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ANTIMICROBIAL ACTIVITY OF SOME ESSENTIAL OILS ON *CANDIDA* GENUS CLINICAL ISOLATES

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Aims. *The purpose of the work was to study the qualitative composition of the concomitant opportunistic pathogenic microbiota in the patients suffering from pulmonary tuberculosis; analyze the sensitivity of the isolates to essential oils and antibiotics.* ***Methods.*** *For antibiotic susceptibility and antimicrobial activity testing was used disc diffusion method. The analysis of essential oils was carried out by Gas Chromatography method.* ***Results.*** *The study showed that microscopic Candida genus fungi were the dominating representative of the satellite microbiota isolated from the sputum – they were plated in 70% cases. Most of the strains, even antibiotic-resistant ones, were ascertained to be susceptible to the essential oil of Thymus vulgaris L. The essential oils of Hyssopus officinalis L. and Rossmarinus officinalis L. were shown to be characterized by moderate antibacterial activity. The sensitivity to Mentha piperita L. and Savlia officinalis L. was strain-specific.* ***Conclusion.*** *By the level of antimycotic activity, the essential oils may be classified in a descending line beginning with Thymus vulgaris L. showing the most expressed antimicrobial activity, down to Hyssopus officinalis L., Rossmarinus officinalis L., Mentha piperita L., and Salvia officinalis L. The essential oil of Matricaria recutita L. showed no antimicrobial activity. The obtained results have proved the actuality of further studies of the impact of essential oils upon microorganisms, including those with multiple resistances to medical antibacterial preparations.*

Keywords: Candida strains, essential oils, antibiotic resistant microoganisms, antimycotic activity.

The significance of opportunistic pathogenic bacteria in the development of inflammatory diseases and complications is shown to be continuously growing over the past several decades. Of them, spread of antibiotic-resistant agents of opportunistic infections has become a most burning problem [1].

Under such conditions, it becomes especially important to perform research aimed at the search for alternative anti-microbial materials. The sources for such materials are the plants that have for a long time been used in popular and traditional medicine. The plant-based materials, essential oils in particular, often demonstrate a high level of direct antimicrobial activity. Essential Oils (EOs) are complex natural mixtures of volatile secondary metabolites isolated from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) by hydro or steam distillation and by expression. The major components of EOs are mono and sesquiterpenes, as well as carbohydrates, alcohols, ethers, aldehydes and ketones which are responsible for the synergic

interaction with antibiotics, due to that EOs antimicrobial activity can be attributed to inhibition or interaction with multiple targets in the microbial cell [2, 3]. Essential oils, which are promising anti-bacterial remedies [4, 5] used in cosmetology, medicine, food industry, etc. [6]. The antimicrobial activity of essential oils are often related to their major components, even though the presence of minor compounds and the ratio between active constituents also play crucial role due to synergistic effect.

Another advantage for the use of plant-based material is their antioxidant and immunomodultaing activity, because resistance to such antimicrobial-active plant-based materials is formed much less frequently.

The contingent of TB patients receiving a protracted course of therapy with antibiotic and chemotherapeutic materials deserves serious attention as such that creates the preconditions for formation of antibiotic-resistant strains referred to the facultative microbiota of human organisms. At the same time, it is shown that the spread of complications caused by the not susceptible to antibiotic *Candida* genus fungi is presently taking on special significance.

The purpose of the work was to study the qualitative composition of the concomitant opportunistic pathogenic microbiota in the patients suffering from pulmonary tuberculosis; analyze the sensitivity of the isolates to essential oils and antibiotics.

Materials and methods. Our examination has been performed on the basis Microbiological laboratory of the Department of Genetics, Plant Physiology and Microbiology of Uzhhorod National University (Ukraine); Laboratory of Department of Ecology, Faculty of Humanities and Natural Sciences, University of Presov (Slovakia); Regional Clinicall Territial Medical Associaciation"Phthisiology", Uzhhorod (Ukraine).

Isolation of microorganisms. To detect the opportunistic pathogenic microorganisms, we performed a bacteriological study of the sputum of 40 pulmonary tuberculosis patients, viz.: to identify microscopic fungi – on Sabouraund Dextrose Agar (Himedia) medium; to identify streptococci and *Neisseria* – blood agar, *Enterobacteriaceae* − Endo agar (Farmaktiv, Ukraine). We identified the yeasts based on macromorphology, micromorphology, and physiological and biochemical tests [7].

