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PRELIMINARY STUDIES ON THE FORMULATION OF VAGINAL SUPPOSITORIES WITH LIPOSOMAL OREGANO OIL

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ABSTRACT

Infections with *Candida* species represent a large percentage of the total number of fungal infections in the vaginal mucosa. Conventional treatments with antifungal medicinal substances have proven to be low in efficiency, so we are currently looking for alternative products based on plant extracts (these being more effective according to existing studies but also non-toxic). Our study aimed to obtain the oil of *Origanum vulgare* L. and its characterization from the point of view of antimicrobial activity, known for its rich composition in active principles such as carvacrol (a compound with proven antifungal activity). Also, the plant *Origanum vulgare* L. was characterized from a macro- and microscopic point of view. The oregano oil obtained was included in the liposomal formula and characterized from the point of view of appearance, surface electric charge, and size, respectively. The liposomal oil was also included in the formula for the preparation of vaginal suppositories. These were characterized organoleptically and from the point of view of the release of active principles. The future perspectives are to test *the formulated pharmaceutical forms in vitro and then in vivo*.

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Introduction

Candida albicans are considered one of the most aggressive fungal strains of the *Candida* genus [1]. According to specialist studies, this characteristic is attributed to the fact that this fungus can create a biofilm at the level of the infected tissue or mucosa [2].

The ability to form a biofilm refers to the fact that *Candida albicans* is able to create a dense cellular population, continuously growing with a very high division rate, which adheres perfectly to the mucous membrane or infected tissue and is practically protected by defense factors of the body or even by antifungal medicinal substances due to the formation of a protective "capsule" [3]. For this reason, conventional treatments (antifungals such as fluconazole and nystatin) for *Candida* sp. no longer work as it is able to create resistance [4].

According to recent studies, the adhesion of the biofilm created by *Candida* species represents the biggest problem in the treatment scheme. Therefore, the innovative treatment implemented must, in the first phase, destroy this adhesion, or in other words, destroy the membrane created by the existing surface proteins at the colony level and then destroy the fungus [5, 6].

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The newest treatments highlight this ability to destroy the adhesion of candida at the level of the mucosa or the affected tissue due to the compounds present in various volatile oils, such as *Origanum vulgare* oil [7].
As the extensive phytochemical composition of oregano oil and the increased ability to destroy fungal strains of the genus *Candida* are known in the specialized literature [8], the aim of our work was to develop an innovative pharmaceutical preparation that can be used in the treatment of vaginal candidiasis.
Starting from this premise in our work, the *Origanum vulgare* L. plant was characterized from a macroscopic, microscopic point of view, antimicrobial activity. The obtained oil was initially included in the liposomal formula and then in vaginal suppositories. The characterization of the formulated liposomes and the release of the included oil from the vaginal suppositories were also monitored.

Materials and Methods

Botanical Analysis of the Species Origanum vulgare L.
The morphological study of the species *Origanum vulgare* L. identified and purchased from Oradea, Bihor county, was carried out with the help of atlases and illustrated botanical determinants. Any parts of the plant that had spots or were worn and damaged were removed. Only healthy and clean plant parts were analyzed.

Macroscopic Analysis of the Botanical Parts of Origanum vulgare L.
According to the 10th edition of the Romanian Pharmacopoeia, macroscopic control establishes the morphological characteristics of plant products, observed with the naked eye or with a magnifying glass, as well as those that can be determined by perceiving smell and taste. The macroscopic analysis refers to the analysis of the appearance, color, smell, and in some situations, taste, being practically an organoleptic analysis [9].

Microscopic Analysis of the Botanical Parts of Origanum vulgare L.
The microscopic study at the level of the vegetative organs (stem, leaf) was carried out on transversal sections, clarified and colored. The identification and differentiation of the structure of the characteristic elements (tectors bristles, secretory structures) were possible by the different coloring of the cell membranes under the action of chemical dyes. The cellulosic membrane was colored red, and the lignified one yellow [10].
The sections are stained (Congo Red) for 1-3 minutes, then the excess dye is washed off several times with distilled water. The preparations are analyzed with the Optika B350 analysis microscope.

