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Study of Antioxidant and Antimicrobial Activity of Chios Mastic Gum Fractions (Neutral, Acidic) Before and After Encapsulation in Liposomes

Olga Gortzi[1]*, Vasilios Athanasiadis[1], Stavros Lalas[1], Ioanna Chinou[2] and John Tsaknis[3]

[1]*Department of Food Technology, Technological Educational Institute of Thessaly, Terma N. Temponera str., GR-43100, Karditsa, Greece*
[2]*Division of Pharmacognosy and Chemistry of Natural Products, Department of Pharmacy, University of Athens, Panepistimiopolis-Zografou, GR-15771, Athens, Greece*
[3]*Department of Food Technology, Technological Educational Institute of Athens, Agiou Spyridonos str., GR-12210, Egaleo, Athens, Greece*

Abstract

Mastic is a well-known natural resin from the trunk and branches, of *Pistacia lentiscus* var. chia (Anacardiaceae), which is grown as endemic only in the Greek island of Chios. During this work, a total mastic gum extract was prepared after removal of the contained insoluble polymer in order to ameliorate solubility and enhance in vivo activity. To overcome the drawbacks (i.e solubility, bioavailability, etc.) of mastic gum extracts (acidic and neutral fraction), the selection of a suitable carrier is crucial. Three different methods of preparation, thin-film evaporation, freezing-thawing, and ethanol injection were used for the preparation of liposomes consisting of Phosphatidylcholine (PC) and Cholesterol (CH). The effect of PC: CH molar ratio on the percentage of mastic extract encapsulated was investigated. Mastic gum extracts components-liposomes interaction was studied using Fourier transform infrared (FT-IR) spectroscopy. The effects of different preparation methods on the physicochemical properties of colloidal systems were evaluated by means of surface morphology, field emission Scanning Electron Microscopy (SEM), and size distribution using a particle size analyzer. For the determination of the antioxidant activity two methods were used: I) The Rancimat method where the protection factor was determined for each sample and compared with known antioxidants. II) Differential Scanning Calorimetry (DSC) where the temperature of oxidation for each sample was determined. Moreover, the crude extract (EtOAc-MeOH) of mastic, as well as, its acidic and neutral fractions was assayed against a panel of 9 human and food pathogenic gram (±) bacteria and fungi.

Keywords: Chios mastic gum; Antioxidant activity; Antimicrobial activity; Encapsulation; Liposomes

Introduction

Chios mastic gum, the resin obtained as an exudate from the trunk and branches of *Pistacia lentiscus* var. chia, has found extensive use in pharmaceutical products and as a nutritional supplement. The oral absorption of crude resin (containing a high percentage of an insoluble and sticky polymer of poly-β-myrcene) is poor due to its low water-solubility and reduces the bioavailability of the contained active compounds [1]. It has been used in traditional Greek medicine for various gastrointestinal disorders while the plant has been mentioned by famous ancient Greek physicians (Dioscorides, Theophrastos, etc.) recommending its healing properties.

Therefore, the objective of the present study was to determine the possibility to encapsulate Chios mastic gum in liposomes. The methods of liposome preparation and characterization were also determined. Finally, the antioxidant stability and antimicrobial activity of the liposomes preparations were evaluated.

Three different methods of preparation, thin-film evaporation, freezing-thawing, and ethanol injection were used for the preparation of liposomes consisting of Phosphatidylcholine (PC) and Cholesterol (CH). The effect of PC:CH molar ratio on the percentage of mastic extract encapsulated was investigated. Mastic gum extracts components-liposomes interaction was studied using Fourier Transform Infrared (FT-IR) spectroscopy. The effects of different preparation methods on the physicochemical properties of colloidal systems were evaluated by means of surface morphology, field emission Scanning Electron Microscopy (SEM), and size distribution using a particle size analyzer. For the determination of the antioxidant activity two methods were used: I) The Rancimat method where the protection factor was determined for each sample and compared with known antioxidants. II) Differential Scanning Calorimetry (DSC) where the temperature of oxidation for each sample was determined. Moreover, the crude extract (EtOAc-MeOH) of mastic, as well as, its acidic and neutral fractions was assayed against a panel of 9 human and food pathogenic gram (±) bacteria and fungi.

