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Abstract

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EFFECT OF EUGENOL EMULSION IN POLYSORBATE-80 ON CLINICAL STRAINS OF CANDIDA ALBICANS

Candida albicans occupies a dominant position in the list of causative agents of candidal lesions of the ear. The development of new antifungal agents, an alternative source of which can be herbal essential oils and their components, remains a priority. One such agent with antiseptic, anti-inflammatory, and analgesic action is eugenol which is a phenol substance. Therefore, this article was aimed to study the effectiveness of the antifungal action of eugenol emulsified in Polysorbate-80 against clinical strains of *C. albicans* isolated from patients with external otomycosis.

The study was performed using 6 clinical strains of *C. albicans* isolated from the ear canal of patients with otitis externa. The diagnosis of fungal disease was established based on the results of clinical and laboratory (microscopical and mycological) studies of pathological material.

Analysis of mycological research showed that mainly representatives of the genus Aspergillus and Penicillium were revealed and only in 7% there were *Candida* genus fungi. *C. albicans* remained the dominant species of clinical significance. The results of our studies showed a high level of antifungal activity of eugenol on all clinical strains of *C. albicans*, including a remarkable inhibitory and fungicidal effect. At postmycostatic concentrations, the eugenol caused partial inhibition of reproduction of the clinical strains of fungi, which was replaced by a subsequent increased cell reproduction rate.

So, the investigation has shown that *C. albicans* is the dominant species among fungi of the *Candida* genus in the structure of the microbial profile of otomycoses. The eugenol, emulsified in Polysorbate-80, has a high antifungal effect against clinical strains of *C. albicans*. At postmycostatic concentrations, the eugenol caused partial inhibition of reproduction of the clinical strains of fungi, which was replaced by a subsequent increase cell reproduction rate.

Keywords: eugenol, polysorbate-80, otomycosis, *C. albicans*, colony-forming units, minimal inhibitory concentration, minimal fungicidal concentration.

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Резюме

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ВПЛИВ ЕМУЛЬСІЇ ЕВГЕНОЛУ В ПОЛІСОРБАТІ-80 НА КЛІНІЧНІ ШТАМИ ГРИБІВ CANDIDA ALBICANS

В переліку збудників кандидозних уражень вуха *Candida albicans* займає домінуючу позицію. Пріоритетною і актуальною залишається розробка нових протигрибкових засобів, альтернативним джерелом яких можуть бути ефірні олії рослин та їх компоненти. Одним із таких засобів з антисептичною, протизапальною і знеболюючою дією є речовина класу фенолів – евгенол. Тому, метою даного дослідження було вивчення ефективності протитгрибкової дії євгенолу, емульгованого в полісорбаті-80 на клінічні штами *C. albicans,* виділені від хворих на зовнішній отомікоз.

Дослідження було проведено на 6 клінічних штамах *C. albicans,* виділених із слухового проходу хворих на зовнішній отит. Діагноз грибкового захворювання встановлювали на підставі результатів клініко-лабораторного, мікроскопічного та мікологічного досліджень патологічного матеріалу.

Аналіз мікологічного дослідження показав, що переважно висівались представники роду *Aspergillus* та *Penicillium* і лише у 7% гриби роду *Candida.* Домінуючим видом, який мав клінічне значення, залишався *C. albicans.* Результати наших досліджень показали високий рівень протигрибкової активності евгенолу на всі клінічні штами *C. albicans,* у тому числі виражену як інгібуючу, так і фунгіцидну дію. В постмікостатичних концентраціях евгенол викликав часткове пригнічення розмноження досліджуваних клінічних штамів грибів, яке змінювалось наступним підвищенням інтенсивності темпів самовідтворення.

В результаті проведених досліджень було встановлено, що C. albicans є домінуючим видом серед грибів роду Candida в структурі мікробного профілю отомікозів. Евгенол, емульгований в полісорбати-80, проявляє високу протигрибкову дію на клінічні штами *Candida albicans.*

В постмікостатичних концентраціях евгенол викликає часткове пригнічення розмноження досліджуваних клінічних штамів грибів, яке змінювалось наступним підвищенням інтенсивності темпів самовідтворення.

