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Olive leaves: From waste to active in pharmaceutical micro and nanoformulations

European project aimed at valorizing waste from olive oil production,
to make products of pharmaceutical, cosmetic and food interest

LEAF4VALUE

OLIVE LEAF MULTI-PRODUCT CASCADE BASED BIOREFINERY

From an under-used biomass in the primary sector to tailormade solutions for high added value international market applications

JULY 1ST 2021 — **JUNE 30TH 2024**

36 months

Coordinated by: NATAC GROUP (ES)
Overall budget: € 5.687.060
Project partners: 16
Countries: 9 EU countries
Grant Agreement: 101023256

Bio-based Industries Consortium
BBI JU
Co-funded by the Horizon 2020 programme of the European Union

GRANT AGREEMENT Nº 101023256

16 PARTNERS & 9 EUROPEAN COUNTRIES INVOLVED

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OLEAF4VALUE covers the whole value chain

1. Olive biomass: Smart dynAmic Multi-valorisation-route BIOrefinery (SAMBIO) for the cascade valorisation of the olive leaf biomass according to its physicochemical composition, particularly modulated by specific pretreatments to produce target products

2. Biorefining: Advanced green extraction and isolation technologies are used to sequentially separate all fractions and compounds of value, with a zero-waste approach.

3. Applications: Enzymatic biotransformation and nanoencapsulation technologies will be applied to develop tailor made prototypes according to end user market needs from high value sectors: food, feed, health, cosmetic, pharma and chemical industries.

4. Market: Large companies from these sectors within the consortium will guarantee a good market-oriented approach throughout the project.

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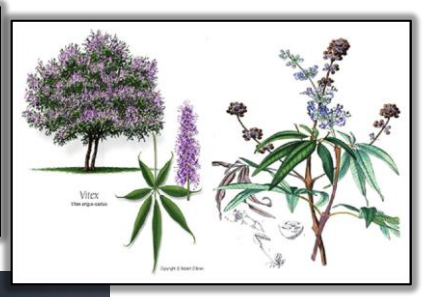
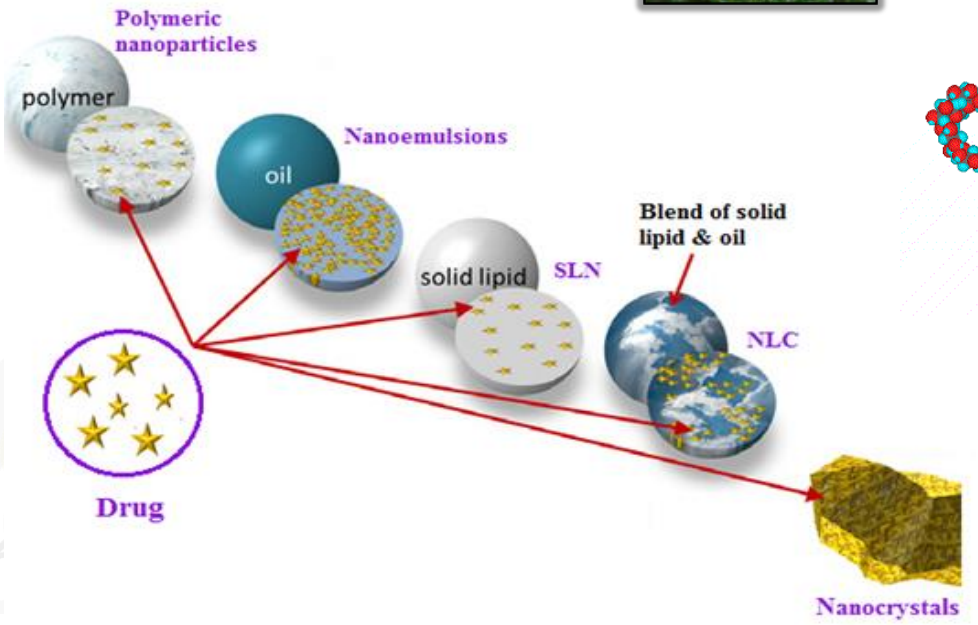
University of Florence (UNIFI)

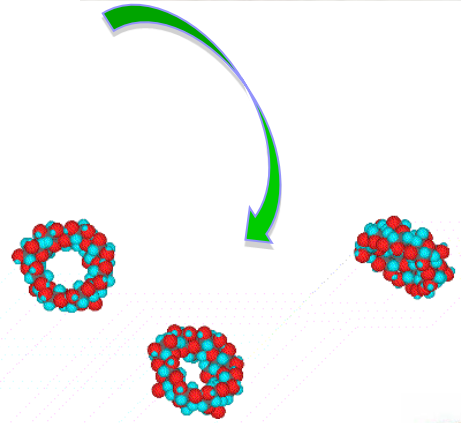


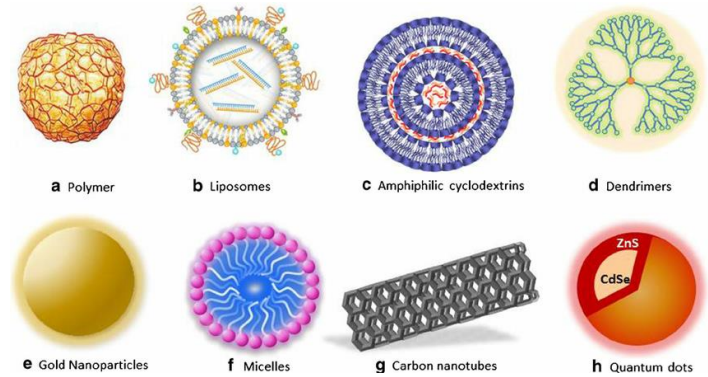












- Increase solubility
- Protection against chemical and biological degradation processes
- Increased permeation and cellular internalization
- Modified release
- Site-specific delivery through superficial modifications
- Reduction of side effects deriving from systemic distribution

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Role of UNIFI in OLEA4VALUE project

- Micro/Nanoformulations to improve technological performance of olive bioactive compounds.
- *In vitro* evaluation of influence of optimized formulations on solubility and permeability of olive bioactive compounds and olive leaf fractions.

Microemulsions
Polymeric micelles

Solid dispersions
Co-ground products

In vitro Solubility test
Dissolution test
PAMPA and Caco-2 cells

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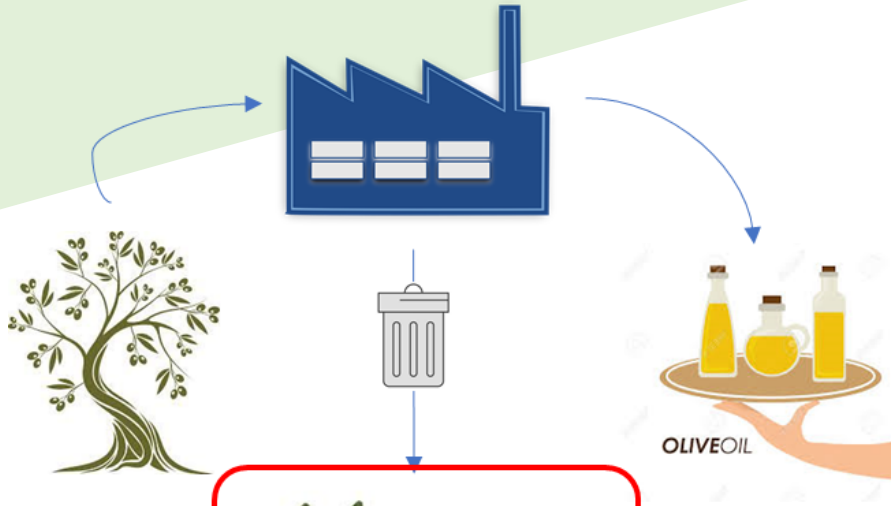
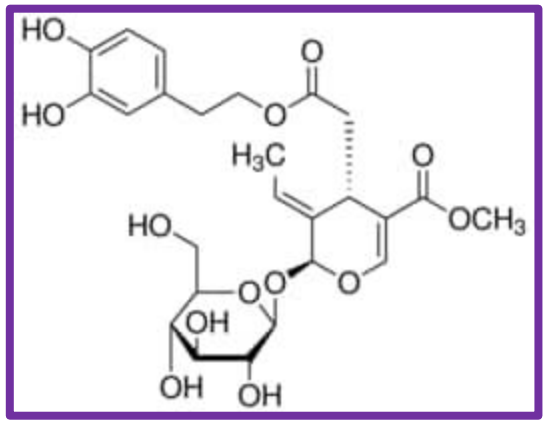
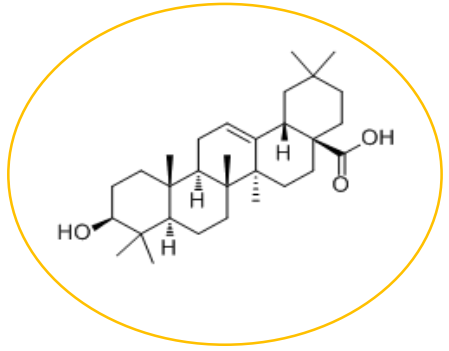
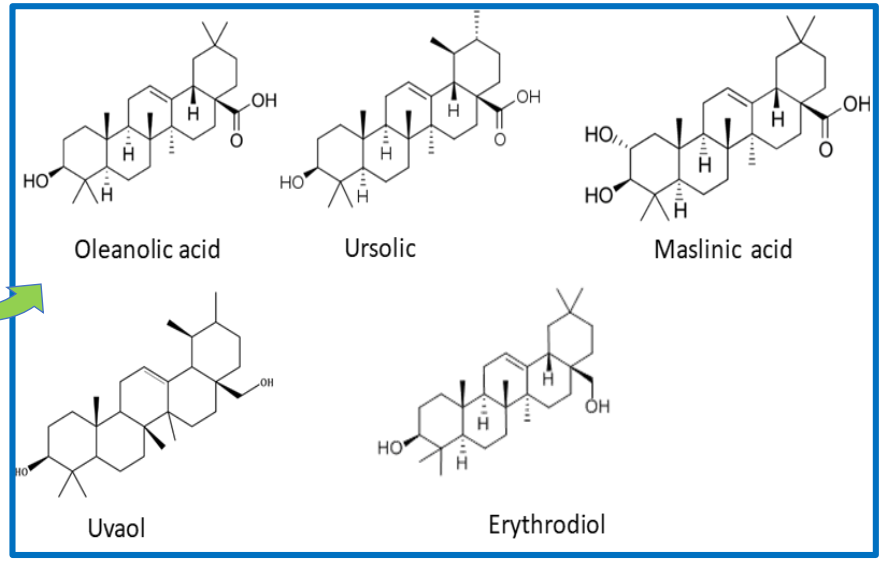




