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1 **Evaluation of the antimicrobial and antioxidant properties of synthetic phenolipids**

2

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21 **Abstract**

22 Functionalization of phenolic compounds by lipophilization allowing the synthesis of new
23 compounds with improved biological properties. This study aims to investigate the
24 antimicrobial effects of eight synthesized phenolipids, derived from bioactive coumaric and
25 ferulic acid esters, against *Bacillus subtilis*, *Escherichia coli*, and *Candida albicans*.
26 Antioxidant activity, as well as antimicrobial capacity were evaluated through DPPH assays
27 and classical disc agar diffusion, respectively, concomitantly with the evaluation of cellular
28 viability using flow cytometry assay. Antioxidant activities results showed that ferulic acid
29 esters exhibit higher antiradical activity than coumaric acid esters. Results of agar diffusion
30 assay revealed that, 1-methylpropyl coumarate and 1-methylpropyl ferulate (1-MPF) have an
31 effective antimicrobial activity with Minimum Inhibitory Concentrations (MICs) values
32 between 0.9 – 3.5 mM against all tested microorganisms. Also, flow cytometry showed that 1-
33 MPF and decyl coumarate exhibited significant bactericidal activity ($p < 0.05$), with almost
34 63% of dead *Bacillus subtilis* cells. Compared to the commonly used agar method, flow
35 cytometry proved to be a fast and straightforward analytical tool to assess antimicrobial
36 activity while providing information on inhibition type. These results suggest the applicability
37 of phenolipids as potent lipophilic bioactive molecules to protect food products and cosmetic
38 formulations from oxidative stress and microbial spoilage.

39

40 **Keywords:** Phenolipids, Antimicrobial activity, Antioxidant activity, Flow cytometry

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44 **1. Introduction**

45 Food and cosmetic products are susceptible to deterioration caused by lipid oxidation and
46 microbial spoilage. Both phenomena have a significant impact on food products with a loss of
47 nutritional value and a change in the taste of food products ¹, as well as on cosmetic
48 formulations with discoloration, texture alteration, changes in activities, and enhanced toxicity
49 ². The use of preservatives and antioxidants is one of the most common methods for limiting
50 microbial contamination and preventing lipid oxidation ².

51 Natural phenolic acids from plants, such as *p*-coumaric and ferulic acid, constitute one of the
52 wider classes of secondary metabolites ³ and are known for their bioactivities (*e.g.*,
53 antimicrobial, antioxidant, antiviral, and antimutagenesis) ⁴⁻⁶. For example, it has been
54 reported that the antimicrobial mechanisms of phenolic acids can be attributed to their
55 capacity to permeabilize and destabilize the plasma membrane, inhibit enzymes involved in
56 cell metabolism by the oxidized product, and repress the synthesis of nucleic acids ⁷⁻⁹. Their
57 antimicrobial and antioxidant bioactivities explain the important role of phenolic acids in food
58 supplementation and cosmetics. However, the major drawback limiting their efficiency in
59 formulations such as emulsions and foams is their low solubility in both water and
60 hydrophobic media ¹⁰. To overcome this limitation, one can graft a lipophilic moiety onto the
61 phenolic acid through several reactions (esterification, transesterification, amidation, and
62 etherification) ^{11,12} using chemical, chemo-enzymatic, and enzymatic approaches ¹³⁻¹⁶
63 depending on the chemical structure and reactivity of the reactants. It has been reported that,
64 in most of the cases, the biological, emulsifying, antimicrobial and antioxidant properties ^{3,17-}
65 ²² of phenolipids are enhanced compared to their acid counterparts. Durand et al in 2017 have
66 reviewed the interesting biological properties of phenolic compounds and their derivatives ²³.
67 Furthermore, it has been reported that the health benefits of phenolipids are attributed to the
68 phenolic motif present in amphipathic phenolipids which preserves their beneficial

69 antioxidant and biological effects. The hydrophobic domain confers special physicochemical
70 properties to the phenolipids, allowing interaction with nonpolar compounds ²⁴. Moreover, it
71 has been observed, in a previous work, that lauryl ferulate obtained through the esterification
72 of ferulic acid with lauryl alcohol displayed antibacterial activity against *S. aureus*, *L.*
73 *monocytogenes*, and *E. coli* ²⁵. Moreover, Merkl *et al.* showed that butyl esters of phenolic
74 acids inhibit the growth of *B. subtilis* and *S. cerevisiae* ²². In these studies, antimicrobial
75 activity was quantified either by the size of the inhibition diameter or the minimum inhibitory
76 concentration (MIC). Although these methods provide valuable information, each has its own
77 limitations (*e.g.*, the need of high concentrations of molecules, time-consuming, diffusion
78 issues with the hydrophobic molecules) ²⁶.

79 To further study in-depth antimicrobial activity of a compound, flow cytometry is
80 recommended. This method has been used for a long time to determine the effects and modes
81 of action of chemical or microbiological agents on microbial cells. Flow cytometry provides
82 information on the nature of the inhibitory effect (bactericidal and bacteriostatic) and the cell
83 damage imposed on the microorganisms tested ²⁷. Many authors investigated the
84 antimicrobial activities of several molecules using this methodology ^{28–31}. The rapid detection
85 of damaged cells using this approach depends on the use of appropriate dyes staining ³².
86 Therefore, propidium iodide (PI), with its fluorescent and intercalating properties, is widely
87 used as a DNA stain. Several studies have reported the effectiveness of flow cytometry as a
88 tool for antibacterial testing of essential oils against *Listeria monocytogenes*, using combined
89 staining with PI and carboxyfluorescein diacetate (cFDA) for membrane damage evaluation
90 and esterase activity detection, respectively ³³. Consequently, in addition to the lysed cells,
91 three populations (*i.e.*, dead, viable and injured cells) can be clearly discriminated in a timely
92 fashion and with good reproducibility (2–6 h compared to 24–72 h for the microdilution or
93 agar methods).

94 This work focused on the evaluation of the potential of eight synthetic phenolipids as
95 antimicrobial agents against three selected microorganisms - *Escherichia coli*; a Gram-
96 negative bacteria, *Bacillus subtilis*; a Gram-positive bacteria and *Candida albicans*; a yeast -
97 using both agar diffusion test and flow cytometry. To the best of our knowledge, this is the
98 first report on the determination of the antimicrobial activity of a diversity of phenolipids
99 using flow cytometry. In addition to antimicrobial activity, antioxidant activity was evaluated
100 using the DPPH method.

