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Liposomal incorporation of carvacrol and thymol isolated from the essential oil of *Origanum dictamnus* L. and *in vitro* antimicrobial activity

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ABSTRACT

The chemical composition of the essential oils from *Origanum dictamnus* L. (wild and organic cultivated plant) was analysed by GC–MS. Carvacrol, thymol, *p*-cymene, and γ-terpinene were identified as major constituents and isolated from both samples. The above components were successfully encapsulated in phosphatidyl choline-based liposomes and the possible improvement of their antioxidant and antimicrobial activities was tested against selected microbia. The antimicrobial properties of the oils were tested by a diffusion technique against four Gram positive and four Gram negative bacteria and three human pathogenic fungi, as well as the food-borne pathogen, *Listeria monocytogenes*. The percentage of the encapsulated carvacrol, the major component of the oil, was determined by GC–FID. In order to investigate any possible synergistic or antagonistic effect between carvacrol/thymol and carvacrol/γ-terpinene, the antimicrobial activities of the mixtures, were also determined before and after encapsulation in liposomes. All tested compounds presented enhanced antimicrobial activities after the encapsulation. Finally, in all cases, their antioxidant activity using differential scanning calorimetry was studied, in order to gain knowledge about their oxidation stability.

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1. Introduction

Essential oils are aromatic oily liquids, obtained from plant material (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) (Burt, 2004). An extensive body of research has demonstrated that essential oils and their main components possess a wide spectrum of biological activity, which may be of great importance in several fields, from food chemistry to pharmaceutics (Cristiani et al., 2007). It is well known that the essential oils obtained from oregano, a traditional Mediterranean spice, posses strong antimicrobial activity due to their very high contents of monoterpenes and oxygenated compounds, such as γ-terpinene, and *p*-cymene, thymol and carvacrol.

Origanum dictamnus L., also known as Dittany, is a rare endemic plant of Greece, growing wild on the rocky mountains of Crete Island (South Greece) (Polunin, 1997). The plant has been widely known since the time of Aristotle for its healing properties on wounds and against difficult labour of women (Thanos, 1994). The major components of its essential oil are carvacrol, γ-terpinene and *p*-cymene (Chorianopoulos et al., 2004; Harvala, Menounos, & Argyriadou, 1987; Skoula, Gotsiou, Naxakis, & Johnson, 1999). The essential oil of *O. dictamnus* L., and its major component, carvacrol, is well known for their antimicrobial activity (Sivropoulou et al., 1996). Carvacrol is a common component of the oils from oregano, thyme, marjoram and summer savory, generally recognised as a safe food additive. It is frequently used in several products as a flavouring and/or as an antimicrobial agent, showing a broad-spectrum of activities against bacteria, yeasts and fungi (Knowles, Roller, Murray, & Naidu, 2005; Ultee, Kets, Alberda, Hoekstra, & Smid, 2000).

The essential oils are hydrophobic and their primary site of activity is the membrane. They accumulate in the lipid bilayer according to a partition coefficient that is specific for the compound applied, leading to disruption of the membrane structure and function (Pol & Smid, 1999).

The antimicrobial activity of the essential oils can be attributed to the contained monoterpenes that, due to their lipophilic character, act by disrupting the microbial cytoplasmic membrane, which thus loses its high impermeability for protons and bigger ions. When the disturbance of membrane integrity occurs, then its functions are compromised, not only as a barrier but also as a matrix for enzymes and as an energy transducer (Cristiani et al., 2007). Unfortunately, most natural compounds are biologically instable, poorly soluble in water and they distribute poorly to target sites. Currently, some novel methods have been introduced in order to improve their stability and their bioavailability, among which is the use of

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liposomal encapsulation (Shoji & Nakashima, 2004). Microencapsulation reduces reactivity with the environment (water, oxygen, light), decreases the evaporation or the transfer rate to the outside environment, promotes handling ability, masks taste and enhances dilution to achieve a uniform distribution in the final product when used in very small amounts (Gibbs, Kermasha, Alli, & Mulligan, 1999; Versic, 1988). Now, natural compounds are considered as health-promoting ingredients in food industry (Shoji & Nakashima, 2004). The use of liposomes, in order to deliver food flavours and nutrients, can also provide the necessary protection against their oxidation, while the incorporation of food antimicrobials could aid in the protection of food products against growth of spoilage and pathogenic microorganisms (Taylor & Davidson, 2005).