Antibiotic susceptibility testing. For antibiotic susceptibility testing was used disc diffusion method according by EUCAST (Eurepean Committee on Antimicrobial Susceptibility Testing). Inoculum of *Candida* isolates was prepared from 24 hours old culture grown on Sabouraund Dextrose Agar (Himedia) and incubated 35±2 єC. Colonies are suspended in 5 ml of sterile 0,85 % Saline. Yeast inocula 100 µL in physiological solution were adjusted to the equivalent of 0.5 McFarland standards and spread on the surface of SDA agar.

Sterile filter paper disks (6 mm in diameter) with Nystatin (50mcg), Itroconazole (10mkg), Fluconazole (25mcg), Ketoconazole (10mkg), Voriconazole (1 mcg), Klotrimazole (10mrg), Miconazole (50 mkg) were placed on the plate previously inoculated with a microbial suspension and incubated at 35±2 єC for 48 hour. Size of inhibition zone diameters surrounding filter papper disc was measured and compared to the Zone Diameter Interpretive Standards.

Disc diffusion method. To determine the antimicrobial activity of essential oils as test cultures were used the typical strain of yeast from the American Type Culture Collection, USA *Candida albicans* ATCC 885-653; 19 clinical strains *Candida* isolated from the sputum of people with tuberculosis, which taken antibiotic therapy during long period. Antimicrobial activity was determined using disk diffusion method, using 6 mm sterilized filter paper discs [7, 8]. Cultures of *Candida* were previously grown on the elective nutrient media Sabourand Dextrose Agar (SDA) 30 C 48 h.

Sterile filter paper disks (6 mm in diameter) impregnated with 10 μL of essential oil were placed on the SDA plate previously inoculated with a microbial suspension and incubated at 35±2 C for 48 hour. The diameters of the inhibition zones were measured in millimeters including diameter of disc. Each antimicrobial assay was perfomed in at least triplicate.

The essential oils of the following plants were used: *Rossmarinus officinalis* L.*, Thymus vulgaris* L.*, Menta piperita* L.*, Matricaria chamomila* L.*, Hyssopus officinalis* L.*,* (produced by «Calendula», Nova Lubovna, Slovakia).

Isolation of the essential oil. Each sample of the plant parts with weight of 10 g was grounded in a blender. The essential oil from this raw-material were prepared by hydro-distillation (2 hrs) in a Clevenger-type apparatus according to the European Pharmacopoeia and a mixture of hexane and diethyl ether (1:1) was used as a collecting solvent. The essential oils stored under N_2 at +4 °C in the dark space before their composition identification.

GC-FID analyses. The analysis of the essential oils was carried out using the Gas Chromatograph Varian 3090, connected to MS Saturn 2100T integrator. The following operating conditions were used: capillary column: RX-5MS, 30 m x 0.250 mm i.d., film thickness: 0.25 μm, carrier gas: Helium, adjusted to a flux of 1.5 ml/min, injection and FID-detector temperatures: 220 °C respectively 250 °C, a capacity of sample injection: 2 μl, MS-detector with automatic injector type 1177.

Components were identified by their GC retention times, and the resulting values were comparable to those of literature. Oil component standards for comparison were supplied by Extrasynthese, Merck, Fulka, Sigma a Roth.

Statistical analysis. Results of experiment we used statistical solfware STATGRAPHIC with the calculation of averages (M) and their error (m), standard deviation (δ).

Results. The results of the bacteriological examination of the TB patients' sputum showed that opportunistic pathogenic microflora was isolated from the sputum of 30 patients, out of 40 examined. The study showed that microscopic *Candida* genus fungi were the dominating representative of the satellite microflora isolated from the sputum – they were plated in 70% cases. The microorganisms isolated from other patients were *Neisseria* genus bacteria (3 cases) and solitary cases of *Escherichia coli, Pseudomonas aeruginosa,* and *Streptococcus* genus bacteria. In two cases, bacterial and microscopic fungal associations *Neisseria spp.+C. albicans; C. albicans+Aspergillus* were identified.