101 2. Materials and methods

102 2.1 Chemicals

103 *p*-Hydroxybenzaldehyde was purchased from TCI (Tokyo, Japan). Vanillin, Meldrum's acid,
104 butan-2-ol, octan-2-ol, decanol, hexadecanol, and DPPH were purchased from Sigma-Aldrich
105 (St.Louis, MO, USA). The solvents were purchased from Fisher Scientific (Hampton, NH,
106 USA) and used as received. All chemicals were used as received without further purification.

107 Characterization of synthesized phenolipids protocols are described in the Supplementary
108 Materials.

109 2.2 Phenolipids chemical synthesis

110 Eight phenolipids; 1-methylpropyl coumarate (1-MPC), 1-methylpropyl ferulate (1-MPF), 1-
111 methylheptyl coumarate (1-MHC), 1-methylheptyl ferulate (1-MHF), Decyl coumarate (DC),
112 Decyl ferulate (DF), Hexadecyl coumarate (HC), Hexadecyl ferulate (HF) were synthesized
113 according to the procedure described by Peyrot *et al.*³⁴ with some modifications. Meldrum's
114 acid (1 g, 7 mmol) was mixed with the corresponding alcohol (butan-2-ol, octan-2-ol,
115 decanol, hexadecanol) (7 mmol, 1 eq) at 95 °C and stirred at 100 rpm for 3 h. After cooling at
116 room temperature, the mixture was partitioned between ethyl acetate and aqueous sodium
117 bicarbonate solution. The aqueous layer was then acidified with concentrated HCl until pH =

118 1. The monoester was extracted with ethyl acetate, dried over anhydrous MgSO₄, then filtered
119 and concentrated under reduced pressure.

120 The corresponding malonate monoester, *p*-hydroxybenzaldehyde or vanillin (1.0 equiv) and
121 L-proline (0.5 equiv) were mixed in ethanol. The mixture was stirred at 250 rpm overnight at
122 90 °C. After cooling at room temperature, ethanol was evaporated under vacuum and
123 phenolipids were purified using a flash prep LC system puriFlash[®] 4100 from Interchim
124 (Montluçon, France) equipped with prepacked silica column (30 µm, Interchim PF-Si30-HP)
125 using cyclohexane/ethyl acetate eluent (90/10, v/v).

126 **2.3 Antimicrobial activity**

127 **2.3.1 Agar diffusion test**

128 The antimicrobial activity of the samples was assessed against *E. coli*, *B. subtilis* and *C.*
129 *albicans* using an agar diffusion assay developed in the laboratory. 9 mm diameter discs made
130 of cotton linter fiber (Grosseron, Coueron) were used. Precultures of the microorganisms were
131 performed in LB (*E. coli* and *B. subtilis*) and PDB (*C. albicans*) media and incubated at 30 °C
132 and 180 rpm for 18 h. Microbial suspensions presenting an OD equal to 1 were prepared, and
133 LB and PDA agar media were inoculated with 100 µL of these microbial suspensions.
134 Spreading was performed using sterile glass beads. Minimum inhibitory concentration (MIC)
135 was determined by preparing phenolipids in ethanol 40% at different concentrations 5,000%,
136 2,500%, 1,250%, 0,625%, 0,310%, 0,156%, 0,078%, 0,040%, 0,020%, w/v. For each dose,
137 four discs were placed on each plate, with 30 µL of each phenolipid. MICs were determined
138 as the lowest concentrations of the compounds required to inhibit microbial growth. A
139 negative control plate containing two discs soaked in ethanol 40% and two positive controls,
140 ampicillin and nystatin antibiotics, were prepared. All the plates were incubated at 30 °C for
141 24 h. The procedure was performed in duplicate.

142 2.3.2 Flow cytometry analysis

143 To determine the bactericidal, fungicidal, bacteriostatic, or fungistatic effects of phenolipids,
144 membrane cell integrity and esterase activity were analyzed by flow cytometry using double
145 cell staining with propidium iodide (PI) and carboxyfluorescein diacetate succinimidyl ester
146 (cFDA-SE) for *B. subtilis* and *C. albicans* cells.

147 *B. subtilis* and *C. albicans* were maintained at -80 °C in 30% glycerol-LB or PDB media. The
148 two strains were inoculated in 250 mL flasks containing 25 mL of LB or YEPD media and
149 incubated overnight in a Thermo MaxQ 4000 shaker set at 30 °C and 180 rpm. These cultures
150 were used to inoculate 50 mL baffled flasks to an initial OD_{620 nm} of 1: one containing 10 mL
151 of LB or YEPD and 2% (w/v) Tween 80 (the control) and one with 10 mL LB or YEPD, 2%
152 (w/v) Tween 80, and phenolipid. The concentrations of different phenolipids tested were
153 chosen based on the results obtained in the agar diffusion tests (MIC in Table 2) depending on
154 their maximal solubility in the medium (equal or twice the MIC). Cultures were grown under
155 the same conditions described above. Samples (400 µL) were collected from each culture
156 after 24 h of incubation.

157 The staining protocol was adapted from the method described by Combes et al. 2020³⁵.
158 Samples were diluted to approximately 10⁶ cells.mL⁻¹ in McIlvaine buffer (pH 7) and
159 centrifuged for 4 min at 10 000 rpm at 4 °C. One milliliter of cell suspension was stained by
160 adding 20 µL cFDA-SE (Chemchrom V8, Biomérieux) diluted ten times in acetone and
161 incubated for 10 min at 30 °C, after which 20 µL of 1 mg.mL⁻¹ commercial PI solution
162 (Sigma-Aldrich) was added and samples were incubated again for 10 min at 30 °C. Stained
163 samples were analyzed with a Sysmex Cyflow® Space flow cytometer (Partec, France)
164 equipped with an argon 488 nm laser and four filters: forward-angle light scatter (FSC), side-
165 angle light scatter (SSC), both combined with a diode collector, a 536 nm band-pass filter
166 (526–546 nm) to collect the green fluorescence of cFDA (FL1 channel) and a 670 nm band-

167 pass filter to collect the red fluorescence of PI (FL3 channel) with photomultiplier tubes. Data
168 acquisition, instrument control, and data analysis were performed using FloMax® software
169 (version 2.9, Partec, France). The procedure to determine the fluorescent signal and cellular
170 viability was described in supplementary materials. An example of preculture and sample
171 fluorescence images is shown in Fig S4.