The overall objective of the present research was to use *O. dictamnus* as a source of carvacrol and thymol, which are its main compounds, and, furthermore, to check any possible improvement of their antioxidant and antimicrobial activity, by encapsulating them in phosphatidyl choline-based liposomes, against selected human pathogenic bacteria and fungi, as well as against *Listeria monocytogenes*, a food-born microbial pathogen.

Thus, the essential oils from wild and organic cultivated *O. dictamnus* L. both growth in Crete island (Greece), were extracted and their chemical composition was determined. Organically cultivated *O. dictamnus* L., to our knowledge, has never before been studied. The main constituents of the oils: carvacrol (42.9–51.7%) and its isomer, thymol were isolated. The antimicrobial activities of (*a*) the oils, (*b*) the pure compounds, (*b*1) carvacrol, (*b*2) thymol, (*b*3) γ-terpinene, and their mixtures, (*c*1) carvacrol/thymol (6/1), and (*c*2) carvacrol/γ-terpinene (3/1) were evaluated, before and after encapsulation in liposomes. The percentage of the encapsulated carvacrol was also evaluated. The mixtures of carvacrol with thymol and γ-terpinene were also studied, since all compounds act on the cytoplasmic membrane and an additive or synergistic effect could be expected. Finally, the antioxidant activity of the above samples was studied using differential scanning calorimetry (DSC), in order to gain knowledge about their oxidation stability. The observed changes in the thermodynamic and antimicrobial properties of liposomes upon entrapment of carvacrol, thymol, and the mixture, carvacrol/thymol (6/1), were determined.

2. Materials and methods

2.1. Plant material

The aerial parts of the wild *O. dictamnus* specimen were collected from Heraclion region (Crete island, Greece) and offered by E. Kastrinakis (summer, 2004), while the sample of organic cultivation, was a kind offer from G.S. Stivaktakis ("Knossos Aromatic Plants" company) also from Heraclion region in Crete.

2.2. Isolation procedure

The dried aerial parts of *O. dictamnus* (400 g for each specimen) were subjected to hydro-distillation for 4 h, in 2 L of water, using a Clevenger-type apparatus, with a water-cooled oil receiver to reduce formation of artifacts due to overheating during hydro-distillation. The essential oils were collected and dried over anhydrous sodium sulphate (Panreac Quimica S.A. Barcelona, Spain) and stored at 4–6 °C.

2.3. Gas chromatography

GC analyses were carried out on a Perkin–Elmer Clarus 500 gas chromatograph, with a RTX-5 (30 m × 0.25 mm × 0.25 μm) fused capillary column and equipped with a flame ionisation detector (FID). The column temperature was programmed from 60 to 280 °C at a rate of 3 °C/min. The injector and detector temperatures were programmed at 230 and 300 °C, respectively. Helium was used as carrier gas, flow rate 1 ml/min.

2.4. Gas chromatography–mass spectrometry

GC–MS of the essential oils and of the isolated fractions was carried out using a Hewlett Packard 5973–6890 GC–MS operating in EI mode (equipped with a HP-5 MS 30 m × 0.25 mm × 0.25 μm film thickness capillary column). Helium (1 ml/min) was used as carrier gas in both cases. Injection temperature was set at 290 °C and detector temperature at 300 °C. The initial temperature of the column was 60 °C for 5 min; then it was raised to 280 °C at 3 °C/min, and held there for 30 min.

2.5. Identification of components

All components were identified by comparison of their mass spectra with those obtained from authentic samples and/or the NIST/NBS, NIST02, Wiley 575 mass spectral database. They have been also confirmed by comparison of their retention indices (RI) (Van den Dool & Kratz, 1963) and retention times (RT), with either those of authentic compounds or with published data (Adams, 2001; Massada, 1976).

2.6. Fractionation of essential oil and isolation of pure compounds

The isolation procedure was carried out according to Harvala et al. (1987). Essential oil (4.0 ml) were subjected to column chromatography using a glass column (1.5 cm i.d. and 22 cm in length) filled with silica (Kieselgel 60, 70-230 Mesh, Merck Ltd, Germany). The elution solvent was hexane (1300 ml) and ethylacetate (400 ml) (Lab Scan Analytical Sciences, HPLC – purity). Two fractions were obtained. The first (Fraction A) was 272.0 mg, while the second (Fraction B) was 957 mg. Both fractions were analysed with GC and GC–MS in order to identify the isolated components and their relative quantities. Carvacrol was isolated from Fraction B, where it was the major component (84.8%), through preparative TLC (Merck 5715) using a solvent system (toluene/ethyl acetate, 93/7). The purity of the isolated compound was double checked through GC-MS (RI, RT, Peak enrichment), showing, especially for carvacrol, a purity over 99%. Main compounds from Fraction A (i.e. γ-terpinene) were also isolated by TLC (solvent system). Finally, thymol was obtained from commercially available essential oil (Vioryl Inc. Athens, Greece) of *Origanum*.

