Aspects Regarding the Influence of Concentration of Components with Antifungal Activity from Some Essential Oils

GABRIELA ISOPENCU[1, 2, *], MARIANA FERDES[1]
[1]Politehnica University of Bucharest, Dept. of Chemical Engineering, 1-7 Polizu Str., 011061, Bucharest, Romania
[2] University of Agricultural Sciences and Veterinary Medicine, 3-5 Calea Mănăştur, 400372, Cluj-Napoca, Romania

In this work we studied the antimicrobial action of essential oils based on their chemical composition. For the experimental study were used essential oils obtained from dill (Anthum gruveoles), basil (Ocimun basilicum), thyme (Thymus vulgaris) and coriander (Coriandrum sativum), specific plants for our geographical area (area Romania). Fungus strains used belong to the classes Aspergillus sp. (A. niger, A oryzae, A. ochraceus), Fusarium sp (Fusarium oxysporum). The experimental study aims to follow the development of colonies of mold growing on synthetic medium of malt, by monitoring the size and appearance of the colony formed. Experiments were carried out maintaining a specific microclimate for growth of microorganisms, rich in essential oils. It is found that for the type Aspergillus, essential oils induces a latency period in the growth of the colonies, from 48 to 96 h and the antifungal activity for Fusarium oxysporum is quite small, even to high quantity of oil.

Keywords: active substances, esential oils, fungal strains, antimicrobial activity

Essential oils from natural sources are a complex of different chemical compounds (liquid or solid), dissolved to form homogeneous solution and their therapeutic effect is resulting from the joint action of the elements (active or inactive). Inactive components can influence the speed of adsorption, reaction and biological availability of active compounds. Also, the composition of essential oils from natural sources is influenced by genotype, chemotype, geographic origin of plants that produce them and, not least, the conditions of agricultural environment in which plants grow [1].

The main components of volatile oils are substances of class terpenoids (hydrocarbon terpenes and oxygenated derivatives alcohols, aldehydes, ketones, ethers, acids, esters, phenols and derivatives thereof), specific substances of the vegetable area. In addition to terpenoids are found, in smaller quantities, organic chemicals such as olefins and acetylene, saturated aliphatic hydrocarbons, aromatic hydrocarbons, alcohols, aldehydes, ketones, phenols, acids, esters, lactones, carotenoids, flavones, lignans, derived nitrogen or sulfur.

Volatile oils are sensitive to the action of oxidizing agents. Presence in the composition of unsaturated hydrocarbons and compounds with oxidant functions make the oxygen in the air to produce irreversible changes on them, especially if his action is activated by light and temperature. Peroxides and polymers formed in this process distort the quality of essential oils [1, 2].

The essential oil undergoes compositional changes during biological development of plants as a result of biochemical processes that occur in plants: in the young, evolving plants, essential oils are rich in hydrocarbon terpenes and compounds with simplest molecule; in the mature plants, the reproductive organs are rich in oils with oxygenated compounds. For the plants, the essential oils is assumed to have a role in cell metabolism as antioxidants and hydrogen donors, and some components of those, such as alcohols and ketones, represents a moderator of intracellular oxidative processes and weather protection [1-4].

The great diversity of essential oils obtained from natural sources (using different methods of preparation) requires studies on their antimicrobial and antioxidant activities, due to specific chemical composition.

This article presents the antimicrobial action of the essential oils obtained from local plants such as dill (*Anthum gruveoles*), thyme *(Thymus vulgaris)*, basil (*Ocimun basilicum*) and coriander *(Coriandrum sativum)* upon some strains of saprophytic fungi, plants rotting agents, such as *Aspergilius* sp *(A. niger, A oryzae, A. ochraceus*) and *Fusarium* sp *(Fusarium oxysporum).*

Experimental part

Essential oils analyzed, in terms of antifungal activity in experimental research, are purchased from the firm specialized in the field. We used specific plant oils that come from the area of our country. The main chemical characteristics of these oils are listed below.

Dill essential oil **(*Anthum gruveoles*).**

Dill essential oil is found in proportion of 2.5 to 4% in the branches and leaves and around 3-6% in seeds. The essential oil contains between 30-60% carvone, 33% limonene, alpha-phellandrene (20.61%) including terpine, myristicin and isomyristicin, and other substances such as benzyl ether, ascorbic acid, carotene, B1, B2, folic acid, maltose, xylose, sucrose, pectin, etc.[5].

Basil essential oil **(*Ocimun basilicum*)**

Basil oil, extracted from the flowering plant, contains mainly linalool (41.21%), estragole (22.17%) and sesquiterpene hydrocarbons category such as (E)-α-Bergamotene (7.56%) less than 1% remaining substances (eg. A- Caryophyllene, Germacrene D-Selinene β, α-Zingiberene, Bicyclogermacrene, α-Murolene, Germacrene A γ-Oxygenated sesquiterpenes Cadinene as, Γ-Cadinol). [6]

Thyme essential oil (Thymus vulgaris)

Chemical composition of volatile oil of thyme is very different, primarily in terms of quantities of the basic

* *email: g_isopencu@chim.upb.ro; Tel: +40213969*

components, thymol and carvacrol. These components may be missing or are minor components of essential's oils, function of plant chemotype. As bioactive components, depending on chemotype, like in thyme herba (whole plant) and thyme aetheroleum (essential oil) encountered: carvacrol (20-40%), tymolol (10-20%), p-cimen (10-30%), α terpinil acetate (5-15%), linalool (2-10%), γ-terpinene (1-5%), β-Caryophyllene (1-5%), terpinene-4-ol (1-5%), geraniol (1-3%) and other substances in smaller concentrations (ie. camfen, mircen, fenchen, limonene, p-sabinene, terpinolen, α and β-phellandren) [7, 8].

