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PATHOGENIC MICROORGANISMS OF MEDICINAL HERBAL DRUGS

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Abstract - All the parts of plants (root, leaf, flower) naturally have a high level of microorganisms, bacteria and fungi, especially molds. Microbial contamination could be a result of inappropriate harvesting, cleaning of the raw plant material, unhygienic processing of the plants, unsuitable transport and storage. After examination of over 40 dried medicinal plant species, the lowest microbial quality was determined for *Maydis stigma*, *Mentha* leaf and herb, *Equisetum* herb, *Calendula* flower, *Urtica* leaf, *Melissa* leaf, *Serpylli* herb, *Chamomilla* flower etc. Although mixed infections are recorded with different types of fungus, *Fusarium* was observed as the most dominant genus in most of the tested drugs, followed by *Aspergillus* and *Alternaria*. In addition to these fungi species from the following genera were identified: *Phoma*, *Cephalosporium*, *Nigrospora*, *Cladosporium*, *Epicoccum*, *Gliocladium*, *Myrothecium*, *Cercospora, Phomopsis, Verticillium, Dreschlera (=Bipolaris), Rhizoctonia, Septoria, Trichoderma, Curvularia, Stachybotrys, Trichothecium, Puccinia, Botrytis*, *Mucor* and *Rhizopus* sp., depending on plant species.

Key words: Herbal drugs, microbial contamination, bacteria, molds, mycotoxins

INTRODUCTION

Herbal drugs are crude preparations of various kinds of medicinal plants involving a dried plant or any of its parts, such as the leaf, stem, root, flower, or seed. Many of them are believed to have medicinal properties and are used to treat minor illnesses and disturbances. They are promoted as natural and safe and are therefore the preferred choice.

Since they are natural products, all parts of the plants can be degraded by bacteria and fungi, especially molds. Unscientific methods of cultivation and collection, inappropriate harvesting and cleaning, unsuitable transportation, prolonged drying and storage, inadequate hygiene of producers and congenital climatic conditions render the raw plant materials prone to infestations and exposed them to many microbial contaminants. Raw materials are most often degraded by microorganisms before harvesting, during handling and after prolonged storage (Mathe, 1995; Kenneth, 1989). The presence sufficient numbers of microbes can be harmful to consumers. As a result of fungal contamination, the risk of mycotoxin production, especially aflatoxin, should be taken into consideration in the manufacturing process because of the proven mutagenic, carcinogenic, teratogenic, neurotoxic, nephrotoxic, immunosuppressive activities (Refai, 1988; Scimeca, 1995; FAO, 2000; Höhler, 2000)

The microbial quality of herbal drugs has to be co-coordinated with the regulations of the European Pharmacopoeia 6th Edition and Regulations of medical safety of dietary ingredients. Despite several reports of fungal contamination and aflatoxin production in

foodstuff, limited research has been carried out on the microbial contamination of drug-plant samples.

A few authors have indicated microbial contamination of medicinal plants from various parts of the world. Halt (1998) isolated a wide spectrum of fungi including *Aspergillus*, *Penicillium*, *Alternaria*, *Cladosporium*, *Rhizopus* and *Mucor* species from Croatian herbal teas and medicinal plants. Jankovic et al. (2005) found that most of the fungal species found in the oregano herb (*Origanum vulgare* L.) were from the genus *Aspergillus*, and less from the genus *Alternaria*, *Rhizopus* and *Penicillium*. Examination of the microbial quality of mint has shown that the most abundant fungi were from *Penicillium, Alternaria* and *Fusarium* according to Stojadinov J. (1998), or *Fusarium* and *Verticillium* (Pavlović et al., 2000; Stević et al., 2004), as well as *Alternaria alternata, Aspergillus flavus, A. ochraceus, Penicillium cyclopium, Fusarium culmorum, F. equiseti, F. semitectum* and *Septoria menthae.*

A perusal of different reports on fungal and mycotoxin contamination of raw materials indicates that there is no uniformity in the association of fungal species with raw materials. This may be because of the presence of specific secondary metabolites in different herbal drugs, which may be fungitoxic in nature for some fungal species and provide chemical resistance against them (Dubey et al., 2008).

