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Reevaluation of Antimicrobial and Antioxidant Activity of *Thymus* spp. Extracts before and after Encapsulation in Liposomes

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ABSTRACT

The antioxidant and antimicrobial activity of four *Thymus* species (*boissieri, longicaulis, leucospermus,* and *ocheus*) extracts were determined. Two methods (Rancimat and malondialdehyde by high-performance liquid chromatography) were used to measure the antioxidant action in comparison with common commercial antioxidants, including butylated hydroxytoluene and α-tocopherol. The extracts that presented high antioxidant activity were encapsulated in liposomes and their antioxidant action was again estimated. Thermal-oxidative decomposition of the samples (pure liposomes and encapsulating extracts) was studied using the differential scanning calorimetry method. The modification of the main transition temperature for the lipid mixture and the splitting of the calorimetric peak in the presence of the antioxidants were also demonstrated by differential scanning calorimetry. All extracts showed antioxidant and antimicrobial activities. Some extracts showed superior or equal antioxidant activity to α-tocopherol. When the extracts were encapsulated in liposomes, their antioxidant as well as antimicrobial activities proved to be superior from the same extracts in pure form.

The excessive use of chemical preservatives, many of which are suspected for unwanted side effects, increases the consumer pressure on food manufacturers to remove them from their products or to adopt "natural" alternatives *(4)*. The use of plants as antioxidants in processed foods is becoming increasingly important in the food industry as an alternative to synthetic antioxidants *(13)*. Extracts of many plants, such as spices, herbs, cocoa shells, coffee beans, oats, tea, beans, sesame oil, tomatoes, rose hips, osage orange, amla fruit, onions, peppers, olive leaves, and soybeans, were shown to have various degrees of antioxidant activity in different fats and oils *(5)*. The extracts of many plants exhibit antibacterial and antifungal properties *(16)*. There is considerable research interest in the possible use of natural products, such as essential oils and extracts of edible and medicinal plants, herbs, and spices, for the development of alternative food additives in order to prevent the growth of pathogens or to delay the onset of food spoilage *(4)*.

Thymus spp. are perennial herbaceous plants and subshrubs that are woody at the base and contain numerous branches. The leaves are about 3 to 8 mm long. The white to pale purple, tubular, two-lipped flowers are arranged in whorled clusters. There are over 300 species of this hardy genus that are native to southern Europe and Asia *(8)*, more than 60 of which are native to Europe. To the Ancient Greeks, thyme was an emblem of bravery. Women would embroider a bee hovering over thyme on scarves, which were presented to their champions. Thyme is a traditional culinary ingredient in many dishes. The most widely used medicinal thymes are *Thymus vulgaris* and *Thymus serpyllum,* the latter having a more sedative effect. Herbal practitioners prescribe *T. vulgaris* in cases of dry cough, laryngitis, bronchitis, asthma, urinary infection, and chronic gastritis. It is applied externally for fungal infections, rheumatism, arthritis, tonsillitis, and gum infections. Reports concerning antimicrobial and antioxidant properties of many *Thymus* spp. have been numerously reported in the literature, and the essential oils have frequently remained as the focus of many studies *(4, 14, 23)*. It is noteworthy, however, that the antibacterial activities of the aforementioned essential oils against foodborne bacteria are limited, prohibiting their commercial application in food preservation or the extension of the processed food shelf life. Thus, there is vigorous research activity directed toward the discovery of more potent essential oils against foodborne bacteria *(4)*. This problem, however, can be overtaken by the use of liposomes.

Liposomes have been used in drug delivery in a wide range of applications such as chemotherapy, blood surrogates, and skin treatment products *(6)*. They resemble the lipid membrane part of cells. Numerous biological processes in living organisms depend on the action of small unilamellar liposomes. Liposomes are spherical particles that encapsulate a fraction of the solvent in which they freely diffuse (float) into their interior *(19)*. This ability helped in overtaking the problem of reduced solubility of lipids in many plant extracts. Liposomes have one, several, or mul-

