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Initial Investigations into the Development of Vaginal Suppositories Incorporating Liposomal Oregano Oil

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ABSTRACT

Infections caused by *Candida* species account for a significant proportion of fungal infections affecting the vaginal mucosa. Traditional antifungal treatments have shown limited efficacy, which has led to the search for alternative products based on plant-derived extracts, which are often more effective and non-toxic according to existing research. Our research focused on the extraction of essential oil from *Origanum vulgare* L. and the evaluation of its antimicrobial properties, known for its high content of bioactive compounds such as carvacrol, which has proven antifungal effects. The *Origanum vulgare* L. plant was also analyzed through macro- and microscopic methods. The oregano oil was encapsulated in a liposomal formulation and evaluated for various properties, including appearance, surface charge, and particle size. The liposomal oil was then used to prepare vaginal suppositories, which were evaluated organoleptically and for their ability to release active compounds. Future work will include in vitro testing of the developed formulations, followed by in vivo evaluation.

Keywords: Quality control, *Origanum vulgare* L. oil, Vaginal suppositories, Liposomes

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Introduction

Candida albicans is regarded as one of the most virulent strains within the *Candida* genus [1]. Research indicates that its aggressiveness is due to its ability to form a biofilm on infected tissues or mucosal surfaces [2]. This biofilm formation allows *Candida albicans* to create a dense population of cells that proliferate rapidly and adhere strongly to the affected tissue, where they are shielded from the body's immune responses as well as from antifungal medications by a protective "capsule" [3]. As a result, conventional antifungal treatments like fluconazole and nystatin become less effective, as the fungus develops resistance [4].

Recent studies emphasize that the primary challenge in treating *Candida* infections is the biofilm's adhesion, which must first be disrupted before the fungus itself can be eradicated. Therefore, effective treatments must target the disruption of the biofilm at the cellular level, breaking down the adhesion of surface proteins within the colony before eliminating the fungus [5, 6].

Modern therapeutic approaches focus on this capacity to disrupt the biofilm's adhesion to mucosal or infected tissue, with promising results from volatile oils, such as *Origanum vulgare* oil [7]. Given the well-known

phytochemical composition of oregano oil and its proven efficacy against *Candida* species [8], the goal of our study was to develop a novel pharmaceutical formulation for treating vaginal candidiasis.
In this context, our work involved the characterization of the *Origanum vulgare* L. plant through both macroscopic and microscopic analysis, alongside evaluating its antimicrobial activity. The extracted oil was first incorporated into a liposomal formulation and subsequently used in the preparation of vaginal suppositories. The liposomal formulation and the release profile of the oil from the vaginal suppositories were thoroughly evaluated throughout the study.

Materials and Methods

Botanical characterization of Origanum vulgare L.
The morphological analysis of *Origanum vulgare* L., sourced from Oradea, Bihor County, was carried out with the help of botanical reference books and illustrated identification guides. Any plant material that showed signs of wear, damage, or discoloration was discarded, and only healthy, clean portions of the plant were selected for further examination.

Visual inspection of Origanum vulgare L. plant parts
According to the Romanian Pharmacopoeia (10th edition), macroscopic evaluation involves identifying the plant's morphological characteristics, which can be observed with the naked eye or with the aid of a magnifying glass, as well as those detectable through odor and taste. This form of analysis focuses on the plant's overall appearance, color, smell, and occasionally taste, essentially providing a sensory examination [9].

Microscopic study of Origanum vulgare L. plant parts
The microscopic analysis of vegetative organs, such as the stem and leaf, was performed by preparing cross-sectional slices, which were then clarified and stained. The presence of distinct structural elements, like secretory glands and tectorial bristles, was determined through the use of different chemical dyes, which colored cellulose membranes red and lignified ones yellow [10]. The samples were treated with Congo Red dye for 1-3 minutes, followed by thorough washing with distilled water to remove any excess dye. The stained sections were then observed under an Optika B350 microscope.