Experimental

SPME procedure & GC-MS analysis

The HS-SPME was performed with Carboxen/polydimethylsiloxane coated fiber (75μm coating) from Supelco, attached in a manual SPME fiber holder (57330-U, Supelco, Germany). For SPME extraction, 1 g of powdered mustic gum in glass vial (15 mL) closed with PTFE coated silicone rubber septum was used. The GC-MS analysis was carried out using a Hewlett Packard 5973-6890 GC-MS system operating on EI mode (ionization energy 70 eV) equipped with an HP 5MS 30 m x 0.25 mm x 0.25 μm film thickness capillary column. In the injector port the temperature was set at 200°C and the SPME fiber was inserted using splitless mode. The fiber was removed after 10min, in order to completely release all substances. The carrier gas was helium (flow rate 1 mL/min) and the temperature program started at 40°C for 3 min and gradually increased to 150°C at a ramp of 4°C/min and then to 250°C at 10°C/min. Identification was succeeded by using spectral library (wiley 275) and comparison with bibliography.

***Corresponding author:** Olga Gortzi, Department of Food Technology, Technological Educational Institute (T.E.I.) of Thessaly, GR-43100, Karditsa, Greece, Tel: +30-24410-76454; Fax: +30-24410-41080; E-mail: ogortzi@teilar.gr

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Methods of liposome preparation

Thin-Film Evaporation (TFE): The method used was a modification of that reported by Dua et al. (2012). Lipid phase consisting of a mixture of PC (10 mg/mL) and CH (2 mg/mL) was dissolved in chloroform. Then, the solvent was removed under reduced pressure using a rotary evaporator at 35°C. Finally, a thin film of dry lipid was formed on the wall of the flask. This thin film was dissolved in an aqueous phase (H2O d.d. + 0.3% NaCl) at 35°C. To complete the dispersion a vortex mixer and an ultrasonic bath were used. Then, the mixture was heated at 35°C for 45 min. The liposomal system was centrifuged for 10 min at 5000 rpm and the supernatant phase was freeze dried for 12 h. After MLV (Multi Lamellar Vehicles) preparation the dispersion was subjected to sonication for 30 min in order to form SLV (Single Lamellar Vehicles) liposomes.

Freezing-Thawing (FT): The method used was a modification of that reported by Dua et al. (2012). After MLV preparation the dispersion was subjected to sonication for 30 min in order to form SUV (Small Unilamellar Vehicles) liposomes.

Ethanol Injection (EI): The method used was a modification of that reported by Dua et al. [2]. After MLV preparation the dispersion was subjected to sonication for 30 min in order to form SUV liposomes.

Methods of characterization

FT-IR: Spectra in the transmission mode were carried out at the region of 4000-400 cm-1. Potassium bromide (KBr) pellets were prepared by gently mixing 6 mg sample powder with 120 mg KBr using an hydraulic press.

SEM: Samples were lyophilized, coated with gold and palladium using a vacuum evaporator, and examined using SEM at 20 kV accelerating voltage.

Size distribution: Liposome particle size was analyzed at 25°C.

Methods for determining antioxidant activity

Rancimat: About 3.0 g of sample oil were weighed into the glass vessel and the appropriate amount for each sample liposome or known antioxidant (BHT or α-tocopherol) was added. The conditions were set at 90ºC and 15 L/h. The protection factor (PF) was calculated as PF = (induction time with antioxidant)/(induction time without antioxidant). A protection factor greater than one indicates inhibition of lipid oxidation. Higher PF value indicates better the antioxidant activity.

DSC: The thermal behavior was studied by heating of about 5.0 mg of each individual sample in a covered sample pan under nitrogen gas flow. The investigations were carried out at a temperature range of 25-580°C and a heating rate of 10°C min^{-1}.