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tiple concentric membranes. They are constructed of polar lipids that are characterized by having a lipophilic and hydrophilic group on the same molecules *(2, 11)*. Uniquely, liposomes can encapsulate both hydrophilic and lipophilic materials (plant extracts). It is also important that liposomes encapsulating extracts possess new physicochemical characteristics and bioactivity *(20)* that can enhance extracts' original activity. Typical liposome compositions include lecithins (phosphatidyl cholines) and kephalins (phosphatidyl ethanolamines), often containing negatively charged lipids, such as phosphatidyl serine and phosphatidyl inositol. In addition to ceramides, such as sphingomyelin, sterols (cholesterol, ergosterol, sitosterol, etc.) are also included. Liposomes have emerged as a tool for food product developers after years of being little more than a laboratory curiosity. However, their lack of stability and high cost were considered as barriers for acceptance in the food industry. These problems have now been largely overcome *(6)*. Liposomes can be effective carriers for nutritionally valuable ingredients. For example, liposomes, as a microstructural component of breast milk, may play an important role in enhanced nutrient absorption, colloidal stability, and immunogenicity *(6)*. Other applications include iron fortification of food *(27)*; emulsification for the preparation of bread *(1)*; help in the control of flavor, texture, and other qualities in food products, such as delivering both flavors and oils that are trapped inside the liposomes (such flavors and oils are released then into the mouth) *(1)*; reduction of the ripening time of cheese by 30 to 50%; fermentation of and improvement in the flavor of beer *(6, 7)*; and many others.

In this work, we evaluated the antioxidant and antimicrobial activity of extracts from *Thymus* spp. (indigenous in Greece) and compared them with known synthetic butylated hydroxytoluene (BHT) or natural (α-tocopherol) antioxidants and antimicrobial agents (netilmicin and 5-flucytocine). The fractions were also encapsulated in liposomes and their modified activities were reevaluated. Liposomes were applied because, due to their unique properties, they are able to enhance the performance of products by increasing ingredient solubility (easier incorporation of water-soluble compounds into oil-based products), thereby improving bioavailability and in vivo and in vitro stability *(6)*.

MATERIALS AND METHODS

***Thymus* spp.** Aerial parts of *Thymus longicaulis* C. Presl. subsp. *chaubardii* (Boiss. & Heldr.) Jalas were collected in June 2000 and identified by E. Kalpoutzakis from the mountain Kithaironas (elev. 1,100 m) and then deposited at the Herbarium of the Division of Pharmacognosy (Chemistry of Natural Products, Athens, Greece). The plant is widespread from southeast France and Sicily to northeast Anatolia (Turkey).

Thymus boissieri Halacsy var. *boissieri* was collected in June 2000 and identified by E. Kalpoutzakis from the mountain Timphi (Ipiros, Greece) (elev. 1,900 m) and then deposited at the Herbarium of the Division of Pharmacognosy (Chemistry of Natural Products). The plant is endemic of Greece and Anatolia (Turkey).

Thymus leucospermus Hartvig was collected in June 2000 and identified by E. Kalpoutzakis from the mountain Timphi (Ipiros, Greece) (elev. 1,850 m) and then deposited at the Herbarium of the Division of Pharmacognosy (Chemistry of Natural Products). The plant is endemic of Greece and Anatolia (Turkey).

Thymus ocheus Heldr. & Sart. ex Boiss. from the mountain Ochi (East Greece, elev. 750 m) was collected in June 2001 and identified by E. Evergetis and then deposited at the Herbarium of the Division of Pharmacognosy (Chemistry of Natural Products). The plant is endemic of Greece and especially mount Ochi.

The dried plant materials were extracted *(25)* with dichloromethane (CH_2Cl_2) or methanol (MeOH) and the extracts were concentrated under vacuum (rotary evaporator).

The evaluation of the antioxidant activity was carried out using the Rancimat method, the determination of malondialdehyde (MDA) by high-performance liquid chromatography (HPLC) (after oxidation with the accelerated UV method) and differential scanning calorimetry (DSC).

Rancimat. The method used was adapted from Lalas and Tsaknis *(10)*. Two and one-half grams of sunflower oil and added antioxidant (pure extract, BHT, α-tocopherol; Sigma Chemicals Company Ltd., St. Louis, Mo.) (in various concentrations) was accurately weighed into the reaction vessel of the Rancimat 679 (Metrhom LTD, Herisau, CH 9101, Switzerland) along with another vessel with a sample of sunflower oil without antioxidant (control) (Elais S.A., Athens, Greece). One milliliter of the appropriate solvent (methanol or dichloromethane) was added in order to dissolve the antioxidant and mixed well. The reaction vessels were placed in a Rancimat 679. The conditions were set at 90°C and 15 liters/h. The protection factor (PF) was calculated as follows: PF = (induction period with antioxidant)/(induction period without antioxidant). A protection factor greater than 1 indicates inhibition of the lipid oxidation. The higher the value, the better the antioxidant activity *(9)*.