Extraction process of Origanum vulgare L. oil
The aerial parts of *Origanum vulgare* L. were dried in an oven at 65 °C until they reached a constant weight. After drying, the plant was ground into a powder, and the essential oil was extracted using a Soxhlet extractor. Petroleum ether was employed as the solvent in this process, and the extraction occurred at 65 °C for 7 hours. The solvent was then removed by rotary evaporation using the Heidolph Laborota 4000, set at 90 rpm, for 30 minutes [11, 12].

Antimicrobial evaluation of Origanum vulgare L. oil
The antimicrobial efficacy of oregano oil was tested against both gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and gram-negative bacteria (*E. coli*), as well as *Candida albicans*. Bacteria were cultured on nutrient agar plates using the spread plate method and incubated at 37 °C for 24 hours [13, 14]. The oil was diluted in mineral oil at ratios of 1:1, 1:10, and 1:20, and carvacrol, a known active compound in oregano oil, was used as a control at similar dilutions [15]. After 24 hours, the zones of inhibition were measured, and the experiment was repeated three times to ensure accuracy.

Formulation, preparation, and characterization of vaginal suppositories with liposomal oregano oil

Preparation and characterization of liposomal oregano oil for inclusion in vaginal suppositories
To prepare the oily phase, a mixture of phosphatidylserine and cholesterol was selected in a 3:1 ratio [14, 16, 17]. These lipids were dissolved using a solvent system of chloroform and methanol at a 3:2 ratio [18]. The oregano oil was incorporated into this lipid blend at a concentration of 50 µg/ml. Following the solubilization, the mixture was evaporated with a Heidolph Rotary Evaporator, Laborota 4000, to eliminate the solvents, leaving a thin lipid film on the walls of the flask [19]. After evaporation, the lipid film was left at ambient temperature for 24 hours

to ensure the complete removal of solvent traces, before being hydrated with fifteen ml of phosphate buffer (pH = 7.6) to form the hydrophilic phase [20].
As the hydration occurred, liposomes were formed spontaneously, and their size was minimized through sonication for thirty minutes and subsequent centrifugation at 10,000 rpm for 40 minutes [16]. The resulting liposomal formulation was analyzed for its size, shape, and surface charge [21].

Microscopic evaluation of liposomal structure

To confirm the structural characteristics of the liposomes containing oregano oil, optical microscopy was employed. A 40× objective lens was used with an Olympus CX40 inverted light microscope, coupled with a Hitachi CCD camera, to capture the images in phase contrast mode [18].

Size and zeta potential characterization of liposomes via DLS

Dynamic light scattering (DLS) analysis was performed to measure the diameter, distribution, and zeta potential of the liposomes. A Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) was used for these measurements. For size determination, polystyrene cells with a 1 cm optical path length were utilized, while zeta potential was assessed with disposable folded capillary cells [22]. All experiments were conducted in triplicate to ensure reproducibility.

Process of preparing vaginal suppositories

The formulation of vaginal suppositories was carried out in three distinct stages, as illustrated in **Figure 1**.