Methods for determining antimicrobial activity

In vitro antibacterial studies of crude extract (EtOAc-MeOH) of mastic, as well as, its acidic and neutral fractions were carried out by the dilution method by measuring the MIC values against: *Staphylococcus aureus, S. epidermidis, Pseudomonas aeruginosa, Enterobacter cloacae, Klebsiella pneumoniae, Escherichia coli, Candida albicans, C. tropicalis* and *C. glabrata* (Table 2).

Results and Discussion

HS-SPME and GC-MS analysis

Through HS-SPME analysis α-pinene (25.6%), verbenone (14.0%), β-cymene and verbenene appeared as the most abundant constituents, representing 58% of the total, among the 27 identified volatile components of the mastic (Table 1).

Encapsulation Efficiency (EE)

Trapping efficiencies for AMGE (acidic mastic gum extract) and NMGE (neutral mastic gum extract) in colloidal carriers were determined by GC-MS and the results are presented below (Table 2).

Particle size distribution

The mean particle size and polydispersity of liposomes obtained with different methods and loaded with AMGE and NMGE are summarized in Table 2.

The freezing-thawing circles caused an amount of entrapped AMGE

RT	Constituents	%
1.52	butane	1.8
2.11	3-methyl-2-buten-1-ol	0.7
4.95	3-methyl-2-buten-1-al	<0.5
5.22	2-methylpent-2-en-4-one	<0.5
10.07	α-pinene	25.6
10.79	verbenene	8.6
11.70	β-pinene	0.7
12.16	6-methyl-5-hepten-2-one	<0.5
13.04	2-methylanisole	6.5
13.68	o-cymene	1.0
13.87	p-menth-4(8)-ene	1.4
15.60	cis-linalool oxide	<0.5
16.18	trans-3-caren-2-ol	<0.5
16.81	trans-β-ocimene	<0.5
17.48	α-terpinene	<0.5
18.39	1(7),5,8-o-menthatriene	2.8
18.46	1-ethyl-2,3-dimethyl benzene	0.8
18.66	1,2-dimethyl-4-ethyl benzene	6.0
19.19	pinocarvone	4.9
20.46	myrtenal	3.0
21.03	verbenone	14.0
21.36	β-cymene	9.8
22.05	p-mentha-1,5,8-triene	<0.5
23.68	trans-α-ocimene	2.0
23.77	camphene	<0.5
23.88	p-cymene	<0.5
33.05	unknown	0.5

Table 1: Mastic gum analysis through HS-SPME and GC-MS

and NMGE to leak from the bilayers, which lead to lower encapsulation efficiency for FT liposomes. Therefore, FT-IR, Rancimat and DSC studies are given only for the two other liposome preparations.

FT-IR

The characteristic functional groups with their frequencies were determined (Table 3).

The first aspect was detected in the vibration band located between 3600 and 3000 cm^{-1} (Figure 1). This band is related to O-H stretching vibration and is much more pronounced in the liposomes formulations EI and TFE than in pure AMGE and NMGE. This indicates that the OH groups of AMGE and NMGE components created hydrogen bonds with the containing phospholipids. These bonds are probably related to the interaction among the O-H groups from AMGE and NMGE components and the polar groups of the phospholipids.

A second important characteristic was related to the peaks at 1236 cm^{-1} and 1720 cm^{-1}, (Figure 1). The first one is related to the stretching of P=O bond of the polar heads of phospholipids. The absorption band attributes to phosphate O-P-O asymmetric stretching vibrations and can also provide important clues about hydration and hydrogen bonding interactions at the surfaces phospholipid assemblies and AMGE components. Also, in the case of the stretching of C=O of ester groups, observed modifications (decreases) reflect differences in the degrees of hydration and/or hydrogen bonding to the ester carbonyl groups in the case of AMGE loaded liposomes (Table 4).

SEM

The SEM photograph of optimized formulation revealed that particles were roughly spherical. Additionally, an uniformity was observed. An average particle size below 120 nm could be achieved and reproduced (Figure 2).