2.7. Preparation of liposomes

The liposomes were prepared as described by Gortzi, Lalas, Chinou, and Tsaknis (2006), using common lipid compositions (Abdalla & Roozen, 2001) They contained egg L-*a*-phosphatidylcholine (PC) (10 mg/ml) and cholesterol (C) (2 mg/ml) or PC (5 mg/ml) and C (1 mg/ml) were prepared by the mechanical shaking technique (thin film method). PC and C were obtained from Sigma Chemicals Company Ltd. (St. Louis, USA). The 99% purity of the lipids was verified via thin-layer chromatography on silicic acid-coated plates (Merck, Darmstadt, Germany), as described previously (New, 1990). The mixture of lipids was dissolved in chloroform/methanol (3:1) (HPLC purity) in a 50 ml round-bottom flask and the organic solvent was removed by rotary evaporator until a thin film was formed on the walls. When carvacrol or carvacrol/thymol (6/1) or carvacrol/γ-terpinene (3/1) were used (in a quantity of 1.00 mg/ml) as antioxidant or antimicrobial agent, it was dissolved in methanol and then mixed in a round-bottom flask

with PC and C. The organic solvents were evaporated under a stream of nitrogen at 35 °C (above the lipid transition temperature). The lipid film was suspended in 2 ml of a phosphate buffer saline solution (PBS) (pH 7.4) (Sigma Chemicals Company Ltd) and vigorously vortexed for 15 min. Sonication of the preparation (in order to reduce the size and homogenise liposomes) was carried out in a bath-type sonicator (Branson Ultrasonics, Danbury, CT, USA). This suspension was allowed to hydrate for 2 h in the dark at room temperature in order to anneal any structural defects. The compound-incorporated vesicles were separated from the unincorporated compounds by ultracentrifugation. Vesicular dispersions were spun in an ultracentrifuge at 4 °C and 30,000 rpm for 60 min. The supernatant was removed and vesicles were reconstituted in distilled water to prepare multilamellar vesicles (MLV).

2.8. Quantitative analysis of the encapsulated carvacrol in MLV

Samples were analysed on the GC–FID Perkin–Elmer Clarus 500 previously described. The following temperature programme was used: 1 min at 75 °C, followed by an increase in temperature to 130 °C at a rate of 30 °C/min, which was held for 6 min, followed by a heating to 260 °C at a rate of 30 °C/min, which was held for 15 min.

All samples were dissolved in 1.0 ml of pentane and 2 μl of the supernatant were injected into the GC–FID for analysis. The chromatograms were recorded with a TotalChrom integrator. For the integration, a calibration curve was constructed using various concentrations of pure carvacrol (Fluka Inc.). Quantitative control was carried out, using the calibration curve of the carvacrol standards.

2.9. Determination of antioxidant activity using (DSC)

The antioxidant activity of carvacrol and the mixtures: carvacrol/thymol (6/1) and carvacrol/γ-terpinene (3/1) was estimated using DSC before and after encapsulation in liposomes. A Perkin–Elmer DSC-6 calorimeter (Perkin Elmer Corp., Norwalk, CT, USA) was employed to study the sample oxidation stability. The method used was adapted from Gortzi et al. (2006).

The starting temperature of oxidation was determined as the onset temperature of the oxidation peak. The temperature programme was: heat from 30 to 180 °C (100 °C/min), hold for 1 min at 180 °C and finally heat from 180 to 400 °C (10 °C/min).

2.10. Antimicrobial activity

The antimicrobial activities of the oils, the pure substances carvacrol, thymol and their mixtures carvacrol/thymol (6:1) carvacrol/γ-terpinene, before and after liposomal encapsulation, were determined by the diffusion technique (disc method) according to previous reported methods (Meliou & Chinou, 2005). The tests were conducted against four Gram positive bacteria: *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus mutans* (ATCC 31989) and *Staphylococcus viridans* (ATCC 19952), four Gram negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047) and *Klebsiella pneumoniae* (ATCC 13883), three human pathogenic fungi: *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13801) and *Candida glabrata* (ATCC 28838), as well as against the food-pathogen, *Listeria monocytogenes* (11994). Standard antibiotics netilmicin and intraconazole (both from Sanofi, Diagnostics Pasteur, Paris, France) were used in order to control the sensitivity of the tested bacteria and fungi, respectively. The experiments were repeated three times and the results (mm of zone of inhibition) were expressed as average values.

