

Article

Lipase-Catalyzed Synthesis of Phloretic Acid Esters with Geraniol and Myrtenol: Reaction-Level Sustainability Indicators and Preliminary In Vitro Antimicrobial Evaluation

Bartłomiej Zieniuk ¹, Şuheda Uğur ¹, Magdalena Rudzińska ², Julia Wojciechowska ³
and Eliza Gruczyńska-Sękowska ^{1,*}

¹ Department of Chemistry, Institute of Food Sciences, Warsaw University of Life Sciences-SGGW, 159C Nowoursynowska St., 02-776 Warsaw, Poland; bartlomiej_zieniuk@sggw.edu.pl (B.Z.); suheda_ugur@sggw.edu.pl (Ş.U.)

² Faculty of Food Science and Nutrition, Poznan University of Life Sciences, 28 Wojska Polskiego St., 60-637 Poznan, Poland; magdalena.rudzinska@up.poznan.pl

³ Independent Researcher, 05-500 Warsaw, Poland; juwojciechowska1999@gmail.com

* Correspondence: eliza_gruczynska@sggw.edu.pl

Abstract

The development of antimicrobial compounds using biocatalytic synthesis routes is of interest in sustainability-oriented chemical and food-related research. In this study, phloretic acid was enzymatically esterified with two naturally occurring terpenoid alcohols, geraniol and myrtenol, using *Candida antarctica* lipase B as a biocatalyst under mild conditions. The study combined terpenoid and phenolic molecular building blocks with a biocatalytic synthesis route and evaluated the resulting esters as preliminary in vitro antimicrobial candidates. The resulting esters were isolated in moderate yields, reaching 65.51% for geranyl 3-(4-hydroxyphenyl)propanoate and 54.50% for myrtenyl 3-(4-hydroxyphenyl)propanoate. Their antimicrobial activity, together with that of the parent compounds, was evaluated against a limited panel comprising two Gram-positive bacteria, two Gram-negative bacteria, and two yeast species, by determining the minimum inhibitory concentration (MIC) and minimum microbicidal concentration (MMC). The tested terpenoid alcohols showed notable antifungal activity, whereas esterification was associated with a marked increase in activity against the tested *Staphylococcus aureus* PCM 2054 strain. Both phloretic acid terpenyl esters showed the lowest measured MIC and MMC values against *S. aureus*, at 0.227 and 0.455 mM, respectively. These MIC values represented 16- and 64-fold decreases relative to the corresponding parent terpenoid alcohols and phloretic acid, respectively. Geranyl 3-(4-hydroxyphenyl)propanoate was selected for time-kill analysis because both esters showed the same measured MIC and MMC values against *S. aureus* PCM 2054, whereas the geranyl ester provided a higher isolated yield, more favorable calculated reaction-stage process descriptors, and a slightly lower geometric mean MIC across the tested microbial panel. In the time-kill experiment, G4HPP at 0.25 and 0.50 mM, corresponding to approximately 75.6 and 151.2 mg/L, respectively, produced an estimated 3-log reduction in viable *S. aureus* PCM 2054 counts after approximately 4.7 and 4.3 h. A small increase in viable counts was observed between 8 and 24 h. Supporting in silico analysis indicated that esterification increased predicted lipophilicity and decreased predicted aqueous solubility, accompanying the microorganism-dependent changes observed in the antimicrobial activity profile. Overall, the synthesized esters should be regarded as preliminary in vitro antimicrobial candidates displaying greater in vitro activity against the tested *S. aureus* PCM 2054 strain than the corresponding parent compounds. Broader strain-panel studies and evaluation in food-relevant matrices are required before any potential food-preservation application can be considered.



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1. Introduction

The growing demand for safer and more sustainable food systems has intensified the search for naturally derived or structurally modified antimicrobial compounds that could complement or partially replace conventional synthetic preservatives [1,2]. Such compounds are attracting increasing attention because they may combine antimicrobial activity with consumer interest in clean-label food products [3,4]. The development of new antimicrobial candidates also remains relevant in view of food spoilage, foodborne microorganisms, and the increasing tolerance of microorganisms to commonly applied preservation treatments [5].

Among plant-derived bioactive compounds, phenolic acids and terpenoids are of particular interest for their broad biological activities, including antimicrobial effects [6,7]. Phloretic acid, also known as 3-(4-hydroxyphenyl)propanoic acid, is a naturally occurring phenolic acid that has attracted attention as a molecular building block for functional derivatives with potential applications in food, pharmaceutical, and materials sciences [8,9]. Its antimicrobial activity has already been demonstrated, and previous studies have shown that structural modifications of phloretic acid, especially esterification, can significantly alter, and in some cases improve, its biological properties [10,11]. In particular, lipophilic esters of phloretic acid have been reported to exhibit enhanced activity against selected bacterial strains, indicating that esterification may modify antimicrobial activity and microorganism-dependent susceptibility rather than uniformly improve activity against all microorganisms [11].

Terpenoid alcohols are another important class of naturally occurring compounds with recognized antimicrobial potential. Geraniol, an acyclic monoterpene alcohol, is known for its antibacterial and antifungal activity and has been investigated as a bioactive constituent of essential oils and plant-derived antimicrobial systems [12]. Myrtenol, a bicyclic monoterpene alcohol, has also been reported to inhibit the growth of selected microorganisms, including *Staphylococcus aureus* [13,14]. Both compounds are therefore attractive candidates for structural coupling with phenolic acids. Combining a phenolic acid moiety with a terpenoid alcohol may yield hybrid molecules with altered polarity, lipophilicity, and membrane affinity, which in turn may affect antimicrobial performance [15,16].

The effect of increased lipophilicity may depend strongly on microbial cell-envelope architecture. In Gram-negative bacteria, the lipopolysaccharide-containing outer membrane constitutes an additional permeability barrier that can restrict the access of hydrophobic molecules to the cytoplasmic membrane [17,18]. Gram-positive bacteria lack this outer membrane, which may facilitate interactions between lipophilic compounds and the cytoplasmic membrane after passage through the peptidoglycan layer. Nevertheless, lipophilicity alone does not determine susceptibility, which may also be influenced by peptidoglycan organization, teichoic acids, membrane composition, efflux systems, compound dispersion, and microorganism-specific physiology [19,20].

From a sustainability perspective, it is important that new antimicrobial candidates not only be derived from bio-based building blocks but also be synthesized via routes that may be conducted under comparatively mild reaction conditions and without a strong mineral acid catalyst. In this context, enzymatic esterification is particularly attractive because it can be performed under relatively mild reaction conditions, offers good selectivity, and avoids the use of strong mineral acid catalysts typically associated with classical

esterification [15,21]. However, mild reaction conditions alone do not demonstrate that an entire process is environmentally preferable. The assessment of such reactions should also consider substrate conversion, isolated yield, catalyst loading, solvent consumption, downstream processing, and mass-based reaction indicators. Therefore, the present study evaluates selected reaction-level sustainability indicators without claiming that the complete synthesis and isolation process is inherently more sustainable than alternative routes.

Geranyl 4-hydroxyphenylpropanoate, corresponding chemically to G4HPP investigated in the present study, was previously synthesized in a study focused on crude freeze-dried extracellular lipase preparations from *Yarrowia lipolytica*, with commercial immobilized CALB used as a comparative biocatalyst. Its antibacterial and antioxidant properties were also evaluated [22]. Accordingly, neither the synthesis nor the antimicrobial activity of G4HPP should be regarded as previously unexplored.

The present study was designed to extend this earlier work rather than repeat it. In particular, it introduces the corresponding myrtenyl ester, M4HPP, and evaluates both esters using a complementary microbial panel comprising Gram-positive bacteria, Gram-negative bacteria, and yeasts. The selected panel differed from that used previously, in which G4HPP had already been evaluated against *E. coli* and other bacterial strains [22]. The current work additionally compares the esters with phloretic acid and their corresponding terpenoid alcohols under the same assay conditions, characterizes the time-dependent activity of G4HPP against *S. aureus* PCM 2054, evaluates selected reaction-level sustainability indicators, and examines exploratory relationships between predicted physicochemical descriptors and antimicrobial endpoints.

Accordingly, the aim of the present study was to synthesize phloretic acid esters with geraniol and myrtenol using immobilized CALB, characterize the isolated products via ^1H NMR spectroscopy, evaluate selected reaction-level sustainability indicators, and compare their preliminary in vitro antimicrobial activity with that of the corresponding parent compounds. We hypothesized that esterification with structurally different terpenoid alcohols would modify compound lipophilicity and aqueous behavior, producing microorganism-dependent changes in antimicrobial activity rather than a uniform enhancement across all tested bacteria and yeasts. The study represents an initial in vitro evaluation and was not designed to establish preservative efficacy in food matrices.

2. Materials and Methods

2.1. Chemicals and Reagents

Phloretic acid (3-(4-hydroxyphenyl)propanoic acid, 98%), geraniol (98%), myrtenol (98%), and immobilized lipase B from *Candida antarctica* (CALB) on acrylic resin were purchased from Sigma-Aldrich (St. Louis, MO, USA). According to the supplier, the recombinant lipase (product L4777) was expressed in *Aspergillus niger*, immobilized on acrylic resin, and had a declared activity of ≥ 5000 U/g. Methyl *tert*-butyl ether (MTBE, HPLC grade), isooctane (2,2,4-trimethylpentane, 99.5%), sodium bicarbonate, anhydrous magnesium sulfate and dichloromethane were obtained from Avantor Performance Materials Poland S.A. (Gliwice, Poland). Silica gel 60 for column chromatography was obtained from Merck KGaA (Darmstadt, Germany). All chemicals and solvents were used as received without further purification.

