Encapsulation of *Olea europaea* leaf polyphenols in liposomes: how to transform biomass waste into valuable resources with antimicrobial activity

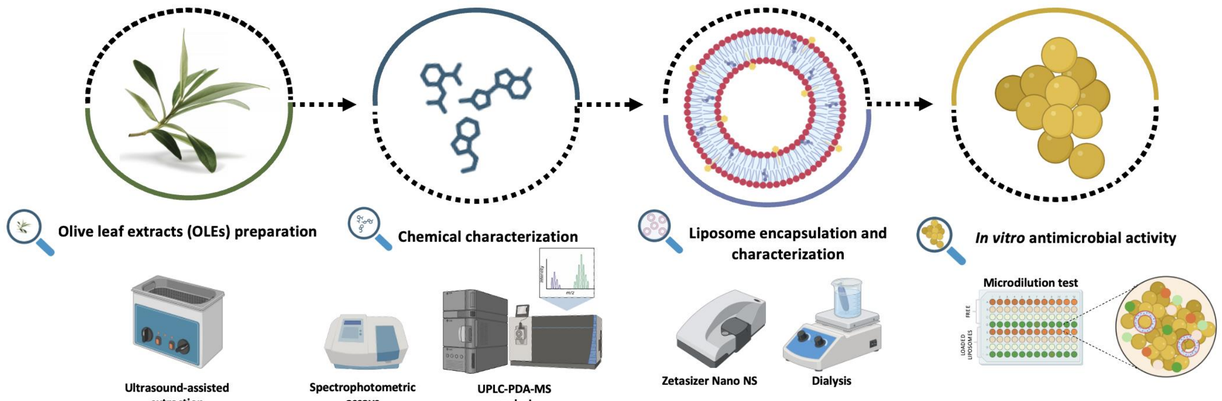
Giuliana Prevete, Enrica Donati, Laura Lilla, Francesca Mariani and Marco Mazzonna

CNR-Institute for Biological Systems (ISB), Territorial Research Area Rome 1, Strada Provinciale 35d, n. 9, 00010 - Montelibretti (Rome), Italy

Email: marco.mazzonna@cnr.it

INTRODUCTION

In recent decades, the overuse of antibiotics in clinical, agricultural and animal production setting, has created strong selective pressure on bacterial species, leading to the emergence of antimicrobial-resistant strains [1]. Consequently, new and effective strategies are necessary to expand the panel of currently available therapeutic tools. An attractive alternative involves the use of bioactive compounds such as polyphenols derived from biomass wastes, for example olive leaves, representing a circular economy approach in which waste biomasses are converted into valuable products [2]. *Olea europaea* leaves are rich in polyphenols with antioxidant, antimicrobial, anti-inflammatory and cytoprotective activities. However, their medical application is limited by poor pharmacokinetic features, resulting in low systemic absorption. To overcome these limitations, liposomes as delivery systems [3] for natural antimicrobial polyphenols have been developed to improve their pharmacokinetic profiles and enhance their antimicrobial activity.



OLEs PREPARATION AND CHEMICAL CHARACTERIZATION

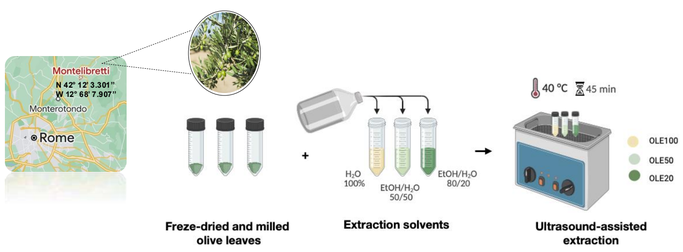


Figure 2. Graphical representation of the extraction protocol used (Created with BioRender.com).

Table 1. Yield of extraction, total phenolic content (TPC) and antioxidant capacity (TEAC) of OLEs.