Polyphenols extract



Oleanolic Acid (OA)



Pentacyclic triterpenes-enriched extract

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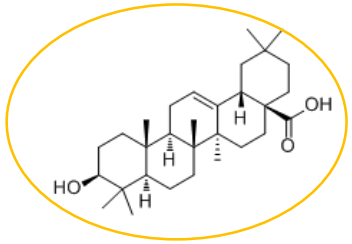
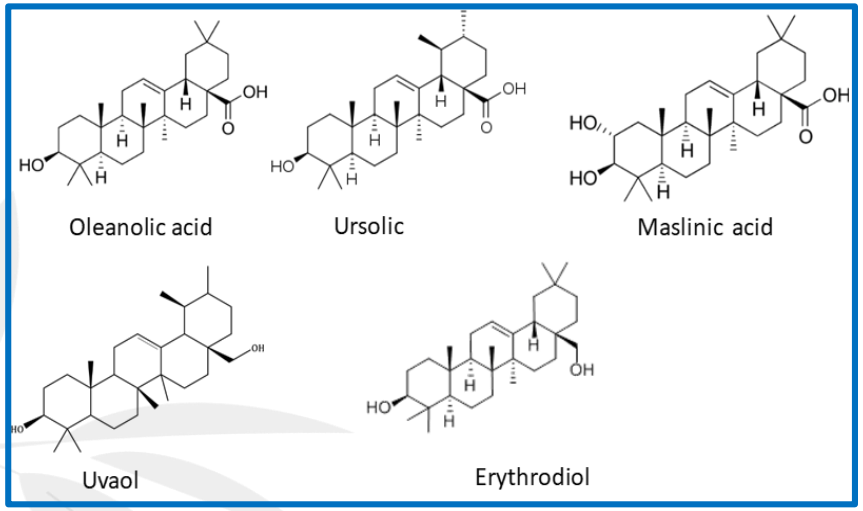
Microemulsions

Systems of water, oil, surfactant which form an optically isotropic and thermodynamically stable solution

Oleanolic Acid (OA)



Pentaciclic triterpenes-enriched extract (TTP70)



Solubility of OA in water < 1 μg/mL
Solubility of Extract in water: TTPs ~ 7 μg/mL

Solubility in ME 1 or 3 mg/mL

- **ME developed**
  - **Capmul ME OA 1 mg/mL**
  - **Capmul ME VR2-001 Extract 1 mg/mL**
  - **Nigella ME OA 3 mg/mL**
  - **Capryol ME VR2-001 Extract 1 mg/mL**
  - **Nigella ME VR2-001 Extract 3 mg/mL**
  - **Capryol ME VR2-001 Extract 2 mg/mL**

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De Stefani, Vasarri, Salvatici, Grifoni, Quintela, Bilia, Degl'Innocenti, Bergonzi. Pharmaceutics 2022, 14, 2232
Vasarri, Degl'Innocenti, Albonetti, Bilia, Bergonzi. Int. J. Mol. Sci. 2023, 24, 12113

MEs' composition

Capmul PG8 5.95 % w/w
Transcutol HP 37.20 % w/w
Tween 20 15.98 % w/w
Water 40.48 % w/w

1 mg/mL of OA
1 mg/mL of NATAC extract

Transcutol HP: Diethylene glycol monoethyl ether
Capmul PG8: Propylene glycol monocaprylate

Nigella oil 2% w/w
IPM 2% w/w
Transcutol 30% w/w
Cremophor EL 16% w/w
Water 50% w/w

3 mg/mL of OA
3 mg/mL of NATAC extract

IPM: Isopropyl Myristate
Cremophor EL: PEG-35 Castor Oil

Transcutol HP 36% w/w
TPGS 4% w/w
Capryol 90 5% w/w
Water 55% w/w

1 mg/mL of NATAC extract

Transcutol HP 67% w/w
TPGS 7.5% w/w
Capryol 90 8% w/w
Water 17% w/w

2 mg/mL of NATAC extract

TPGS: D-a-Tocopherol polyethylene glycol 1000 succinate
Capryol® 90 - Propylene glycol monocaprylate

De Stefani, C.; Vasarri, M.; Salvatici, M.C.; Grifoni, L.; Quintela, J.C.; Bilia, A.R.; Degl'Innocenti, D.; Bergonzi, M.C. Pharmaceutics 2022, 14, 2232.
M. Vasarri, D. Degl'Innocenti, L. Albonetti, A.R. Bilia, M.C. Bergonzi. IJMS 2023 24,12113

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n° 101023256

Stability studies

Storage stability + 25°C and + 4°C:

Physical stability: DLS and ELS, Chemical stability: HPLC-DAD analysis

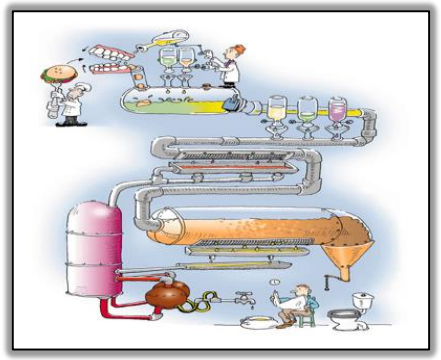
All formulations are physically and chemically stable for 8 weeks

Gastro-intestinal stability study in SGIF:

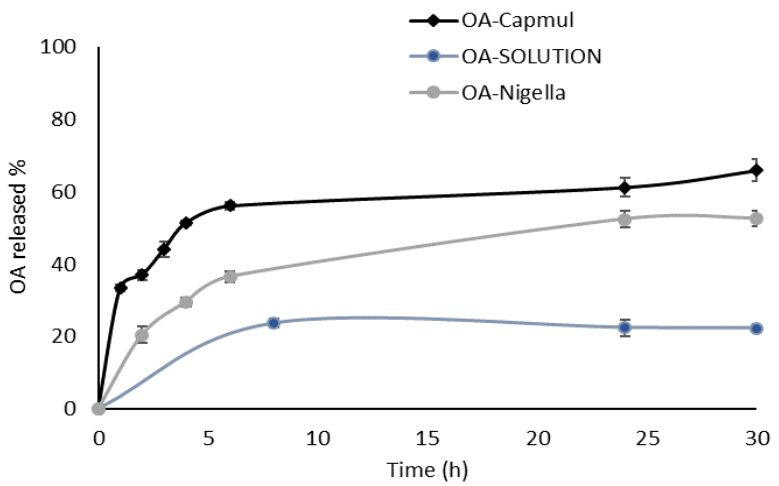
Physical stability: DLS and ELS.

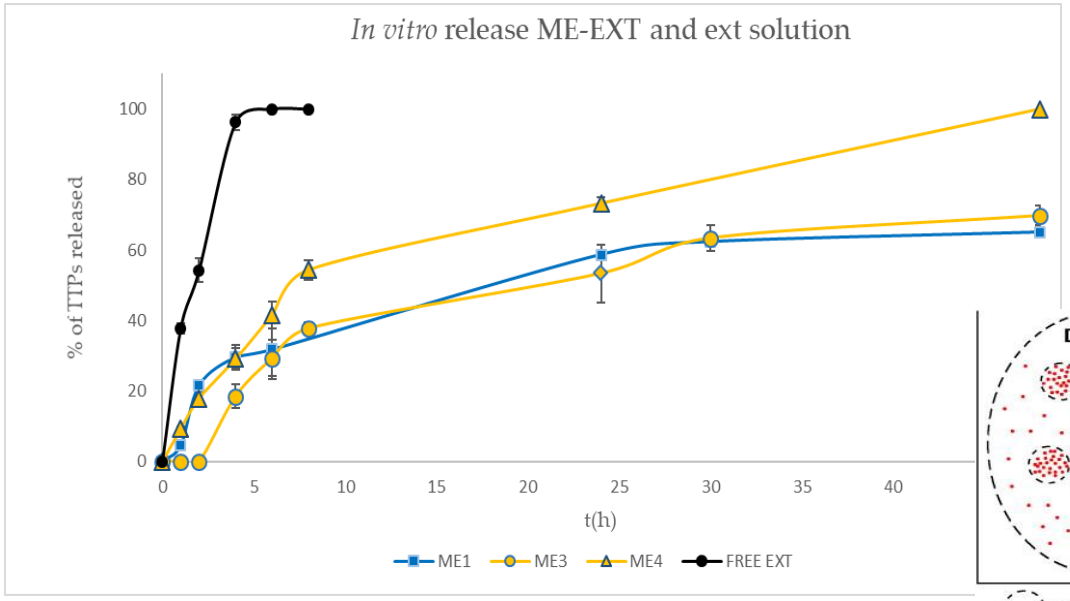
SGF, pH 1.2: 2 g of NaCl and 7 mL of HCl per liter of deionized water

