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Nanoencapsulation of *Origanum vulgare* essential oil into liposomes with anticancer potential

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Origanum vulgare L. essential oil possesses a wide spectrum of biological activities. Nanoencapsulation of *O. vulgare* essential oil into liposomes seems to be a promising strategy to maintain and improve these biological properties. This research was carried out to develop a suitable liposomal formulation for the effective encapsulation of *O. vulgare* essential oil in order to improve the antioxidant and cytotoxic activities. The characterization of liposomal nanocarriers was conducted in terms of size, zeta potential, and encapsulation efficiency. An MTT assay was used to assess the cytotoxic activity of the prepared and characterized *O. vulgare* essential oil liposomes in MCF-7 cancer cell lines. Antioxidant activity was determined by assessing DPPH scavenging activity. *O. vulgare* essential oil exerted cytotoxic activity with an IC_{50} of 50 µg/ml. The essential oil of *O. vulgare* was effectively encapsulated in liposomes, with no significant change observed among the formulations. The antioxidant activity was significantly enhanced after encapsulating the essential oil in liposomes. *Origanum vulgare* essential-oil-loaded Phospholipon 90H liposomes demonstrated considerably increased cytotoxic activity against MCF-7 cells, whereas Lipoid S100 liposomes showed no significant differences from the non-encapsulated essential oil. Phospholipon 85G liposomes had the least cytotoxic impact. As a result, liposomes containing *O. vulgare* essential oil may be promising nanocarriers for the development of anticancer agents.

1. Introduction

Multidrug resistance, severe side effects, and high costs associated with current cancer therapies necessitate the development of more effective and less harmful interventions. The current goal of many researchers is to identify anti-cancer agents from natural sources (Buyel 2018; Khan and Gurav 2018, Gezici and Şekeroğlu 2019). The use of natural components to treat cancer is considered as a critical objective of herbal medicine. Medical plants have been recognized as a rich source of anticancer compounds for many years (Li and Weng 2017; Wang et al. 2019).

Plants provide over 70% of the drugs used in conventional cancer treatment. As a result, the use of complementary therapies is desirable in order to naturally activate the immune system and to reduce chemotherapy side effects (Iqbal et al. 2017; Goldberg 2019; Huang et al. 2019). Essential oils can include up to 100 ingredients in varying proportions; two or three principal constituents account for up to 70% of the total, with additional elements present in minor amounts. The highly concentrated components in essential oils are most often responsible for their bioactivity; however, research has shown that chemicals found in tiny percentages can also improve the efficiency of this activity. Similarly, the biological activity of an essential oil has been associated with the phytochemical structures of its components (Leyva-López et al. 2017).

Origanum vulgare is a plant in the mint family Lamiaceae with highly regarded economic and medicinal worth. Many studies have shown that *O. vulgare* essential oil has a wide range of biological properties, displaying antioxidant, antibacterial, and cytotoxic activities against several human cancer cell lines. These activities indicate that it might be utilized as both a preventive and adjuvant component in cancer therapy (Leyva-López et al. 2017; Ali et al. 2020).

Because the phytochemical composition and biological activity of essential oils vary based on circumstances associated with their growth habits and geographic locations, these cannot be generalized and must be determined for each essential oil individually. Essential oils also have limited therapeutic uses due to their high volatility, poor stability, water solubility, and bioavailability. As a result, essential oil nanoencapsulation seems to be a potential technique for addressing these shortcomings, while also enhancing bioavailability and stability (Leyva-López et al. 2017; Sebaaly et al. 2015; Nagaraju et al. 2021; Wang et al. 2019).

Liposomes are promising nanocarriers that can be used to protect biomolecules from the environment. They have the added advantages of being non-toxic and biodegradable. These nanocarriers have been shown to influence the physicochemical properties and thus the biological activities of a wide range of natural compounds, including essential oils, as nanocarrier compositions. They have also been shown to improve the physicochemical and biological properties of natural molecules, as well as their bioavailability and stability (Sebaaly et al. 2015; Hammoud et al. 2019; Kyriakoudi et al. 2021).

Recently increased interest has been expressed in employing liposomes as nano-drug delivery vehicles in cancer therapy. These are able to safely transport anticancer medications to cancer cells and facilitate their successful delivery to the target location. Liposomes have an amphiphilic phospholipid bilayer that resembles a typical cell membrane, which allows their seamless integration among other liposomes and the cell membrane and, hence, their

successful cellular absorption (Yan et al. 2020). They are also well known for their ease of production and administration, as well as their low production costs (Hammoud et al. 2019; Yan et al. 2020; Beltrán-Gracia et al. 2019; Nasr et al. 2021).

While plenty of research has been and is being carried out on essential oils, their encapsulation in nanocarriers and their biological activity require more study. Several studies have reported that the physicochemical properties of essential-oil-loaded nanocarriers are based on the type and ratio of the nanocarriers as well as the type of essential oil (Cimino et al. 2021).

The particle size and surface charge of essential-oil-loaded nanocarriers are critical parameters, as these lower the essential oil volatility and increase the membrane contact, the cellular absorption, and thereby the biological activity. Furthermore, high nanocarrier encapsulation efficiency is critical for addressing all concerns related to essential oil solubility and chemical stability, as well as for increasing the efficacy of essential-oil-loaded nanocarriers, leading to increased therapeutic efficacy. The preparation procedure, type of essential oil, vesicle composition, nanocarrier content, and storage stability can all impact these characteristics (Aguilar-Perez et al. 2021; Hammoud et al. 2019).

Table 1: Chemical composition of *O. vulgare* essential oil

[a] *RT (min) = Retention Time*
[b] *KIL = Kovats Retention Index*

No.	RT[a] (min)	KIL[b]	Components	% *
1	8.067	932	α-Pinene	0.82
2	8.633	946	Camphene	0.21
3	9.852	988	Myrcene	0.99
4	10.567	1002	α-Phellandrene	0.22
5	10.968	1014	α-Terpinene	0.97
6	11.351	1020	*p*-Cymene	7.29
7	12.667	1054	γ-Terpinene	4.31
8	13.108	1065	*cis*Ssabinene hydrate	0.24
9	13.703	1086	Terpinolene	0.10
10	14.252	1095	Linalol	0.24
11	17.502	1165	Borneol	0.28
12	17.851	1174	Terpinen-4-ol	0.58
13	19.161	1186	α-*Terpineol*	0.10
14	20.283	1232	Thymol, ethyl ether	0.31
15	22.022	1233	Pulegone	0.43
16	22.652	1289	Thymol	1.29
17	23.882	1298	Carvacrol	71.41
19	26.714	1387	β-Burbonene	0.11
20	28.254	1417	trans-Caryophyllene	1.69
21	28.952	1439	Aromadendrene	0.14
22	29.678	1452	α-Humulene	0.29
23	30.417	1478	γ-Murrolene	0.16
24	31.750	1505	β-Bisabolene	0.77
25	31.973	1513	γ-Cadinene	0.14
26	32.156	1522	δ-Cadinene	0.30
			Total	**93.39**

* The percentages of compounds were obtained by FID peak-area normalization.
The percentage composition of the oils was computed by the normalization method from the GC peak areas, calculated as the mean of three samples, without correction factors.