Determination MDA by HPLC. The determination of MDA was carried out after accelerated oxidation of sunflower oil under UV light. The oxidation method used was adapted from Lalas and Tsaknis *(10)*. Ten grams of sunflower oil and added antioxidant (pure extract, BHT, or α-tocopherol in concentrations of 100 and 150 ppm, which are under the maximum permitted dose of BHT for food [ie., 200 ppm]) was accurately weighed into a glass petri dish (87 mm inside diameter and 15 mm in height) and mixed well. The dishes were placed (half immersed) in a water bath (50°C) directly exposed to UV light produced by a lamp situated 50 cm above (General Electric 260 nm UV Germicidal lamp G25T8, 25 W, 45 cm in length and 3 cm in diameter) for 12 h. Then, the oils were stored in dark glass bottles with nitrogen at −16°C until analysis (MDA by HPLC) performed on the same day. The method used for the determination of MDA was adapted from Tsaknis et al. *(24)*. HPLC was performed using a Waters System, which consisted of a Waters 600E HPLC pump and Waters 486 Tunable Absorbance Detector (Millipore Corporation, Waters Chromatography Division, Milford, Mass.; detection limit: 6.3×10^{-8} mol/kg of oil).

Preparation of lipid vehicles. Liposomes containing phosphatidyl choline (PC) (10 mg/ml) and cholesterol (C) (2 mg/ml) (for determination of antioxidant and antimicrobial activity) or PC (10 mg/ml), C (2 mg/ml), and phosphatidyl glycerol (PG) (1 mg/ml) (for determination of antimicrobial activity) were prepared by the mechanical shaking technique (thin film method) *(15)*. The mixture was dissolved in chloroform-methanol (3/1) and the organic solvents were removed by a rotary evaporator. When *Thymus* spp. extracts were used (in a quantity of 1.6 mg) as antioxidants or antimicrobial agents, they were dissolved in dichloro-

