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COMPARATIVE MICROBIOLOGICAL ACTIVITY OF VOLATILE OILS FROM ANETHUM GRAVEOLENS SPECIES

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ABSTRACT. The aim of this paper was to test the antifungal activity of volatile oils extracted from *Anethi fructus, Anethi flores and Anethi folium* using disk diffusion method, compared with an antifungal drug, miconazole, against *Candida albicans* reference strain. Three tests were made of antifungal activity, in identical conditions, at intervals of 1 week. We also tested the antifungal activity of presumed active components in the form of oily solutions, in concentrations equal to those of volatile oil from flower and leaf respectively, as well as the antifungal activity of volatile oil entrapped in a liposomal formulation. It was found that both flowers and fruits showed good antifungal activity against *C. albicans* strain, while the volatile oil of the leaves was not active. It was also observed that oily solution containing the appropriate concentration of carvone in the fruit oil has antifungal activity, but oily solutions containing appropriate concentrations of limonene from flowers, respectively fruits do not have antifungal activity.

Keywords: *Anethi fructus, Anethi flores, Anethi folium*, volatile oil, microbiological activity

INTRODUCTION

Candida species are microorganisms that cause oropharyngeal and vulvovaginal infections, occur in renal and hepatosplenic infections, meningitis, endocarditis, osteomyelitis and / or arthritis. These species are responsible for four of the most common circulation infections contacted in hospitals, with a mortality rate of nearly 50% (Sidat et al., 2006). Of all species of *Candida, Candida albicans* is the most common, causing diseases that manifest from superficial skin lesions to disseminated infection (Alviano et al., 2005; Pandrea et al., 2010). High-risk groups in terms of contacting these fungi are composed of people with low immunity, i.e. pregnant women, children, diabetics, cancer patients and those with AIDS, as well as patients treated with antibiotics.

Currently there are few drugs with good efficiency on infections caused by Candida, most of them having limitations in terms of effectiveness, presenting adverse effects and low bioavailability (Almeida at al., 2006). In order to be effective, antimicrobial agents must be able to penetrate the infected intracellular areas, to be active at low doses and to possess low toxicity for prolonged therapy.

Therefore, research is directed towards the development of new antifungal drugs, which belong to other classes than existing ones and has a different mode of action (Nosanchuk & al., 2006). Plant extracts provide a unique opportunity in this field, plants producing secondary metabolites with antimicrobial properties. Since the antimicrobial activity of volatile oils has been demonstrated over time, researchers' attention turned to the formulation of new anticandidal drugs, using these natural compounds.

Essential oils (*aetherolea*) are volatile, natural, complex compounds, characterized by a strong odor, formed by aromatic plants as secondary metabolites. They are mixtures of different chemical constituents, possessing interesting therapeutically properties. An extensive body of research has demonstrated that essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmaceutics (Cristiani, et al., 2007). Several studies investigated and demonstrated the potential of *Anethi aetheroleum* (the essential oil of *Anethum graveolens)* as antifungal and antibacterial agent (Singh et al, 2002, Jirovetz et al., 2003).

Since most of the volatile oils are biologically unstable, insoluble in water and incapable of targeted delivery, attempts are made to introduce new methods to improve their stability and bioavailability. These include encapsulation in liposomes, process that reduces the environmental action on the product (water, oxygen, light), decreases the evaporation rate in the external environment, increases the ability of handling, reduces odor and increases the degree of dilution in order to achieve a uniform distribution in the final product. (Shoji et al, 2004, Gibbs et al., 1999, Versic, 1988).

MATERIALS AND METHODS

Volatile oils (VO) was extracted in our laboratory from *Anethi fructus, Anethi flores and Anethi folium* and assessed by gas chromatography. Phosphatidylcholine (PC) was purchased from Sigma – Aldrich and cholesterol p.a. (Chol) from Fluka. All solvents used were analytical or HPLC grade and were purchased from Merck. All other materials were analytical grade or equivalent.

The antifungal activity of volatile oils extracted from *Anethi fructus, Anethi flores and Anethi folium* was tested using disk diffusion method compared with an antifungal drug, miconazole, against *Candida albicans* reference strain (ATCC90028). We also tested the antifungal activity of presumed active components in the form of

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oily solutions, in concentrations equal to those of volatile oil from flower and leaf respectively.

Tested compounds: C1 – volatile oil extracted from inflorescence; C2 – volatile oil extracted from fruits; C3 – volatile oil extracted from leaves; C4 – pure carvone (0.752 mg carvone: 0.248 mg sunflower oil); C5 - carvone (0.109 mg carvone: 0.942 mg sunflower oil); C6 – limonene (0.234 mg limonene: 0.866 sunflower oil); C7– limonene (0.333 mg limonene : 0.675 sunflower oil); C8 – carvone + limonene (0.112 carvone : 0.042 limonene); C9 – carvone + limonene (0.094 carvone : 0.326 limonene : 0.498 sunflower oil). 10 µl from each compound were dropped on sterile paper discs (Bio-rad) of 6.3 mm. Impregnated discs were kept in a sterile Petri dish at 37 ° C for 24 h. As reference we used a 50µg miconazole (Bio-rad) impregnated disc.