The objective of this investigation was to determine the microbial quality (presence of bacteria and fungi) of the herbal drugs that are mostly used in the manufacture of various products, especially teas. The samples were either taken from the warehouse of the Institute of Medicinal Plant Research "Dr Josif Pančić" from Belgrade before the manufacturing process, or from finished products of herbal drugs (teas). The degree of their contamination by bacteria and fungi in relation to a specified limit of the European Pharmacopoeia was examined. The main subject of this thesis is the isolation and identification microbial species (bacteria and molds) from herbal samples with the worst microbial quality.

MATERIALS AND METHODS

Evaluation of the microbial quality of plant material

The microbial quality of herbal drugs and their herbal medicinal products were tested according to the regulation of Pharmacopoeia (European Pharmacopoeia, 2006) and Regulations. The tests were used for the quantitative evaluation of mesophilic bacteria and fungi that grow aerobically and anaerobically.

Aerobic plate count determination

All supplements were tested as follows: 10 g of each sample were aseptically removed and transferred to sterile blender jars. Subsequently, each sample was blended in 90 ml of 0.1% peptone (saline) for 45 sec. Because the plant samples are recognized to have a significant microbial contamination, serial dilutions were prepared so that the number of colony forming units (CFUs) in the Petri dishes would be less than 300. Duplicate 1 ml aliquots of each dilution sample were pipetted onto two separate sterile Petri dishes (9 cm in diameter); 20 ml of a liquid nutritive agar medium suitable for the cultivation of bacteria or Sabouraud agar for the cultivation of fungi, were added. After solidification of the soft agar, the Petri plates were incubated at 35°C for bacteria and 25°C for fungi, for three and five days respectively. The number of microorganisms in each sample was evaluated by multiplying the average number of colonies per plate by the dilution used. Colonies were counted and the counts were expressed as colony forming units per gram (CFU $g^{-}1$). For the detection of certain bacteria, we used tests for specific microorganisms according to Pharmacopoeia and Regulations. Mold isolates were purified on PDA (potato dextrose agar) and further sub-cultured on malt extract agar (MEA) for microscopic examination and identification. Identification of plant pathogenic fungi isolated from the herbal drugs was carried out using standard determinants.

Identification of fungi

The determination of fungal colonies formed on the

medium was based on the macroscopic and microscopic characteristics of the isolates. Macroscopic features include the appearance and speed of development of colonies on PDA medium, pigmentation and other substrates. Microscopic features include the presence or absence of microconidia, form and manner of formation of microconidia and conidia cells, the appearance of macroconidia, the presence or absence of chlamydosporia, sclerotia, the biometric value characteristics of the reproductive organs of fungi in the culture and the host, and the facultative fungi. For obligate parasites we prepared native preparations and examined the fresh material under a microscope. The standard determinants of Ainsworth et al., (1973); Boot (1971), Gerlach and Nirenberg (1982), Brown (1987), Burgess et al. (1994); Pitt (1979); Raper and Feenel (1965); Elis and Elis, 1997 were used. The identification of the most frequent *Fusarium* species was guided by attention to some generally accepted principles (Nelson et al., 1983).

RESULTS AND DISCUSSION

Depending on the level and possibilities of microbial contamination, a final control of the microbial quality of medicinal herbal drugs was organized. In some herbal drug samples we found that the level of microbial contamination, which is mainly caused by a large number of viable aerobic bacteria or/and increased number of fungi, especially molds, clearly exceeded the tolerable limit according to criteria of Pharmacopoeia and Regulations. Mixed infections were frequently noted.

The presence of a large number of pathogenic bacteria, including *Escherichia coli* (mostly in geranium herb, thyme herb, mint leaves, herb of horsetail, nettle leaves, elderflower, lime flower etc.) was also observed. Contamination with *E. coli* may be a result of either the habitats (proximity of settlements and animals that could contaminate the herbs with urine and feces) or the poor hygiene of the workers.

The dominant bacteria in most of the tested samples belong to the *Bacillus* and *Clostridia* genus, and their high number could be explained by the fact that these bacteria produce spores which are resistant to harsh processing, elevated heat and dry conditions. Therefore, they can survive for a long time on the product in a dormant state. In addition, part of the bacterial pollution may have originated from the personal handling of the raw herbal materials during processing, especially if hygienic conditions were not followed, and from the processing plants' environment.