172 **2.4 Antioxidant activity**

173 The antioxidant activity of phenolipids was determined following the method of Peyrot *et al.*,
174 using DPPH (2,2-diphenyl-1-picrylhydrazyl) to provide EC₅₀ values^{of 33}. The DPPH assay
175 was performed in a 96-well microplate. To a well containing 10 µL of the sample at different
176 concentrations ranging from 400 to 12.5 µM, 190 µL of DPPH solution at 200 µM in ethanol
177 was added. Two negative controls (ethanol and DPPH solutions) were used. The
178 disappearance of DPPH free radicals was monitored every 5 min for 7.5 h at 520 nm using a
179 microplate reader (Epoch2®; BioTek®). The use of different concentrations of phenolipids
180 yielded the EC₅₀ value, which corresponds to the amount of antioxidant necessary to reduce
181 half of the initial population of DPPH radicals.

182 **2.5 Statistical analysis**

183 All experiments were performed in duplicate and the data are presented as the average value ±
184 standard deviation (SD). Data were analyzed by analysis of variance (ANOVA) using the R
185 software to determine significant differences ($p < 0.05$).

186

187 **3. Results**

188 **3.1 Chemical synthesis of phenolipids**

189 The most common synthetic approach to access phenolipids is through conventional methods
190 such as Fischer esterification between an aliphatic alcohol and protected phenolic acids.
191 Nevertheless, this procedure involves the use of toxic compounds and protection and
192 deprotection steps, operations that conduct in general to moderate yields^{5,37}. Herein, a
193 synthetic strategy is presented in which the synthesis of eight phenolipids was performed
194 using a Knoevenagel-Doebner condensation reaction with Meldrum's acid, aliphatic alcohols
195 and benzaldehyde derivatives (4-hydroxybenzaldehyde or vanillin) as starting materials. This
196 two-step synthesis of phenolipids allows the production of the targeted phenolipids in a more
197 environmentally friendly way. This method does not require any protection and deprotection
198 steps and was previously described by Peyrot *et al.* for the synthesis of sinapic acid esters³⁶.

199 First, as synthetic intermediaries, monomalonates were readily synthesised in high yields by
200 simple heating Meldrum's acid at 95 °C in presence of alcohols (butan-2-ol, octan-2-ol,
201 decanol and hexadecanol) without any solvent. The L-proline-mediated Knoevenagel-
202 Doebner condensation was then performed in presence of the aforementioned monomalonates
203 and benzaldehyde derivatives in ethanol to synthesise the corresponding phenolipids average
204 too high in yields ranging from 16% to 77%, the lower yields being obtained for decyl
205 coumarate and ferulate (28% and 16%, respectively) (Fig.1).

206 The purified phenolipids (coumaric and ferulic acid esters) were characterized using ¹H and
207 ¹³C NMR (NMR spectra are available in Figure S1 as a supplementary material) and their
208 purity was further confirmed by GC-MS analysis (Figure S2 & S3 supplementary data).

209 **3.2 Antimicrobial activity of phenolipids**

210 **3.2.1 Agar diffusion test**

211 The results of the antimicrobial effect of the eight phenolipids measured using the agar
212 diffusion method are shown in Table 2. This inhibitory effect was supported by the

213 appearance of a clear halo around the discs after 24 h of incubation (Table S1). In this study,
214 the activity was characterized by measuring the minimum inhibitory concentration (MIC).

215 Based on the results reported in Table 2, it appears that out of the eight phenolipids tested,
216 only four molecules (1-MPC, 1-MPF, 1-MHC, and DC) were effective against the three
217 strains (Table 2). A general overview of the results indicated that of all the molecules tested,
218 coumaric or ferulic acids (Table 2) were less effective than their respective esters (1-MPC, 1-
219 MHC, DC, 1-MPF) (Table 2) against the three strains investigated. The MICs of coumaric
220 acid and ferulic acid varied between 76 and 90 mM and 64 and 98 mM, respectively (Table
221 2). However, those for coumaric and ferulic acid esters ranged from 0.9 to 12 mM (Table 2),
222 demonstrating that the lipophilization of coumaric and ferulic acids increase the antimicrobial
223 activities up to 100 times. It is noteworthy to mention that, of the four efficient phenolipids,
224 three are coumaric acid esters. Moreover, both 1-methylpropyl esters (coumarate and ferulate)
225 presented a particularly low and similar MIC regardless of the strain tested.

226 Finally, the results showed that *E. coli* was more resistant to the action of some coumaric acid
227 esters (1-MHC and DC), while the growth of *the B. subtilis* strain was inhibited by the four
228 phenolipids (1-MPC, 1-MPF, 1-MHC, and DC) esterified with fatty alcohols with carbon
229 chains ranging from C₄ to C₁₀.

230

231 **3.2.2 Flow cytometry**

232 The flow cytometry data after 24 h of incubation are shown in Fig. 2 & 3. After double
233 staining with PI and cFDA-SE, for each culture, four subpopulations were identified based on
234 the values measured with the positive control (where the majority of the cells were dead in the
235 presence of ethanol) and the negative control (when the microbes were cultivated in their
236 optimal media): viable (esterase active cells measured by cFDA-SE labelling), dead

237 (membrane-damaged cells measured by PI labelling), injured (double-stained PI and cFDA-
238 SE cells), and unmarked cells (Fig. 3 a & b). As *E. coli* seems to be more resistant to most of
239 the phenolipids according to the results obtained in agar diffusion test, we decided to test their
240 effectiveness only against *B. subtilis* and *C. albicans*.

241 Each of the eight phenolipids was evaluated at a specific concentration based on their
242 maximal solubility in the medium and their MIC measured in the agar diffusion test (twice the
243 MIC when below maximal solubility and once the MIC otherwise) (Table 1).