The study revealed the high proportion of resistant strains among *Candida* sp. (tabl.1).

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Table 1

Antibiotics sensitivity of *Candida* strains classified as diameter of growth inhibition zones, M±m

Clinical *Candida* isolates	Nystatin	Itroconazole	Fluconazole	Ketoconazole	Voriconazole	Klotrimazole	Miconazole
C. albicans 144	18.00±0.70	-	14.00±1.00	-	9.50±0.50	10.00±0.40	17.50±0.80
C. tropicalis 181	12.00±0.80	-	18.50±0.60	16.00±0.60	15.00±0.40	17.00±0.75	25.00±1.00
C. albicans 186	16.00±1.00	-	11.00±0.70	8.50±0.50	10.75±0.50	16.50±0.80	24.00±1.50
C. albicans 187	18.00±0.75	-	7.00±0.55	-	11.75±0.25	18.75±0.50	18.00±0.90
C. albicans 193	18.00±0.80	-	8.50±0.50	9.00±0.40	10.50±0.80	-	7.75±0.50
C. albicans 200	-	-	12.00±0.50	-	11.50±1.00	-	8.50±0.25
C. albicans 210	-	-	9.50±0.25	16.50±0.80	10.75±0.80	-	26.00±1.25
C. albicans 214	22.00±1.20	-	11.50±0.80	12.00±0.55	9.00±0.80	-	12.00±1.00
C. albicans 218	20.50±1.50	-	8.00±0.20	16.75±0.85	9.00±0.50	16.00±0.80	-
C. albicans 219	18.00±1.00	-	8.50±0.20	-	11.50±0.60	-	10.00±0.80
C. albicans 220	20.00±1.50	-	10.00±0.25	-	12.00±0.50	-	8.00±0.45
C. albicans 222	20.50±1.75	-	13.50±0.75	-	8.50±0.90	-	8.50±0.60
C. albicans 223	22.75±1.80	-	13.50±0.55	-	11.50±0.75	-	10.00±0.80
C. tropicalis 236	16.50±1.20	8.00±0.40	11.00±0.50	8.00±0.25	12.75±0.55	-	20.50±1.00
C. tropicalis 237	16.50±0.80	10.00±0.20	10.00±0.25	-	8.00±0.85	12.50±0.50	16.00±0.80
C. albicans 244	16.00±1.25	-	13.00±0.40	-	10.00±0.70	8.75±0.75	16.75±0.50
C. krusei 259	10.25±0.50	12.00±0.30	12.00±0.25	14.00±1.00	8.50±0.40	14.00±0.90	14.50±0.60
C. krusei 260	8.50±0.45	14.00±0.30	11.00±0.40	12.75±0.50	18.50±0.50	12.00±0.60	16.75±0.75
C. krusei 264	12.50±0.65	14.00±0.40	12.00±0.50	14.00±0.80	14.00±0.35	14.50±1.00	14.50±0.75

The microbial sensitivity test showed that 13 out of the 19 isolates were nystatin-sensitive; 3 isolates were low sensitive, 3 resistant. All isolates were resistant or low-sensitive to fluconazole and itraconazole; 90% strains were voriconazole-resistant. Only one of the 19 *Candida* strains was low sensitive to Fluconazole; all the rest were resistant to it: zones of growth retardation varied from 7.00±0.55 to 18.50±0.60 mm. As for Voriconazole, one strain proved sensitive; 2 low-sensitive; the others were resistant to that antibiotic material: maximum zone of growth retardation equaled to 15.00±0.40 mm. No zones of growth retardation were identified in 14 isolates against Itroconazole; only 4 strains showed zones of growth retardation within 8.00±0.40 to 14.00± ±0.40 mm. No zones of growth retardation were identified in 9 isolates against Clotrimazole; however zones of growth retardation for Clotrimazole other 10 isoletes varied from 10.00±0.40 to 18.75±0.50 mm. The sensitivity of the isolates to Miconazole was somewhat higher: four strains had a sensitivity zone of 20 and more mm, and only one strain was absolutely insensitive to the material. Eight strains were shown not to express sensitivity to Ketonazole; three strains were sensitive to the material. One isolate isolated from a patient with the recurrent disease was established to be resistant to all antibiotics tested; one was not sensitive or low-sensitive.