The Method of Obtaining the Oil of Origanum vulgare L.
The aerial parts of the plant *Origanum vulgare* L. were subjected to oven drying at a temperature of 65°C until constant weight. The uprooted plant was pulverized by crushing and used to obtain oregano oil using a soxhlet apparatus. Petroleum ether was used as a solvent to extract the oil. The Soxhlet apparatus was run at 65°C for 7 hours. After this, the solvent was evaporated using the Heidolph Rotary Evaporator, Laborota 4000, for 30 minutes at 90 rpm [11, 12].

The Antimicrobial Activity of Origanum vulgare L. oil
Gram-positive bacterial strains (*Enterococcus faecalis* and *Staphylococcus aureus*) were used to test the antimicrobial activity of oregano oil. The antimicrobial activity was also tested on gram-negative bacterial strains such as *E. coli* and fungi of the *Candida albicans* type. Bacterial microorganisms were cultured on nutrient agar using the spread plate technique and incubated for 24 hours at 37°C [13, 14]. Bacterial strains were grown in Mueller-Hinton plates at 37°C. The applied oregano oil samples were diluted with oily solvent using the following oregano: mineral oil ratios (O: U): 1:1; 1:10; 1:20. Carvacrol was used as a standard (in the same rations), being one of the predominant compounds of oregano oil according to the specialized literature [15]. Twenty-four hours after the application of the samples, the inhibition diameter was measured, and the test was performed in triplicate.

Formulation, Preparation, and Characterization of Vaginal Suppositories with Liposomal Oregano Oil
Formulation and Characterization of Liposomes with Oregano That Will Be Included in Your Vaginal Suppositories
For the formulation of the oily phase, phosphatidylserine and cholesterol were used in a ratio of 3:1 [14, 16, 17]. These lipid molecules were solubilized using the solvent mixture chloroform: methanol in a 3:2 v/v ratio [18]. Oregano oil was included in the lipid phase at 50 µg/ml volume.
After the solubilization process, which takes place with a slight heating, the lipid mixture is subjected to evaporation using the Heidolph Rotary Evaporator, Laborota 4000 rotavapor until the total removal of the organic solvents and the formation of the lipid film on the walls of the vessel [19]. To remove possible traces of organic solvents, the formed lipid film is kept at room temperature for 24 hours, after which it is hydrated with 15 ml of phosphate buffer pH 7.6, which constitutes the hydrophilic phase [20].
In this formulating phase, liposomes are formed spontaneously, and their size is reduced by sonication for 30 minutes and centrifugation for 40 minutes at 10,000 rpm [16].
After these steps, the formulated liposomes were characterized in terms of shape, size, and surface electrical charge [21].

Microscopic Characterization of the Formed Liposomes

To be able to confirm the shape and appearance of the liposomes with oregano oil included, according to the authors, Miere *et al*. optical microscopy can be used. Thus, an Olympus CX40 (Tokyo, Japan) inverted light microscope was used through a 40× objective in phase contrast mode, and images were captured by a Hitachi CCD camera [18].

Characterization of the Size and the Electric Surface Potential of the Liposomes by DLS

Dynamic light scattering (DLS) was applied to determine the diameter, distribution, and Zeta potential of the formulated liposomes using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Polystyrene cells with an optical path of 1 cm were used for diameter measurements. Disposable folded capillary cells were used to determine the electrical surface charge (Zeta potential) [22]. All measurements were done in triplicate.

Formulation of Vaginal Suppositories

The preparation of vaginal suppositories was carried out in three stages **(Figure 1)**.