While studies have shown that liposomal nanoencapsulation of essential oils maintain/boosts biological activities, no studies have assessed the impact of different liposomal formulations with varying physicochemical properties on the cytotoxicity and antioxidant activity of the same *O. vulgare* essential oil. According to our knowledge, no data have been reported on the cytotoxicity and antioxidant activities of lipid-based liposomal nanocarriers loaded with *O. vulgare* essential oil on MCF-7 breast cancer cell lines.

Our study objective was to define an appropriate liposomal formulation for the effective encapsulation of *O. vulgare* essential oil in order to maintain/improve the antioxidant and cytotoxic activities. For this reason, *O. vulgare* essential oil was encapsulated in three different liposomal nanocarriers to investigate the effect of these nanosystems on DPPH scavenging activity of *O. vulgare* oil, as well as the MTT cytotoxic activity in MCF-7 human cancer cells.

2. Investigations and results

2.1. Essential oil extraction

The average essential oil yield for *O. vulgare* samples was 1.47±0.03 %.

2.2. Quantification of the essential oil constituents

GC/MS phytochemistry investigations of *O. vulgare* essential oil revealed the yield and chemical composition presented in Table 1. The 26 identified constituents represent 93.39% of the total oil content. The main components of *O. vulgare* essential oil were: carvacrol (71.41%), *p*-cymene (7.29%), and γ-terpinene (4.31%). The relative content (%) is the relative share of each component from the essential oil obtained by applying the normalization approach to the GC/FID peak areas that was used to quantify the components without correction factors.

2.3. Determination of particle size, pdi, and Zeta potential

The particle size, polydispersity index (PDI), and zeta potential of the blank- and essential-oil-loaded liposomal batches were determined using a Malvern Zetasizer Nano ZS and are presented in Table 2. The samples were analyzed in triplicate and presented as a mean value ± SD. Blank Phospholipon 85G liposomes exhibited the smallest particle size values, (81±1.9 nm) as compared to blank Phospholipon 90H (161 ± 3.6 nm) and blank Lipoid S100 (270 ± 1.5 nm). Regarding the essential oil-loaded liposomes, the composition of the phospholipids and cholesterol was fixed at a proportion of 10:5 (w/w). *Origanum vulgare* essential oil was added at a concentration of 2.5 mg/ml. Table 2 presents the physicochemical properties of the blanks and the *O. vulgare* essential-oil-loaded Phospholipon 85G, Lipoid S100, and Phospholipon 90H liposomes.

2.4. Encapsulation efficiency

Origanum vulgare essential oil was highly encapsulated into Lipoid S100, Phospholipon 85G, and Phospholipon 90H liposomes. Among the three formulations used, the highest EE value was obtained for the Lipoid S100 liposomes (EE% 85.5 ± 2.4), followed by the Phospholipon 90H liposomes (84.4 ± 4.1) and then the Phospholipon 85G (83.5 ± 3.5), with no significance differences observed ($p > 0.05$).

2.5. Morphological observations

The morphology of the liposome can impact the liposome efficiency and, therefore, this morphology is also an excellent indicator for liposome performance. Scanning electron microscopy was employed to examine the size, size uniformity, and particle morphology of the produced liposomes. The shape of the outer surface revealed the presence of nanocarriers with spherical forms. Liposomes were homogeneous and varied in size from nano- to micrometers, according to the SEM images (Fig. 1).

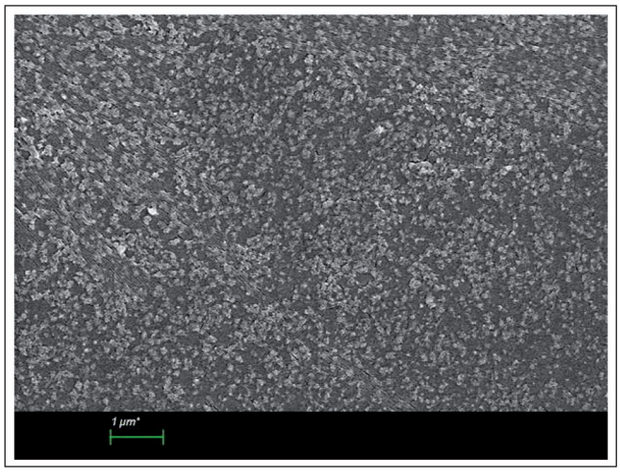
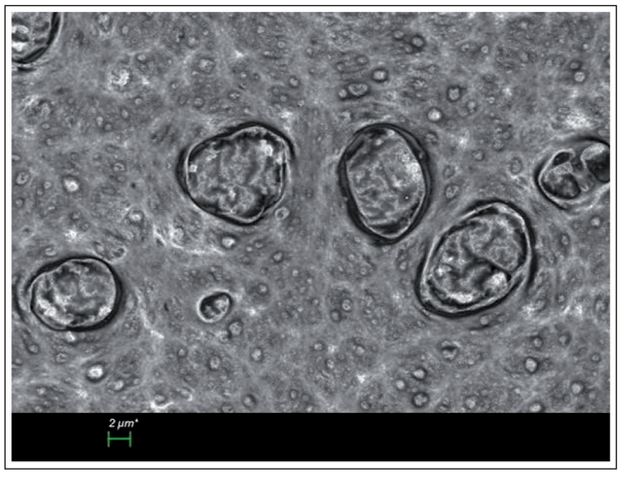
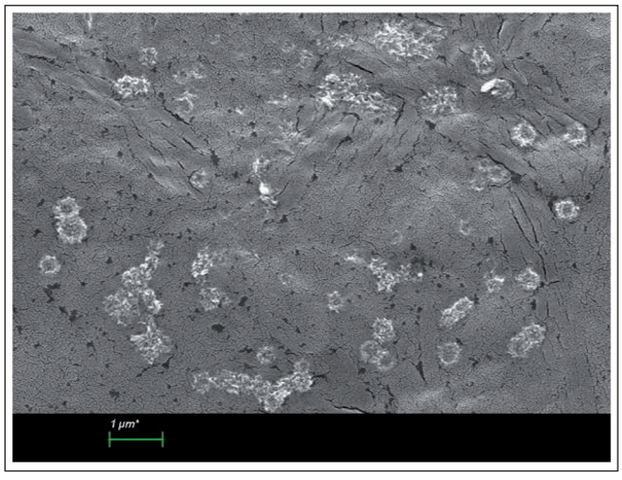


Fig. 1: SEM images of *O. vulgare* essential-oil-loaded (a) Lipoid S100, (b) Phospholipon 85G, and (c) Phospholipon 90H liposomes.