2.2. Microorganisms and Culture Conditions

The antimicrobial activity of the tested compounds was evaluated against six microorganisms, including four bacterial strains and two yeasts. The bacterial strains *Enterobacter cloacae* PCM 2848, *Serratia marcescens* PCM 549, *Bacillus cereus* PCM 482, and *Staphylococcus aureus* PCM 2054 were obtained from the Polish Collection of Microorganisms of the

Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (Wrocław, Poland). The yeast strain *Y. lipolytica* KKP 379 was obtained from the Collection of Industrial Microorganisms of the Institute of Agricultural and Food Biotechnology—State Research Institute (Warsaw, Poland). *Rhodotorula mucilaginosa* was isolated at the Department of Chemistry, Institute of Food Sciences, Warsaw University of Life Sciences—SGGW (Warsaw, Poland). The in-house *R. mucilaginosa* isolate was identified through sequencing of the internal transcribed spacer (ITS) region. The isolate was not assigned a separate laboratory strain designation and was not deposited in a public culture collection.

The microorganisms were stored at $-20\text{ }^{\circ}\text{C}$ until use. Microbial cultures were maintained as glycerol stocks at $-20\text{ }^{\circ}\text{C}$. Before antimicrobial testing, bacterial strains were revived on Mueller–Hinton agar for 24 h at $37\text{ }^{\circ}\text{C}$, whereas yeast strains were revived on potato dextrose agar (PDA) for 48 h at $28\text{ }^{\circ}\text{C}$. Two successive passages were performed under the corresponding microorganism-specific culture conditions before preparation of the test inocula.

The following microbiological media were used in the study: Mueller–Hinton broth and agar, potato dextrose agar (PDA), tryptic soy agar (TSA), and tryptic soy broth (TSB). All microbiological media were purchased from Graso Biotech (Starogard Gdański, Poland) and prepared according to the manufacturer's instructions using distilled water unless otherwise stated. Sabouraud broth was prepared using glucose at 40 g/L and peptone at 10 g/L, and the pH was adjusted to 5.6 before sterilization. Sabouraud agar contained the same concentrations of glucose and peptone supplemented with 15 g/L agar. All media were sterilized by autoclaving at $121\text{ }^{\circ}\text{C}$ for 15 min.

2.3. Lipase-Catalyzed Esterification of Phloretic Acid

Lipase-catalyzed esterification of phloretic acid with geraniol or myrtenol was carried out using the commercial immobilized CALB preparation described in Section 2.1. The corresponding reaction schemes are presented in Figure 1. Phloretic acid (0.005 mol) and the corresponding terpenoid alcohol (0.0075 mol) were introduced into 100 mL Erlenmeyer flasks to obtain an acid-to-alcohol molar ratio of 1:1.5. The commercial immobilized CALB preparation was added at 10% of the total substrate mass, corresponding to approximately 0.199 g for the reaction with geraniol and 0.197 g for the reaction with myrtenol. These values refer to the total mass of the commercial immobilized preparation and not to the mass of pure enzyme. The reaction medium consisted of 30 mL of an MTBE/isooctane mixture (1:1, *v/v*). This solvent composition was selected based on preliminary work as a practical compromise between substrate solubility and the catalytic performance of the immobilized CALB preparation. The solvent ratio was not systematically re-optimized in the present study. The flasks were fitted with glass stoppers, additionally sealed with Parafilm, and incubated in an orbital shaker at $37\text{ }^{\circ}\text{C}$ and 250 rpm for 120 h. After completion of the reaction, the immobilized biocatalyst preparation was removed by filtration, and the solvent was evaporated under reduced pressure. Yield values were obtained from three independently prepared reaction batches, each of which was subjected separately to the same work-up, chromatographic isolation, and yield determination procedure. No enzyme-free reaction control was performed; therefore, uncatalyzed background conversion under the applied conditions was not experimentally evaluated.

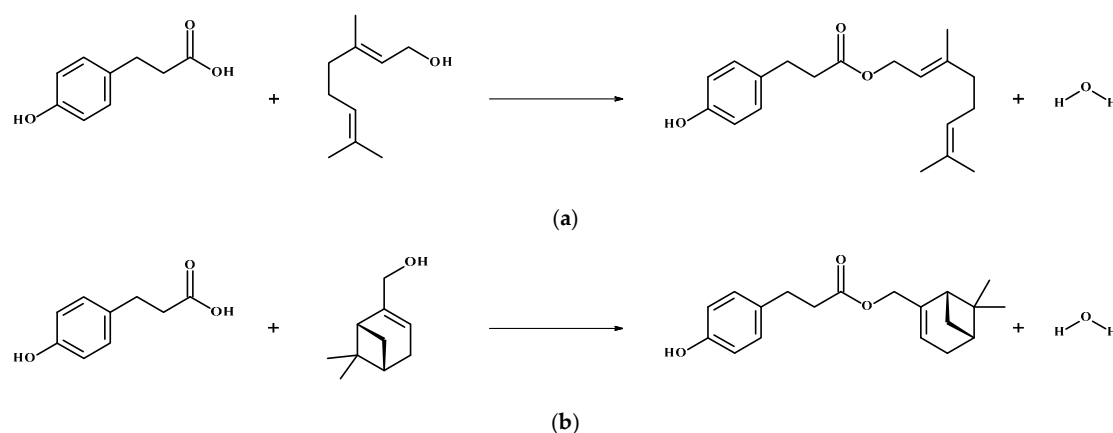


Figure 1. Reaction scheme of the lipase-catalyzed esterification of phloretic acid with selected terpenoid alcohols: (a) geraniol and (b) myrtenol.

2.4. Chromatographic Isolation and ^1H NMR Characterization of the Ester Products

After removal of the reaction solvent, the crude reaction mixture was treated with 10 mL of 8% aqueous sodium bicarbonate and 10 mL of dichloromethane and stirred for 30 min. After phase separation, the aqueous phase was extracted twice more with 10 mL portions of dichloromethane. The combined organic phases were washed with distilled water and dried over approximately 2 g of anhydrous magnesium sulfate for 10 min. The drying agent was removed by filtration, and the organic phase was concentrated under reduced pressure. The resulting crude product was subjected to silica-gel column chromatography using a glass column measuring 400 mm in length and 20 mm in internal diameter, with a nominal capacity of 125 mL. Approximately 35 g of silica gel 60 (0.040–0.063 mm; 230–400 mesh) was used as the stationary phase. Dichloromethane was used as the isocratic eluent. Fractions of 10 mL were collected and monitored by thin-layer chromatography using dichloromethane as the mobile phase. The TLC plates were visualized under UV light at 254 nm. Fractions exhibiting the TLC profile assigned to the target ester were combined and concentrated under reduced pressure to obtain the isolated ester products. G4HPP and M4HPP were obtained as viscous liquids. Based on the mean isolated yields, the corresponding mean isolated product masses were approximately 0.991 g for G4HPP and 0.819 g for M4HPP. The isolated products were weighed and used to determine the yield (Equation (1)):

$$\text{Yield}(\%) = \frac{m_{\text{isolated product}}}{m_{\text{theoretical product}}} \times 100 \quad (1)$$

where $m_{\text{isolated product}}$ is the mass of the isolated ester product, and $m_{\text{theoretical product}}$ is the theoretical mass of the product calculated from the stoichiometry of the reaction.

The isolated ester products were characterized by ^1H NMR spectroscopy. Spectra were recorded on a Bruker AVANCE 300 MHz spectrometer (Bruker, Billerica, MA, USA) using CDCl_3 as the solvent. The obtained spectroscopic data were consistent with the proposed structures. Complete spectra, integrations, enlarged spectral regions, and coupling constants for clearly resolved signals are provided in the Supplementary Materials. Proton chemical shifts in the obtained esters are reported below as δ values (ppm) relative to tetramethylsilane (TMS) as the internal standard.

Geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP):

^1H NMR (300 MHz, CDCl_3) δ 7.06 (d, J = 8.4 Hz, 2H, ArH), 6.74 (d, J = 8.7 Hz, 2H, ArH), 5.31 (m, 1H, CH=), 5.08 (m, 1H, CH=), 4.80 (s, 1H, OH), 4.59 (d, J = 7.2 Hz, 2H,

CH₂OCO), 2.88 (t, J = 7.8 Hz, 2H, ArCH₂), 2.59 (t, J = 7.7 Hz, 2H, CH₂COO), 2.11–2.00 (m, 4H, 2 × allylic CH₂), 1.69 (s, 6H, 2 × CH₃), 1.60 (s, 3H, CH₃).

Myrtenyl 3-(4-hydroxyphenyl)propanoate (M4HPP):

¹H NMR (300 MHz, CDCl₃) δ 7.06 (d, J = 8.4 Hz, 2H, ArH), 6.75 (d, J = 8.7 Hz, 2H, ArH), 5.52 (m, 1H, CH=), 4.72 (br s, 1H, OH), 4.45–4.44 (m, 2H, CH₂OCO), 2.88 (t, J = 7.8 Hz, 2H, ArCH₂), 2.59 (t, J = 7.7 Hz, 2H, CH₂COO), 2.42–2.02 (m, 5H), 1.28 (s, 3H, CH₃), 1.17 (m, 1H), 0.81 (s, 3H, CH₃).

2.5. Green Metrics and Process Descriptors

Atom economy (AE, Equation (2)) was calculated as:

$$AE(\%) = \frac{MW_{\text{product}}}{\sum MW_{\text{reactants}}} \times 100 \quad (2)$$

where MW_{product} is the molecular weight of the ester product and $\sum MW_{\text{reactants}}$ is the sum of molecular weights of the stoichiometric reactants participating in the esterification reaction.

Reaction mass efficiency (RME) was calculated as follows:

$$RME(\%) = \frac{m_{\text{isolated product}}}{\sum m_{\text{reactants}}} \times 100 \quad (3)$$

where m_{isolated product} is the mass of the isolated ester product and $\sum m_{\text{reactants}}$ is the total mass of stoichiometric reactants charged into the reaction.

Catalyst productivity was expressed as micromoles of isolated product obtained per gram of immobilized *C. antarctica* lipase B (CALB) preparation per hour and was calculated according to Equation (4):

$$\text{Productivity}(\mu\text{mol/g immobilized CALB preparation/h}) = \frac{n_{\text{product}}(\mu\text{mol})}{m_{\text{CALB}}(\text{g}) \times t(\text{h})} \quad (4)$$

where n_{product} is the amount of isolated ester expressed in micromoles, m_{CALB} is the mass of immobilized enzyme preparation used in the reaction, and t is the reaction time.