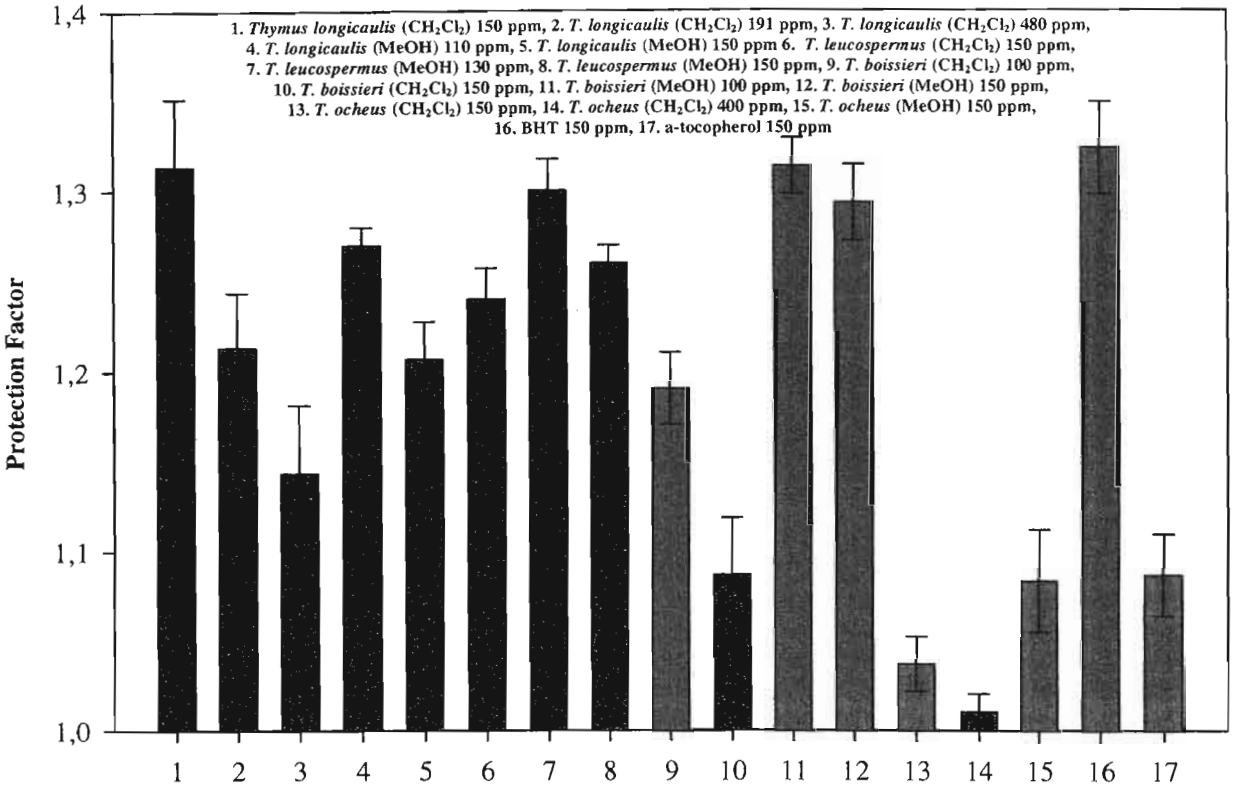


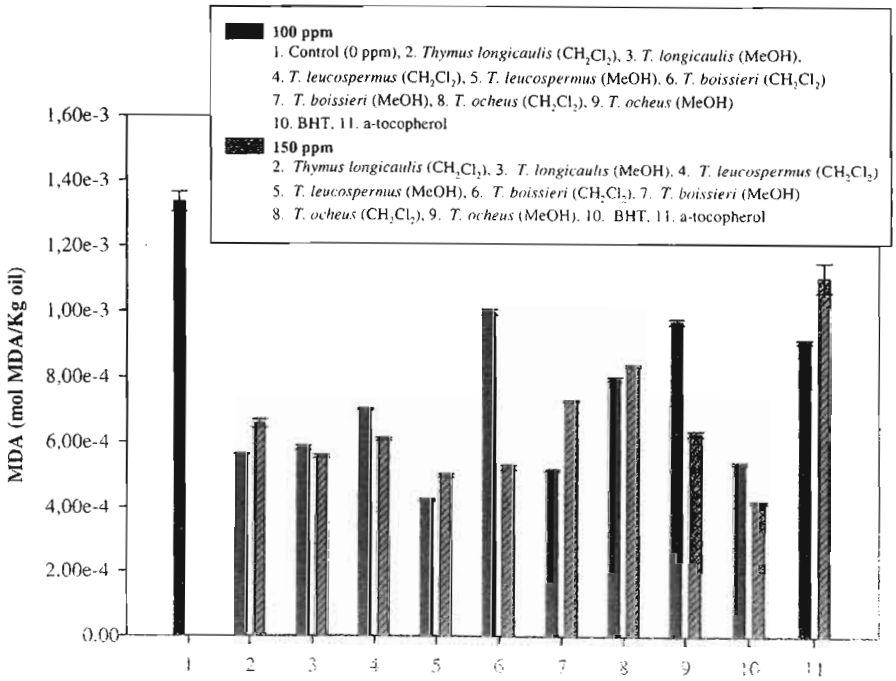
FIGURE 1. *Protection factor of* Thymus *spp. pure extracts in sunflower oil. Values are means of triplicate determinations.*

methane-methanol (2/1) and then mixed in a round bottom flask with PC and C or PC, C, and PG, respectively. 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 phosphorus buffer saline (pH 7.4) (Sigma) and vigorously vortexed for 15 min at 35°C. This suspension was allowed to hydrate for 2 h in the dark at room temperature and then centrifuged at 6,500 rpm at 4°C (Sorvall General-Purpose RC-3 Automatic Refrigerated Centrifuge, Ivan Sorvall, Inc., Newtown, Conn.) in order to not dispose incorporated molecules of extracts or lipids from the liposome suspension (supernatant). The liposomes obtained were multilamellar vehicles (MLV).

The prepared liposomes were freeze-dried with a Virtis model (Sentry 5L, Virtis Company, Inc., Gardiner, N.Y.).

DSC. The extracts that presented high antioxidant activity (as determined by Rancimat and MDA) were encapsulated in liposomes and their antioxidant action was again estimated using

FIGURE 2. *Malondialdehyde (MDA) concentration in sunflower oil after UV accelerated method with various concentrations of pure extracts (100 and 150 ppm). Values are means of triplicate determinations.*



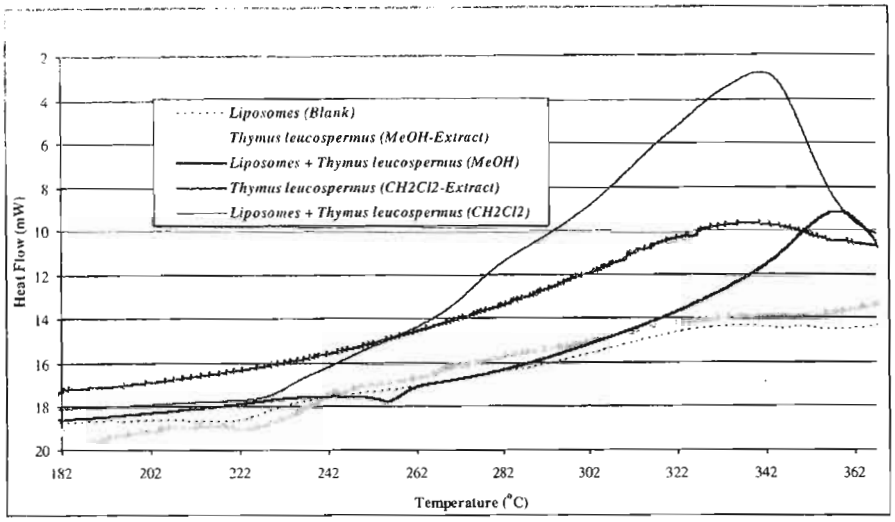


FIGURE 3. *DSC oxidation (DSC using heat flow and temperature) of* Thymus leucospermus *pure extracts and encapsulated in liposomes. Values are means of triplicate determinations.*

DSC. A Perkin Elmer DSC-6 calorimeter (Perkin Elmer Corp., Norwalk, Conn.) was used to study the phase transition characteristics of MLV liposomes and the oxidation stability of the samples.