Coriander essential oil (Coriandrum sativum)

The essential oil is found in leaves and seeds of *C. sativum*, with different composition, depending on the source of production. Thus, the amount of volatile oil in leaf is smaller and contains about 44 compounds, acids and alcohols mostly aromatic: 2 decenoic acid (30.8%), E-11-tetradecenoic acid (13.4%), capric acid (12.7%), undecyl alcohol (6.4%), tridecanoic acid (5.5%) and undecanoic acid (7.1%). In *C. sativum* seeds were found 53 compounds, of which the most important are: linalool (37.7%), geranyl acetate (17.6%) and γ-terpinene (14.4%) [9].

Common mold species are found currently in food and cause them contamination, degradation, through altering the nutritional and organoleptic properties. The microorganisms used as indicators of antifungal activity of volatile oils are four fungal strains from the collection of the Laboratory of Microbiology, Faculty of Applied Chemistry and Materials Science:

- *Aspergillus niger* (*ATCC 15475*) belonging to septate class of fungi, is a widespread contaminant of various substrates. It is forming colony with color brown-black and radial shape. Several selected strains are used for the extraction of enzymes: amylase, protease, glucozoxidaze, pectolytic enzymes, invertase or to obtain organic acids: citric acid, lactic acid. *Aspergillus niger* especially contaminate food containing oligosaccharides, starch and protein [10, 11].

- *Aspergillus oryzae* (*ATCC 11498*) it is a mold forming beige-orange colony. Is rightly called "the arsenal of enzymes" because it is known more than 200 enzymes produced by this mold and those are obtained with high purity. This mold is used to obtain enzymes amylolytic - type koji for saccharification of the rice starch substrates and for obtaining fermented beverages, such as sake [10].

- *Aspergillus ochraceus (AM.PR)* forms large colonies, at first white, circular, cream-colored, fluffy-looking profile and the colony edges are profiled and smooth. Growth environment is represented by the soil, grains and salted food. Mold secretes a toxin which affect human kidney, ochratoxin A, which can cause disease, such as Balkan nephropathy. The toxin is produced under optimal growth at 25°C and high humidity. Other toxins that can be produced by this mold include penicillic acid, xanthomegnin and viomellein. All these toxins are considered to be kidney and liver toxins [10].

- *Fusarium oxysporum* (*MUCL 791*) is a septate mold that is found widely in soil, on plants, the seeds of cereals, lettuce, peanuts, soybeans, peas, beans, cotton, potato, apple, beet and corn. Molds belonging to the genus *Fusarium* species include common soil saprophytic and pathogenic species of parasitic higher plants. Species of the genus: *F. graminearum, F. monilliforme, F. tricinctum F. nival*, etc., cause brown rot of citrus fruits, figs wet rot, rot grains (barley, wheat) and produce mycotoxins (tricothecene) [10].

Cultivation of microorganisms was performed on a synthetic medium of malt: malt - dextrose (17 g/L) and agar (20 g/L). Sterile medium was distributed in Petri plates and inoculated with 2 mL of microbial suspension. On the cover of Petri plates were poured the quantities of 1, 5, 10 μL of essential oil in an air volume of approx. 65 mL, thus creating a specific microclimate for growth of microorganisms. For each strain of mold was kept a witness control for the normal growth on the selected microbial medium. Plates were isolated with tape, in order to prevent the emission of essential oil vapour out of the area of microbial growth. Petri plates were incubated at a temperature of 25°C for two weeks, during which it was measured periodically the diameter of colonies formed.

Results and discussions

Experimental research follows to determine the antimicrobial activity of essential oils mentioned above, on the strains of microorganisms selected, by evaluating the following parameters:

- growth rate of microorganisms, ν, in the presence of essential oils as determined by the relationship:

$$\nu = \frac{d_c}{\tau} \qquad (1)$$

- efficiency of inhibition of essential oil on growth rate of microorganisms, E_{exp}.

$$E_{exp} = \frac{\nu_M - \nu}{\nu_M} \cdot 100 \qquad (2)$$

Antimicrobial activity of essential oils was determined through analyzing the characteristics of mold's, by measuring the diameter of colonies formed and the calculation of colony development parameters: speed of growth rate and effective inhibition (table 1 and 2).

To highlight the antifungal action of essential oils is presented dimensions evolution of mold colonies in the atmosphere of essential oils, compared with the development of control samples (fig. 1-4).