(a) *O. vulgare* essential-oil-loaded Lipoid S100 liposomes. From this picture, small vesicles can be seen.



(b) *O. vulgare* essential-oil-loaded Phospholipon 85G liposomes. The insertion of essential oil into Phospholipon 85G increased vesicle sizes and size distribution. Vesicles ranged in size from 100 nm to 8 µm in diameter. The vesicles were seen to be non-perfect spheres.



(c) *O. vulgare* essential-oil-loaded Phospholipon 90H liposomes The particle sizes ranged from 150 to 600 nm. The vesicles were spherical with a restricted size distribution. The insertion of essential oil caused changes in the size, size distribution, and shape of the vesicles. More spherical vesicles with narrow particle size distributions were created using Phospholipon 90H.

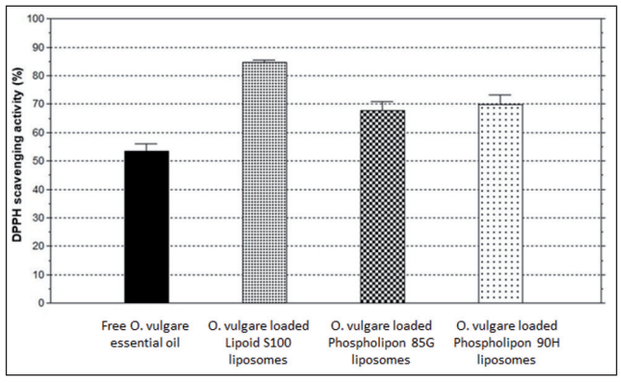


Fig. 2: DPPH scavenging activity of free *O. vulgare* essential oil and *O. vulgare* essential-oil-loaded Lipoid S100, Phospholipon 85G, and Phospholipon 90H liposomes. Each bar represents means±SD ($n = 3$).

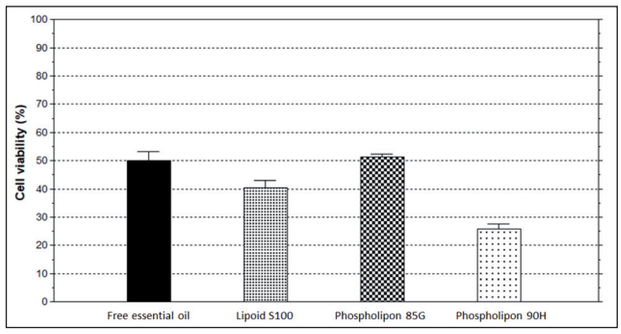


Fig. 3: MTT cytotoxic activity of free *O. vulgare* essential oil and *O. vulgare* essential-oil-loaded Lipoid S100, Phospholipon 85G, and Phospholipon 90H liposomes. Each bar represents means±SD ($n = 3$).

2.6. Antioxidant activity

The *in vitro* antioxidant activity of *O. vulgare* essential oil and *O. vulgare* essential-oil-loaded liposomes was determined using the DPPH radical scavenging assay. The results are presented in Fig. 2. The free *O. vulgare* essential oil exhibited a good DPPH free radical scavenging activity of 53.52±4.27 %. Among all batches tested, *O. vulgare* essential-oil-loaded Lipoid S100 liposomes showed the best DPPH free radical scavenging activity of 84.67±1.53 %. This activity was significantly higher different from the activities of *O. vulgare* essential-oil-loaded Phospholipon 85G and *O. vulgare* essential-oil-loaded Phospholipon 90H liposomes: 67.77±5.36 % ($p < 0.01$) and 69.93±5.68 % ($p < 0.05$), respectively. No significant differences were observed between the *O. vulgare* essential-oil-loaded Phospholipon 85G and 90H liposomes ($p > 0.05$).

2.7. Cell toxicity assay

In this study, the cytotoxicity of the free *O. vulgare* essential oil, empty liposomes, and essential-oil-loaded liposomes was examined in human MCF-7 cancer cell lines. The MTT test was used to measure the cell viability, and the results are shown in Fig. 3. After 24 h, viability was determined using various concentrations of *O. vulgare* essential oil (0.39 to 200 µg/ml). The IC_{50} of the free *O. vulgare* essential oil was 25 µg/ml. Free *O. vulgare* essential oil showed measurable cytotoxicity and reduced the cell viability to 50.10 %. Nanoencapsulation of *O. vulgare* essential oil into Phospholipon 90H liposomes significantly ($p < 0.001$) enhanced the cytotoxic activity (reduction of cell viability to 25.89%). No significant differences ($p > 0.05$) were observed between free *O. vulgare* essential and *O. vulgare* essential-oil-loaded Phospholipon 85G or *O. vulgare* essential-oil-loaded Lipoid S100 liposomes, with cell viability reduction to 51.22% and 40.41%, respectively.

Table 2: Characteristics of blank and essential-oil-loaded liposomes in terms of mean particle size, PDI, zeta potential, and encapsulation efficiency.

Phospholipon 85G

Batch composition (w/w/w)	Liposome batches	Mean size (nm) ± SD (n = 3)	Polydispersity index (PDI) ± SD (n = 3)	Zeta potential (mV) ± SD (n = 3)	Encapsulation efficiency of *O. vulgare* essential oil (%) ± SD (n = 3)
Ph 85G/Chol/*O. vulgare* essential oil (10:5:0)	Blank liposomes	81 ± 1.9	0.30 ± 0.01	-35.4 ± 0.9	
Ph 85G/Chol/*O. vulgare* essential oil (10:5:2.5)	*O. vulgare* essential-oil-loaded liposomes	89 ± 0.91	0.28 ± 0.01	-26.9 ± 0.9	83.5 ± 3.5

[a] Ph 85G: Phospholipon 85G, [a] Chol: cholesterol.