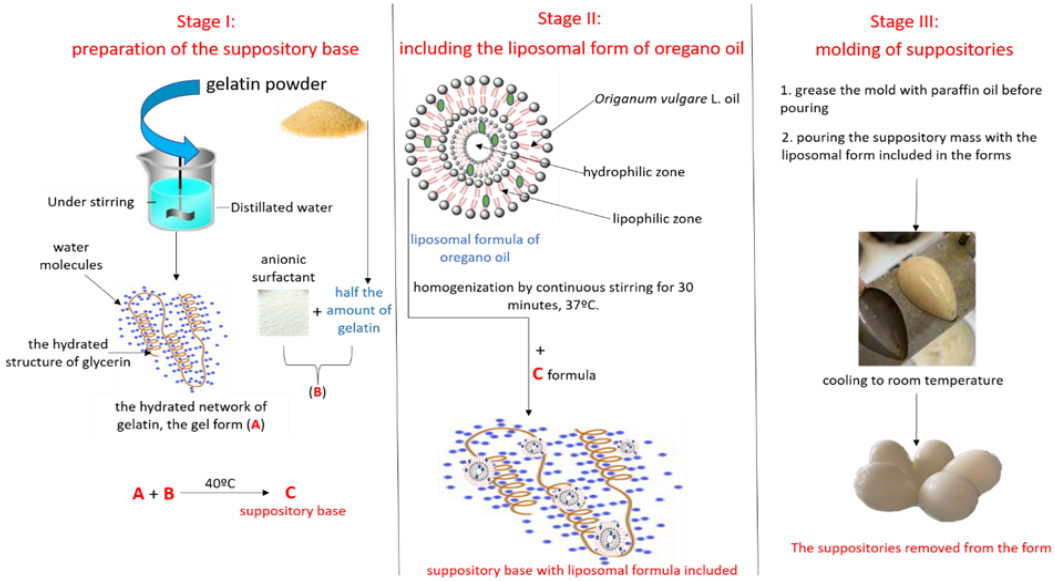


Figure 1. The stages of preparation of vaginal suppositories with *Origanum vulgare* L. oil in the liposomal form.

Briefly, in the first stage, the suppository base was prepared, which consisted of 10.5 g of gelatin. Part of the gelatin is placed to hydrate, and the rest is homogenized separately with surfactant (3.5 g). The two parts of gelatin are homogenized while warm (in a water bath). 52.5 g of glycerin is added to the homogenous mixture previously heated to the same temperature as the gelatin [23, 24].

In the second step, add liposomal oregano oil (0.5 g) and homogenize.

The third stage is casting the vaginal suppository in a form previously lubricated with paraffin oil. These quantities of the substances used correspond to the formulation of a 100g vaginal suppository base.

Suppository Quality Control

The quality control of the suppositories was carried out following the rules provided by the Romanian Pharmacopoeia, 10th edition, and the quality conditions imposed by the European Pharmacopoeia, 7.1 edition [9].

In order to verify the quality of vaginal suppositories with liposomal oregano oil, the following were observed: their appearance, mass uniformity, dissolution behavior, disaggregation, and dosing [9].

Determination of the Appearance and the Uniformity of the Mass of Vaginal Suppositories

According to the Romanian Pharmacopoeia, suppositories must have a homogeneous appearance and retain their shape and consistency at room temperature. In the longitudinal section, examined with a magnifying glass (4.5 x), it must not present agglomerations of particles, crystals, or air bubbles [9].

Twenty suppositories were weighed to determine the average uniformity of the vaginal suppository mass [9].