Rancimat method

Based on the protection factor, the results are a comparative study of the antioxidant activity of the samples, liposomes and known antioxidants (BHT or α-tocopherol).

Preparation Method	Fractions	EE %	Mean particle size (nm)	Polydispersity
FT	AMGE	11.13 ± 0.43	145.6 ± 3.9	0.346 ± 0.005
	NMGE	10.03 ± 0.38	138.7 ± 4.1	0.317 ± 0.002
TFE	AMGE	13.26 ± 0.34	125.1 ± 3.4	0.333 ± 0.004
	NMGE	12.40 ± 0.22	129.1 ± 3.8	0.301 ± 0.004
EI	AMGE	16.83 ± 0.42	114.8 ± 4.0	0.367 ± 0.005
	NMGE	15.30 ± 0.31	116.1 ± 2.0	0.205 ± 0.003

Table 2: Effect of different preparation methods and loading capacity on particle size distribution of AMGE and NMGE liposomes (EE%: percentage of encapsulation efficiency)

Functional Group Names	Type of Vibration	Empty TFE	TFE AMGE loaded	TFE NMGE loaded	Empty EI	EI AMGE loaded	EI NMGE loaded	AMGE	NMGE
Alcohol	**O-H** stretch	**3444.87**	**3452.58**	**3431.36**	**3448.72**	**3451.62**	**3439.08**	**3446.79**	**3450.65**
Ester	**C=O** stretch	1741.72	**1739.79**	1741.72	1741.72	1741.72	1741.72	**1708.93**	**1705.07**
Phosphate ester	**P=O** stretch	1240.23	**1236.37**	1240.23	1240.23	1240.23	1240.23	**1246.02**	**1244.95**

Table 3: FT-IR interpretation of TFE and EI preparations (empty and AMGE & NMGE loaded)

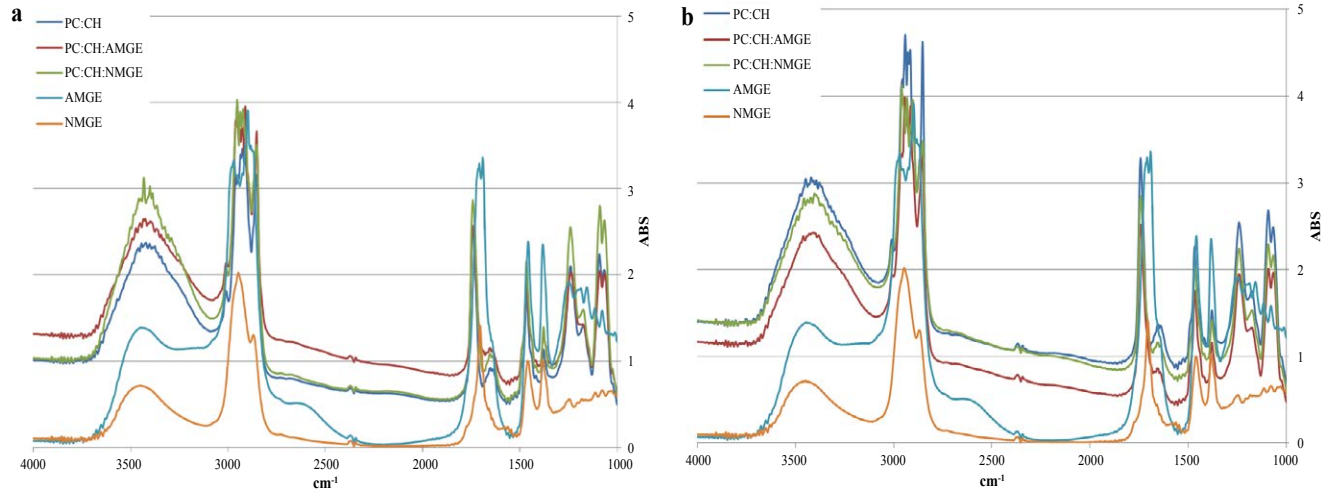

Figure 1: Comparative FT-IR graphs of TFE (a) and EI (b) preparations.