Lipoid S100

Batch composition (w/w/w)	Liposome batches	Mean size (nm) ± SD (n = 3)	Polydispersity index (PDI) ± SD (n = 3)	Zeta potential (mV) ± SD (n = 3)	Encapsulation efficiency of *O. vulgare* essential oil (%) ± SD (n = 3)
L-S100/Chol/*O. vulgare* essential oil (10:5:0)	Blank liposomes	270 ± 1.5	0.22 ± 0.05	-4.2 ± 0.7	
L-S100/Chol/*O. vulgare* essential oil (10:5:2.5)	*O. vulgare* essential-oil-loaded liposomes	319 ± 20.5	0.23 ± 0.01	-8.4 ± 0.3	85.5 ± 2.4

[a] L-S100: Lipoid S100, [a] Chol: cholesterol.

Phospholipon 90H

Batch composition (w/w/w)	Liposome batches	Mean size (nm) ± SD (n = 3)	Polydispersity index (PDI) ± SD (n = 3)	Zeta potential (mV) ± SD (n = 3)	Encapsulation efficiency of *O. vulgare* essential oil (%) ± SD (n = 3)
Ph 90H/Chol/*O. vulgare* essential oil (10:5:0)	Blank liposomes	161 ± 3.6	0.38 ± 0.03	-11.6 ± 0.1	
Ph 90H/Chol/*O. vulgare* essential oil (10:5:2.5)	*O. vulgare* essential-oil-loaded liposomes	133 ± 2.4	0.16 ± 0.02	-9.0 ± 0.1	84.4 ± 4.1

[a] Ph 90H: Phospholipon 90H, [a] Chol: cholesterol.

3. Discussion

In this work, the effects of the nanoencapsulation of *O. vulgare* essential oil into three different liposomal formulations on maintaining/improving the antioxidant and cytotoxic activity were investigated.

The phytochemical results show that *O. vulgare* essential oil has a high content of carvacrol and lower contents of *p*-cymene, γ-terpinene, and thymol. These results are in agreement with those of previous studies, where carvacrol was determined as the main component of oregano essential oils, even at different levels (12–88%) (Laothaweerungsawat et al. 2020; Papajani et al. 2015). According to several studies, the phenols carvacrol and thymol and their precursors *p*-cymene and γ-terpinene account for over 70% of *Origanum* essential oil (Fitsiou et al. 2016). This might suggest that the hereditary features of carvacrol synthase are constantly active (Khan et al. 2019). The results conflict with those of previous studies, which showed thymol and *p*-cymene as having higher percentages than carvacrol (Shafiee-Hajiabad et al. 2014; Béjaoui et al. 2013). The *O. vulgare* oil yield and volatile compositions, however, is known to be affected by changes in growing conditions, geographic regions, time of harvest, and the growth stage at harvest, which can explain the differences reported in the main constituents and their concentrations (Morshedloo et al. 2018; Mancini et al. 2014; Khan et al. 2019; Chahla et al. 2021; Basholli-Salihu et al. 2017).

In this study, the average size, polydispersity index (PDI), and zeta potential of the blank and essential-oil-loaded liposomes were measured. When vesicles were loaded with *O. vulgare* essential oil, the size increased as compared to empty liposomes in all liposomal batches. This result is in agreement with results reported earlier showing that the increase in nanoliposome size is related to the encapsulation of the oil (Ali et al. 2020). These results can be attributed to *O. vulgare* essential oil complexation in the bilayer, which may diminish the phospholipid cohesiveness, a behavior that has already been reported for liposomes (Sebaaly et al. 2015). The PDI values of the blank and the *O. vulgare* essential-oil-loaded Phospholipon 85G liposomes imply that the liposomes had a uniform size distribution. The *O. vulgare* essential-oil-loaded Phospholipon 90H liposomes showed the lowest PDI values, implying that these systems are highly homogeneous. Meanwhile, the PDI values of blank and *O. vulgare* essential-oil-loaded Lipoid S100 liposomes were not significantly different from those of the Phospholipon 85G liposomes. Furthermore, no significant variations in the zeta potentials between the blanks and *O. vulgare* essential-oil-loaded liposomes were observed. Similar results have been reported by other authors (Faraji et al. 2020). Likewise, the composition of the lipid vesicle membrane, and especially the type of phospholipids, cholesterol content, preparation method, and the kind of essential oil, may affect the liposome size and the encapsulation efficiency (Sebaaly et al. 2015; Faraji et al. 2020).

The results reveal that *O. vulgare* essential oil was efficiently encapsulated, and no notable changes were observed among all liposomal formulations. Liposomes prepared with Lipoid S100 had the highest encapsulation efficiency among the formulations, followed by Phospholipon 90H and Phospholipon 85G liposomes. These findings show that encapsulating *O. vulgare* essential oil into prepared liposomes resulted in high levels of encapsulation efficiency, regardless of the saturated or unsaturated composition of the phospholipids. These results might potentially be due to the addition of cholesterol during the preparation procedure, as cholesterol reduces membrane permeability, favoring encapsulation. Similarly, additional investigations have found that the nanocarrier membrane components interact with the hydroxyl groups contained within essential oils, increasing the encapsulation efficiency (Aguilar-Perez et al. 2021; Nasr et al. 2021).

Regarding the antioxidant activity, the results of the present study show that free *O. vulgare* essential oil exerted a good DPPH free radical scavenging activity. Earlier reported studies have shown that oregano essential oil containing carvacrol, thymol, and *p*-cymene as the primary constituents have significant antioxidant activity due to the fact that these components form chemical complexes with metal ions and free radicals (Lombrea et al. 2020; Coccimiglio et al. 2016). Moreover, other monoterpenes, and especially oxygenated ones, can also exhibit significant antioxidant activities, suggesting that this activity is related to synergism among several essential oil components (Radünz et al. 2021; Kosakowska et al. 2021).

Encapsulation of *O. vulgare* essential oil into liposomes resulted in the maintenance and increase of DPPH scavenging activity for *O. vulgare* essential oil. The results of this study indicate that Lipoid S100 formulations significantly enhanced the antioxidant activity of *O. vulgare* essential oil, while Phospholipon 85G and Phospholipon 90H liposomes were able to maintain the antioxidant activity. These results are in agreement with those from previous studies which confirmed that liposomal formulations preserve and enhance the antioxidant activity of the essential oil (Sebaaly et al. 2015; Risaliti et al. 2019). According to earlier findings, antioxidant activity is improved by the liposomes' membrane-antioxidant complex, which depends on the particle size, zeta potential, and the overall composition of the essential oil (Nasr et al. 2021).

Biological activities of essential oil are affected by the factors influencing the oil's chemical composition (Chahla et al. 2021); therefore, these factors should be considered when determining the biological properties of plants harvested in different countries. Thus, the results cannot be generalized and must be determined for each essential oil individually.

