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Cytotoxic activity of liposomal *Thymus capitatus* essential oil on HT-29 human colorectal cancer cell line

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Multidrug resistance, severe side effects, and high cancer treatment costs are still well-known issues and remain an open challenge. These factors reduce the therapy's efficiency and safety, seriously affecting human health. Developing therapeutic approaches based on plant extracts, especially based on essential oils with cytotoxic and antioxidant properties, could be of efficacious strategies. This work incorporated *Thymus capitatus* essential oil (TEO) in liposomes. *Thymus capitatus* is a plant native to the northern region of Albania and found specifically in the Mediterranean region. TEO has several biological activities and cytotoxic properties. Due to its volatility, poor solubility, and chemical instability, however, its applicability is restricted. Incorporation into liposomes enables its effective use because the exposure time to the active compounds can be extended, increasing its efficacy against colorectal cancer cell lines, as highlighted in *in vitro* studies. TEO demonstrated detectable cytotoxic action against HT-29 colorectal cancer cells, and this action could be enhanced by applying various formulations of TEO-loaded liposomes to this cell line. Among the tested nanosystems, TEO-Phospholipon 90H liposomes showed more significant cytotoxic effects than TEO-Lipoid S100 liposomes and TEO-Phospholipon 85G liposomes. TEO-Phospholipon 90 H liposomes also maintained its physicochemical stability for six months at 25 °C. This research suggests that TEO, particularly when encapsulated in TEO-Phospholipon 90 H liposomes, may offer a promising therapeutic approach. However, these findings are based on *in vitro* studies and further *in vivo* research is needed to validate the efficacy and safety of this approach in clinical settings.

1. Introduction

Colorectal cancer is among the most common forms of cancer, and it has one of the highest incidence rates worldwide. According to the WHO, more than 1.93 million cases of this cancer were reported in 2020 (WHO 2022). The chance of having this type of cancer increases with age and typically affects adults over 50 years of age. Over 330,000 cases in the over-45 age group were registered in Europe in 2020. People aged 20–44 presented a lower number of cases (i.e., 6,880 cases). Colorectal cancer is the second most common cause morbidity from cancer in males and the third most common cause in women (World Cancer Research Fund International 2023). According to the literature, the predisposition for developing this cancer can be up to 35% due to genetic factors. Moreover, 50% of patients treated with current therapies face the possibility of a recurrence of the pathology (Anitha et al. 2016).

Current cancer therapies have several drawbacks, including multidrug resistance, environmental impacts, high production costs, and severe side effects (American Cancer Society 2023). These drawbacks necessitate developing alternative or complementary therapeutic strategies, focusing on effective active compounds and safer, cost-effective delivery systems like liposomes for patients. If thoroughly researched, these may also be competitive in terms of therapeutic performance (Sebaaly et al. 2015; Bolouri et al. 2022). Among the therapeutic strategies currently available, the development of therapeutic approaches employing herbal products is auspicious (Bolouri et al. 2022). The mechanism of action of many therapeutic agents is derived from studying components found in various medicinal plants, and the therapeutic potential of these agents has a long history of documentation. Therefore, researchers have attempted to identify new cancer therapeutic approaches based on natural sources (Khan et al. 2017; Buyel 2018; Gezici and Şekeroğlu 2019).

Essential oils (EOs) extracted from different plant organs are complex mixtures of natural compounds that possess various biological activities. These EOs are mixtures of monoterpenes and sesquiterpenes. Essential oils generally comprise 20–60 natural products; the type and concentration of these components vary depending on the plant species, growth conditions, geographic location, harvest time, and climate. The phytochemical composition of these oils is critical because it determines their biological action. Although research on EOs for anticancer therapeutic purposes is still in its early stages, approximately half of the traditional chemotherapeutic agents are of plant origin, with approximately 25% derived directly from plants and 25% represented by chemically modified versions of plant products (Sebaaly et al. 2015; Jampilek and Kralova 2022; Bolouri et al. 2022).

Thymus genus essential oils are known for their bioactive compounds. Despite extensive research on their biological effects, further studies are needed to fully understand the potential of EOs from this genus (Arrais et al. 2023). EOs extracted from plants are showing, for example, cytotoxic, antioxidant, antimicrobial, antifungal, antiseptic, and anti-inflammatory properties. The cytotoxic and antioxidant activities can exert significant therapeutic and economic benefits (Pérez-González et al. 2019; Jabraeili et al. 2022). Moreover, they can be used to preserve food and have pleasant aroma. This makes them appealing subjects for scientific

studies in medical and food chemistry research. These biological activities are thought to be derived from the EOs diverse composition, where each compound can influence the type and strength of the biological action (Ramos da Silva et al. 2021).

Thymus capitatus, commonly referred to as conehead thyme or Persian hyssop, is a native plant of the Mediterranean and is part of the *Lamiaceae* family. Traditionally, *T. capitatus* has been employed for an array of health issues such as respiratory problems, cancer, skin conditions, diabetes, and heart and digestive ailments. It is also known for its antimicrobial, antiseptic, pain-relieving, and calming properties (Maniki et al. 2023).

Extensive studies on the phytochemical composition of *T. capitatus* essential oil (TEO) have revealed that it contains a variety of bioactive substances including flavonoids, terpenoids (particularly, monoterpenes and sesquiterpenes), and phenolic acids. These compounds have the ability to interact with biological systems in various ways, which could enhance the oils potential to combat against cancer cells. Thymol and carvacrol, in particular, stand out as significant contributors to the medicinal advantages of the plant (Nasr et al. 2021; Maniki et al. 2023).

The therapeutic efficacy of TEO appears to be influenced by a combination of its constituents rather than a single compound. These compounds individually exhibit antioxidant, antimicrobial, and cytotoxic properties. However, the overall efficacy of the essential oil is likely due to the synergistic effects main and other minor components. This synergy enhances the oil's biological properties, making it more effective than its individual compounds alone. This phenomenon is common in natural products, where the combined action of various compounds provides a more potent therapeutic effect than any single compound (Džamić et al. 2015; Pérez-González et al. 2019).

Moreover, according to previous findings, the cytotoxic activity of EOs is not conditionally directly related to the components that have the highest concentrations. The phytochemical components of the EOs interact with cells at different concentrations. As a result, even trace components may play a role in the overall cytotoxic activity of EOs (Pérez-González et al. 2019; Hassan et al. 2019; Blažíčková et al. 2022).

Since the phytochemical composition and biological activity of EOs differs depending on how the plant has grown (wild or cultivated), its geographical location, and the harvest time, research data cannot be generalized. Each EO should be studied individually. Unfortunately, the use of EOs is generally limited due to their low stability, low aqueous solubility, low bioavailability, and sensitivity to heat, light, oxygen, and moisture (Pérez-González et al. 2019; Ramos da Silva et al. 2021; Kryeziu et al. 2022). As a result, these factors have the potential to limit potential therapeutic applications of EOs (Ramos da Silva et al. 2021).

Encapsulating EOs in nanosystems is a successful strategy to overcome these limitations. Nanosystems that affect or improve the physicochemical properties and the biological activities (e.g., bioavailability) are increasingly being used to encapsulate various biomolecules, and this trend is expected to continue (Ramos da Silva et al. 2021).