In our two-year examination, the highest microbial contamination was recovered from samples of mint, corn silk and marigolds, which is probably due to primary contamination in the field as a consequence of the manner of harvesting. Corn silk is thrown to the ground during the harvesting of corn, but for marigold there is an assumption that the contamination comes from the dust that rises from the ground during preparation of the herbs that are grown with this medicinal plant. It is believed that the secondary contamination of mint generally occurs during the post-harvest period in the course of separation of the leaves from herb which takes place mainly on the ground. Among other isolated bacteria, *Staphylococcus aureus* was the most frequent in the *Althaeae radix* samples.

The biggest problem, in terms of the microbiological safety of herbal drugs, was that of mold contamination. The level of contamination varied over a wide range, from a minimum exceeding the allowable limit, to a significantly large contamination over the upper limit. The lowest microbial quality, in terms of molds, was determined for the following herbal drugs: 82% of the samples of corn silk (*Maydis* stigma) were contaminated with fungi; mint leaf and herb (*Menthae* folium et herb) - 74% samples; horsetail herb (*Equiseti* herb) - 62%; marigold flowers (*Calendula* flowers) - 56%; nettle leaf (*Urtica* folium) - 52%; chamomile flower - 47%; linden flower (*Tilia* flower) - 40%; lemon balm leaf (*Melissa* folium) - 38%; wild thyme herb (*Serpylli* herb) - 37.5%; yarrow herb (*Millefolii* herb) - 30% (shown in Fig. 1). The level of contamination with molds was generally above 10^5 CFU/g in the samples of the

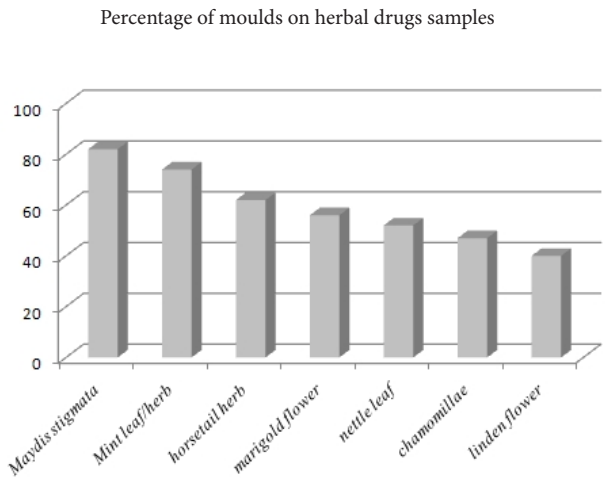

Fig. 1. Percentage of molds on herbal drugs samples. Images of mold species isolated and identified in some herbal drugs

first five herbal drugs, and very often above 10^6 in the samples of corn silk, mint and marigold.

The highest degree of contamination with molds was detected in corn silk ($\geq 10^6$ CFU/g). We extracted the corn silk at different times during the ripening of the cob and concluded that the silk was contaminated while in its fresh, non-skimmed form, probably due to the high percentage of moisture in the cob. Additionally, secondary contamination certainly was caused by the throwing of the silk on the ground during the corn harvest.

In support of these results are those of Martins et al. (2001) which recorded the highest level of contamination in mint, corn silk and chamomile (> 10^7CFU/g sample). According to these authors, almost all samples (96.8%) of corn silk were contaminated with *Bacillus cereus* and *Clostridium perfringens*. In the case of molds, *Fusarium* spp., *Penicillium* spp., *Aspergillus flavus* and *A. niger* were dominant in all samples.

Apart from corn silk, mint and marigold, we found that most commonly contaminated drugs include horsetail and nettle. Almost all of the samples were contaminated with mixed infections of bacteria and fungi (mostly molds). The values for both parameters significantly exceeded the specified limits. We also noted an ongoing contamination of nettle and horsetail samples with *E. coli*, which further affects the poor quality of drugs. As these medicinal herbs are mostly collected from natural habitats (spontaneous flora) such as neglected areas, the edges of forests, river banks, streams and along fences and roads where there are plenty of animals, it is supposed that the cause of high contamination with this coliform bacteria is through animal feces and urine. Humid environments with dust are also ideal for microbial growth, especially molds.