Ключові слова: евгенол, полісорбат-80, отомікоз, C. albicans, колонієутворюючі одиниці, мінімальна інгібуюча концентрація, мінімальна фунгіцидна концентрація.

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Introduction/Вступ

Opportunistic mycoses of the ENT organs remain one of the urgent medical problems, the number of which is constantly growing, including fungal lesions of the ear. According to the literature, the otomycosis (OM) proportion among inflammatory diseases of the ear is 20% [1, 2, 3]. The fungi that cause otomycosis belong to the group of opportunistic pathogens in the vast

majority of cases and cause disease only under the influence of factors that determine the reproduction of fungi and ensure the transition from saprophytic to the virulent way of life**.** The leading role in the development of this disease is played by mycelial fungi of the genus *Aspergillus (A. niger, A. versicolor, A. fumugatus, A. flavus, A. clavatus, A. terreus),* that is 65% in the structure of otitis of fungal etiology and 10% are fungi of the *Penicillium* genus (*P. chrisogenum, P. nidulans, P. notatum, P. tardum, P. glaucus, P. puberulum, P. Citrinum*). Representatives of the genera *Mucor, Alternaria, Kladosporium* are isolated in 3–5% under conditions of otomycosis. [4]. Yeast-like fungi of the *Candida* genus (*C. albicans, C. tropicalis, C. stellatoidea, C. pseudotropicalis, C. glabrata, C. brumpti, C. parapsillosis, C. krusei*) are 24% of the total number of mycoses [5, 6]. Micromycetes of this genus are characterized by a wide range of virulence and persistency factors, as well as morphological, structural, and biochemical properties, which determine adaptive resilience. It is why yeast-like fungi can survive under the constant action of innate and adaptive immunity factors. [7] Candidiasis is caused by *C. albicans* in 90% of cases when the strains are characterized by significant adhesive, dermonecrotic, and hemolytic properties.

The growing role of fungi (including members of the genus *Candida*) in the etiology of the ENT infection in modern medical practice, has led to the widespread and active use of antifungal drugs with different chemical compositions. However, their use, in addition to the development of side effects, may be accompanied by the formation of acquired resistance of micromycetes to antifungal drugs. The mechanisms of the acquired resistance to antifungals are studied sufficiently and clarified in scientific sources. Resistance to polyene antibiotics is explained by blocking the synthesis of ergosterol at the stage of conversion of zymosterol to ergosterol. The emergence of resistance to one of the antibiotics may simultaneously lead to the decrease in sensitivity to several other groups of antifungal drugs. Azole resistance genes – CDR and PDR, multiresistance and fluconazole gene – MDR, fluconazole specific genes – FCY1, FCY2, FLU1 and FLR1 are described. Most of them encode cellular transport systems of azoles' active excretion outside the fungal cell. The lability of enzyme systems of the fungus cell wall can lead to mutations that can cause the possibility of resistance to these drugs [8, 9]. The elaboration of new antimicrobial agents remains to be in priority and relevance [10]. Essential oils (EO) of plants and their components may be an alternative source of it.

The eugenol (a phenol class substance) is one of such substances with antiseptic, anti-inflammatory and analgesic effects, which was first isolated from plants of the genus Eugenia. The eugenol is part of a large number of essential oils: clove (up to 85%), eugenol basil oil (70–80%), coluria oil (70–80%), and oils of other plants. In addition to the pronounced antibacterial action, eugenol has anthelmintic and antiprotozoal properties. Scientific sources reflect the fungicidal action of eugenol and essential oils, which include this substance, on mycelial and yeast-like fungi**.** The antimicrobial activity of eugenol was studied with this substance soluted in alcohol or dimethyl sulfoxide. [11,12].

Our previous studies results have shown the effectiveness of the action of the eugenol emulsion in polysorbate-80 on the reference strains of *C. albicans*. [13]. Polysorbate-80 as an emulsifier of eugenol is characterized by significant emulsifying, solubilizing properties, easily decomposes in the environment, has no side effects.

The aim of the article was to study the effectiveness of the emulsified in polysorbate-80 eugenol's antifungal action on the clinical strains of *C. albicans* isolated from patients with otitis externa.