	S. aureus	*S. epidermidis*	*P. aeruginosa*	*E. cloacae*	*K. pnaumioniae*	*E. coli*	*C. albicans*	*C. tropicalis*	*C. glabrata*
Crude extract	0.04	0.05	0.21	0.25	0.34	0.18	0.73	0.56	0.32
Acidic fraction	0.19	0.20	0.27	0.38	0.47	0.25	1.10	0.98	0.78
Neutral fraction	0.45	0.52	0.73	0.88	1.00	0.64	1.25	1.00	0.85
Amphotericin	-	-	-	-	-	-	$1.0•10^{-3}$	$0.5•10^{-3}$	$0.4•10^{-3}$
5-flucytocine		-	-	-	-	-	$0.1•10^{-3}$	$1.0•10^{-3}$	$10•10^{-3}$
Amoxicillin with Clavulanic acid	$0.5•10^{-3}$	$5.0•10^{-3}$	$1.0•10^{-3}$	$1.0•10^{-3}$	$1.6•10^{-3}$	$1.2•10^{-3}$	-	-	-

Table 4: Antimicrobial activity MIC (mg/ml) of the mastic gum

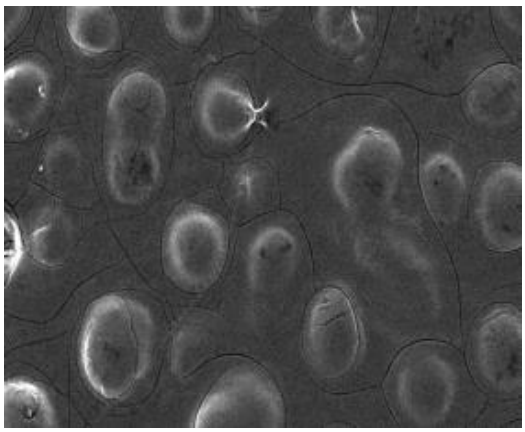

Figure 2a: Empty EI

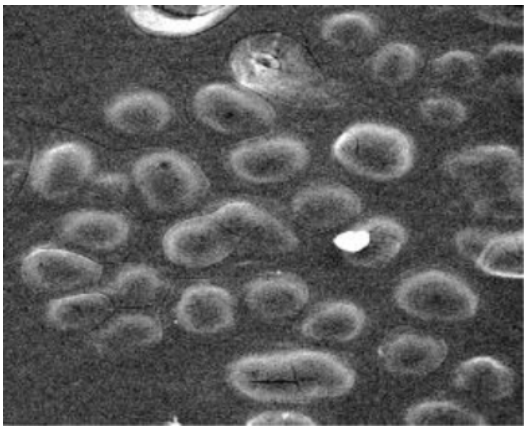

Figure 2b: NMGE loaded EI

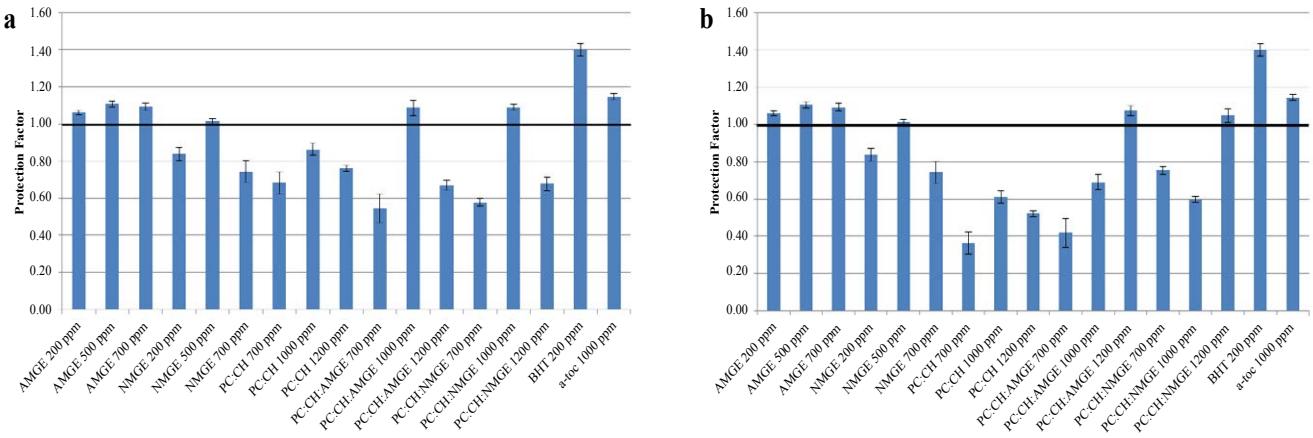

Figure 3: Protection factor of TFE (a) and EI (b) preparations and known antioxidants (BHT and α-tocopherol) as determined by the Rancimat method.

DSC

Thermal oxidative decomposition of AMGE and NMGE, TFE and EI preparations (empty and AMGE or NMGE loaded liposomes) was studied by the DSC method using the onset temperature (To) of curves at the point where the auto-oxidation process begins. The results were in line with those reported by other researchers who also suggested that NMGE proved low oxidation stability. The oxidative stability of both extracts was increased after encapsulation and depended on the method of preparation used (TFE and EI) (Figure 3).