After determining the herbal drugs that expressed a higher contamination with molds, we started with the isolation and identification of mold species. We investigated the fungal status in mint (*Mentha piperita* L.), corn silk (*Maydis* stigma), field horsetail (*Equisetum arvense* L.), nettle (*Urtica dioica* L.) and marigold (*Calendula officinalis* L.). The fungal profiles of the tested and most contaminated herbal drugs are summarized in Table 1.

It can be seen from Table 1 that a wide spectrum of fungi (including *Fusarium*, *Aspergillus*, *Penicillium*, *Alternaria*, *Rhizopus, Mucor, Cladosporium*, *Phoma* species and others) was isolated from the chosen herbal medicinal drugs.

Among the fungi so far identified, those belonging to *Fusarium* and *Aspergillus* were predominant in most of the drugs, except marigold where *Mucor* and *Rhizopus* were the most abundant. In addition to these fungi, in most samples we identified species from the genus *Alternaria.*

In addition to reducing their quality and usefulness, the presence of fungi in medicinal plants could, under certain conditions, also lead to the secretion the toxic metabolites, mycotoxins which possess very powerful mutagenic and carcinogenic effects. Mycotoxins are thermostable and cannot be destroyed by cooking. They have a cumulative ability and are difficult to eliminate from the organism.

Mycotoxic-producing molds can be classified into three groups according to the time of develop-

Table 1. Identified molds of the tested herbal drugs

Herbal drugs	Identified fungi
Maydis stigma	*Mucor sp.* *Fusarium* sp.; *F. proliferatum; F. verticillioides (=F. moniliforme); F. taphsini; F. subglutinans; F. graminearum; F. nygamai* *Alternaria* sp. *Aspergillus niger, A. flavus* *Penicillium* sp. *Cephalosporium* sp. *Cladosporium* sp. *Epicoccum purpurascens* *Nigrospora* sp. *Rhizopus* sp. *Epicoccum purpureus*
Menthae folium and herba	*Dreschlera (=Bipolaris) tetramera* *Aspergillus flavus, A. niger, Aspergillus* sp. *Fusarium verticillioides; F. sporotrichioides; Fusarium* sp., *F. proliferatum* *Cercospora* sp. *Mucor* sp. *Penicillium notatum, Penicillium* sp. *Phoma* sp. *Phomopsis* sp. *Verticillium dahliae, V. cynobarium* *Rhizoctonia solani* *Septoria* sp. *Trichoderma viride* *Gliocladium* sp. *Curvularia lunata* *Septoria* sp. *Cladosporium* sp. *Rhizopus* sp. *Alternaria alternata*
Equiseti herba	*Fusarium solani, F.equiseti, F. tricinctum, F. graminearum. F. oxysporum, Fusarium* sp. *Cladosporium* sp. *(herbarum)* *Aspergillus niger, A. flavus, Aspergillus* sp. *Chaetomium* sp. *Stachybotrys* sp. *Alternaria* sp. *Penicillium* sp. *Epicoccum* sp. *Myrotechium* sp.
Urtica folium	*Fusarium semitectum, F. verticillioides, F. sporotrichioides, F. verti, F. graminearum* *Gliocladium roseum* *Aspergillus niger* *Phoma* sp. *Trichothecium roseum* *Puccinia* sp. *Septoria* sp. *Alternaria alternata*, *A. tenuissima* *Botrytis* sp. *Rhizopus* sp.
Calendulae flos	*Mucor* sp. *Rhizopus* sp. *Alternaria alternata, A. tenuissima, Alternaria* sp. *Myrothecium veruccaria* *Fusarium proliferatum, F. oxysporum, F. verticillioides,*

ment and contamination of the plants: field molds (*Alternaria*, *Fusarium*, *Cladosporium*, *Helminthosporium*), barn and storage molds (*Aspergillus*, *Penicillium*) and advanced decay molds (*Mucor*, *Rhizopus*, *Sordaria*). It is believed that all of the important and known mycotoxins are produced by *Aspergillus, Fusarium* and *Penicillium* species.

