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1 **Selection of nerolidol among a series of terpenic and phenolic compounds for**
2 **its potent activity against *Lactobacillus fermentum* ATCC 9338**

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22 **Abstract**

23 Essential oil components are widely used for their antibacterial activity against spoilage
24 microorganisms in food. *Lactobacillus fermentum* is a Gram-positive lactic acid bacteria
25 responsible for the deterioration of various food products, including beverages and dairy
26 products. In this study, 17 terpenic and 11 phenolic compounds were screened against the food
27 spoilage microorganism *Lactobacillus fermentum* ATCC 9338. The antibacterial activity of the
28 tested compounds was dependent on hydrophobicity and particular chemical features. Nerolidol
29 solubilized in dimethylsulfoxide exhibited the highest antibacterial activity and showed low
30 minimal inhibitory (MIC: 25 μ M) and minimal bactericidal (MBC: 50 μ M) concentrations.
31 Moreover, no viable cells were detected within 16 h of incubation at 50 μ M. The important
32 antibacterial activity of nerolidol against *L. fermentum* is probably related to the high
33 hydrophobicity, the aliphatic chain length, and the presence of the hydroxyl group.
34 Hydroxypropyl- β -cyclodextrin/nerolidol inclusion complex showed MIC and MBC values of
35 100 and 200 μ M, respectively. The total bacterial kill was observed after 12 h of incubation. The
36 results obtained with the inclusion complex are probably due to the time required to allow
37 nerolidol to be released from the inclusion complex.

38 **Keywords:** Cyclodextrins; *Lactobacillus fermentum*; nerolidol; phenolic compounds; terpenes.

39 **List of abbreviations:** CFU: colony forming unit; DMSO: dimethylsulfoxide; HP- β -CD:
40 hydroxypropyl- β -cyclodextrin; HPLC: high performance liquid chromatography; LAB: lactic
41 acid bacteria; MBC: minimal bactericidal concentration; MIC: minimal inhibitory concentration;
42 Ner: nerolidol.

44 **1. Introduction**

45 *Lactobacillus fermentum* is an ubiquitous Gram-positive, rod-shaped, anaerobic, thermo-
46 acidophilic lactic acid bacteria (LAB), which can originate from plants, animals, meat, dairies,
47 fruits, and cereals. *L. fermentum* is an obligate heterofermentative bacteria which ferments
48 various types of sugars (lactose, fructose, maltose, sucrose) under anaerobic conditions. In
49 addition to lactic acid, *L. fermentum* produces acetic acid, ethanol, carbon dioxide, and minor by-
50 products, such as diacetyl, hydrogen peroxide, and different alcohols (e.g. 3-methylbutanol),
51 esters (e.g. ethyl acetate), and carbonyls (e.g. nonanal) compounds. *L. fermentum* is not harmful
52 to humans and its presence is necessary for the fermentation of different food products, including
53 sourdough, cocoa, and certain beverages. Certain *L. fermentum* strains are probiotic agents and
54 express outstanding health-promoting characteristics when consumed [1]. Nonstarter lactic acid
55 bacteria, including *L. fermentum*, cause defects in certain food products, such as slits or cracks in
56 hard cheeses, lack of flavors, or bloated packaging in dairy products [2–4]. *L. fermentum* also
57 spoils various types of beverages and can grow in fruit juice, leading to the production of
58 undesirable compounds [5–9]. It is one of the LABs responsible for the desired malolactic
59 fermentation in wine, resulting in the conversion of malic acid into lactic acid, acetate, succinate,
60 and carbon dioxide [10]. However, the presence of LABs could lead to wine spoilage as the
61 control of bacterial growth and malolactic fermentation is difficult to achieve, thus altering the
62 wine organoleptic properties [10–12]. The growth of heterofermentative LABs (such as *L.*
63 *fermentum*) in wine causes an increased acidity, cloudiness, and mousy odor [13]. On the other
64 hand, *L. fermentum* can grow in beer, as it is resistant to hop-compounds [14], leading to beer
65 spoilage [14,15] and aroma alteration [14].

66 Different strategies have been adopted to overcome the microbial spoilage of food, including the
67 addition of chemical additives and physical treatments. Physical treatments include various
68 preservation techniques, such as thermal, ultraviolet light, ultrasound, pulsed electric field, and
69 high hydrostatic pressure technologies [16]. However, the application of these treatments is
70 limited due to changes in the organoleptic properties of the food product [17–19] and sometimes
71 due to high cost [20]. On the other hand, chemical preservatives used against food microbial
72 spoilage, including benzoates, sorbates, propionates, nitrates, and nitrites, can cause allergic
73 responses and could be converted to potential carcinogens [21]. The high demand of fresh and
74 “safe” food, free of synthetic additives and contaminants, have increased the interest of using
75 natural products for food preservation. Natural antimicrobials may derive from plants, animals,
76 and microorganisms. Plant essential oils are largely exploited due to their wide spectrum of
77 antimicrobial activity against spoilage bacteria and food-borne pathogens [22]. Plant
78 antimicrobials include different chemical classes, among which are saponins, tannins, flavonoids,
79 terpenes, simple phenols, and phenolic acids [23,24].

80 The majority of natural antimicrobials are hydrophobic and poorly stable, which limit their use in
81 aqueous media. Solvents, such as dimethylsulfoxide (DMSO) [25], dimethylformamide [26], and
82 ethanol [27, 28], are used to dissolve hydrophobic compounds in aqueous solutions. However,
83 the use of organic solvents in food products is not desirable. Various encapsulation systems
84 (nanoemulsions, liposomes, nanoparticles, solid lipid nanoparticles, cyclodextrins, etc.) were
85 introduced to the food industry as a novel strategy to overcome the poor water solubility of food
86 antimicrobials and to enhance their stability in food matrices [29,30]. Cyclodextrins are natural
87 oligosaccharides widely used in food products for their safety and their ability to deliver
88 hydrophobic compounds (e.g. antioxidants, antimicrobials, etc.) [31]. Moreover, different

89 derivatives of cyclodextrin have been synthesized to enhance the aqueous solubility of native
90 cyclodextrins [32].

91 The objective of this study is to select a natural potent antibacterial agent against *L. fermentum*.

92 The selected antibacterial is then complexed with hydroxypropyl- β -cyclodextrin (HP- β -CD) and
93 the obtained inclusion complex is tested against the chosen bacterium in culture medium.