Liposomes are nanocarriers that can be used for encapsulation purposes and the controlled transport of various biocompounds in the human body, but are non-toxic, non-allergic, and biodegradable. Furthermore, they are cost effective, can improve the oil's bioavailability, and effectively transport anticancer agents into tumor cells (Perumalsamy et al. 2022). Liposomes are known to modify the physicochemical and biological characteristics of various natural substances. Additionally, they enhance the physical and biological properties, bioavailability, and stability of essential oils (Sebaaly et al. 2015; Hammoud et al. 2019; Kyriakoudi et al. 2021).

A common method for encapsulating essential oils into liposomes is the ethanol injection technique, favored for its simplicity, affordability, and speed (Gouda et al. 2021). This method also avoids the use of harmful solvents and intense forces, which preserves the integrity of both the liposomes and the encapsulated molecules. The efficacy of the ethanol injection method can be modulated by adjusting factors such as phospholipid concentration, cholesterol concentration, and the ethanol to water ratio. These adjustments can be made to optimize the production of small, uniform liposomes (Sebaaly et al. 2015; Hammoud et al. 2021).

Scientists have investigated these novel LS-based formulations in various biological models, including human cancer cell lines, increasingly in recent years. However, more research is needed to further clarify the biological activity of the oils encapsulated in liposomes. The liposome particle size, Polydispersity Index (PDI), surface charge, morphology, and Encapsulation Efficiency (EE%) are all crucial parameters that need to be investigated when studying the efficiency and stability of EOs contained in LS (Jabraeili et al. 2022).

Although preliminary research has shown that nanodelivery systems can maintain or improve EO bioactivity, a comprehensive study has not yet been carried out to investigate the impact of different LS formulations on the cytotoxic and antioxidant activity of EOs from the *Thymus* genus or specifically *T. capitatus*.

This study is the first to evaluate the cytotoxic effects of nanoencapsulated TEO, extracted from wild plants in northern Albania, on the HT-29 human colorectal cancer cell line. The HT29 cell line is widely used because of its physiological relevance to human colorectal cancer, its sensitivity to therapeutic interventions, and the comprehensive pre-existing research data available for reference (Pérez-González et al. 2019; Hassan et al. 2019; ATCC® 2022).

The main goal of this research was to develop liposomes that could be used for the delivery of TEO to preserve and enhance its biological activities (i.e., cytotoxic and antioxidant activities) while improving the physicochemical properties (i.e., stability) as a successful therapeutic approach. For this reason, TEO was encapsulated in three different liposomal (LS) nanocarriers (TEO-Lipoid S100 LS, TEO-Ph 85G LS, and TEO-Ph 90H LS) to investigate the effect of these nanosystems on the DPPH (2,2-diphenyl-1-picrylhydrazyl)-scavenging activity of TEO, and the cytotoxic activity was studied in HT-29 human colorectal cancer cells by using an MTT assay.

2. Investigations, results and discussion

2.1. Essential oil composition

The average EO yield for TEO samples was 1.9±0.05%. The EO phytochemical composition varies depending on plant growth conditions, geographical region, climate, genetic variations, extraction method, and plant parts used, all factors that affect their biological properties. Therefore, the essential oil's phytochemical composition and biological activity cannot be generalized and must be determined for each one individually (Hussain et al. 2013; Papajani et al. 2015; Sarrou et al. 2017; Pérez-González et al. 2019).

In this work, the phytochemical composition and chromatogram of TEO was determined by GC/FID/MS, and the results are presented in Table 1 and Fig. 1. Thymol (74.50%), *p*-cymene

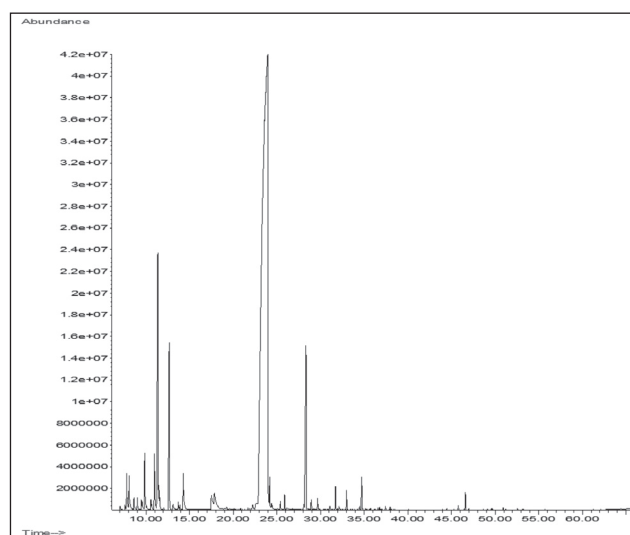

Fig. 1: GC/FID/MS chromatogram of ***T. capitatus*** essential oil.

Table 1: Chemical composition of *T. capitatus* essential oil.

No.	RT[a] (min)	KIL[b]	Compound	%[c]
1	7.79	924	β-pinene	0.19
2	8.07	932	α-terpinene	1.09
3	8.63	946	camphene	0.24
4	9.57	974	γ-terpinene	3.18
5	9.85	988	myrcene	1.1
6	10.56	1002	α-phellandrene	0.27
7	10.97	1014	α-thujene	0.64
8	11.35	1020	*p*-cymene	6.19
9	12.6	1054	thymol	74.5
10	13.1	1065	cis-sabinene hydrate	0.17
11	13.7	1086	terpinolene	0.13
12	14.28	1095	linalool	0.9
13	17.5	1165	borneol	0.66
14	17.85	1174	terpinen-4-ol	1.05
15	19.25	1186	α-terpineol	1.05
16	22.23	1239	carvone	0.21
17	23.93	1289	α-pinene	0.69
18	24.38	1356	eugenol	0.16
19	25.88	1370	carvacrol acetate	0.12
20	28.31	1417	trans-caryophyllene	4.02
21	29.67	1452	α-humulene	0.18
22	31.72	1505	β-bisabolene	0.33
23	32.99	1506	α-(Z)-bisabolene	0.28
24	34.72	1582	caryophyllene oxide	0.57
			Total	**97.92**

[a] RT (min) = Retention time
[b] KIL = Kovat's retention index
[c] The percentages of compounds were obtained by FID peak-area normalization. The percentage composition of the oil was computed by the normalization method from the GC peak areas, calculated as the mean of three samples without correction factors.

(6.19%), *trans*-caryophyllene (4.02%), γ-terpinene (3.18%), myrcene (1.10%), and α-terpinene (1.09%) were identified as the main components. These results align with other studies indicating that thymol is a major constituent of *T. capitatus* EO in different scientific reported data (Goudjil et al. 2020; Kowalczyk et al. 2020; Jabraeili et al. 2022).

Thymol was found to be the most plentiful EO component in *T. capitatus* and *T. vulgaris* collected from different countries (Jabraeili et al. 2022). In contrast, carvacrol was reported as the main component of EO from *T. capitatus* collected in Italy (Džamić et al. 2015; Gonçalves et al. 2017).

These differences in the main components and their concentrations are shown to be related to factors influencing the phytochemical composition of the EO of the same plant (Khan et al. 2017; Basholli-Salihu et al. 2017; Manconi et al. 2018; Morshedloo et al. 2018; Benbrahim et al. 2021). Furthermore, it has been reported that EOs from Mediterranean climate regions generally are containing more monoterpenes than those from continental regions (Tammar et al. 2019; Niksic et al. 2021). Our findings contribute by offering insights into these data.