Partial reaction-stage process mass intensity (PMI, Equation (5)) was calculated as follows:

$$PMI = \frac{\sum m_{\text{all input materials}}}{m_{\text{product}}} \quad (5)$$

where $\sum m_{\text{all input materials}}$ is the total mass of materials included in the reaction-stage calculation and m_{product} is the mass of isolated product. In the present study, partial reaction-stage PMI was calculated using the masses of the stoichiometric reactants, the commercial immobilized CALB preparation, and the reaction solvents. The aqueous sodium bicarbonate solution, dichloromethane used during work-up, anhydrous magnesium sulfate, silica gel, and chromatographic eluent were not included. No solvent-recovery credit was applied, and the reaction solvents included in the calculation were conservatively assumed to become waste.

The partial reaction-stage E-factor (Equation (6)) was calculated as:

$$E - \text{factor} = \frac{m_{\text{waste}}}{m_{\text{product}}} \quad (6)$$

where m_{waste} is the difference between the total mass of the materials included in the reaction-stage calculation and the mass of the isolated product. Because the same system boundaries were applied to both metrics, the partial reaction-stage E-factor was numerically equivalent to the partial reaction-stage PMI minus one.

2.6. Antimicrobial Activity Assay

The antimicrobial activity of phloretic acid, geraniol, myrtenol, and their corresponding synthesized esters was evaluated using an adapted broth microdilution procedure in sterile 96-well microplates. Individual stock solutions of each tested compound were prepared in ethanol at a concentration of 640 mM. To prepare the highest test concentration, 10 μ L of the corresponding ethanolic stock solution was mixed with 90 μ L of the appropriate liquid growth medium. An 11-step twofold dilution series was then prepared. Each well initially contained 100 μ L of the corresponding compound dilution. Subsequently, 10 μ L of microbial inoculum was added, resulting in a final well volume of 110 μ L.

Following inoculation, the final compound concentration series was 58.18, 29.09, 14.55, 7.27, 3.64, 1.82, 0.909, 0.455, 0.227, 0.114, and 0.0568 mM. The maximum final ethanol concentration in the wells was 9.09% (*v/v*). The vehicle control contained ethanol at 9.09% (*v/v*), corresponding to the maximum concentration present in the test wells, and showed growth comparable to the untreated growth control. Microbial suspensions were prepared from fresh cultures and adjusted turbidimetrically to a 0.5 McFarland standard. The suspensions were then diluted tenfold in the appropriate growth medium, and 10 μ L of the diluted suspension was added to each test well. The final viable inoculum density was not independently verified by colony counting.

Bacterial strains were tested in Mueller–Hinton broth and incubated at 37 °C for 16–18 h. Yeast strains were tested in Sabouraud broth and incubated at 28 °C for 48 h. MIC was determined visually as the lowest nominal concentration of the tested compound at which no visible microbial growth was observed relative to the corresponding growth control. Growth controls containing inoculated medium without the tested compound, sterility controls containing uninoculated medium, vehicle controls, and compound-only controls containing medium and the tested compound without microbial inoculum were included. No reference antibiotic or antifungal agent was included. Occasional compound-related turbidity, precipitation, or surface-film formation was observed during the assays, although their occurrence was not systematically recorded for individual compound–concentration combinations. Compound-only controls were used to distinguish compound-related optical effects from visible microbial growth during visual endpoint assessment. Because the extent of compound dissolution or dispersion in the assay media was not quantified, the reported concentrations are presented as nominal assay concentrations. All assays were conducted in three independent biological replicates. Identical MIC and operational MMC endpoints were obtained in all three replicates for every compound–microorganism combination. Therefore, the values reported represent both the modal endpoints and the complete observed replicate ranges, with no between-replicate variation.

Following MIC determination, 3 μ L aliquots from wells showing no visible microbial growth were transferred directly onto the corresponding agar medium without prior dilution or chemical neutralization. The plates were incubated under the microorganism-specific conditions described above. The operational minimum microbicidal concentration (MMC) was defined as the lowest nominal compound concentration from which no colonies were recovered. Because samples were plated without prior dilution or neutralization, antimicrobial carry-over onto the agar surface cannot be excluded.

2.7. Time-Kill Assay and Growth Curves

The time-kill assay was conducted with geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP) against *S. aureus* PCM 2054. G4HPP was selected for further kinetic characterization because of its higher isolated yield and a slightly lower geometric mean MIC across the tested microorganism panel. Experiments were conducted in 500 mL flat-bottom round flasks containing 100 mL of tryptic soy broth (TSB). A separate 100 mM stock solution

of G4HPP was prepared in ethanol, corresponding to 30.24 mg/mL. Aliquots of 250 and 500 μ L of this stock solution were added to obtain final G4HPP concentrations of 0.25 and 0.50 mM, corresponding to 75.6 and 151.2 mg/L, respectively. The corresponding final ethanol concentrations were 0.25 and 0.50% (*v/v*). A bacterial suspension prepared from a fresh culture was adjusted turbidimetrically to a 0.5 McFarland standard. One milliliter of the undiluted suspension was added to each experimental culture. The volume of TSB was adjusted so that the final culture volume, including the G4HPP stock solution and bacterial inoculum, was 100 mL. The viable cell count measured immediately after inoculation was approximately 6.32 log CFU/mL. An untreated bacterial culture and an ethanol vehicle control containing 0.50% (*v/v*) ethanol, corresponding to the maximum solvent concentration used in the G4HPP-treated cultures, were incubated under identical conditions. The vehicle control showed growth comparable to that of the untreated control. The MIC of G4HPP was not independently determined in TSB; therefore, the time-kill conditions are reported exclusively as the absolute concentrations of 0.25 and 0.50 mM.

Cultures were incubated aerobically at 37 °C with orbital shaking at 150 rpm. Samples were collected at 0, 2, 4, 8, and 24 h. At each sampling time, decimal serial dilutions were prepared in sterile physiological saline. Aliquots of 1 mL were analyzed using the pour-plate method on tryptic soy agar (TSA). One plate was prepared for each selected dilution from each biological replicate. Plates were incubated at 37 °C for 16–18 h, after which colonies were counted. Viable cell counts were expressed as log CFU/mL.

In parallel, bacterial growth was monitored by measuring optical density at 600 nm. Samples collected at the same time points were transferred to cuvettes and measured spectrophotometrically. TSB containing G4HPP without bacterial inoculum and TSB containing the corresponding amount of ethanol without bacteria served as optical blanks. These controls showed no detectable contribution to OD₆₀₀ under the conditions used. Nevertheless, viable cell counts were considered the primary endpoint, whereas OD₆₀₀ served as a complementary indicator of culture turbidity. All experiments were performed in three independent biological replicates, and the results are presented as mean $\pm$ SD.

2.8. In Silico Physicochemical Analysis

Physicochemical descriptors of phloretic acid, geraniol, myrtenol, and their corresponding esters were predicted using the SwissADME web tool [23]. The descriptors analyzed included molecular weight (MW), number of rotatable bonds, topological polar surface area (TPSA), Consensus Log P, ESOL Log S, and ESOL solubility class. These parameters were used to support the interpretation of the observed antimicrobial activity and to assess changes in lipophilicity, polarity, and predicted aqueous solubility associated with esterification.

2.9. Calculations and Descriptive Analysis

The operational MMC/MIC ratio (Equation (7)) was calculated for each compound–microorganism pair as follows:

$$\text{MMC/MIC ratio} = \frac{\text{MMC}}{\text{MIC}} \quad (7)$$

where MIC is the nominal minimum inhibitory concentration and MMC is the operational minimum microbicidal concentration determined under the applied spot-plating conditions. The operational MMC/MIC ratios were calculated solely as descriptive numerical parameters. Because MMC was determined by direct spot plating without prior dilution or chemical neutralization, potential antimicrobial carry-over cannot be excluded. Therefore, the calculated ratios were not used to assign definitive microbicidal or microbiostatic classi-

fications. When the operational MMC exceeded the highest final concentration evaluated, namely 58.18 mM, the corresponding MMC/MIC ratio was expressed as a lower-bound inequality rather than as an exact numerical value.

To summarize the antimicrobial activity of each compound across all tested microorganisms, the geometric mean MIC (Equation (8)) was calculated as follows:

$$\text{Geometric mean MIC} = \left(\prod_{i=1}^n \text{MIC}_i \right)^{1/n} \quad (8)$$

where n is the number of tested microorganisms and MIC_i is the MIC value obtained for the i -th microorganism.

The effect of esterification on antimicrobial activity was expressed as fold change in MIC and calculated according to Equation (9):

$$\text{Fold change} = \frac{\text{MIC}_{\text{parent compound}}}{\text{MIC}_{\text{corresponding ester}}} \quad (9)$$

In this study, fold change was calculated relative to the parent terpenoid alcohol and, separately, relative to phloretic acid. Values above 1 indicated improved activity after esterification, values equal to 1 indicated no change, and values below 1 indicated reduced activity.

Antimicrobial activity against *S. aureus* was additionally expressed as pMIC (Equation (10)), calculated as the negative decimal logarithm of MIC values expressed in molar concentration (mol/L):

$$\text{pMIC} = -\log_{10}(\text{MIC}) \quad (10)$$

For descriptive summarization of the mean time-kill trajectories, the reduction in viable cell counts was expressed relative to the initial inoculum according to Equation (11):

$$R_t = \log\text{CFU}_0 - \log\text{CFU}_t \quad (11)$$

where R_t is the log reduction at time t , $\log\text{CFU}_0$ is the mean initial viable count, and $\log\text{CFU}_t$ is the mean viable count at time t .

The time required to reach a 3-log reduction ($t_{3\log}$) was estimated by linear interpolation between two adjacent time points surrounding the 3-log reduction threshold according to Equation (12):

$$t_{3\log} = t_1 + \frac{3 - R_1}{R_2 - R_1} (t_2 - t_1) \quad (12)$$

where t_1 and t_2 are consecutive sampling times, and R_1 and R_2 are the corresponding log reduction values. Because $t_{3\log}$ was estimated from the mean viable-count trajectory rather than calculated separately for each biological replicate, it was treated solely as a descriptive estimate and was reported to one decimal place.