94 Therefore, a wide range of terpenic and phenolic phytochemicals was screened against the
95 bacterium, under its optimal growth conditions. The tested compounds belong to different

96 subclasses and possess variable structures. Seventeen terpenes were tested, among which eight
97 monoterpene hydrocarbons (camphene, *p*-cymene, limonene, α -phellandrene, α -pinene, β -

98 pinene, α -terpinene, γ -terpinene), a sesquiterpene hydrocarbon (β -caryophyllene), four
99 monoterpene alcohols (borneol, geraniol, linalool, menthol), a sesquiterpene alcohol (nerolidol),

100 two monoterpene esters (bornyl acetate, linalyl acetate), and a monoterpene ketone (camphor)
101 (Fig. 1). In addition, eleven phenols were tested, among which four phenylpropenes (*trans*-

102 anethole, eugenol, isoeugenol, estragole), a phenylpropene ester (eugenyl acetate), two
103 hydroxycinnamic acids (*p*-coumaric acid, *trans*-ferulic acid), a phenol ether (anisole), a

104 methylphenol (*o*-cresol), a flavonoid (quercetin), and a stilbenoid (resveratrol) (Fig. 2).
105 Nerolidol (Ner) showed the most potent antibacterial activity against *L. fermentum* among the 28

106 tested compounds. The potency of nerolidol solubilized in DMSO and hydroxypropyl- β -
107 cyclodextrin/nerolidol (HP- β -CD/Ner) complex was assessed against *L. fermentum* by

108 determining the minimal inhibitory concentration (MIC) and the minimal bactericidal
109 concentration (MBC) values under the optimal conditions for *L. fermentum* growth. The time

110 required for both forms of nerolidol to cause a total bacterial kill was determined by time-kill
111 analysis.

112 2. Materials and methods

113 2.1. Materials

114 β -Caryophyllene, geraniol, isoeugenol (98% mixture *cis* and *trans*), nerolidol (98%, mixture of
115 *cis* (40%) and *trans* (60%) isomers), (-)- β -pinene, ethanol, and DMSO were purchased from
116 Sigma-Aldrich (Missouri, United States). *trans*-Anethole (99%) and linalyl acetate were
117 purchased from Sigma-Aldrich (Madrid, Spain). Anisole, camphene, *o*-cresol, eugenol, eugenyl
118 acetate, (-)-menthol, and α -phellandrene were purchased from Sigma-Aldrich (Schnelldorf,
119 Germany). (-)-Borneol, (-)-bornyl acetate, *p*-cymene, (R)-(t)-limonene, linalool, (+)- α -pinene, α -
120 terpinene, and γ -terpinene were purchased from Sigma-Aldrich (Buchs, Switzerland). (\pm)-
121 Camphor and *trans*-ferulic acid were purchased from Sigma-Aldrich (Hong Kong, China).
122 Quercetin was purchased from Sigma-Aldrich (Haryana, India). *p*-Coumaric acid was purchased
123 from Sigma-Aldrich (Irvine, United kingdom). Hydroxypropyl- β -cyclodextrin was purchased
124 from Wacker-Chemie (Lyon, France). De Man, Rogosa, and Sharpe (MRS) broth and MRS agar
125 were purchased from Laboratorios Conda (Madrid, Spain).

126 2.2. Bacterial strain and culture

127 *L. fermentum* ATCC 9338 was purchased from American Type Culture Collection (*Manassas*,
128 Virginia, USA). *L. fermentum* cultures were routinely maintained at 4 °C on MRS agar. Before
129 each antimicrobial assay, fresh cultures were prepared in sterile MRS broth and incubated at 37
130 °C for 22 h under anaerobic conditions. A bacterial suspension was prepared by diluting the
131 bacterial culture in MRS broth to a final concentration of 25×10^5 colony forming unit (CFU)/ml.

132 2.3. Screening of natural terpenic and phenolic compounds for antibacterial activity

133 2.3.1. Screening rounds

134 Twenty-eight natural terpenic and phenolic compounds were screened for their antibacterial
135 activity against *L. fermentum* at 3500, 500, 250, and 100 μM . The tested molecules were
136 dissolved in DMSO, except β -caryophyllene and camphene which were dissolved in ethanol, and
137 the obtained solutions were homogenized by hand agitation prior to each test. Antibacterial agent
138 solutions (25 μl) were mixed with MRS broth (4.8 ml) in glass culture tubes (20 x 100 mm). The
139 tubes were then inoculated with 200 μl of a diluted *L. fermentum* suspension (25×10^5 CFU/ml)
140 to yield a bacterial concentration of 10^5 CFU/ml at baseline. Bacterial cultures (5 ml) exempt of
141 any agent, or containing 25 μl of DMSO or ethanol, served as controls. All cultures were
142 incubated at 37 °C for 22 h under anaerobic conditions. Screening rounds were conducted with
143 bioactive compounds at 3500, 500, and 250 μM , successively. After each screening round,
144 molecules demonstrating an anti-proliferative activity against *L. fermentum* at a given
145 concentration were identified. Whereas, compounds demonstrating a bactericidal effect against
146 *L. fermentum* were selected for another screening round at a lower concentration. The
147 compounds exhibiting a total bactericidal activity or a total anti-proliferative activity against *L.*
148 *fermentum* at 250 μM were screened at 100 μM . Each test was performed in triplicate and under
149 sterile conditions.

150 2.3.2. Determination of the anti-proliferative activity of molecules

151 The anti-proliferative activity of the molecules against *L. fermentum* was assessed by UV-visible
152 spectroscopy at 660 nm using Uviline 9100-9400 spectrophotometer (GmbH, Germany). The
153 optical density of each tube was measured and compared to the control. The percentage of
154 bacterial proliferation inhibition was calculated as follows:

155
$$\text{Bacterial proliferation inhibition (\%)} = 100 \times \left[1 - \frac{OD_{660m}}{OD_{660c}} \right],$$

156 where OD_{660m} and OD_{660c} are the optical densities of the tubes containing the molecule and the
157 control tube, respectively. Each test was done in triplicate.

158 2.3.3. Determination of the bactericidal activity of the natural molecules

159 Cultures showing a total inhibition of bacterial proliferation were analyzed in duplicate by
160 spreading an aliquot of 100 μ l on MRS agar. The bacterial concentration in the control was
161 determined by enumeration. The bactericidal capacity was then evaluated according to the
162 decrease in the initial bacterial concentration and calculated as follows:

163
$$\text{Bacterial kill (\%)} = 100 \times \left[1 - \frac{[bac]_m}{[bac]_c} \right],$$

164 where $[bac]_m$ and $[bac]_c$ are the bacterial concentrations in the tube containing the molecule and
165 the control tube, respectively.