2.2. *Evaluation of liposome particle size, PDI, and zeta potential*

Three different liposomal formulations were prepared, and liposome particle size, PDI, and ZP of the blank and TEO-LS samples were evaluated. The results are summarized in Table 2. The liposome particle size of the empty liposomes prepared with different phospholipids varied significantly ($p < 0.001$) among the different liposomal formulations. The liposome particle sizes of blank LS containing Ph 90 LS and Ph 85G LS were 161±3.60 nm and 81±1.90 nm, respectively. Meanwhile, the blank Lipoid S100 LS had the largest diameter (270±1.50 nm).

Encapsulating TEO within their structures significantly increased the liposome particle size of Lipoid S100 LS to 580±48.04 nm ($p < 0.001$). In contrast, the liposome particle size of TEO-Ph 85G LS and TEO-Ph 90 LS showed only minor changes, with no significant changes as compared to the blank LS: 72±1.09 nm and 175±1.31 nm, respectively ($p > 0.05$). Our findings do not entirely align with those of other studies (Sebaaly et al. 2015; Kryeziu et al. 2022). The reason for these liposome particle size changes may be that the oils can affect the membrane of the liposomes; these differences are associated with differences in the structures of different liposomal formulations and may be attributed to the intercalation of TEO in the liposome bilayer of different liposomal

Table 2: Characteristics of blank and *T. capitatus* essential oil-loaded liposomes in terms of the mean liposome particle size, polydispersity index (PDI), zeta potential (ZP), and encapsulation efficiency (EE%).

	Liposome batches[1]	Mean size (nm) ± SD ($n = 3$)	PDI ± SD ($n = 3$)[2]	ZP (mV) ± SD ($n = 3$)[3]	EE% of TEO (%) ± SD ($n = 3$)[4]
Lipoid S100 LS	Blank LS Ph/Chol/EO (10/5/0)	270 ± 1.50	0.22 ± 0.05	– 4.20 ± 0.70	
	TEO-LS Ph/Chol/EO (10/5/2.5)	580 ± 48.04	0.83 ± 0.02	- 11.70 ± 0.21	86.07 ± 5.60
Ph 85G LS	Blank LS Ph/Chol/EO (10/5/0)	81 ± 1.90	0.30 ± 0.01	- 35.40 ± 0.90	
	TEO-LS Ph/Chol/EO (10/5/2.5)	72 ± 1.09	0.37 ± 0.04	- 33.90 ± 0.30	81.42 ± 6.97
Ph 90H LS	Blank LS Ph/Chol/EO (10/5/0)	161 ± 3.60	0.38 ± 0.03	- 11.60 ± 0.10	
	TEO-LS Ph/Chol/EO (10/5/2.5)	175 ± 1.31	0.66 ± 0.02	- 22.80 ± 0.15	83.17 ± 4.05

[1] LS: liposome, Ph: phospholipon, Chol: cholesterol, EO: essential oil
[2] PDI: polydispersity index
[3] ZP: zeta potential
[4] EE%: encapsulation efficiency
Data are expressed as a mean value ± standard deviation (n = 3).

structures, leading to different reduction scales of the cohesion between phospholipids (Sebaaly et al. 2015; Manconi et al. 2018; Aguilar-Pérez et al. 2021).

In terms of uniformity, the blank Lipoid S100 LS performed the best PDI value (0.22±0.05), followed by the blank Ph 85G LS (0.30±0.01), with no significant differences ($p > 0.05$). Blank Ph 90H LS showed the highest PDI value (0.38±0.03), with a significant difference ($p < 0.01$) with blank Lipoid S100 LS and no significant difference with blank Ph 85G LS (p > 0.05).

The encapsulation of TEO was associated with a significant increase in the PDI values for TEO-Lipoid S100 LS: 0.83±0.02 ($p < 0.001$) and TEO-Ph 90H LS, 0.66±0.02 ($p < 0.001$), as compared to empty liposomes, thus leading to polydisperse formulations. The PDI value of TEO-PH 85G LS was generally satisfied with no significant difference from blank PH 85G LS: 0.37±0.04 ($p > 0.05$), demonstrating a slight polydisperse LS. These results agree with previous findings and suggest that the PDI parameter is significantly affected by the content, concentration, and characteristics of each ingredient used in the preparation (Danaei et al. 2018; Hammoud et al. 2021).

The ZP for blank Ph 85G LS was – 35.40±0.90 mV; for blank Ph 90H LS, – 11.60±0.10 mV; and for blank Lipoid S100 LS, – 4.20±0.70 mV, which are in line with earlier findings, demonstrating that that the surface charge of phospholipid based liposomes is anionic (Celia et al. 2013; Hammoud et al. 2021). After TEO encapsulation into PH 85G LS (ZP was shown to be: – 33.90±0.30 mV), no significant differences were observed as compared to the blank Ph 85 G LS ($p > 0.05$). The ZP significantly decreased to – 22.80±0.15 mV ($p < 0.001$) for TEO-Ph 90H LS. On the other hand, TEO-Lipoid S100 LS showed that, despite a significant difference in ZP as compared to their blank form, ZP values remained low after encapsulation: – 11.70±0.21 mV ($p < 0.001$). These changes in the ZP values after encapsulation are in accordance with previous studies and could be related to differences in the distribution and interactions of TEO with the liposomal bilayer, which alter the charge on the surface. These data suggest that the composition of the lipid vesicle membrane, and particularly the type of phospholipids, cholesterol content, and preparation method, can influence the liposome particle size, PDI, ZP, and EE% (Sebaaly et al. 2015; Hammoud et al. 2021).

2.3. Evaluation of the encapsulation efficiency

The results of this study show that LS provided a high EE% when encapsulating TEO and that no significant difference was seen among the three liposomal formulations used (Table 2).

TEO-Lipoid S100 LS (86.07±5.60 %) had the highest EE%, followed by TEO-Ph 90H LS (83.17±4.05 V), and TEO-Ph 85G LS (81.42±6.97 %) ($p > 0.05$). Previously, similar findings on EE% have been reported in the literature. However, some results with significantly lower EE% rates have been reported (Sebaaly et al. 2015; Cui, Zhao, and Lin 2015; Aguilar-Pérez et al. 2021). This indicator was reported to vary depending on the composition and concentration of the oil's constituents and the LS itself (Sebaaly et al. 2015; Kryeziu et al. 2022). The nature of the encapsulated substance(s) is a critical parameter that can influence the interaction of the oil components with the LS membrane. Because the EE% is related to the membrane permeability, the EE% of different species' oils can differ for the same LS (Pérez-González et al. 2019; Ramos da Silva et al. 2021).

Furthermore, the type/nature of the phospholipid used and the concentration of phospholipid and cholesterol have been shown to influence the physicochemical properties of the nanoparticles and the EE% (Sebaaly et al. 2015).

Similarly, studies have shown that the LS membrane components interact with EO hydroxyl groups, thus increasing the EE%. The low EE% values for LS containing certain EOs may be related to the degree of saturation of the LS lipid membrane with the EO components (Kaddah et al. 2018; Aguilar-Pérez et al. 2021; Nasr et al. 2021).