3. Results and discussion

3.1. Essential oil components

Origanum species are characterised by the presence of phenolic compounds (Chorianopoulos et al., 2004; Skoula et al., 1999). The aromatic monoterpenes mainly presented in the genus *Origanum* are: *p*-cymene, thymol, carvacrol and their precursor, γ-terpinene (Skoula et al., 1999), previous chemical analyses on common oregano (*Origanum vulgare* spp. *hirtum*) and *O. dictamnus* have shown that thymol was the major constituent of the essential oil from *O. vulgare*, while its isomer carvacrol was the major component of the essential oil from *O. dictamnus* (Chorianopoulos et al., 2004). *p*-Cymene and γ-terpinene also appeared as major components of the essential oil from *O. dictamnus* (Sivropoulou et al., 1996; Skoula et al., 1999).

The high amount of carvacrol found in common oregano (*O. vulgare* ssp. *hirtum*), in previous studies has also been observed in several other Greek wild populations of this taxon and it should be noted that, in some cases, thymol, instead of carvacrol, is the major component of the Greek oregano essential oils. The hydrodistillation of the aerial parts of the two samples of *O. dictamnus* (wild and organic cultivated) gave, for both cases, an orange–yellow oil in good yield (0.41% and 0.46% w/w, respectively), with distinct odour. Carvacrol, in accordance with previous literature data (Chorianopoulos et al., 2004; Harvala et al., 1987; Lagouri, Blekas, Tsimidou, Kokkini, & Boskou, 1993) proved to be the major component for both analysed samples (Table 1) (51.7% and 42.9%, respectively). The biosynthetic precursors of carvacrol, the monoterpene hydrocarbons *p*-cymene (8.78–10.1%) and γ-terpinene (14.1–9.20%), were also present in high amounts.

3.2. Fractionation of the essential oils

The fractionation method of *O. dictamnus* essential oil was based on previous studies (Harvala et al., 1987) concerning the isolation of the main components, carvacrol and thymol. As expected, the GC–MS analysis of both fractions showed that Fraction B consisted mainly of the following alcohols and phenols (Table 1): linalool, terpinene-4-ol, thymol, carvacrol, while Fraction A contained the rest components of the essential oil. The phenols, carvacrol and its isomer thymol, added up to 84.8% of total components of Fraction B.

3.3. Quantitative analysis of encapsulated carvacrol in liposome vesicles

To our knowledge there are no previous references to the efficient encapsulation of thymol and carvacrol in liposomes and their *in vitro* successful application. In this study the two phenol isomers were successfully encapsulated in liposomes PC/C (10/2) and PC/C (5/1). The quantitative analysis of liposome vesicles containing carvacrol, showed that only a small amount of the starting quantity of carvacrol was incorporated. From the 1.07 mg of pure carvacrol added for the incorporation a percentage of only 4.16% (0.045 mg/ml) was encapsulated inside the liposome vesicle.

3.4. Antioxidant activity of thymol and carvacrol before and after their encapsulation in liposomes

It has been established that thymol and carvacrol inhibit the peroxidation of liposome phospholipids in a concentration-dependent manner (Aeschbach et al., 1994). The inhibition of oxidation by the essential oils from *Origanum* plants was highly dependent on the contents of carvacrol and thymol (Lagouri et al., 1993).

Table 1
Chemical constituents (% of total) of both essential oils from *Origanum dictamnus*