The overall viable-count trajectory was summarized using the area under the time-kill curve (AUC), calculated by the trapezoidal rule according to Equation (13):

$$\text{AUC} = \sum_{i=1}^{n-1} \frac{(y_i + y_{i+1})}{2} (t_{i+1} - t_i) \quad (13)$$

where y_i is the mean viable count expressed as $\log \text{CFU/mL}$ at time t . AUC values are reported as $(\log_{10}\text{CFU/mL}) \cdot \text{h}$.

The percentage reduction in the area under the time-kill curve relative to the untreated control was calculated according to Equation (14):

$$\text{AUC reduction vs control(\%)} = \frac{\text{AUC}_{\text{control}} - \text{AUC}_{\text{treated}}}{\text{AUC}_{\text{control}}} \times 100 \quad (14)$$

where $\text{AUC}_{\text{control}}$ is the AUC calculated from the mean viable-count trajectory of the untreated control and $\text{AUC}_{\text{treated}}$ is the AUC calculated from the corresponding mean trajectory of the G4HPP-treated culture.

Log reductions, $t_{3 \log}$, AUC values, and percentage AUC reductions were calculated from the mean viable-count data obtained in three independent biological experiments. These parameters were used as descriptive summaries of the mean kinetic trajectories and were not subjected to inferential statistical comparison between the tested concentrations. A formal detection limit was not prospectively established; therefore, no censoring or substitution based on an assumed detection threshold was applied.

2.10. Statistical Analysis

All quantitative data are presented as mean $\pm$ standard deviation (SD), where applicable. Differences in isolated yields among the synthesized esters were evaluated using one-way analysis of variance (ANOVA) with Tukey's post hoc test. Differences were considered statistically significant at $p < 0.05$. Statistical analyses were performed using Statistica 13.3 (TIBCO Software Inc., Palo Alto, CA, USA).

Spearman's correlation coefficients were calculated solely to visualize rank-order patterns between selected predicted physicochemical descriptors and antimicrobial endpoints within the investigated dataset. The analysis included only five structurally related compounds that did not constitute an independent or systematically varied chemical series, and several antimicrobial endpoints contained tied values. No correction for multiple comparisons was applied. Therefore, the coefficients were treated exclusively as descriptive, dataset-specific values and were not used to infer general associations, causal relationships, predictive performance, quantitative structure–activity relationships, or antimicrobial mechanisms. The graphical presentation of the Spearman correlation matrix was prepared using the online platform ChiPlot [24].

3. Results and Discussion

In recent years, considerable attention has been devoted to hydroxycinnamic acids, the synthesis of their esters and other derivatives, and the evaluation of their biological activity [25–27]. In contrast, other phenolic acids, such as phloretic acid, have attracted much less interest, even though their chemical structure suggests potential antioxidant and antimicrobial properties. In the present study, phloretic acid esters with selected terpenoid alcohols were synthesized via lipase-catalyzed esterification. The selection of geraniol and myrtenol as substrates was based on their natural origin and suitability for biocatalytic esterification, because primary alcohols are generally more favorable substrates for lipase-catalyzed esterification than secondary or tertiary alcohols [28].

As shown in Figure 2, both esters were obtained in moderate isolated yields. Geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP) was obtained in a significantly higher yield than myrtenyl 3-(4-hydroxyphenyl)propanoate (M4HPP), at $65.51 \pm 1.85\%$ and $54.50 \pm 2.39\%$, respectively. Under the applied reaction and isolation conditions, geraniol afforded a higher mean isolated ester yield than myrtenol. The higher yield of G4HPP may be related to the more flexible acyclic structure of geraniol, which could facilitate enzyme–substrate interactions compared with the more rigid bicyclic structure of myrtenol. This interpretation

agrees with reports that CALB-catalyzed transformations are affected by steric factors and that bulky substituents can limit efficient binding in the lipase stereospecificity pocket [28].

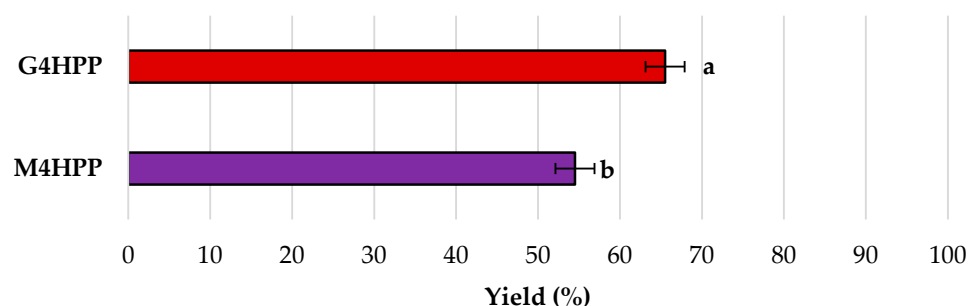


Figure 2. Isolated yields of geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP) and myrtenyl 3-(4-hydroxyphenyl)propanoate (M4HPP) obtained from three independently prepared esterification batches, each subjected separately to the same work-up and chromatographic isolation procedure. Data are presented as mean $\pm$ SD ($n = 3$). Different lowercase letters (a, b) indicate statistically significant differences between means at $p < 0.05$.

Crystallographic studies of CALB have revealed a relatively restricted active-site entrance and a stereospecific alcohol-binding pocket, indicating that substrate geometry and productive positioning are key determinants of catalytic performance [29,30]. Structural studies have also demonstrated conformational mobility in the helices surrounding the active-site entrance, which may influence substrate access to the catalytic pocket [31]. The flexible acyclic structure of geraniol may allow it to sample a broader range of conformations and facilitate productive positioning of its hydroxyl group near the catalytic center. In contrast, the rigid bicyclic framework of myrtenol may limit the number of accessible productive orientations and impose greater steric constraints during binding. However, no docking, molecular dynamics simulations, or enzyme–substrate structural analyses were performed for these alcohols in the present study. This explanation should therefore be regarded as a plausible structural interpretation rather than a demonstrated binding mechanism. Moreover, because the reported values represent isolated yields, differences in product recovery and chromatographic isolation may also have contributed to the observed results.

A preliminary evaluation of the synthesis using green metrics is presented in Table 1. In both cases, the calculated atom economy exceeded 94%, reflecting the favorable stoichiometry of the esterification reaction. However, the remaining metrics revealed differences between the two esters. G4HPP showed a higher reaction mass efficiency (49.75%) than M4HPP (41.52%), consistent with its higher isolated yield. Similarly, catalyst productivity was higher for G4HPP (136.40 $\mu\text{mol/g}$ immobilized CALB preparation/h) than for M4HPP (113.46 $\mu\text{mol/g}$ immobilized CALB preparation/h), indicating more efficient use of the biocatalyst in the synthesis of the geranyl ester.

The calculated partial reaction-stage mass metrics were lower for G4HPP than for M4HPP. The partial reaction-stage PMI values were 23.93 and 28.93, and the corresponding partial E-factor values were 22.93 and 27.94, respectively. These differences primarily reflected the higher isolated yield of G4HPP under otherwise comparable reaction conditions. These values should not be interpreted as a complete sustainability assessment because they exclude the aqueous work-up, extraction, drying, and chromatographic isolation stages. They also do not account for solvent hazard, energy consumption, catalyst reuse, or potential solvent recovery. At the same time, the obtained PMI and E-factor values indicate that, despite the advantages of the enzymatic route, the current protocol still involves substantial material input, mainly due to the use of organic solvents. From a sustainability perspective, the relevance of the present approach should be assessed primarily at

the reaction-concept level. Green chemistry principles provide an important conceptual framework, but several authors have emphasized that the greenness of a process should be supported by quantitative metrics rather than inferred solely from the use of a catalyst or bio-based substrates. In particular, mass-based indicators such as PMI and E-factor are widely used to evaluate process material intensity and waste generation and to guide future optimization [32,33].

Table 1. Reaction-level sustainability indicators and process descriptors for the CALB-catalyzed synthesis of G4HPP and M4HPP, including isolated yield, atom economy, reaction mass efficiency, catalyst productivity, partial reaction-stage process mass intensity, and partial reaction-stage E-factor.

Compound	Yield (%)	Atom Economy (AE, %)	Reaction Mass Efficiency (RME, %)	Productivity ($\mu\text{mol/g}$ Immobilized CALB Preparation/h)	Partial Reaction-Stage PMI	Partial Reaction-Stage E-Factor
G4HPP	65.51	94.38	49.75	136.40	23.93	22.93
M4HPP	54.50	94.34	41.52	113.46	28.93	27.94

Partial reaction-stage PMI and E-factor include the stoichiometric reactants, commercial immobilized CALB preparation, and reaction solvents. Aqueous work-up materials, extraction solvent, drying agent, silica gel, and chromatographic eluent were excluded. No solvent-recovery credit was applied.

In this context, the lipase-catalyzed synthesis described here remains relevant to sustainability because it combines bio-based substrates with a biocatalytic route performed under mild reaction conditions. Similar sustainability-oriented studies on enzymatic esterification have shown that biocatalytic processes can be framed as biocatalytic alternatives that may offer operational advantages under appropriately optimized process conditions, especially when they reduce catalyst-related hazards, operate under milder conditions, and limit by-product formation [34,35]. At the same time, the literature shows that the actual green profile of such processes depends strongly on the complete process design, including solvent use, work-up, and purification strategy [34].

In contrast to recent examples of biocatalytic ester synthesis performed in solvent-free media or in recyclable reaction systems designed to reduce downstream waste, the present procedure still relies on organic solvents and chromatographic purification. Therefore, while the reaction itself is conceptually aligned with green catalysis, additional improvements in solvent selection, solvent loading, and downstream processing would be necessary to strengthen the method's overall sustainability profile.