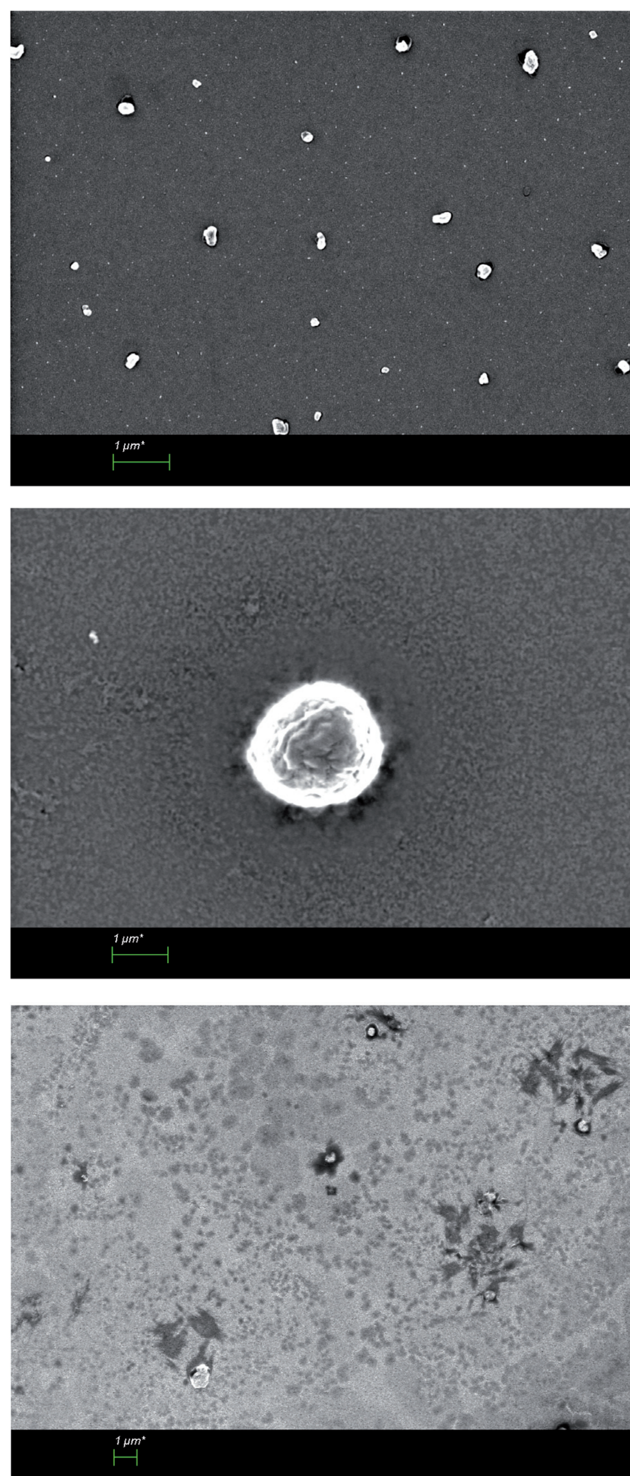

Fig. 2: SEM images of ***T. capitatus*** essential oil loaded (a) Lipoid S100, (b) Phospholipon 85G, and (c) Phospholipon 90H liposomes.
(a) *T. capitatus* essential oil-loaded Lipoid S100 liposomes. The diameters of vesicles varied from 100 to 700 nm, and the shapes of the vesicles had distorted spherical shapes.
(b) *T. capitatus* essential oil-loaded Phospholipon 85G liposomes. The vesicles were seen to be non-perfect spheres.
(c) *T. capitatus* essential oil-loaded Phospholipon 90H liposomes. These liposomes produced spherical sized vesicles after essential oil encapsulation.

2.4. Evaluation of the morphology

A scanning electron microscope (Carl Zeiss) was used to examine diameter, uniformity, and morphology of the prepared LS. Fig. 2 depicts the nanoparticles, represented by mostly spherical shapes, present on the LS outer surface. The SEM images show that the LS are mostly homogeneous and range in particle size from nano (10^{-9}) to micro (10^{-6}).

Morphologically, TEO-Lipoid S100 LS containing oil had spherical or irregular spherical shapes and were 100–700 nm in diameter. Similarly, TEO-Ph 85G LS ranged from 100 nm to several μm in diameter and had imperfect spherical shapes. Despite having more stable bonds in their structures due to their more highly hydrogenated nature (i.e., these contain phospholipids with saturated fatty chains), TEO-Ph 90H LS produced almost identical results and had diameters ranging from 150–600 nm (Fig. 2a–c). Furthermore, when we compare the findings of this study to those found cited in the literature, we find that these are comparable. Liposomes were spherical in both cases (Aguilar-Pérez et al. 2021).

2.5. Cytotoxic potential

TEO and TEO-LS cytotoxic activities were evaluated *in vitro* using the HT-29 cancer cell line. The viability of the cells was determined using the MTT assay, and the results are presented in Figs. 3 and 4. Our study found that TEO, as the active compound, significantly reduced the viability of HT-29 cancer cells when delivered using LS. The IC_{50} for TEO was measured to be 65 μg/mL for the HT-29 cell line. Similar previous studies have shown that *Thymus* plant essential oils are cytotoxic to a variety of human cancer cells, including colon cancer cells (HCT-116 and CaCo-2), breast cancer cells (MCF-7), and lung cancer cells (A-549) (Hassan et al. 2019; Pérez-González et al. 2019; Blažíčková et al. 2022). According to previous findings, the cytotoxic action of EOs is not conditionally directly related to the components that have the highest concentrations. The phytochemical components of the EO interact with cells at different concentrations. As a result, even trace components may play a role in the overall cytotoxic activity of EOs (Hassan et al. 2019; Pérez-González et al. 2019; Blažíčková et al. 2022).

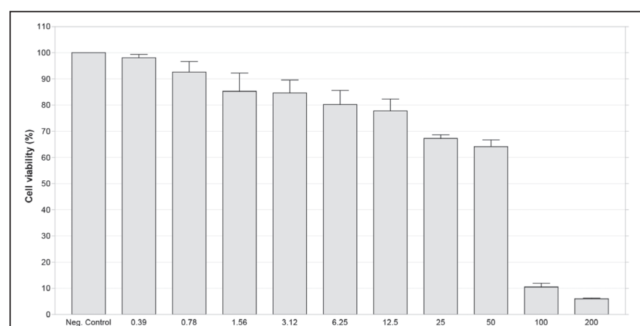

Fig. 3: Viability of HT-29 Cancer Cell Line after treatment with different concentrations of TEO (0.39, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100, 200 μg/mL). Each bar represents means ± SD.

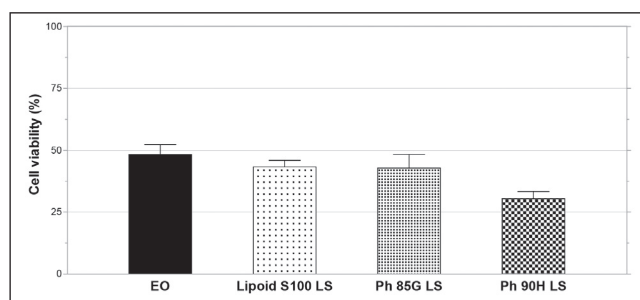

Fig. 4: Cytotoxicity of Free TEO and nanoencapsulated TEO on HT-29 Cancer Cell line (65 μg/mL). The chart shows cell viability post-treatment with free essential oil and essential oil encapsulated in Lipoid S100 liposomes, Phospholipon 85G liposomes, and Phospholipon 90H liposomes, as assessed by the MTT method. Each bar represents mean ± SD.