166 2.4. Preparation of HP- β -CD/Ner inclusion complex

167 HP- β -CD/Ner inclusion complex was prepared by freeze-drying, as previously described by Azzi
168 et al. [33]. Briefly, an aqueous solution of HP- β -CD (25 mM) containing an excess of nerolidol
169 was kept under magnetic stirring at 300 rpm for 24 h at room temperature. The suspension was
170 then filtered (0.45 μ m, cellulose acetate membrane) to remove the excess of nerolidol. The
171 filtrate was frozen at -80 $^{\circ}$ C, and lyophilized. HP- β -CD/Ner complex in powder form was stored
172 at 4 $^{\circ}$ C until usage. The amount of nerolidol encapsulated in the cyclodextrin cavity was
173 determined by HPLC as described previously by Azzi et al. [33], and the result was expressed as
174 mass of nerolidol (μ g) per mg of powder (μ g_{Ner}/mg_{powder}).

175 2.5. Study of the antibacterial activity of nerolidol and HP- β -CD/Ner complex

176 2.5.1. Determination of MIC and MBC values

177 Bacterial cultures were prepared as previously described in section 2.3.1, in presence of nerolidol
178 dissolved in DMSO, and added at a final concentration ranging from 0.1 to 3500 μ M. The MIC
179 was determined as the minimal concentration at which no bacterial growth was observed in MRS
180 broth, whereas the MBC was determined as the minimal concentration at which no bacterial
181 growth was observed on agar. Each experiment was performed in triplicate and under sterile
182 conditions. On the other hand, HP- β -CD was investigated for its capacity to replace DMSO for
183 nerolidol solubilization in aqueous solution. The inclusion complex was tested at a final
184 concentrations of nerolidol ranging between 50 and 4000 μ M. Bacterial cultures (5 ml) exempt
185 of any agent, or containing DMSO (25 μ l) or blank HP- β -CD added in similar amounts to that of
186 the inclusion complex, served as controls.

187 2.5.2. Time-kill analysis

188 Time-kill assay was performed in triplicate on nerolidol and HP- β -CD/Ner complex. Cultures
189 were prepared as described in section 2.3.1. Nerolidol solubilized in DMSO and the complex
190 were added to cultures at their respective MBC values. Starting from an initial bacterial
191 concentration of 10^5 CFU/ml (5 log CFU/ml), bacterial growth was followed during 22 h in
192 cultures maintained at 37 °C under anaerobic conditions. The viable plate count was determined
193 at different time intervals using the spread plate method [34]. Therefore, 100 μ l of samples with
194 appropriate dilutions was spread on MRS agar. The plates were incubated under *L. fermentum*
195 optimal growth conditions for 22 h. The colonies were then counted and the bacterial
196 concentration was determined.

197 2.6. Statistical Analysis

198 Statistical analysis was performed using the Student T test. *P* values equal or less than 0.05 were
199 considered statistically significant.

200 3. Results

201 3.1. Antibacterial activity of terpenic and phenolic compounds

202 Terpenic compounds exhibited a significantly higher antibacterial activity against *L. fermentum*
203 compared to phenolic compounds, with α -terpinene being the only exception. In fact at 3500 μ M,
204 16 among the 17 tested terpenes were bactericidal, whereas α -terpinene strongly inhibited the
205 proliferation of *L. fermentum* (89.1%) (Table 1). Eugenol and its ester derivative eugenyl acetate
206 were the only phenolic compounds exhibiting a strong bactericidal activity against *L. fermentum*
207 at 3500 μ M. Indeed, eugenol and eugenyl acetate exhibited a bactericidal activity of 100% and
208 95%, respectively, within 22 h of incubation at 37 °C under anaerobic conditions (Table 2).
209 Among the phenolic compounds, *trans*-anethole, estragole, and isoeugenol showed a significant
210 anti-proliferative activity against *L. fermentum* at the highest concentration (3500 μ M), where
211 bacterial growth inhibition was higher than 88% (Table 2). At this concentration, *p*-coumaric
212 acid, *trans*-ferulic acid, and anisole showed a weak bacterial growth inhibition of 21.9, 17.3, and
213 16.1%, respectively (Table 2). Whereas, quercetin, *o*-cresol, and resveratrol possessed no
214 antibacterial activity against *L. fermentum* (Table 2). In fact, quercetin and resveratrol were
215 insoluble in MRS at this concentration, which was marked by their strong precipitation.

216 The stronger antibacterial property of terpenes compared to phenolic compounds was highlighted
217 by the strong bactericidal and anti-proliferative effect of different studied terpenes at 500 μ M.
218 Indeed, at this concentration, the two phenolic compounds, eugenol and eugenyl acetate, showed
219 no antibacterial activity against *L. fermentum* (Table 2). On the other hand, β -pinene, bornyl

220 acetate, linalyl acetate, and nerolidol exhibited a total bactericidal activity at 500 μ M (Table 1).
221 Camphene, *p*-cymene, limonene, α -phellandrene, α -pinene, and γ -tepinene showed a total
222 inhibition of *L. fermentum* proliferation but were not bactericidal (Table 1). Furthermore, β -
223 caryophyllene, geraniol, and menthol showed a bacterial inhibition percentage higher than 75%,
224 compared to control, whereas borneol demonstrated a weak anti-proliferative activity (28.5%)
225 (Table 1). However, linalool and camphor, which exhibited a bactericidal activity against *L.*
226 *fermentum* at 3500 μ M, showed no antibacterial activity at 500 μ M (Table 1).

227 When β -pinene, bornyl acetate, linalyl acetate, and nerolidol were tested at 250 μ M, nerolidol
228 was the only compound exhibiting a bactericidal activity (100%) (Table 1). Whereas, β -pinene,
229 bornyl acetate, and linalyl acetate showed a strong anti-proliferative activity against *L.*
230 *fermentum* at 250 μ M, as no bacterial growth was observed in MRS broth within 22 h of
231 incubation (Table 1). Nerolidol maintained a strong bactericidal activity (100%) against *L.*
232 *fermentum* at 100 μ M, whereas, β -pinene strongly inhibited bacterial proliferation (~91%), and
233 bornyl acetate and linalyl acetate showed no antibacterial activity (Table 1). Thus, among the 28
234 tested compounds, nerolidol was retained for further studies as it was the most effective studied
235 compound against *L. fermentum*.