To date, this is the first study to evaluate the cytotoxic activity of essential oil extracted from wildcrafted *O. vulgare* from Albania on the MCF-7 cancer cell lines. According to our findings, this *O. vulgare* essential oil effectively reduced the cell viability of MCF-7 cancer cell lines.

The cytotoxicity of *O. vulgare* essential oil may be attributed to the main constituents (carvacrol, *p*-cymene, γ-terpinene, and thymol). Oregano essential oil and its main components have been reported to show cytotoxic activities in different human breast cancer cell lines (Pérez-González et al. 2019; Coccimiglio et al. 2016; Elshafie et al. 2017). Carvacrol, a key component of *O. vulgare* essential oil, may have a considerable impact on the oil's cytotoxic activity. The anticancer properties of carvacrol have been widely evaluated (Rojo-Ruvalcaba et al. 2020; Sampaio et al. 2021). Unlike these findings, the essential oil of *O. compactum* (with carvacrol, *p*-cymene, and γ-terpinene as main components) was shown to be non-toxic against MCF-7 cancer cell lines (Babili et al. 2011; Leyva-López et al. 2017). Moreover Hussain et al. (2011) reported that *O. majorana* (with terpinen-4-ol as the main component and no carvacrol) was shown to be more cytotoxic against MCF-7 cancer cell lines than *O. vulgare* (with carvacrol as the main component). These findings suggest that the cytotoxicity of essential oils is not always related to their primary active constituents. Therefore, the synergistic effects of several components, including those found in trace amounts, may contribute to the overall cytotoxicity of the essential oil.

These data show that the genus *Origanum* is a major source of bioactive compounds with cytotoxic potential against breast and colon malignancies (Leyva-López et al. 2017; Pérez-González et al. 2019).

The lipid-soluble nature and low molecular weights of solitary components of the investigated oregano essential oil may clarify the capacity of these compounds to move rapidly through the cell membrane and affect the lipid membrane layers, resulting in higher cell membrane fluidity and, ultimately, the destruction of the cytoplasmic content (Jerônimo et al. 2021; Leyva-López et al. 2017; Coccimiglio et al. 2016; Elshafie et. 2017).

In this study, the cytotoxic effect of nanoencapsulated *O. vulgare* essential oil was further evaluated. The incorporation of *O. vulgare* essential oil into liposomes resulted in changes in MCF-7 breast cell line viability. According to our knowledge, the cytotoxic activity of the different liposomal formulations of *O. vulgare* essential oils on the MCF-7 cancer cell lines has not been previously studied. As a result, an evaluation of the cytotoxic action of several liposomal preparations of the same essential oil is crucial. *Origanum vulgare* essential-oil-loaded Phospholipon 90H liposomes exerted the highest impact on MCF-7 cell lines, with a significant decrease in cell viability observed when compared to the effects of the free essential oil. With regard to the cytotoxicity toward MCF-7 cell lines, no significant differences between free *O. vulgare* essential oil and *O. vulgare* essential-oil-loaded Lipoid S100 liposomes were observed, whereas essential-oil-loaded Phospholipon 85G liposomes exhibited the lowest cytotoxic activity.

One possible explanation for the observed enhancement of anticancer efficacy with the liposomal delivery system is the increased intracellular uptake of the liposomes in comparison to the essential oil. Studies have previously reported that, in comparison to free essential oil, liposomes have a larger and distinct intracellular absorption rate, which might indicate the enhancement of cytotoxic activity. Utilizing liposomes to deliver a compound can be seen in various studies as a means of increasing intracellular accumulation (Celia et al. 2013; Elshafie et al. 2017). Furthermore, the differences observed among the three liposomal formulations may be attributed to the physical and chemical instability of liposomes containing unsaturated components, which can reduce the liposome efficacy (Nakhaei et al. 2021).

Based on the findings of this study, the essential oil of *O. vulgare* might be considered an antioxidant and cytotoxic candidate for future study as a natural food preservative or for use in the pharmaceutical industry. Along with its antioxidant and cytotoxic effects, *O. vulgare* oil may be used as a valuable source of carvacrol, as the plant is normally easily accessible and can be cultivated.

After liposomal encapsulation, the antioxidant and activities were maintained and improved, thus highlighting the importance of liposomal carriers for the future development of *O. vulgare* as an anticancer agent. These findings suggest that liposomes containing *O. vulgare* essential oil might have a promising therapeutic potential as a cancer treatment strategy. Moreover, the study findings suggest the need and importance for further *in vivo* studies to ascertain the cytotoxic activities of free and nanocarrier-loaded *O. vulgare* essential oil in order to define safe dosage levels and to determine the mechanism of action, which is crucial for future pharmaceutical applications.

4. Experimental

4.1. Materials

Lipoid S100 (94% soybean phosphatidylcholine, 3% lysophosphatidylcholine, and residual solvents), Phospholipon 85G (85% soybean phosphatidylcholine, 3.3% lysophosphatidylcholine, and residual solvents) and Phospholipon 90H (90% hydrogenated soybean phosphatidylcholine, 4% lysophosphatidylcholine, 2% triglycerides, and residual solvents) were supplied by Lipoid GmbH, Germany. 2,2-diphenyl-1-picrylhydrazyl (DPPH), hexane, absolute ethanol (HPLC grade), (-)-cholesterol 95% were obtained from Sigma-Aldrich, Germany. The dialysis membrane ZelluTrans/ROTH T1 (MWCO: 3500) was acquired from Zellu Carl Roth GmbH+Co, Germany). MCF-7 human breast cancer cell lines were obtained from the American Type Culture Collection (ATCC).

4.2. Plant material and essential oil extraction

Plant material of *O. vulgare* was collected from the northern zone of Albania. The plant material was identified by Professor Skerdilaid Xhulaj, University of Tirana, and voucher specimens were deposited at the herbarium of the University of Tirana. The plants were dried at room temperature. The air-dried plant samples were subjected to hydrodistillation using a Clevenger-type apparatus and following this procedure: 20 g of the plant material subjected to hydrodistillation with 500 ml of distilled water for 3 h in order to obtain essential oil at a rate of 2-3 ml/min. The resulting essential oil was dried over anhydrous sodium sulfate and stored at 4 °C as described by Papajani et al. (2015).