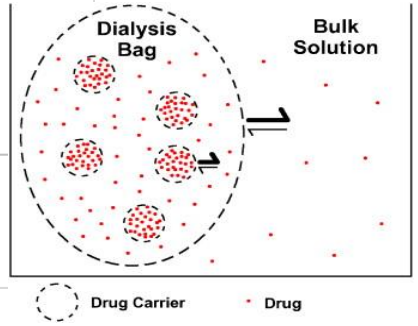
SIF, pH 6.8: 6.805 g of KH_2PO_4 and 0.896 g of NaOH per liter of deionized water



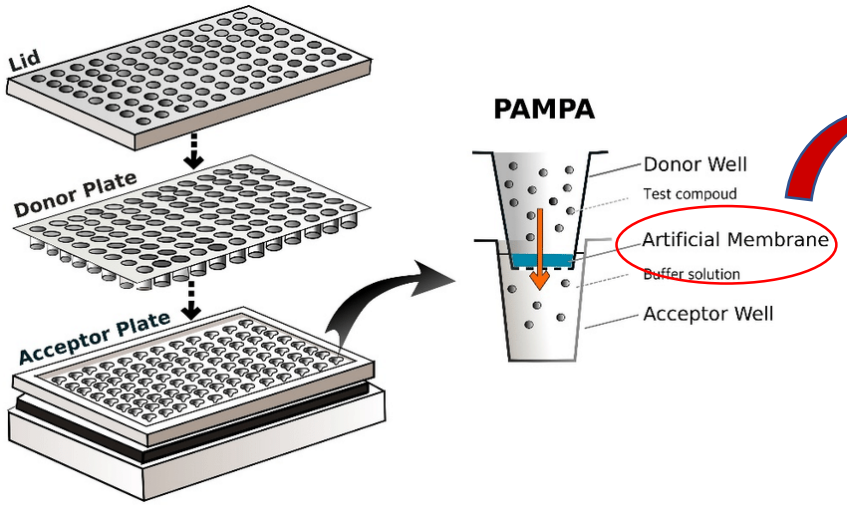
In vitro studies







PAMPA parallel artificial membrane permeability assay



Donor compartment: formulation (diluted in PBS), or OA in SDS 0.5% w/v
Acceptor compartment: PBS/EtOH 95:5 or 70:30

Lecithin 10 mg/mL + Cholosterol 8 mg/mL in 1,7-octadiene (10 mL)

OA Pe 2.7 x 10^{-7} ± 1.4 x10-8 cm/s

TTP70 extract (in PBS:EtOH 90:10) no permeation for 6 h. Recovery > 95%

ME 1-OA Pe 5.7 x10^{-6} ± 4.0 x10^{-7} cm/s at 1 h. Recovery 98%

ME1 TTP70 extract Pe TTPs 4.31x10^{-06} ± 2.01 10^{-07} cm/s at 1 h. Recovery 90%

ME 2-OA Pe 4.7 ± 0.04 x10^{-5} cm/s at 1 h. Recovery 94%

ME3 TTP70 extract (1 mg/mL) Pe TTPs 2.7 x 10-6 ± 3.70 x 10-8 cm/s at 2h. Recovery 82%

ME4 TTP70 extract (2 mg/mL) Pe TTPs 1.2 x 10-7 ± 3.8 x 10-8 cm/s at 2 h. Recovery 100%

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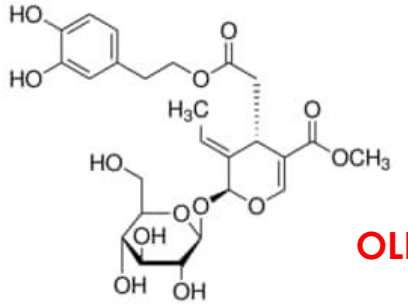
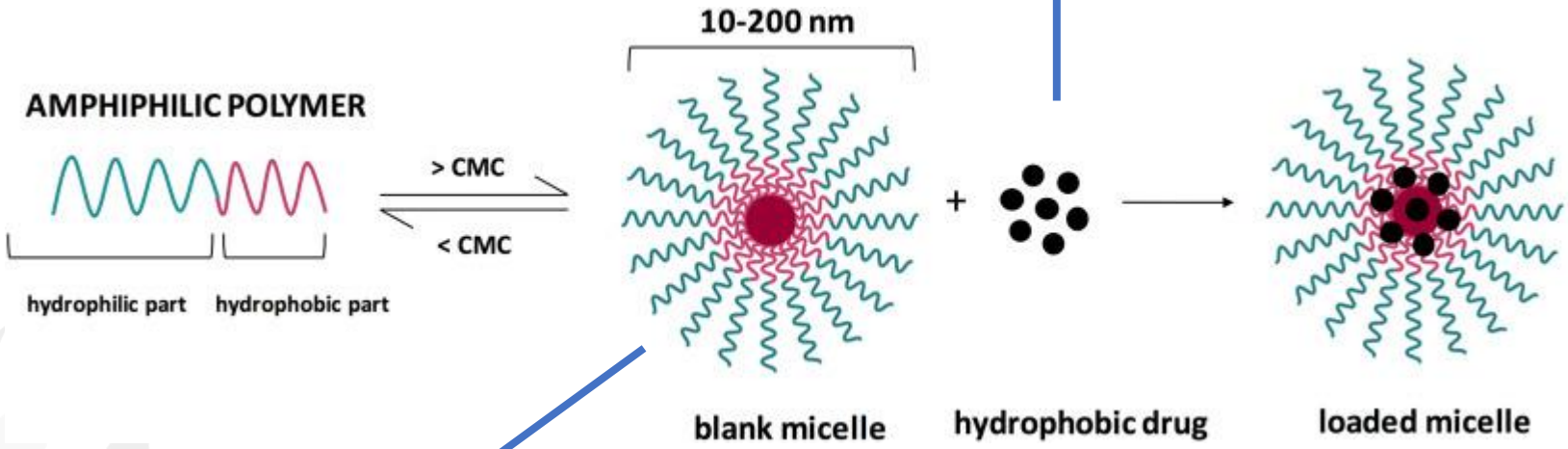
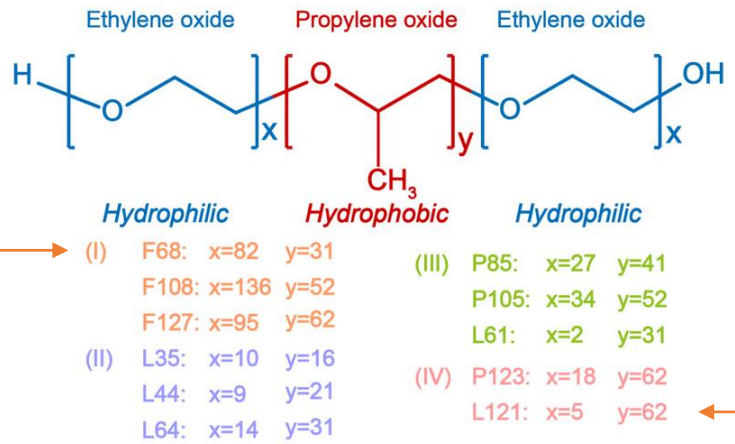
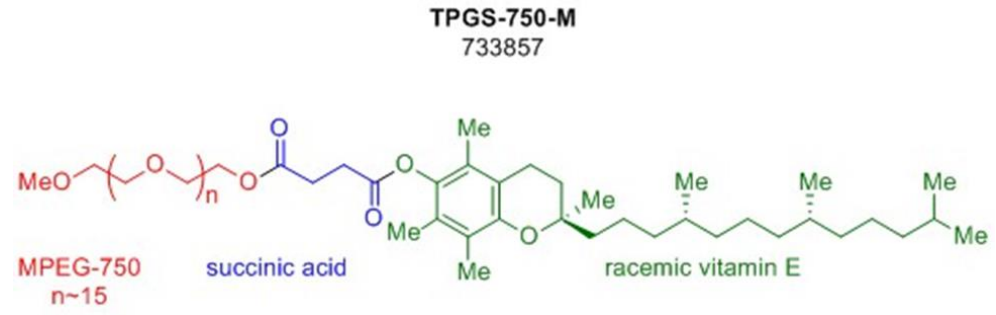
Polymeric micelles



OLE40, polyphenols extract







D-α-tocopheryl polyethylene glycol succinate (Vitamin E TPGS or simply TPGS)

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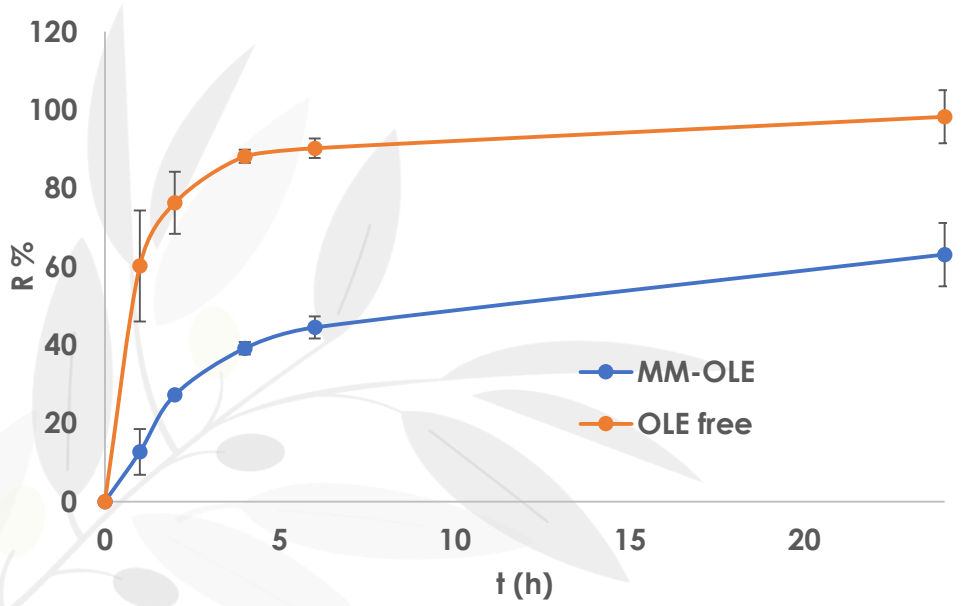
Pluronic L121: Pluronic P188: TPGS 2:2:1

