Nanostructured lipid carriers (NLC) for the delivery of natural molecules with antimicrobial activity: production, characterization and *in vitro* studies

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25 **ABSTRACT**

26 This study describes the preparation, characterization and *in vitro* activity of nanostructured lipid
27 carriers (NLC) encapsulating natural molecules with antimicrobial activity, namely plumbagin,
28 hydroquinon, eugenol, alpha-asarone and alpha-tocopherol. NLC were prepared by melt and
29 ultrasonication method and characterized by Cryo-TEM for morphology and SdFFF for dimensional
30 distribution and active encapsulation yields. *In vitro* tests conducted on bacteria, fungi and human
31 cell culture. *In vitro* tests demonstrated that plumbagin is strongly toxic towards *F. oxysporum*
32 especially when active molecules are loaded on NLC. Plumbagin was completely non toxic on
33 cyanobacterial model strain up to a threshold over which cell viability was completely lost. NLC
34 loaded with active molecules showed a lower toxicity as compared to their free form on human
35 cultured cells. Notwithstanding further studies have to be performed, these systems can be potentially
36 proposed to control phytopathogenic organisms.

37

38

39 **Abbreviations:** Nanostructured lipid carriers: NLC; cryogenic transmission electron microscopy:
40 cryo-TEM; photon correlation spectroscopy: PCS; Sedimentation Field Flow Fractionation: SdFFF;
41 Plumbagin: PB, Hydroquinon: HQ, Eugenol: EU, α-Asarone: aA; α-Tocopherol: aT; Methanol:
42 MeOH; dimethyl sulfoxide: DMSO.

43

44 **Keywords:** Nanoparticles, Nanotechnology, Formulation, Nanostructured lipid carriers.

45 **Introduction**

46

47 The long-term use of synthetic agrochemicals has led European governors and institutions to strongly
48 suggest and support research to find new eco-friendly solutions for agricultural practices reducing
49 environmental pollutants and increasing the use of bio-based products for pest control (Copping and
50 Duke, 2007; Duke et al., 2008; Dayan et al., 2009).

51 Despite the proved antimicrobial activity against phytopathogens of several natural extracts or pure
52 natural compounds (Balestra et al 2009; Dan et al., 2010; Pradhanang et al., 2003; Slusarenko et al.,
53 2008; Varvaro et al., 2001, Cowan, 1999; Scalbert et al., 1991; Shihabudeen et al., 2010; Tsuchiya et
54 al., 1996), their in-field application points out some toughness, mainly represented by the low
55 solubility in water of the extracts, their instability and also by their handling and storage
56 characteristics. A possible effective solution to these critical points is represented by the use of micro-
57 or nano- encapsulation strategies that may minimize the natural bioactive compound(s) degradation
58 optimizing the efficacy as pest-controller. Micro- or nano- particles can be constituted by non-toxic,
59 chemically inert (inactive), biocompatible polymer or lipid matrix able to alloy both bioactive
60 compound(s) and excipients without altering their chemical and physical properties (Battaglia et al.,
61 2012; Nysrtom and Fadeel, 2012; Cortesi et al., 2002; Esposito et al., 2002; Takei et al., 2008; Glenn
62 et al., 2010; Gonzales et al., 2015).

63 In the present study, lipid-based nanoparticles, namely nanostructured lipid carriers (NLC), are
64 considered as a versatile tool with a high potential of applications. Indeed, NLC can solubilize a
65 number of molecules with different physico-chemical properties in a biocompatible and
66 biodegradable matrix with well-established safety profiles. Moreover, lipid-based nanoparticles can
67 significantly contribute in the field of green nanosystems due to their derivation from natural source.
68 The production of NLC was carried out using blends of solid (e.g. triglycerides) and liquid (e.g.
69 tricaprylin) lipids at room and body temperatures (Saupe et al., 2005) obtaining an imperfect and

70 disordered lipid matrix able to accommodate poor water-soluble compounds (Yoon et al., 2013)
71 maintaining their physical stability for long periods of time.
72 Starting from these premises, the main target of the current study was to produce by standard
73 protocols NLC characterized in terms of morphology, inner structure, dimensional distribution and
74 encapsulation yield (Jores et al., 2004). NLC were then checked as encapsulation and solubilisation
75 tool for natural bioactive pure compounds - namely Plumbagin, Hydroquinone, Eugenol, α-Asarone
76 and α-Tocopherol - chosen for their physical-chemical characteristics and their known in vitro
77 bioactivity (Kumar et al., 2013; Xu et al., 2015; Rajput et al., 2014; Teixeira et al., 2016). Therefore,
78 the aim and novelty of the research is to check the opportunity to use NLC as encapsulating
79 technology for bioactive natural compounds to be used in agriculture, trying to make bio-based
80 products competitive in the market of agricultural treatments products.
81
82
83 **Materials and methods**
84 ***Materials***
85 Plumbagin (PB), Hydroquinone (HQ), Eugenol (EU), α-Asarone (aA) and α-Tocopherol (aT) were
86 purchased from Sigma-Aldrich (Saint Louis, MO, USA) for checking and comparing their biological
87 activities as pure and microencapsulated compounds. The copolymer poly (ethylene oxide) (PEO, a)
88 -poly (propylene oxide) (PPO, b) (a=80, b=27) (poloxamer 188) was from BASF ChemTrade GmbH
89 (Burgbernheim, Germany). Caprylic/capric triglycerides (Miglyol 812 N, miglyol) were from Cremer
90 Oleo Division (Witten, Germany). Tristearin (stearic triglyceride), polysorbate 80 and all other
91 chemicals were from Sigma-Aldrich (Milano, Italy).
92
93 ***Preformulatory study***