The study of susceptible of *Candida* genus fungi to antibiotics showed that resistance of the isolates did not depend upon the length of the patients' antibiotic treatment. Antibiotic-resistant isolates were detected in fresh patients as well as in those suffering from the recurrent disease. Other authors also showed cases of isolation of *C. albicans* to Nystatin, Fluconasole and Voriconazole isolated from faeces [7].

Antimicrobial activity of essential oils. According to results, it has been found that essential oils of *Thymus vulgaris* L. have significant antimicrobial activity to *Candida* spp. isolates: zones of growth retardation varied from 32.50±0.50 to 75.00±1.50 mm (tabl. 2). What was more, all isolates were sensitive to the given essential oil, even those that were resistant to the antibiotics.

A little lesser effect was registered with application of *Hyssopus officinalis* L. and *Rossmarinus officinalis* L. Zones of growth retardation for the fungi isolates varied from 10 to 16 mm. A certain antimycotic effect was observed with the use of *Salvia officinalis* L. however the sensitivity indices to this oil varied considerably. In particular, for this oil the smallest index was 7 mm, and the biggest 12 mm. Not all fungi isolates showed sensitivity to *Menta piperita* L. All yeast strains were resistant to essential oils of *Matricaria recutita* L., and neither of the strains showed any zones of growth retardation.

Chemical composition of EOs were as follows:

Rosmary essential oil (*Rossmarinus officinalis* L.): α-pinene 19±1%; camphene 9±1%; β-pinene 5±1%; cineole 25±1%; p-cymene 17±1%; camphor 19±1%; bornylacetate < 2%; α-terpineole 2.5±0.2%; borneole 2.0±0.2%.

Pippermint essential oil (*Mentha piperita* L.): limonene 2,5±0,2%; cineole 5.2±0.2%; menthone 24±1%; menthofuran 3.2±0.2%; isomentone 3.8±0.2%; menthyl acetate 4.1±0.2%; isopulegol less than 0.1%; menthol 39±1%; pulegone 1.1±0.1%; carvone 0.3±0.1%; cineole 2.1±0.1%.

Table 2

Antimicrobial activity of the 6 essential oils against *Candida* strains using agar disc diffusion in mm (M±m).

Strains of *Candida albicans*	*Rossmarinus officinalis* L.	*Thymus vulgaris* L.	*Menta piperita* L.	*Matricaria chamomila* L.	*Hyssopus officinalis* L.	*Salvia officinalis* L.
C.albicans ATCC 885-653	12.00±1.00	70.00±1.50	15.00±1.15	-	11.5±0.20	10.00±0.40
C. albicans 144	13.00±0.50	38.00±0.80	10.00±0.25	-	12.00±0.80	11.00±0.35
C. tropicalis 181	12.00±0.25	40.00±1.00	11.50±0.60	-	13.00±0.70	10.00±0.10
C. albicans 186	13.00±0.41	34.50±0.90	12.50±0.35	-	12.00±0.30	10.00±0.20
C. albicans 187	15.00±0.80	32.50±0.50	16.00±0.85	-	13.50±0.50	9.00±0.30
C. albicans 193	15.00±0.50	70.50±1.50	11.00±1.00	-	15.50±1.25	12.00±0.50
C. albicans 200	14.00±0.55	50.00±0.75	12.00±0.50	-	13.00±0.40	12.50±0.20
C. albicans 210	15.00±0.30	55.00±1.00	11.00±0.40	-	12.00±0.35	11.00±0.35
C. albicans 214	15.00±0.25	65.00±1.00	9.50±0.30	-	14.00±0.40	9.00±0.20
C. albicans 218	11.00±0.40	50.00±0.80	10.50±0.50	-	13.00±0.25	11.00±0.20
C. albicans 219	15.00±0.50	55.00±0.80	10.00±0.70	-	13.00±0.55	7.00±0.10
C. albicans 220	15.70±0.45	51.50±0/75	11.00±0.55	-	15.00±0.35	9.00±0.45
C. albicans 222	16.50±1.00	75.00±1.50	8.00±0.45	-	16.00±1.00	10.00±0.60
C. albicans 223	14.00±0.75	54.00±1.00	9.50±0.65	-	12.00±0.40	9.50±0.25
C. tropicalis 236	9.00±0.50	43.50±0.75	9.00±0.25	-	13.00±0.45	8.00±0.15
C. tropicalis 237	9.00±0.30	50.00±0.50	10.00±0.30	-	10.00±0.20	10.00±0.20
C. albicans 244	12.00±0.40	49.00±0.65	9.00±0.30	-	12.00±0.25	9.00±0.10
C. krusei 259	11.00±0.25	60.00±1.25	11.00±0.25	-	11.00±0.30	10.00±0.40
C. krusei 260	10.00±0.40	42.00±0.65	12.00±0.45	-	15.00±0.65	11.00±0.40
C. krusei 264	11.00±0.30	48.00±0.65	9.50±0.50	-	13.00±0.25	11.00±0.20