- L121 200 mg
- P188 200 mg
- TPGS 100 mg
- OLE 15 mg/mL

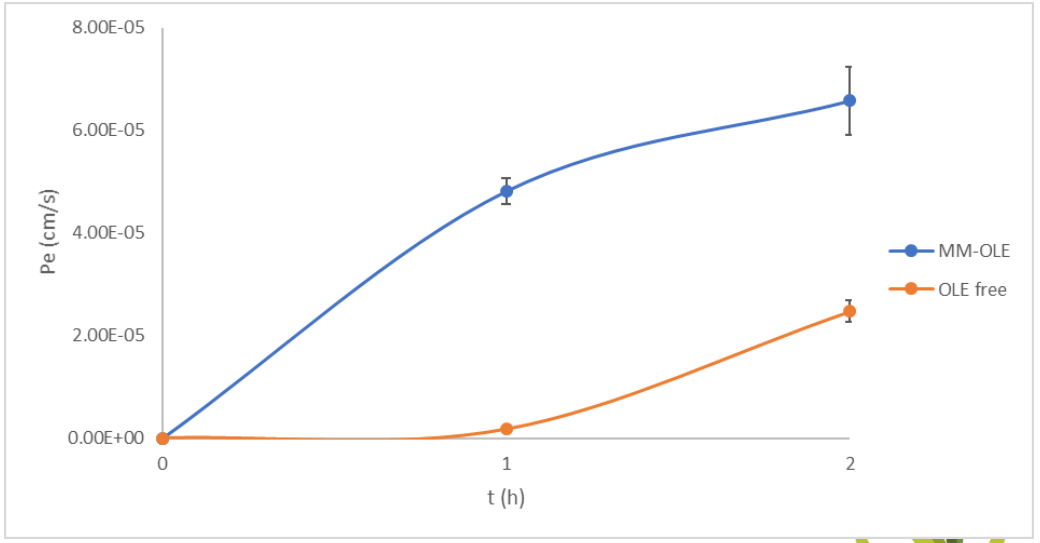
Micelles
Size 14.21+/- 0.14
PdI 0.19 +/-0.05
Recovery % : 91.55 +/- 4.55
EE% : 65.82% +/-1.11

Micelles after freeze-drying process
Size 15.97 +/- 1.13
PdI 0.24 +/-0.04
Recovery % : 88.12 +/- 2.07
EE% : 64.71% +/- 2.82

In vitro release study
Release medium: PBS and tween 80 (0.5% w/v)



PAMPA
Donor compartment: formulation or OLE inPBS, Acceptor compartment: PBS



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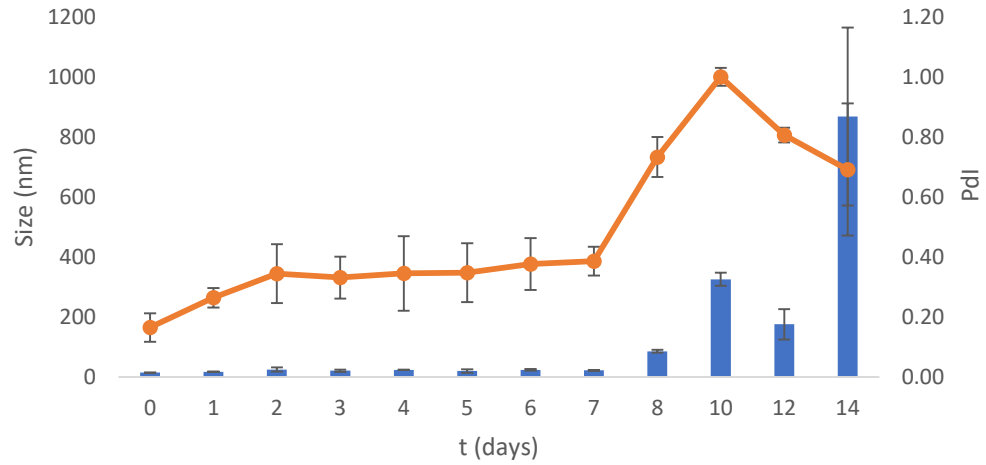
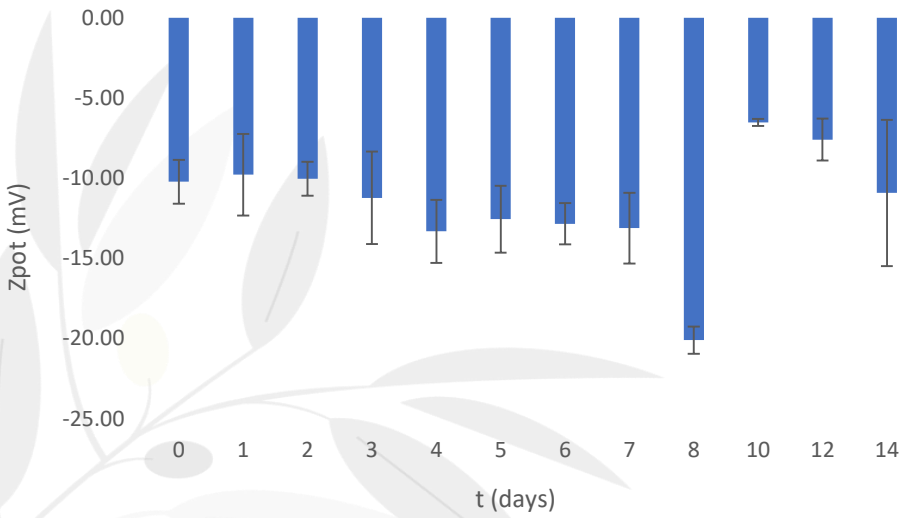
Stability studies

Storage stability at + 4°C:
Physical stability: DLS and ELS, Chemical stability: HPLC-DAD analysis