A/A	Component	*O. dictamnus* wild	*O. dictamnus* cultiv.	RT[a]	RI[b]	Method of identification
1.	*trans*-2-hexenal	–	0.13	3.87	798	a,b,d
2.	α-Thujene	1.44	1.17	5.93	930	a,b,d
3.	α-Pinene	0.63	0.59	6.16	936	a,b,d
4.	Camphene	0.15	0.12	6.63	949	a,b,d
5.	β-thujene	–	0.27	7.59	972	a,b,d
6.	Sabinene	0.22	0.51	7.68	974	a,b,d
7.	β-Pinene	0.12	–	8.36	988	a,b,d
8.	1-Octen-3-ol	0.27	–	8.39	989	a,b,d
9.	Myrcene	1.63	0.98	8.42	990	a,b,d
10.	3-Octanol	0.11	–	8.47	991	a,b,d
11.	α-Phellandrene	0.33	–	8.88	999	a,b,d
12.	δ-3-carene	0.09	–	8.90	1009	a,b,d
13.	α-Terpinene	2.82	2.39	9.53	1014	a,b,c,d
14.	π-Cymene	8.78	10.1	10.84	1041	a,b,d
15.	Limonene	0.48	–	10.86	1042	a,b,c,d
16.	*trans*-β-ocimene	0.10	–	11.01	1045	a,b,d
17.	γ-Terpinene	14.1	9.20	12.57	1073	a,b,d
18.	*cis*-Sabinene Hydrate	1.07	0.88	12.64	1075	a,b,d
19.	Terpinolene	0.10	0.13	13.14	1083	a,b,d
20.	Linalool	1.10	2.49	14.27	1101	a,b,d
21.	Borneol	–	0.37	16.27	1147	a,b,c,d
22.	iso-borneol	0.42	–	16.25	1146	a,b,d
23.	*trans*-Sabinene hydrate	–	1.23	16.30	1147	a,b,d
24.	Terpinen-4-ol	0.46	1.36	17.16	1165	a,b,d
25.	*cis*-Dihydrocarvone	0.09	–	17.89	1179	a,b,d
26.	*trans*-Dihydrocarvone	0.11	–	17.93	1180	a,b,d
27.	Carvacrol methyl ether	0.28	–	22.66	1290	a,b,d
28.	L-Carvone	1.52	–	22.70	1291	a,b,c,d
29.	Thymol	0.13	0.61	23.78	1312	a,b,c,d
30.	Carvacrol	51.7	42. 9	24.08	1318	a,b,c,d
31.	Dihydrocarvyl acetate	0.27	–	25.04	1336	a,b,d
32.	α-Cubebene	0.38	0.48	25.41	1343	a,b,d
33.	*cis*-Carvyl acetate	0.14	–	25.50	1344	a,b,d
34.	α-Copaene	2.24	2.91	27.57	1381	a,b,d
35.	β-Bourbonene	0.19	–	27.89	1386	a,b,d
36.	β-Cubebene	0.32	1.45	27.99	1388	a,b,d
37.	4,8-alpha-epoxy caryophyllene	2.6	–	28.90	1404	a,b,d
38.	β-Caryophyllene	–	3.88	29.03	1408	a,b,d
39.	α-Humulene	0.14	0.34	30.47	1449	a,b,d
40.	Epi-bicyclosesquiphellandrene	0.34	1.36	30.89	1460	a,b,d
41.	γ-Cadinene	–	0.15	31.13	1467	a,b,d
42.	Germacrene D	0.47	1.07	31.56	1478	a,b,c,d
43.	β-Bisabolene	0.42	0.55	32.75	1509	a,b,d
44.	α-Amorphene	–	0.16	32.75	1514	a,b,d
45.	δ-Cadinene	1.07	2.33	33.35	1524	a,b,d
46.	α-Cadinene	–	0.26	33.72	1533	a,b,d
47.	Trimethylbicyclo-octan-2-one	–	1.47	34.98	1564	a,b,d
48.	Caryophyllene oxide	0.21	1.11	35.42	1574	a,b,d
49.	T-cadinol	0.12	1.03	38.35	1640	a,b,d
Total		97.2	94.2			

[*] Compounds listed in order of elution from a HP-5 MS column. a, retention time; b, retention index; c, peak enrichment; d, mass spectra.
[a] Retention times (min).
[b] Retention indices (RI) on HP-5 MS capillary column.

Previous studies (Yanishlieva, Marinova, Gordon, & Raneva, 1999) have shown that thymol is a better antioxidant in lipids (two lipid systems-purified triacylglycerols of lard and sunflower oil) than is carvacrol, due to the greater steric hindrance of its phenolic group. Therefore, in order, to increase the knowledge on compound interaction with lipid membrane, the antioxidant activities of thymol and carvacrol, before and after their encapsulation in PC-based liposomes, were investigated in this study.