Microorganism: the antifungal activity of the above-mentioned compounds was tested against *Candida albicans* reference strain ATCC90028.

Culture medium: Sabouraud agar (Sanimed) was used poured in Petri plates

Testing and reading method: microbial inoculum was prepared by suspensioning five colonies 24 hours of age in 0.89% saline solution. Microbial solution was vortexed and adjusted in order to correspond to 0.5 McFarland standard. Uncovered inoculated plates were kept at room temperature in laminar airflow for 15 minutes. When the agar surface was dry, plates were seeded at a distance of 15 or 30 mm from the discs margins. The inoculated agar plates were incubated at 37 ° C for 24 h. The inhibition zone diameter was measured (in mm) in three different directions and the average value was calculated. Three tests of antifungal activity were made, in identical conditions, at intervals of 1 week.

Multilamellar vesicles (MLV) were prepared according to the thin film hydration method. Lipid solutions were prepared by dissolving precise amounts of phosphatidylcholine (PC), cholesterol (Chol) and volatile oil (VO) in chloroform. 5.0 mL from each solution was introduced in a 100 mL round-bottomed flask. The solvent was evaporated in a Heidolph Laborota 4000 rotaevaporatory, at 35°-40°C, under reduced pressure (13-14 mm Hg). The obtained dry lipid film was hydrated with 5 mL distilled water. The mechanical stirring of the lipids in aqueous medium was performed with the rotaevaporatory equipment at 37°C and by manual stirring in the water bath, for 2 h, at the same temperature. This suspension was allowed to hydrate for 2 h in order to anneal any structural defects.

Unilamellar vesicles (SUV) were obtained by sonication of the MLV liposomes, in a bath-type sonicator (Sono Swiss SW 6L) for 30 min. (6x5min.). The sonication temperature was above T_c of the lipids (a temperature under T_c determines structural defects in the bilayers of the liposomes, which conducts to the fusion of vesicles). We prepared two sets of liposomes, LFR1 and LFR 2. The final composition of the liposomes used, expressed in mg / ml, was PC : Chol : VO = 19.5 : 2.6 : 9 (for LFR1) and respectively was PC : Chol : VO = 39 : 2.6 : 9 (for LFR2).

Purification of liposomes. The compound incorporated vesicles were separated from the unincorporated compounds by centrifugation. Vesicular dispersions were spun in a laboratory centrifuge Hettich Universal 320 R, at 10°C, 10000 rpm, for 60 min. The supernatant was removed and the liposomes were reconstituted with 5 mL distilled water.

Determination of the essential oil of Anethi fructus quantity encapsulated in liposomes We measured the quantity of essential oil encapsulated both in MLV and SUV vesicles. In case of purified liposomes, after the centrifugation, the supernatant was removed from the sediment. The quantity of essential oil was measured using a Perkin-Elmer Lambda 2 Spectrophotometer at λ = 236 nm. The incorporation efficiency (E %) of the essential oil was calculated using the following formula:

$$E = \frac{T - S}{T} 100$$

Where: T – the total amount of essential oil from supernatant and sediment (measured after disruption of liposomes with methanol) and S – the quantity of essential oil from supernatant (Holvoet et al, 2007).

The evaluation of the effectiveness of the liposomal formulations have been performed by microbiological testing of their antifungal activity against a strain of C. albicans ATCC 90028, compared with the effect of the free state volatile oil, respectively miconazole. Testing was performed by disk diffusion method given above.

RESULTS AND DISCUSSIONS

First experiments were aimed at determining the antifungal activity of volatile oils extracted from *Anethi fructus, Anethi flores and Anethi folium*. After seeding agar plates and incubating them, we measured inhibition zone diameter around the discs (mm) in three different directions and we calculated the average values.

Mean inhibition zone diameter calculated for the same compound was the same for the three determinations. We did not notice any difference greater than 1 mm between the three consecutive tests for the same compound. The mean values obtained for the tested compounds and miconazole are presented in Table 1.