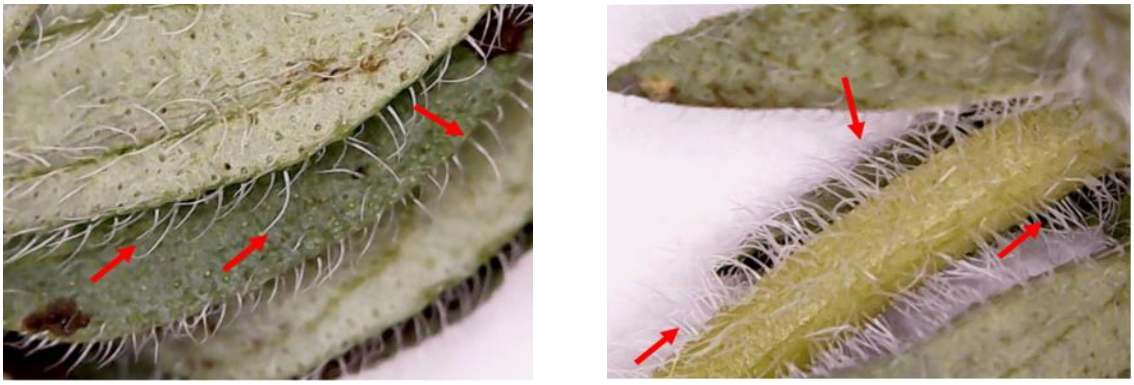
The Dissolution Behavior and Release of Oregano Oil

In order to determine the solubility of the vaginal suppository and the release of the liposomal oregano oil, a volume of 500 mL medium with a neutral pH (distilled water) and an acidic pH = 4 (HCl) was inserted into 2 of the cylindrical vessels of the device (Dissolution tester Electrotab TDT – 08L). The acidic pH chosen is similar to the vaginal pH, and the media were previously heated to 37 ºC (body temperature). The sample, vaginal suppositories with liposomal oregano oil, was placed in

the lower part of the cylindrical vessel at a blade rotation speed of 37 rpm. Four samples of 5 mL of each dissolution medium (acidic and neutral) were taken in 4 different time intervals (15min, 30min, 45min, and 60min) [24].
After sampling, the samples were tested to determine the degree of oregano oil release by titration with a 0.1N NaOH solution.

Results and Discussion

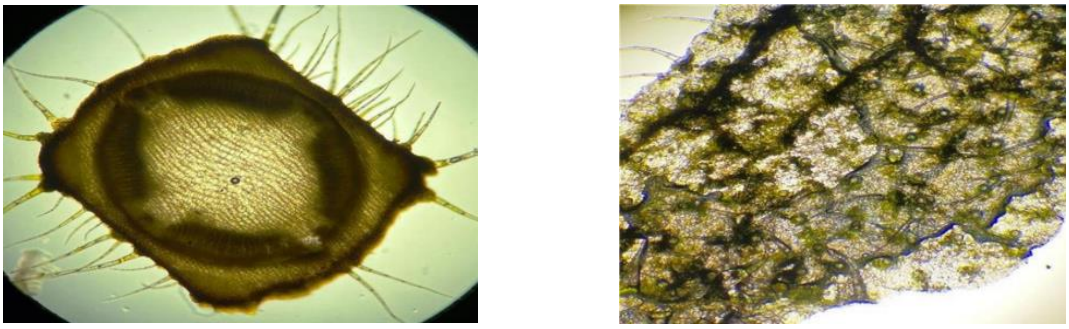
Macroscopic Analysis of the Botanical Parts of Origanum vulgare L.
In the macroscopic analysis, the aerial parts of Origanum vulgare L. can be highlighted, namely the stem covered with hairs visible to the naked eye **(Figure 2b)** and the leaves covered by hairs and secretory structures on the dorsal side **(Figure 2a)**.



a) b)

Figure 2. Macroscopic images of the dorsal part of the leaves of *Origanum vulgare* L. a) and the tulip of the oregano plant, b) The red arrows highlight the presence of tectorial and secretory bristles present in vast numbers.