244 Sample analysis after incubation of the strains without phenolipids, used as the negative
245 control, showed a small number of PI-stained cells with red fluorescence (4% and 6% dead
246 cells for *B. subtilis* and *C. albicans*, respectively). Almost all cells were single-stained with
247 green fluorescence (cFDA-SE) (67% and 71% for *B. subtilis* and *C. albicans*, respectively),
248 meaning that most of cells were alive in this sample (Fig. 2a and c & Fig. 3a and b). In
249 contrast, in the presence of ethanol as a positive control, most *B. subtilis* cells were labelled
250 with PI (82% of cells), whereas for *C. albicans*, 38% of injured cells, 35% of unmarked cells,
251 and only 21% of dead cells were observed (Fig. 2b, c, and d & Fig. 3a and b).

252 Sample analysis after incubation with phenolipids revealed differences in their antimicrobial
253 properties. The results in Figure 1 show that only four compounds (1-MPC, 1-MPF, 1-MHC,
254 and DC) were bioactive against the two strains *B. subtilis* and *C. albicans*. These results also
255 demonstrate that *B. subtilis* is more sensitive to the action of phenolipids than *C. albicans*
256 when looking at the dead cells (Fig. 2c & Fig. 3a). Indeed, 1-MPF and DC were the most
257 active compounds against *B. subtilis*, without any significant differences ($p > 0.05$), and
258 showed clear bactericidal effects. The two molecules acted on enzymatic activity by reducing
259 the percentage of cells labeled with cFDA-SE (7% and 6% of viable cells, respectively,
260 against 66% for the negative control) (Fig. 2a & Fig. 3a). Furthermore, the treatment of cells
261 with the two molecules affected cell membrane integrity, thereby increasing the percentage of

262 PI labelling (61% and 62% of PI-labelled dead cells, respectively) compared to the negative
263 control (4% of cells labelled with PI) (Fig. 2c). Notably, no significant differences were
264 observed between DC and the positive control ($p > 0.05$) (Fig. 2c) showing its efficiency
265 against the *B. subtilis* strain. In contrast, 1-MPC and 1-MHC increased the percentage of PI
266 labelling cells (32% of dead cells) against *B. subtilis* without affecting esterase activity (40%
267 and 32% of viable cells, respectively) (Fig. 2a & c). Thus, these molecules damaged the cell
268 membrane without inhibiting enzymatic activity (Fig. 2a and c), and showed clear
269 bacteriostatic effects.

270 The four phenolipids active against *B. subtilis* were less active against *C. albicans*, except for
271 1-MPF, which demonstrated a fungistatic effect as it was the only one that showed a
272 significant difference from the negative control (without molecules) ($p < 0.05$) (Fig. 2c & Fig.
273 3b).

274 The antimicrobial activities of 1-MHF and DF against *C. albicans* are shown in Fig. 2b & Fig.
275 3b and demonstrate that almost half of the cells were injured with double staining green and
276 red fluorescence (51% and 49%, respectively). This indicates that both molecules act as
277 fungistatic agents that affect the membrane integrity without affecting the enzymatic activity
278 of the cells. The other molecules, *i.e.*, HC and HF, do not exhibit any effect against the two
279 strains, with viable cells ranging from 60 to 66% for *B. subtilis* and from 57 to 64% for *C.*
280 *albicans* (Fig. 2a & Fig. 3 a and b).

281

282 **3.3 Antioxidant activity of phenolipids**

283 The EC_{50} (the amount in nmol of synthesized compounds required to reduce 50% of free
284 radicals) of the different phenolipids, as well as that of butylated hydroxyanisole (BHA,
285 reference compound) are shown in Figure 4.

286 Results in Figure 4. a showed that coumaric acid and its esters are less effective ($EC_{50} > 240$
287 nmol) than ferulic acid and its esters ($8.7 \text{ nmol} < EC_{50} < 15.6 \text{ nmol}$). Ferulic acid showed a
288 higher antioxidant capacity than its esters (Fig. 4b). To evaluate the potential of the
289 synthesized molecules as antioxidants, their EC_{50} values were compared to those of BHA, a
290 molecule commonly used as an antioxidant in the pharmaceutical, food, and cosmetics fields,
291 but recognized as potentially carcinogenic³⁸. Although none of the molecules outperformed
292 BHA (4.4 nmol), they remain competitive as additives of choice for the cosmetics and food
293 industries because of their interesting multiproperties (antioxidant and antimicrobial) with
294 regard to the toxic nature of BHA.

295

296 4. Discussion

297 In this paper, we report the evaluation of the antimicrobial activity of eight coumaric and
298 ferulic acid esters. The antimicrobial activities of these compounds were assessed using agar
299 diffusion and flow cytometry assays. It appears that lipophilization of phenolic acids can
300 significantly improve their antimicrobial activity - up to 80-90 times - on the tested bacteria or
301 yeast. Among the eight synthesized phenolipids, only four showed the ability to inhibit the
302 growth of bacteria and yeast on agar plates. Of the four active phenolipids, those with shorter
303 alkyl chains on the ester moiety (*i.e.*, 1-MPC and 1-MPF) proved to be the most efficient,
304 exhibiting antimicrobial properties regardless of the degree of substitution of their phenolic
305 core (coumaric or ferulic). The effect of the phenolic moiety was observable for longer alkyl
306 chains, with only coumaric-based phenolipids, 1-MHC and DC prevented the growth of the
307 tested bacteria and yeast. It is important to note that longer alkyl chains lead to a decrease in
308 antimicrobial activity, as phenolipids with the longest alkyl chain (*i.e.*, HC and HF) did not
309 show any inhibition effect. Moreover, it appears that the phenolipid inhibition profile of *C.*
310 *albicans* is different from that of the gram-positive bacteria *B. subtilis* because their cell walls

311 have different patterns. Indeed, the wall of gram-positive bacteria is composed of a large layer
312 of peptidoglycan, whereas *the C. albicans* cell wall is a two-layered structure consisting of a
313 hydrophilic mannan layer encompassing a β -glucan-chitin skeleton that is responsible for the
314 strength and shape of the cell wall ³⁹. Furthermore, gram-negative *E. coli* were more resistant
315 to the action of some phenolipids in the agar diffusion test. This can be explained by the cell
316 envelope, which is formed by a thin layer of peptidoglycan surrounded by an outer envelope
317 made up of hydrophilic lipopolysaccharides that can prevent the penetration of phenolipids
318 with a rather hydrophobic character ³.

