“Study of antioxidant and antimicrobial activity of Chios mastic gum fractions (neutral, acidic) before and after encapsulation in liposomes”

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- 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.
- 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.

- 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).
- 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.
- 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.

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

✓ Liposomes 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 90 ^{o}C 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 ^{o}C and a heating rate of 10 ^{o}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

Results & 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).

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

RT	Constituents	%
10.07	**α-pinene**	25.6
10.79	**verbenene**	8.6
11.70	β-pinene	0.7
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

- 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.

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

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

- The freezing-thawing circles caused an amount of entrapped AMGE 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

Fig. 1a. Comparative FT-IR graphs of TFE preparation

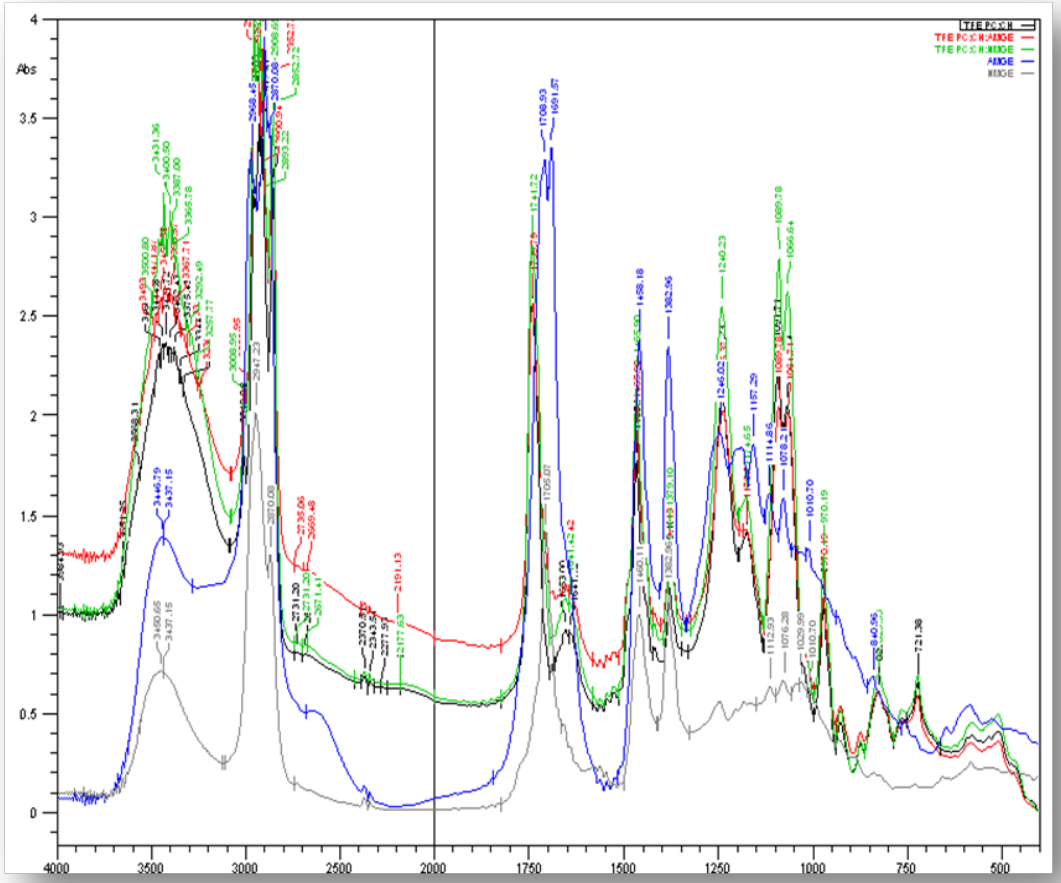
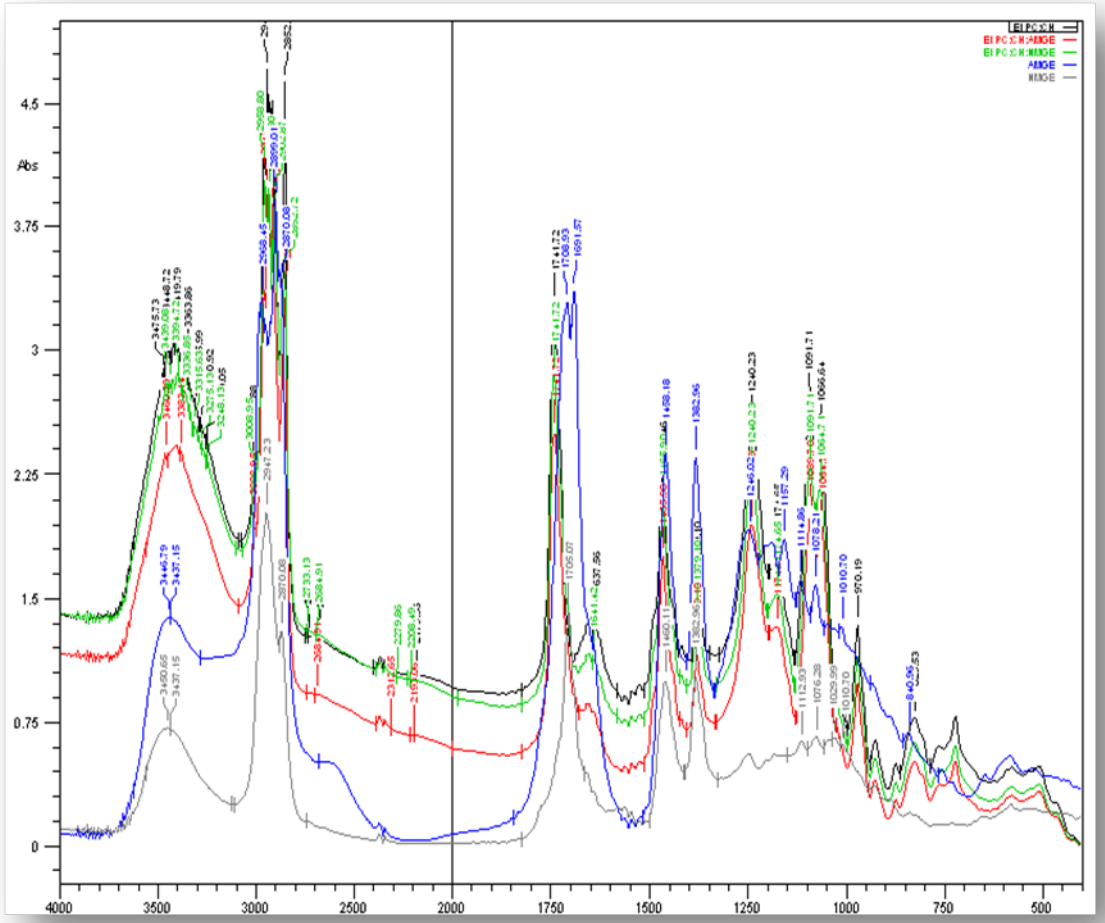