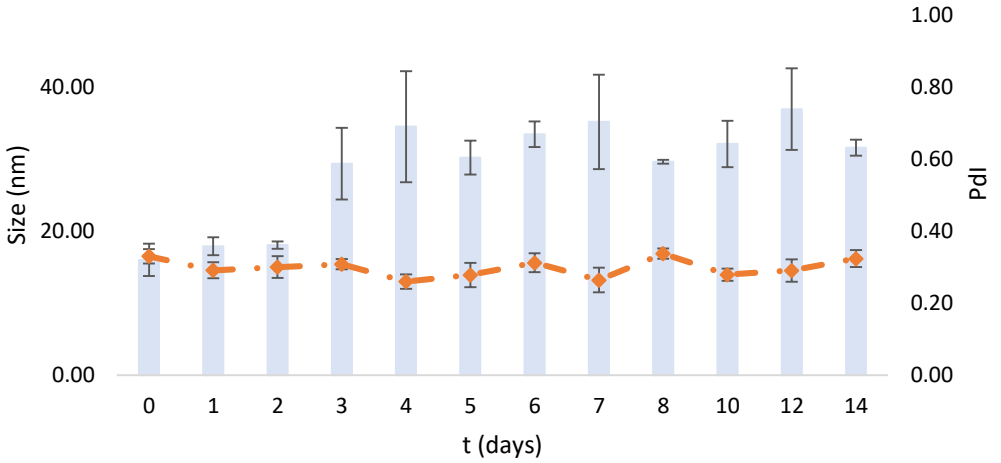
Colloidal dispersion

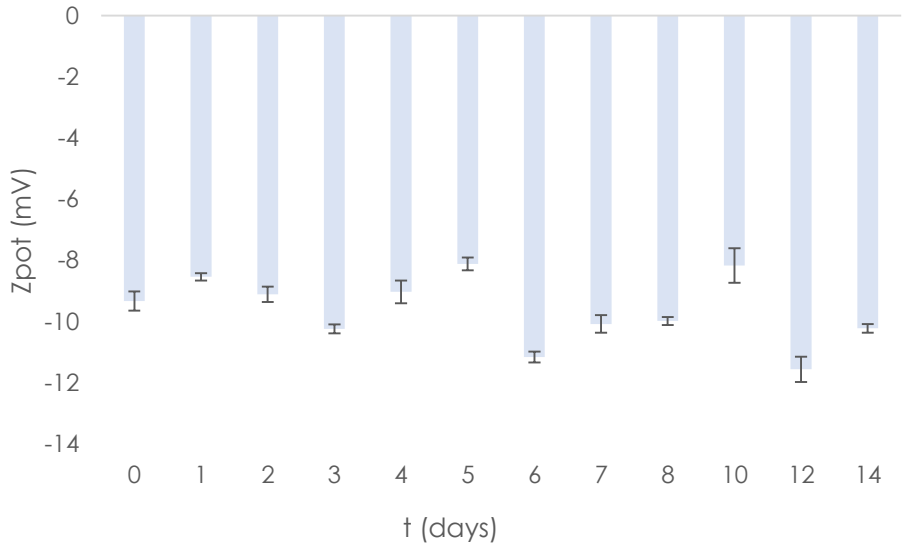




days	EE%	ds
1	68.24	2.56
7	60.39	4.78
14	57.34	3.65

Freeze-dried product





days	EE%	ds
1	63.45	0.98
7	61.38	2.42
14	61.12	3.18

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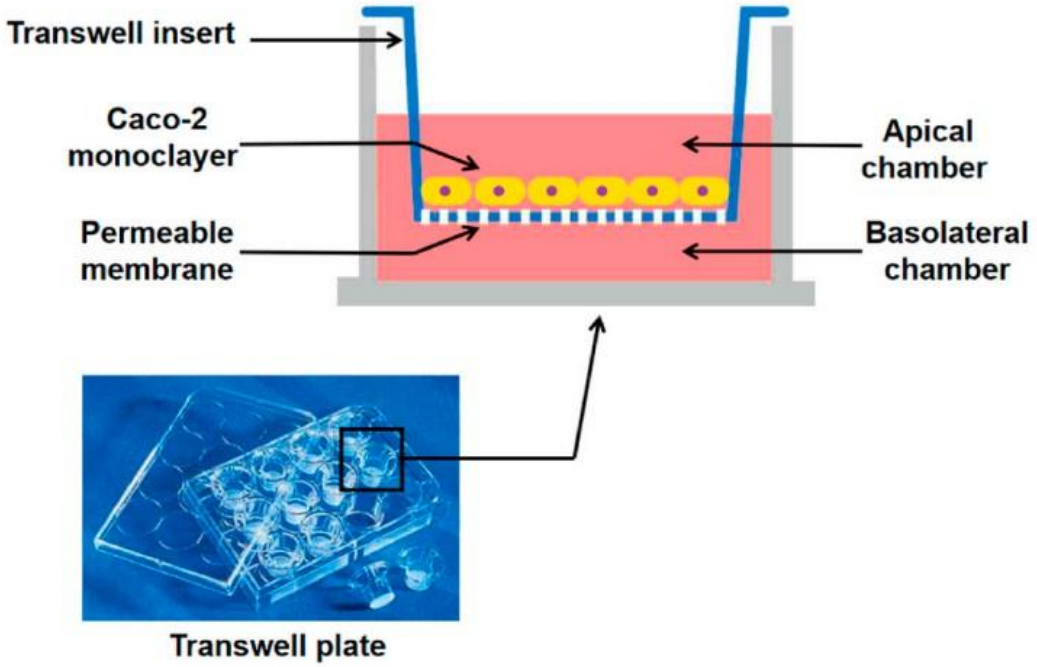
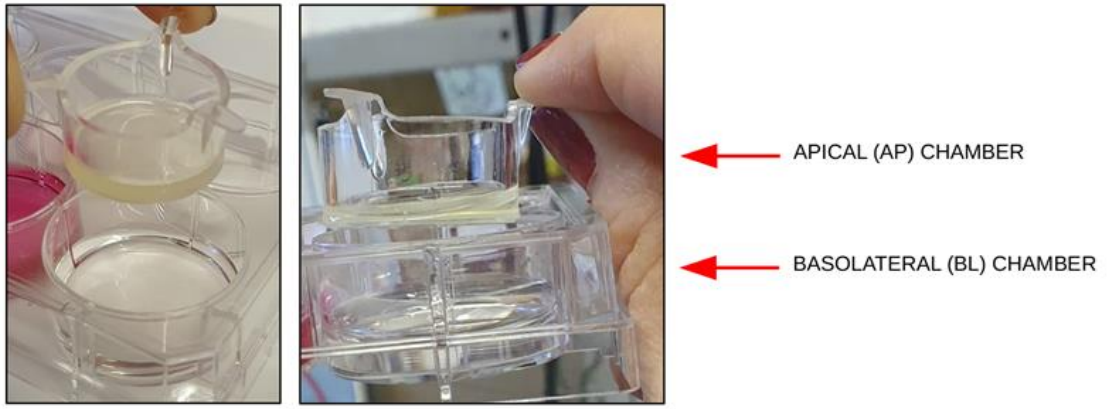
Caco-2 cells permeability test





Papp OLE 40	1.10 ± 0.03 x 10-6 cm/s
Papp PM OLE 40	2.18 ± 0.18 x 10-6 cm/s

Recovery 86-90%
P% (LY) 1.30 and 1.15%

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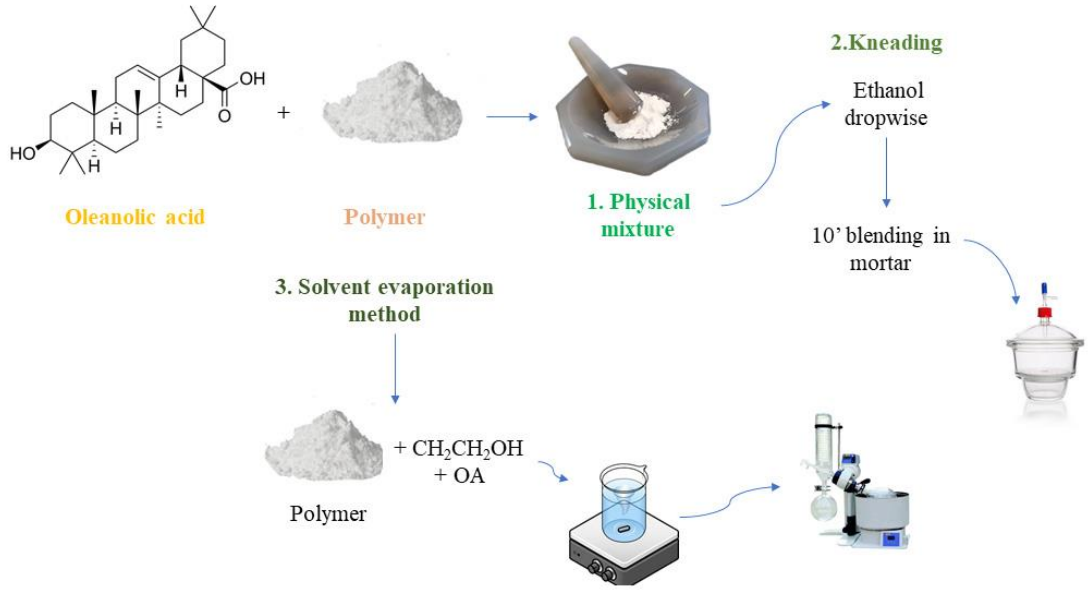
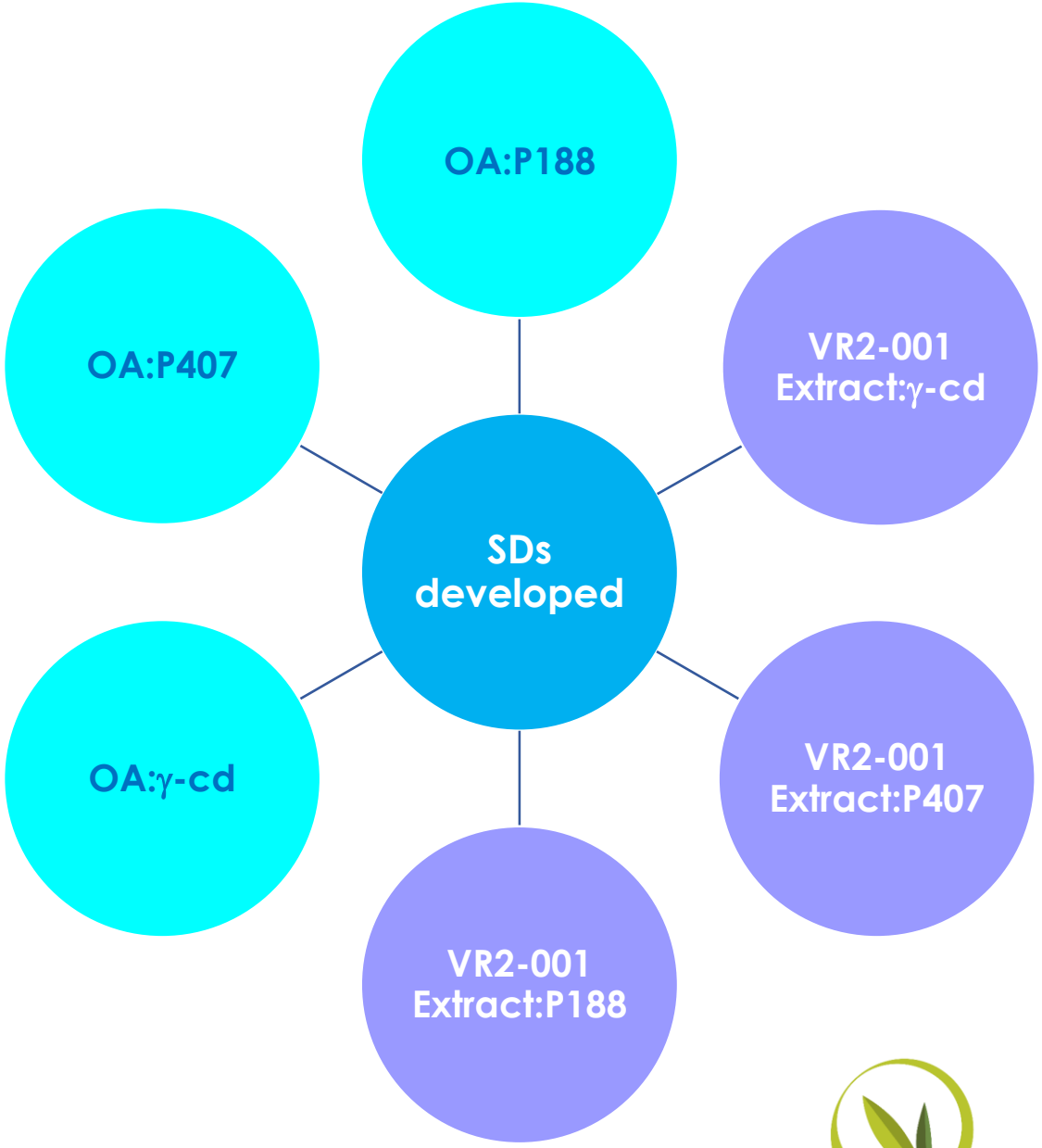
Solid dispersions

Solid dispersion is the dispersion of one hydrophobic active ingredient in an inert hydrophilic carrier in the solid state prepared using different methods. In these systems, the drug is combined with a water-soluble polymer to produce a single-phase amorphous mixture of the drug and the polymer.