As stated, mixed infections were recorded in all drug samples, but the most common (in percentage) belonged to typical field mycopopulations such as the species from *Fusarium* and *Alternaria* genera. In addition to this fungus, we also identified storage molds in most of tested herbal species (*Penicillium*, *Aspergillus*) and advanced decay molds (*Mucor* and *Rhizopus*) which were dominant in marigold and corn silk.

Fusarium species were the most prevalent in the tested samples. Their spores survived the drying conditions and can remain dormant for several months, possibly years, on the dried herb. During this time, if the moisture of the product were to increase to levels allowing spore germination, significant mold growth and possibly mycotoxin production could occur.

Potentially toxigenic *Fusarium* species, *F. sporotrichioides*, *F. semitectum*, *F. oxysporum*, *F. solani*, *F. equiseti* and *F. proliferatum*, were determined, so it is necessary to pay special attention to them not only because they are a potential producer of a large number of toxins, but because they are connected with various diseases in humans and animals. *F. verticillioides* (formerly known as *F. moniliforme*) and *F. proliferatum* are the main source of fumonisins in maize (Marasas et al., 2001).

As can be seen from the table, we isolated and identified the largest number of mold species from the mint samples (*Mentha piperita* L.). Apart from the most abundant species from *Fusarium* and *Aspergillus* genera, we also identified *Alternaria alternata*, *Phoma* sp., *Penicillium* sp., *Cladosporium* sp., *Cercospora* sp, *Phomopsis* sp, *Verticillium dahliae* and *V. cynobarium, Bipolaris tetramera, Rhizoctonia solani, Septoria* sp., *Trichoderma viride, Curvularia lunata*, *Gliocladium* sp., *Rhizopus* sp. and *Mucor* sp.

The results of this study matched the results of previous investigations of mint from plantations throughout Serbia and Europe where it was observed that almost all of the samples were contaminated with molds and aerobic mesophilic bacteria (Stojkovic, 1998; EHIA, 2003). Contamination with the coliform bacteria, *E. coli* was also frequently noted. Pavlović et al. (2000; 2006) found that the most significant fungi causing serious commercial damage to peppermint are from the genus *Fusarium* (*F. semitectum*, *F. subglutinans*, *F. culmorum*, *F. proliferatum*, *F. equiseti*) and *Verticillium* or some other species: *Alternaria* spp., *Penicillium* spp., *Aspergillus* species (*A. niger, A. flavus*, *A. candidus,* and smaller amounts of *A. ochraceus, A. fumigatus*), *Trichothecium roseum, Trichoderma* and others. Omurtag and Yazicioglu (2004) reported that *Fusarium* could also produce the toxin fumonisin B1 (160 ppb) on the mint herb.

Mint leaves are very hygroscopic and susceptible to contamination, especially by fungus, so that fast drying is essential after harvesting, as well as rigorous control of external conditions (temperature and humidity) at all stages of production and storage to minimize the potential for further secondary contamination. It is believed that significant contamination comes in the post-harvest period during the separation of leaves from the herb, because this mostly take place on the ground.

In our investigation, besides *Fusarium*, *Aspergillus* species were wide-spread in the tested drugs and mostly occurred in mint, horsetail, nettle and corn silk. Among the *Aspergillus* species, the *A. niger* group was the most frequently encountered and widely distributed in the tested drugs. *A. niger* is an extremely common species, but only a few strains appear to be producers of ochratoxin A, so this species may be of much less importance than *A. flavus*. The potentially toxigenic fungi *A. flavus* was determined in corn silk, mint and horsetail at low levels. The isolation of *A. flavus* from drugs is of the highest concern because these organisms are known to produce aflatoxins and ochratoxin, respectively (Pitt and Hocking, 1997; Riba et al., 2008).