Fig. 1b. Comparative FT-IR graphs of EI preparation



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

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

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

- 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.

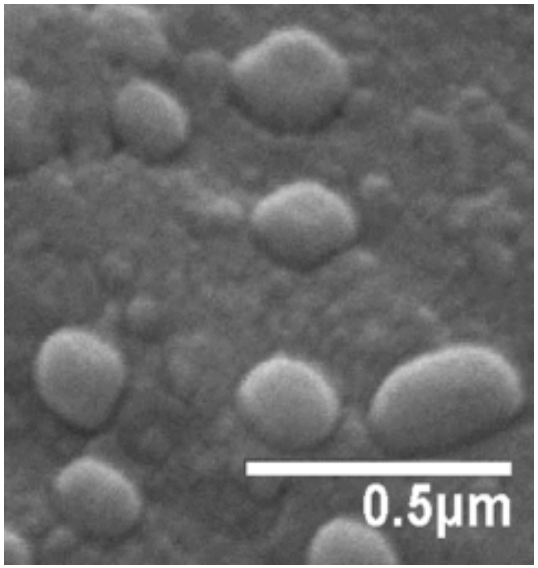
- ✓ These bonds are probably related to the interaction among the O-H groups from AMGE and NMGE components and the polar groups of the phospholipids.
- A second important characteristic was related to the peaks at 1236 cm-1 and 1720 cm-1, (Figure 1).
- ✓ The first one is related to the stretching of 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.

