural practices, the use of resistant varieties obtained through conventional breeding, cross protection and chemical control. While these methods have been successful in some cases, indeed there is a need for new approaches. Furthermore some fungicides are being withdrawn from the market because of their undesirable effects on the environment. Whereas this information is interesting with regards to economic aspects, such as the share of fungicides costs in the output or in the variable costs, the information is less useful with regards to environmental aspects: i.e., the amounts spent on fungicides do say little about the types and quantities used. Over recent decade, producers have used synthetic fungicides as the main tool to control this problem. It has been estimated that over 23 million kg of these synthetic fungicides are used annually worldwide and it is generally accepted that production and marketing of plants would be not possible without their use (Martinez-Romero et al., 2008). New strategies for disease control are therefore urgently required. The development of novel control strategies for plant diseases is particularly important for pathogens that are difficult to control using existing methods.

More recently the scientific community has turned its attention to secondary metabolites from actinobacteria and its exploitation for various purposes which include therapeutic, environmental and industrial applications. With developing microbial resistance and need for safe and cost-effective antifungal drugs, screening of some other source, i.e. micro- and macro-fungi, mosses, for potential bioactive secondary metabolites becomes necessary. Particularly desirable is the discovery of novel prototype therapeutic agents representing new chemical classes, that operate by different modes of action compared to existing agents. We were developed suitable approves during the last several years in order to find new solutions in the aim to discover new antifungal drugs either by testing already existing medical compounds, compounds from natural sources such as plants, micro- and

macrofungi, or by combination of chemical compounds with natural one. Researchers also strive to elucidate the underlying biology of fungal microorganism both *in vitro* and *in vivo*.

The majority of our results are focused on investigation of antifungal activity of natural products isolated from plants, especially essential oils. We used conventional research methods for testing of antifungal activity and introduced some modification for the corresponding class of compounds and microorganisms in which we operate. For example, for testing of compounds in small quantity, we recommend using a microdilution method, and microathmosphere method for testing of volatile compounds. Bioautogaphic method on TLC plates is suitable for testing of plant and fungi extracts and fractions. Considering the fact that the fungi (micro and macro) may be the source of new biologically active substances, special attention in our laboratory is given to the research of biological activities of fungal metabolites and their application in protection and treatment of diseases caused by fungi and environmental protection. The broad diversity of the fungi, as well as their easy acquisition make them especially interesting for natural products screening program. The fungi possess high capacity of bio-synthesizing various metabolites possessing different structural and pharmacological characteristics. Many medicinal and therapeutic properties are attributed to the presence of active substances in fungi. Some such compounds are investigated because their known triggering mechanisms important for fungi, while other compounds are tested blindly for their antifungal properties.

Finally, research should be kept up in order to uncover as much potentially interesting data as possible, including toxicity against animal or human cells, mechanisms of action, effects *in vivo*, positive and negative interactions with common fungicides and so forth. Currently, these studies have produced a compounds suitable for the clinical trial stage. In summary, it is our belief that the study of plant, fungi and other natural sources as antimicrobial agents is necessary but the use of a appropriate method for investigation is essential. Finally, our results could be suggested for further clinical tests and for getting new information and possible application. To this should be given high priority.

7. Conclusion and future trends

Natural product-based fungicides are generally considered safer than synthetic herbicides, because of their relatively short environmental half-life and they are not harmful. The recent resurgence of interest in natural sources of bioactive compounds may, in part, be attributed to improved methods and instrumentation that has greatly reduced the time and effort required in natural product discovery programs. This interest is also associated with several other factors, including the realization that nature has already selected for very specific biological activities, that many natural compounds have yet to be discovered, and that the biological activities of relatively few of the known natural products have been characterized. All of the described and possible secondary metabolites have some kinds of inherent activity but in many cases these activities have not yet been discovered. Only the methods to detect their possible, perhaps until now unknown type of activity, has to be developed. There is no reason to suppose that the majority of the natural products including fungal metabolites should not exhibit some kind of biological function.