The method for the determination of phase transition was adapted from Socaciu et al. *(18).* The phase transition of all samples (pure liposomes and encapsulating extracts) was determined against a reference (empty crucible). For all measurements, 100 μl of liposomal suspension was weighed and transferred to the sample compartment. Samples were heated in temperature range of −10 to 70°C at a scanning rate of 0.5°C/min. Five cycles of heating and cooling were used to equilibrate the samples before recording the definitive calorimetric curve. All determinations were performed in triplicate. The enthalpy change ΔH (J/g) or Tc-values were calculated by computer processing of the experimental data and represented as mean ± standard deviation values (data not shown).

Oxidative stability was determined using the method of Tan and Che Man *(22).* Samples of 4 mg were placed in DSC aluminum crucibles with closed lids that were perforated by a 1-mm-diameter hole in the center in order to allow the sample to be in contact with the oxygen stream. The purge gas foaming in the reaction atmosphere was oxygen. The starting temperature of oxidation was determined as the onset temperature of the oxidation peak. The temperature program was as follows: heat from 30 to 180°C (100°C/min), hold for 1 min at 180°C, and finally heat from 180 to 390°C (10°C/min).

Antimicrobial bioassay. The antibacterial activities of the extracts (only for *T. longicaulis* and *T. ocheus*) were determined, using the diffusion technique of Bauer-Kirby (disc method) *(3),* by measuring the zone of inhibition against four gram-positive bacteria, including *Staphylococcus aureus* (ATCC 25923), *Streptococcus mutans* (ATCC 31989), *Streptococcus viridans* (ATCC 19952), and *Staphylococcus epidermidis* (ATCC 12228); four gram-negative bacteria, including *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922), *Enterobacter cloacae* (ATCC 13047), and *Klebsiella pneumoniae* (ATCC 13883); three human pathogenic fungi, including *Candida albicans* (ATCC 10231), *Candida tropicalis* (ATCC 13801), and *Candida glabrata* (ATCC 28838); and against the food-pathogen bacteria *Listeria monocytogenes.* Standard antibiotics netilmicin (Sanofi, Diagnostics Pasteur, Paris, France) and 5-flucytocine (Sanofi, Diagnostics Pasteur) were used in order to control the sensitivity of the tested bacteria and fungi, respectively. The tested compounds were dissolved in MeOH. For each experiment a control disc with pure solvent was used as blind control. All the paper discs had a diameter of 6 mm and were deposited on the surface

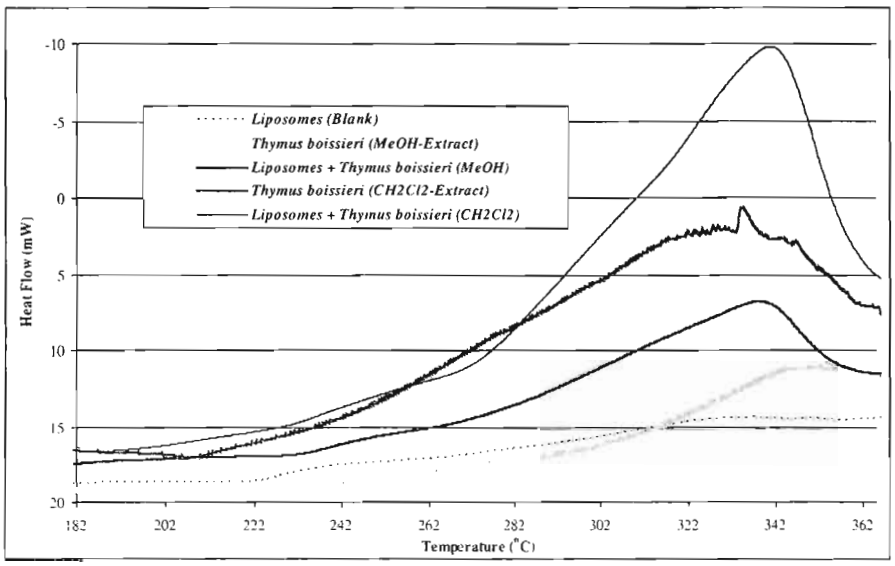


FIGURE 4. *DSC oxidation (DSC using heat flow and temperature) of* Thymus boissieri *pure extracts and encapsulated in liposomes. Values are means of triplicate determinations.*