319 Several studies have shown that the lipophilization of phenolic acids can improve their
320 hydrophilic-lipophilic balance to generate phenolipids with better antimicrobial properties
321 ^{22,23}. The MICs of ferulic acid ester (1-MPF) obtained against the three strains evaluated in the
322 agar diffusion test (Table 2) were in accordance with the MICs reported in the literature.
323 Merkl and co-workers obtained an MIC of 5 mM against the *E. coli* strain and 2.5 mM against
324 *B. cereus* and *S. cerevisiae* ²², while antimicrobial activity of phenolipids has been previously
325 reported with successful results *vs. E. coli, B. subtilis* and *C. albicans* ^{3,22,40}. However, the
326 present study differs from previous ones by the diversity of synthesized phenolipids,
327 associated with both agar diffusion and flow cytometry assays for better understanding of the
328 correlation between the chain length of phenolipids and their antimicrobial activity.

329 Monitoring of cFDA and PI cell staining with flow cytometry allowed clear discrimination of
330 cell subpopulations between viable, injured, dead and unlabelled cells. Flow cytometric
331 assessment highlighted the succession of cell changes that occurred in *B. subtilis* and *C.*
332 *albicans* populations, revealing physiological heterogeneity within some phenolipids-treated
333 cell populations compared to non-treated control cells. Among the eight phenolipids studied
334 by flow cytometry, four molecules (1-MPC, 1-MPF, 1-MHC and DC) were found effective
335 against *B. subtilis.*, thus confirming the agar diffusion test results determined by halo diameter

336 (Table 2) while allowing to discriminate between bactericidal/bacteriostatic and
337 fungicidal/fungistatic phenolipids. 1-MPF and DC can be considered as bactericides, affecting
338 membrane integrity and cause loss of esterase activity of cells leading to about 60% *B. subtilis*
339 death and less than 5% viable bacteria. This phenomenon is called the ‘phoenix effect’ which
340 corresponds to the formation of pores in the membrane caused by the molecules ²⁸. In order to
341 understand the mode of action, changes in the cell wall, monitoring the DNA damage along
342 with the leakage of the internal content of the cells could be performed. Moreover, the
343 minimal bactericidal concentration (MBC) could be determined since its significant to
344 determine their potential application ability in food items and cosmetic formulations.

345 All other combinations, 1-MPC and 1-MHC with *B. subtilis* and 1-MPF bioactive phenolipid
346 against *C. albicans*, showed a bacteriostatic or fungistatic effect, meaning that viable and
347 injured cells vary from 20% to 40% while dead cells do not exceed 10%.

348 However, concerning the antioxidant activity, it depends upon the molecular structure of the
349 compounds ⁴¹. In addition to fatty alcohol carbon chain length effect, the impact of the degree
350 of substitution of the phenolic moiety on antioxidant activity was also assessed (coumaric and
351 ferulic acids) (Figure 4). It is well known that the antiradical activity of phenolic acids and
352 their derivatives is improved depending on the degree of substitution of the phenolic moiety
353 (caffeic > sinapic > ferulic > coumaric) ^{5,42} as the result of greater resonance stabilization ⁴¹.
354 Therefore, the efficacy based on radical scavenging assay of the evaluated antioxidants is
355 expected to be highest for ferulic acid than coumaric acid. The obtained results confirmed this
356 ranking in activity for the evaluated phenolic compounds (Fig. 4a & b). Moreover, Sorensen
357 *et al.* and Kikuzaki *et al.* have also reported that ferulic acid had higher DPPH scavenging
358 activity compared to coumaric acid ^{13,43}. The results obtained by Mathew *et al.* (2015) and
359 Peyrot *et al.* (2020) confirmed the lower efficacy of coumaric acid as an antioxidant compared
360 to ferulic acid ^{36,44}.

361 In this study, ferulic acid showed higher activity than its alkyl esters (Fig. 4b). This decrease
362 in radical-scavenging activity of lipophilized compared to free phenolic acid may be
363 attributed to the increased hydrophobicity of the synthesised phenolipids, therefore, their low
364 solubility in polar media (ethanol). These findings are similar to the observations reported by
365 Laguerre *et al.* (2010) and Garrido *et al.* which reported lower IC₅₀ values of ferulic acid than
366 its alkyl esters ^{42,45}. Moreover, Weng *et al.* (2020) obtained the same results with 1-
367 monocafferol glycerol (1-MCG) and its esters and reported that phenolic acid esters are more
368 effective in lipid dispersions, micellar solutions or in dispersions of liposomes. As an
369 example, it has been proven that lipophilization of ferulic acid into 2-ethylhexylferulate
370 allows its use in emulsion-based cosmetic formulation, showing a better inhibitory effect on
371 lipid oxidation than ferulic acid ⁴⁶. Regarding these results, it could be assumed that ferulic
372 acid and its alkyl esters have potential applications as food and cosmetic preservatives and
373 functional food additives. However, further research is needed in order to assess the
374 effectiveness and the safety of the synthesized phenolipids in food systems and cosmetic
375 formulations. For instance, other antiradical and antioxidant tests, such as ABTS ⁴⁷, FRAP ⁴⁸,
376 ORAC ⁴⁹, TAC ⁵⁰ could be performed to determine the activities of the synthesized
377 phenolipids in different media.

378 To conclude, this study demonstrates that the full expression of phenolipids' potential is
379 conditioned, not only by their intrinsic structural features, but also by the environment where
380 the molecules were located. On the one hand, shorter lipophilic moieties are associated with
381 an increase of the antimicrobial potential of the phenolipids, no matter the considered
382 microorganism (bacteria or fungi). On the other hand, only ferulic acid and its derivatives
383 exhibited an antiradical activity (compared to coumaric acid derivatives), decreasing with
384 longer alkyl chains. Therefore, synthesized phenolipids in an environmentally friendly way in

385 comparison with commercially ones appear as excellent bioactive ingredients capable of
386 limiting oxidative stress and microbial alteration in food products and cosmetic formulations.

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392 Marne (France), and the Grand Reims (France).

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394 **Conflict of Interest Statement**

395 The authors declare that they have no competing interests.

396

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550 **Figure legends**

551 **Fig.1:** Structures of synthesized phenolipids using L-proline mediated Knoevenagel-Doebner
552 condensation.