Analyzing the antifungal activity of essential oils and the charts of the microorganisms growth parameters, are evidenced the following conclusions:

- dill oil shows antifungal activity against *Aspergillus niger* and *Aspergillus oryzae* strains at a concentration of 1 μL oil/65 mL air; concentration required for *Aspergillus ochraceus* is 5μL oil/65 mL air and for inhibition of *Fusarium oxysporum* are required concentrations higher than 10 μL oil/65 mL air in the microbial growth environment. Antimicrobial action is due to its high polyphenols content; those are in percentage of 10.6-14.3% w/w - determined by the Folin -Ciocalteu method [12]. *Aspergillus* species are more sensitive at D-limonene and D-carvone, compounds existing in high concentration in dill oil, which is why the amount of oil required for inhibition is less.

- basil-oil substances have antifungal activity through methyl-eugenol and t-anethole [13], inhibition of microbial growth was determined for concentrations of 5 μL oil / 65 mL air for *Aspergillus ochraceus* and *Apergillus niger*; for *Fusarium oxysporum* and *Aspergillus oryzae* required concentration is 10 μL oil/65 mL air in the microbial growth environment.

- thyme oil inhibits the activity of *Apergillus niger* and *Aspergillus oryzae* of 1 μL oil/ 65mL air and for *Aspergillus ochraceus* 5 μL oil /65 mL air; for *Fusarium oxysporum* strain at a concentration of essential oil of 10 μL oil/65 mL air; components which are directly involved in antifungal activity are carvacrol and thymol as phenolic substances [14].

No. crt.	Essential oil	Microbial strain	Growth rate, mm/h			
			Control sample	Oil - 1μL	Oil - 5μL	Oil - 10μL
1	*Dill*	***MUCL 791***	0.42	0.30	0.28	0.26
2		***ATCC 15475***	0.24	0.12	0.12	0.09
3		***AM.PR***	0.13	0.08	0.07	0.04
4		***ATCC 11498***	0.29	0.16	0.10	0.07
5	*Basil*	***MUCL 791***	0.29	0.20	0.14	0.04
6		***ATCC 15475***	0.15	0.09	0.04	0
7		***AM.PR***	0.15	0.12	0.04	0.01
8		***ATCC 11498***	0.25	0.20	0.14	0.06
9	*Thyme*	***MUCL 791***	0.29	0.20	0.14	0.04
10		***ATCC 15475***	0.15	0.06	0	0
11		***AM.PR***	0.15	0.13	0.03	0
12		***ATCC 11498***	0.21	0.03	0	0
13	*Coriander*	***MUCL 791***	0.33	0.26	0.19	0.01
14		***ATCC 15475***	0.17	0.15	0.12	0.05
15		***AM.PR***	0.15	0.11	0.08	0.01
16		***ATCC 11498***	0.21	0.15	0.12	0.03

Table 1
GROWTH RATE DEVELOPMENT OF MOLDS CULTURE IN THE PRESENCE OF ESSENTIAL OILS

No. crt.	Essential oil	Microbial strain	Efficiency, E_{exp} %		
			Oil - 1μL	Oil - 5μL	Oil - 10μL
1	*Dill*	***MUCL 791***	29	33	38
2		***ATCC 15475***	50	50	63
3		***AM.PR***	38	46	69
4		***ATCC 11498***	45	66	76
5	*Basil*	***MUCL 791***	31	52	86
6		***ATCC 15475***	40	73	100
7		***AM.PR***	20	73	93
8		***ATCC 11498***	20	44	76
9	*Thyme*	***MUCL 791***	31	52	86
10		***ATCC 15475***	60	100	100
11		***AM.PR***	13	80	100
12		***ATCC 11498***	86	100	100
13	*Coriander*	***MUCL 791***	21	42	97
14		***ATCC 15475***	12	29	71
15		***AM.PR***	27	47	93
16		***ATCC 11498***	29	43	86

Table 2
EFFICIENCY OF INHIBITION OF FUNGI CULTURE BY ESSENTIAL OILS

- coriander oil is active antifungal for concentrations higher than 10 μL oil/65 mL air in the microbial growth environment, for all studied strains, because the content of carvacol, eugenol and their precursors are in low concentration (less than 1% w/w) [11].

The morphological characteristics of the mold strains are modified when the colonies are growing in essential oil atmosphere. In figures 5-8 are presented the differences between the control sample and the sample with essential oil, poured on the cover of the Petri plate, regarding the dimensions and colony morphology for *Aspergillus ochraceus*.

As can be seen, dill oil not only affects colony size but also its appearance (from puffy, septed and radial colony, now looks creamy, compact, with irregular borders).