94 NLC were prepared by melt and ultrasonication method (Esposito et al., 2008). Briefly, 0.25 g of
95 lipid mixture was melted at 80 °C. The lipid mixture was constituted of tristearin/miglyol 2:1 w/w.
96 4.75 ml of an aqueous poloxamer 188 solution (2.5 % w/w) at 80 °C were poured into the vial
97 containing the molten lipids. The mixture was then emulsified at 15000 rpm, 80 °C for 1 min, using
98 a high-speed stirrer (Ultra Turrax T25, IKA-Werke GmbH & Co. KG, Staufen, Germany). The
99 emulsion was subjected to ultrasonication (Microson TM, Ultrasonic cell Disruptor) at 6.75 kHz for
100 15 min and then cooled down to room temperature.
101 In the case of active containing NLC, 0.2% w/w (with respect to total weight of dispersion) of each
102 active (i.e. PB, HQ, EU, aA and aT) was added to the lipid mixture and dissolved before addition to
103 the aqueous solution. Then the preparation was conducted as above described. The obtained
104 dispersions were stored at room temperature and protected from the light until used.
105
106 ***Characterization of NLC***
107 *Cryo-Transmission Electron Microscopy*
108 Samples vitrified as previously described (Esposito et al., 2008) were transferred to a Zeiss
109 EM922Omega transmission electron microscope for imaging using a cryoholder (CT3500, Gatan).
110 Throughout the examination, the sample temperature was kept below -175 °C. Specimens were
111 examined with doses of about 1000-2000 e/nm^2 at 200 kV. Images were recorded digitally using a
112 CCD camera (Ultrascan 1000, Gatan) by mean of an image processing system (GMS 1.9 software,
113 Gatan).
114
115 *Photon Correlation Spectroscopy (PCS)*
116 Submicron particle size analysis was performed using a Zetasizer Nano Series, Nano SP90 (Malvern
117 Instr., Malvern, England) equipped with a 5 mW helium neon laser with a wavelength output of 633
118 nm. Glassware was cleaned of dust by washing with detergent and rinsing twice with water for

119 injections. Measurements were made at 25 °C at an angle of 90°. Data were interpreted using the
120 CONTIN method (Pecora, 2000).
121
122 *Sedimentation Field Flow Fractionation Analysis*
123 A sedimentation field flow fractionation analysis (SdFFF) system (Model S101, FFFractionation,
124 Inc., Salt Lake City, UT, USA) was employed to determine the size distribution of particles by
125 converting the fractograms, i.e. the graphical results (Contado et al., 1997). Fifty microliter samples
126 were injected through a 50 µl Rheodyne loop valve. As mobile phase demineralized water pumped
127 at 2.0 ml/min was used. After the SdFFF system fractions of 3 ml, being the collecting time 90 sec,
128 were automatically collected by a Model 2110 fraction collector (Bio Rad laboratories, UK).
129
130 ***Active content in NLC***
131 The encapsulation efficiency (EE) and loading capacity (LC) of NLC were determined by
132 centrifugation followed by dissolution of nanoparticles in methanol as previously described (Puglia
133 et al., 2013). 100 µl of each NLC batch was loaded in a centrifugal filter (Microcon centrifugal filter
134 unit YM-10 membrane, NMWCO 10 kDa, Sigma Aldrich, St Louis, MO, USA) and centrifuged
135 (Spectrafuge™ 24D Digital Microcentrifuge, Woodbridge NJ, USA) at 8,000 rpm for 20 min. The
136 amount of active in both the lipid and the hydrophilic phase was determined by high performance
137 liquid chromatography (HPLC), analyses as below reported. The encapsulation parameters were
138 determined as follows.
139 $$EE = L_{active} / T_{active} \times 100 \quad (1)$$
140 $$LC = L_{active} / T_{lipid} \times 100 \quad (2)$$
141 where L_{active} is the amount of active encapsulated in NLC; T_{active} and T_{lipid} are the total weight of active
142 and lipid used for the NLC preparation, respectively.
143

144 ***HPLC Procedure***

145 The HPLC apparatus consisted of a two-plungers alternative pump (Jasco, Japan), an UV-detector
146 operating at the λ reported in Table 1, and a 7125 Rheodyne injection valve. RP-HPLC analysis was
147 performed using a stainless steel C-18 reverse-phase column (150×4.6 mm) packed with 5 μm
148 particles (Zorbax® Eclipse XDB - Agilent, USA). A pre-column filter Alltima C18 5μm (7.5x4.6
149 mm) was mounted above the column. Samples of 50 μl were injected through a 50 μl Rheodyne loop
150 valve. For each active the HPLC conditions are summarized in Table I.