Solvent	Extract	Yield of extraction (%)*	TPC§ ($mg_{Gallic_Acid}/g_{leaves}$)*	TEAC# t_{1min} ($mmol_{Trolox}/g_{leaves}$)*	TEAC# t_{4min} ($mmol_{Trolox}/g_{leaves}$)*
H_2O 100%	**OLE100**	33 ± 1	17.9 ± 0.6	0.16 ± 0.02	0.17 ± 0.03
EtOH/H_2O 50/50	**OLE50**	40 ± 2	24 ± 3	0.28 ± 0.02	0.31 ± 0.03
EtOH/H_2O 80/20	**OLE20**	41 ± 1	26.7 ± 0.8	0.34 ± 0.03	0.39 ± 0.05

* Data are expressed as mean ± standard deviation (n = 3, three different samples).
§ TPC (Total Phenolic Content) assessed by Folin-Ciocâlteu assay.
# TEAC (Trolox Equivalent Antioxidant Capacity).

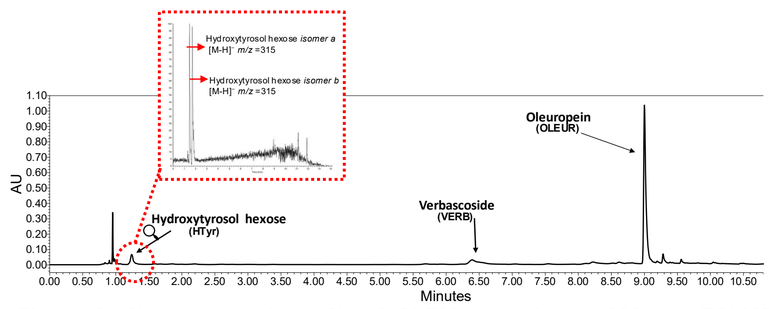


Figure 3. Chromatographic profile of OLE20, detection at 280 nm. ESI-MS detection in negative scanning mode for *m/z* = 315 is shown in the red frame.

LIPOSOME ENCAPSULATION AND PHYSICOCHEMICAL CHARACTERIZATION

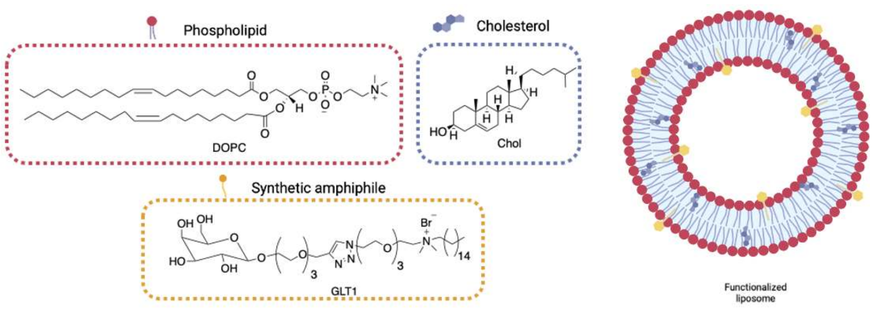
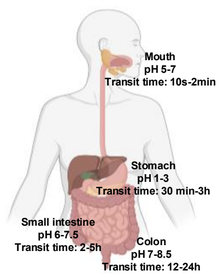


Figure 4. Schematic representation of developed liposomes (Created with BioRender.com).



Formulation	pH 2.9 (30 min-3h)	5.7 (1-3min)	6.4 (3h)	7.4	8.1 (24h)
1a	✓	✓	✓	✓	✓
2a	✓	✓	✓	✓	✓
1b	✓	✓	✓	✓	✓
2b	✓	✓	✓	✓	✓
1c	✓	✓	✓	✓	✓
2c	✓	✓	✓	✓	✓
1d	✓	✓	✓	✓	✓
2d	✓	✓	✓	✓	✓

Figure 5. Stability of developed liposomes under pH variations (pH 7.4 = reference value).

Figure 7. OLEUR still entrapped in liposomes over time under forced release conditions.

Table 2. Physicochemical features of empty and loaded neutral and galactosylated liposomes (10 mM in total lipids) in PBS (pH 7.4).