The antimicrobial activity data in Table 2 indicate that esterifying phloretic acid with terpenoid alcohols did not uniformly increase activity across all tested microorganisms but instead produced a marked shift in the activity profile. Phloretic acid itself showed only moderate activity, with MIC values ranging from 3.64 to 14.55 mM, whereas the parent terpenoid alcohols displayed distinct activity patterns. Geraniol was the most active against the tested yeasts, with MIC values of 0.227 mM against *Y. lipolytica* and 0.455 mM against *R. mucilaginosa*, while myrtenol showed a broader but overall weaker activity profile. In contrast, esterification improved antibacterial activity against *S. aureus* under the tested conditions. Both geranyl 3-(4-hydroxyphenyl)propanoate and myrtenyl 3-(4-hydroxyphenyl)propanoate exhibited identical MIC and MMC values of 0.227 and 0.455 mM, respectively, representing the highest anti-*S. aureus* activity observed in the tested group.

Table 2. Minimum inhibitory concentration (MIC) and operational minimum microbicidal concentration (MMC, in parentheses) values of phloretic acid, terpenoid alcohols, and their enzymatically synthesized esters against the selected microorganisms.

Compound	<i>E. cloacae</i> PCM 2848	<i>S. marcescens</i> PCM 549	<i>B. cereus</i> PCM 482	<i>S. aureus</i> PCM 2054	<i>Y. lipolytica</i> KKP 379	<i>R. mucilaginosa</i>
Phloretic acid	7.27 (14.55)	3.64 (7.27)	7.27 (>14.55)	14.55 (29.09)	7.27 (14.55)	14.55 (29.09)
Geraniol	3.64 (3.64)	3.64 (3.64)	3.64 (>14.55)	3.64 (3.64)	0.227 (0.909)	0.455 (0.909)
Geranyl 3-(4-hydroxyphenyl)propanoate	7.27 (14.55)	3.64 (14.55)	3.64 (>14.55)	0.227 (0.455)	1.82 (1.82)	0.909 (0.909)
Myrtenol	3.64 (7.27)	2 (7.27)	3.64 (>14.55)	3.64 (14.55)	3.64 (7.27)	3.64 (7.27)
Myrtenyl 3-(4-hydroxyphenyl)propanoate	7.27 (14.55)	7.27 (14.55)	3.64 (>14.55)	0.227 (0.455)	3.64 (3.64)	3.64 (7.27)

Abbreviations: MIC, minimum inhibitory concentration; MMC, minimum microbicidal concentration. Values are expressed in mM. For each compound–microorganism combination, identical MIC and operational MMC endpoints were obtained in all three independent biological replicates. MMC values were determined by direct transfer of 3 μ L aliquots from growth-negative wells onto agar without prior dilution or chemical neutralization. Potential antimicrobial carry-over cannot be excluded; therefore, MMC values should be interpreted as operational estimates.

The antimicrobial results should also be considered in light of the limited aqueous solubility predicted for the synthesized esters. Although ethanol vehicle controls and compound-only controls were included, complete dissolution of the compounds in the assay media was not experimentally demonstrated. Occasional turbidity, precipitation, or surface-film formation was observed during the assays, but these effects were not systematically documented for individual compound–concentration combinations. Therefore, the reported MIC and MMC values refer to nominal concentrations, and the freely dissolved concentrations may have been lower than the amounts added to the wells. Limited and variable compound dispersion may also have affected the visual assessment of microbial growth.

At the same time, the esters did not outperform the parent compounds against all microorganisms. For *B. cereus*, no improvement was observed after esterification, as all terpenoid-derived compounds showed the same MIC of 3.64 mM and MMC values above 58.18 mM. Likewise, the antifungal activity of geraniol decreased after esterification, particularly against *Y. lipolytica*, where the MIC increased from 0.227 mM for geraniol to 1.82 mM for its phloretic acid ester. These findings indicate that esterification did not generally enhance antimicrobial potency but produced a microorganism-dependent change in the activity profile, with the most pronounced improvement observed against the tested *S. aureus* PCM 2054 strain.

Esterification produced the largest decrease in nominal MIC against *S. aureus* PCM 2054, whereas no comparable improvement was observed against *B. cereus* PCM 482. This difference indicates that the observed response cannot be explained solely by the Gram-positive character of the tested microorganisms or by the absence of a lipopolysaccharide-containing outer membrane. In *S. aureus*, wall teichoic acids contribute to cell-surface physicochemical properties and can influence interactions with and susceptibility to lipophilic antimicrobial compounds [19,36]. By contrast, members of the *B. cereus* group possess structurally diverse secondary cell-wall polysaccharides, and some strains additionally form S-layers whose protein composition can modify surface charge, hydrophilicity, and interactions with the surrounding environment [37,38]. These differences may influence the adsorption and availability of lipophilic compounds at the bacterial surface, but they do not provide a direct mechanistic explanation for the susceptibility pattern observed in the present study.

The increased predicted lipophilicity of G4HPP and M4HPP may promote association with the cytoplasmic membrane once the compounds reach it. However, increased lipophilicity was also accompanied by lower predicted aqueous solubility, which may

reduce compound dispersion and the freely available concentration in the assay medium. Lipophilicity alone therefore cannot account for the different responses of *S. aureus* and *B. cereus*. The present findings should be interpreted as a microorganism-dependent activity pattern rather than as evidence of general selectivity toward Gram-positive bacteria.

The mechanism underlying the greater susceptibility of the tested *S. aureus* strain was not examined experimentally. Confirmation would require studies of cellular uptake, membrane permeabilization, membrane potential, leakage of intracellular components, and additional strains of both species.

The operational MMC/MIC ratios summarized in Table 3 ranged from 1 to 4 for most compound–microorganism combinations. For *B. cereus* PCM 482, the operational MMC exceeded the highest concentration evaluated for all tested compounds, and the corresponding ratios were expressed as lower-bound inequalities. Because the MMC procedure involved direct spot plating without prior dilution or neutralization, these ratios are presented only as descriptive numerical parameters and do not establish whether the observed effects were definitively microbicidal or microbiostatic.

Table 3. Operational MMC/MIC ratios, MIC ranges, and geometric mean MIC values of the tested compounds against selected microorganisms.

Compound	<i>E. cloacae</i> PCM 2848	<i>S. marcescens</i> PCM 549	<i>B. cereus</i> PCM 482	<i>S. aureus</i> PCM 2054	<i>Y. lipolytica</i> KKP 379	<i>R. mucilaginosa</i>	MIC Range (mM)	Geometric Mean MIC (mM)
Phloretic acid	2	2	>8	2	2	2	3.64–14.55	8.16
Geraniol	1	1	>16	1	4	2	0.227–3.64	1.62
Geranyl 3-(4-hydroxyphenyl)propanoate	2	4	>16	2	1	1	0.227–7.27	1.82
Myrtenol	2	4	>16	4	2	2	1.82–3.64	3.24
Myrtenyl 3-(4-hydroxyphenyl)propanoate	2	2	>16	2	1	2	0.227–7.27	2.89

Abbreviations: MIC, minimum inhibitory concentration; MMC, minimum microbicidal concentration. Operational MMC/MIC ratios were calculated using the final nominal assay concentrations. Ratios based on operational MMC values exceeding the highest tested concentration of 58.18 mM are presented as lower-bound inequalities. Because MMC was determined by direct transfer of 3 μ L aliquots onto agar without prior dilution or chemical neutralization, potential antimicrobial carry-over cannot be excluded. Therefore, the ratios are presented solely as descriptive numerical parameters and were not used to assign definitive microbicidal or microbiostatic classifications.

A contrasting activity pattern was observed against *Y. lipolytica*, for which esterification was associated with higher nominal MIC values than those of the corresponding parent compounds. This observation further indicates that increased lipophilicity does not uniformly enhance antimicrobial activity. The yeast cell wall is a multilayered, cross-linked structure composed predominantly of glucans, mannoproteins, and chitin, which surrounds the plasma membrane [39]. In *Y. lipolytica*, glucan-linked and glycosylated cell-wall proteins additionally contribute to the organization and surface properties of the cell envelope [40].

Increased lipophilicity may favor partitioning into the plasma membrane once a compound reaches it. However, esterification also increased molecular size and predicted lipophilicity while decreasing polarity and predicted aqueous solubility. These changes may have reduced the dispersion and freely dissolved concentrations of G4HPP and M4HPP in the aqueous assay medium, thereby limiting their availability at the yeast plasma membrane. Studies of homologous phenolic esters have similarly shown that antifungal activity does not increase continuously with lipophilicity and may exhibit a microorganism-dependent optimum [41].

Nevertheless, compound uptake, binding to the yeast cell wall, membrane accumulation, and membrane damage were not evaluated in the present study. Therefore, reduced

aqueous availability and restricted access to the plasma membrane should be regarded as possible explanations rather than demonstrated mechanisms.

However, the most informative parameter was the geometric mean MIC, which highlighted differences in overall activity profiles. Geraniol showed the lowest geometric mean MIC (1.62 mM), followed by geranyl ester (1.82 mM), whereas phloretic acid displayed the weakest overall activity (8.16 mM). This confirms that although geraniol retained the strongest broad-spectrum activity, particularly owing to its pronounced antifungal effect, esterification yielded derivatives with more selective antibacterial activity.

The MIC ranges in Table 3 also reflect this shift in selectivity. Geraniol showed the broadest activity range at lower concentrations (0.227–3.64 mM). In contrast, both esters ranged from 0.227 to 7.27 mM, indicating that they retained high activity against selected strains but did not universally improve. The combined analysis of Tables 2 and 3 shows that phloretic acid esterification with terpenoid alcohols is best interpreted not as a strategy for broadly enhancing antimicrobial activity, but as a means of modifying microorganism-dependent activity profiles. Within the microbial panel evaluated, the most pronounced increase was observed against *S. aureus* PCM 2054. Among the two esters, the geranyl derivative was especially attractive as a lead compound because it combined the same high anti-*S. aureus* potency as the myrtenyl ester with a lower geometric mean MIC and stronger activity against the tested yeasts.