To achieve this goal the following tasks were solved:

1) to determine the etiological role of *C. albicans* in the structure of otitis externa;
2) to determine the minimal inhibitory and fungicidal concentrations (MIC, MFC) of the eugenol emulsified in polysorbate-80 for clinical strains of *C. albicans*;
3) to determine the reproductive intensity of *C. albicans* in postmycostatic concentrations of eugenol.

Materials and Methods. To achieve this goal, 89 patients with otitis externa, aged 18–65 years, who were treated at the municipal enterprise "2nd City Clinical Hospital of Poltava City Council" were examined. To study the microbial profile of the pathogens of otitis externa, pathological material was obtained from the external auditory meatus using probes or Folkman's spoon with subsequent cultivation and identification, respectively to the Order of the Ministry of Education and Science of the USSR №535 dated 22.04.1985 "On the unification of microbiological

(bacteriological) research methods used in clinical diagnostic laboratories of medical institutions" and "Guidelines for the use of unified microbiological (bacteriological) research methods in clinical diagnostic laboratories" [14]. The diagnosis was established based on clinical and laboratory examination, which included a careful collection of complaints and anamnesis, microscopy of pathological material, seeding it on elective nutrient media with subsequent identification and counting of colony-forming units (CFU/ml).

To identify the fungi of *C. albicans*, microscopy of the slides was performed first with an 8 × lens and then 40 ×. Gram-stained smears showed dark purple round or oval budding yeastlike cells, pseudohyphae, and chlamydoconidia in the test material. The ability of pathogens to form germ tubes, chlamydospores, to decompose, glucose maltose, and galactose to acid and gas were taken into consideration for mycological identification of C. albicans Also the CFU were taken into account if it exceeded 10^3/ml.

Eugenol (manufactured by Latus LLC, Ukraine) was used as the main test substance.

Emulsifier polysorbate-80, which is capable to provide high-quality distribution of the active substance to the emulsion, was used to obtain the emulsion. To prepare the working solution, eugenol was emulsified in polysorbate-80 in the ratio of 1:1. Quantitative determination of the minimum inhibitory concentration (MIC) of eugenol for fungal culture was investigated by the method of sequential macro-dilutions of the working solution [15] in Sabouraud liquid medium in the range from 0.1 to 0.00313 vol.%. This range of dilutions was selected based on literature data about the anticandidal activity level of eugenol in different solvents, [11, 16] as well as on own previous studies of the eugenol emulsion in Polysorbate-80 [13].

The inoculum was prepared by obtaining a suspension of 5 typical colonies of the clinical strain of *C. albicans* daily culture in Sabouraud liquid medium accordingly to the 0.5 McFarland standard [15]. 100 µl of the inoculum was added to 1 ml of eugenol emulsion with its concentration in the range from 0.1 to 0.00313 vol.%. The samples were incubated at 37°C for 48 hours. The experimental series contained all the components in the appropriate ratios. All components except eugenol were added to the emulsifier control. Culture controls contained nutrient medium and inoculum of clinical strains.

MIC was determined based on the absence of visible growth of fungi in a liquid nutrient medium. Next, the contents of the tubes with no visible signs of microbial growth were transplanted onto a dense Sabouraud medium to determine the fungistatic and fungicidal action of eugenol**.**

A quantitative evaluation of the effect of eugenol on fungal cells was performed by determining the number of CFU/ml. To do this, we made retransplants from test and control tubes after 48 hours of cultivation at 37°C [17] on a dense nutrient medium Sabouraud in Petri dishes by the sector method. The samples were incubated at 37°C for 24 hours.

Statistical analysis of the obtained results was performed using the standard software packages "SPSS 13.0" and "Microsoft Excel 2010". The presence of differences between the studied indexes was assessed by Student's t-test. The results were considered statistically significant at $p < 0.05$. Statistical significance of the difference between the indexes of colony formation, obtained at different doses of substance X, was determined by the Mann–Whitney method because the distribution of the obtained indicators did not correspond to normal.

