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Abstract

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

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

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

1. Introduction

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

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

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

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

The objective of this study is to select a natural potent antibacterial agent against *L. fermentum*. The selected antibacterial is then complexed with hydroxypropyl-β-cyclodextrin (HP-β-CD) and the obtained inclusion complex is tested against the chosen bacterium in culture medium. Therefore, a wide range of terpenic and phenolic phytochemicals was screened against the bacterium, under its optimal growth conditions. The tested compounds belong to different subclasses and possess variable structures. Seventeen terpenes were tested, among which eight monoterpene hydrocarbons (camphene, *p*-cymene, limonene, α-phellandrene, α-pinene, β-pinene, α-terpinene, γ-terpinene), a sesquiterpene hydrocarbon (β-caryophyllene), four monoterpene alcohols (borneol, geraniol, linalool, menthol), a sesquiterpene alcohol (nerolidol), two monoterpene esters (bornyl acetate, linalyl acetate), and a monoterpene ketone (camphor) (Fig. 1). In addition, eleven phenols were tested, among which four phenylpropenes (*trans*-anethole, eugenol, isoeugenol, estragole), a phenylpropene ester (eugenyl acetate), two hydroxycinnamic acids (*p*-coumaric acid, *trans*-ferulic acid), a phenol ether (anisole), a methylphenol (*o*-cresol), a flavonoid (quercetin), and a stilbenoid (resveratrol) (Fig. 2).

Nerolidol (Ner) showed the most potent antibacterial activity against *L. fermentum* among the 28 tested compounds. The potency of nerolidol solubilized in DMSO and hydroxypropyl-β-cyclodextrin/nerolidol (HP-β-CD/Ner) complex was assessed against *L. fermentum* by determining the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) values under the optimal conditions for *L. fermentum* growth. The time required for both forms of nerolidol to cause a total bacterial kill was determined by time-kill analysis.

2. Materials and methods

2.1. Materials

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

2.2. Bacterial strain and culture

L. fermentum ATCC 9338 was purchased from American Type Culture Collection *(Manassas*, Virginia, USA). *L. fermentum* cultures were routinely maintained at 4 °C on MRS agar. Before each antimicrobial assay, fresh cultures were prepared in sterile MRS broth and incubated at 37 °C for 22 h under anaerobic conditions. A bacterial suspension was prepared by diluting the 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 x 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:

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

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

2.3.3. Determination of the bactericidal activity of the natural molecules

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

$$\text{Bacterial kill (\%)} = 100\ x\ [1 - \frac{[bac]m}{[bac]c}],$$

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

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

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

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 µM. 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 µM. Bacterial cultures (5 ml) exempt
185 of any agent, or containing DMSO (25 µl) 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 µl 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.

2.6. Statistical Analysis

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

3. Results

3.1. Antibacterial activity of terpenic and phenolic compounds

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

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

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

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

3.2. Antibacterial activity of nerolidol

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

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

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

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

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

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

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

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

4. Discussion

4.1. Antibacterial efficiency of nerolidol

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.3.2. Structure-activity analysis of terpenic compounds

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

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

The structural features of nerolidol are in line with the previous findings. Indeed, the antibacterial activity of aliphatic terpene alcohols was demonstrated to be dependent on the hydrophobic chain length starting from the carbon connected to the hydroxyl group [38,61]. In fact, farnesol (C_{12}) exhibited a stronger antibacterial activity than nerolidol (C_{10}) followed by geraniol (C_8), whereas linalool (C_6) 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 (C_{12}), nerolidol (C_{10}), and plaunotol (C_{11}),
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 (C_{14}) [61], geranylgeraniol, and phytol (C_{16}) [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 (C_{10}; sesquiterpene) exhibited a stronger antibacterial activity than
386 geraniol (C_8; monoterpene), the latter being more potent than linalool (C_6; 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

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

5. Conclusion

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

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Conflict of Interest

The authors declare no conflict of interest.

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Figure legends

Fig.1. Chemical structure of terpenic compounds.

Fig. 2. Chemical structure of phenolic compounds.

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

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

	Borneol	**Bornyl acetate**	**Camphene**	**Camphor**	**β-Caryophyllene**	***p*-Cymene**	**Geraniol**	**Limonene**	**Linalool**	**Linalyl acetate**	**Menthol**	**Nerolidol**	**α-Phellandrene**	**α-Pinene**	**β-Pinene**	**α-Terpinene**	**γ-Terpinene**
LogP	3.01 [70] 2.85 [79]	3.86 [71]	4.22 [70] 4.56 [76]	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] 3.5 [70]	3.93 [70]	3.4 [70] 3.38 [78] 3.3 [71]	5.36 [74] 5.33 [77]	4.55 [76]	4.83 [75] 4.49 [76] 4.48 [70] 4.27 [79]	4.16 [70] 4.42 [76] 4.35 [79]	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 ± 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