Such a pattern aligns with earlier reports indicating that esterification of phenolic acids may enhance antimicrobial activity by increasing lipophilicity and, consequently, strengthening interactions with microbial cell envelopes. In a previous study on geranyl 4-hydroxyphenylpropanoate, esterification of 4-hydroxyphenylpropanoic acid with geraniol also increased antibacterial activity relative to the precursor acid [22]. In this context, the present results suggest that lipophilization of phloretic acid was accompanied by greater activity against the tested *S. aureus* PCM 2054 strain, which might be associated with altered interactions between the more hydrophobic ester derivatives and the bacterial cell envelope [42,43]. However, the absence of a comparable improvement against *B. cereus* PCM 482 indicates that the observed effect cannot be generalized to Gram-positive bacteria and cannot be explained by Gram classification alone.

As shown in Table 4, the effect of esterification varied strongly with the reference compound and the tested microorganism. Relative to the parent terpenoid alcohols, both esters produced the most pronounced improvement against *S. aureus*, with a 16-fold decrease in MIC, whereas no improvement was observed against *B. cereus*, and activity was reduced in some other cases, particularly for the geranyl ester against yeasts. In contrast, relative to phloretic acid, both esters showed a 64-fold improvement against *S. aureus* and a 2-fold improvement against *B. cereus*. The geranyl ester also showed greater gains against the tested yeasts than the myrtenyl ester. These results indicate that esterification did not produce a universal increase in antimicrobial potency. Instead, the largest decrease in MIC within the tested microbial panel was observed against *S. aureus* PCM 2054.

The selected physicochemical descriptors predicted using SwissADME (Table 5) showed that esterification of phloretic acid with terpenoid alcohols markedly altered the molecular profiles of the resulting derivatives. Compared with the parent compounds, both esters had substantially higher molecular weights and more rotatable bonds, reflecting increased structural complexity. At the same time, esterification increased lipophilicity, as indicated by higher Log P values, and decreased predicted aqueous solubility, as reflected by lower Log S values.

The predicted decrease in aqueous solubility provides possible physicochemical context for the antimicrobial results but cannot be directly related to the nominal MIC values because the dissolved compound concentrations were not experimentally determined.

Table 4. Effect of esterification on antimicrobial activity expressed as fold change in MIC relative to the parent terpenoid or phloretic acid.

Ester	Comparator	<i>E. cloacae</i> PCM 2848	<i>S. marcescens</i> PCM 549	<i>B. cereus</i> PCM 482	<i>S. aureus</i> PCM 2054	<i>Y. lipolytica</i> KKP 379	<i>R. mucilaginosa</i>
Geranyl 3-(4-hydroxyphenyl)propanoate	vs geraniol	0.50	1.00	1.00	16.00	0.13	0.50
Geranyl 3-(4-hydroxyphenyl)propanoate	vs phloretic acid	1.00	1.00	2.00	64.00	4.00	16.00
Myrtenyl 3-(4-hydroxyphenyl)propanoate	vs myrtenol	0.50	0.25	1.00	16.00	1.00	1.00
Myrtenyl 3-(4-hydroxyphenyl)propanoate	vs phloretic acid	1.00	0.50	2.00	64.00	2.00	4.00

Note: Fold change was calculated as MIC of the comparator compound divided by MIC of the corresponding ester. Values > 1 indicate improved activity after esterification, values = 1 indicate no change, and values < 1 indicate reduced activity.

Table 5. Selected physicochemical descriptors of the tested compounds predicted using SwissADME [23].

Compound	MW (g/mol)	Rotatable Bonds	TPSA (Å ²)	Consensus Log P	ESOL Log S	ESOL Class
Phloretic acid	166.17	3	57.53	1.31	−1.77	Very soluble
Geraniol	154.25	4	20.23	2.74	−2.78	Soluble
Geranyl 3-(4-hydroxyphenyl)propanoate	302.41	9	46.53	4.34	−4.48	Moderately soluble
Myrtenol	152.23	1	20.23	2.40	−2.75	Soluble
Myrtenyl 3-(4-hydroxyphenyl)propanoate	300.39	6	46.53	3.96	−4.87	Moderately soluble

Abbreviations: MW, molecular weight; TPSA, topological polar surface area; Log P, octanol/water partition coefficient; Log S, predicted aqueous solubility; ESOL, estimated solubility.

These changes align with the biological data and suggest that esterification did not merely produce larger molecules but shifted the balance between polarity and hydrophobicity in a way that may influence microbial interactions. In particular, the two esters showed the lowest measured MIC values against *S. aureus* PCM 2054 among the tested compounds and also displayed higher predicted Consensus Log P values than phloretic acid and the parent alcohols. Thus, esterification clearly shifted the compounds toward a more hydrophobic physicochemical profile.

Geraniol retained the lowest geometric mean MIC value, which was largely driven by its pronounced antifungal activity, even though its predicted lipophilicity was lower than that of the esters. This indicates that an increase in Log P alone did not guarantee stronger activity across all tested microorganisms. Instead, the physicochemical changes introduced by esterification appear to have favored a more selective antibacterial profile, particularly against *S. aureus*, rather than a general enhancement of antimicrobial potency.

An exploratory Spearman analysis was used to visualize rank-order patterns between selected predicted physicochemical descriptors and antimicrobial endpoints within the investigated five-compound dataset (Figure 3). The largest absolute coefficients involved Consensus Log P, pMIC against *S. aureus* PCM 2054, and the geometric mean MIC for bacteria. Consensus Log P showed a coefficient of $\rho = 0.949$ with pMIC against *S. aureus* PCM 2054, and a coefficient of $\rho = -0.900$ with the geometric mean MIC for bacteria. These values describe only the ordering of the five compounds included in the present dataset. Because the compound set was very small, structurally related, and not systematically varied, and because several antimicrobial endpoints contained tied values, the coefficients do not establish a general physicochemical–activity relationship, predictive model, causal association, or antimicrobial mechanism.

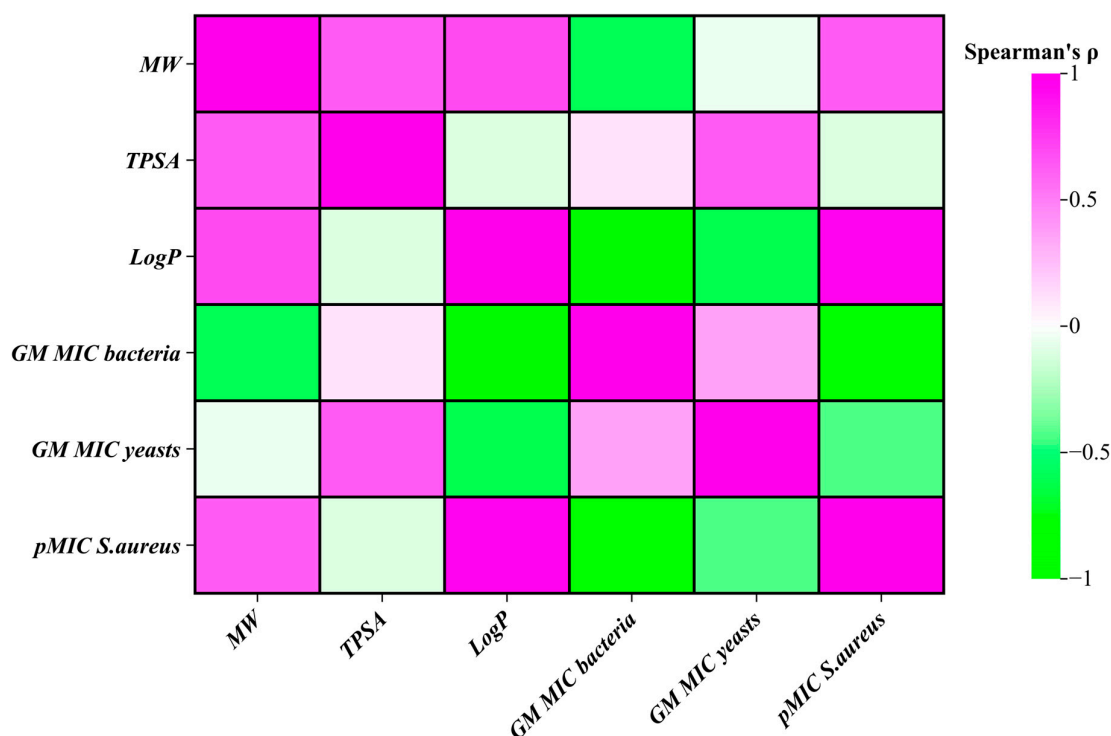


Figure 3. Spearman rank-correlation matrix for selected predicted physicochemical descriptors and antimicrobial endpoints calculated for five structurally related compounds. GM MIC bacteria and GM MIC yeasts represent the geometric mean nominal MIC values for the tested bacterial and yeast strains, respectively. The coefficients are presented solely as descriptive rank-order patterns within this specific five-compound dataset. Owing to the small sample size, tied antimicrobial endpoints, structural relatedness of the compounds, and absence of correction for multiple comparisons, the coefficients should not be interpreted as confirmatory evidence, a predictive relationship, a quantitative structure–activity model, or an antimicrobial mechanism. Abbreviations: MW, molecular weight; TPSA, topological polar surface area; LogP, octanol/water partition coefficient; GM MIC, geometric mean minimum inhibitory concentration; pMIC, negative logarithm of the minimum inhibitory concentration expressed in molar units.

Within this five-compound dataset, the coefficient between Consensus Log P and the geometric mean MIC for yeasts was numerically smaller in absolute magnitude ($\rho = -0.616$) than the coefficients involving the bacterial endpoints. However, these numerical differences should not be interpreted as evidence that lipophilicity is more closely related to antibacterial than antifungal activity, because the analysis lacks sufficient sample size and statistical power for such comparisons.