The presence of *Aspergillus* and *Penicillium* species on herbal drugs could mean that there was some growth of these organisms established before the complete drying of the drugs. Since aspergilla are capable of growing at low water content, in order to avoid such growth and the possible production

Figures 2 -7. Images of mold species isolated and identified in some herbal drugs

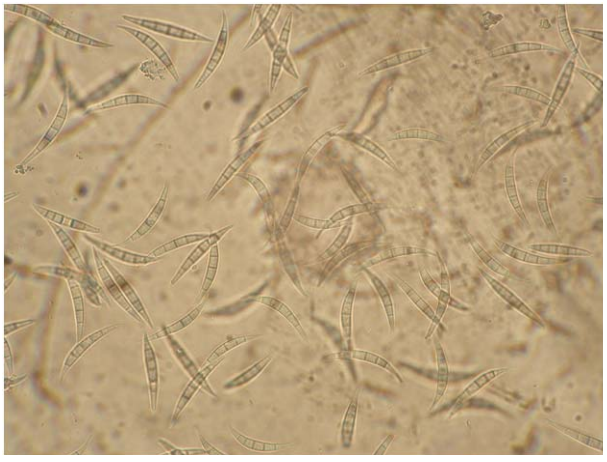

Fig. 2. Macroconidia of *Fusarium equiseti*

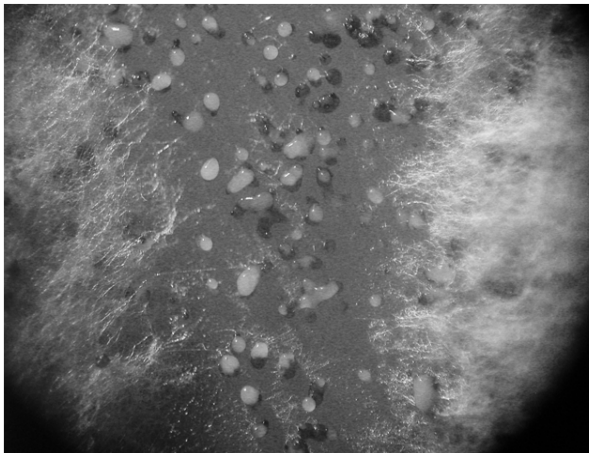

Fig. 5. *Phoma* sp. on PDA (nettle)

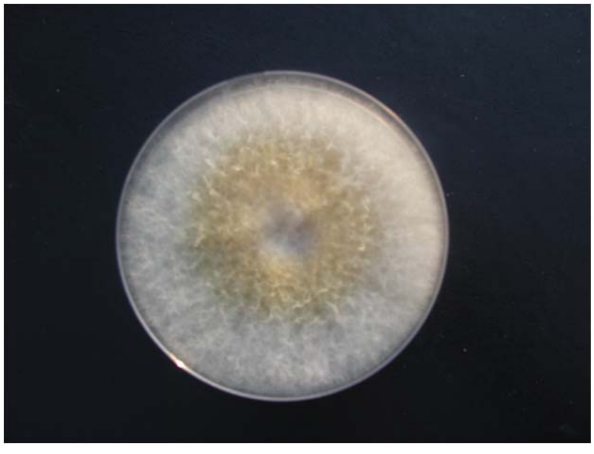

Fig. 3. *Fusarium equiseti* - colony on PDA

Fig. 6. Mixed infection of several species of fungi

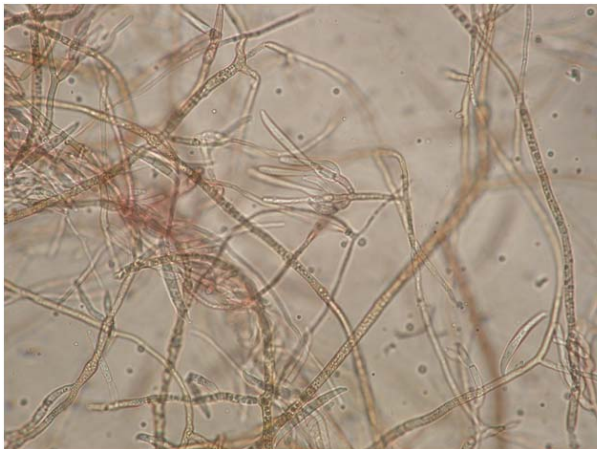

Fig. 4. *Fusarium graminearum - in situ.*

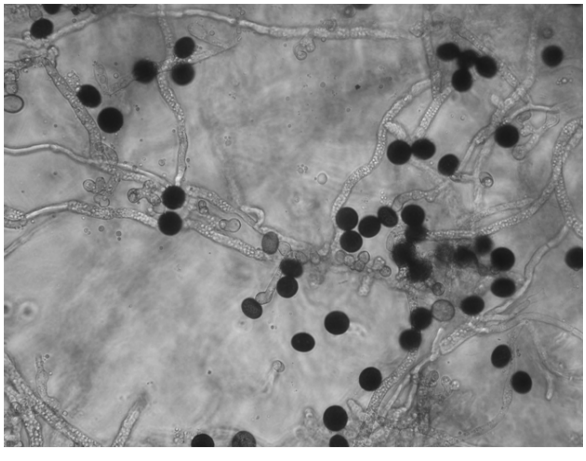

Fig. 7. *Nigrospora* sp.