151

152 **In vitro** ***release studies***

153 *In vitro* release studies were performed using the dialysis method. Typically, 1.5 ml of NLC
154 suspension were placed into a dialysis tube (8 cm) (molecular weight cut off 10,000-12,000; Medi
155 Cell International, England), then placed into 30 ml of receiving phase constituted of phosphate buffer
156 (100 mM, pH 7.4) and ethanol (70:30, v/v) and shaken in a horizontal shaker (MS1, Minishaker, IKA)
157 at 175 rpm at room temperature (23-25 °C). Samples of receiving phase were withdrawn at regular
158 time intervals, and analyzed by HPLC method as above described. Fresh receiving mixture was added
159 to maintain constant volume. The encapsulation efficiency of each active was determined four-fold
160 in independent experiments and the mean values ± standard deviations were calculated.
161 The obtained release data were fitted to the following semiempirical equations describing Fickian
162 dissolutive and diffusional release mechanisms, as previously indicated by Esposito et al. (2005).
163 Precisely,

164 $$Mt/M\infty = K_{diff}\, t^{0,5} + c' \quad (3)$$

165 $$1 - Mt/M\infty = e^{-K_{diss}}\, t + c \quad (4)$$

166 where Mt is the active fraction released at the time t, M∞ is the total active content of the analyzed
167 amount of NLC, K, c and c' are coefficients calculated by plotting the linear forms of the indicated

168 equations. The release data up to the plateau of percent were used to produce theoretical release
169 curves.
170
171 **In vitro *activity against phytopathogens***
172 *Clavibacter michiganensis subsp. nebraskense (ATCC 27822), Pseudomonas syringae pv. syringae*
173 *(ATCC 19310), Agrobacterium tumefaciens (DMS 30207), Agrobacterium vitis (DMS 6583)* were
174 used as phytopathogen bacterial strains. For testing the antifungal capacity, *Alternaria alternata*
175 (SIAPA, Italy) and *Fusarium oxysporum f. sp. radicis licopersici* (SIAPA, Italy) were employed.
176 The culture media – all purchased from Oxoid (http://www.oxoid.com/uk/) – were: Tryptone Soya
177 Broth for *C. michiganensis subsp. nebraskense* and *P. syringae pv. syringae*; Nutrient Broth for *A.*
178 *tumefaciens* and *A. vitis;* Potato Dextrose Broth for *A. alternata* and *F. oxysporum f. sp. radicis*
179 *licopersici.*
180 The antimicrobial activity was assessed in triplicate by the microdilution broth method in 96-well
181 microplates (Microplate reader - Model 680 XR, BIO-RAD) as Minimum Inhibitory Concentration
182 (MIC) values of the pure and microencapsulated compounds (Furtado et al., 2014). Pure and
183 microencapsulated compounds PB, HQ, EU, aA and aT were dissolved to reach a final concentration
184 of 0.1 mg/ml in the specific culture media for each micro-organism and dimethyl sulfoxide (2%;
185 DMSO). This solution was then progressively diluted following a 1:2 ratio for each test dilution to
186 determine the MIC values considered as the lowest concentration of each compound (pure and
187 microencapsulated) that inhibits the growth of the microorganisms. Each well contained a final
188 volume of 200 µL with 2x107 CFU/ml and 1x5x103 spores/ml, for bacterial and fungal strains
189 respectively.
190 The microplates with bacterial strains were incubated under gentle shaking (110 rpm) for 24 h at
191 28°C. Before (time 0) and after incubation (24h), the microplates were read at 615 nm to check the
192 bacterial growth. Then, 40 µL of the dye 2,3,5-triphenyl-tetrazolium chloride (20 mg/ml) were added

193 in each well. Absorbance was recorded at 415 nm (time 0) and after 2 h of further incubation to check
194 bacterial viability. DMSO (2%) and chloramphenicol (0.1 mg/ml) were used as negative and positive
195 control respectively.
196 To check the antifungal activity, the microplates were incubated under gentle shaking (110 rpm) for
197 5 days at 26°C. After incubation, the fungal growth was visually determined. DMSO (2%) was used
198 as negative control.
199