553 **Fig.2:** Flow cytometry data after double staining with PI and cFDA, and after 24 h incubation with
554 phenolipids and two strains *B. subtilis* and *C. albicans*. (a) Viable cells; (b) Injured cells; (c) Dead
555 cells; (d) Unmarked cells. Experiment performed in duplicate for the controls and the most effective
556 phenolipids (1-MPC, 1-MPF, 1-MHC, DC). Significant differences ($p < 0.05$) between bars, as
557 determined by analysis of variance (ANOVA) and Tukey HSD at a 95% confidence level, are
558 indicated by distinct labels. Lowercase letters and capital letters were used to the analysis of *B. subtilis*
559 and *C. albicans*, respectively.

560 **Fig.3:** Flow cytometry dot plots for a single replicate of FL1 (cFDA, green fluorescence) vs. FL3 (PI,
561 red fluorescence) of a: *Bacillus subtilis* (Bs) and b: *Candida albicans* (Ca) after 24 h of cultivation,
562 following treatment with eight phenolipids and untreated cells (positive and negative controls).

563 **Fig.4:** Radical scavenging properties of *p*-hydroxycinnamic acids and their alkyl esters in ethanol
564 calculated as EC_{50} . (a) CA: coumaric acid; CAE: coumaric acid esters, (b) FA: ferulic acid; 1-MPF: 1-
565 methylpropylferulate; 1-MHF: 1-methylheptylferulate; DF: decylferulate; HF: hexadecylferulate.
566 BHA: butylated hydroxyanisol was included as a positive control in this essay. Error bars indicate the
567 standard deviation ($n = 3$).

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573 **Tables**574 **Table 1.** The concentrations of different phenolipids tested in flow cytometry.

Phenolipids	Concentrations % (m/v)	
	<i>B. subtilis</i>	<i>C. albicans</i>
1-MPC	0.16	0.04
1-MPF	0.16	0.16
1-MHC	0.04	0.16
1-MHF	0.16	0.16
DC	0.32	0.16
DF	0.32	0.16
HC	0.08	0.16
HF	0.08	0.16

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576 **Table 2.** Agar diffusion test for coumaric acid (CA), ferulic acid (FA), 1-methylpropyl coumarate (1-
577 MPC), 1-methylpropyl ferulate (1-MPF), 1-methylheptyl coumarate (1-MHC), 1-methylheptyl ferulate
578 (1-MHF), decyl coumarate (DC), decyl ferulate (DF), hexadecyl coumarate (HC), hexadecyl ferulate
579 (HF) against *E. coli*, *B. subtilis* and *C. albicans*. MIC: minimum inhibitory concentration – performed
580 in duplicate.

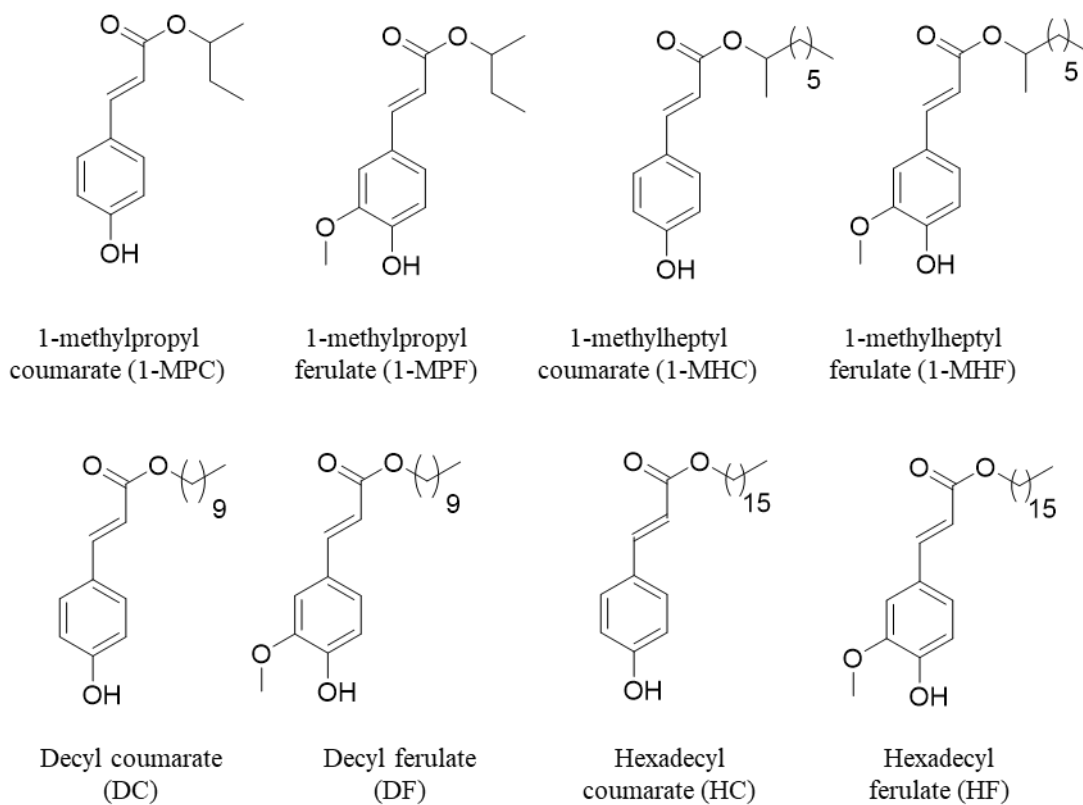
Strains	MIC of the compounds (mM)									
	CA	FA	1-MPC	1-MPF	1-MHC	1-MHF	DC	DF	HC	HF
<i>E. coli</i>	76.1	64.4	1.8	3.5	12.0	ND	12.0	ND	ND	ND
<i>B. subtilis</i>	76.1	64.4	3.5	1.8	0.9	ND	11.3	ND	ND	ND
<i>C. albicans</i>	90.0	64.4	0.9	1.8	1.8	ND	12.0	ND	ND	ND