Auto-oxidation of fats, fatty acids and lipids is a well-established exothermic process and methods of thermal analysis, e.g. DSC, are valuable for the study of the thermostability and thermo-oxidation while the kinetic parameters of the non-inhibited and inhibited fatty acid oxidation can also be determined (Gortzi et al., 2006). The antioxidant activity is evaluated by the extrapolated temperature at the start of the oxidation process (Litwinienko, Kasprzycka-Guttman, & Studzinski, 1997), based on the measurements of the incubation period. Fig. 1 presents the DSC curves of *O. dictamnus* essential oils, pure components and their mixtures, before and after their encapsulation in liposomes (PC/C, 10/2 and 5/1). An exothermic peak was observed in the range 258–392 °C, related to the auto-oxidation process of the samples. Using the curves, the onset temperature at which the auto-oxidation process begins was determined (Gortzi et al., 2006) as follows: 362 °C (empty liposomes 5:1), 372 °C (empty liposomes 10:2), 258 °C (carvacrol), 273 °C (thymol), 302 °C (carvacrol/thymol-6/1), 392 °C (liposomes 5/1+carvacrol/thymol 6/1) and 382 °C (liposomes 10/2+carvacrol/thymol 6/1).

The tested compounds presented better antioxidant action when encapsulated than when in pure form. The temperature, at the beginning of the oxidation reaction of the encapsulated samples, was significantly higher. The modified antioxidant action after encapsulation was expected since the complex: liposome

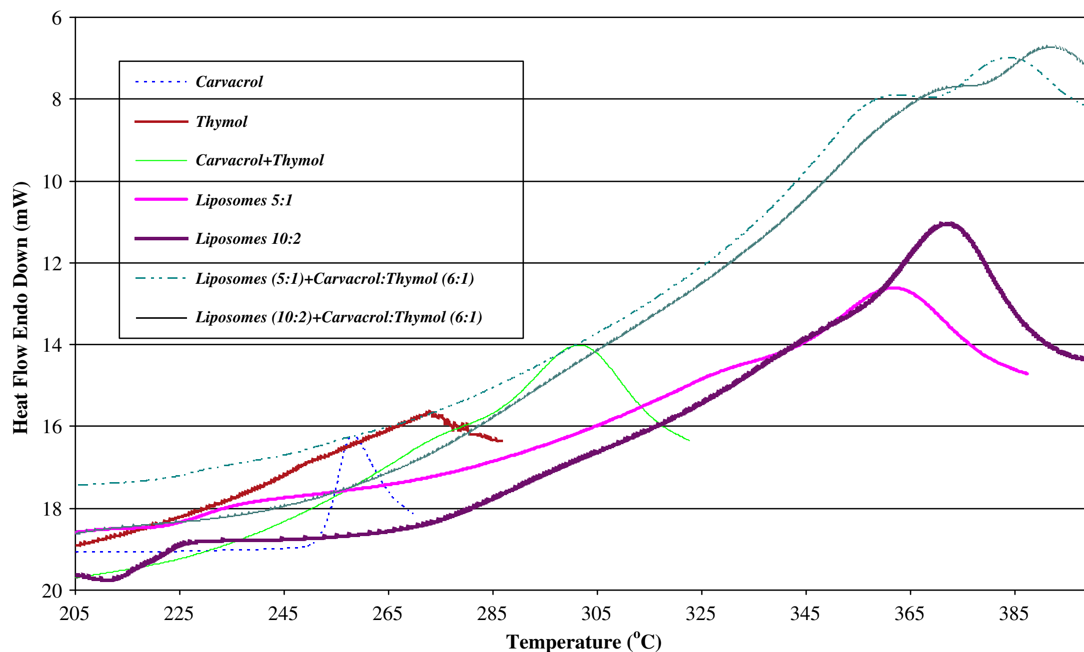

Fig. 1. DSC oxidation (DSC-heat flow/temperature) of pure compounds encapsulated in liposomes.

membrane-antioxidants, possesses new physicochemical characteristics and bioactivity, depending on: structure, size and z-potential of the preparation (Gortzi et al., 2006; Sulkowski, Pentak, Nowak, & Sulkowska, 2005). The antioxidant action of pure liposomes (control sample) appeared to be much lower than that of the liposome preparations with encapsulated compounds, implying that the encapsulation of the components and the possible link of the lipophilic compounds in lipid bilayers stabilized the liposome membranes.