Lipophilization of phenolic compounds has been recognized as a simple and effective strategy for modifying their physicochemical and biological properties, particularly by improving their performance in hydrophobic systems and altering their antimicrobial behavior [15]. As highlighted in the review by Arzola-Rodríguez et al. [44], phenolipids are amphiphilic derivatives in which the phenolic core retains the beneficial biological properties of the parent compound, while the lipophilic moiety enables stronger interactions with nonpolar environments, including cellular membranes. This interpretation is supported by the experimental study by Ham et al. [45], which showed that lipophilizing *p*-coumaric and ferulic acids significantly enhanced antimicrobial activity, with MIC values for the corresponding esters decreasing from 64–98 mM for the parent acids to 0.9–12 mM for selected phenolipids. The authors concluded that lipophilization increased antimicrobial activity 80–100-fold, depending on the derivative and the tested microorganism [45]. In the context of the current study, these reports provide broader literature context for con-

sidering lipophilicity as one of several factors that may influence antimicrobial activity. However, the present five-compound dataset does not establish that the increase in predicted lipophilicity caused the observed differences in susceptibility. The antimicrobial changes observed after esterification were microorganism-dependent rather than universal, indicating that predicted lipophilicity alone was insufficient to explain or predict the complete activity profile.

Both terpenoid esters showed the same measured nominal MIC and operational MMC values against *S. aureus* PCM 2054, namely 0.227 and 0.455 mM, respectively. Therefore, the selection of G4HPP for time-kill analysis was not based on superior activity against the target strain. G4HPP was selected because it was obtained in a higher isolated yield, showed more favorable calculated reaction-stage process descriptors, and had a slightly lower geometric mean MIC across the tested microbial panel than M4HPP (1.82 versus 2.89 mM). These criteria supported its selection for extended kinetic evaluation but should not be interpreted as evidence of a substantially broader antimicrobial spectrum.

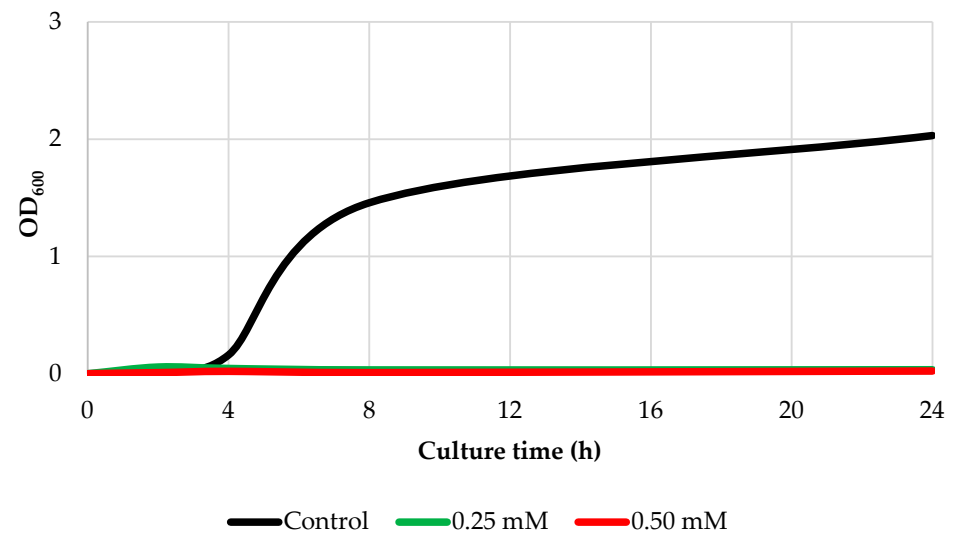
As shown in Figure 4a, G4HPP clearly suppressed the growth of *S. aureus* PCM 2054, as monitored by optical density measurements. In the untreated control, OD₆₀₀ increased progressively over time, indicating normal bacterial growth. In contrast, cultures treated with G4HPP at both 0.25 and 0.50 mM showed a marked reduction in turbidity throughout the experiment, confirming substantial inhibition of biomass development. The inhibitory effect was evident during the early stages of incubation and persisted up to 24 h. Only a small difference was observed between 0.25 and 0.50 mM, suggesting that even the lower concentration was sufficient to substantially suppress bacterial growth under the applied conditions.

The OD₆₀₀ measurements showed lower culture turbidity in the presence of G4HPP than in the untreated control and were qualitatively consistent with the viable-count results. Compound-only and vehicle blanks did not produce a detectable contribution to OD₆₀₀ under the applied conditions. Nevertheless, because optical density does not distinguish viable cells from non-viable cells or cellular debris, the CFU measurements were treated as the primary measure of the antibacterial effect.

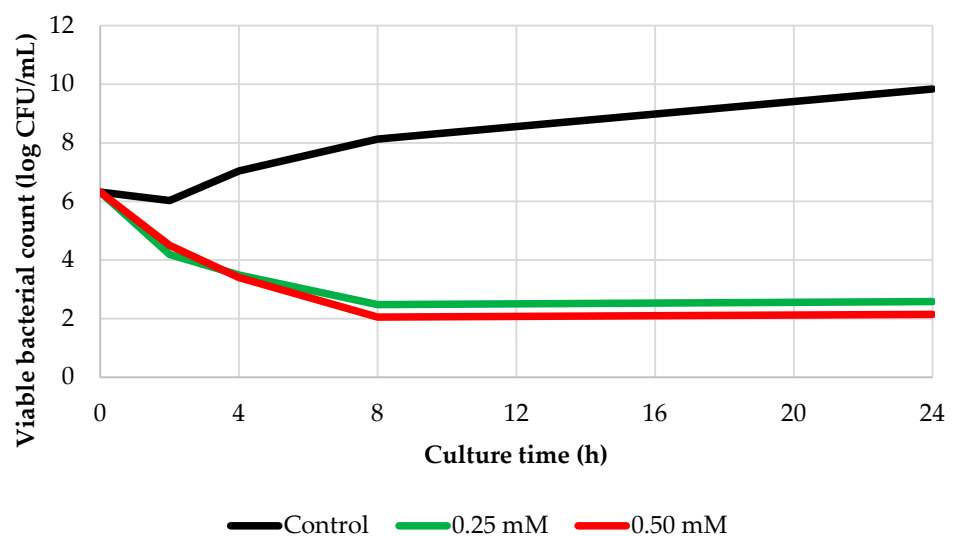
Viable-count measurements confirmed a pronounced reduction in the number of culturable cells in the G4HPP-treated cultures. At 0.25 mM, the viable count decreased by 2.13, 2.83, and 3.84 log units after 2, 4, and 8 h, respectively, relative to the initial inoculum. At 0.50 mM, the corresponding reductions were 1.82, 2.91, and 4.26 log units. Thus, a reduction exceeding 3 log units was observed between 4 and 8 h at both tested concentrations. Between 8 and 24 h, a small increase in viable counts was observed in both treated cultures. Consequently, the reductions after 24 h were 3.73 log units at 0.25 mM and 4.17 log units at 0.50 mM. Although the viable counts remained substantially below the initial inoculum and the untreated control, this slight increase indicates that the reduction was not completely sustained at the maximum level observed after 8 h.

These kinetic effects are summarized in Table 6. Based on linear interpolation between the experimental sampling points, the estimated times required to achieve a 3-log reduction were 4.7 h at 0.25 mM and 4.3 h at 0.50 mM. The numerically shorter estimated time at 0.50 mM suggests a modest concentration-related difference under the applied conditions; however, these descriptive estimates were not subjected to confirmatory statistical comparison. In addition, compared with the untreated control, the differences were 5.64 and 7.25 log units at 8 and 24 h, respectively, for 0.25 mM, and 6.07 and 7.68 log units for 0.50 mM. The overall antibacterial effect over time, expressed as AUC, was lower for the treated cultures than for the control, with AUC values of 70.64 and 63.24 (logCFU/mL)·h at 0.25 and 0.50 mM, respectively. This corresponded to AUC reductions of 64.58% and 68.29% relative to the untreated control. Taken together, the results shown in Figure 4 and

Table 6 indicate that G4HPP produced a rapid reduction in viable counts of *S. aureus* PCM 2054 under the experimental conditions tested. A ≥ 3 -log reduction was achieved between 4 and 8 h at both tested concentrations. However, a small increase in viable counts was observed between 8 and 24 h, indicating limited regrowth or reduced maintenance of the maximal effect during prolonged incubation. Because compound stability and bacterial persistence were not investigated, the basis of this observation cannot be determined from the present data.



(a)



(b)

Figure 4. Effect of geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP) on the growth and viability of *S. aureus* PCM 2054 in tryptic soy broth. (a) Growth curves based on optical density measurements at 600 nm. (b) Time-kill curves based on viable bacterial counts. G4HPP was tested at nominal concentrations of 0.25 and 0.50 mM, corresponding to 75.6 and 151.2 mg/L, respectively. The MIC was not independently determined in TSB. Data are presented as mean $\pm$ SD from three independent biological replicates.

Table 6. Time-kill parameters of geranyl 3-(4-hydroxyphenyl)propanoate against *S. aureus* PCM 2054 in TSB at nominal concentrations of 0.25 and 0.50 mM, expressed as log reduction relative to the initial inoculum, log difference relative to the untreated control, and descriptive kinetic parameters.

Parameter	Time (h)	0.25 mM	0.50 mM
Δ log reduction	2	2.13	1.82
	4	2.83	2.91
	8	3.84	4.26
	24	3.73	4.17
Δ log vs. control	8	5.64	6.07
	24	7.25	7.68
$t_{3 \log}$ (h)		4.7	4.3
AUC ((log CFU/mL)·h)		70.64	63.24
AUC reduction vs. control (%)		64.58	68.29

The concentrations of 0.25 and 0.50 mM correspond to 75.6 and 151.2 mg/L, respectively. The MIC was not independently determined in TSB. The kinetic parameters are descriptive estimates calculated from mean viable-count data obtained in three independent biological replicates. The $t_{3 \log}$ values were estimated by linear interpolation between the sampling points surrounding the 3-log reduction threshold and are reported to one decimal place. The AUC of the untreated control was 199.41 (log CFU/mL)·h and was used to calculate the percentage AUC reductions. No inferential statistical comparison between the tested concentrations was performed.