Microscopic Analysis of the Botanical Parts of Origanum vulgare L.
Figure 3a represents the cross-section through the tetrahedral stem of the *Origanum vulgare* L. plant. You can see the unistratified epidermis from the outside to the inside, which shows epidermal cells tightly joined together. The outer walls of the epidermis are slightly convex and show pluricellular bristles on the entire surface. In the four edges of the stem, the angular collenchyma is noticeable, a characteristic aspect of plants from the *Lamiaceae* family [25].
Under the epidermis, the secretory parenchymal tissue is evident, having a role in photosynthesis. The pericycle is the next layer of cells with thin, cellulosic walls. In the central cylinder, *Origanum vulgare* L. presents several free-woody, mixed fascicles arranged next to the angular collenchyma.
Figure 3b represents a skinning section of the leaves of *Origanum vulgare* L. followed by staining with synthetic dye (Congo Red).
Under the upper epidermis, the palisade parenchyma is differentiated, consisting of photosynthesizing cells. The epidermis consists of a single row of cells with a highly developed cuticle covered with wax. The mesophyll is made up of a palisade tissue, rich in chloroplasts, made up of 2-3 rows of elongated cells closely joined together.
We identify here the presence of large, polyhedral cells, less rich in chlorophyll, with a larger diameter than the surrounding cells, which contain a large amount of vacuolar juice and make up the aquifer tissue. Different formations are found on the surface of the epidermis: stomata, secretory structures, and peri tectors.
Tector bristles are outgrowths of epidermal cells, which elongate and divide perpendicularly to the epidermis. In *Origanum vulgare* L., they are multicellular, filamentous, and unbranched. Also, at the leaf level, we find the presence of secretory structures of the volatile oil of oregano. These structures have circular shapes of different sizes arranged on the entire surface of the leaf blade.



a) b)

Figure 3. Transverse sections through the stem of *Origanum vulgare* L. a) and the leaf of *Origanum vulgare* L. b) Images are highlighted using the 40X magnification objective.

The Antimicrobial Activity of Origanum Vulgare L. oil

In the specialized literature, it is highlighted that during the maturation of the *Origanum vulgare* L. plant, the composition of the volatile oils changes [26]. In young plants, the volatile oils contain mainly terpenic hydrocarbons and compounds with simpler molecules, while the reproductive organs contain essential oils richer in oxygenated compounds [15]. The chemical structures of natural compounds are very varied, and they are known for their increased antibacterial and antifungal properties [27].

Several *in vitro* studies have shown that the most active compounds in volatile oils are phenols (carvacrol, thymol, eugenol), aldehydes, and terpenic alcohols (terpineol) [15]. *Thymus vulgaris* oils, *Origanum vulgare*, *Melaleuca alternifolia*, *Cinnamomum* sp., *Eugenia carryophyllata, Eucalyptus globulus* are known for their composition rich in these compounds [6]. For example, carvacrol (the most abundant compound in *Origanum vulgare* L. oil) acts specifically on some strains of *Staphylococcus aureus* and *S. epidermidis*, preventing biofilm formation [28, 29].

For this reason, carvacrol was used as a positive control in the analysis of the antimicrobial capacity of the oregano oil obtained. The results obtained on gram-positive and gram-negative bacterial strains and fungi such as *Candida albicans* are highlighted compared to carvacrol in **Table 1**.

Table 1. The antimicrobial activity of the oil obtained from *Origanum vulgare* L. compared to carvacrol.

Strains / Samples	Enterococcus faecalis	Staphylococcus aureus	Escherichia coli	Candida albicans
	Inhibition diameter ± SD (mm)			
***Origanum vulgare* L. oil 1:1**	17±0.9	25±0.8	24±0.9	30±08
***Origanum vulgare* L. oil 1:10**	17±0.75	23±0.9	24±1.0	30±0.10
***Origanum vulgare* L. oil 1:20**	12±0.8	20±1.1	20±1.0	30±1.1
Carvacrol 1:1	18±1.0	32±1.0	25±1.2	35±1.0
Cavacrol 1:10	14±0.9	26±1.2	25±1.3	30±1.2
Carvacrol 1:20	10±0.9	16±0.9	23±0.9	30±0.9

According to **Table 1**, it can be stated that the antibacterial and antifungal activity of the tested oil is comparable or higher (due to the synergic effect of all the compounds present in the oil) to the activity of the pure substance (carvacrol) used as a standard.