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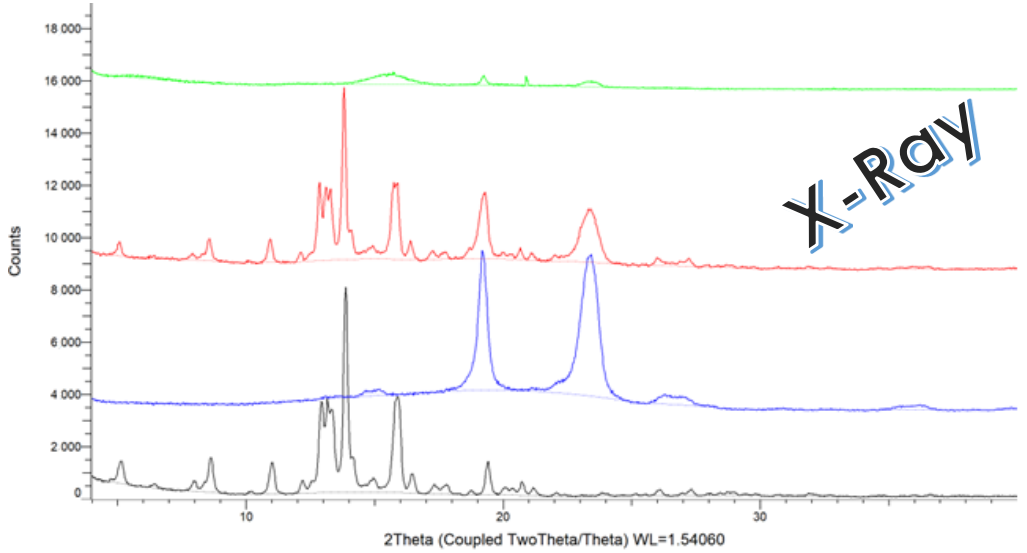
De Stefani, C.; Lodovichi, J.; Albonetti, L.; Salvatici, M.C.; Quintela, J.C.; Bilia, A.R.; Bergonzi, M.C. Molecules 2022, 27, 3042.

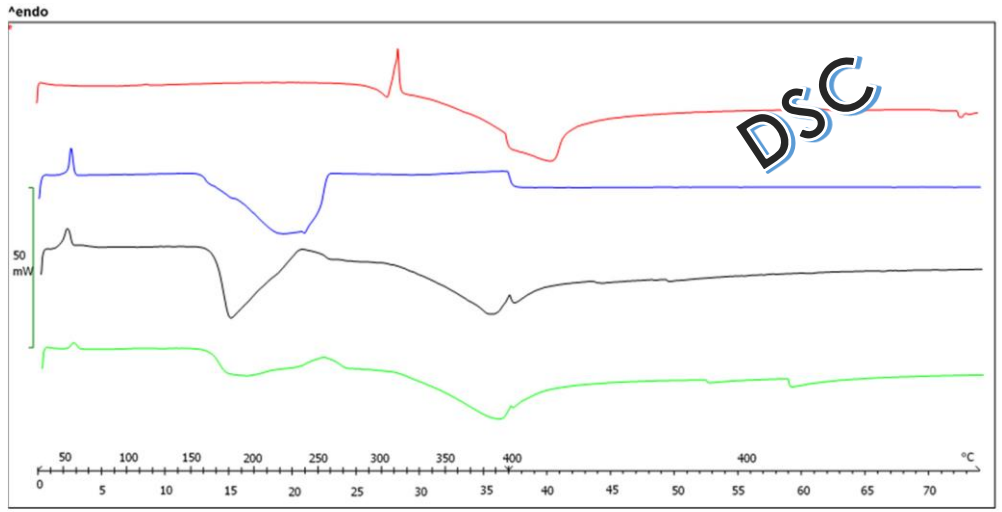
SD's characterization

Table 2. Solubility of OA in hydrophilic polymers, using 1:2 drug-to-polymer weight ratio and different preparation methods (PM: physical mixture; K: kneading; SEM: solvent evaporation method).

Method	Polymer	Solubility (μg/mL)
PM	Poloxamer 188	14 ± 2
	Poloxamer 407	32 ± 0.7
	PEG 4000	6 ± 0.8
	PEG 6000	5 ± 0.8
	γ-CD	66± 3
K	Poloxamer 188	79 ± 1
	Poloxamer 407	130 ± 7
	PEG 4000	12 ± 2
	PEG 6000	9 ± 2
	γ-CD	7 ± 0
SEM	Poloxamer 188	190 ± 42
	Poloxamer 407	170 ± 28
	PEG 4000	10 ± 1
	PEG 6000	12 ± 3
	γ-CD	145 ± 4



OA (black), Poloxamer 407 (blue), PM1:1 (red), and SEM1:1 (green).



OA (red), Poloxamer 407 (blue), PM OA-P407 1:2 (black), and SEM OA-P407 1:2 (green)

De Stefani, C.; Lodovichi, J.; Albonetti, L.; Salvatici, M.C.; Quintela, J.C.; Bilia, A.R.; Bergonzi, M.C. Molecules 2022, 27, 3042.

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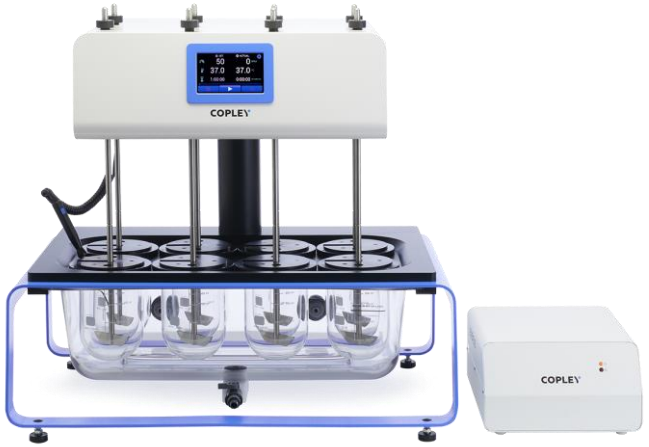
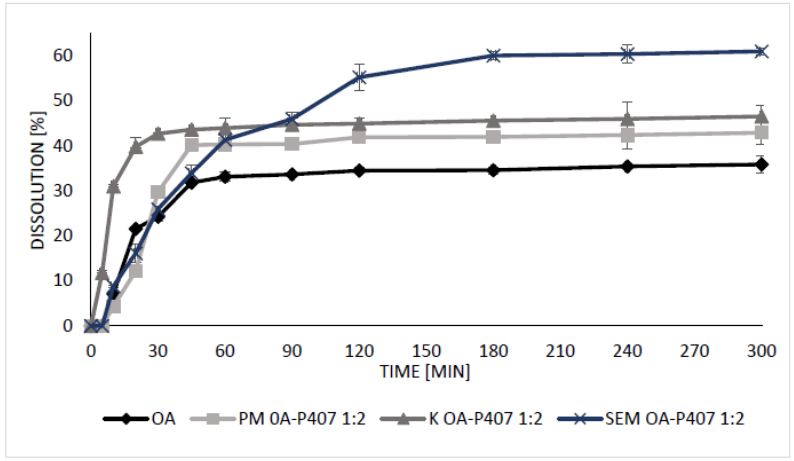
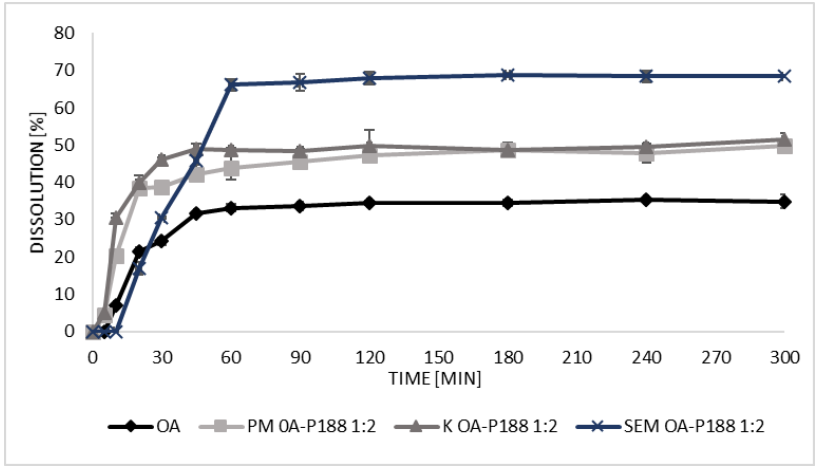
OA SD Dissolution test