3.5. Antimicrobial activity of the essential oils

In the present study, the antimicrobial activities of essential oils isolated from *O. dictamnus,* (wild and organically cultivated specimen), pure carvacrol and thymol, and the mixtures of carvacrol with thymol (6/1) and γ-terpinene (3/1) were determined before and after encapsulation in liposomes (Table 2). The essential oils from both assayed species showed comparable activities against all tested microbial strains. Pure compounds (carvacrol, thymol), proved more active than the oil, and their antimicrobial activities were significantly increased after their encapsulation in liposomes. Thymol exhibited stronger activity than did carvacrol, against most microbial types while no inhibition was found in controls, consisting of empty liposomes.

Various studies, concerning *Origanum* species and *O. dictamnus* in particular, have shown that their oils possess strong antimicrobial activity; this activity could be attributed to their high percentage of phenolic compounds and, specifically, carvacrol, thymol, *p*-cymene and their precursor γ-terpinene (Sivropoulou et al., 1996). Most studies, investigating the action of essential oils against food spoilage organisms and food-borne pathogens, generally agree that they are slightly more active against Gram positive than Gram negative bacteria (Burt, 2004). A small quantity of carvacrol (4.16%) was successfully encapsulated in liposomes (Table 3) and appeared to be more active than the pure natural compound which was present in higher quantity. In fact, a small quantity of about 25.0×10^{-8} g/ml (encapsulated in liposomes) showed equal or improved activity than did the pure compound at the concentration of 6.0×10^{-3} g/ml.

In previous studies, it has been showed (Ultee et al., 2000) that carvacrol interacts with membrane, where it dissolves in the phospholipid bilayer and is assumed to align between the fatty acid chains. The interaction of lipophilic compounds with the phospholipid membrane components causes dramatic changes in the structure of the membrane (Ultee et al., 2000). This distortion of the physical structure would cause expansion and destabilization of the membrane, increasing membrane fluidity which, in turn, would increase passive permeability (Ultee et al., 2000). The amphipathicity of phenolic compounds such as thymol and carvacrol can explain their interactions with biomembranes and thus the antimicrobial activity (Cristiani et al., 2007).

In fact, the hydrophilic part of the molecule interacts with the polar part of the membrane, while the hydrophobic benzene ring and the aliphatic side chains are buried in the hydrophobic inner part of the bacterial membrane (Cristiani et al., 2007). Furthermore, the involvement of the hydroxyl group in the formation of hydrogen bonds and the acidity of these phenolic compounds may have other possible explanations.

The results lead us to speculate that the antimicrobial activity of monoterpenes may be the result of this mechanism and it is significantly influenced by liposome physicochemical characteristics (namely composition, size and charge) and by the composition of the bacterial membrane. In the present study, the two monoterpenes stabilized the liposomes membranes instead of swelling them. The increase in the onset temperature, with the entrapment of monoterpenes, led us to conclude, that encapsulated compounds, at this low studied concentration, act as stabilizers of the PC/Chol-based vesicles. A possible explanation is that the addition of cholesterol, in the liposome composition used, caused changes in the degree of head group dissociation and probably in the interaction with lipophilic compounds. A cholesterol molecule will be oriented with its steroid nucleus among the fatty acyl chains of phospholipid molecules and its hydroxyl group facing towards the water face. Therefore, cholesterol is often added to liposomes to improve their *in vivo* and *in vitro* stability (Chan, Chen, Chiu, & Lu, 2004). In addition, PC-liposomes can interact with cells in many ways (inter-membrane transfer, contact release, absorption, fusion, phagocytosis). The mechanism of interaction depends

Table 2
Antimicrobial activities (mm of zone of inhibition) of *Origanum dictamnus* (cultivated and wild) essential oils, pure carvacrol and thymol before and after encapsulation in liposomes