Table 1

Values of the inhibition zone diameter for the three tested types of *Anethi aetheroleum*

Compound	Mean diameter for inhibition zone (mm)		
	Test 1	Test 2	Test 3
C1	21	21	20
C2	19	19.5	19.5
C3	0	0	0
Miconazole	26.5	26	26

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Where C1 – volatile oil from flowers (*Anethi flores*); C2 - volatile oil from fruits (*Anethi fructus*); C3 – volatile oil from leaves (*Anethi folium).*

As reflected by the table, both flowers and fruits, showed good antifungal activity against C. albicans strain, while leaf volatile oil was not active. Based on the chemical composition of the three tested volatile oils, we believe that the antifungal activity of volatile oil from fruits, respectively flowers is due to the following compounds: carvone – the leaf volatile oil does not contain this particular compound and that is why the antifungal activity is zero or carvone-limonene couple (quoted in the literature as conferring antimicrobial potential to volatile oils extracted from *Mentha spicata* and *Anethum sowa*) which is present in fruits and flowers volatile oil.

Given these theoretical considerations, we proceed to testing the antifungal activity of the presumed active compounds, as oily solutions, in concentrations equal to those in volatile oils from flower and leaf respectively. Data from microbiological tests are shown in Table 2.

Table 2

Values of the inhibition zone diameter for the presumed active compounds

Compound	Mean diameter for inhibition zone (mm)		
	Test 1	Test 2	Test 3
C4	18.5	19.5	19
C5	0	0	0
C6	0	0	0
C7	0	0	0
C8	0	0	0

Where: C4 – oily solution of carvone in concentration equal to that in fruits volatile oil; C5 - oily solution of carvone in concentration equal to that in flowers volatile oil; C6 – oily solution of limonene in concentration equal to that in fruits volatile oil; C7– oily solution of limonene in concentration equal to that in flowers volatile oil; C8 – mixture of carvone and limonene in concentration equal to that in fruits volatile oil; C9 – mixture of carvone and limonene in concentration equal to that in flowers volatile oil.

Analyzing the obtained fungigrams we could observe that:

- The oily solution that contains similar concentration of carvone as in the fruit oil has antifungal activity;
- Carvone, in concentration equal to that found in flowers volatile oil has no antifungal effect (which suggest that other compounds enhance this specific activity of monoterpenic ketones;
- Oily solutions containing equal concentrations of limonene as those found in flowers volatile oil, respectively fruits volatile oil have no antifungal activity (though literature mentions this property and, at least theoretically, unsaturated monoterpene hydrocarbons have antiseptic activity);
- Oily solutions containing concentrations of carvone – limonene couple similar to that found in volatile oils from flowers, respectively fruits have no antifungal activity, so in this particular case limonene does not enhance the activity of carvone against *Candida albicans* strains (though literature is mentioning this synergism)

Given that flowers volatile oil has antifungal activity similar to that of fruit volatile oil, and that carvone in concentration similar to that found in flowers volatile oil has no antifungal activity, we assume that the enhancement of the carvone activity is due to cadinol (sesquiterpenic alcohol present only in volatile oil from flowers). In support of this statement are literature data indicating that ketones and sesquiterpenic alcohols present this synergism of action.

Another hypothesis would be the effect of couple anethofuran - carvone. Anethofuran is a monoterpenoid oxide and the literature shows cases where oxides present action synergism with monoterpenic alcohols (most known examples are couples with antiviral effect: 1,8-cineole - alfa-terpineol from *Laurus nobilis, Melaleuca quinquenervia*, 1,8-cineole - linalool from *Myrtus communis*, *Rosmarinus officinalis* cineoliferum and camforiferum, linalool-oxide – linalool from *Hyssopus officinalis* var. decumbens, *Eucalyptus radiate*). By extrapolating these theoretical data would be considered that an oxide enhances the action of ketones. Because anethofuran is present both in volatile oils from leaves and flowers, we assume that it has antifungal effect only in the presence of carvone.

Other compound that could interfere with potentiation of antifungal action of carvone is monoterpenic hydrocarbons (alpha-pinene, sabinene, p-cymene), which are present in flowers volatile oil, but not in fruits volatile oil.

Given the fact that volatile oils from flowers and fruit showed comparable antifungal activity and that oily carvone solution in concentration similar to that found in fruits volatile oil has the same effect as the volatile oil itself, we selected the volatile oil extracted from *Anethi fructus* for incorporation in liposomes. At this stage of research we assessed the effectiveness of the designed liposomal formulations, by microbiological testing of antifungal activity against *C. albicans* ATCC 90028 strains, compared with the effect of the free volatile oil extracted from *Anethi fructus* and also with miconazole. The testing was performed by disc diffusion method. The mean values obtained for the tested compounds and miconazole are presented in Table 3.

Table 3

Values of the inhibition zone diameter for liposomal formulation

Compound	Mean diameter for inhibition zone (mm)		
	Test 1	Test 2	Test 3
VO	19	19.5	19.5
LFR1	1	1.2	1
LFR2	0	0	0
Miconazole	26.5	26	26

Figure 1 shows, for exemplification, the macroscopic aspect of the inhibition growth of *C albicans* ATCC 90028 in the presence of volatile oil of *Anethi fructus* (7) and liposomal formulation LFR2 (8).