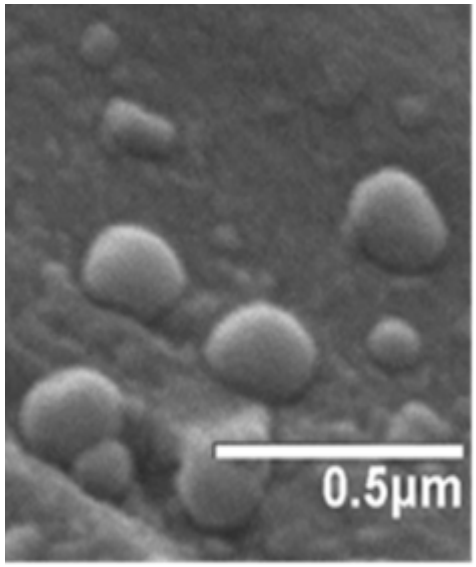
SEM

- 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.

Empty EI



NMGE loaded EI



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).

Fig. 2a. Protection factor of TFE preparation and known antioxidants (BHT and α-tocopherol) as determined by the Rancimat method.

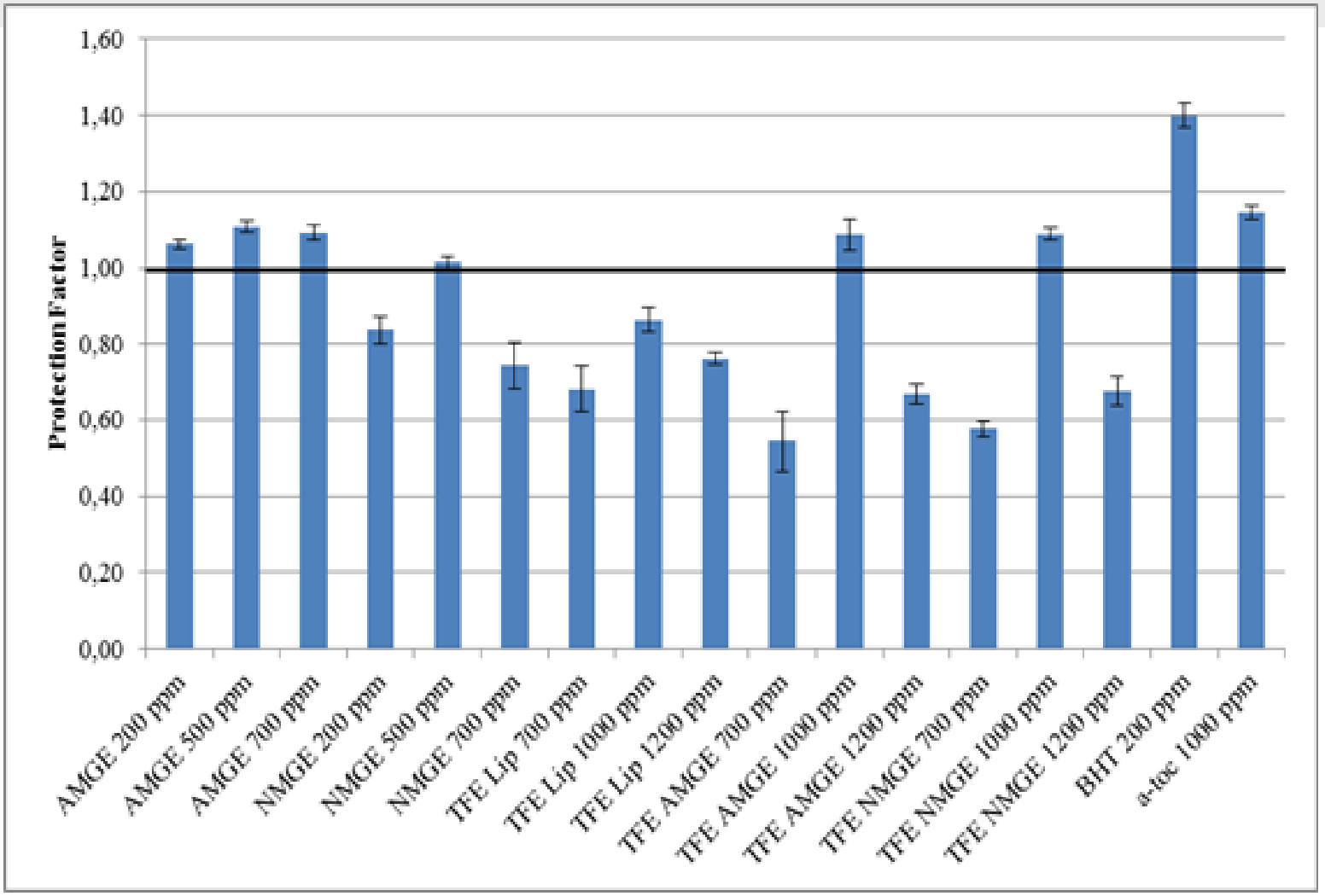
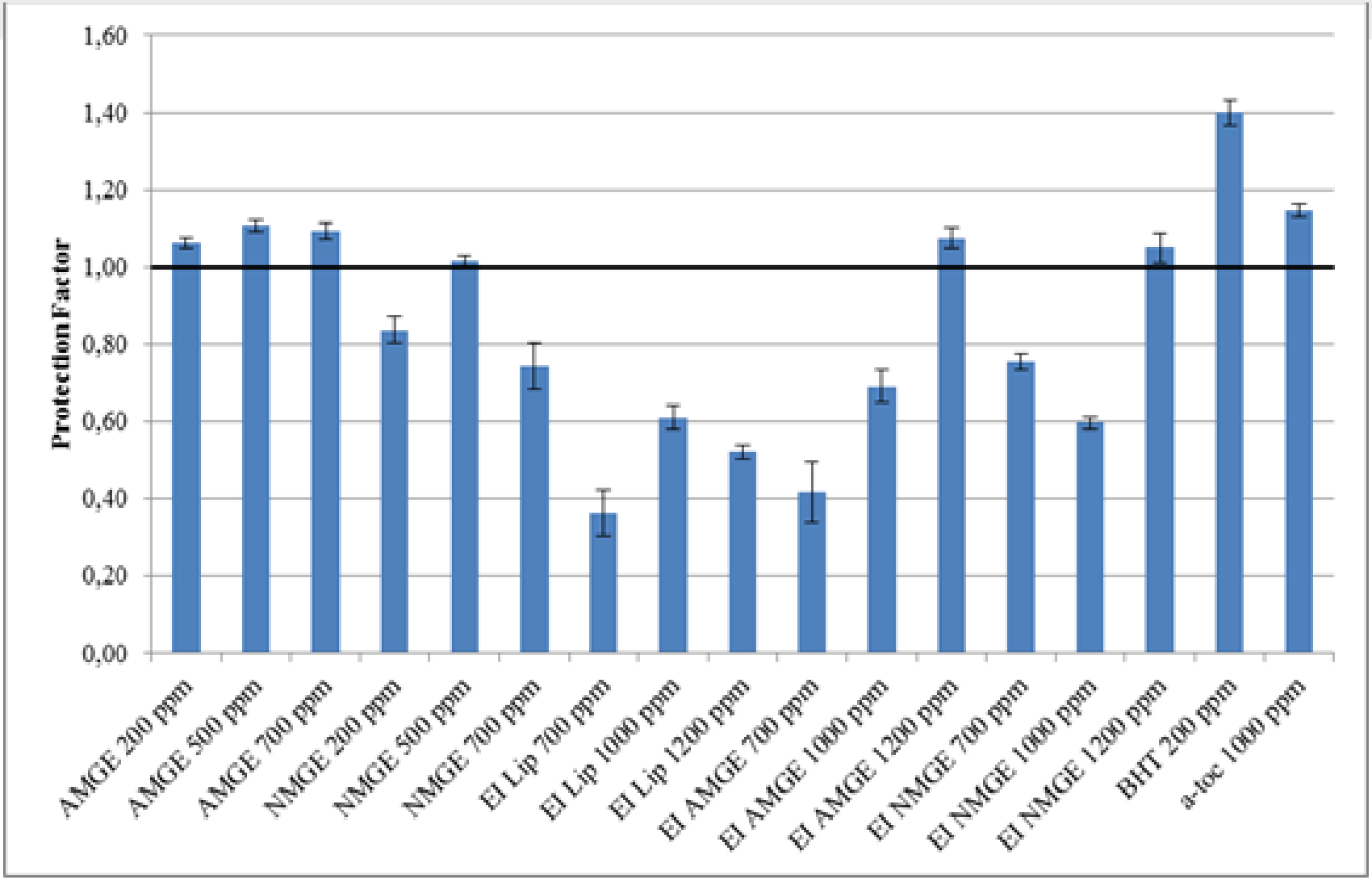


Fig. 2b. Protection factor of EI preparation and known antioxidants (BHT and α-tocopherol) as determined by the Rancimat method.