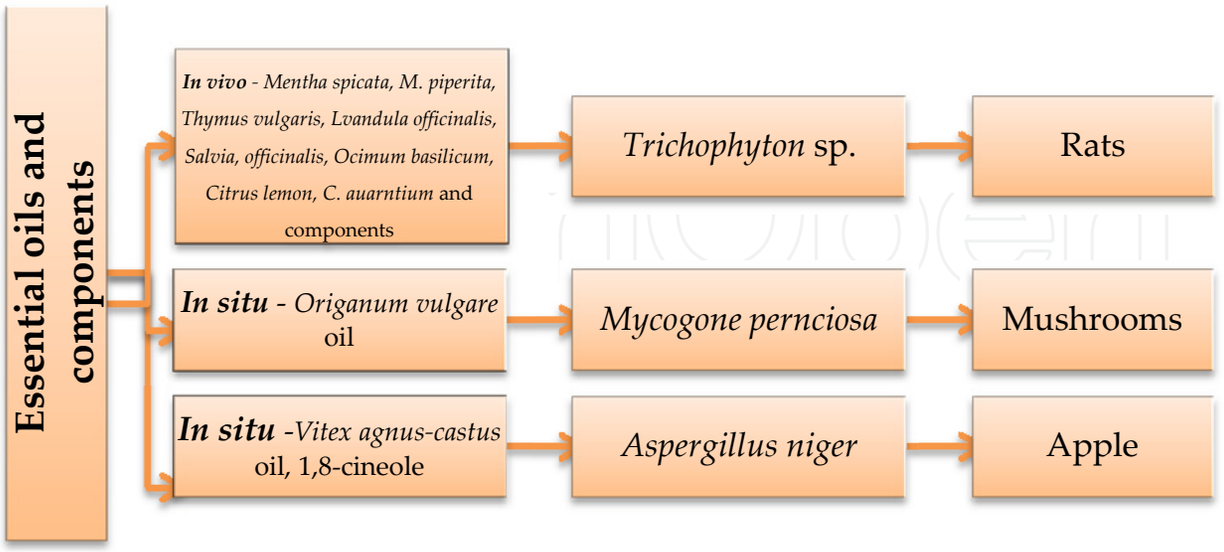

Figure 4. *In situ* and *in vivo* testing of antifungal activity of essential oils and components against pathogenic microfungi

Hundreds of presently known bioactive metabolites originally was discovered as "inactive", natural product and their activity was only discovered later, investigating them with new more specific methods, or reisolated them (sometimes from different species). In our days, in fact, there would not be any reason to talk about "bioactive" or "inactive" secondary metabolites and treat them separately. Moreover, the study of natural products may lead to the discovery of novel target sites, and/or new classes of chemistry that can be developed for pathogen management. Structural diversity has been, and still remains, an invaluable source of lead compounds in developing novel products. A recent study on complementary synthetic and natural products confirmed that the later generally have higher molecular weights than the former. Such diversity may be useful to the synthetic chemist in developing new classes of fungicides. One indirect and important benefit of the chemical composition and structural characteristics of natural products (the absence of "unnatural" ring structures and the low content of heavy atoms) is that most of them are rapidly degraded in the natural environment to benign products. In addition to their structural features, natural products tend to have different target sites from conventional fungicides.

Our results contribute to the development of safe, effective, and inexpensive formulations and processes to reduce the presence of pathogens. The antifungal compounds identified by us as the most active against major pathogens are candidates for future studies of synergism, compatibility and activity in different systems. Isolation and identification of natural active components may include a multitude of different extractions, chemical modifications, and increase knowledge of their mechanisms of action. As essential part of obtaining natural fungicides is the development of bioassays. The development of fungal resistance to synthetic drugs poses a serious long-term trait to plant, animal and human

health and environmental requirements. This could also possess as significant financial issues. The advantage of natural products compared to synthetic is not only in their non toxic characteristics but also in low costs. Growing of medicinal and aromatic plants and fungi is well established and in most cases economically justified. Identification and isolation of active components from plants and fungi is also good elaborated. Natural products with antifungal activity usually operate in very small concentrations, especially essential oils, and the for further application small amount are needed. All together makes them relative cheap and available as antifungal agents.

The future of fungicide management will probably be significantly influenced by research on natural products. Modern instrumentation has simplified the isolation and identification of lead compounds from which fungicides will be derived. The reviewed studies clearly demonstrate that natural products from plants and fungi present great potential for medical procedures and for the food, cosmetic, agricultural and pharmaceutical industries.

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