As can be seen from the results shown in Figure 1, the percentage of patients with otitis externa caused by monocultures of fungi was 25% in the structure of infectious pathology of this disease. Analysis of mycological research showed that mainly representatives of the genus Aspergillus and Penicillium were obtained. The *Candida* genus fungi were obtained in 7% of it only. The *C. albicans* remained the dominant clinically significant species of the genus. 6 clinical strains of *C. albicans* fungi were isolated from the auditory canal of patients.

The obtained results confirm the literature data on the role of fungi in the case of otitis. Determining the fungal landscape of otitis externa is important for the application of adequate etiotropic treatment.

To date, a wide range of antifungal chemotherapeutics has been proposed and used [1, 18]. However, the dissemination of resistant strains encourages the search for and development of new effective antimicrobials. One of such drugs is the substance we are studying is eugenol.

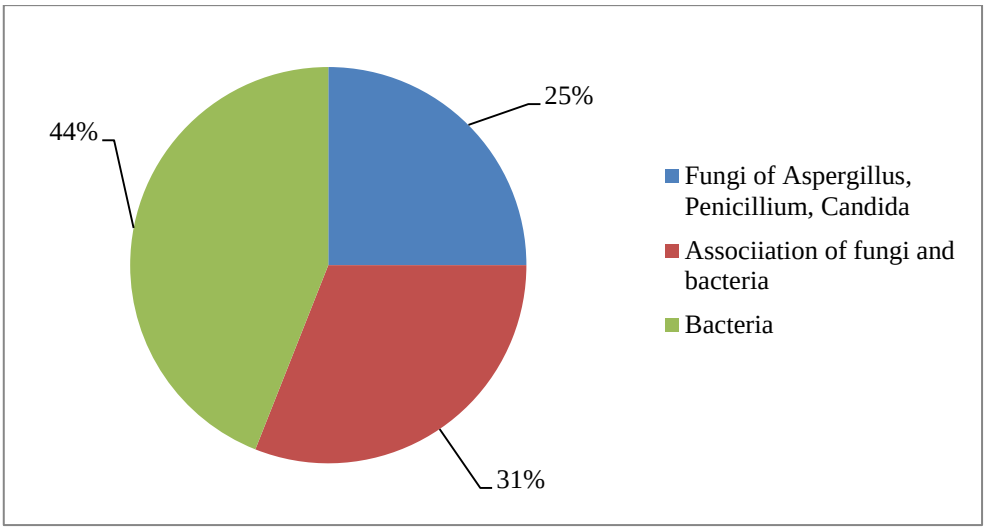

Figure 1 – The microbial landscape of the otomycosis

The study of the antifungal activity of eugenol in polysorbate-80 by serial dilutions showed the heterogeneity of *C. albicans* clinical strains based on their sensitivity to this substance (Table 1).

In previous studies, the MIC of the eugenol in polysorbate-80 for the reference strain of *C. albicans* ATCC 885-653 was 0.025 vol.% [13]. According to the results shown in table 1, the clinical strains of *C. albicans* were characterized by heterogeneity in terms of sensitivity to eugenol. As the result, the MIC of eugenol for strains 3 and 6 was determined at the concentration of 0.025 vol.% and for clinical strains 1 2 4 5 MIC was higher and was equal to 0.05 vol.%. The obtained results indicate a fairly high level of antifungal efficacy of eugenol against clinical strains.

Table 1 – The minimum inhibitory concentrations of the eugenol emulsion in polysorbate-80 for clinical strains of C. albicans in Sabouraud liquid medium

Vol.% / Strains of the studied micromycetes	0.1	0.05	0.025	0.0125	0.00625	0.00313	Culture control
C. albicans 1	-	-	+	+	+	+	+
C. albicans 2	-	-	+	+	+	+	+
C. albicans 3	-	-	-	+	+	+	+
C. albicans 4	-	-	+	+	+	+	+
C. albicans 5	-	-	+	+	+	+	+
C. albicans 6	-	-	-	+	+	+	+
Emulsifier control	-	+	+	+	+	+	+

Note: "+" – visible growth of culture; "-" – no visible growth

Table 2 presents the results of the MFC research. The results of the study revealed an increase in all clinical strains with concentrations in the range of 0.025–0.00313 vol.%. Thus, the minimum fungicidal concentration of the eugenol for clinical strains was 0.05 vol.%. The fungicidal activity was not detected in the control of the emulsifier with these dilutions.