Antimicrobial activity

All tested extracts of mastic, as well as, its acidic and neutral fractions showed a very interesting antimicrobial profile against all 9 assayed microorganisms. Moreover, the crude extract appeared as the most active, where it exhibited the strongest activity against Gram

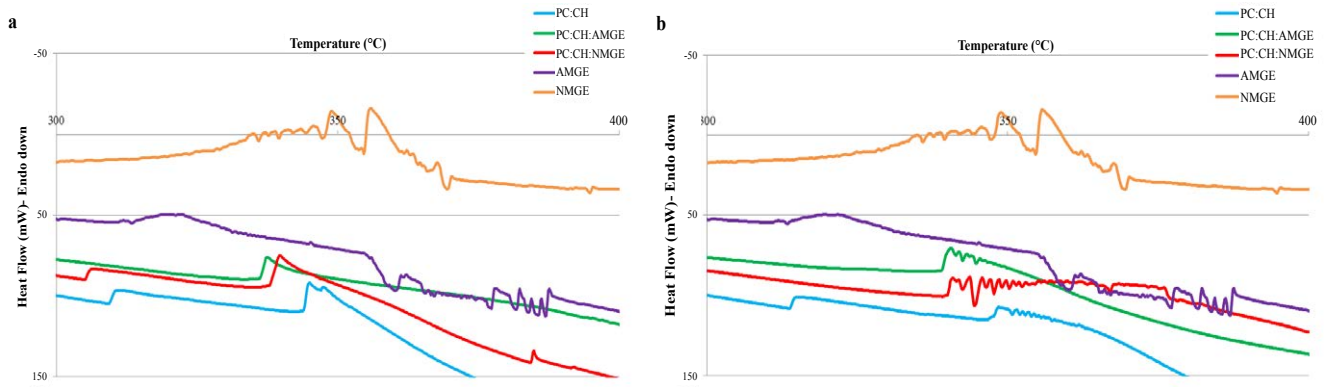

Figure 4: Thermal profile of TFE (a) and EI (b) preparations as determined by the DSC.

positive human pathogenic bacteria (MIC values 0.05-0.20 mg/mL) (Figure 4).

Conclusion

During current study, colloidal systems (liposomes) containing AMGE and NMGE were successfully prepared with three different methods (TFE, FT, EI). The comparison of preparation methods (using their physicochemical properties) revealed that lipid based carriers prepared by the TFE and EI methods showed better encapsulating efficiency. From the experimental results it is concluded that the method of preparation has an impact on the release rate of constituents (i.e. terpenes, pinenes, etc.).

The encapsulated fractions of mastic gum (especially the acidic one) presented higher antioxidant activity in comparison to the non-encapsulated ones.

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