The cytotoxic action of EOs is not conditionally directly related to the main components. The phytochemical components of the EO interact with cells at different concentrations. As a result, even trace components may play a role in the overall cytotoxic activity of EOs (Pérez-González et al. 2019; Hassan et al. 2019; Blažíčková et al. 2022)

The biological properties of essential oils (EOs) are primarily linked to their major compounds. However, it has been noted that these primary compounds can interact with less prevalent compounds within the EOs, potentially leading to a synergistic effect that enhances the overall activity of the EO (Savelev et al. 2003; Sabater-Jara et al. 2019). This synergy is particularly notable in the context of the anticancer properties of thymol and carvacrol, which have been extensively researched before (Pérez-González et al. 2019; Rojo-Ruvalcaba et al. 2020; Sampaio et al. 2021). In contrast, essential oils containing carvacrol, p-cymene, and γ-terpinene as key components have been found to be non-toxic to cancer cell lines, as reported (Babili et al. 2011; Leyva-López et al. 2017). Furthermore, the major component of Thyme essential oil has been shown to possess less potent biological activity in comparison to the oil in its entirety (Hussain et al. 2013).

In addition, essential oils, including thyme oil, have demonstrated cytotoxic effects *via* several mechanisms like apoptosis, cell cycle arrest, and the disruption of cell membranes. The specific nature of these mechanisms can vary based on the oil's composition and the type of cell being targeted. The advantage of utilizing essential oils lies in their diverse array of potentially bioactive compounds, though this diversity also necessitates thorough characterization for their effective use (Sabater-Jara et al. 2019; Niksic et al. 2021).

Incorporating TEO in LS altered cell viability of the cancer cell line used in this study. The results demonstrate that the EO-loaded LS out-performed free EO in terms of cytotoxic activity *in vitro*. To our knowledge, no previous research has evaluated the cytotoxic effect of various TEO LS compositions on the HT-29 cancer cell line. Encapsulation of TEO into Ph 90H LS significantly improved the cytotoxic activity ($p < 0.01$). As shown in Fig. 4, TEO-Ph 90H LS was significantly more cytotoxic than free TEO. At the same time, TEO-Lipoid S100 LS and TEO-Ph 85G LS activities did not differ significantly from free TEO activity ($p > 0.05$). Blank formulations did not show any activity.

These results are in line with previously reported results, indicating the improvement of EO activity after LS encapsulation. The enhanced cytotoxic activity observed for TEO-Ph 90H LS may be explained by the increased ability of TEO to be trapped inside the cell, compared to the non-encapsulated form of the EO. In fact, a number of investigations have demonstrated an enhanced intracellular accumulation of a compound when encapsulated in LS (Celia et al. 2013; Nakhaei et al. 2021). Additionally, prolonged EO stability following LS encapsulation and the sustained presence of EO from LS through controlled release may explain the enhancement of EO LS's cytotoxic activity (Marslin, Khandelwal, and Franklin 2020; Jampilek and Kralova 2022).

While Lipoid S100 LS and Ph 85G LS could maintain cytotoxic activity of TEO, TEO-Ph 90 LS significantly improved cytotoxic activity ($p < 0.01$). The differences observed in the different types of LS (Lipoid S100 LS, Ph 85G LS, and Ph 90H LS) could be attributed to various factors, including physicochemical instability caused by excipients with unsaturated fatty acid chains, which may reduce the LS efficacy, as previously reported (Nakhaei et al. 2021; Kryeziu et al. 2022).

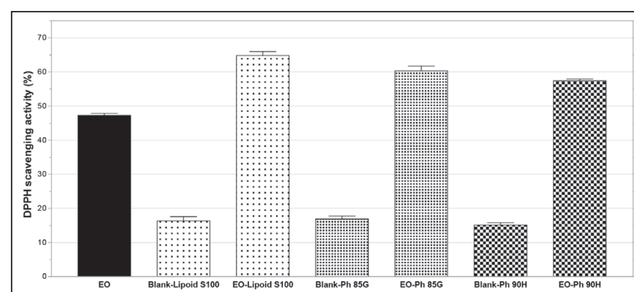

Fig. 5: Antioxidant activity of Free ***T. capitatus*** (65 μg/mL), blank liposomes (Blank-lipoid S100, Blank-Phospholipon 85G and Blank-Phospholipon 90H liposomes) and ***T. capitatus*** essential oil-loaded liposomes (EO-Lipoid S100, EO-Phospholipon 85G and EO-Phospholipon 90H liposomes). Each bar represents ± SD (n = 3).

2.6. Antioxidant potential

In this study, the DPPH radical scavenging assay was used to investigate the antioxidant activities of unencapsulated TEO and TEO-LS. Figure 5 depicts the antioxidant activities represented as a percentage. The results show that TEO exhibited an antioxidant activity of 47.28±0.58 %.

The encapsulation of TEO into LS resulted in a significant improvement of this biological activity, regardless of the liposomal formulation used. TEO-Lipoid S100 LS were found to be the most effective, enhancing the antioxidant activity to 64.84±1.16 % ($p < 0.001$), followed by TEO-Ph 85G LS (60.37±1.38 %), and then TEO-Ph 90 LS (57.44±0.52 %) ($p < 0.001$), demonstrating that the different degrees of saturation in the fatty acid chains in their structures did not have a significant impact on the antioxidant activity. These findings are supported by data previously reported in the literature, which confirm the improved antioxidant activity of EO liposomes as compared to free EOs (Sebaaly et al. 2015; Battista et al. 2020; Kryeziu et al. 2022).

The antioxidant-liposome membrane complex, which depends on the phytochemical composition of EO, ZP, and liposome particle size, has been found to affect antioxidant activity (Nasr et al. 2021). Because of their ability to form chemical complexes with free radicals, the compounds thymol, carvacrol, and *p*-cymene (as central components of TEO) have good antioxidant activity. Furthermore, other monoterpenes in the oil have been shown to have antioxidant activity, implying that this biological activity results from interactions among the oil's components (Manconi et al. 2018; Radünz et al. 2021).

2.7. Stability studies

The storage stability of a liposomal nanosystem must be thoroughly understood to assess its viability as a delivery system for therapeutic agents, particularly for clinical applications and large-scale production. As a result, changes in the LS physicochemical properties were measured after six months. The liposome particle size, ZP, and storage conditions have been shown to influence LS stability (Cornier et al. 2017).

The liposome particle size, PDI, and ZP of TEO-LS before and after six months of storage at 25 °C were evaluated in this study. Table 3 shows that TEO-Ph 90H LS remained stable regarding the liposome particle size ($p > 0.05$). No significant differences in liposome particle size were observed after six months of storage, and the average liposome particle size was 204±0.64 nm ($p > 0.05$). Even though PDI and ZP of TEO-Ph 90H LS changed significantly to 0.36±0.02 ($p < 0.001$) and – 29.76±0.52 mV ($p < 0.001$), respectively, these values remained low, as required for stability.

After six months of storage, TEO-Lipoid S100 LS and TEO-Ph 85G LS showed less favorable stability results. The liposome particle size of TEO-Lipoid S100 LS and TEO-Ph 85G LS increased significantly to 4789±108.90 nm and 2579±67.03 nm ($p < 0.001$), PDI values were shown to increase to 1.00±0.01 ($p < 0.001$) and 0.92±0.06 ($p < 0.001$), respectively, while ZP values were – 9.03±0.72 mV ($p < 0.001$) and – 9.05±0.36 mV ($p < 0.001$), respectively. These data indicate that these two LS were unstable during six months of storage.

The stability of LS is attributed to the preparation method, including the ratio between the components of the respective LS (Nasr et al. 2021). Larger particles tend to aggregate, because they are more susceptible to the influence of Brownian motion; therefore, they have more opportunities to collide. In general, smaller nanoparticles have a lower tendency to aggregate. ZP also plays an essential role in the stability and aggregation behavior of LS. This may explain previously reported data indicating that nanosystems that contain smaller vesicles are, in some cases, less stable (Danaei et al. 2018).

