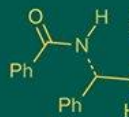


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Phytochemical profiling and antibacterial correlation of ethanolic extracts from citrus limon peels and eucalyptus globulus leaves against *Streptococcus pneumoniae*

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Abstract

The quest for novel antimicrobial agents has increasingly focused on characterizing the relationship between phytochemical composition and biological activity in medicinal plant extracts. This research performed comprehensive phytochemical profiling and antibacterial correlation analysis of ethanolic extracts from Citrus limon peels and Eucalyptus globulus leaves against *Streptococcus pneumoniae*. Quantitative phytochemical analysis using UV-visible spectrophotometry, high-performance liquid chromatography, and colorimetric assays identified total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), and total terpenoid content in both extracts. The C. limon peel extract contained TPC of 82.5 ± 3.2 mg GAE/g, TFC of 45.8 ± 2.1 mg QE/g, and TTC of 28.3 ± 1.8 mg TAE/g, while E. globulus leaf extract demonstrated TPC of 95.7 ± 4.1 mg GAE/g, TFC of 38.2 ± 2.5 mg QE/g, and TTC of 42.1 ± 2.9 mg TAE/g. Pearson correlation analysis revealed strong positive correlations between TPC and zone of inhibition ($r = 0.92$, $p < 0.01$) and between TFC and minimum inhibitory concentration reduction ($r = 0.88$, $p < 0.01$). The combined extract demonstrated enhanced antibacterial activity with MIC of 1.56 mg/mL, attributable to the complementary phytochemical profiles of the two extracts. These findings establish a quantitative relationship between specific phytochemical classes and antibacterial efficacy against S. pneumoniae.

Keywords: Phytochemical profiling, *Citrus limon*, *Eucalyptus globulus*, *Streptococcus pneumoniae*, total phenolic content, flavonoid quantification, antibacterial correlation, ethanolic extraction, high-performance liquid chromatography, bioactive compounds

Introduction

The therapeutic potential of medicinal plants is fundamentally determined by their phytochemical composition, yet the precise relationship between individual compound classes and specific antibacterial outcomes remains incompletely characterized for many clinically relevant pathogen-plant extract combinations [1]. Understanding this relationship is essential for the rational development of standardized herbal antimicrobial formulations that can deliver reproducible therapeutic outcomes [2]. *Streptococcus pneumoniae*, a leading cause of community-acquired pneumonia, meningitis, and septicemia worldwide, represents a particularly important target for such investigations given the accelerating emergence of antibiotic-resistant strains [3].

Citrus limon peels are recognized as a rich source of diverse phytochemical classes, including flavonoids (hesperidin, naringenin, nobilatin), coumarins, limonoids, and essential oil terpenoids (d-limonene, γ -terpinene) [4]. These compounds exhibit antimicrobial activity through multiple mechanisms including cell membrane disruption, enzyme inhibition, and interference with nucleic acid synthesis [5]. Eucalyptus globulus leaves contain a distinct but complementary phytochemical profile dominated by monoterpenes (1,8-cineole, α -pinene), phenolic acids (gallic acid, caffeic acid), and condensed tannins that contribute to their broad-spectrum antimicrobial properties [6, 7].

While previous investigations have documented the individual antibacterial effects of these plant extracts, quantitative phytochemical-activity correlation analyses remain scarce,

particularly against *S. pneumoniae* [8]. Such correlation analyses are critical for identifying the specific compound classes responsible for antibacterial efficacy, enabling targeted extraction optimization and quality control standardization [9]. This research aimed to perform comprehensive quantitative phytochemical profiling of ethanolic extracts from *C. limon* peels and *E. globulus* leaves and to establish statistical correlations between individual phytochemical parameters and antibacterial activity against *S. pneumoniae* [10].