581 ND: not determined.

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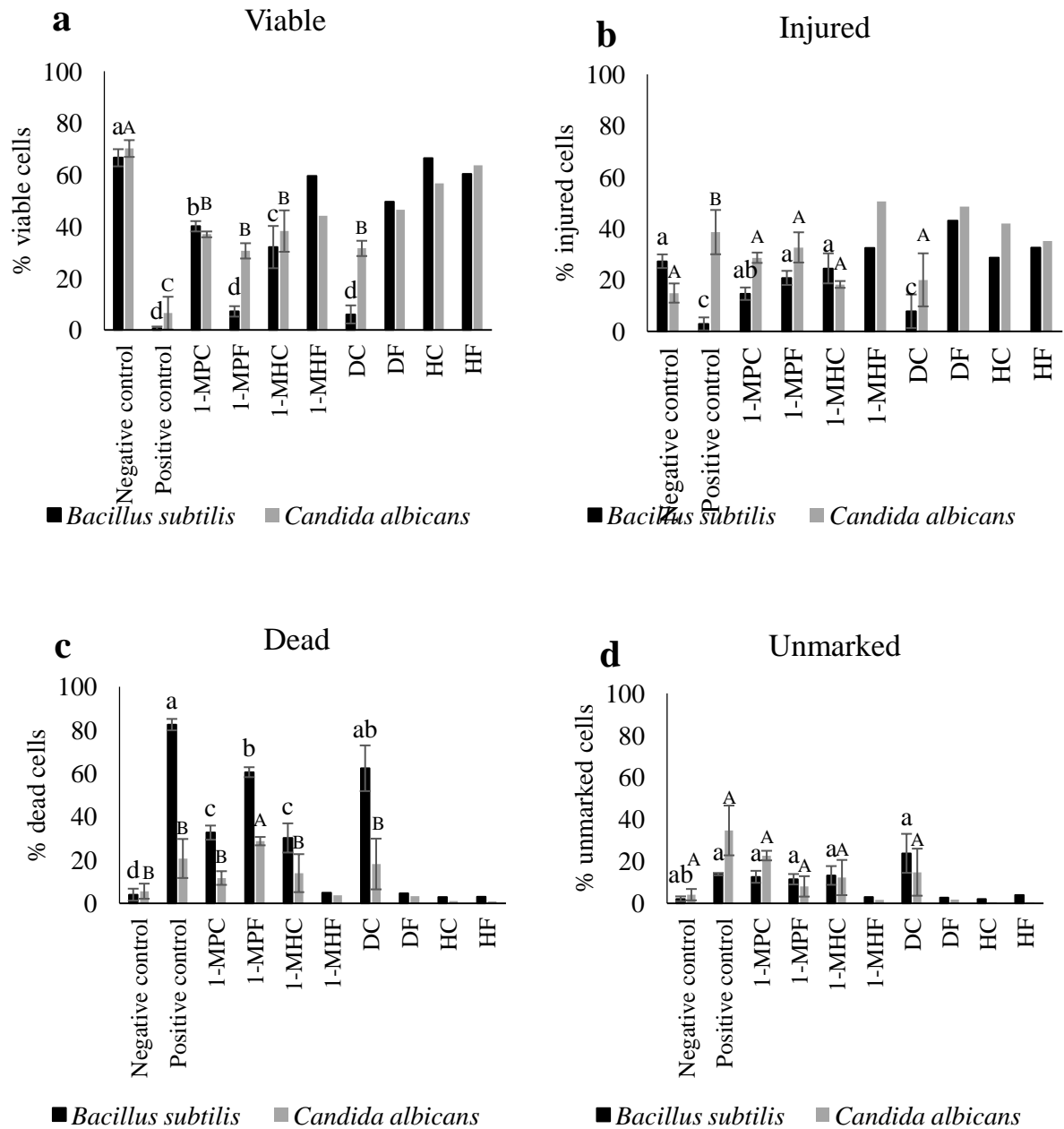
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Figures