Authors Gitea *et al*. 2021, *Origanum vulgare* L. oil is rich in carvacrol, thymol, sabinene, γ-terpinene, p-cymene, p-cymene, and thymoquinone. Other chemicals frequently detected in *Origanum* species are the monoterpenes geraniol, linalool, linalyl acetate, myrcene, camphene, camphor, borneol, and sesquiterpenes. Phenolic p-hydroxybenzoic and hydroxycinnamic acids have been identified in *Origanum* plants. Most *in vitro* studies highlight antimicrobial, antifungal, antibacterial, antiparasitic, and antiviral activity [15].

The antibacterial mechanism of action is based on the destabilization of the cell membrane, the denaturation of plasma proteins, and the inactivation of some bacterial enzymes. Lipophilic compounds in the oil volatiles interact with the polysaccharides, fatty acids, and phospholipids in the structure of the cell membrane. This makes it more permeable, thus determining the loss of cellular constituents and, therefore, the death of the bacterium [30].

Antiviral activity has also been demonstrated against *Hepatitis A* and *Herpes simplex* viruses [31, 32]. Antifungal activity is increased not only on *Candida* species but also on *Aspergillus* species. Antiparasitic activity has been shown to be present in *Coccidium* sp [33, 34].

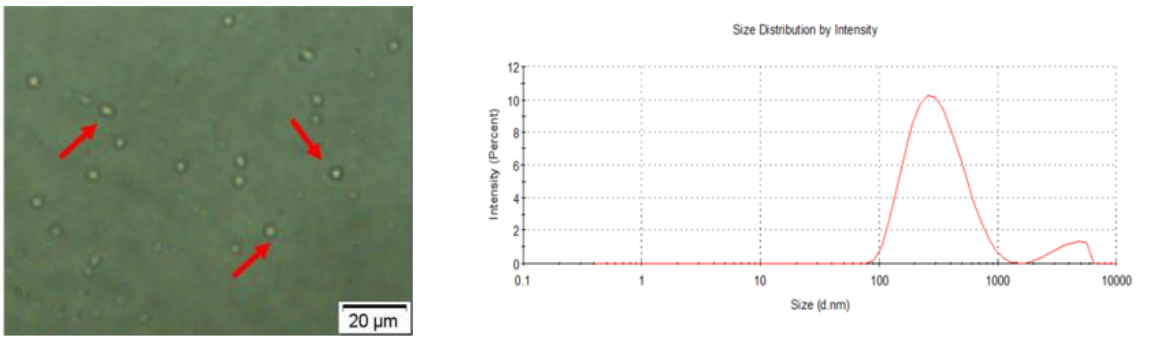
Microscopic Characterization and DLS Analyses of Liposomes with Oregano Oil

Oregano oil liposomes were formed by the lipid film hydration method [35]. This method is practically based on two stages. The first stage is the formulation of the lipid film, which is made up of phosphatidylserine and cholesterol in a ratio of 3:1 and oregano oil in a quantity of 50 μg/ml. The second stage consists of the hydration of the formed film with the hydrophilic phase consisting, in this case, of the phosphate buffer pH 7.6 [18].

At the moment of hydration, liposomes are formed spontaneously, and oregano oil is included. In order to reduce the size of the liposomes so that their stability is increased, the mixture is sonicated and centrifuged according to the method described in the materials and methods chapter. According to specialized literature, the liposomes formed by this method are called giant-type liposomes and are multilamellar [36].

To demonstrate the successful formation of liposomes, optical microscopy was applied to the matte emulsion, and DLS analyzes were performed.

The characteristic round or spherical shape of oregano oil liposomes can be seen in **Figure 4a**. It is also observed that they are uniformly dispersed in the microscopic field. **Figure 4b** shows the graph obtained by DLS, which provides information on the size of the formed liposomes. They have a size between 90 nm and 1μm, the great majority being in the range of 200-500 nm. The electric charge of the surface is negative (-20.43 V), which means increased stability of liposomes with oil of oregano formulated over time [37-41].



a) b)

Figure 4. a) microscopic image of *Origanum vulgare* L. oil liposomes formulated, b) Graph of the size distribution of the formulated liposomes. The red arrows indicate the liposomes in the microscopic field, observing the round or spherical shape.