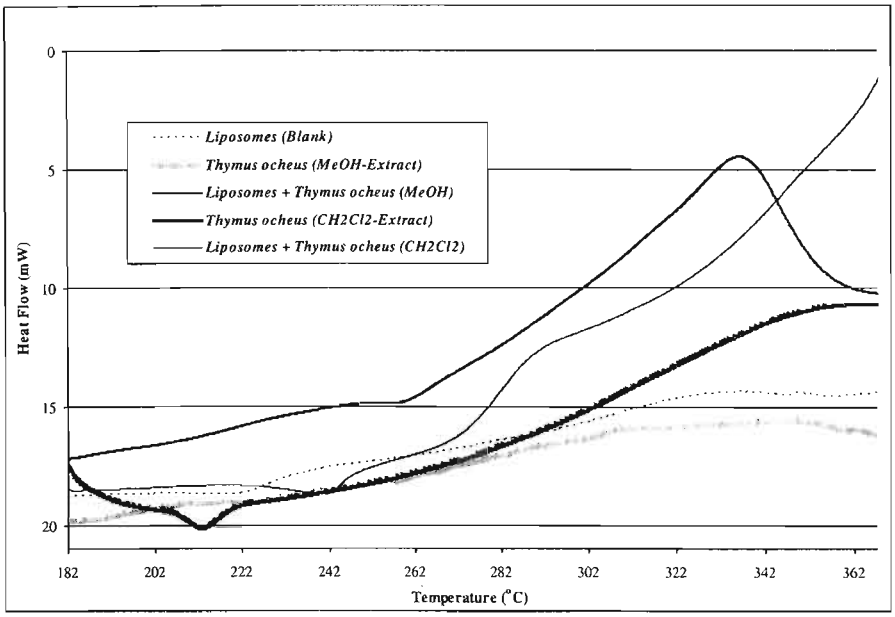


FIGURE 5. *DSC oxidation (DSC using heat flow and temperature) of* Thymus ocheus *pure extracts and encapsulated in liposomes. Values are means of triplicate determinations.*

of the seeded Trypticase soy agar (Scharlau Chemi S.A., Barcelona, Spain) petri dishes. Six microliters of a solution of 1 mg/ml of the extracts was put on the discs. The plates were inoculated with the tested organisms to give a final cell concentration of 10^7 CFU/ml and incubated for 48 h at 37°C. The fungi were grown on Sabouraud's agar (Pronadisa, Conda Lab, Madrid, Spain) at 25°C for 48 h. The experiments were repeated three times and the results (millimeter of zone of inhibition) were expressed as average values.

Scanning electron microscopy (SEM). Electron microscopy micrographs of liposomes were taken using a JEOL JSM-6360 scanning electron microscope (JEOL Ltd., Tokyo, Japan). The solution of liposome bilayers was placed on aluminum stubs (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and sputter coated with gold. The micrographs were made after total drying of the liposome layer.

Statistical analysis. Results, means, and standard deviations of three simultaneous assays were carried out in all methods. Statistical significance of the differences between mean values was assessed by the ANOVA test.

RESULTS AND DISCUSSION

The antioxidant action of *Thymus* spp. pure extracts was determined using Rancimat and MDA by HPLC. The antioxidant actions were compared with those of common commercial antioxidants BHT and α-tocopherol. All the extracts (Figs. 1 and 2) showed antioxidant action. However, none of them showed significantly ($P < 0.05$) higher activity than BHT. Most extracts showed superior action than α-tocopherol (significant at $P < 0.05$). As it appears in Figures 1 and 2, the increase of antioxidant activity was not proportional to the concentration. As observed by Schuler *(17)*, the activity of certain antioxidants does not increase linearly with the increase of their concentration. High levels of addition can even act pro-oxidantly.