236 3.2. Antibacterial activity of nerolidol

237 Nerolidol was the only molecule that exhibits a bactericidal activity against *L. fermentum* at a
238 low concentration (100 μ M) (Tables 1 and 2). Therefore, the study was taken further to
239 determine the MIC and MBC values of nerolidol. Nerolidol exhibited a strong antibacterial
240 activity against *L. fermentum* with low MIC (25 μ M; 5.56 mg/l) and MBC (50 μ M; 11.12 mg/l)
241 values. Moreover, *L. fermentum* survival was approximately 1% at a nerolidol concentration of

242 35 μM . However, the adopted MBC was 50 μM , as no bacterial growth was observed at this
243 concentration.

244 The antibacterial activity profile (bactericidal or bacteriostatic) of nerolidol was evaluated using
245 the MBC to MIC ratio. The *in vitro* antimicrobial activity of nerolidol can be described as
246 bactericidal as the MBC to MIC ratio (MBC/MIC= 2) is lower than 4 [35].

247 3.3. Antibacterial activity of HP- β -CD/Ner complex

248 The amount of nerolidol in the freeze-dried inclusion complex, determined by HPLC, was 40
249 $\mu\text{g}_{\text{Ner}}/\text{mg}_{\text{powder}}$. The antibacterial activity of HP- β -CD/Ner complex was investigated for the first
250 time against *L. fermentum* in culture medium under the optimal conditions for bacterial growth.
251 HP- β -CD without nerolidol had no effect on the bacterial growth (data not shown). The MIC and
252 MBC values of HP- β -CD/Ner against *L. fermentum* were 100 and 200 μM , respectively. At 50
253 μM , a 4 log increment was observed in the bacterial concentration within 22 h of incubation
254 (data not shown).

255 3.4. Nerolidol and HP- β -CD/Ner complex time-kill analysis

256 A time-kill analysis was conducted to determine the time required to achieve a total bacterial kill
257 in presence of nerolidol solubilized in DMSO at 50 μM and HP- β -CD/Nero complex at 200 μM
258 (MBC). Free nerolidol exhibited a bactericidal activity against *L. fermentum* within the first few
259 hours of incubation (Fig. 3). Indeed, a 1.44 log reduction of *L. fermentum* concentration was
260 observed within 4 h of incubation. The bacterial concentration continued to decrease over time,
261 as a 2.82 log reduction was obtained after 10 h. At 16 h of incubation, no viable cells were
262 observed.

263 In presence of HP- β -CD/Ner complex (200 μ M), the bacterial concentration decreased by 1.22
264 log and 2.44 log after 4 and 10 h, respectively, and a total bacterial death was obtained within 12
265 h (Fig. 3).

266 **4. Discussion**

267 4.1. Antibacterial efficiency of nerolidol

268 Plants produce a wide range of antimicrobial agents highly desired by consumers due to their
269 natural origin. However, many of these antimicrobials are only effective at high concentrations
270 (for example at millimolar range), thus exhibiting a weak activity compared to common
271 antibiotics [36]. In this study, nerolidol exhibited the highest antibacterial activity among the 28
272 tested terpenic and phenolic compounds. The outstanding antibacterial potency of nerolidol
273 against *L. fermentum* was marked by the low MIC (25 μ M; 5.56 mg/l) and MBC (50 μ M; 11.12
274 mg/l) values, as well as the rapid bactericidal activity (Fig. 3). This compound acts by disrupting
275 and by damaging the bacterial cell membrane, and by interfering with genes responsible for the
276 microbe pathogenicity [37]. Besides, Brehm-Stecher and Johnson [27] demonstrated the
277 permeabilizing effect of nerolidol on *L. fermentum* membrane. The permeabilization of the
278 bacterial membrane leads to the leakage of the cytoplasmic molecules, thus causing cell lysis
279 [38]. Also, the disruption of the bacterial membrane would allow the permeation of exogenous
280 molecules into the bacterial cytoplasm [27]. Moreover, Brehm-Stecher and Johnson [27]
281 suggested that the permeabilizing activity of nerolidol may be due to its structural resemblance to
282 the lipids of the bacterial membrane. This was previously highlighted by Cornwell and Barry
283 [39] which attributed the enhancement of skin penetration by nerolidol to its long hydrocarbon
284 tail which promotes the interaction of the molecule with the interior of the cell bilayer.

285 Nerolidol is a sesquiterpene alcohol widely used in the food industry as a flavoring agent. It
286 demonstrated a potent antimicrobial activity against some fungi, in addition to different Gram-
287 positive and Gram-negative bacterial strains, such as *Staphylococcus aureus* (MIC: 3.9 mg/l; 50
288 mg/l) [26,40,41], *Streptococcus mutans*, *Propionibacterium acnes* (MIC: 25 mg/l) [26],
289 *Salmonella enterica* (MIC: 15.6 mg/l) [41], *Trichophyton mentagrophytes* (MIC: 12.5 mg/l) [26],
290 and *Aspergillus niger* (15.6 mg/l) [41]. On the other hand, nerolidol was able to enhance the
291 susceptibility of *S. aureus* and *Escherichia coli* to antibiotics, including ciprofloxacin,
292 erythromycin, gentamicin, vancomycin [42], and amoxicilline/clavulanic acid [43]. In this study,
293 nerolidol antibacterial potency against *L. fermentum* (MIC: 25 μ M; 5.56 mg/l) was weaker than
294 that of gentamicin [44] and chloramphenicol [45], and close to that of novobiocin [46] and N-
295 alkyl dimethylbenzyl ammonium chloride [47] (Table 3). On the other hand, *L. fermentum* strains
296 were less sensitive to well-known antibiotics including vancomycin [45,48], teicoplanin [45],
297 streptomycin [44,45,48], erythromycin, tobramycin, clindamycin, kanamycin, polymixin B
298 [44,46,48], metronidazole, and nitrofurantoin [49] (Table 3).

299 4.2. Antibacterial assay of HP- β -CD/Ner complex

300 Cyclodextrin inclusion complexes have been widely studied for their capacity to enhance the
301 stability and solubility of antimicrobials [28,50,51].

302 Some studies reported similar antibacterial activities of free and complexed antimicrobials (e.g.
303 clarithromycin, chlorogenic acid, peptide CM4) [28,50,52], while others proved modifications of
304 the potency of the antimicrobial agent following complexation with cyclodextrins. Compared to
305 the free form, the inclusion complex of *Hyptis martiusii* essential oil [53] and coriander essential
306 oil [51] showed a lower antimicrobial activity, whereas that of carvacrol showed a higher
307 antimicrobial activity [54].