200 **In vitro *activity on algae***

201 The cyanobacterium *Synechococcus elongatus*, strain PCC 6301, was grown at 24 ± 1 °C under 14-h
202 days (150 μmol m^{-2} s^{-1} PAR) and 10-h nights as previously described (Demuner et al., 2013). Late
203 log-grown cells were settled by centrifugation 5 min at 4,000 x *g*, and used to inoculate 96-well plates,
204 0.2 ml per well, to an initial density of about 1.0 mg L^{-1} chlorophyll. Aliquots (2 μL) of suitable
205 dilutions of either 50 mg ml^{-1} active stock solutions in DMSO or active -containing NLC dispersions
206 encapsulating the same molecules were added to obtain final concentrations ranging from 0.25 to 250
207 mg l^{-1}. A complete randomized design with eight replications was adopted. Cell growth in each well
208 was followed for one week by daily determination of absorbance using a Ledetect 96 plate reader
209 (Labexim, Lengau, Austria) equipped with a LED plugin at 660 nm, subtracting turbidity at 750 nm.
210 Following logarithmic transformation of data, growth constants were calculated, and expressed as
211 percent of the mean value for controls treated with the same volume of DMSO or non-active-
212 containing NLC dispersions. Mean values ± SE over replicates are reported. The concentrations
213 causing 50% inhibition (IC_{50}) of cyanobacterial growth and their confidence limits were estimated by
214 nonlinear regression analysis using Prism 6 for Windows, version 6.03 (GraphPad Software).
215

216 **In vitro *activity on human cells***

217 Human lung epithelial A549 cells (ATCC; Manassas, VA) were grown in F-12K nutrient mixture
218 (Invitrogen, Carlsbad, CA), supplemented with 10% Foetal Calf Serum (FCS) and 1%
219 penicillin/streptomycin on Vitrogen-coated (Collagen Corporation, Palo Alto, CA) Costar clear
220 Transwells (0.4-mm pore size; Costar Corporation, Cambridge, MA) until a confluent monolayer was
221 established (approximately 4 × 106 cells/well).
222 Human keratinocytes HaCaT cells (a cell line gift from Dr. F. Virgili) were grown in Dulbecco's
223 modified Eagle's medium High Glucose (Lonza, Milan, Italy), supplemented with 10% FCS, 100
224 U/ml penicillin, 100 lg/ ml streptomycin and 2 mM L-glutamine as previously described (Sticozzi et
225 al., 2012). HaCaT cells were treated with different NLC doses (1 and 10 mM) at different time points
226 (1 and 24 h).
227 Free actives were dissolved in ethanol at a concentration of 10 mM as a stock solution then diluted to
228 the required concentrations directly in the medium. The final concentration of ethanol in culture
229 medium did not exceed 0.1% (v/v).
230 Viability studies were performed 24 h after the treatments and determined by cytofluorimetric assay
231 Muse Count & Viability Kit (Millipore Corporation, Billerica, MA, USA). Briefly, 380 μL with Muse
232 Count & Viability working solution was added to cells ($1x10^6$ cell/ml), and 20 μL of this cell
233 suspension was incubated for 5 min at room temperature in the dark. L-lactate dehydrogenase (LDH)
234 release was measured by enzymatic assay as previously described (Sticozzi et al., 2012) following
235 the kit instructions (EuroClone, Milan, Italy). All tests were performed in triplicate and repeated at
236 least three times.
237
238 **Results**
239 ***Preparation of NLC***
240 The molecules considered in the present study, namely PB, HQ, EU, A and T were selected on
241 the basis of their physico-chemical characteristics, such as the low solubility in water (see Table 1),

242 and also of their *in vitro* bioactivity (Hammer et al., 1999; Naz et al., 2007; Prabuseenivasan et al.,
243 2006; Pereira et al., 2007). The production of bio-friendly NLC was performed by a two-step protocol
244 based on the emulsification of the molten lipid phase in an aqueous phase containing poloxamer 188
245 under high speed stirring at 15000 rpm, followed by treatment with probe ultrasound.
246 After preparation, the dispersions appeared white milky and free of aggregates and sediment. In the
247 case of PB nanoparticle, due to the color of the molecule, the milky dispersion is yellow colored
248 (Figure 1). Serial dilutions of the dispersions gave rise to a progressive decrease of the scattering
249 intensity and of the milkiness.
250 The determination of the EE and LC was performed after centrifugation of the dispersions. As
251 reported in Table 2, apart from HQ whose EE was around 31%, all the preparation showed an almost
252 quantitative encapsulation within the lipid matrix of the nanoparticles, as proved by the very high EE.
253 The behavior of the LC results was in agreement with those obtained for EE.
254
255 ***Characterization of dispersions***
256 *Cryo-TEM*
257 In order to shed light on the general morphology and internal structure of the dispersed particles in
258 NLC dispersions, Cryo-TEM analyses were conducted. Figure 2 shows cryo-TEM images of NLCs,
259 including samples of empty NLC (A), PB-NLC (B), EU-NLC (C), HQ-NLC (D), aA-NLC (E) and
260 aT-NLC (F). As reported in literature, the use of blends of lipids in solid (i.e. tristearin) and liquid
261 state (i.e. miglyol) leads to the formation of imperfect lipid matrix structures with the absence of
262 clearly identifiable oil droplets in the NLC samples (Saupe et al., 2005; Esposito et al., 2012). In all
263 panels are visible platelets oriented at 0° and 180° appearing as ellipsoidal platelet-like crystalline
264 particles and dark structures edge-on viewed. No significant differences are appreciable between
265 empty and active loaded nanoparticles indicating that the presence of the active compound does not
266 affect the NLC aspect.