In Figures 3 through 6 the DSC curves of *Thymus* spp. extracts and liposomes suspensions in the absence and in the presence of extracts are given. Thermal oxidative decomposition of pure extracts and liposome preparations were studied. In comparison to the Rancimat method, DSC

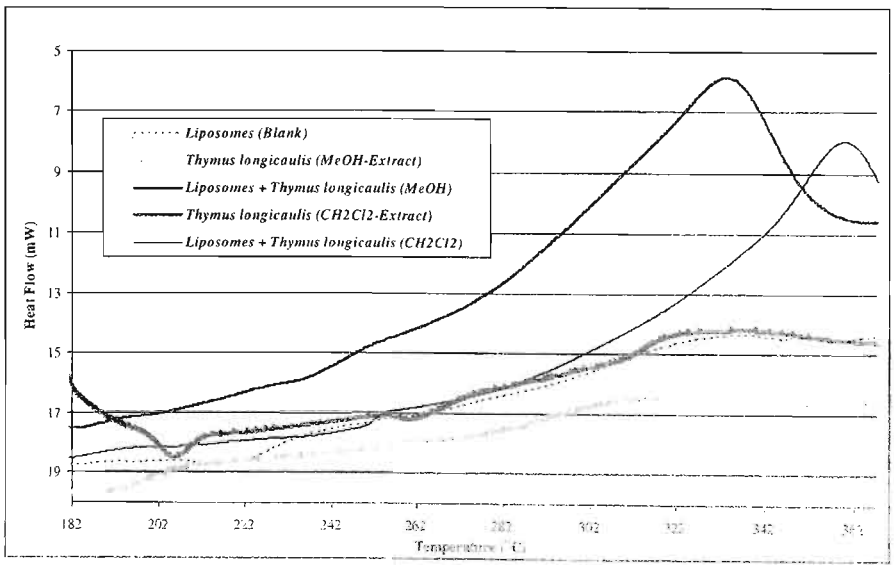


FIGURE 6. *DSC oxidation (DSC using heat flow and temperature) of* Thymus longicaulis *pure extracts and encapsulated in liposomes. Values are means of triplicate determinations.*

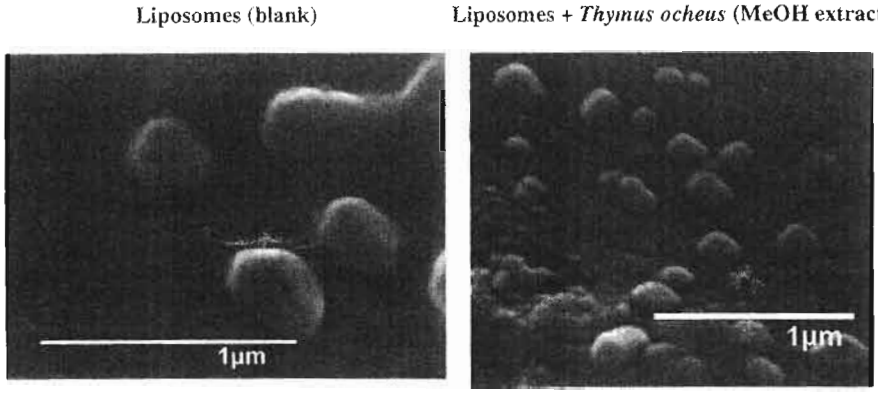


FIGURE 7. *Scanning electron micrographs of MLV liposomes before and after encapsulation of* Thymus ocheus *extract obtained by thin film method (pH 7.4, room temperature).*

is concluded to be useful as a method employing milder conditions and sorter time of analysis and which can be applied for the evaluation of oxidative stability of samples containing volatile antioxidants and other lipid systems containing water *(26)*. An exothermic peak is observed in the range of 150 to 370°C related to auto-oxidation process of the samples. Using the curves, the onset temperature at which the auto-oxidation process begins is determined *(12)*. As it appears in Figures 3 through 6, the addition of extract encapsulated in liposomes had a more intense antioxidant action (significant at $P < 0.05$) than itself in pure form. The shapes of DSC plots of liposome preparations are similar to those of the pure extract oxidation curves. However, at the same heating rate, the temperature of the start of the oxidation reaction is significantly ($P < 0.05$) higher. The modified antioxidant action of extract during its encapsulation was expected since the complex (liposome membrane–fraction) possesses new physicochemical characteristics and bioactivity depended on the structure, size, and z-potential of the preparation *(20)*. Also, the antioxidant action of pure liposomes (no extract added) of the same lipid composition appeared much lower (significant at $P < 0.05$) than that of the extract, implying that the encapsulation of the components of the extract in the aqueous part of liposomes and the possible link of the lipophilic in lipid bilayers stabilized the liposome membranes.

Morphological studies using SEM were performed in order to verify vesicle formation and to provide knowledge on their shape and structure since these are related to the behavior (physicochemical properties) of liposomes *(21)*. SEMs (Fig. 7, only for *T. ocheus*) showed that the liposome vesicle, obtained by the thin film method, were spherical in shape and, in majority, were less than 250 ± 20 nm in diameter. The tendency of the liposomes to aggregate was also observed. The method applied allowed us to obtain liposomes of the above diameter and of regular spherical structure. However, in order to clarify the physicochemical behavior of these structures more studies should be performed (e.g., studies in different temperature and in solution of different pH value).