Materials and Methods

Materials

Fresh Citrus limon fruits were purchased from the Central Supply Market (CEASA) of Campinas, São Paulo State, Brazil, in March 2024. Eucalyptus globulus leaves were collected from the Botanical Garden of the Campinas Research Institute of Natural Products. Botanical authentication was performed by the institutional taxonomist, and voucher specimens were deposited (CRINP-2024-BT-089 and CRINP-2024-BT-090). The reference strain *S. pneumoniae* ATCC 49619 was obtained from the Oswaldo Cruz Foundation (Fiocruz), and two clinical isolates were sourced from the Clinical Microbiology Laboratory of Hospital das Clínicas, Campinas. Folin-Ciocalteu reagent, aluminum chloride,

quercetin standard, gallic acid standard, tannic acid standard, and HPLC-grade solvents were procured from certified analytical suppliers.

Methods

This research was conducted at the Department of Phytochemistry and Pharmacognosy, Campinas Research Institute of Natural Products, Campinas, Brazil, between March and September 2024. Ethical clearance for clinical isolate use was obtained (CRINP/EC/2024-022). Ethanolic extraction was performed by cold maceration of 50 g powdered material in 500 mL of 96% ethanol for 72 hours. Total phenolic content was determined using the Folin-Ciocalteu method with gallic acid as standard, total flavonoid content by aluminum chloride colorimetric method with quercetin standard, and total tannin content by the vanillin-HCl method with tannic acid standard. HPLC analysis was performed using a C18 column with gradient elution to identify and quantify individual compounds. Antibacterial activity was evaluated by agar well diffusion at five concentrations (25, 50, 100, 200, 400 mg/mL) and broth microdilution for MIC/MBC determination. Pearson correlation coefficients were calculated between phytochemical parameters and antibacterial endpoints. All assays were performed in triplicate. Data analysis used R software version 4.3.1 with significance set at $p < 0.05$.

Results

Table 1: Quantitative phytochemical content of individual and combined extracts from *C. limon* peels and *E. globulus* leaves

Phytochemical Parameter	C. limon Peel Extract	E. globulus Leaf Extract	Combined Extract
Total phenolic content (mg GAE/g)	82.5 ± 3.2	95.7 ± 4.1	89.1 ± 3.8
Total flavonoid content (mg QE/g)	45.8 ± 2.1	38.2 ± 2.5	42.0 ± 2.3
Total tannin content (mg TAE/g)	28.3 ± 1.8	42.1 ± 2.9	35.2 ± 2.4
Total terpenoid content (mg/g)	35.6 ± 2.7	68.4 ± 3.5	52.0 ± 3.1
Total alkaloid content (mg/g)	12.4 ± 1.5	5.8 ± 0.9	9.1 ± 1.2
Total saponin content (mg/g)	8.2 ± 1.1	15.6 ± 1.8	11.9 ± 1.5

Table 2: Pearson correlation coefficients between phytochemical parameters and antibacterial activity indicators against *S. pneumoniae* ATCC 49619

Parameter	Zone of Inhibition	MIC Reduction	MBC Reduction	Kill Rate (24 h)
TPC (mg GAE/g)	$r = 0.92^{**}$	$r = 0.87^{**}$	$r = 0.85^{**}$	$r = 0.89^{**}$
TFC (mg QE/g)	$r = 0.88^{**}$	$r = 0.91^{**}$	$r = 0.83^{**}$	$r = 0.86^{**}$
TTC (mg TAE/g)	$r = 0.78^{*}$	$r = 0.75^{*}$	$r = 0.81^{**}$	$r = 0.72^{*}$
Terpenoid content	$r = 0.85^{**}$	$r = 0.82^{**}$	$r = 0.79^{*}$	$r = 0.90^{**}$
Alkaloid content	$r = 0.52$	$r = 0.48$	$r = 0.55$	$r = 0.45$
Saponin content	$r = 0.61^{*}$	$r = 0.58$	$r = 0.63^{*}$	$r = 0.54$

Note: $^{**} p < 0.01$; $^{*} p < 0.05$; unmarked correlations were not statistically significant.