Thyme essential oil (*Thymus vulgaris* L.): p-cymene 40±2%; thymol 32±2%.

Matricaria oil (*Matricaria recutita* L.): bisabololoxides 6.0±0.2%; bisabolol 42±1%; chamazulene 2.3±0.2%; benzopyrene < 10 ppb.

Sage essential oil (*Salvia officinalis* L.): cineole 14±1%; thujone 30±1%; borneole 7.5±0.5%.

Hyssop essential oil (*Hyssopus officinalis* L.): α-pinene 15±1%; pinocampfene 35±2%; isopinocampfene 20±1%.

Discussion. The study revealed the high proportion of resistant strains among *Candida* sp. isolated from sputum of people with tuberculosis. According to results, it has been found that essential oils of *Thymus vulgaris* L., *Menta piperita* L., *Rossmarinus officinalis* L. have significant antimicrobial activity to *Candida* sp. Thereby, we have established the most distinguished antimycotic effect of essential oils of *Thymus vulgaris* L. According to other authors, the essential oil of this plant is also efficient against typical strains of *C. albicans* [9], *Clostidium* genus bacteria isolated from different places [10]. The results of the studies of other authors have pointed to sensitivity of microscopi fungi to essential oils of *Satureja intermedia* [11].

Most plants contain several compounds with antimicrobial properties for protection against aggressor agents, especially microorganisms. Active compounds found in some plants have antiseptic action; for example, thyme has thymol and carvacrol, clove has eugenol and isoeugenol, and oregano has carvacrol and terpineno. The action mechanisms of natural compounds are related to disintegration of cytoplasmic membrane, destabilization of the proton motive force, electron flow, active transport and coagulation of the cell content. Not all action mechanisms work on specific targets, and some sites may be affected due to other mechanisms. [12]. Nuzhat T. et all [13, 3] reported that antifungal activity of EOs can be attributed to the presence and synergism of some components such as carvacrol, α-terpinyl acetate, p-cymene, thymol, pinene, linalool which have antimicrobial activity. Thymus oils and their components have presented fungicidal effect by disruption of cell membrane and suppression of germ tube formation. The study shows [2, 3] the effect of essential oils, including of thyme, upon biofilms. Carvacrol and thymol, two biocidal compounds, present in thyme (*Thymus vulgaris* L.) oil have an important antimicrobial effect on biofilms formed by *S. aureus*, *S. epidermidis* and *Salmonella enterica serovar typhimurium*. Also, *Candida albicans*, *C. glabrata* and *C. parapsilosis* biofilms were treated with carvacrol, geraniol and thymol producing inhibition in biofilm formation in > 75%.

The obtained results have proved the actuality of further studies of the impact of essential oils upon bacterial isolates, including those with multiple resistances to medical preparations.

Conclusions. The study showed that microscopic *Candida* genus fungi were the dominating representative of the satellite microbiota isolated from the sputum – they were plated in 70% cases. According to results, it has been found that essential oils of *Thymus vulgaris* L. have significant antimicrobial activity to clinical *Candida* isolates. The essential oils of *Hyssopus officinalis* L. and *Rossmarinus officinalis* L. were shown to be characterized by moderate antibacterial activity. The sensitivity to

Mentha piperita L. та *Salvia officinalis* L. was strain-specific. By the level of antimycotic activity, the essential oils may be classified in a descending line beginning with *Thymus vulgaris L.* showing the most expressed antimicrobial activity, down to *Hyssopus officinalis* L., *Rossmarinus officinalis* L., *Mentha piperita* L., and *Salvia officinalis* L. The essential oil of *Matricaria recutita* L. showed no antimicrobial activity.