267

268 *PCS and SdFFF analyses*

269 Table 3 summarizes the dimensional size distribution of NLC as determined by PCS measurements.
270 Notwithstanding all the prepared NLC formulations showed a bimodal distribution characterized by
271 a Z-average mean diameter ranging between 200 and 240 nm, they possess the appropriate
272 dimensional characteristics for the environmental distribution by spraying or nebulization.
273 NLC's size distribution was also determined by SdFFF method that allows to measure the particle
274 size distribution within the sample, by retaining particles of different diameter and eluting them at a
275 calculable time (Merkus et al., 1989). Indeed an observed retention time can be directly related by
276 theory to the mass of the eluting particles and transformed into a size and the UV signal into a mass
277 frequency function (Contado et al., 1997; Esposito et al., 2008). The obtained fractograms are thus
278 converted into PSD plots according to well-proven equations.
279 Since particle in SdFFF are sorted based on their specific mass, knowing the particle density and by
280 supposing a spherical shape, the sizes represent diameter of an equivalent sphere (Bunjes, 2005). The
281 NLC's density was calculated as previously reported (Esposito et al., 2015). In Figure 3 the PSD plots
282 of three NLC samples taken as an example, namely PB-NLC, EU-NLC and aT-NLC, are reported. In
283 general it can be seen that the three samples subjected to SdFFF are characterized by a size
284 distribution superimposable to that obtained by PCS. Particularly, all the three samples show a quite
285 narrow peak around 100 nm and a secondary broad population spanning between 170 nm up to 600-
286 800 nm. It is interesting to underline that PCS and SdFFF are based on completely different rational
287 principles. Indeed, PCS assesses the size considering the diffusion of the scattered light, thus the
288 obtained results depend on the particles' position when the laser ray hits them. On the other hand
289 SdFFF "weighs" in some extent the particles being considered as compact spheres of uniform density.
290 In this respect the complementary evaluation of size distribution by PCS and SdFFF are important to
291 obtain realistic information about particle size.

292 SdFFF was also employed to obtain information about the encapsulation of active compounds. The
293 amount of each molecule encapsulated in the NLC was determined by HPLC after diluting with the
294 mobile phase, by injecting the eluate exiting from the SdFFF channel collected during the
295 fractionation. The injection results are reported in Figure 3 (square dots), where it is clearly evident
296 that each active is entirely associated with NLC.
297 Concerning stability studies, macroscopic aspect of the formulations and their size stability was
298 evaluated over a period of six months from preparation. The formulations were sealed in glasses vials
299 and maintained at room temperature up to six months. No changes in macroscopic aspect (i.e.
300 precipitation, agglomeration or phase separations) were detectable. In addition, as reported in Table
301 4, the produced NLC maintain almost unchanged their dimensions.
302
303 **In vitro *release of active from NLC***
304 In order to obtain quantitative and qualitative information about the active encapsulated in NLC,
305 namely PB-NLC, EU-NLC, aA-NLC and aT-NLC, an *in vitro* release profile was determined by a
306 dialysis method (Figure 4). Since the tested natural molecules are scarcely soluble in water, their
307 diffusion kinetic in aqueous physiological receptor phases is undetectable. Therefore as suggested by
308 literature, a non-physiological receptor phase composed of phosphate buffer and ethanol (70:30) was
309 used in order to allow the establishment of the sink conditions and to sustain permeant solubilization
310 (Siewert et al., 2003). The theoretical release curves were determined according to the linear form of
311 Eqs. (3) and (4), mimicking a diffusive and a dissolutive model, respectively (Esposito et al., 2005).
312 The comparison between the theoretical curves calculated from equations (3) and (4) and the
313 experimental curves obtained for NLC showed that the experimental curves are superposable to the
314 theoretical curves referring to diffusive kinetics (data not shown). This was confirmed also by R and
315 R^2 values found by linearization of Eq. (3) reported in Table 5.
316

317 ***Antimicrobial activity against phytopathogens***

318 PB, EU, aA, aT, both as pure compounds and encapsulated in NLC, were tested for antimicrobial
319 activity against phytopathogenic bacteria and fungi (Table 6). The choice of the kind of
320 microorganisms was driven by their particular incidence as etiological agents for important arboreal
321 and herbaceous cultures (Schumann and D'Arcy, 2009). In particular, the bacterial strains belonging
322 to *Clavibacter michiganensis* subsp. *nebraskense* (CM; Leaf freckles disease), *Pseudomonas*
323 *syringae pv. syringae* (PSS; Necrotic leaf spots, stem cankers and other symptoms related to the
324 different plant and part infected), *Agrobacterium tumefaciens* (AT; crown gall disease),
325 *Agrobacterium vitis* (AV; crown gall of grape disease) were used, while *Alternaria alternata* (AL;
326 leaf spot symptoms and other diseases related to the different plant infected) and *Fusarium oxysporum*
327 *f. sp. radicis licopersici* (FO; severe necrotrophic symptoms) were employed as phytopathogen fungi.
328 HQ, EU, aA, αT did not evidenced interesting bioactivities both as pure and micro-encapsulated
329 showing growth inhibitory properties only at concentrations equal or higher than 200 μg/ml against
330 all employed microorganisms. PB showed instead valuable results against the bacterial strains AT,
331 AV and CM with MIC values ranging from 6.3 to 12.5 μg/ml as pure molecule, and from 12.5 to 25
332 μg/ml in microencapsulated form. PSS strain was more resistant to both the treatments with PB and
333 PB-NLC showing equal results (MIC=100 μg/ml). Similar sensitivity was evidenced by the fungal
334 phytopathogen AL, but the strongest and most interesting evidences were given by FO, in particular
335 by those cultures treated with PB-NLC (MIC=12.5 μg/ml) showing a sensitivity about 8-folds higher
336 than those treated with the sole pure PB (MIC=100 μg/ml).