This work demonstrated that thyme essential oil (TEO) exhibited a measurable *in vitro* cytotoxic activity toward HT-29 colorectal cancer cell line and that the encapsulation of TEO in liposomes enhanced the cytotoxic activity as compared with free TEO.

Among the three prepared liposomal nanosystems, TEO-Ph 90H LS generally demonstrated better cytotoxic activity than TEO-Lipoid S100 and TEO-Ph 85G LS. Moreover, TEO PH 90 H LS remained physicochemically stable during six months of storage at 25 °C.

These results emphasize the role of LS in enhancing the delivery and efficacy of TEO, as an active anti-cancer agent, in therapeutic applications. The study's findings indicate that additional *in vitro* research should be carried out to confirm the cytotoxic effects of free and encapsulated TEO in LS. This research would enable the establishment of safe dosage levels and the determination of the mechanism of action, aspects which are critical for future pharmaceutical applications. Overall, this study contributes to a broader understanding of the properties of TEO and its potential applications. Our study adds to this body of knowledge by demonstrating its cytotoxic potential against colorectal cancer cells and highlighting the benefits of using LS encapsulation to enhance its efficacy. This suggests that TEO, particularly when encapsulated in LS, could be a promising candidate for further research and development in cancer therapy.

3. Experimental

3.1. Materials

MTT (Thiazolyl blue tetrazolium bromide), nutrient medium (Dulbecco's Minimum Essential Medium (DMEM):HAMS F12 (1:1), DMSO, Trypsin-EDTA, DPPH

Table 3: Characteristics of blank and *T. capitatus* essential oil-loaded liposomes in terms of mean liposome particle size, PDI, and zeta potential.

	Time interval	Mean size (nm) ± SD ($n = 3$)	PDI ± SD ($n = 3$)[3]	ZP (mV) ± SD ($n = 3$)[4]
Lipoid S100 LS[1]	t_0	580 ± 48.04	0.83 ± 0.02	- 11.70 ± 0.21
	t_6	4789 ± 108.9	1.00 ± 0.01	– 9.03 ± 0.72
Ph 85G LS[2]	t_0	72 ± 1.09	0.37 ± 0.04	- 33.90 ± 0.30
	t_6	2579 ± 67.03	0.92 ± 0.06	– 9.05 ± 0.36
Ph 90H LS	t_0	175 ± 1.31	0.66 ± 0.02	- 22.80 ± 0.15
	t_6	204 ± 0.64	0.36 ± 0.02	- 29.76 ± 0.52

[1] LS: liposome
[2] Ph: phospholipon
[3] PDI: polydispersity index
[4] ZP: zeta potential
Data are expressed as a mean value ± standard deviation ($n = 3$).

(2,2-diphenyl-1-picrylhydrazyl), hexane, cholesterol 95 % and absolute ethanol (HPLC grade) were obtained from Sigma-Aldrich. Lipoid S100 (soybean phosphatidylcholine 94 %, lysophosphatidylcholine 3 %, and residual solvents), Phospholipon 85G (soybean phosphatidylcholine 85%, lysophosphatidylcholine 3.3 %, and residual solvents), and Phospholipon 90H (hydrogenated soybean phosphatidylcholine 90%, lysophosphatidylcholine 4 %, and residual solvents) were supplied by Lipoid. The dialysis membrane ZelluTrans/ROTH T1 (MWCO: 3500) was acquired from Zellu Carl Roth.

3.2. Plant material and essential oil extraction

Plant material of *T. capitatus* was collected from the northern zone of Albania in May, with the aerial parts of the plants specifically utilized to extract essential oil. Professor Skerdilaid Xhulaj (University of Tirana) identified the plant material, and voucher specimens were deposited at the University of Tirana Herbarium. Plant samples were air-dried at ambient room temperature and then subjected to hydrodistillation using a Clevenger-type apparatus for essential oil extraction. This procedure was followed: 20 g of the plant material was subjected to hydrodistillation with 500 mL of distilled water for 3 h at 100 °C to obtain EO at a 2-3 mL/min collection rate. The resultant EO was dried over anhydrous sodium sulfate (Sigma-Aldrich) and stored at 4 °C as described in the literature (Papajani et al. 2015).

3.3. GC/FID/MS analysis of T. capitatus essential oil

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

3.4. Liposome preparation

The ethanol injection technique was used to prepare a blank sample and the TEO-LS as described in the literature with minor changes (Sebaaly et al. 2015). Phospholipids (Lipoid S100, Phospholipon 85G or Phospholipon 90H, 10 mg/mL), TEO (2.5 mg/mL), and cholesterol (5 mg/mL) were dispersed in absolute ethanol (10 mL) by stirring with a magnetic bar. Using a syringe pump (LA-100, Landgraf), the organic phase was carefully injected into 20 mL of Milli-Q water at a flow rate of 1 mL/min. When the alcoholic solution came into contact with the water phase, liposomes formed spontaneously. Following the injection, we stirred the resulting LS suspension at room temperature for 15 min to ensure homogeneity. We then removed the ethanol by dialysis, employing ZelluTrans/ROTH T1 membranes, in a dialysis process involving 1000 mL of dialysis medium, stirred continuously at 200 rpm for 16 h. Blank LS were considered the batches that did not contain TEO. Each LS was prepared in triplicate to ensure reproducibility. Before further analyses were performed, all LS batches were stored at 4 °C.

3.5. Characterization of the liposomes

3.5.1. Liposome Particle size, PDI, and zeta potential measurements

TEO-LS were investigated for their liposome particle size, PDI, zeta potential (ZP), encapsulation efficiency (EE%), and morphology by using SEM. In this study, a Malvern Zetasizer Nano ZS Model was used (i.e., ZEN3500, Malvern), to determine the mean liposome particle size, PDI, and ZP of the blank and TEO-LS samples. After 2 min of equilibration time, one mL of the prepared samples was placed in the folded capillary cell (Malvern), and ZP measurements were conducted using Smoluchowski's equation. All analyses were performed in triplicate at 25 °C after a 2-min equilibration time. The means±SD (n = 3) were used to express the data.

3.5.2. Determination of encapsulation efficiency

The encapsulation efficiency was determined by applying the technique described in the literature with minor modifications (Natrajan et al. 2015; Borges et al. 2018). The liposomal suspension was centrifuged for 30 min at 4,000 rpm. The collected supernatant (2 mL) was mixed with hexane (2 mL). The amount of encapsulated EO was quantified by using a UV 1800 UV-Vis spectrophotometer, measuring at λ = 306 nm (Shimadzu). The experiment was performed in triplicate. The EO concentration was defined by determining the standard curve obtained with various concentrations of *T. capitatus* EO diluted in hexane. The encapsulation efficiency was expressed as a percentage and calculated as follows:

(EE%) = (Total oil − Free oil) / (Total oil) * 100

3.5.3. Scanning Electron Microscopy (SEM)

The morphology of vesicles was visualized using a scanning electron microscope (SEM) Carl Zeiss 300. The VP analysis was carried out at a 25-kV accelerating voltage. The samples were put on lamellar plates for SEM analysis and allowed to dry at room temperature for 72 h. Before visualization, all the samples were coated with Au-Pd for 30 seconds to increase the conductivity by using a Quorum Q150 RES modular coating system.