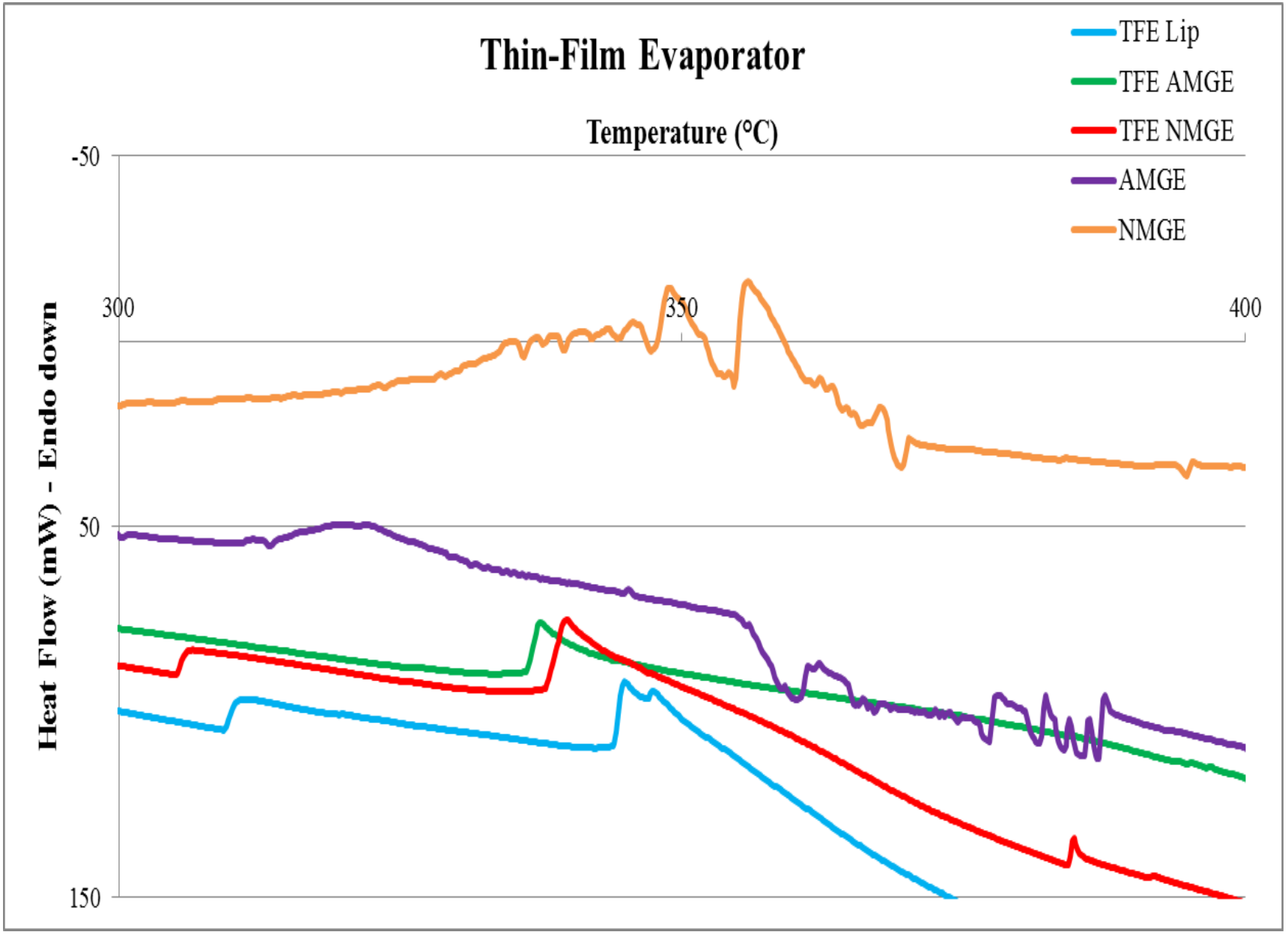


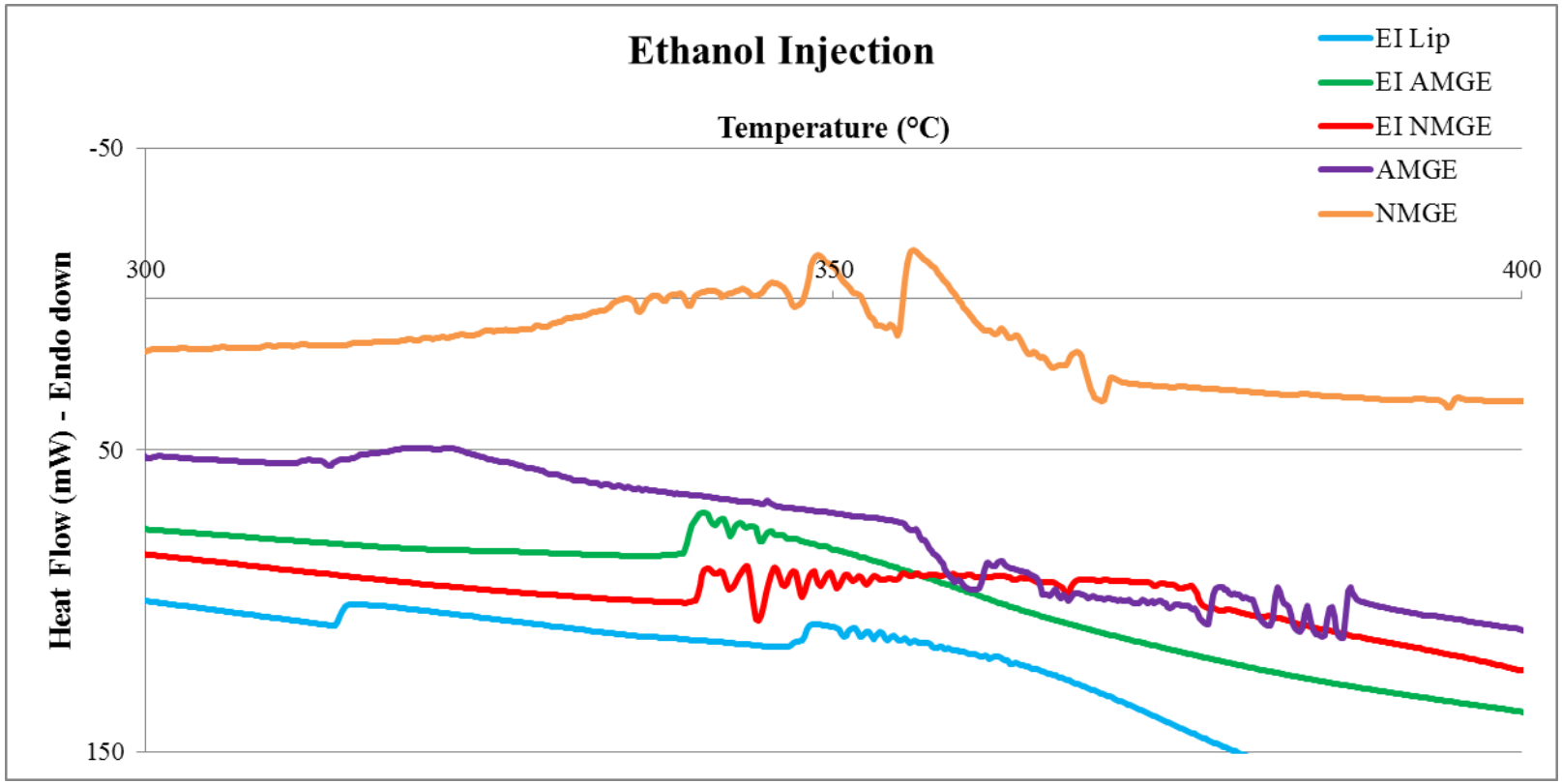
DSC

- 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).

The following figures show Thermal profile of TFE and EI preparation as determined by the DSC.


Thin-Film Evaporator
Temperature (°C)
-50
300
350
400
50
150
Heat Flow (mW) - Endo down
TFE Lip
TFE AMGE
TFE NMGE
AMGE
NMGE


Ethanol Injection
Temperature (°C)
-50
300
350
400
50
150
Heat Flow (mW) - Endo down
EI Lip
EI AMGE
EI NMGE
AMGE
NMGE

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

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

	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	-	-	-	-	-	-	$1.0 \cdot 10^{-3}$	$0.5 \cdot 10^{-3}$	$0.4 \cdot 10^{-3}$
5-flucytocine		-	-	-	-	-	$0.1 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$10 \cdot 10^{-3}$
amoxicillin with clavulanic acid	$0.5 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$1.6 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}$	-	-	-

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.

Acknowledgements

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