Component	Lipid component	Microbial strain											
		S. aureus	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*	*S. mutans*	*S. viridans*	*C. albicans*	*C. tropicalis*	*C. glabrata*	*L. monocytogenes*
O. dictamnus wild	–	18.0 ± 0.10	17.0 ± 0.07	12.0 ± 0.20	11.0 ± 0.05	8.3 ± 0.20	9.1 ± 0.03	16.0 ± 0.04	12.0 ± 0.20	10.2 ± 0.27	10.6 ± 0.22	10.3 ± 0.22	12.0 ± 0.20
O. dictamnus cultivated	–	17.1 ± 0.05	17.1 ± 0.02	10.2 ± 0.10	12.0 ± 0.10	10.0 ± 0.10	10.1 ± 0.10	15.0 ± 0.05	13.0 ± 0.04	9.0 ± 0.03	11.0 ± 0.1	13.0 ± 0.02	12.1 ± 0.20
Thymol 100%	–	13.2 ± 0.02	12.1 ± 0.33	11.3 ± 0.22	10.1 ± 0.01	11.1 ± 0.03	11.1 ± 0.03	14.2 ± 0.05	14.1 ± 0.33	8.1 ± 0.09	10.2 ± 0.06	11.1 ± 0.04	10.1 ± 0.05
Thymol 100%	PC:C[a] (5:1 mg/ml)	15.7 ± 0.22	15.0 ± 0.67	13.3 ± 0.22	12.0 ± 0.01	10.1 ± 0.02	12.1 ± 0.00	16.3 ± 0.22	16.0 ± 0.22	11.7 ± 0.22	11.1 ± 0.00	11.1 ± 0.00	15.1 ± 0.67
Carvacrol 100%	–	15.0 ± 0.01	12.1 ± 0.23	10.1 ± 0.4	10.2 ± 0.42	10.1 ± 0.50	12.2 ± 0.05	13.0 ± 0.08	12.0 ± 0.81	10.0 ± 0.08	11.0 ± 0.05	11.1 ± 0.22	12.0 ± 0.20
Carvacrol 100%	PC:C (5:1 mg/ml)	16.0 ± 0.01	16.1 ± 0.03	12.1 ± 0.04	12.3 ± 0.67	11.0 ± 0.51	14.0 ± 0.90	17.0 ± 0.45	17.1 ± 0.05	11.0 ± 0.50	12.0 ± 0.06	13.1 ± 0.08	16.0 ± 0.05
Carvacrol 100%	PC:C(10:2 mg/ml)	15.2 ± 0.01	12.1 ± 0.22	12.1 ± 0.30	12.3 ± 0.22	10.3 ± 0.22	12.0 ± 0.10	14.0 ± 0.10	15.1 ± 0.01	11.1 ± 0.03	11.0 ± 0.03	12.3 ± 0.22	14.0 ± 0.01
Carvacrol/Thymol (6:1)	–	14 ± 0.01	14.1 ± 0.03	10.3 ± 0.22	10.3 ± 0.04	10.1 ± 0.05	12.1 ± 0.42	12.3 ± 0.4	12.2 ± 0.01	10.1 ± 0.01	11.2 ± 0.00	11.1 ± 0.03	14.0 ± 0.41
Carvacrol/Thymol (6:1)	PC:C(5:1 mg/ml)	17.0 ± 0.33	16.1 ± 0.33	13.3 ± 0.22	12.0 ± 0.50	13.0 ± 0.50	14.1 ± 0.55	17.1 ± 0.33	17.0 ± 0.30	12.1 ± 0.04	14.1 ± 0.3	14.1 ± 0.05	17.0 ± 0.30
Carvacrol/γ-terpinene(3/1)	–	13.3 ± 0.22	12.2 ± 0.23	11.1 ± 0.01	10.1 ± 0.01	10.1 ± 0.03	11.0 ± 0.22	11.3 ± 0.22	12.0 ± 0.24	9.2 ± 0.1	10.2 ± 0.22	11.2 ± 0.22	12.1 ± 0.33
Carvacrol/γ-terpinene (3/1)	PC:C (10:2 mg/ml)	13.0 ± 0.33	13.1 ± 0.33	12.3 ± 0.22	10.2 ± 0.22	9.2 ± 0.00	11.3 ± 0.04	13.4 ± 0.05	14.1 ± 0.22	9.3 ± 0.22	10.4 ± 0.01	11.4 ± 0.01	14.1 ± 0.40
Netilmicin	–	21	25	20	23	22	24	24	25	–	–	–	22
Intraconazole	–	–	–	–	–	–	–	–	–	20	22	23	–

Results are means of triplicate determinations.
[a] PC, phosphatidylcholine; C, cholesterol.

Table 3
Encapsulation efficiency of liposome suspensions

Lipid composition	Type of liposome	Dry weight of preparation (mg)	Concentration of encapsulated carvacrol (mg/ml)	% of encapsulation
PC:C (5:1 mg/ml	MLVs	11	0.045	4.16

on the cell type (cell wall/membrane composition), as well as the liposome membrane physicochemical characteristics. The use of liposomal formulation improves the cellular transport and releases the active component inside the cell (Shoji & Nakashima, 2004).

The dramatically increased antimicrobial activity, after the encapsulation in liposomes, can promote the use of the above-mentioned natural products as potent preservative and conservation agents, not only in the food industry but also in cosmetics and medical preparations.

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