ND: Not determined

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

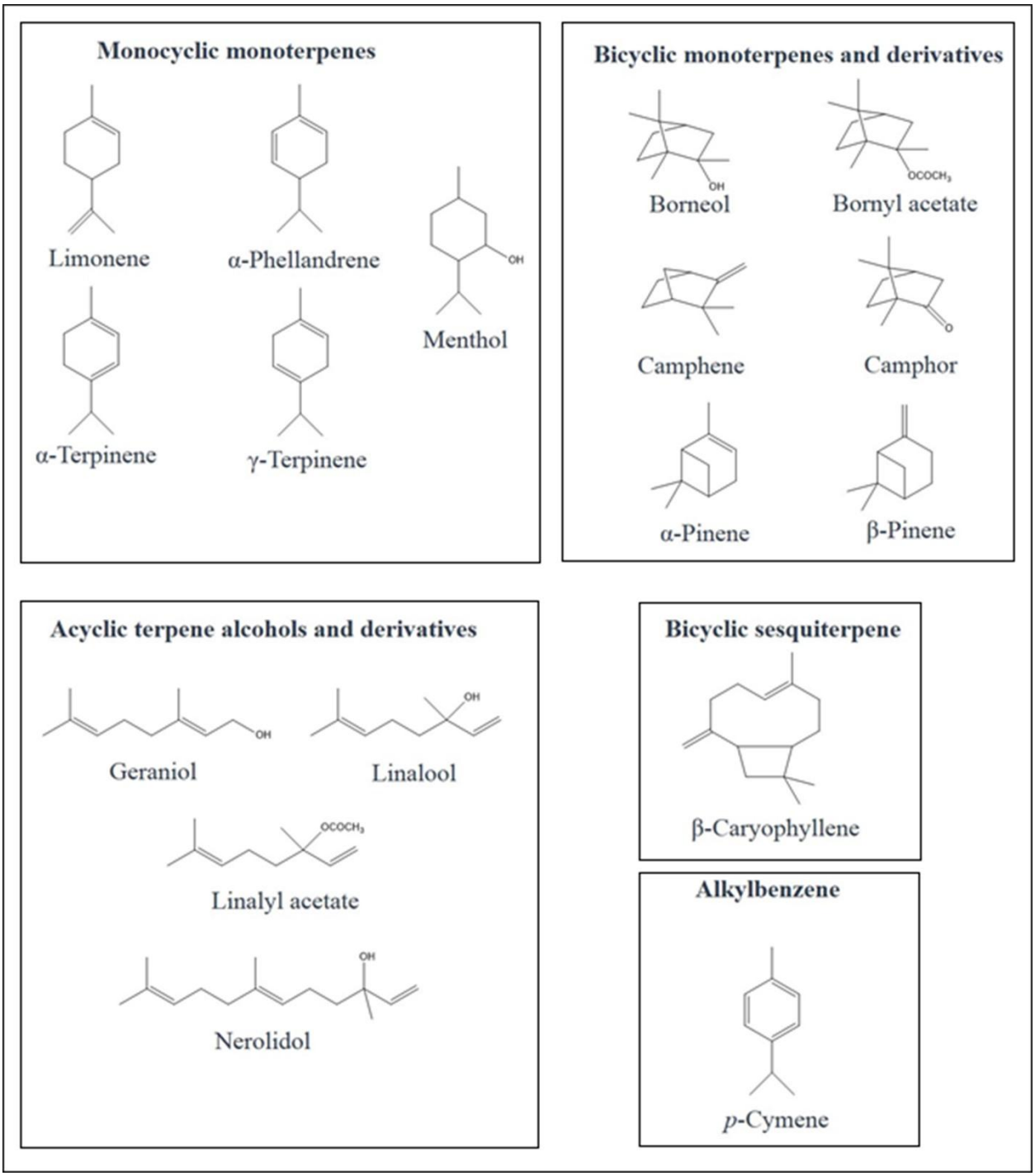
	***trans*-Anethole**	**Anisole**	***p*-Coumaric acid**	***o*-Cresol**	**Estragole**	**Eugenol**	**Eugenyl acetate**	***trans*-Ferulic acid**	**Isoeugenol**	**Quercetin**	**Resveratrol**
LogP	3.31[80] 3.0961[82]	2.11[81]	1.43[82]	1.95[73] 1.975[86]	3.13[80] 2.818[82]	2.40[83] 2.45[72] 2.29[87] 2.73[78]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

ND: Not determined

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]