4.3. Gas chromatography/mass spectrometry assay

The chemical composition of the essential oils was analyzed on the Agilent 7890A Gas Chromatography system equipped with an FID detector as well as the Agilent 5975C Mass Quadrupole detector. For that purpose, an HP-5 ms capillary column (30 m × 0.25 mm, film thickness 0.25 µm) was used. The analytical conditions were as follows: initial oven temperature 60 °C (0 min) increased at a rate of 3 °C/min to 240 °C (1 min) and then on to 280 °C at a rate of 10 °C/min (1 min); helium as carrier gas at a flow rate of 1 ml/min; injector temperature 220 °C and that of the FID detector, 270 °C. Each sample was injected at a split ratio of 1:1.
MS conditions: Ionization voltage was 70 eV, ion source temperature was 230 °C, transfer line temperature was 280 °C, and the mass range was 50–550 Da. Scan mode was used when operating MS. The compounds were identified utilizing a combination of homologous series of normal alkanes (C9–C25) examined under Automated Mass Spectral Deconvolution and Identification System (AMDIS) conditions, as well as calculated Kovats (retention) indices from the literature (Adams 2007). Mass spectra of each ingredient were compared to those stored in the Wiley, Adams, and NIST databases, as well as mass spectra from the literature, to confirm their identification. The normalization approach of the GC/FID peak areas was used to quantify the components without correction factors.

4.4. Preparation of the liposomes

The ethanol injection technique was used to prepare blank and *O. vulgare* essential-oil-loaded liposomes as described by Sebaaly et al. (2015) with minor changes. The appropriate amounts of Phospholipon 85G, Lipoid S100, and Phospholipon 90H (10 mg/ml), cholesterol (5 mg/ml), and *O. vulgare* essential oil (2.5 mg/ml) were dispersed in absolute ethanol (10 ml) by stirring with a magnetic bar. The attained organic phase was then injected using a syringe pump (LA-100, Landgraf Laborsysteme GmbH, Germany) into a proper volume of Milli-Q water (20 ml) at a flow rate of 1 ml/min. Liposomes formed spontaneously when the alcoholic solution came into contact with the water phase. Afterward, the liposomal suspension was kept at room temperature for 15 min while stirring. The ethanol was then removed using dialysis membranes (ZelluTrans/ROTH T1, Germany), which were suspended at a release volume of 1000 ml and were stirred at 200 rpm for 16 h. Blank liposomes were considered as the batches that did not contain *O. vulgare* essential oil. All batches were prepared three times. Before further analysis was performed, all liposome batches were stored at 4 °C.

4.5. Characterization of the liposomes

4.5.1. Particle size, PDI, and zeta potential analysis

Origanum vulgare essential-oil-loaded liposomes were studied for particle size, PDI, zeta potential, encapsulation efficiency, and SEM morphology. In this study, a Malvern Zetasizer Nano ZS Model: ZEN3500 (Malvern, UK) was used to determine the mean particle size, PDI, and zeta potential of blank and *O. vulgare* essential-oil-loaded liposomes. The prepared samples were analyzed without dilution or filtration step. The means ± SD (n = 3) were used to express the data.
Zeta potential (ZP) is another vital factor that predicts the stability of the nanocarrier through the determination of its overall electrostatic behavior. ZP was assessed by applying Smoluchowski's equation. One ml of nanocarrier was placed in the folded capillary cell, and the ZP was measured. All analyzes were performed in triplicate at 25 °C after a 2-min equilibration time.

4.5.2. Encapsulation efficiency

The encapsulation efficiency was determined by applying the technique described by Natrajan et al (2015), with minor modifications. The liposomal suspension (2 ml) was added to hexane (2 ml), and the mixture was vortexed. Following that, a 30-min centrifugation step at 4,000 rpm was carried out. The supernatant was then collected, and the amount of EO was quantified using the Shimadzu UV 1800, UV-Vis spectrophotometer, at λ = 306 nm. The experiment was performed in triplicate, and the concentration of essential oil was defined by determining the standard curve obtained with various concentrations of *O. vulgare* essential oil diluted in hexane. The encapsulation efficiency was expressed as a percentage and calculated as follows:
EE% = (Total oil − Free oil) * 100 / (Total oil)

4.5.3. Scanning Electron Microscopy (SEM)

Morphology of vesicles was visualized using a scanning electron microscope (SEM) Carl Zeiss 300. The VP analysis was performed at an accelerating voltage of 25 kV. The samples were dropped on lamellar and dried at room temperature for 72 h. Before visualization, all the samples were coated with Au–Pd for 30 seconds to increase the conductivity via QUORUM Q150 RES.

4.6. Evaluation of biological activities

4.6.1. DPPH scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH)-scavenging activities of free *O. vulgare* essential oil and *O. vulgare* essential-oil-loaded liposomes were determined according to a previously described method with minor modifications (Seblaay et al. 2015; Brand-Williams et al. 1995). A 1-ml aliquot of each sample (*O. vulgare* essential oil or *O. vulgare* essential-oil-loaded liposome, 500 µg/ml) was mixed with 2 ml of DPPH ethanolic solution (0.125 mM). Mixtures were incubated at 25 °C in the dark for 10 min and then centrifuged at 15,000 rpm for 15 min. The supernatant absorption was measured at 517 nm with an 1800 UV-Vis spectrophotometer (Shimadzu Company, Japan). The blank sample of *O. vulgare* essential-oil-loaded liposomes was prepared by mixing 1 ml of blank liposomes and 2 ml of DPPH ethanolic solution. All measurements were performed in triplicate, and the results were expressed as mean value ± SD.
The DPPH scavenging activity percentage was calculated as follows:
Scavenging activity (%) = Sample absorbance value / Control absorbance value × 100