337

338 ***Activity on a cyanobacterial model strain***

339 PB, EU, aA, aT, both as pure compounds and encapsulated in NLC, were tested *in vitro* on
340 cyanobacterial strain and human cells. Cyanobacteria, also known as blue-green algae, are ubiquitous
341 photosynthetic prokaryotes that can be found in both terrestrial ecosystems and freshwaters, and are

342 particularly abundant under eutrophic conditions, as those in most cultivated fields. For these reasons,
343 cyanobacterial strains are well suitable to assess non-target effects of phytochemicals. The addition
344 of increasing levels of PB, EU and aA to the culture medium of a *Synechococcus sp*. model strain
345 caused a marked inhibition of bacterial cell proliferation (Figure 5), with concentrations inhibiting
346 growth by 50% (IC_{50}) equal to 60 ± 6, 36 ± 3 and 2 ± 1 μg/ml, respectively. When the same natural
347 compounds were administered as encapsulated in NLC dispersions, in all cases an increased toxicity
348 was evident, with IC_{50} values of 16 ± 2, 15 ± 1 and 0.4 ± 0.2 μg/ml for EU, aA and PB, respectively.
349 Interestingly, while for PB and EU a progressive, dose-dependent toxicity was found, in the case of
350 PB the addition was ineffective up to a concentration threshold, over which cell viability was
351 completely lost. A behavior completely different was observed for aT since, as expected, no toxic
352 effect was detected for aT, either in the free or NLC form.
353

354 ***Effect on human cell lines of NLC loaded with active compounds***

355 Experiments on human cells were conducted testing the formulations that showed toxic activity on
356 phytopathogens and cyanobacteria. Concerning human keratinocytes as it is shown in Figure 6,
357 treatment with aA and EU either as free compound or microencapsulated forms did not affect
358 significantly cell viability at the doses raging from 10.41 to 104.1 and 8.21 to 82.1 μg/ml respectively.
359 Of note is that when EU was loaded in NLC, the higher doses (82.1 μg/ml) significantly reduced
360 cellular viability of about 35%. A completely different response was observed with PB treatment,
361 indeed either alone or loaded in NLC PB resulted extremely toxic to keratinocytes ranging from the
362 doses of 9.41 to 94.1 μg/ml.
363 In the case of human lung epithelial cells, as it is shown in Figure 7 treatment with aA affected cellular
364 viability in lung cell only at the higher doses (104.1 μg/ml) but this effect was abolished when it was
365 loaded in NLC. Similar pattern was also noted for EU, where at the doses of 82.1 decreases cell
366 viability of about 50%, while once loaded in NLC this effect was eliminate.

367 Pretreatment with PB, as only active compound, significantly affects cellular viability at all doses of
368 about 55%. This effect was abrogated when PB was loaded in NLC, demonstrating a completely no
369 toxic cellular effect.

370

371 **Discussion**

372 In recent years, the need to use more eco-friendly and natural-derived tools in agricultural treatments
373 for pest control has become increasingly urgent due to dramatic environmental issues and effects on
374 human and animal health caused by agrochemicals. Several researches were aimed to find plant
375 extracts, fractions or pure natural compounds active against plant pathogens. Moreover, the strong
376 need to find a diffusion tool effective in preserving the bioactive molecule and in promoting at the
377 same time the biological activity lowering toxicity was given (Friedman et al., 2002; Balestra et al.,
378 2009; Quattrucci et al., 2013; Pradhanang et al., 2003; Slusarenko et al., 2008; Dan et al., 2010). In
379 light of these premises, nanoparticles enable better penetration into tissues for instance influencing
380 the effect of the carried molecule by inducing faster penetration or direct contact, or increasing the
381 exposure time of the biological active molecule to the phyto-pathogen(s) (Nel et al. 2006, 2009;
382 Margulis-Goshen and Magdassi 2012). Eco-friendly nanoparticles are needed, by employing
383 biocompatible solvents and renewable materials (i.e. lipids or polymers), or organic-solvent-free
384 processes. This study was undertaken in order to provide an eco-friendly and effective encapsulation
385 strategy for natural bioactive molecules to obtain a NLC-Biomolecule tool. The obtained system was
386 studied *in vitro* using different strategies and biological models to check the biological activity against
387 phytopathogens (fungi and bacteria), the potential toxicological impact involving environment (algal
388 model system) and humans (human cell lines) (Kah et al. 2013).
389 The nanoencapsulation of the selected active molecules for appropriate parameters allowed the
390 improvement of their solubility in aqueous formulation, solving the drawbacks associated with their
391 use up to at least 6 months from preparation. In addition, PCS and SdFFF techniques applied to