Taken together, the results obtained for G4HPP demonstrate that this ester exerts a rapid and sustained antibacterial effect against *S. aureus*. The low MIC and MMC values were further supported by growth-curve and time-kill analyses, which showed marked suppression of bacterial growth, a rapid decline in viable counts, and high AUC reductions relative to the untreated control. These findings characterize the time-dependent activity of G4HPP against the tested *S. aureus* PCM 2054 strain but do not demonstrate that esterification improved killing kinetics relative to phloretic acid, geraniol, or M4HPP, because those compounds were not included in the kinetic experiment. Previous studies on other phenolic esters have reported time-dependent antibacterial effects and membrane-related mechanisms. These literature observations provide context for the present findings but do not establish that G4HPP acts through the same mechanism or that its killing kinetics are superior to those of its parent compounds. Experimental studies on alkyl ferulate esters showed that hexyl ferulate exhibited strong antibacterial activity against *Listeria monocytogenes*, and both growth curves and time-kill assays confirmed substantial inhibition of bacterial growth, accompanied by cell lysis and membrane disruption [46]. Likewise, alkyl gallates displayed chain-length-dependent antibacterial activity, with octyl gallate exerting a pronounced bactericidal effect associated with membrane damage and intracellular oxidative stress [47].

Comparable trends were also reported for rosmarinic acid esters. Suriyarak et al. [48] demonstrated that esterification of rosmarinic acid markedly altered antimicrobial performance in a chain-length-dependent manner, with dodecyl rosmarinate showing the strongest activity against *S. carnosus*. Importantly, time-kill assays revealed that the ester reduced viable counts more efficiently than the parent acid and that its effect varied with the bacterial growth phase. In a subsequent study [49], the same authors further showed that esterification increased the compound's affinity for bacterial membranes and reduced its dependence on environmental factors such as pH and salt concentration.

Additional support comes from studies on gallic acid derivatives, in which methyl gallate was evaluated against *S. aureus* strains using time-kill analysis. Although this derivative offers only a modest increase in lipophilicity relative to longer-chain phenolipids, it still confirms that phenolic esters can produce time-dependent anti-staphylococcal effects and induce bacterial surface damage [50]. More recently, octyl gallate has also been shown to fit this broader pattern of phenolipid-like antimicrobial behavior. Xu et al. [51] reported potent anti-MRSA activity of octyl gallate, including a low MIC, rapid killing,

inhibition of biofilm formation, and practical efficacy in pork preservation. Multi-omics and computational analyses suggested a multi-target mechanism involving membrane transport, ribosomes, and amino acid metabolism [51]. Together with earlier studies on alkyl gallates and ferulate or rosmarinate esters, these reports show that lipophilic phenolic derivatives may display strongly microorganism-dependent antibacterial properties. In the present study, however, enhanced activity was demonstrated only against the tested *S. aureus* PCM 2054 strain and should not be generalized to Gram-positive bacteria or to other *Staphylococcus* strains.

A limitation of the present study is that structural characterization of the synthesized products relied solely on ^1H NMR spectroscopy, without complementary ^{13}C NMR or high-resolution mass spectrometric analysis. Furthermore, no objective chromatographic or quantitative NMR purity assessment was available for the exact batches used in the antimicrobial assays. Although the products were isolated by silica-gel column chromatography and their ^1H NMR spectra were consistent with the proposed structures, the presence of minor residual substrates or purification-related impurities cannot be completely excluded. Therefore, the antimicrobial findings should be regarded as preliminary and should be confirmed in future studies using compounds subjected to orthogonal structural identification and quantitative purity assessment.

A further limitation is that the microbial panel included only one *S. aureus* strain. Consequently, the greater activity observed against *S. aureus* PCM 2054 cannot be generalized to *S. aureus* as a species, to other *Staphylococcus* species, or to Gram-positive bacteria more broadly. Future studies should evaluate a broader panel of food-associated and clinical isolates, including biofilm-forming and antimicrobial-resistant strains, to assess the reproducibility and practical relevance of the observed activity.

The antimicrobial evaluation was limited to broth-based assays using a small panel of laboratory strains. Food-matrix performance, stability, toxicity, sensory effects, effective food dose, and regulatory feasibility were not evaluated. Furthermore, the time-kill experiment included only G4HPP and an untreated control; consequently, it did not allow direct comparison of killing kinetics with the parent compounds, M4HPP, or a reference preservative. The results should therefore be interpreted as preliminary in vitro evidence requiring further food-related validation.

The antimicrobial assay also had methodological limitations. The actual dissolved concentrations of the tested compounds were not quantified, and occasional turbidity, precipitation, or surface-film formation may have affected compound availability and visual MIC assessment. Moreover, the final viable inoculum density was standardized turbidimetrically but was not independently verified by colony counting. MMC was determined by direct transfer of 3 μL aliquots onto agar without prior dilution or neutralization, and antimicrobial carry-over could therefore have contributed to the apparent absence of colony growth. Accordingly, the MIC values represent nominal concentrations, whereas the MMC values and MMC/MIC classifications should be regarded as preliminary operational estimates.

From a process-development perspective, future work should evaluate deep eutectic solvents as alternative reaction media for CALB-catalyzed synthesis of phloretic acid esters. Such systems could reduce the use of volatile organic solvents and improve the solubilization of phenolic substrates [52]. However, their environmental advantage should be verified experimentally by assessing enzyme performance, viscosity and mass-transfer limitations, product isolation, solvent recovery and reuse, energy demand, and complete process mass indicators [53].

4. Conclusions

In this study, two phloretic acid esters with geraniol and myrtenol were obtained via CALB-catalyzed esterification under mild reaction conditions. Among the synthesized derivatives, geranyl 3-(4-hydroxyphenyl)propanoate (G4HPP) was isolated in higher yield than the corresponding myrtenyl ester and exhibited more favorable reaction-level indicators, including higher reaction mass efficiency and catalyst productivity, along with lower partial reaction-stage PMI and E-factor values. However, these indicators did not include the complete downstream isolation process, energy demand, solvent hazard, solvent recovery, or catalyst reuse. Therefore, they should be interpreted as preliminary reaction-level descriptors rather than evidence that the complete process is more sustainable than conventional synthesis.

Biological evaluation showed that esterification of phloretic acid with the selected terpenoid alcohols did not uniformly increase antimicrobial activity across the tested microbial panel. The most pronounced effect was observed against *S. aureus* PCM 2054, for which both esters showed identical MIC and MMC values of 0.227 and 0.455 mM, respectively. These MIC values were 16-fold lower than those of the corresponding parent terpenoid alcohols and 64-fold lower than that of phloretic acid. Because only one *S. aureus* strain was evaluated, these findings should not be interpreted as demonstrating general anti-staphylococcal activity or selectivity.

In the time-kill experiment, G4HPP at 0.25 and 0.50 mM (75.6 and 151.2 mg/L, respectively) produced a ≥ 3 -log reduction in viable *S. aureus* PCM 2054 counts within approximately 4–5 h. However, phloretic acid, geraniol, M4HPP, and a reference preservative were not included in the kinetic experiment. Therefore, these results characterize the time-dependent activity of G4HPP under the applied conditions but do not demonstrate that esterification improved killing kinetics. A small increase in viable counts between 8 and 24 h was also observed.

The in silico analysis indicated that the synthesized esters had higher predicted lipophilicity and lower predicted aqueous solubility than the parent compounds. Among the five structurally related compounds, these physicochemical changes coincided with microorganism-dependent differences in antimicrobial activity; however, the observed patterns should be regarded as exploratory and do not establish a quantitative structure–activity relationship or mechanism.

Overall, the synthesized esters should be considered preliminary in vitro antimicrobial candidates. The present broth-based assays do not establish their efficacy as food preservatives. Further studies should evaluate a broader panel of *S. aureus* strains and other food-relevant microorganisms, and address activity in food matrices, compound stability, safety, sensory effects, effective dose, and regulatory feasibility before any potential food-preservation application can be considered.

Overall, the synthesized esters should be considered preliminary in vitro antimicrobial candidates rather than established food preservatives. Further studies should include broader strain panels, food-matrix validation, compound stability and safety, sensory effects, dose–response relationships, and regulatory considerations. From a process-development perspective, deep eutectic solvents should be evaluated as alternative reaction media for CALB-catalyzed esterification. Their potential to reduce volatile-solvent use and improve substrate solubilization should be assessed alongside enzyme performance, mass-transfer limitations, downstream isolation, solvent reuse, energy demand, and complete process mass indicators.

Supplementary Materials: The following supporting information can be downloaded at <https://www.mdpi.com/article/10.3390/su18157965/s1>. File S1: G4PP-Raw NMR data; File S2: M4PP-Raw NMR data.

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Abbreviations

The following abbreviations are used in this manuscript:

AE	Atom economy
ANOVA	Analysis of variance
AUC	Area under the curve
CALB	Lipase B from <i>Candida antarctica</i>
CFU	Colony-forming unit
ESOL	Estimated SOLubility
FID	Free induction decay
G4HPP	Geranyl 3-(4-hydroxyphenyl)propanoate
GM MIC	Geometric mean minimum inhibitory concentration
HPLC	High-Performance Liquid Chromatography
ITS	Internal transcribed spacer
Log P	Logarithm of the partition coefficient
Log S	Logarithm of predicted aqueous solubility
M4HPP	Myrtenyl 3-(4-hydroxyphenyl)propanoate
MIC	Minimum inhibitory concentration
MMC	Minimum microbicidal concentration
MRSA	Methicillin-Resistant <i>Staphylococcus aureus</i>
MTBE	Methyl <i>tert</i> -butyl ether
MW	Molecular weight
NMR	Nuclear magnetic resonance
OD ₆₀₀	Optical density at 600 nm
PDA	Potato dextrose agar
pMIC	Negative decimal logarithm of MIC expressed in mol/L
PMI	Process mass intensity
RME	Reaction mass efficiency
SD	Standard deviation
TLC	Thin-layer chromatography
TMS	Tetramethylsilane
TPSA	Topological polar surface area
TSA	Tryptic soy agar
TSB	Tryptic soy broth
UV	Ultraviolet

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