4.6.2. MTT cytotoxic activity

MCF-7 lines used in the experiments were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured and passaged in a sterile culture room and a laminar cabinet. Cells were inoculated into flasks in EMEM: DMEM: HAMS F12 nutrient medium containing 5% newborn bovine serum (FBS), 100 IU/ml penicillin, 10 mg/ml streptomycin, and 1% L-glutamine, and inoculated into flasks at 37 °C at 95% humidity. These flasks were placed in an incubator containing 5% CO_2. Cells proliferating and adhering to the flask base were passaged three days apart. During the passage process, the medium in the flask was removed, and trypsin-EDTA at 37 °C was added to it; this was then kept for 5 min for the cells to rise up from the flask base. The cell and trypsin mixture that separated from the base was transferred into 15 ml centrifuge tubes and centrifuged at 2,500 g for 2.5 min. After that, the supernatant was removed, and the cells remaining at the bottom were mixed with the medium and then inoculated into new flasks. MCF-7 cells were seeded in 96-well plates (180 µl per well). Cells seeded on plates were incubated for 24 h at 37 °C in an Incubator and kept in a 5% CO_2 atmosphere. At the end of the incubation period, 20 µl of *O. vulgare* essential oil was tested at ten different concentration levels to obtain a final concentration between 0.39–200 µg/ml, and the IC_{50} was calculated. Separately, 20 µl of Phospholipon 90H, Phospholipon 85G, and Lipoid S100 liposomes were administered to the cells. After a 24-h incubation period, MTT solutions (20 µl/200 µl per well of a 5-mg/ml solution) were pipetted in each well, and the plates were incubated for 4 h at 37 °C. At the end of the 24 h application period, 20 µl of the MTT solution prepared at 5 mg/ml was added to each well. It was incubated for 2 h at 37 °C in an incubator with 5% CO_2. Finally, the medium plate on the cells in the wells was removed by inversion. 200 µl of DMSO was added to each well of the plate. The optical densities of the cells in the wells were read at λ = 492 nm in a microplate reader. According to the determined absorbance values, the % cell viability and the dose (LD_{50}) at which 50% of the cells were alive were calculated according to the following formula:
% Cell viability: Sample absorbance value / control absorbance value * 100
For each analysis, the untreated control was normalized to 100%, and the treatments were expressed as the percentage of the control.
The entire cytotoxicity assay was carried out in triplicate on separate days. Results are expressed as the mean value ± SD.

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References

Adams RP (2007) Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry, 4th ed., USA, p. 9-31.

Aguilar-Pérez KM, Medina DI, Narayanan J, Parra-Saldívar R, Iqbal H (2021) Synthesis and nano-sized characterization of bioactive Oregano essential oil molecule-loaded small unilamellar nanoliposomes with antifungal potentialities. Molecules 26: 2880.

Ali H, Al-Khalifa AR, Aouf A, Boukhebti H, Farouk A (2020) Effect of nanoencapsulation on volatile constituents, and antioxidant and anticancer activities of Algerian Origanum glandulosum Desf. essential oil. Sci Rep 10: 1–9.

Babili FE, Bouajila J, Souchard JP, Bertrand C, Bellvert F, Fouraste I, Moulis C, Valentin A (2011) Oregano: chemical analysis and evaluation of its antimalarial, antioxidant, and cytotoxic activities. J Food Sci 76: 512–518.

Basholli-Salihu M, Schuster R, Hajdari A, Mulla D, Viernstein H, Mustafa B, Mueller M (2017) Phytochemical composition, anti-inflammatory activity and cytotoxic effects of essential oils from three Pinus spp. Pharm Biol 55: 1553–1560.

Béjaoui A, Chaabane H, Jemli M, Boulila A, Boussaid M (2013) Essential oil composition and antibacterial activity of Origanum vulgare subsp. glandulosum Desf. at different phenological stages. J Med Food 12: 1115–1120.

Beltrán-Gracia E, López-Camacho A, Higuera-Ciapara I, Velázquez-Fernández JB, Vallejo-Cardona AA (2019) Nanomedicine review: clinical developments in liposomal applications. Cancer Nanotechnol 10: 1–40.

Brand-Williams W, Cuvelier ME, Berset CL (1995) Use of a free radical method to evaluate antioxidant activity. LWT--Food Sci. Technol 28: 25–30.

Buyel JF (2018) Plants as sources of natural and recombinant anti-cancer agents. Biotechnol. Adv 36: 506–520.

Celia C, Trapasso E, Locatelli M, Navarra M, Ventura CA, Wolfram J, Carafa M, Morittu VM, Britti D, Di Marzio L, Paolino D (2013) Anticancer activity of lipo-