The methanolic as well as the dichloromethane extracts of the *Thymus* spp. have also been studied for their antimicrobial activities, before and after encapsulation in liposomes (Table 1). The methanolic extracts of *T. longicaulis* and *T. ocheus* appeared as the most active ones against most of the studied microorganisms (8- to 11-mm zones of inhibition). In general, the gram-negative bacteria appeared as the most resistant ones as well as *C. albicans* among the tested fungi. After the encapsulation, the exhibited antimicrobial activities appeared much stronger (significant at $P < 0.05$), mostly against the gram-positive bacteria as well as against the foodborne pathogen *L. monocytogenes* (10- to 15-mm zones of inhibition). The antimicrobial properties of plant extracts have been of great interest in both academia and the food industry, because of their possible use as natural additives emerged from a growing tendency to replace synthetic antimicrobial agents with natural ones *(23)*. Due to their strong antimicrobial activity, the extracts from the herbal parts of *Thymus* spp. used in our study could be considered a natural source that can be freely used in the food industry as a culinary herb. Especially, the dramatically increased antimicrobial activity after the encapsulation in liposomes can promote the use of the abovementioned *Thymus* extracts as potent preservatives and conservation agents not only in the food industry but also in cosmetics and medical preparations.

The present study demonstrated the potential antioxidant and antimicrobial food preservative ability of four *Thymus* spp. (*boissieri, longicaulis, leucospermus,* and *ocheus*). Especially, to our knowledge, the antioxidant and antimicrobial activity of all species (apart from *T. longicaulis*) were studied for the first time. These aromatic plants, commonly used as spices in food or in a hot drink after boiling in water, are feasible as they are considered safe. The *Thymus* spp. are self-growing plants, hardy, extremely tolerant to drought, and can easily grow on rocky arid soil. The production of extracts and their exploitation as potential natural antioxidant and food preservative could be of economic benefit. The encapsulation in liposomes modified the activities of the extracts. Both the antimicrobial and antioxidant activity seemed to improve and their commercial application in food preservation could be considered. However, further investigation should be carried out on the modified solubility of extract, as well as the rate of release of its antioxidant-antimicrobial components from liposomes.

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TABLE 1. *Antimicrobial activities (millimeter of zone of inhibition) of* Thymus *spp. extracts before and after encapsulation in liposomes*[a]

Tested compound	Liposome preparation[b]	*S. aureus*	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pneumoniae*	*E. coli*	*S. mutans*	*S. viridans*	*C. albicans*	*C. tropicalis*	*C. glabrata*	*L. monocytogenes*
T. longicaulis													
CH_2Cl_2 extract	PC:C, 10:2	11 (0.1)	12 (0.1)	12 (0.2)	—	—	—	11 (0.1)	11 (0.2)	—	10 (0.1)	10 (0.2)	11 (0.1)
MeOH extract	(mg/ml)	14 (0.2)	14 (0.2)	11 (0.2)	10 (0.1)	12 (0.3)	11 (0.1)	14 (0.3)	14 (0.2)	11 (0.2)	10 (0.1)	10 (0.2)	14 (0.2)
MeOH extract	—	10 (0.2)	11 (0.2)	10 (0.1)	10 (0.2)	10 (0.1)	10 (0.3)	9 (0.1)	10 (0.1)	8 (0.1)	9 (0.1)	9 (0.1)	10 (0.1)
CH_2Cl_2 extract	—	9 (0.1)	8 (0.1)	9 (0.1)	9 (0.1)	9 (0.1)	—	—	—	—	8 (0.1)	8 (0.1)	—
T. acheus													
MeOH extract	—	10 (0.1)	12 (0.1)	11 (0.2)	10 (0.2)	9 (0.1)	9 (0.1)	11 (0.1)	11 (0.2)	8 (0.1)	9 (0.1)	9 (0.1)	10 (0.3)
MeOH extract	PC:PG:C, 10:1:2 (mg/ml)	14 (0.3)	15 (0.1)	12 (0.2)	11 (0.1)	10 (0.1)	10 (0.2)	15 (0.3)	15 (0.3)	10 (0.1)	12 (0.2)	12 (0.2)	15 (0.3)
Netilmicin	—	21 (0.3)	25 (0.4)	20 (0.2)	23 (0.1)	22 (0.4)	24 (0.5)	24 (0.3)	25 (0.1)	—	—	—	22 (0.3)
5-Flucytocine	—	—	—	—	—	—	—	—	—	18 (0.3)	19 (0.2)	20 (0.2)	—

[a] Results are means of triplicate determinations. Standard deviations are given in parentheses.
[b] PC, phosphatidyl choline; C, cholesterol; PG, phosphatidyl glycerol.

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