The results of our previous [13] and current research that is presented in this article confirm the data about the high level of antifungal activity of eugenol for either reference strain or clinical ones.

Table 2 – The minimum fungistatic and fungicidal concentrations of the eugenol emulsion in polysorbate-80 for clinical strains of C. albicans

Vol.% / Strains of the studied micromycetes	0.1	0.05	0.025	0.0125	0.00625	0.00313	Culture control
C. albicans 1	-	-	+	+	+	+	+
C. albicans 2	-	-	+	+	+	+	+
C. albicans 3	-	-	+	+	+	+	+
C. albicans 4	-	-	+	+	+	+	+
C. albicans 5	-	-	+	+	+	+	+
C. albicans 6	-	-	+	+	+	+	+
Emulsifier control	+	+	+	+	+	+	+

Notes: "+" – the growth of culture; "-" – no growth

It is known that the mechanism of antimicrobial activity of eugenol is carried out by influence on the synthesis of the cell wall components (beta-glucans, chitin, mannans), by influence on important membrane-bounded enzymes [11, 16]. The effect on the shell structures of *C. albicans* is considered to be practically significant, as it means the possibility of influence on such pathogenicity factors as adhesive properties (reducing the ability of the fungus to colonize the tissues of the host organism) [19, 20, 21].

To shed light on the effect of the eugenol on clinical strains of *C. albicans* in concentrations less than mycostatic, cultures were reseeded from liquid nutrient media to dense Sabouraud media by the sector method with the followed determination of CFU/ml. The results of the sector technique are presented in Table 3.

The CFU/ml index was equal to $28.5 \times 10^6 \pm 9.6. \times 10^6$ in the controls of clinical strains. The amount of the CFU/ml was decreased under the action of eugenol in concentrations from 0.025 v.% (volume %) to 0.0125 v.%. It indicates a negative effect of eugenol in these concentrations on clinical strains of fungi.

Table 3 – Indexes of the clinical strains of C. albicans CFU/ml on Sabouraud medium under conditions of cultivation with different concentrations of the eugenol emulsion in polysorbate-80 , M ± m (n = 6)

Vol.% / Strains of the studied micromycetes	0.1	0.05	0.025	0.0125	0.00625	0.00313	Culture control
C. albicans	-	-	$20.33*10^3 \pm 9.52*10^3$, $p<0.00^5$	$36.67*10^4 \pm 15.37*10^4$, $p<0.005$	$14.17*10^6 \pm 72.36*10^6$, $p> 0.05$	$21.00*10^7 \pm 9.27*10^7$, $p <0.05$	$28.50*10^6 \pm 9.71*10^6$

Note: p – an index of the statistically significant difference of the experimental data with the control group, obtained by the Mann–Whitney method

The CFU/ml was increased significantly (10-fold in comparison with the controls) with the eugenol concentration of 0.00313 vol.%. This difference was statistically significant.

In our opinion, this was a result of the eugenol influence on the bond strength between cells. It was decreased and cells were more separable during shaking and, as a result of this, the cultures activated a genetically determined potential. Finding out the real cause of this phenomenon requires additional researches. However, it is unconditional, that these experimental parameters differ from the control sample; therefore, there is a certain effect of eugenol in these dilutions.

Conclusions/Висновки

As a result of the research the following was established:

1. *C. albicans* is the dominant species among fungi of the genus Candida in the structure of the microbial profile of otomycoses. The percentage of patients with otitis externa caused by *C. albicans* was 6% in the structure of infectious pathology of this disease.

2. The eugenol emulsified in Polysorbate-80 has antifungal effect in concentrations to 0.025 vol.%–0.05 vol.%, depending on the clinical strain of *Candida albicans*.

3. The postmycostatic concentrations of eugenol caused partial inhibition of reproduction of clinical strains of fungi, which then was replaced by a subsequent cell's reproduction rate increasing.

Prospects for future research/Перспективи подальших досліджень

Further studies will be focused on the effect of eugenol on the pathogenicity factors of the reference and clinical strains of *C. albicans*.

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