somal bergamot essential oil (BEO) on human neuroblastoma cells. Colloids Surf B Biointerfaces 112: 548–553.
Chahla B, Salih BM, Adriana B, Viviana M, Guido F, Sergio S, Federica C, Rosaria N, Marina P, Abdelmounaim K, Asma Z (2021) Chemical composition and biological activities of Oregano and Lavender essential oils. Appl. Sci 12: 5688.
Cimino C, Maurel OM, Musumeci T, Bonaccorso A, Drago F, Souto EM, Pignatello R, Carbone C (2021) Essential oils: pharmaceutical applications and encapsulation strategies into lipid-based delivery systems. Pharmaceutics 13: 327.
Coccimiglio J, Alipour M, Jiang ZH, Gottardo C, Suntres Z (2016) Antioxidant, antibacterial, and cytotoxic activities of the ethanolic Origanum vulgare extract and its major constituents. Oxid Med Cell Longev 2016.
Donsì F, Ferrari G (2016) Essential oil nanoemulsions as antimicrobial agents in food. J Biotechnol 233: 106–120.
Elshafie HS, Armentano MF, Carmosino M, Bufo SA, De Feo V, Camele I (2017) Cytotoxic activity of Origanum vulgare L. on hepatocellular carcinoma cell line HepG2 and evaluation of its biological activity. Molecules 22: 1435.
Faraji Z, Shakarami J, Varshosaz J, Jafari S (2020) Encapsulation of essential oils of Mentha pulegium and Ferula gummosa using nanoliposome technology as a safe botanical pesticide. J. Appl. Biotechnol. Rep 7: 237–242.
Fitsiou E, Anestopoulos I, Chlichlia K, Galanis A, Kourkoutas I, Panayiotidis MI, Pappa A (2016) Antioxidant and antiproliferative properties of the essential oils of Satureja thymbra and Satureja parnassica and their major constituents. Anticancer Res 36: 5757–5763.
Gezici S, Şekeroğlu N (2019) Current perspectives in the application of medicinal plants against cancer: novel therapeutic agents. Anticancer Agents Med Chem 19: 101–111.
Goldberg MS (2019) Improving cancer immunotherapy through nanotechnology. Nat Rev Cancer 19: 587–602.
Hammoud Z, Gharib R, Fourmentin S, Elaissari A, Greige-Gerges H (2019) New findings on the incorporation of essential oil components into liposomes composed of lipoid S100 and cholesterol. Int J Pharm 561: 161–170.
Huang XL, Li XJ, Qin QF, Li YS, Zhang WK, Tang HB (2019) Anti-inflammatory and antinociceptive effects of active ingredients in the essential oils from Gynura procumbens, a traditional medicine and a new and popular food material. J Ethnopharmacol 239: 111916.
Hussain AI, Anwar F, Rasheed S, Nigam PS, Janneh O, Sarker SD (2011) Composition, antioxidant and chemotherapeutic properties of the essential oils from two Origanum species growing in Pakistan. Rev. Bras. Farmacogn 21: 943–952.
Iqbal J, Abbasi BA, Mahmood T, Kanwal S, Ali B, Shah SA, Khalil AT (2017) Plant-derived anticancer agents: A green anticancer approach. Asian Pac J Trop Biomed 7: 1129–1150.
Jerônimo LB, da Costa JS, Pinto LC, Montenegro RC, Setzer WN, Mourão RH, da Silva JK, Maia JG, Figueiredo PL (2021) Antioxidant and cytotoxic activities of Myrtaceae essential oils rich in terpenoids from Brazil. Nat. Prod. Commun 16: 1934578X21996156.
Khan T, Gurav P (2018) PhytoNanotechnology: Enhancing delivery of plant based anti-cancer drugs. Front Pharmacol 8: 1002–1020.
Khan M, Khan ST, Khan M, Mousa AA, Mahmood A, Alkhathlan HZ (2019) Chemical diversity in leaf and stem essential oils of Origanum vulgare L. and their effects on microbicidal activities. AMB Express 9: 1–15.
Kosakowska O, Węglarz Z, Pióro-Jabrucka E, Przybył JL, Kraśniewska K, Gniewosz M, Bączek K (2021) Antioxidant and antibacterial activity of essential oils and hydroethanolic extracts of Greek oregano (O. vulgare L. subsp. hirtum (Link) Ietswaart) and common oregano (O. vulgare L. subsp. vulgare). Molecules 26: 988.
Kyriakoudi A, Spanidi E, Mourtzinos I, Gardikis K (2021) Innovative delivery systems loaded with plant bioactive ingredients: Formulation Approaches and Applications. Plants 10: 1238.
Laothaweerungsawat N, Sirithunyalug J, Chaiyana W (2020) Chemical compositions and anti-skin-ageing activities of Origanum vulgare L. essential oil from tropical and mediterranean region. Molecules 25: 1101.
Leyva-López N, Gutiérrez-Grijalva EP, Vazquez-Olivo G, Heredia JB (2017) Essential oils of oregano: Biological activity beyond their antimicrobial properties. Molecules 22: 989.
Li FS, Weng JK (2017) Demystifying traditional herbal medicine with modern approach. Nat Plants 3: 1-7.
Lombrea A, Antal D, Ardelean F, Avram S, Pavel IZ, Vlaia L, Mut AM, Diaconeasa Z, Dehelean CA, Soica C, Danciu C (2020) A recent insight regarding the phytochemistry and bioactivity of Origanum vulgare L. essential oil. Int J Mol Sci 24: 9653.
Mancini E, Camele I, Elshafie HS, De Martino L, Pellegrino C, Grulova D, De Feo V (2014) Chemical composition and biological activity of the essential oil of Origanum vulgare ssp. hirtum from different areas in the Southern Apennines (Italy). Chem Biodivers 11: 639–651.
Morshedloo MR, Mumivand H, Craker LE, Maggi F (2018) Chemical composition and antioxidant activity of essential oils in Origanum vulgare subsp. gracile at different phenological stages and plant parts. J. Food Process. Preserv 42: e13516.
Nagaraju PG, Sengupta P, Chicgovinda PP, Rao PJ (2021) Nanoencapsulation of clove oil and study of physicochemical properties, cytotoxic, hemolytic, and antioxidant activities. J. Food Process Eng 44: e13645.
Natrajan D, Srinivasan S, Sundar K, Ravindran A (2015) Formulation of essential oil-loaded chitosan–alginate nanocapsules. J Food Drug Anal 23: 560–568.
Nasr G, Greige-Gerges H, Elaissari A, Khreich N (2021) Liposome Permeability to Essential Oil Components: A Focus on Cholesterol Content. J Membr Biol: 1–15.
Papajani V, Haloci E, Goci E, Shkreli R, Manfredini S (2015) Evaluation of antifungal activity of Origanum vulgare and Rosmarinus officinalis essential oil before and after inclusion in β-cyclodextrine. Int J Pharm Pharm Sci 7: 270–273.
Pérez-González C, Pérez-Ramos J, Méndez-Cuesta CA, Serrano-Vega R, Martell-Mendoza M, Pérez-Gutiérrez S (2019) Cytotoxic activity of essential oils of some species from Lamiaceae family. In: Istifli ES (ed.) Cytotoxicity-Definition, Identification, and Cytotoxic Compounds, 1st ed., United Kingdom, p. 29-44.
Radünz M, Camargo TM, dos Santos Hackbart HC, Alves PI, Radünz AL, Gandra EA, da Rosa Zavareze E (2021) Chemical composition and in vitro antioxidant and antihyperglycemic activities of clove, thyme, oregano, and sweet orange essential oils. LWT--Food Sci. Technol 138: 110632.
Risaliti L, Kehagia A, Daoultzi E, Lazari D, Bergonzi MC, Vergkizi-Nikolakaki S, Hadjipavlou-Litina D, Bilia AR (2019) Liposomes loaded with Salvia triloba and Rosmarinus officinalis essential oils: In vitro assessment of antioxidant, antiinflammatory and antibacterial activities. J. Drug Delivery Sci. Techno 51: 493–498.
Rojo-Ruvalcaba BE, García-Cobián TA, Pascoe-González S, Campos-Bayardo TI, Guzmán-García LM, Gil-Gálvez MC, Escobar-Millán Z, Huerta-García E, García-Iglesias T (2020) Dose-dependent cytotoxicity of the Origanum vulgare and carvacrol on triple negative breast cancer cell line. MDPI Proceedings 61: 6.
Sampaio LA, Pina LT, Serafini MR, dos Santos Tavares D, Guimarães AG (2021) Antitumor effects of carvacrol and thymol: a systematic review. Front Pharmacol 12. doi: 10.3389/fphar.2021.702487.
Sebaaly C, Jraij A, Fessi H, Charcosset C, Greige-Gerges H (2015) Preparation and characterization of clove essential oil-loaded liposomes. Food Chem 178: 52-62.
Shafiee-Hajiabad M, Hardt M, Honermeier B (2014) Comparative investigation about the trichome morphology of Common oregano (Origanum vulgare L. subsp. vulgare) and Greek oregano (Origanum vulgare L. subsp. hirtum). J Appl Res Med Aromat Plants 2: 50–58.
Wang J, Zhang HH, Liu F, Zhang YP, Zhao YM (2019) Preparation, characterization and antimicrobial activity of inclusion complexes of Myristica fragrans Hott. essential oil in β-cyclodextrins. Pharmazie 74: 590–594.
Yan W, Leung SS, To KK (2020) Updates on the use of liposomes for active tumor targeting in cancer therapy. Nanomedicine 15: 303–318.