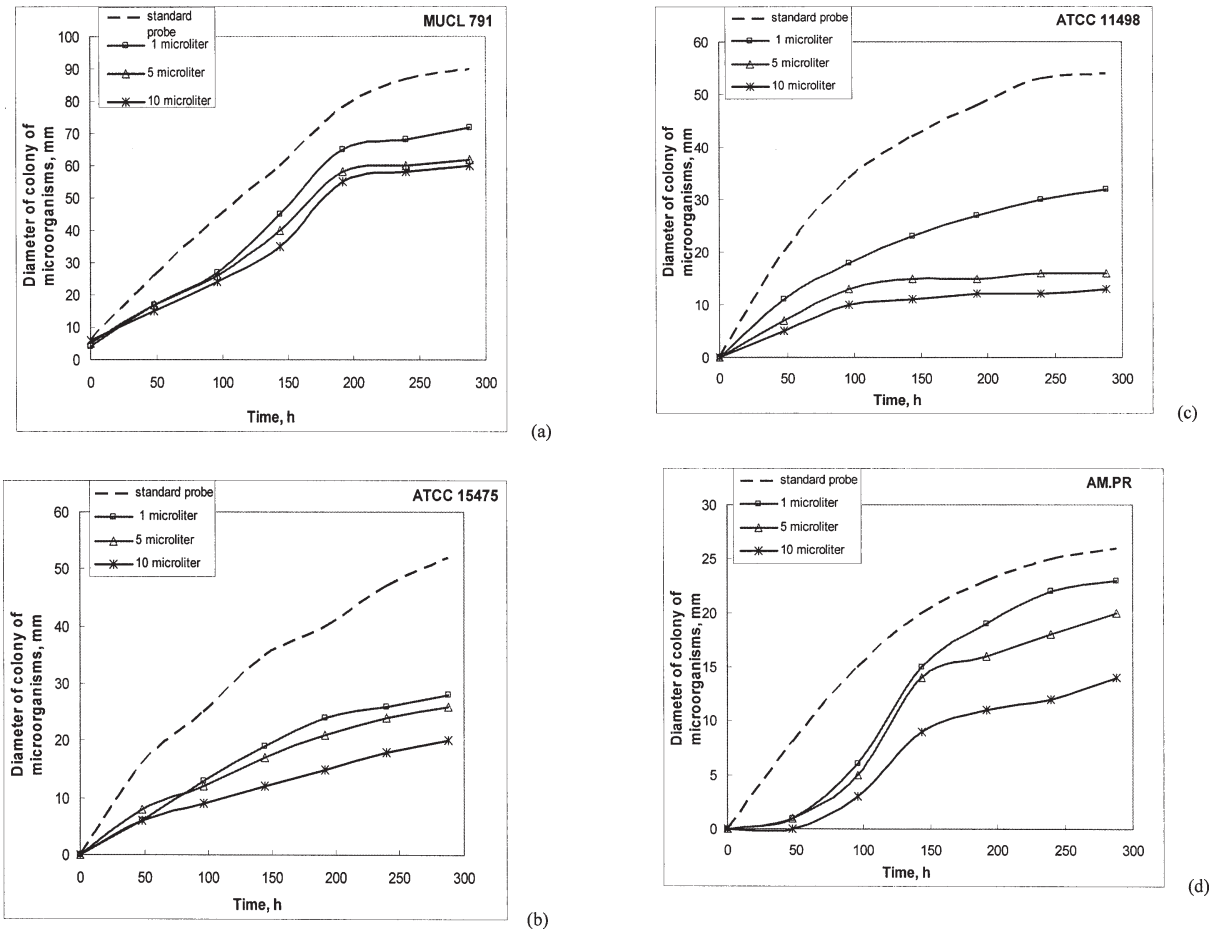

Fig.1. The evolution of microorganism's colony in the presence of different concentrations of dill essential oil (a - *Fusarium oxysporum*, b - *Aspergillius niger,* c - *Aspergillius oryzae,* d - *Aspergillius ochraceus)*