392 investigate the average size of the particles indicated that dimensions slightly increased during 180
393 days of storage time (data not shown). These data allow us to exclude the occurring of agglomeration
394 process during the storage of these formulations. Possibly, the surfactant characteristics of poloxamer
395 present within NLC composition prevent this from happening.
396 Cryo-TEM images revealed nanoparticles in good dispersion, in the nanometric range (according
397 with PCS and SdFFF results) with the characteristic ovoidal and ellipsoidal platelet-like crystalline
398 aspect unaffected by the presence of the active molecule. The analysis of in vitro release of the tested
399 active molecules from NLC suggested that it has to be ascribed mainly to dissolution rather than
400 diffusion rate confirming previously reported results concerning molecules encapsulated in lipid
401 based nanosystems (Esposito et al., 2012).
402 Results of in vitro assays performed against phytopathogenic bacteria and fungi were not promising
403 in view of applicative large scale use in integrated or organic agricultural treatments, except for PB.
404 The results of the pure PB against AT, AV and CM were particularly relevant, but the micro-
405 encapsulation reduced the efficacy from two- to four-folds, limiting the possibility of an effective use
406 of PB in agricultural formulations. Not particularly relevant were the results against PSS, with equal
407 MIC values (100 μg/ml), according to the known evidence of the higher resistance to treatments of
408 Gram negative bacteria than that of Gram positive ones (Chapman et al., 2016 and references therein).
409 The most interesting results were obtained against FO (severe necrotrophic symptoms). In fact,
410 contraryly to what obtained with other microorganisms, PB-NLC showed an efficacy 8-folds higher
411 (MIC=12.5 μg/ml) than that observed in the assays with the pure compound (MIC=100 μg/ml). This
412 result is also relevant for the selective efficacy of the PB-NLC system suggesting the opportunity to
413 perform specific treatments without affecting the non-phytopathogenic fraction of the microbiota
414 pointing out a further eco-friendly aspect of the research approach.
415 Similar results were obtained in vitro with a cyanobacterial model strain. Also in this case all the
416 tested molecules increased their efficacy when administered in NLC (demonstrated by lower IC_{50}

417 values) as compared to the correspondent free form. This suggests that a low uptake may limit their
418 effectiveness, and that lipid encapsulation may help the active principles to reach their targets inside
419 the cells. Consistently, and differently from data obtained for fungi, the addition of PB was
420 completely ineffective up to concentration threshold over which cell viability was completely
421 abolished. Although further experiments are required to shed light on these aspects, this suggests that
422 a higher external concentration of PB is required to allow cell internalization, than to exert cytotoxic
423 effects.
424 Overall, data indicate that the active molecules considered in this study when loaded on NLC showed
425 a lower activity against phytopathogens as compared to their free form suggesting that NLC is able
426 to prevent their possible toxicity by the slower release of the molecules in the tissues. However, the
427 opposite was true for cyanobacterial cells and PB-NLC, thus suggesting that in particular cases their
428 use may provide better results than the free form. The rationale of testing those molecule in
429 keratinocytes and epithelial lung cells was based on the fact that these tissues (skin and respiratory
430 tract) are the main to be exposed to environmental molecules. From this study it is possible to read
431 that the use of NLC reduced the toxicity of the molecules analyzed most likely because they allow
432 the slow release of the active compound, avoiding the cells to be exposed to high dose (often toxic)
433 of natural extract.
434
435 **Conclusions**
436 Taken together, the results described in the present study allow us to potentially propose NLC loaded
437 with natural molecules as an eco-friendly and biocompatible strategy possibly useful in the control
438 of phytopathogenic organisms in agricultural treatments. To this aim, further intensive studies have
439 yet to be performed in order to investigate the efficiency of these systems in greenhouse and in field.
440
441

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624 Table 1 Physico-chemical characteristic and HPLC conditions of the actives considered in the
625 present study

	PB	**EU**	**HQ**	**αA**	**aT**
chemical structure					
chemical formula	$C_{11}H_8O_3$	$C_{10}H_{12}O_2$	$C_6H_4(OH)_2$	$C_{12}H_{16}O_3$	$C_{29}H_{50}O_2$
water solubility	low	low	low	not soluble	not soluble
melting point (°C)	78-79°C	-10° C	172°C	62-63° C	2-4° C
molar mass (g/mol)	188.18	164.21	110.11	208.25	430.71
elution phase (v/v)	MeOH/Water (90:10)	MeOH/Water (60:40)	MeOH/Water (40:60)	MeOH/Water (70:30)	MeOH (100)
flux (ml/min)	0.5	1	0.5	1	1
λ (nm)	254	215	290	258	295
retention time (min)	4	6	5	5	8