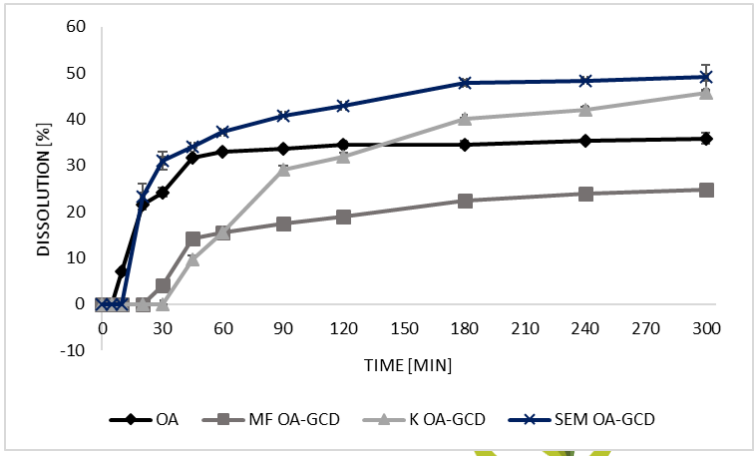
Poloxamer 407



Poloxamer 188



γ-CD



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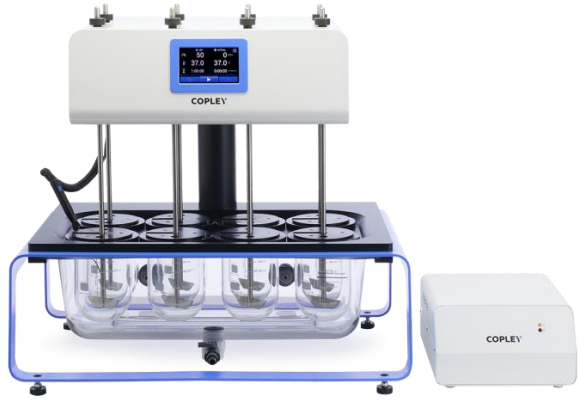
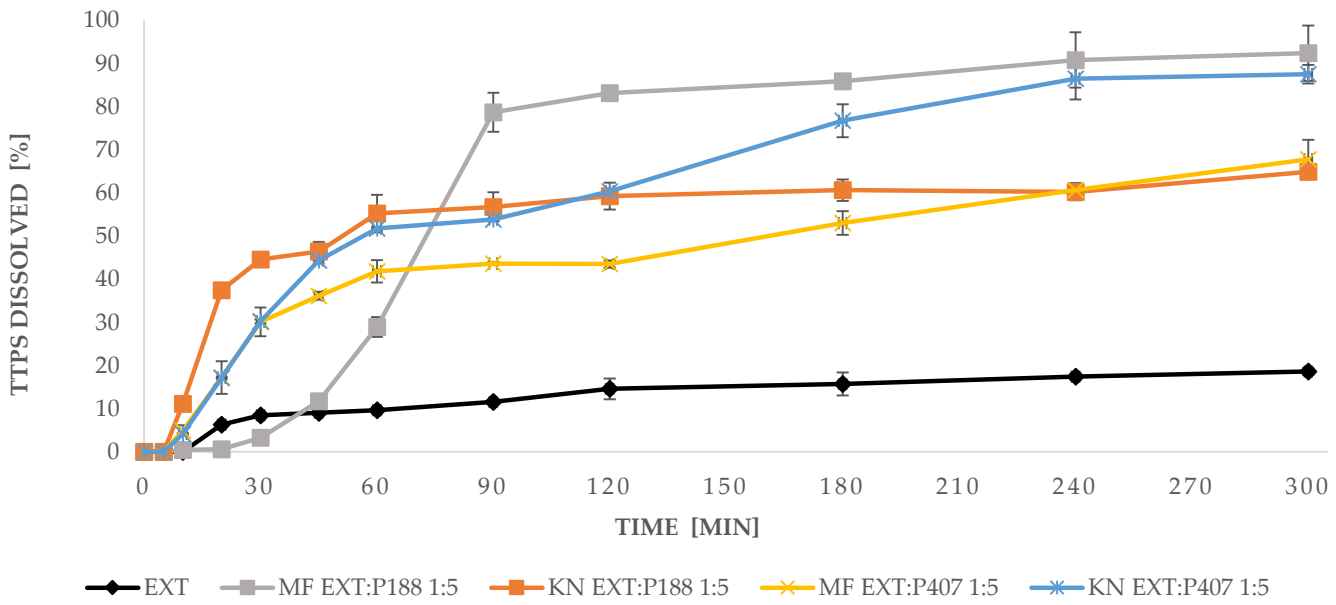
TTP70 SD Dissolution test





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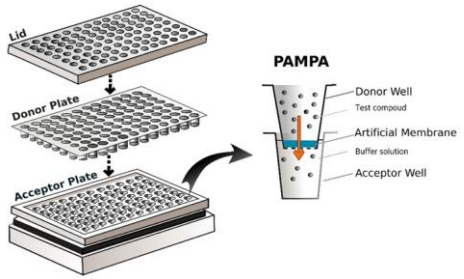
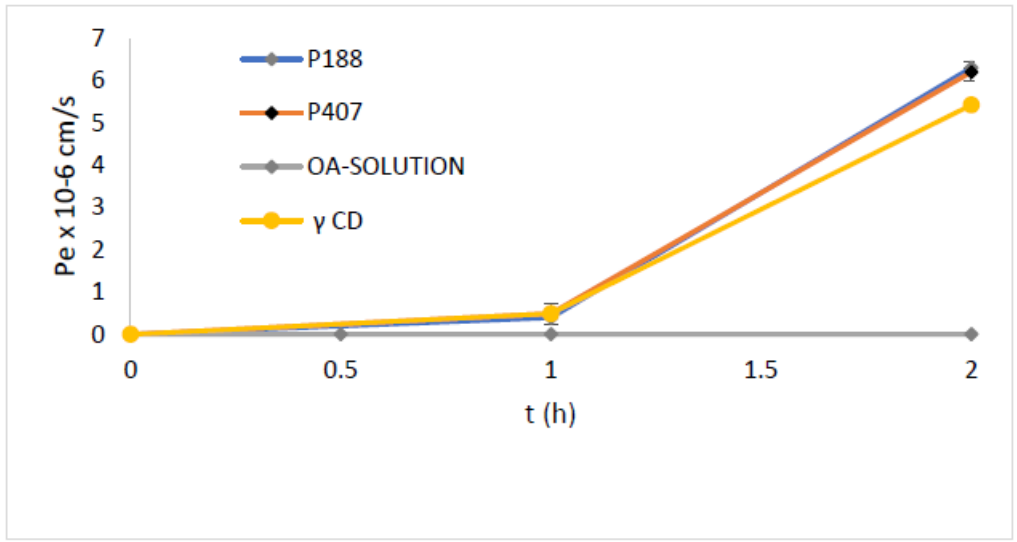
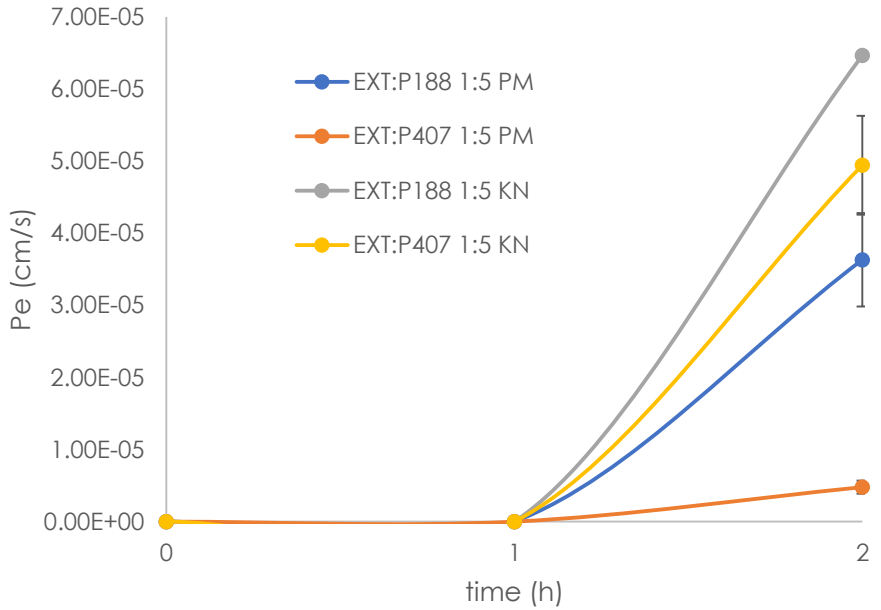
PAMPA







OA Pe 2.7 x 10^{-7} ± 1.4 x10^{-8} cm/s

P407 Pe 6.2 ± 0.22 x 10^{-5} cm/s
P188 Pe 6.3 ± 0.53 x 10^{-5} cm/s
γ-CD Pe 5.43 ± 0.12 x 10^{-5} cm/s

Recovery 85%

TTPs extract no permeation for 6 h.

MF P407 Pe 4.78 x 10-6 ± 9.16 x 10^{-7} cm/s
KN P407 Pe 4.90 x 10-5 ± 6.87 x 10^{-6} cm/s

MF P188 Pe 3.63 x 10-5 ± 6.48 x 10^{-6} cm/s
KN P188 Pe 6.46 x 10-5 ± 6.56 x 10^{-6} cm/s