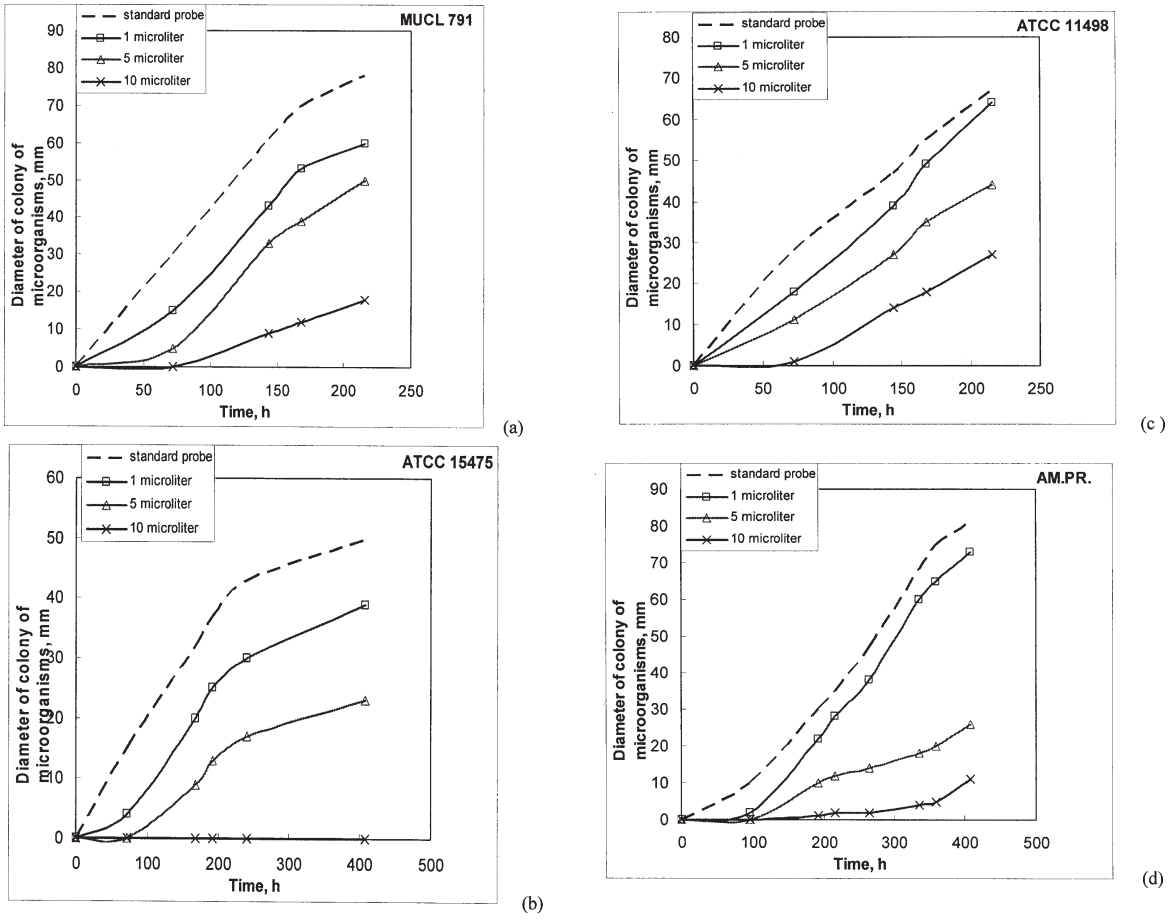

Fig.2. The evolution of microorganism's colony in the presence of different concentrations of basil essential oil (a - *Fusarium oxysporum*, b - *Aspergillius niger,* c - *Aspergillius oryzae,* d - *Aspergillius ochraceus)*

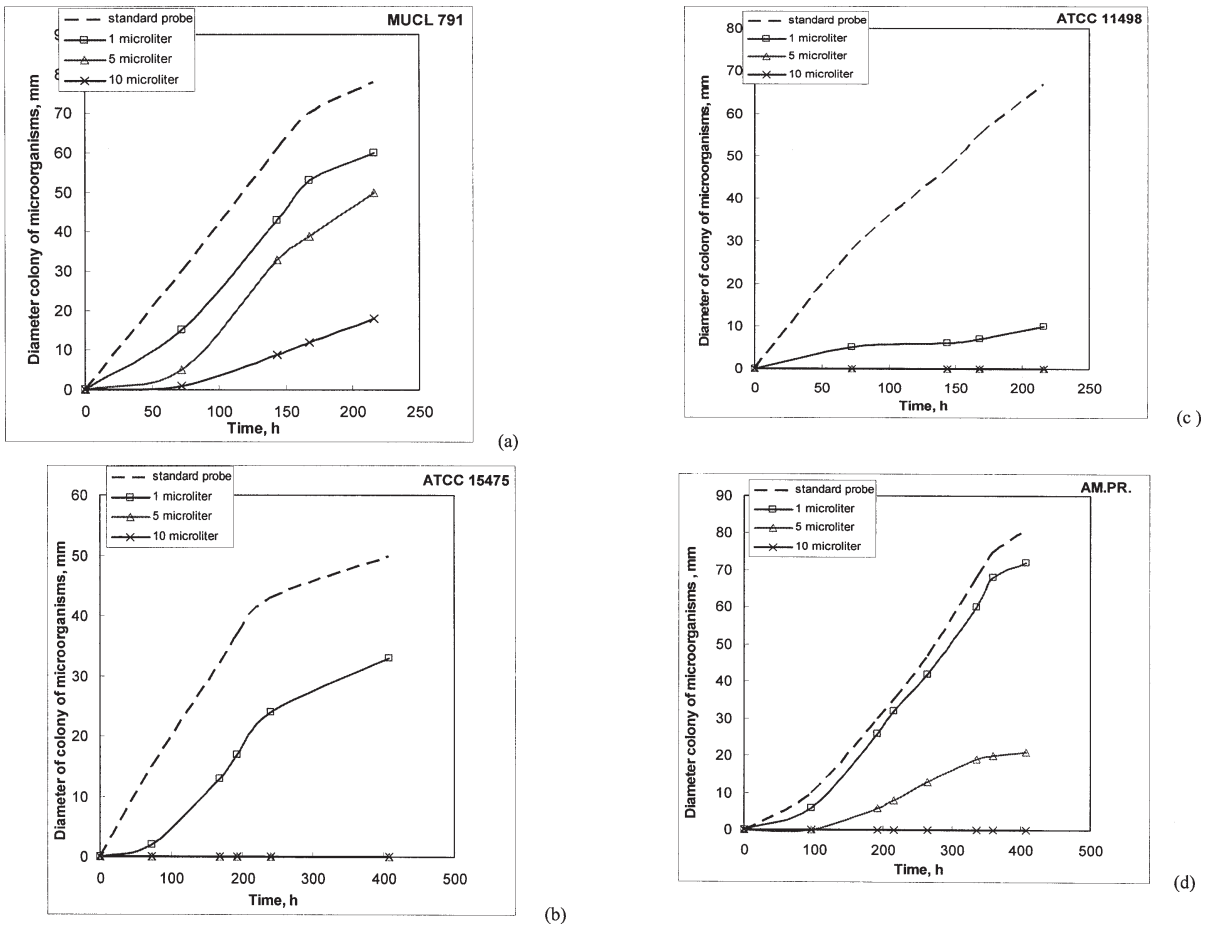

Fig.3.The evolution of microorganism's colony in the presence of different concentrations of thymus essential oil (a - *Fusarium oxysporum*, b - *Aspergillius niger,* c - *Aspergillius oryzae,* d - *Aspergillius ochraceus)*