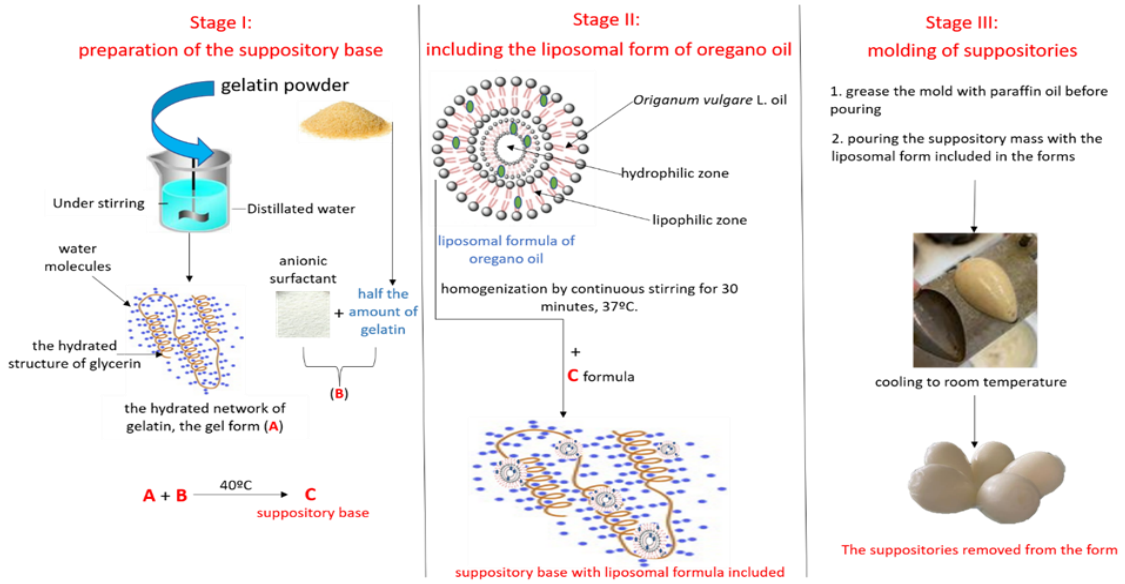


Figure 1. The stages of preparation of vaginal suppositories with *Origanum vulgare* L. oil in the liposomal form.

In the initial stage, the suppository base was formulated using 10.5 g of gelatin. A portion of this gelatin was set aside to hydrate, while the remainder was mixed with 3.5 g of surfactant. These two components were combined under warm conditions in a water bath. To the homogeneous mixture, 52.5 g of glycerin was incorporated, maintaining a temperature similar to that of the gelatin [23, 24].
In the subsequent step, 0.5 g of liposomal oregano oil was added and the mixture was thoroughly homogenized.
The final step involved molding the vaginal suppositories by pouring the prepared mixture into a mold that had been pre-lubricated with paraffin oil. The quantities listed are intended to create 100 g of the vaginal suppository base.

Quality control of the suppositories

The suppositories underwent quality control based on the standards established by the Romanian Pharmacopoeia, 10th edition, and the European Pharmacopoeia, 7.1 edition [9]. The evaluation focused on various aspects of the

suppositories, including appearance, mass consistency, dissolution characteristics, disaggregation, and proper dosage [9].

Evaluation of appearance and mass consistency

As per the guidelines in the Romanian Pharmacopoeia, vaginal suppositories must maintain a consistent, smooth appearance and should retain their structural integrity at room temperature. Upon inspecting a longitudinal section with a 4.5x magnification, the suppositories should be free from particle clusters, crystals, or air bubbles [9]. The mass uniformity was assessed by weighing a batch of 20 suppositories and calculating the average weight to ensure uniformity [9].

Assessment of dissolution and release of oregano oil

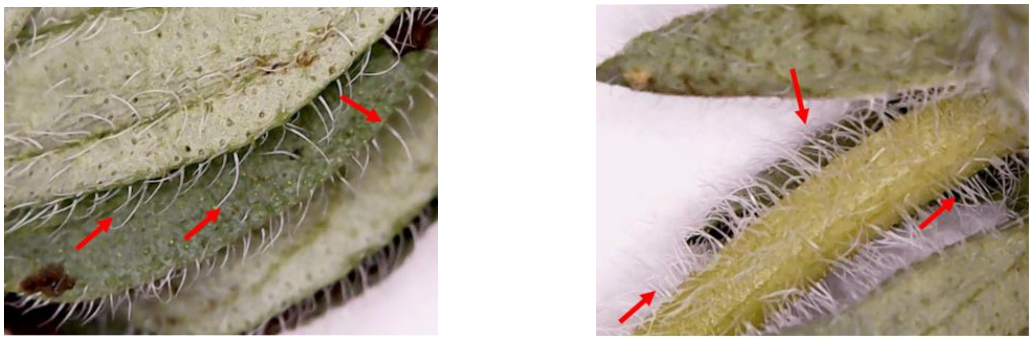
To evaluate the solubility and release of oregano oil from the vaginal suppositories, 500 mL of two different dissolution media were prepared. One medium was neutral (distilled water), and the other had an acidic pH of 4 (HCl), mimicking the vaginal environment. Both media were pre-heated to 37 °C, simulating body temperature. Each suppository was placed in a cylindrical vessel, and the dissolution test was conducted at a constant stirring speed of 37 rpm.