308 The incorporation of nerolidol into HP- β -CD inclusion complex increased the MIC and MBC
309 values by 4 fold. Azzi et al. [33] studied the release of nerolidol from the inclusion complex in
310 water and at room temperature by dialysis. Around 45% of nerolidol were released from HP- β -
311 CD/Ner complex within 8 h, followed by a slow release rate over 7 days [33]. In fact, when HP-
312 β -CD/Ner complex is added at 50 μ M, the actual concentration of nerolidol interacting with *L.*
313 *fermentum* in MRS is significantly lower than 50 μ M during the whole experiment, thus
314 explaining the ability of the inoculated bacteria to proliferate (data not shown). However, at 200
315 μ M the concentration of nerolidol in the culture medium should be approximately 100 μ M after 8
316 to 10 h of incubation, which could explain the rapid drop in the bacterial concentration (Fig. 3).
317 The slow release and the photo-protection of nerolidol provided by the encapsulation systems
318 [33] would prevent and limit the proliferation of spoilage bacteria in food products.

319 4.3. Relationship between the antibacterial activity of terpenic and phenolic compounds and their 320 structural and physicochemical parameters

321 Various parameters may modulate the activity of antibacterial agents, including the drug
322 hydrophobicity, the presence of functional groups, the bacterial cell envelope characteristics, and
323 the incubation conditions (for instance, aeration).

324 4.3.1. The influence of hydrophobicity on the antibacterial activity of molecules

325 It has been reported that molecules with high hydrophobicity exhibit a high affinity to the
326 lipophilic structures in the target microorganisms, such as the bacterial membrane [55].
327 Therefore, they may disrupt the membrane integrity, thus affecting membrane permeability and
328 enzymes activity [55]. However, many exceptions could be revealed from this work, which
329 suggests that the hydrophobicity is not always the key parameter governing the antibacterial

330 activity; and some structural features could be taken into consideration when analyzing the
331 structure-antibacterial activity relationship.

332 Nerolidol, which has shown the strongest antibacterial activity against *L. fermentum*, has a LogP
333 value (5.33-5.36) considerably higher than that of the other tested molecules, except β -
334 caryophyllene (6.87) (Table 1). The latter, showed a modest antibacterial activity against *L.*
335 *fermentum* as it lost its bactericidal potential at 500 μ M (Table 1). In fact, β -caryophyllene have
336 been found to exhibit a weak antibacterial activity against different Gram-positive and Gram-
337 negative bacteria [56].

338 On the other hand, the antibacterial potency of the compounds exhibiting an antibacterial activity
339 at a concentration lower than 500 μ M was influenced by hydrophobicity (Table 1). Indeed, β -
340 pinene showed a higher antibacterial activity than the less hydrophobic molecules, bornyl acetate
341 and linalyl acetate (Table 1). The acetylation of linalool and borneol increased their
342 hydrophobicity and their antibacterial activity (Table 1). Indeed, bornyl acetate and linalyl
343 acetate retained their total anti-proliferative activity at 250 μ M, whereas borneol and linalool lost
344 partially and totally the activity at 500 μ M, respectively (Table 1). Similarly, Knobloch et al.
345 [57] reported a higher inhibition of H⁺-translocation by linalyl acetate, compared to linalool.
346 Also, Dorman and Deans [58] observed a higher antibacterial activity of bornyl acetate compared
347 to borneol against a wide range of bacterial strains, among which *Lactobacillus plantarum*.
348 Moreover, phenolic compounds with LogP values close to or lower than 2, such as anisole (2.11)
349 and *o*-cresol (1.95-1.98), showed no or a weak antibacterial activity against *L. fermentum* (Table
350 2).

351 Acyclic monoterpenoids showed an increased antibacterial activity with the increment of their
352 LogP value. Indeed, linalyl acetate showed a higher antibacterial activity than geraniol, which

353 was more potent than linalool (Table 1). This was also observed for bicyclic monoterpenes, as
354 borneol and camphor had the lowest LogP values, and showed the weakest antibacterial activities
355 (Table 1). However, despite β -pinene not being the most hydrophobic compound in the bicyclic
356 monoterpenes chemical class, it exhibited the strongest antibacterial activity (Table 1).

357 For monocyclic monoterpenes, limonene, α -phelladrene, and γ -terpinene, having higher LogP
358 values than menthol, showed a stronger antibacterial activity (Table 1). α -Terpinene was found
359 to be an exception as it showed the weakest antibacterial potency, despite a high LogP value
360 (Table 1).

361 4.3.2. Structure-activity analysis of terpenic compounds

362 Different studies have demonstrated the role of terpenoid functional groups in the antimicrobial
363 activity. Carvacrol showed a better antimicrobial activity compared to its derivatives, carvacrol
364 methyl ether and *p*-cymene, which lack the hydroxyl group [58]. On the other hand, Kotan et al.
365 [59] reported a better antibacterial activity for alcohol derivatives of oxygenated monoterpenes,
366 when compared to ketone and acetate derivatives.

367 The weak antibacterial activity of β -caryophyllene, despite its high hydrophobicity, may be due
368 to the absence of a hydrophilic functional group in the chemical structure of the molecule. The
369 combination of a lipophilic character of the skeleton and the presence of a hydrophilic functional
370 group was found to be important for the antimicrobial activity of essential oils components [60].

371 The structural features of nerolidol are in line with the previous findings. Indeed, the
372 antibacterial activity of aliphatic terpene alcohols was demonstrated to be dependent on the
373 hydrophobic chain length starting from the carbon connected to the hydroxyl group [38,61]. In
374 fact, farnesol (C₁₂) exhibited a stronger antibacterial activity than nerolidol (C₁₀) followed by
375 geraniol (C₈), whereas linalool (C₆) showed no antibacterial activity against *Staphylococcus*

376 *aureus* [61]. Similarly, Togashi et al. [38] reported a very weak antibacterial activity of geraniol
377 and linalool against *S. aureus*. Additionally, farnesol (C₁₂), nerolidol (C₁₀), and plaunotol (C₁₁),
378 showed a strong antibacterial activity against *S. aureus*, in that order [38,62]. Also, no or weak
379 antibacterial activity against *S. aureus* was reported for alcohols with chains containing more
380 than 12 carbon atoms like farnesylacetol (C₁₄) [61], geranylgeraniol, and phytol (C₁₆) [38].
381 Therefore, to exhibit a potent antibacterial effect, the authors suggested that the chain, starting
382 from the hydroxyl group, should contain from 10 to 12 carbon atoms [38], or less than 12 carbon
383 atoms but as close to 12 as possible [61]. Although the previous studies were conducted on *S.*
384 *aureus*, our study supports the pattern of the antibacterial activity of terpene alcohols against *L.*
385 *fermentum*. Indeed, nerolidol (C₁₀; sesquiterpene) exhibited a stronger antibacterial activity than
386 geraniol (C₈; monoterpene), the latter being more potent than linalool (C₆; monoterpene) (Table
387 1).