[a]Values of MIC_{90}



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760 **Fig. 1**

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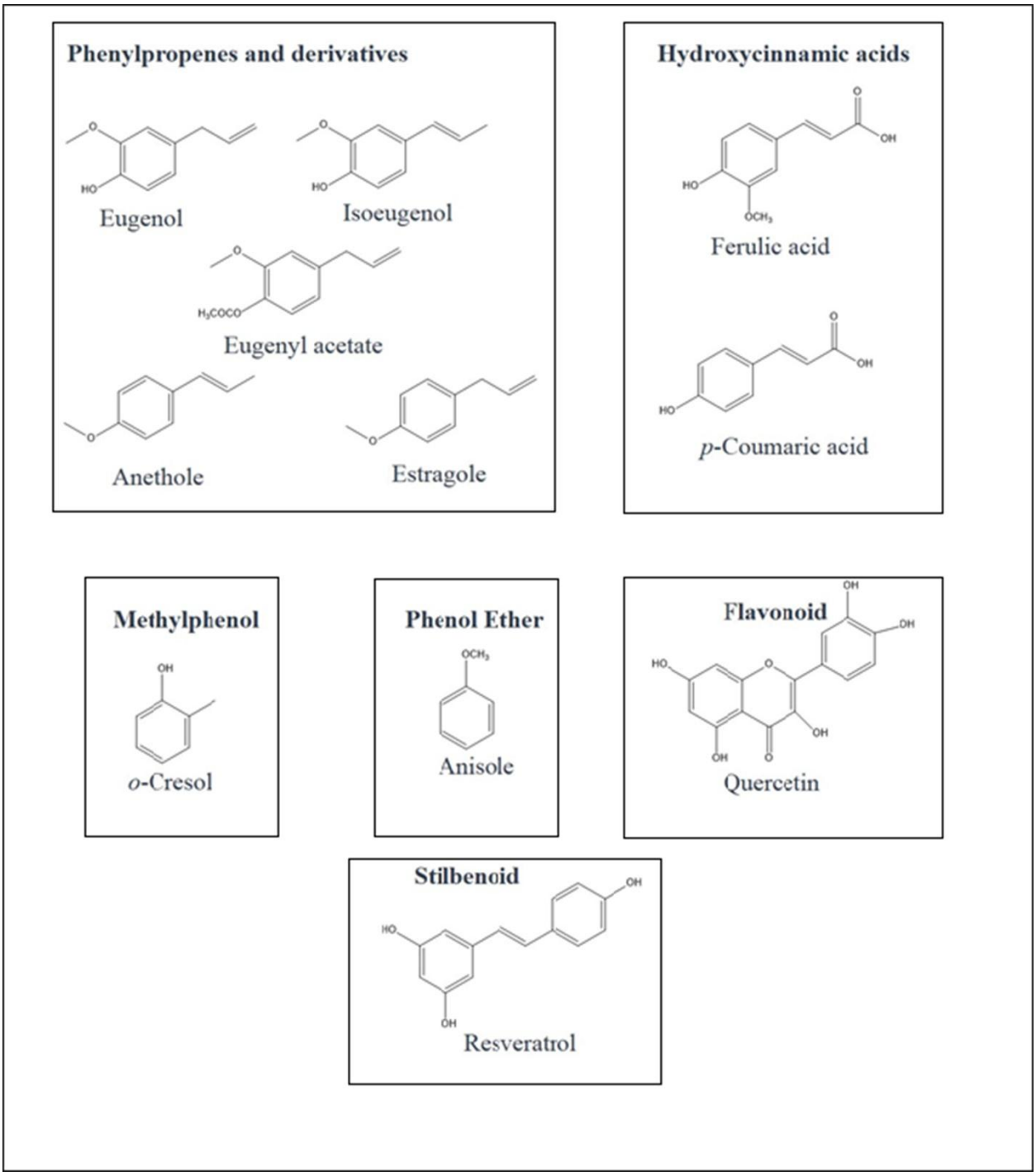

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764 **Fig. 2**

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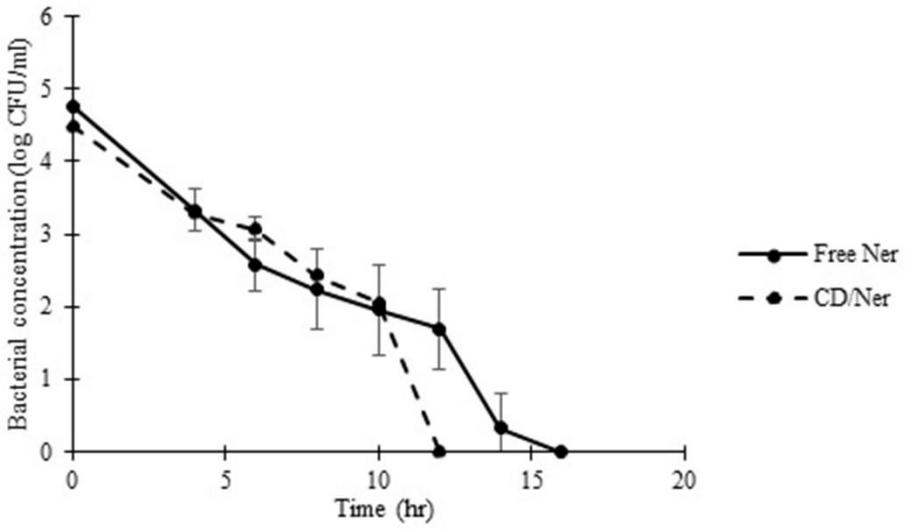

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768 **Fig. 3**

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