At four time intervals—15, 30, 45, and 60 minutes—5 mL samples were collected from each dissolution medium (both acidic and neutral). After sampling, the released oregano oil was quantified using titration with a 0.1N NaOH solution.

Results and Discussion

Macroscopic examination of Origanum vulgare L. plant parts

During the macroscopic examination, the aerial components of *Origanum vulgare* L. were observed, particularly the stem, which is adorned with hair-like structures visible to the unaided eye **(Figure 2b)**. Additionally, the leaves were noted to have a layer of hairs and specialized secretory structures on their upper surface **(Figure 2a)**.



a) b)

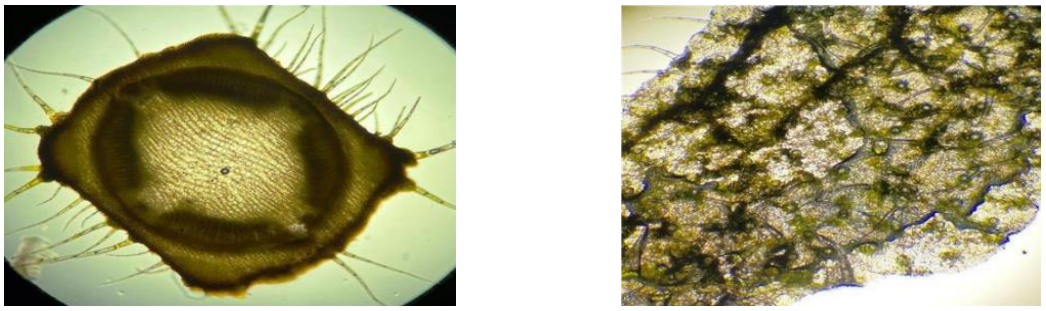
Figure 2. Macroscopic images of the dorsal part of the leaves of *Origanum vulgare* L.; a) the tulip of the oregano plant, and b) the red arrows highlight the presence of tectorial and secretory bristles present in vast numbers.

Microscopic investigation of Origanum vulgare L. plant parts

In **Figure 3a**, a cross-sectional view of the tetrahedral stem of *Origanum vulgare* L. is presented. The outer epidermis consists of tightly packed cells with slightly raised outer surfaces, which are covered in multi-cellular bristles extending across the stem. In the stem's corners, angular collenchyma is visible, which is typical of plants from the Lamiaceae family [25]. Beneath the epidermis, the secretory parenchyma is observable, playing a key role in photosynthesis. The next layer, the pericycle, is made of thin-walled cells. Within the central cylinder of the stem, the plant features several mixed fascicles of free-woody tissue positioned next to the angular collenchyma.

Figure 3b showcases a skinning section of the leaves of *Origanum vulgare* L., which has been stained with Congo Red. Beneath the upper epidermis, the palisade parenchyma is well-defined, consisting of cells specialized for photosynthesis. The epidermis is composed of a single row of cells, coated with a well-developed cuticle and a waxy layer. The mesophyll contains palisade tissue made up of tightly packed, elongated cells that house

numerous chloroplasts. Within the leaf's structure, large polyhedral cells, which have a larger diameter and are less chlorophyll-rich than surrounding cells, form the aquifer tissue and store vacuolar fluid. The epidermis is covered with several types of formations, such as stomata, secretory structures, and tector bristles. These tector bristles are unbranched, multi-cellular, and filamentous, growing perpendicularly from the epidermis. Additionally, the leaves contain circular secretory structures of varying sizes, which are responsible for producing the volatile oil of oregano.



a) b)

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

The antimicrobial activity of Origanum vulgare L. oil

It is well documented that as *Origanum vulgare* L. matures, the profile of its volatile oils undergoes significant changes [26]. In the early growth stages, these oils mainly consist of simpler terpenic hydrocarbons, while the reproductive parts of the plant contain oils that are richer in oxygenated components [15]. The diverse chemical structures of these natural compounds contribute to their strong antibacterial and antifungal activities [27].