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631 Table 2 Encapsulation efficiencies and loading capacities of the active in the produced
632 NLC
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NLC	**Encapsulation efficiency (%)**	**Loading capacity (mg/ml)**
PB	89.1 ± 1.25	4.21 ± 1.02
HQ	31.1 ± 1.84	1.67 ± 0.11
EU	81.4 ± 1.11	3.92 ± 0.71
aA	92.6 ± 0.77	4.63 ± 0.20
aT	82.6 ± 1.87	4.13 ± 0.07

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635 Table 3 Dimensional characteristics of the produced NLC as determined by PCS

	empty-NLC	PB-NLC	HQ-NLC	EU-NLC	aA-NLC	aT-NLC
Z average (nm)	238	206	247	202	203	203
polydispersity index	0.39	0.24	0.19	0.25	0.25	0.26
Mean diameter by intensity (nm)	120 (42%) 415 (58%)	103 (76%) 290 (24%)	147 (52%) 408 (48%)	106 (64%) 410 (36%)	124 (59%) 395 (41%)	123 (66%) 397 (34%)

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640 Table 4 Size of the produced NLC over a period of six months, as determined by PCS

days	empty-NLC (P.I.)	PB-NLC (P.I.)	HQ-NLC (P.I.)	EU-NLC (P.I.)	aA-NLC (P.I.)	aT-NLC (P.I.)
0	238 (0.39)	206 (0.24)	247 (0.19)	202 (0.25)	203 (0.25)	203 (0.26)
30	233 (0.22)	210 (0.19)	236 (0.17)	222 (0.21)	213 (0.23)	210 (0.24)
90	245 (0.23)	217 (0.21)	232 (0.18)	219 (0.22)	210 (0.21)	209 (0.20)
180	248 (0.20)	214 (0.18)	240 (0.19)	215 (0.18)	216 (0.24)	211 (0.23)

641 P.I. : polydispersity index
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649 Table 5 Release kinetic parameters of active release from the produced NLC

Equation	**K**	**c, c'**	***R***	***R*²**
$Mt/M\infty = K_{Diff}t^{0,5}+c'$				
PB	34.007	-0.06889	0.98294	0.96617
EU	29.669	23.569	0.87914	0.77289
aA	52.576	-1.625	0.98964	0.97839
aT	20.642	-1.2494	0.9913	0.98278
$1-Mt=M\infty$ ¼ e^{-Kdiss} t +c				
PB	-0.2481	4.4616	0.97154	0.81449
EU	-0.26024	4.0062	0.86464	0.74760
aA	-0.70225	4.5806	0.9869	0.97397
aT	-0.1229	4.547	0.98279	0.96588

650 K and c - Mathematical coefficients obtained by plotting the linear forms of the indicated equations,
651 *R* - Regression coefficient
652 R^2 - Squared regression coefficient

653 Table 6. MIC (Minimum Inhibitory Concentration; µg/ml) against phytopathogenic bacterial and
654 fungal strains.
655

MIC (µg/ml)	***A. tumefaciens* (AT)**	***A. vitis* (AV)**	***C. michiganensis* (CM)**	***P. syringae* (PSS)**	***Alternaria sp* (AL)**	***F. oxysporum* (FO)**
PB	6.3	12.5	12.5	100	100	100
PB-NLC	12.5	12.5	25	100	100	12.5
EU	200	> 200	200	200	200	> 200
EU-NLC	> 200	> 200	> 200	> 200	200	> 200
aA	> 200	> 200	200	200	200	> 200
aA-NLC	> 200	> 200	> 200	> 200	200	> 200
aT	> 200	> 200	> 200	> 200	> 200	> 200
aT-NLC	> 200	> 200	> 200	> 200	> 200	> 200

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LEGENDS TO FIGURES

Fig. 1 Macroscopic aspect of the produced NLC dispersions: PB-NLC, EU-NLC, HQ-NLC, aA-NLC and aT-NLC.

Fig. 2 Cryo-transmission electron microscopy images (cryo-TEM) of: empty NLC (A), PB-NLC (B), EU-NLC (C), HQ-NLC (D), aA-NLC (E) and aT-NLC (F).

Fig. 3 PSD plots of PB-NLC (A) and EU-NLC (B) and aT-NLC (C).

Fig. 4 *In vitro* release profile of active molecule from PB-NLC (dot), EU-NLC (e), aA-NLC (diamond) and aT-NLC (triangle). Experiments were performed by dialysis method. Data were the mean of 4 experiments ± SD.

Fig. 5 Effect of increasing concentrations of PB (A), EU (B), aA (C) and aT (D) as free form (dot) or encapsulated in NLC (square) on the growth of the cyanobacterium *Synechococcus sp.,* strain PCC 6301.

Fig. 6. Vitality of HaCaT cells after treatment with PB-NLC, EU-NLC and aA-NLC

Fig. 7. Vitality of A549 cells after treatment with PB-NLC, EU-NLC and aA-NLC