3.6. Evaluation of biological activities

3.6.1. Determination of cytotoxicity with an MTT assay

The HT-29 cell line, utilized in the experiments, was procured from the American Type Culture Collection (ATCC®). The cell line was cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and HAM's Nutrient Mixture F12 nutrient medium containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 10 mg/ml streptomycin, and 1% L-glutamine. The flasks were placed in a 5% CO_2 incubator. HT-29 cells were seeded in 96-well plates at a density of $7x10^3$ cells per well (180 μL). Cells seeded on plates were incubated for 24 h at 37 °C in an incubator and kept under a 5% CO_2 atmosphere. The TEO was initially dissolved in DMSO to obtain an initial concentration of 100 mg/mL stock solution and further diluted in medium prior to addition to plates. Then at the end of the incubation period, 20 μL of the diluted sample was added to the cells in the wells at a concentration of 0.39 to 200 μg/mL, and the IC_{50} was calculated.
Separately, 20 μL of TEO-loaded 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 into each well, and the plates were incubated for 4 h at 37 °C. Finally, 200 μL of DMSO were 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 (IC_{50}) at which 50% of the cells were alive were calculated by applying 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, as previously described in the literature (Kryeziu et al. 2022). Results are expressed as the mean value±SD (n = 3).

3.6.2. Determination of antioxidant activity (DPPH radical scavenging assay)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH)-scavenging activities of free TEO and TEO-LS were determined by ap-plying a previously described method with minor modifications (Sebaaly et al. 2015). A 1-mL aliquot of TEO or LS that contained TEO (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 λ = 517 nm with a UV 1800 UV-Vis spectrophotometer (Shimadzu). All measurements were carried out in triplicate, and the findings were expressed as a mean value ± SD.
The DPPH scavenging activity percentage was determined by applying the following equation:
DPPH Scavenging activity (%) = Sample absorbance value / Control absorbance value × 100

3.7. Stability

TEO-loaded liposomes were tested for their physicochemical stability after 6 months of storage at 25 °C. At that time, the liposome particle size, PDI, and ZP were evaluated. The conditions for liposome storage, utilizing the Memmert Climate Chamber ICH110 were as follows: temperature of 25±2 °C and relative humidity of 60±5 %.

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References

Adams RP (2007) Identification of Essential Oil Components by Gas Chromatography/Mass Spectorscopy. 4th ed. Carol Stream, Ill: Allured Pub. Corp.
Aguilar-Pérez KM, Medina DI, Narayanan J, Parra-Saldívar R, Iqbal HMN (2021) Synthesis and nano-sized characterization of bioactive Oregano essential oil molecule-loaded small unilamellar nanoliposomes with antifungal potentialities. Molecules 26: 2880.
American Cancer Society. 2023. Key Statistics for Prostate Cancer | Prostate Cancer Facts." 2023. https://www.cancer.org/cancer/prostate-cancer/about/key-statistics.html.
Anitha A, Maya S, Sivaram, AJ, Mony U, Jayakumar, R (2016) Combinatorial nanomedicines for colon cancer therapy. WIREs Nanomed Nanobiotechnol 8: 151–159.
Arrais A, Bona E, Todeschini V, Caramaschi A, Massa N, Roncoli M, Minervi A, Perin E, Gianotti B (2023) Thymus vulgaris essential oil in beta-cyclodextrin for solid-state pharmaceutical applications. Pharmaceutics 15: 914.
ATCC®. 2022. Human Cells." November 26, 2022. https://www.atcc.org/cell-products/human-cells.
El Babili F, Bouajila J, Souchard JP, Bertrand V, Bellvert F, Fouraste I, Moulis C, Valentin A (2011) Oregano: Chemical analysis and evaluation of its antimalarial, antioxidant, and aytotoxic activities. J Food Sci 76: C512–C518.