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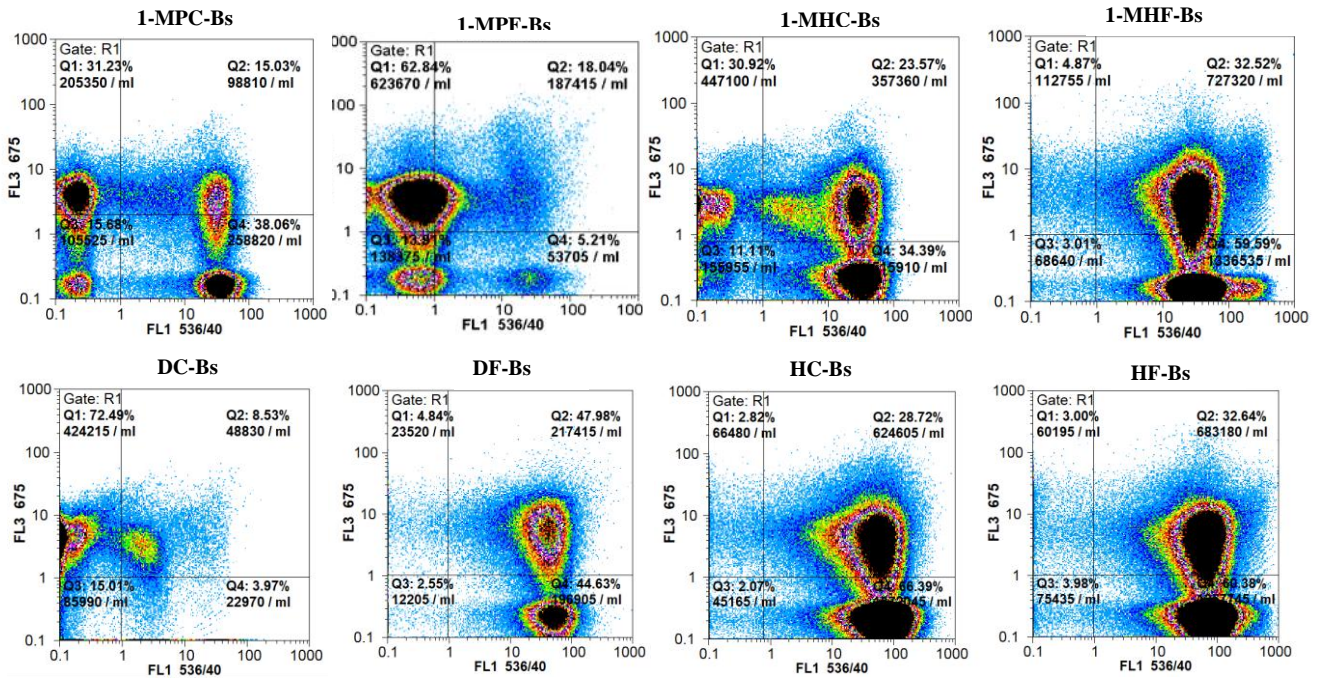
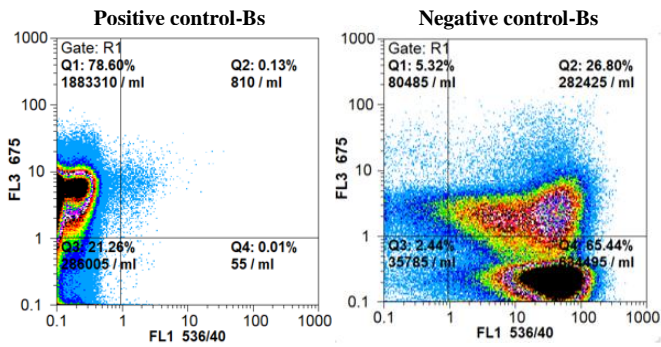
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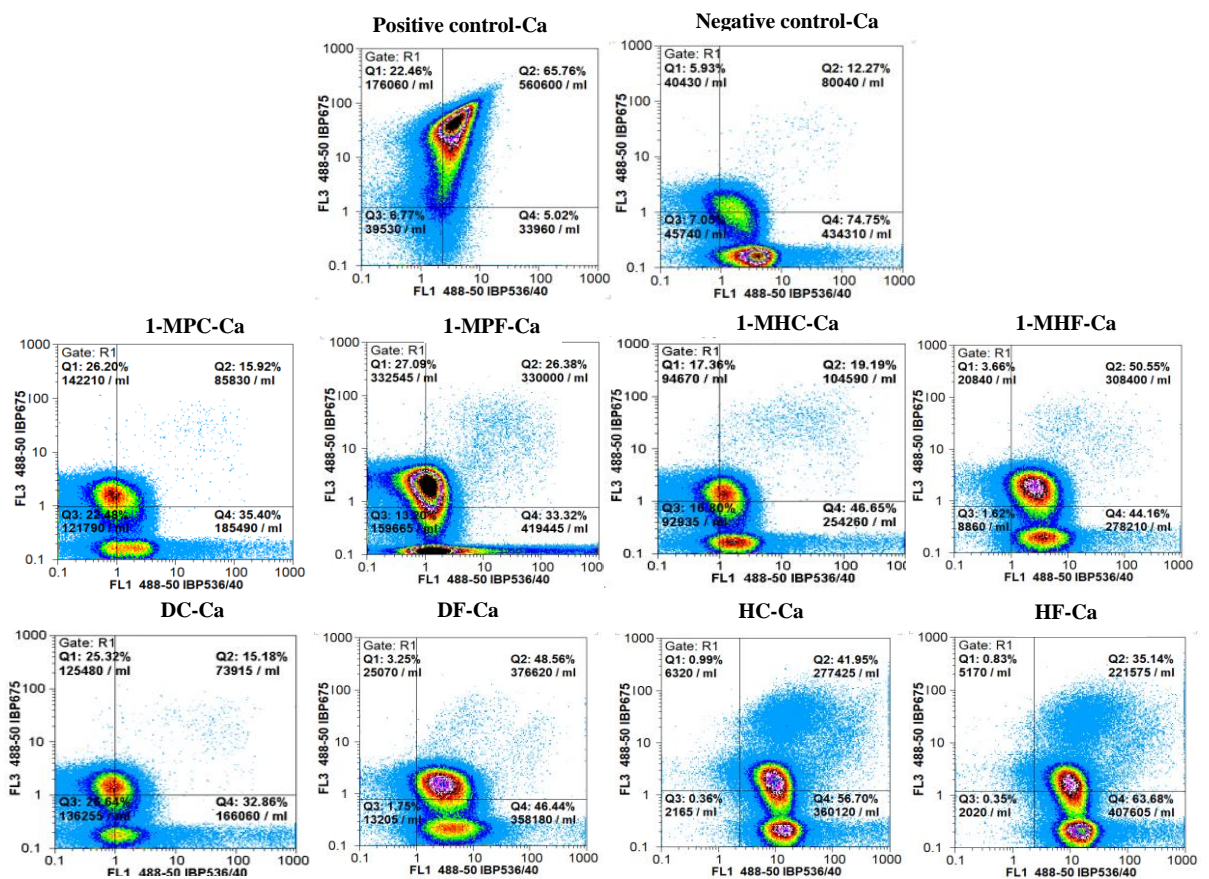


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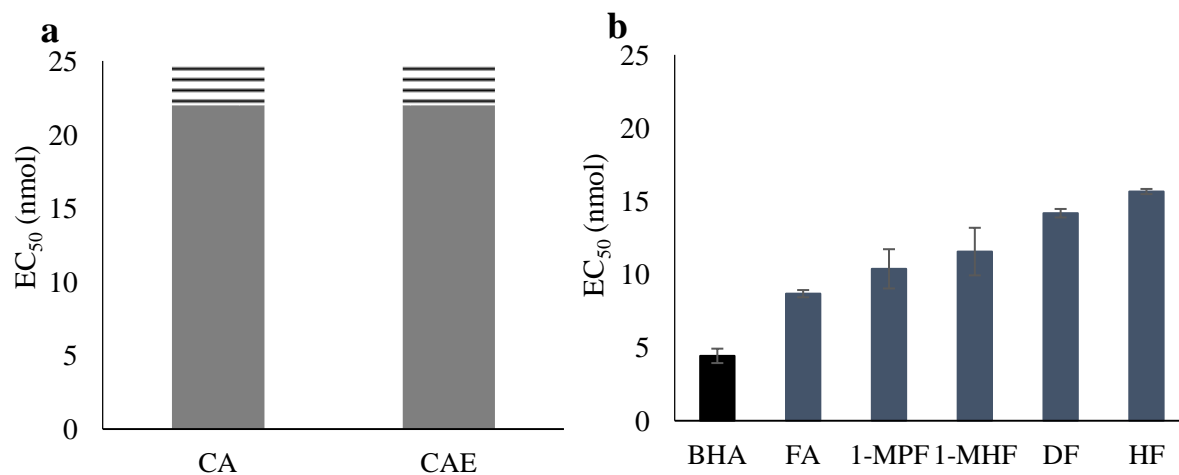


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