388 4.3.3. Structure-activity analysis of phenolic compounds

389 The importance of the propenyl side chain was noted among the tested phenolic compounds, as
390 eugenol, *trans*-anethole, isoeugenol, and estragole, showed a significantly higher antibacterial
391 activity against *L. fermentum* compared to anisole (Table 2). The propenyl side chain is absent in
392 anisole compared to eugenol, *trans*-anethole, isoeugenol, and estragole (Fig. 2), which could be
393 the reason for the weaker antibacterial activity of anisole.

394 On the other hand, eugenol showed the highest antibacterial activity among the tested phenolic
395 compounds against the Gram-positive bacterium *L. fermentum*. However, Gharib et al. [63]
396 reported a higher antibacterial activity for anethole and estragole compared to eugenol, against
397 the Gram-negative bacterium *Escherichi coli*. Indeed, the hydrophobicity of the compound
398 seemed to play a role in the potency of the antibacterial activity against *E. coli*, as anethole and

399 estragole have a higher hydrophobicity than eugenol [63] (Table 2). Whereas, the hydrophobicity
400 of phenylpropenes did not seem to influence their antibacterial activity against the Gram-positive
401 bacterium, *L. fermentum*. Therefore, our results strongly suggest the presence of other factors
402 that modulate the antibacterial activity of phenylpropenes against *L. fermentum*. The higher
403 antibacterial activity of eugenol compared to its ester, eugenyl acetate (Table 2), highlights the
404 importance of the hydroxyl group in the phenolic structure (Fig. 2). Additionally, the absence of
405 the hydroxyl group in *trans*-anethole and estragole structure (Fig. 2) could explain the lower
406 antibacterial activity against *L. fermentum*, compared to eugenol (Table 2). Indeed, the hydroxyl
407 group of eugenol and isoeugenol has been found to reinforce the lipid membrane-fluidizing
408 effect, compared to anethole and estragole [63]. Also, the bacterial membrane characteristics
409 play a crucial role in the differential bacterial susceptibility to a given antibacterial agent [64–
410 66]. In fact, eugenol has previously shown lower MIC values against the Gram-positive bacteria
411 *Staphylococcus aureus* (MIC= 2.5 mg/l) and *Bacillus subtilis* (MIC= 1.25 mg/l), compared to *E.*
412 *coli* (MIC= 5 mg/l) [67].

413 Both ferulic acid and *p*-coumaric acid exhibited a weak anti-proliferative activity against *L.*
414 *fermentum* in MRS broth (pH ~6.2) (Table 2). The antibacterial activity of hydroxycinnamic
415 acids depends on pH, which controls the concentration of their undissociated form. The
416 undissociated form can easily penetrate the cytoplasmic membrane of the bacterium [68]. The
417 pKa values of ferulic acid and *p*-coumaric acid are between 4 and 5, thus a greater proportion of
418 their dissociated forms is found at the pH of the culture medium (~6.2). In fact, the antibacterial
419 activity of ferulic acid and *p*-coumaric acid was found to increase in culture media at pH values
420 below 6 [69]. Moreover, *L. fermentum* is able to metabolize ferulic acid and coumaric acid into

421 less potent metabolites, such as phloretic acid, p-vinyl phenol, or dihydroferulic acid [69]. This
422 also may explain the weak antibacterial activity of both acids against *L. fermentum*.

423 **5. Conclusion**

424 In this study, 28 terpenic and phenolic compounds were screened against *L. fermentum*, an
425 ubiquitously present bacterium which could cause spoilage of different food products. Among
426 the tested compounds, nerolidol exhibited the strongest antibacterial activity marked by the low
427 MIC and MBC values. Moreover, a total bacterial kill was obtained within 16 h in presence of
428 nerolidol (50 μ M). The antibacterial activity of nerolidol was dependent on different factors,
429 including the hydrophobicity of the compound, as well as the position of the hydroxyl group.
430 Compared to nerolidol solubilized in DMSO, HP- β -CD/Ner inclusion complex exhibited 4 fold
431 higher MIC and MBC values and a more rapid bactericidal activity. Indeed, HP- β -CD/Ner
432 inclusion complex was proven effective against *L. fermentum* in culture medium. The high
433 demand for the replacement of synthetic food additives by natural molecules encourages the
434 application of nerolidol in food. Moreover, due to the physico-chemical limitations of the
435 application of natural bioactive components in food, the use of encapsulation systems has
436 recently become a widely investigated novel approach for the delivery of bioactive compounds in
437 food products. Therefore, further studies could be realized to investigate the antibacterial activity
438 of free and encapsulated nerolidol in different food products including fruit juices, alcoholic
439 beverages, and milk products. This evaluation would lead to a better understanding of the
440 antibacterial potency of natural molecules such as nerolidol, under their free and complexed
441 form, in various types of food matrices and under various conditions.

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444 **Conflict of Interest**

445 The authors declare no conflict of interest.

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450 **References**

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719 **Figure legends**

720 **Fig.1.** Chemical structure of terpenic compounds.

721 **Fig. 2.** Chemical structure of phenolic compounds.

722 **Fig. 3.** Nerolidol (50 μ M) and HP- β -CD/Ner complex (200 μ M Ner) time-kill analysis against *L.*
723 *fermentum* at 37 °C under anaerobic conditions.