Recovery > 95%

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Mechanochemical activation

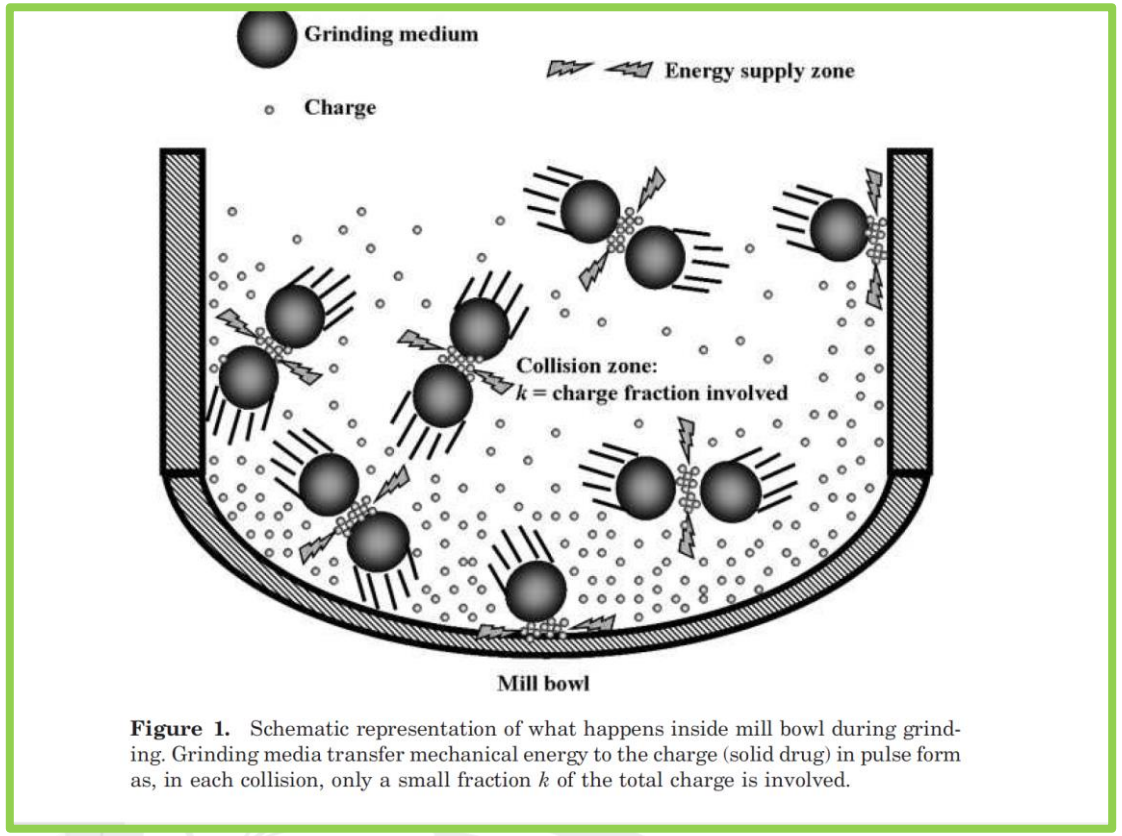
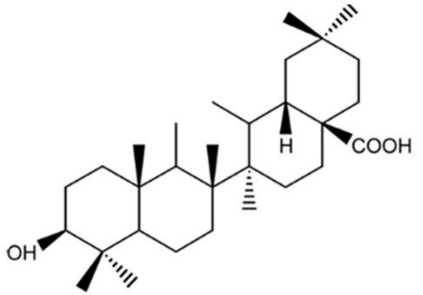


Figure 1. Schematic representation of what happens inside mill bowl during grinding. Grinding media transfer mechanical energy to the charge (solid drug) in pulse form as, in each collision, only a small fraction k of the total charge is involved.



Oleanolic acid (OA)

Stabilizer

Na Cholate

Model: Retsch MM200
Tubes: 25 ml in stainless steel
Sphere: Tempered steel 2x12 mm + 2x5 mm

The mill is the typical energy supplier and grinding and cogrinding are the common processes inducing mechanochemical transformations. There is a complete amorphization of the drug.

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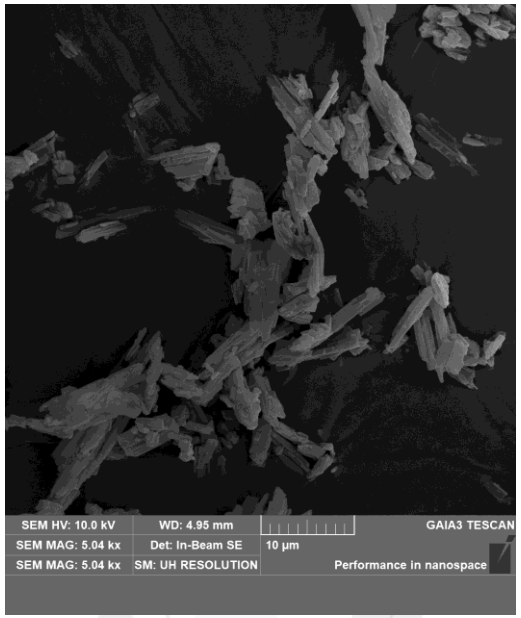
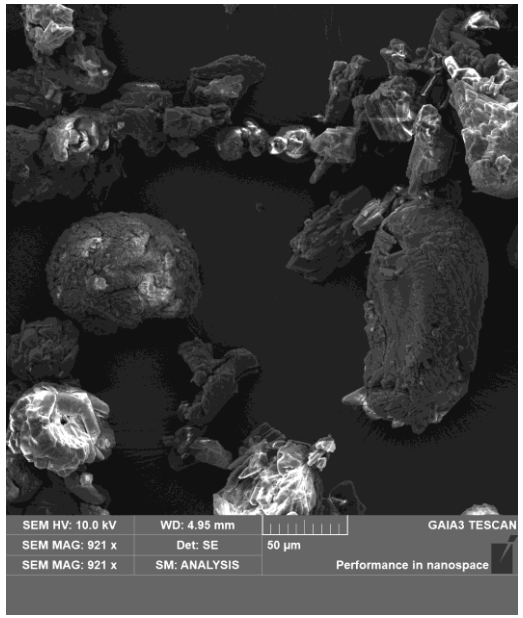
Results: SEM



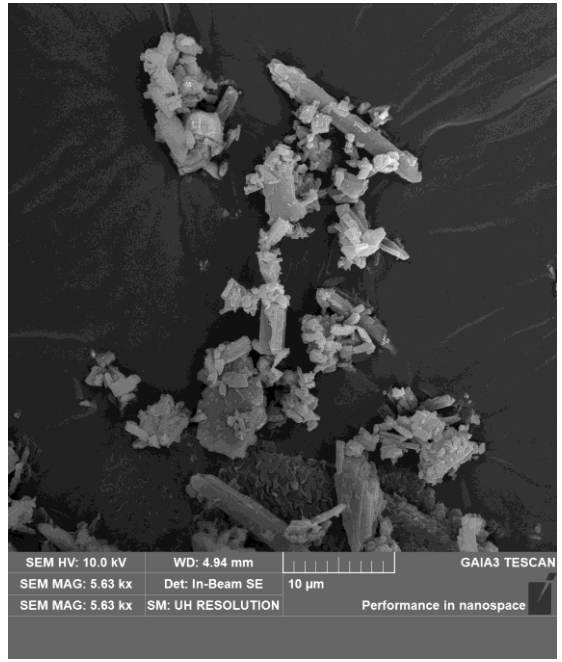
OA

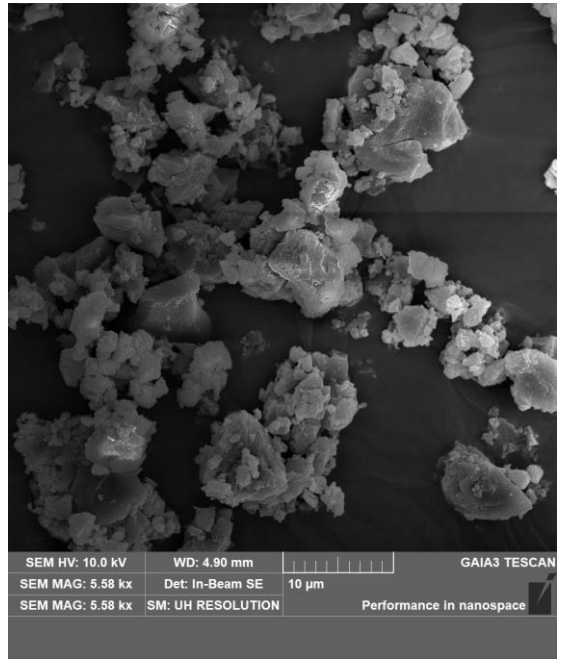


Na cholate

PM

Coground





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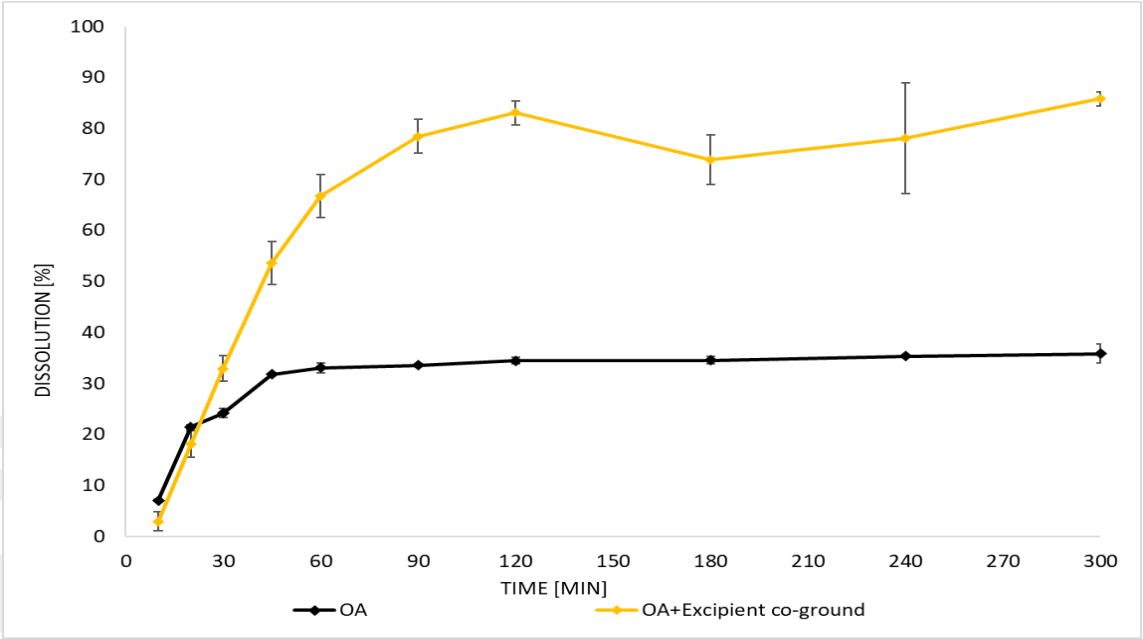
Dissolution test



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Conclusions

- ✓ Micro/Nanoformulations were developed to improve technological performance of olive bioactive compounds.
- ✓ 6 MEs, 6 SDs, 2Co-G for triterpenes and 1 PM for polyphenolic extract.
- ✓ The optimized formulations improved the solubility and *in vitro* permeability of olive bioactive compounds and olive leaf fractions.
- ✓ The formulations improved the bioactivity of compounds in *in vitro* tests.

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Thanks for your kind attention!

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