According to numerous studies, phenolic compounds such as carvacrol, thymol, and eugenol, as well as aldehydes and terpenic alcohols (e.g., terpineol), are identified as the most potent active ingredients in these oils [15]. Plants like *Thymus vulgaris*, *Origanum vulgare*, *Melaleuca alternifolia*, *Cinnamomum* sp., *Eugenia carryophyllata*, and *Eucalyptus globulus* are all recognized for containing significant quantities of these bioactive compounds [6]. Carvacrol, the predominant component in *Origanum vulgare* L. oil, has demonstrated effectiveness against certain strains of *Staphylococcus aureus* and *S. epidermidis*, preventing biofilm formation [28, 29].

As a result, carvacrol was chosen as a standard in evaluating the antimicrobial effects of oregano oil. The comparative antimicrobial results, which include data on both gram-positive and gram-negative bacterial strains and *Candida albicans*, are summarized 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

As per the data presented in **Table 1**, the antimicrobial activity of the oregano oil tested either matches or surpasses that of pure carvacrol, which serves as the standard, likely due to the combined synergistic effects of the various compounds found in the oil. Gitea *et al*. reported that *Origanum vulgare* L. oil is abundant in carvacrol, thymol, sabinene, γ-terpinene, p-cymene, and thymoquinone. Other common compounds found in Origanum species include geraniol, linalool, linalyl acetate, myrcene, camphene, camphor, borneol, as well as sesquiterpenes. Phenolic acids such as p-hydroxybenzoic and hydroxycinnamic acids have also been identified in these plants. Most in vitro investigations underline the presence of significant antimicrobial, antifungal, antibacterial, antiparasitic, and antiviral properties [15].

The antibacterial effect of oregano oil is primarily linked to its ability to destabilize the bacterial cell membrane, denature plasma proteins and deactivate certain enzymes within the bacteria. Lipophilic compounds present in the volatile oils interact with various membrane components such as phospholipids, fatty acids, and polysaccharides. This interaction increases membrane permeability, leading to leakage of cellular contents and ultimately bacterial cell death [30].

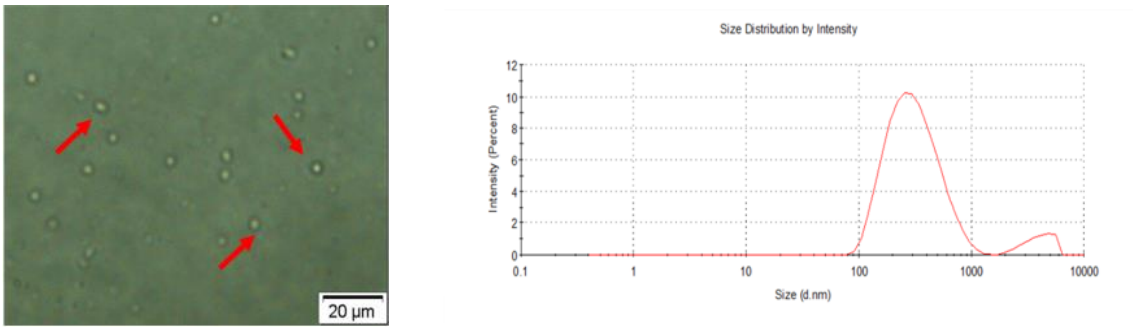
Oregano oil has also demonstrated antiviral efficacy against Hepatitis A and Herpes simplex viruses [31, 32]. In addition to its effects on *Candida* species, the oil exhibits antifungal properties against Aspergillus species, and antiparasitic activity has been observed against *Coccidium* sp. [33, 34].

Microscopic characterization and DLS analyses of liposomes containing oregano oil

The liposomes containing oregano oil were synthesized through the lipid film hydration technique [35]. This method involves two primary stages. In the first stage, a lipid film is created using phosphatidylserine and cholesterol at a 3:1 ratio, along with oregano oil at a concentration of 50 µg/ml. In the second stage, the lipid film is hydrated using a hydrophilic phase composed of phosphate buffer with a pH of 7.6 [18].