of toxic metabolites, care should be taken to dry the product quickly before these molds have the chance to establish any significant growth. Members of the genera *Penicillium* and *Aspergillus* are reported to produce the widest range of mycotoxins, which, with the exception of the mentioned ochratoxin A and aflatoxins, are patulin, citrinin, citreoviridin, griseofulvin, rubratoxin, and penicillic, cyclopiazonic, secalonic, or mycophenolic acids (Ostrp, 2001).

According to our investigation, *Alternaria* species were found in all the tested herbal drugs. The infection and colonization of medicinal plants with these plant pathogens and *Phoma* (identified in mint and nettle), could start in the field and spoilage could become rapid and extensive after harvest, when the plant defenses are weakened or eliminated (PMSP 2002). Alternatively, a few spores carried on healthy leaves from the field could also spoil the product after harvest during transport and storage if the moisture level is sufficient for fungal growth. Both *Alternaria* and *Phoma* species have the ability to produce mycotoxins (Canafoglia et al. 2007; Lugauskas et al. 2006). Therefore, their presence in herbal drugs should be kept as low as possible and the moisture of the product should be maintained at levels that do not allow fungal growth.

In a much smaller number of tested medicinal drugs, species of the genera *Rhizopus* and *Mucor* were detected. These genera were the most frequently encountered molds in the marigold flower samples (found in over 90% of the samples), and slightly less in corn silk. We sporadically isolated the species of other genera: *Cephalosporium* and *Nigrospora* in corn silk; *Cladosporium* (in mint, corn silk, horse tail); *Phoma* (mint, nettle); *Epicoccum* (corn silk, horsetail); *Gliocladium* (mint, nettle); *Myrothecium* (horse tail, marigold); *Cercospora, Phomopsis, Verticillium, Dreschlera (=Bipolaris), Rhizoctonia, Septoria, Trichoderma, Cuvularia* in mint; and *Stahybotrys, Trichotecium roseum, Puccinia, Botrytis* in nettle samples (images of some mold species are shown).

Based on our results, it can be said that the contamination of herbal drugs is a result of contamination during cultivation and harvesting, but also of inadequate drying and prolonged storage. Some of medicinal plants are restricted to special localities, from where they are collected, dried and then distributed to producers where they are stored for future use. The levels of relative humidity and environmental temperature are precisely the conditions that favor fungal colonization of stored plant material. Storage decay of plant products is a common occurrence. Proper storage facilities are not available to many growers of medicinal plants (appropriate relative humidity and air circulation), and cross-contamination and development of microscopic filamentous, naturally occurring saprophytic and pathogenic fungi on herbal drug material occurs. This may result in the accumulation of toxic fungal metabolites, which can be hazardous to humans who unknowingly consume products contaminated by mycotoxins.

Karan et al. (2005) assumed that essential oils from medicinal and aromatic plants and spices could influence the number and composition of molds. As our research has shown that the most contaminated herbal drugs are also those with a low content of essential oils; apart from mint, we can say that it has the greatest impact on the number of molds on the herb.

Microbial contaminations represent a great problem for herbal remedies. Significant yield reduction and the low quality of the drugs as a result of the activities of different fungi, requires more attention because of the danger of the mycotoxins produced by fungi. Therefore, the prevention of mold development and mycotoxin production in medicinal plants in the field and on the dried herbal drugs, as well as in the manufacturing and stored rooms, is of great importance. Previous methods (radioactivity, sterilization by ethylene oxide etc.) are partly unpopular, partly harmful which makes necessary the introduction of new methods of preserving medicinal plants using natural compounds, by so-called bio-control.

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