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Abbreviations used: EO (essential oil).

АНТИМІКРОБНА АКТИВНІСТЬ ДЕЯКИХ ЕФІРНИХ ОЛІЙ НА КЛІНІЧНІ ІЗОЛЯТИ РОДУ *CANDIDA*

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Резюме

Мета. Вивчити склад умовно-патогенної супутньої мікробіоти у хворих на туберкульоз легень та визначити чутливість ізолятів до ефірних олій та антибіотиків. **Методи.** Чутливість мікроорганізмів до антибіотиків та антимікробну активність ефірних олій визначали з використанням диско- дифузійного методу. Біохімічний склад ефірних олій визначали методом газової хроматографії. **Результати.** Дослідження показали, що домінуючими представниками супутньої мікробіоти, ізольованої із мокроти хворих на туберкульоз, були мікроскопічні гриби роду *Candida,* які виділяли у 70 % випадків. Встановлено, що більшість штамів, навіть антибіотикорезистентних, були чутливими до ефірної олії *Thymus vulgaris* L. Помірною антибактеріальною активністю характеризувались ефірні олії *Hyssopus officinalis* L. та *Rossmarinus officinalis* L. Чутливість до *Mentha piperita* L. і *Salvia officinalis* L. була штамоспецифічною. Не виявлено антимікробної дії ефірної олії *Matricaria recutita* L. **Висновки.** За рівнем антимікотичної активності ефірні олії можна розмістити у ряді зниження протимікробної дії від *Thymus vulgaris* L., що характеризувалась найвиразнішою антимікробною активністю, до *Hyssopus officinalis* L., *Rossmarinus officinalis* L., *Mentha piperita* L., *Salvia officinalis* L. Отримані результати вказують на актуальність подальших досліджень впливу ефірних олій на мікроорганізми, у тому числі з множинною резистентністю до антибактеріальних лікарських препаратів.

Ключові слова: Candida, ефірні олії, антибіотикорезистентні мікроорганізми, антимікозна активність.

АНТИМИКРОБНАЯ АКТИВНОСТЬ НЕКОТОРЫХ ЭФИРНЫХ МАСЕЛ НА КЛИНИЧЕСКИЕ ИЗОЛЯТЫ РОДА *CANDIDA*

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Резюме

Цель. Изучить состав сопутствующей условно-патогенной микробиоты у больных туберкулезом легких и определить чувствительность изолятов к эфирным маслам и антибиотикам. **Методы.** Чувствительность микроорганизмов к антибиотикам и антибактериальную активность эфирных масел изучали диско-диффузионным методом. Биохимический состав эфирных масел определяли методом газовой хроматографии. **Результаты.** Исследования показали, что доминирующими представителями сопутствующей микробиоты, изолированной из мокроты больных туберкулезом, были микроскопические грибы рода *Candida,* которые выделяли в 70 % случаев. Показано, что большинство штаммов, в том числе антибиотикорезистентные, были чувствительны к эфирному маслу *Thymus vulgaris* L. Умеренной антибактериальной активностью характеризовались эфирные масла *Hyssopus officinalis* L. и *Rossmarinus officinalis* L. Чувствительность к *Mentha piperita* L. и *Salvia officinalis* L. была штаммоспецифической. Антимикозную активность эфирного масла *Matricaria recutita* L. не обнаружено. **Выводы.** По уровню антимикотической активности эфирные масла можно разместить в ряду снижения антимикробного действия от *Thymus vulgaris* L.*,* характеризующегося наиболее выраженной антимикробной активностью, до *Hyssopus officinalis* L.*, Rossmarinus officinalis* L.*, Mentha piperita* L.*, Salvia officinalis* L. Полученные результаты указывают на актуальность дальнейших исследований влияния эфирных масел на микроорганизмы, в том числе с множественной устойчивостью к антибактериальным лекарственным препаратам.

Ключевые слова: Candida, эфирные масла*,* антибиотикорезистентные микроорганизмы, антимикозная активность.

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