Formulation	Composition	D_h (nm)#	PDI#	ζ-potential (mV)#	EE (%)#
1	DOPC/Chol 8.0:2.0	119 ± 2	0.10 ± 0.02	-3 ± 2	-
1a	DOPC/Chol/OLEUR* 8.0:2.0:1.25	100 ± 2	0.12 ± 0.01	-10 ± 1	73 ± 2
1b	DOPC/Chol/OLE100§ 8.0:2.0	102 ± 1	0.18 ± 0.01	-10 ± 1	26 ± 4
1c	DOPC/Chol/OLE50§ 8.0:2.0	111 ± 1	0.18 ± 0.01	-10 ± 5	32 ± 5
1d	DOPC/Chol/OLE20§ 8.0:2.0	120 ± 1	0.20 ± 0.01	-10 ± 5	43 ± 7
2	DOPC/Chol/GLT1 7.0:2.0:1.0	94 ± 2	0.12 ± 0.01	16 ± 1	-
2a	DOPC/Chol/GLT1/OLEUR* 7.0:2.0:1.0:1.25	79 ± 1	0.14 ± 0.01	14 ± 3	75 ± 5
2b	DOPC/Chol/GLT1/OLE100§ 7.0:2.0:1.0	93 ± 1	0.15 ± 0.01	10 ± 2	36 ± 6
2c	DOPC/Chol/GLT1/OLE50§ 7.0:2.0:1.0	94 ± 1	0.16 ± 0.01	10 ± 3	51 ± 7
2d	DOPC/Chol/GLT1/OLE20§ 7.0:2.0:1.0	119 ± 1	0.20 ± 0.01	9 ± 1	57 ± 13

* [OLEUR]/[total lipids] ratio at the beginning of the preparation is 1/8.
§ OLE/total lipids ratio at the beginning of the preparation is 1/1 (w/w).
# Data are expressed as mean ± standard deviation (n = 3, three different samples).

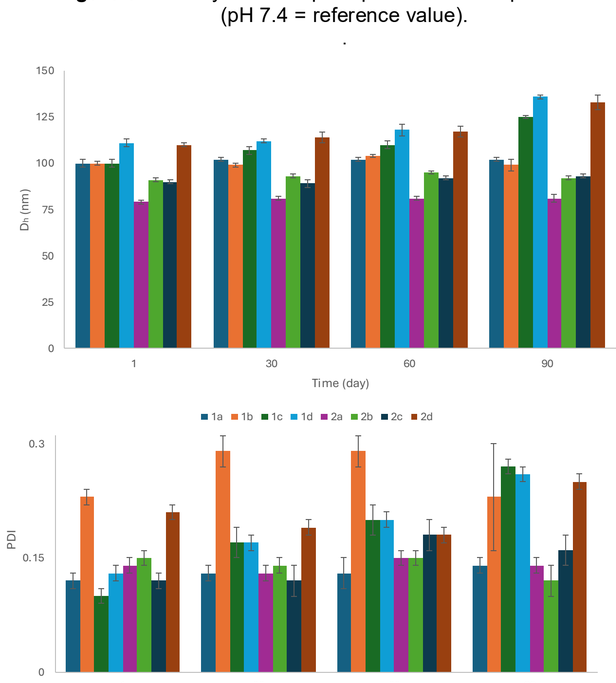


Figure 6. Storage stability of liposomes at 4°C in the dark.

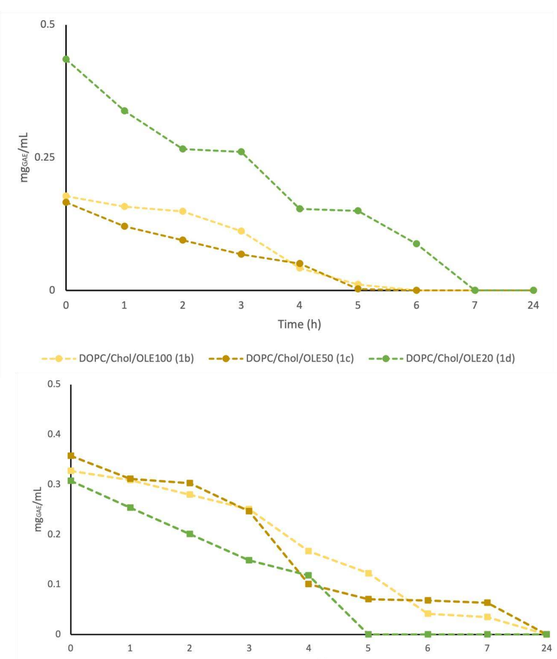


Figure 8. Total phenolic content still encapsulated in OLEs loaded in liposomes over time under forced release conditions.