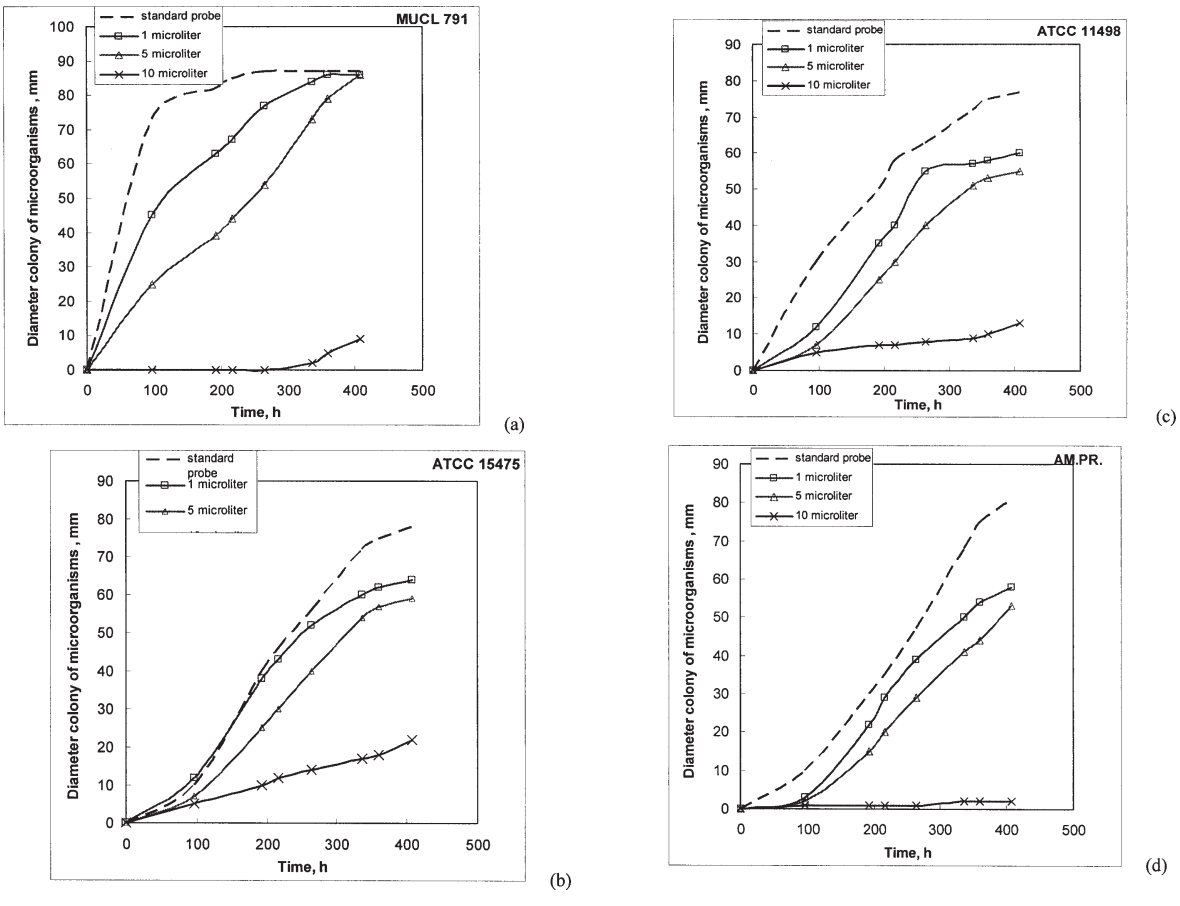

Fig.4. The evolution of microorganism's colony in the presence of different concentrations of coriander essential oil (a - *Fusarium oxysporum*, b - *Aspergillius niger,* c - *Aspergillius oryzae,* d - *Aspergillius ochraceus)*