Upon hydration, the liposomes spontaneously form, encapsulating oregano oil within them. To enhance the stability of these liposomes, the mixture undergoes sonication followed by centrifugation, as described in the methodology section. These liposomes, referred to as giant-type or multilamellar liposomes in the literature, exhibit characteristics typical of this formation process [36].

To confirm the formation of liposomes, optical microscopy was employed to examine the emulsion, and dynamic light scattering (DLS) analysis was conducted. The results, shown in **Figure 4a**, reveal that the liposomes take on a round or spherical shape and are uniformly dispersed within the microscopic field. DLS analysis, depicted in **Figure 4b**, shows the size distribution of the liposomes, which range from 90 nm to 1 µm, with the majority falling between 200 nm and 500 nm. Additionally, the surface charge of the liposomes was found to be negative (-20.43 V), indicating a high level of stability for the oregano oil liposomes over time [37-41].



a) b)

Figure 4. a) microscopic image of *Origanum vulgare* L. oil liposomes formulated, and 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.

According to Mechmechani *et al*. [38], encapsulating volatile oils in various formulations enhances their biological activity and improves their effectiveness in breaking down biofilms formed by microorganisms sensitive to the oil's composition [38]. Additionally, incorporating oregano oil into a liposomal formulation within vaginal suppositories contributes to the greater stability of the oil and protects its components from degradation caused by environmental factors such as humidity, light, and temperature [18]. The decision to use liposomal encapsulation for oregano oil in vaginal suppositories was driven by the need to enhance control over the release of the volatile oil at the vaginal mucosal surface [39]. As noted by De Assis *et al*. [40], encapsulating volatile oils in liposomal structures promotes their steady and controlled release while also reducing their potential toxicity [40, 42].

Suppository quality control

The consistency of vaginal suppository mass was assessed by weighing 20 suppositories and calculating the average weight. The average weight was determined to be 6.9 ± 0.57 g, which falls within the allowable range outlined by the Romanian Pharmacopoeia, 10th edition. The vaginal suppositories were uniform in appearance

when cut open. They retained their shape and consistency at room temperature, exhibited a yellowish-white color, and showed no signs of clumping crystallization, or air bubbles. The scent was characteristic of the ingredients used.

Table 2. The total acid content of the sample at different time intervals is subject to the pH of the dissolution medium; testing the behavior of vaginal suppositories upon dissolution.

Medium pH / Samples	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

At an acidic pH, after 30 minutes, the release of acids from oregano oil rises by 0.8 µg, which remains constant after 45 minutes. By 60 minutes, this value increases to 1.6 µg/ml **(Table 2)**. In contrast, at neutral pH, the oil release from the pharmaceutical forms begins after thirty minutes and stays constant for an additional hour. After that, no active ingredients are detected.
The data analysis suggests that the vaginal suppositories containing liposomal oregano oil meet the required disaggregation time as specified in the Romanian Pharmacopoeia, tenth edition, ensuring proper release of the active component at the targeted application site.

Conclusion

Examination of the aerial parts of *Origanum vulgare* L. under both macro- and microscopic analysis revealed features characteristic of the Lamiaceae family, particularly the tector and secretory bristles, which are responsible for the production of volatile oils. Antimicrobial testing demonstrated that the oil from *Origanum vulgare* L. effectively inhibits various microbial strains, such as *Enterococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. The maximum inhibition was observed with a 1:1 ratio of oregano oil and mineral oil, with results similar to those obtained with carvacrol used as a standard compound.
Oregano oil was successfully incorporated into a liposomal formulation through the lipid film hydration method, producing spherical liposomes with sizes ranging from 90 nm to 1 µm. These liposomes exhibited a negative surface charge, contributing to their enhanced stability.
The vaginal suppositories with liposomal oregano oil were prepared and met the quality standards defined by the Romanian Pharmacopoeia, 10th edition. The release efficiency of active ingredients from these suppositories at an acidic pH, which mimics the pH of the vaginal mucosa, offers a significant advantage in terms of effectiveness. Future steps include conducting in vitro testing, followed by in vivo evaluations of the vaginal suppositories.

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Conflict of Interest: None

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Ethics Statement: None

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