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738 **Table 1: Bactericidal and anti-proliferative activity of terpenes against *L. fermentum*.**

	Borneol	Bornyl acetate	Camphene	Camphor	β -Caryophyllene	<i>p</i> -Cymene	Geraniol	Limonene	Linalool	Linalyl acetate	Menthol	Nerolidol	α -Phellandrene	α -Pinene	β -Pinene	α -Terpinene	γ -Terpinene
LogP	3.01 [70]	3.86 [71]	4.22 [70]	2.74 [70]	6.87 [72]	4.1 [73]	3.56 [70]	4.58 [74] 4.23 [77] 4.57 [75] 4.38 [70] 4.83 [79]	2.97 [75]	3.93 [70]	3.4 [70] 3.38 [78]	5.36 [74]	4.55 [76]	4.83 [75] 4.49 [76] 4.48 [70]	4.16 [70] 4.42 [76]	4.25 [70]	4.5 [75] 4.36 [70]
3500 μM Bacterial kill (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	0	100
Inhibition (%)	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	89.1 \pm 0.5	100
500 μM Bacterial kill (%)	0	100	0	0	0	0	0	0	0	100	0	100	0	0	100	ND	0

Inhibition (%)	28.5 ± 1.8	100	100	0	89 ± 2.4	100	80.9 ± 1.4	100	0	100	75.7 ± 4.4	100	100	100	100	ND	100
250 μM Bacterial kill (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	0	ND	ND
Inhibition (%)	ND	100	ND	ND	ND	ND	ND	ND	ND	100	ND	100	ND	ND	100	ND	ND
100 μM Bacterial kill (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	0	ND	ND
Inhibition (%)	ND	0	ND	ND	ND	ND	ND	ND	ND	0	ND	100	ND	ND	91.4 ± 0.8	ND	ND

739 ND: Not determined

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744 **Table 2: Bactericidal and anti-proliferative activity of phenolic compounds against *L.***
 745 ***fermentum*.**

	<i>trans</i> -Anethole	Anisole	<i>p</i> -Coumaric acid	<i>o</i> -Cresol	Estragole	Eugenol	Eugenyl acetate	<i>trans</i> -Ferulic acid	Isoeugenol	Quercetin	Resveratrol
LogP	3.31[80]			1.95[73]	3.13[80]	2.40[83] 2.45[72]		1.51[73]			3.10 [85]
	3.0961[82]	2.11[81]	1.43[82]	1.975[86]	2.818[82]	2.29[87] 2.73[78] 2.99[70]	2.9[72]	1.249[82]	3.04[70]	1.82 [84]	3.06 [88]
3500 μM											
Bacterial kill (%)	0	0	0	0	0	100	95 \pm 0.47	0	0	0	0
Inhibition (%)	91.4 \pm 1.80	16.1 \pm 0.92	21.9 \pm 0.94	0	84.4 \pm 0.13	100	100	17.3 \pm 0.14	89.3 \pm 0.09	0	0
500 μM											
Bacterial kill (%)	ND	ND	ND	ND	ND	0	0	ND	ND	ND	ND
Inhibition (%)	ND	ND	ND	ND	ND	0	0	ND	ND	ND	ND

746 ND: Not determined

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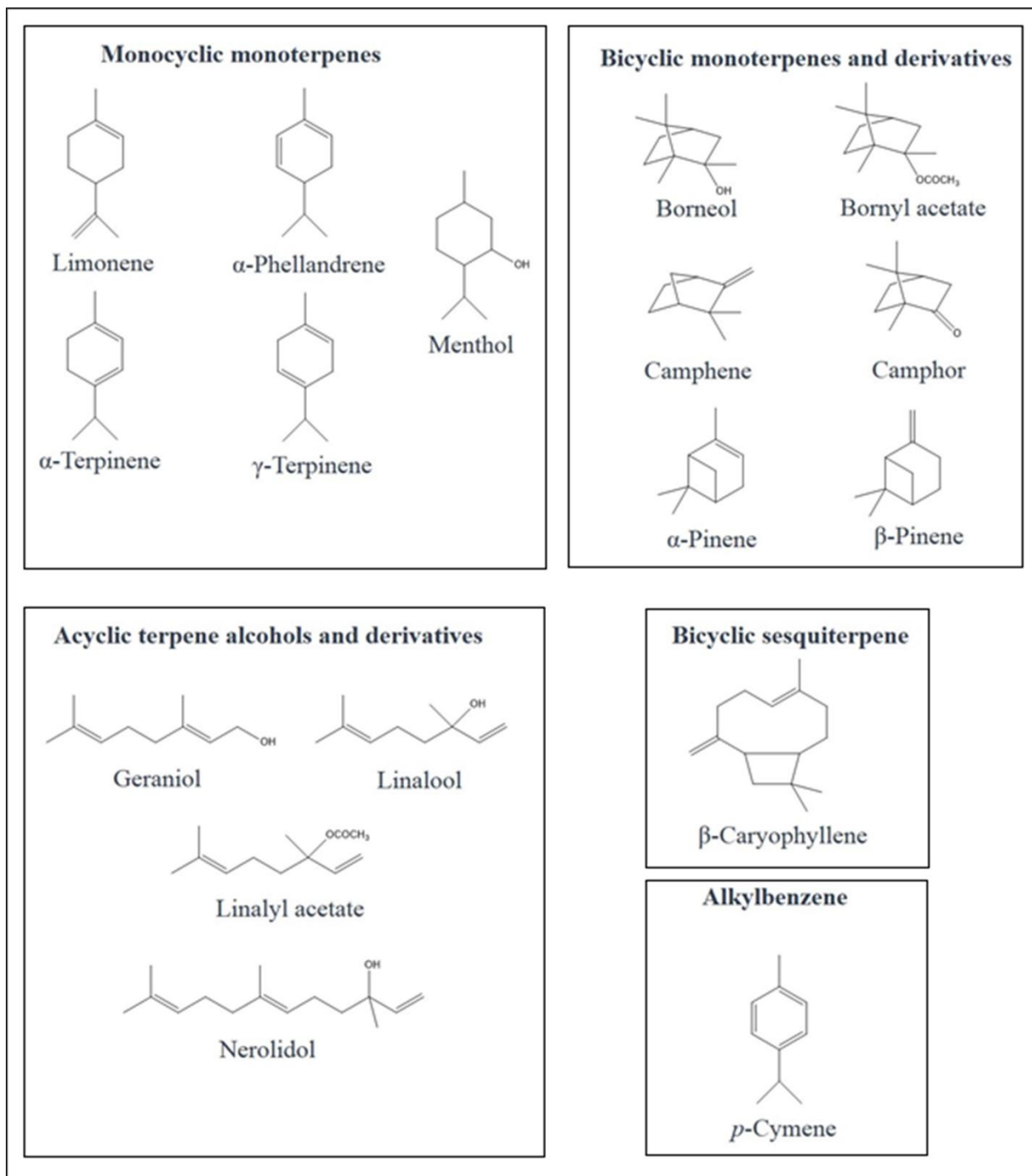
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756 **Table 3: MIC values of common antibiotics against *L. fermentum*.**