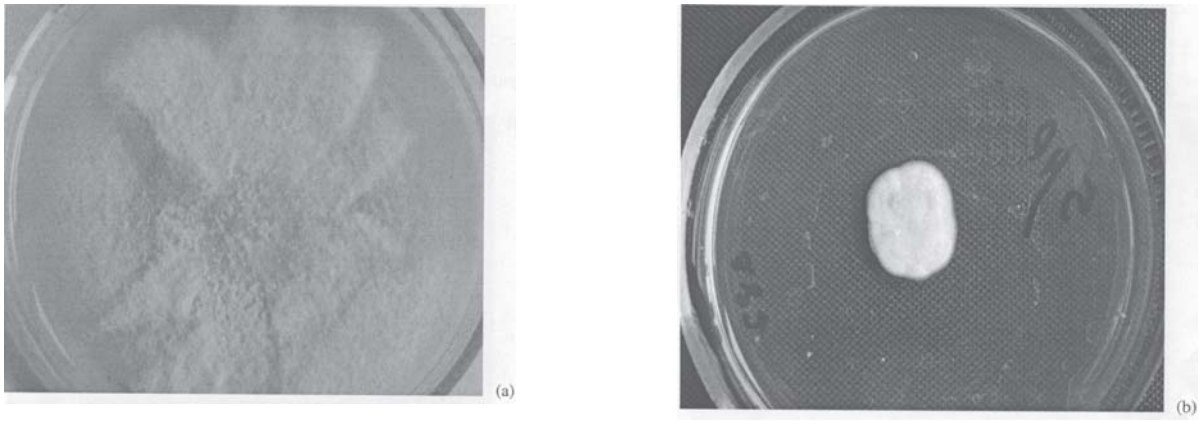

Fig.5. The morphological characteristics of the *Aspergillus ochraceus* in control sample (a) and in sample with 5 μL dill essential oil (b)

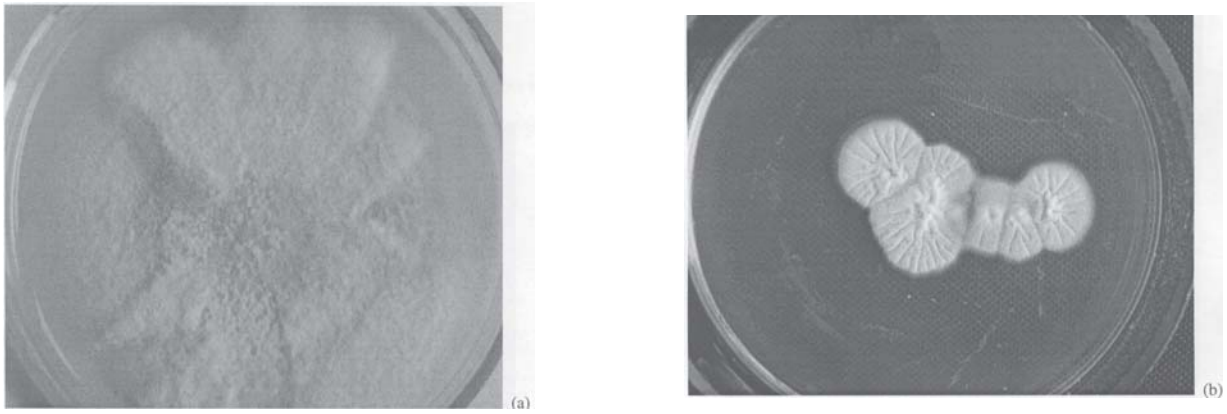

Fig.6. The morphological characteristics of the *Aspergillus ochraceus* in control sample (a) and in sample with 5 μL basil essential oil (b)

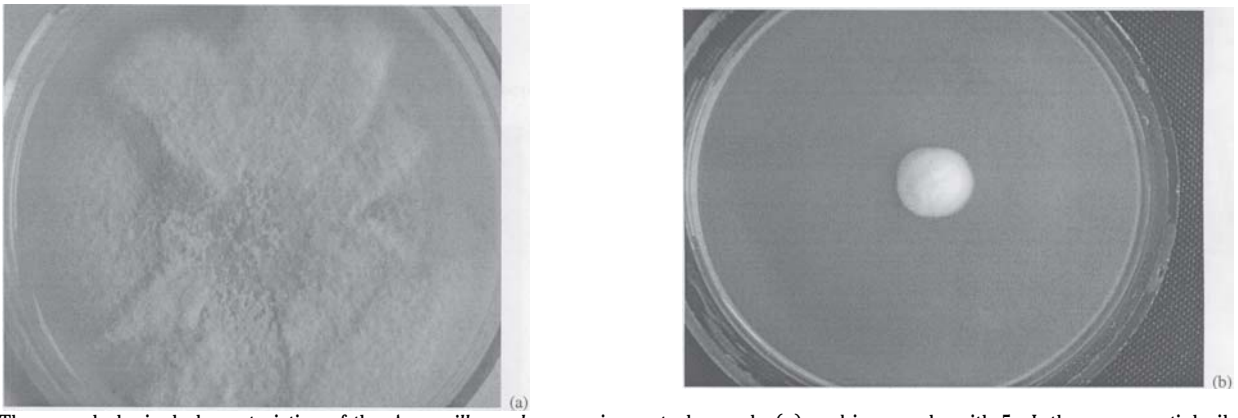

Fig.7.The morphological characteristics of the *Aspergillus ochraceus* in control sample (a) and in sample with 5 μL thyme essential oil (b)