Mechmechani *et al*., 2022 state that the encapsulation of volatile oils in different formulas improves their biological activity and increases the ability to destroy the biofilm created by microorganisms sensitive to the composition of the included volatile oil [38].
Also, the liposomal formulation of the oregano oil that was included in the vaginal suppositories leads to an increase in the stability of the oil and a decrease in the degradation of existing compounds under the influence of storage factors such as light, temperature, humidity, etc. [18].
The idea to include oregano oil in liposomes and the liposomal formula obtained in vaginal suppositories also came from the need to increase the control of the release of volatile oil at the level of the vaginal mucosa [39]. According to the authors Assis K.M.A *et al*., 2020 the inclusion of volatile oils in liposomal formulas leads to a controlled and sustained release of them and also reduces their possible toxic potential [40, 42].

Suppository Quality Control
The uniformity of the mass of vaginal suppositories was tested by consecutively weighing 20 suppositories and determining their average mass. Thus, the average weight of a suppository was 6.9 ± 0.57g, being within limits imposed by Romanian Pharmacopoeia, 10th edition.
The prepared vaginal suppositories have a homogenous appearance in the section. At room temperature, they keep their shape and consistency; they are yellowish-white in color, do not show agglomerations of particles, crystals, or air bubbles, and have a smell specific to the components.

Table 2. Total acid content of the sample at different time intervals subject to the pH of the dissolution medium. Testing the behavior of vaginal suppositories upon dissolution.

Samples \ Medium pH	pH= 4	pH=7	Time (min)
	Acidity index (µg/ml)		
1	32.00	-	15
2	32.80	16.80	30
3	32.90	16.80	45
4	33.60	-	60

In acid pH, an increase of 0.8 µg in the release of acids from oregano oil is observed after 30 minutes, a quantity that remains constant after 45 minutes so that after 60 minutes, it increases to 1.6 µg /ml **(Table 2)**.
The behavior in neutral pH is different because the release of the oil from the pharmaceutical forms takes place after 30 minutes and remains constant for an hour, after which the active principles are not detected.
From the interpretations of the obtained results, it appears that the prepared vaginal suppositories with liposomal oregano oil have an appropriate disaggregation time, according to the provisions of the Romanian Pharmacopoeia, 10th edition, ensuring a good release of the active substance at the place of application.

Conclusion

The macro- and microscopic analysis of the aerial part of *Origanum vulgare* L. carried out highlighted the specific characteristics of the family to which the plant belongs (*Lamiaceae* family) and highlighted the tector and secretory bristles responsible for the production of volatile oils.
The antimicrobial analysis demonstrated the inhibitory activity of the oil obtained from *Origanum vulgare* L. on a wide range of microbial strains such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The diameter of inhibition was demonstrated to be maximum on all microorganisms in the case of the 1:1 ratio (oregano oil: mineral

oil) used and very close to that of carvacrol used as a standard compound.
Oregano oil was successfully included in the liposomal formula by the lipid film hydration method, obtaining spherical-round liposomes with a size between 90nm and 1µm. The liposomal formula has been shown to have a negative surface electric charge, which provides increased stability to the created liposomes.
Through pouring the vaginal suppository mass, the ovules with liposomal oil of oregano were obtained, meeting all the quality conditions imposed by the Romanian Pharmacopoeia ed. X.
An efficiency of release of the active compounds from the vaginal suppositories at an acidic pH was also demonstrated, equal to the pH of the vaginal mucosa (administration area), which gives the obtained pharmaceutical forms a great advantage. Future perspectives include in vitro and later in vivo tests of the obtained vaginal suppositories.

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Conflict of interest: None

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Ethics statement: None

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