IN VITRO ANTIMICROBIAL ACTIVITY: DETERMINATION OF THE *MINIMUM INHIBITORY CONCENTRATION* (MIC) AND *MINIMUM BACTERICIDAL CONCENTRATION* (MBC)

Table 3. Antimicrobial activity of free HTyr, VERB and OLEUR.

Compound	*S. aureus* wild type (ATCC 25923) MIC (μM)	MBC (μM)	MRSA (ATCC 33591) MIC (μM)	MBC (μM)
HTyr	117	130	123	136
VERB	77	82	59	82
OLEUR	139	167	155	174

Table 4. Antimicrobial activity of OLEUR loaded into liposomes.

Formulation	*S. aureus* wild type (ATCC 25923) MIC (μM)	MBC (μM)	MRSA (ATCC 33591) MIC (μM)	MBC (μM)
1a	205	242	213	242
2a	198	239	233	270

1 = DOPC/Chol liposomes; **2** = DOPC/Chol/GLT1 liposomes; **a** = OLEUR.

Table 5. Antimicrobial activity of free and liposome-loaded OLEs.

Formulation	*S.* aureus wild type (ATCC 25923) MIC ($mg_{extract}$/mL)	MIC (μg_{GAE}/mL)	MBC ($mg_{extract}$/mL)	MBC (μg_{GAE}/mL)	MRSA (ATCC 33591) MIC ($mg_{extract}$/mL)	MIC (μg_{GAE}/mL)	MBC ($mg_{extract}$/mL)	MBC (μg_{GAE}/mL)
-	0.24	12.8	0.29	15.4	0.24	12.8	0.31	16.6
1b	n.d.	30.5	n.d.	31.2	n.d.	34.8	n.d.	35.5
2b	n.d.	8	n.d.	8.2	n.d.	8.5	n.d.	8.9
-	0.15	9.3	0.18	11.2	0.14	8.7	0.18	11.2
1c	n.d.	34.4	n.d.	41.9	n.d.	75.5	n.d.	78.2
2c	n.d.	10.8	n.d.	14.1	n.d.	10.8	n.d.	11.2
-	0.18	11.7	0.19	12.4	0.16	10.4	0.18	11.7
1d	n.d.	36	n.d.	37.6	n.d.	57.3	n.d.	58.9
2d	n.d.	8.6	n.d.	9.6	n.d.	8.9	n.d.	9.1

1 = DOPC/Chol liposomes; **2** = DOPC/Chol/GLT1 liposomes; **b** = OLE100; **c** = OLE50; **d** = OLE20; - = OLE in free form; n.d. = not determined.

CONCLUSIONS

- Olive leaf extracts, rich in polyphenols, exhibited significant antimicrobial activity against both bacterial strains, likely due to a synergistic effect among the bioactive compounds.
- Neutral liposome-loaded OLEs (**1b**–**1d**) did not improve antimicrobial activity, resulting in higher MIC and MBC values compared to free-form OLEs. Conversely, galactosylated liposomes enhanced the activity of OLE100 and OLE20 (**2b** and **2d**), although no improvement was observed for OLE50 activity (**2c**).
- The improved antimicrobial activity of formulations **2b** and **2d** is probably related to the presence of GLT1 in the lipid bilayer, which facilitates electrostatic interactions between the cationic liposomes and negatively charged bacteria. Additionally, interaction between galactose residues, exposed on the liposome surface, and bacterial lectins or sugar transporters likely enhance the diffusion and the interaction of the active compounds through bacterial cell walls, amplifying the synergistic effect of OLE polyphenols.
- These findings pave the way for novel strategies to combat drug-resistant infections, a major concern in the era of antibiotic resistance, by exploiting the synergistic effects of botanical polyphenols delivered through functionalized liposomes as targeted drug-delivery systems.

REFERENCES [1] A. Rodríguez-Rojas et al., *Int J Med Microbiol*, **2013**, *303*, 293. [2] M. Aliaño-González et al., *Biomolecules*, **2022**, *12*, 2. [3] T. Lian et al., *J Pharm Sci*, **2001**, *90,* 667.