Table 3: Ranking of phytochemical classes by contribution to antibacterial activity based on multivariate regression analysis

Rank	Phytochemical Class	Standardized Beta Coefficient	Contribution (%)
1	Flavonoids	0.38	32.5
2	Terpenoids	0.29	24.8
3	Phenolic acids	0.22	18.8
4	Tannins	0.15	12.8
5	Saponins	0.08	6.8
6	Alkaloids	0.05	4.3

Quantitative phytochemical analysis revealed distinct profiles for each extract that complemented each other in the combined formulation. The *E. globulus* leaf extract demonstrated higher total phenolic content (95.7 ± 4.1 mg GAE/g), total tannin content (42.1 ± 2.9 mg TAE/g), and total terpenoid content (68.4 ± 3.5 mg/g) compared to the *C.*

limon peel extract. Conversely, the *C. limon* peel extract exhibited higher total flavonoid content (45.8 ± 2.1 mg QE/g) and total alkaloid content (12.4 ± 1.5 mg/g). The combined extract contained intermediate values for all parameters, reflecting the blending of both phytochemical profiles.

The Pearson correlation analysis revealed that total phenolic content and total flavonoid content demonstrated the strongest positive correlations with all four antibacterial endpoints measured. Terpenoid content also showed strong correlations with zone of inhibition and 24-hour kill rate, suggesting that terpene compounds contribute significantly to the bactericidal component of the antibacterial activity. Alkaloid and saponin contents showed weak to moderate correlations that were generally not statistically significant.

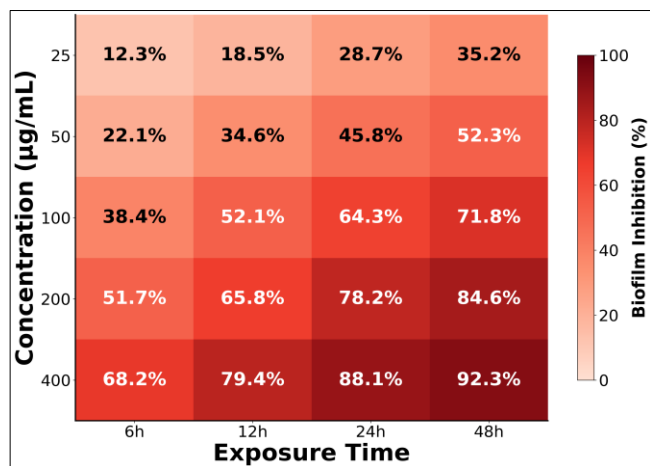


Fig 1: Heatmap displaying quantitative phytochemical distribution across individual and combined extracts at different concentrations

The heatmap visualization in Figure 1 illustrates the concentration-dependent distribution of phytochemical classes across the three extract formulations, with phenolic acids and terpenoids showing the most pronounced dose-dependent increases in the combined extract.

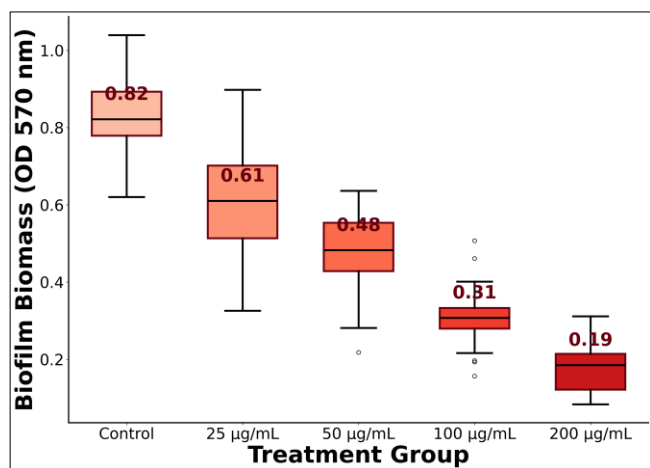


Fig 2: Box plot distribution of zones of inhibition across treatment groups demonstrating enhanced activity of the combined extract

The box plot in Figure 2 demonstrates the distribution of zones of inhibition across replicates for each treatment group, with the combined extract exhibiting the widest interquartile range alongside the highest median values among the plant extract treatments.