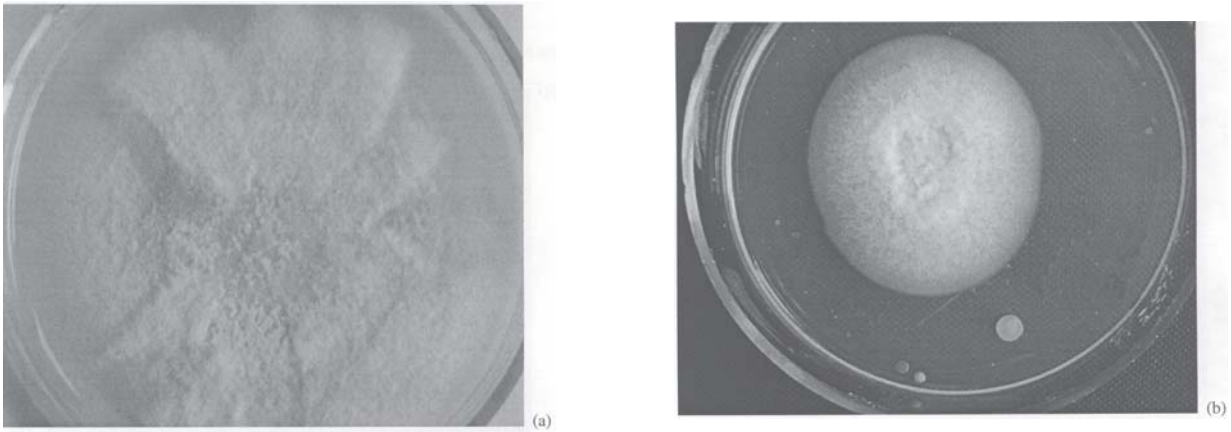

Fig.8. The morphological characteristics of the *Aspergillus ochraceus* in control sample (a) and in sample with 10 μL coriander essential oil (b)

Thyme oil also affects colony morphology and size of mold: look fluffy, without radial ditches, with irregular edges. Oils of basil and coriander affects only size of growing colony, without influence on its morphology.

It can be considered that active ingredients of essential oils, such as tymol, eugenol and carvacrol destroy biological cell membrane by binding to essential enzymes embedded on them, thus preventing the flow of biological substances.

Table 3
THE VALUES OF THE EMPIRICAL EQUATION PARAMETER FOR THE EFFICIENCY OF GROWTH INHIBITION OF THE ESSENTIAL OILS STUDIED

Essential oil	k	a	b
Dill	0.3100	3.0149	-0.2816
Basil	0.1670	3.5696	-0.5855
Thyme	0.2753	1.6959	-0.3088
Coriander	0.1018	6.7736	-0.5905

Experimental data obtained from studying the influence of essential oils on the development of mold strains, allows the correlation of inhibition efficiency, E_u, of a type of oil on all strains of molds, depending on the concentration, c, of used oil in growth space and the duration, τ, of microbial inhibition, after empirical relationship of the form:

$$E_u = k \cdot \left(\frac{c}{c_{min}}\right)^a \cdot \left(\frac{\tau}{\tau_{max}}\right)^b \quad (3)$$

Constants k, a, b were determined by regression from experimental data for each oil studied; the specific constants values obtained are presented in table 3.

In the correlation equation was chosen ratio between the current concentration and minimum inhibition's concentration, $c\backslash c_{min}$, because the latter is a standard parameter for assessing the antimicrobial activity. For the time inhibition parameter, was chosen the duration of the maximum inhibition period, τ_{max}, which can be achieved, with the purpose to be compared with the normal life cycle of the mold colonies analyzed. Reporting to the maximum time, the correlation coefficient is negative, because the inhibition efficiency is directly proportional to the maximum duration of exposure to toxic environment with volatile oils.

The results for the correlation parameters indicates that coriander oil concentration in microbial growth environment is a sensitive parameter to increase the efficiency of inhibition, given the fact that this oil need high concentrations to express his activity.

For the dill and basil oil, the maximum exposure of the microorganisms in atmosphere with essential oil is an important parameter. Thyme oil, as was evidenced by experimental determinations, shows effective inhibition of microbial growth at low concentrations and relatively low durations, which is confirmed by the correlation parameters.

Conclusions

Different behaviour of the fungal strains in the essential oil atmosphere is determined by the different concentration of polyphenols in each type of essential oil utilized in this study.

Following the experimental studies on antifungal activity of the four essential oils selected, it can be concluded with the hierarchy of these oils, as follows: oil of thyme is the most efficient antifungal agent, closely followed by oil of dill; basil oil has a relatively good antifungal activity, which can not be said about coriander oil, which is a weaker antifungal agent.

An empirical equation, which correlates the efficiency of inhibition with oil concentration and growth duration ratio, was determined, for each essential oil analyzed.

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Nomenclature

c - concentration of volatile oil used, g/L
c_{min} - minimum concentration of oil at which is occurring cell growth inhibition, g/L
d_c - colony diameter, mm
E_{exp}^c - efficiency of inhibition experimentally determined, %
E_u - efficiency of inhibition mathematically determined, %
ν - growth rate of the mold in the presence of essential oil, mm/h
ν_M - growth rate of control sample culture, mm/h
τ - duration of colony growth, h
τ_{max} - the longest time to produce the phenomenon inhibition of growth culture, h

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