Basholli-Salihu M, Schuster R, Hajdari A, Mulla D, Viernstein D, 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.
Battista S, Maggi MA, Bellio P, Galantini L, D'Archivio AA, Celenza G, Colaiezzi R, Giansanti (2020 Curcuminoids-loaded liposomes: Influence of lipid composition on their physicochemical properties and efficacy as delivery systems. Colloids Surfaces A 597: 124759.
Benbrahim C, Barka MS, Basile A, Maresca V, Flamini G, Sorbo S, Carraturo F, Notariale R, Piscopo M, Khadir A (2021) Chemical composition and biological activities of Oregano and Lavender essential oils. Applied Sci 11: 5688.
Blažíčková M, Blaško J, Kubinec R, Kozics K (2022) Newly synthesized thymol derivative and its effect on colorectal cancer cells. Molecules 27: 2622.
Bolouri P, Salami R, Kouhi S, Kordi M, Lajayer BA, Hadian J, Astatkie T (2022 Applications of essential oils and plant extracts in different industries. Molecules 27: 8999.
Borges RS, Keita H, Sánchez Ortiz BL, dos Santos Sampaio TI, Ferreira IM, Lima ES, de Jesus Amazonas da Silva M, Pinho Fernandes C, Maciel de Faria Mota Oliveira AE, Cardoso da Conceição E (2018) Anti-inflammatory activity of nanoemulsions of essential oil from Rosmarinus officinalis L.: In vitro and in zebrafish studies. Inflammopharmacology 26: 1057–1080.
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 (2013) Anticancer activity of liposomal bergamot essential oil (BEO) on human neuroblastoma cells. Colloids Surfaces B 112: 548–553.
Cornier J, Owen A, Kwade A, Van de Voorde M (2017) Pharmaceutical Nanotechnology, 2 Volumes: Innovation and Production. John Wiley & Sons.
Cui H, Zhao C, Lin L (2015) The specific antibacterial activity of liposome-encapsulated clove oil and its application in tofu. Food Control 56: 128–134.
Danaei M, Dehghankhold M, Ataei S, Davarani FH, Javanmard R, Dokhani A, Khorasani S, Mozafari MR (2018) Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. Pharmaceutics 10: 57.
Džamić AM, Nikolić BK, Giweli AA, Mitić-Ćulafić DS, Soković MD, Ristić MS, Knežević-Vukčević JB, Marin PD (2015) Libyan Thymus capitatus essential oil: Antioxidant, antimicrobial, cytotoxic and colon pathogen adhesioninhibition properties. J Appl Microbiol 119: 389–399.
Gezici S, Şekeroğlu N (2019) Current perspectives in the application of medicinal plants against cancer: Novel therapeutic agents. Anti-Cancer Agents Med Chem 19: 101–111.
Gonçalves JCR, Andrade de Meneses D, Pereira de Vasconcelos A, Alves Piauilino C, de Castro Almeida FR, Napoli EM, Ruberto G, Machado de Araújo DA (2017) Essential oil composition and antinociceptie activity of Thymus capitatus. Pharm Biol 55: 782–786.
Gouda A, Sakr OS, Nasr M, Sammour O (2021) Ethanol injection technique for liposomes formulation: An insight into development, influencing factors, challenges and applications. J Drug Del Sci Technol 61: 102174.
Goudjil MB, Zighmi S, Hamada D, Mahcene Z, Bencheikh SE, Ladjel S (2020) Biological activities of essential oils extracted from Thymus capitatus (Lamiaceae). South Afr J Bot 128: 274–282.
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.
Hammoud Z, Kayouka M, Trifan A, Sieniawska E, Ben Jemâa JM, Elaissari A, Greige-Gerges H (2021) Encapsulation of α-pinene in delivery systems based on liposomes and cyclodextrins. Molecules 26: 6840.
Hassan HM, Mina SA, Bishr MM, Khalik SMA (2019) Influence of foliar spray of ethephon and water stress on the essential oil composition and impact on the cytotoxic activity of Thymus vulgaris aerial parts. Nat Prod Res 33: 2714–2717.
Hussain AI, Anwar F, Chatha SAS, Latif S, Sherazi STH, Ahmad A, Worthington J, Sarker SD (2013) Chemical composition and bioactivity studies of the essential oils from two Thymus species from the Pakistani flora. Food Sci Technol 50: 185–192.
Jabraeili S, Mirzaei H, Anarjan N, Javadi A, Behnajady MA (2022) Nanoliposomal Thyme (Thymus vulgaris) essential oil: Effects of formulation parameters. Food Sci Technol Int 28: 257–272.
Jampilek J, Kralova K (2022) Anticancer applications of essential oils formulated into lipid-based delivery nanosystems. Pharmaceutics 14: 2681.
Kaddah S, Khreich N, Kaddah F, Charcosset C, Greige-Gerges H (2018) Cholesterol modulates the liposome membrane fluidity and permeability for a hydrophilic molecule. Food Chem Toxicol 113: 40–48.
Khan F, Khan I, Farooqui A, Ansari IA (2017) Carvacrol induces reactive oxygen species (ROS)-mediated apoptosis along with cell cycle arrest at G0/G1 in human prostate cancer cells. Nutrition Cancer 69: 1075–1087.
Kowalczyk A, Przychodna M, Sopata S, Bodalska A, Fecka I (2020) Thymol and thyme essential oil—New insights into selected therapeutic applications. Molecules 25: 4125.
Kryeziu TL, Haloci E, Loshaj-Shala A, Bagci U, Oral A, Stefkov GJ, Zimmer A, Basholli-Salihu M (2022) Nanoencapsulation of Origanum vulgare essential oil into liposomes with anticancer potential. Pharmazie 77: 172–178.
Kyriakoudi A, Spanidi E, Mourtzinos I, Gardikis K (2021) Innovative delivery systems loaded with plant bioactive ingredients: Formulation approaches and applications. Plants 10: 1238.
Leyva-López N, Gutiérrez-Grijalva EP, Vazquez-Olivo G, Basilio Heredia J (2017) Essential oils of Oregano: Biological activity beyond their antimicrobial properties. Molecules 22: 989.
Manconi M, Petretto G, D'Hallewin G, Escribano E, Milia E, Pinna R, Palmieri A, Firoznezhad M, Peris JE, Usach I (2018) Thymus essential oil extraction, characterization and incorporation in phospholipid vesicles for the antioxidant/antibacterial treatment of oral cavity diseases. Colloids Surfaces B 171: 115–122.
Maniki E, Kostoglou D, Paterakis N, Nikolaou A, Kourkoutas Y, Papachristoforou A, Giaouris E (2023 Chemical composition, antioxidant, and antibiofilm properties of essential oil from Thymus capitatus plants organically cultured on the Greek island of Lemnos. Molecules 28: 1154.
Marslin G, Khandelwal V, Franklin G (2020) Cordycepin nanoencapsulated in poly(-lactic-co-glycolic acid) exhibits better cytotoxicity and lower hemotoxicity than free drug. Nanotechnol Sci Appl 13: 37–45.
Morshedloo MR, Reza M, 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 Proc Pres 42: e13516.
Nakhaei P, Margiana R, Bokov DO, Abdelbasset WK, Kouhbanani MAJ, Varma RS, Marofi F, Jarahian M, Beheshtkhoo N (2021) Liposomes: Structure, biomedical applications, and stability parameters with emphasis on cholesterol. Frontiers Bioengin Biotechnol 9:705886.
Nasr G, Ghenwa, Greige-Gerges H, Elaissari A, Khreich N (2021) Liposome permeability to essential oil components: A focus on cholesterol content. J Membrane Biol 254: 381–395.
Natrajan D, Srinivasan S, Sundar K, Ravindran A (2015) Formulation of essential oil-loaded chitosan–alginate nanocapsules. J Drug Anal 23: 560–568.
Niksic H, Becic F, Koric E, Gusic I, Omeragic E, Muratovic S, Miladinovic B, Duric K (2021) Cytotoxicity screening of Thymus vulgaris L. essential oil in brine shrimp nauplii and cancer cell lines. Sci Rep 11: 13178.
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. Cytotoxicity: Definition, Identification, and Cytotoxic Compounds; Istifli, ES, Ila, HB, Eds, 29–43.
Perumalsamy H, Rajeshkumar Shanmugam H, Kim JR, Anandapadmanaban G, Huq M, Dua K, Chellappan DK, Yoon TH, Balusamy SR (2022) Nanoemulsion and encapsulation strategy of hydrophobic Oregano essential oil increased human prostate cancer cell death via apoptosis by attenuating lipid metabolism. Bioinorg Chem Appl 2022:9569226.
Radünz M, Camargo TM, dos Santos Hackbart HC, Correa 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 Technol138: 110632.
Ramos da Silva LR, Ferreira OO, Cruz JN, de Jesus Pereira Franco C, Oliveira Dos Anjos T, Cascaes MM, Almeida da Costa W, Helena de Aguiar Andrade E, Santana de Oliveira M (2021) Lamiaceae essential oils, phytochemical profile, antioxidant, and biological activities. Evid Based Complement Alternat Med 2021:6748052.
Rojo-Ruvalcaba, Brian Enrique, Teresa Arcelia García-Cobián, Sara Pascoe-González, Tannia Isabel Campos-Bayardo, Luz 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. Multidisciplinary Digital Publishing Institute Proceedings 61: 6.
Belén Sabater-Jara A, Pina Funes M, Angeles Pedreño M, Belchí-Navarro S (2019) Essential oils of Thymbra capitata and Thymus hyemalis and their uses based on their bioactivity. Thymus Thymus. IntechOpen; 2020. http://dx.doi.org/10.5772/intechopen.89309 .
Sampaio LA, Santos Pina LT, Serafini MR, dos Santos Tavares D, Guimaraes AG (2021) Antitumor effects of carvacrol and thymol: A systematic review. Frontiers Pharmacol 12: 702487.
Sarrou E, Tsivelika N, Chatzopoulou P, Tsakalidis G, Menexes G, Mavromatis A (2017) Conventional breeding of Greek Oregano (Origanum vulgare ssp. hirtum) and development of improved cultivars for yield potential and essential oil quality. Euphytica 213: 1–16.
Savelev S, Okello E, Perry NSL, Wilkins RM, Perry EK (2003) Synergistic and antagonistic interactions of anticholinesterase terpenoids in Salvia lavandulaefolia essential oil. Pharmacol Biochem Behav 75: 661–668.
Sebaaly C, Jraij A, Fessi H, Charcosset C, Greige-Gerges H (2015) Preparation and characterization of clove essential 0il-loaded liposomes. Food Chem 178: 52–62.
Tammar S, Salem N, Rebey IB, Sriti J, Hammami M, Khammassi S, Marzouk B, Ksouri R, Msaada K (2019) Regional effect on essential oil composition and antimicrobial activity of Thymus capitatus L. J Ess Oil Res 31: 129–137.
WHO. 2022. Fact Sheets: Cancer." July 28, 2022. https://www.who.int/news-room/fact-sheets/detail/cancer.
World Cancer Research Fund International. 2023. Colorectal Cancer Statistics." WCRF International. April 7, 2023. https://www.wcrf.org/cancer-trends/colorectal-cancer-statistics/.