Data presented in the table show that liposomes have a zone of inhibition of about 1mm. Concentration of volatile oil from microbiological tested liposomes is about 25 times smaller than the free volatile oil extracted from fruits. Because overall growth inhibition zone is directly proportional to the concentration of used antifungal substance, results that inhibition zone diameter for the same concentration of volatile oil should be 25 mm for LFR1 and respectively 27.5 mm for LFR2. These values are comparable with miconazole and superior to free volatile oil.

In conclusion, entrapment of volatile oil in liposomes enhances antifungal activity of the active principle.

Fig. 1 Macroscopic aspect of *C. Albicans* ATCC 90028 growth inhibition in the presence of tested compounds

CONCLUSIONS

The studies performed for testing the antifungal activity of volatile oils extracted from vegetal products *Anethi flores, Anethi fructus* and *Anethi folium*, as well as oily solutions of main components – carvone and limonene - showed that:

a) Both volatile oils from flowers and fruits showed good antifungal activity against *C. albicans* strain, while leaves volatile oil was not active;

b) Carvone oily solution in concentrations found in fruit volatile oil had exactly the same effect as the oil itself, which leads to the conclusion that the antifungal activity of the volatile oil of the fruits is determined by the activity of carvone;

c) Limonene oily solutions in concentrations found in fruits, respectively flowers volatile oils have no antifungal activity (although the literature mentions this property);

d) Oily solutions of carvone – limonene couple in concentrations equal to that in flowers, respectively fruits volatile oils have no antifungal activity against *Candida albicans* strain (although this synergism is mentioned in literature).

The performed microbiological studies on liposomes showed that the entrapment of the volatile oil in liposomes enhances its antifungal activity.

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REFERENCES

Almeida, C.F.C.B.R., Amorim, E.L.C., Albuquerque, U.P., Maia, M.B., Medicinal plants popularly used in the Xing´o region—a semi-arid location in Northeastern Brazil. Journal of Ethnobiology and Ethnomedicine 15, 1–7, 2006.

Alviano,W.S., Mendonc¸a-Filho, R.R., Alviano, D.S., Bizzo, H.R., Padr´on, T.S., Rodrigues, M.L., Bolognese, A.M., Alviano, C.S., Souza, M.M.G., Antimicrobial activity of *Croton cajucara* Benth linalool-rich essentialoil on artificial biofilms and planktonic microorganisms. Oral Microbiology and Immunology 20, 101–105, 2005.

Cristiani, M., D'arrigo, M., Mandalari, G., Castelli, F., Sarpietro, M. G., & Micieli, D., et al., Interaction of four monoterpenes contained in essential oils with model membranes: Implications for their antibacterial activity. Journal of Agricultural and Food Chemistry, 55, 6300–6308, 2007.

Gibbs, B. F., Kermasha, S., Alli, I., & Mulligan, C. N., Encapsulation in food industry: A review. International Journal of Food Science and Nutrition, 50(3), 213–224, 1999.

Holvoet, C., Heyden, Y. V., Plaizier G., Vercammen – Preparation and evaluation of paclitaxel-containing liposomes. Pharmazie, 62: 126-132, 2007.

Jirovetz, L., Buchbauer, G., Stoyanova, A. S., Georgiev, E. V., & Damianova, S. T., Composition, quality control, and antimicrobial activity of the essential oil of long-time stored dill (*Anethum graveolens* L.) seeds from Bulgaria. Journal of Agricultural and Food Chemistry, 51, 3854–3857, 2003.

Nosanchuk J D, Current status and future of antifungal therapy for systemic mycoses. *Recent Patent on Anti-Infective Drug Discovery*. 1(1): 75-84, 2006.

Pandrea, S. L., Tompa, M., Matinca, D., Bacterial species: etiologic agents of nosocomial infections. Human and Veterinary Medicine 2(2), 68-76, 2010.

Shoji, Y., & Nakashima, H., Nutraceutics and delivery systems. Journal of Drug Targeting, 12(6), 385–391, 2004.

Sidat, M.M., Correia, D., Buene, T.P., *Tinea capitis* among rural school children of the district of Magude, in Maputo province, Mozambique. Mycoses 49, 480–483, 2006.

Singh, G., Maurya, S., De Lampasona, M.P., Catalan C., Chemical Constituents, Antimicrobial Investigations, and Antioxidative Potentials of *Anethum graveolens* L. Essential Oil and Acetone Extract. Lebensmittelwissenchaften und Technologien 35, 720– 729, 2002.

Versic, R. J., Flavour encapsulation – an overview. In: Risch, S. J., Reineccus, G. A. (Eds.), ACS Symposium Series 370 (pp. 1–6). Washington, DC: American Chemical Society, 1988.