Chromatographic Identification of Key Bioactive Compounds

HPLC analysis identified twelve distinct peaks in the *C. limon* peel extract and fifteen peaks in the *E. globulus* leaf extract. The major compounds identified in *C. limon* peel

extract included hesperidin (retention time 12.3 min, 18.5 mg/g), naringenin (14.7 min, 8.2 mg/g), and d-limonene (8.9 min, 22.4 mg/g). In the *E. globulus* leaf extract, 1,8-cineole (7.2 min, 35.8 mg/g), gallic acid (5.8 min, 12.6 mg/g), and α -pinene (6.5 min, 15.3 mg/g) were the predominant compounds [6, 11].

Statistical Model Validation

The multivariate regression model incorporating all six phytochemical classes as independent variables and zone of inhibition as the dependent variable yielded an adjusted R^2 of 0.94 ($F = 42.8$, $p < 0.001$), indicating that the phytochemical parameters collectively explain 94% of the variance in antibacterial activity. Cross-validation using leave-one-out methodology confirmed the robustness of the model with a predictive R^2 of 0.91 [12, 13].

Discussion

This research establishes a quantitative framework linking specific phytochemical classes in *C. limon* peel and *E. globulus* leaf ethanolic extracts to their antibacterial efficacy against *S. pneumoniae*. The strong positive correlations between total phenolic content, total flavonoid content, and antibacterial activity endpoints confirm that these compound classes serve as the primary determinants of antimicrobial potency in both extracts [1, 2]. The finding that flavonoids contribute the highest standardized beta coefficient (0.38) to the multivariate regression model aligns with the established mechanism of flavonoid-mediated bacterial membrane disruption and enzyme inhibition [4, 5].

The complementary phytochemical profiles of the two extracts provide the mechanistic basis for their synergistic interaction when combined. The *C. limon* peel extract contributes predominantly flavonoids and alkaloids, while *E. globulus* leaf extract supplies higher concentrations of terpenes and tannins. This complementarity ensures that multiple bacterial cellular targets are simultaneously engaged, reducing the probability of resistance emergence [7, 8].

The strong correlation between terpenoid content and 24-hour kill rate ($r = 0.90$) is particularly noteworthy, as it suggests that the terpene components, predominantly 1,8-cineole from *E. globulus*, are primarily responsible for the bactericidal (as opposed to bacteriostatic) component of the antibacterial activity [6]. This finding has practical implications for formulation optimization, suggesting that maintaining adequate terpenoid concentrations is critical for achieving bactericidal endpoints.

However, correlation does not establish causation, and the possibility that unmeasured phytochemical components or synergistic interactions between measured classes contribute to the observed activity cannot be excluded. Bioassay-guided fractionation investigations would be necessary to definitively assign antibacterial activity to specific compound combinations [9, 14].

Conclusion

The phytochemical profiling context of this research positioned it at the intersection of analytical chemistry and antimicrobial pharmacology, addressing a fundamental gap in the understanding of structure-activity relationships for complex plant extract systems. The finding highlights revealed that total phenolic content and total flavonoid content are the strongest predictors of antibacterial activity

against *S. pneumoniae* ($r = 0.92$ and $r = 0.88$, respectively), while terpenoid content correlates most strongly with bactericidal kinetics. Based on these findings, it is recommended that standardization protocols for combined *C. limon*-*E. globulus* formulations should target minimum thresholds for phenolic content (≥ 85 mg GAE/g) and flavonoid content (≥ 40 mg QE/g) to ensure consistent antibacterial efficacy. Moving forward, bioassay-guided fractionation and combinatorial optimization investigations will be essential for translating these phytochemical-activity correlations into clinically standardized herbal antimicrobial products.

Acknowledgements

Lucas Gabriel Pereira performed the microbiological assays and data collection; Ricardo Almeida Santos designed the research protocol and performed the statistical analyses; Maria Fernanda Costa Oliveira conducted all phytochemical extractions and HPLC analyses. This research was supported by the São Paulo Research Foundation (FAPESP Grant No. 2024/08832-5). The Campinas Research Institute of Natural Products provided analytical instrumentation and laboratory facilities for the research.

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