Antibiotic	MIC (mg/l)	MIC (μM)	Reference
Clindamycin	>64	>150.60	[46]
Chloramphenicol	4 ^a	12.38	[45]
Erythromycin	>128 - 256	>174.40 - 348.81	[46]
Gentamicin	4 - 8	8.38 - 16.75	[44]
Kanamycin	64	132.10	[48]
Metronidazole	>40	>233.7	[49]
N-alkyl dimethylbenzyl ammonium chloride	8	25.11	[47]
Nitrofurantoin	15	62.98	[49]
Novobiocin	>16	>26.12	[46]
Polymixin B	64	49.17	[48]
Streptomycin	16 ^a	27.51	[45]
	16	27.51	[48]
	8 - 128	13.76 - 220.09	[44]
	>128	>220.09	[46]
Teicoplanin	>256 ^a	>134.19-163.65	[45]
Tobramycin	>128	>273.79	[46]
Vancomycin	96	66.24	[48]
	>256 ^a	>176.64	[45]

757 ^aValues of MIC₉₀

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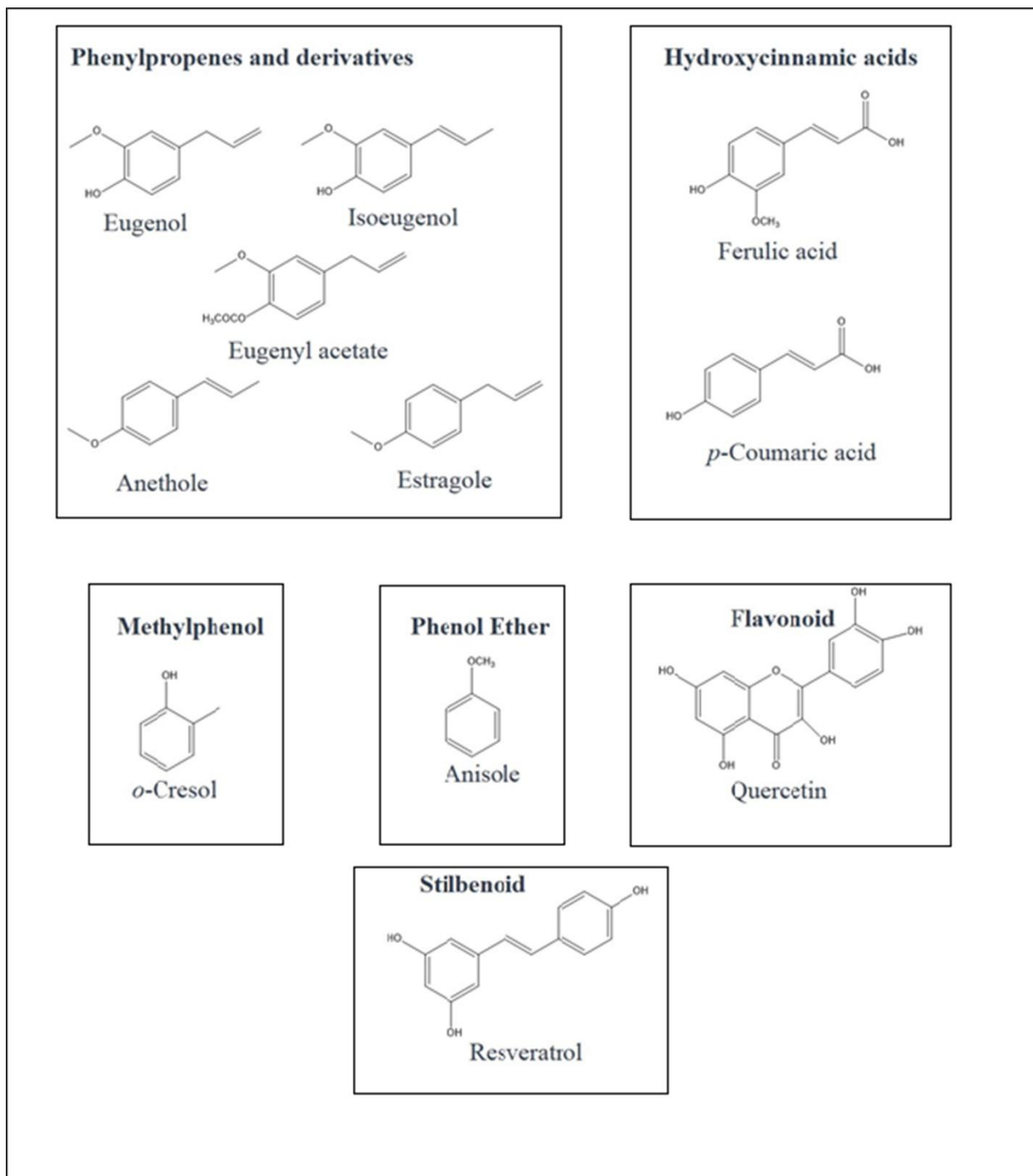


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760 **Fig. 1**

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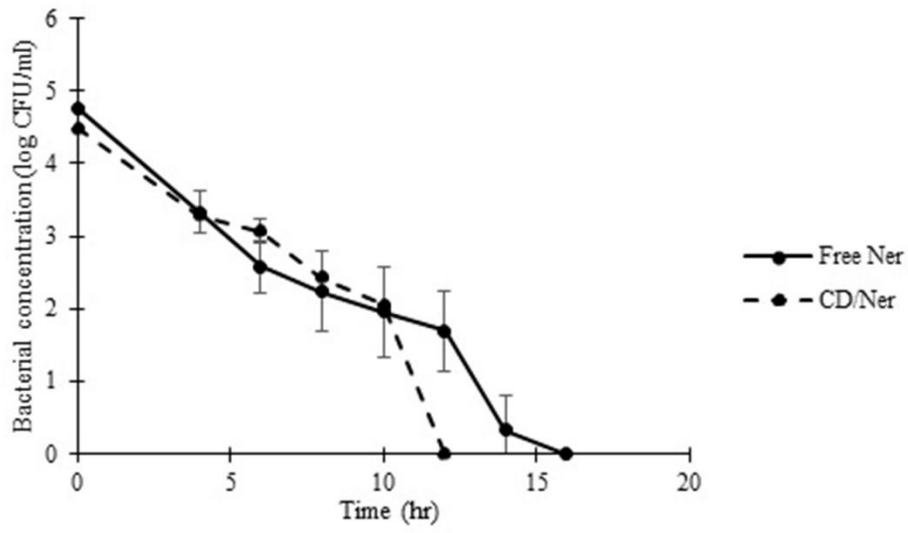


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764 **Fig. 2**

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768 **Fig. 3**

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