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Original article

Effect of a pinocembrin-rich *Lippia origanoides* extract on the induction of antibiotic resistance and SOS responses in *Escherichia coli*

Efecto de un extracto de *Lippia origanoides* rico en pinocembrina en la inducción de la resistencia antibiótica y la respuesta SOS en *Escherichia coli*

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Abstract

Antibiotic resistance is a growing problem due to the rise in diseases associated with resistant pathogens. The bacterial SOS response is a genetic stress-response pathway whose primary role is DNA repair. It can be activated by DNA damage induced by antibiotics and contribute indirectly to the emergence of resistance through mutagenic DNA polymerases, horizontal gene transfer, and the promotion of bacterial persistence and tolerance to antibiotics. Inhibiting this pathway represents a promising strategy to reduce the appearance of resistant variants. Here, we aimed to evaluate the antibacterial, antibiotic-modulating, and SOS-inhibitory capacities of *Lippia origanoides* extract (LOE) and pinocembrin, using the *Escherichia coli* PQ37 strain and the SOS Chromotest assay. Ciprofloxacin was used as the test antibiotic and proved to be a potent inducer of the SOS response in *E. coli*. In co-treatments with this antibiotic, pinocembrin (0.097 mM) exhibited a synergistic effect on the antibiotic's activity, reducing its minimum inhibitory concentration by approximately fourfold. Furthermore, pinocembrin inhibited the ciprofloxacin-induced SOS response in a concentration-dependent manner, with significant inhibition starting at 0.031 mM ($r = -0.87$, $p < 0.05$). These results indicate that pinocembrin can be an adjuvant in combination antibiotic therapy, as it potentiates antibiotic activity while simultaneously suppressing the bacterial SOS response, a mechanism that could minimize the risk of antibiotic resistance development.

Keywords: Antibiotic resistance; SOS response; ciprofloxacin; pinocembrin; inhibition; *Lippia origanoides*.

Resumen

La resistencia antibiótica constituye un problema creciente debido al aumento de enfermedades asociadas con patógenos resistentes. La respuesta SOS bacteriana es una vía génica de respuesta al estrés cuyo papel principal es la reparación del ADN. Dicha respuesta puede activarse por el daño en el ADN causado por antibióticos y contribuye indirectamente a la aparición de resistencia a través de las polimerasas mutagénicas, la transferencia horizontal de genes y la promoción de la persistencia y la tolerancia bacteriana a los antibióticos. La inhibición de esta vía representa una estrategia prometedora para reducir la generación de variantes resistentes. En este contexto, nuestro objetivo fue evaluar las capacidades antibacterianas, moduladoras de antibióticos e inhibitorias de la respuesta SOS del extracto de *Lippia origanoides* (ELO) y la pinocembrina, utilizando la cepa *Escherichia coli* PQ37 y el ensayo SOS Chromotest. El antibiótico evaluado fue el ciprofloxacino, el cual demostró ser un potente inductor de la respuesta SOS en *E. coli*. En los tratamientos concomitantes con este antibiótico, la pinocembrina (0.097 mM) exhibió un efecto sinérgico sobre

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la actividad del antibiótico, reduciendo en cerca de cuatro veces su CMI. Además, la pinocembrina inhibió la respuesta SOS inducida por el ciprofloxacino de forma dependiente de la concentración, observándose una inhibición significativa a partir de 0.031 mM ($r = - 0.87$, $p < 0.05$). Nuestros resultados indican que la pinocembrina puede actuar como coadyuvante en la terapia antibiótica combinada, dado que potencia su actividad a la vez que inhibe la respuesta SOS bacteriana, un mecanismo que podría minimizar el riesgo de desarrollar resistencia a antibióticos.

Palabras clave: resistencia antibiótica; respuesta SOS; ciprofloxacino; pinocembrina; inhibición; *Lippia origanoides*.

Introduction

Antibiotic resistance constitutes one of the most serious public health challenges of this century. In 2021, deaths worldwide associated with antibiotic microbial resistance were estimated to be 1.91 million; these numbers are expected to increase to 8.22 million deaths by 2050 (**Naghavi *et al.***, 2024). The excessive and often indiscriminate use of antibiotics promotes the emergence and spread of resistant strains not only in clinical settings but throughout the food chain; foodborne infections alone account for a substantial proportion of illnesses and diseases, with an estimated 9.4 million people treated annually for this cause in the United States (**Scallan *et al.***, 2010; **de Castro *et al.***, 2025). Of particular concern is the rising prevalence of antibiotic-resistant pathogens in animal meat production processes and markets (**Nhung *et al.***, 2017; **Kumar *et al.***, 2018; **Kasimanickam *et al.***, 2021). Colombia is no exception to this trend, as resistant strains have been isolated from poultry, cattle, milk, and even water sources (**Arenas & Melo**, 2018). Furthermore, antibiotic-resistant pathogens are the most common cause of nosocomial infections (**Alpert**, 2016; **Kaur *et al.***, 2020). These scenarios pose enormous challenges for animal meat production and human health worldwide, underscoring the need to develop alternatives to control infections caused by multidrug-resistant bacteria in different settings.

Recent studies (**Qin *et al.***, 2015; **Mo *et al.***, 2016) have shown that the SOS response in bacteria plays a crucial role in the emergence of antibiotic-resistant variants. This bacterial response is initiated when the cellular genome is damaged, activating different gene pathways that allow DNA damage repair and cell division restoration, which are essential for survival (**Radman**, 1975; **Shinagawa**, 1996). RecA is a positive regulator of this response and has a high affinity for single-stranded DNA regions, which are products of damaged DNA processing. Bound to these regions, the RecA protein produces a nucleofilament structure that promotes the self-cleavage of the LexA repressor, the negative regulator (repressor) of more than 50 genes associated with the SOS response (**Little *et al.***, 1980; **Fernández de Henestrosa *et al.***, 2000). The induction of these SOS genes in bacteria is sequential, and its magnitude varies depending on the affinity of the LexA repressor for the promoter region (SOS box) of each SOS (**Courcelle *et al.***, 2001).

In *Escherichia coli*, the *dinB* and *umuDC* genes, which encode polymerases IV and V, respectively, are induced late during the SOS response. These low-fidelity polymerases (prone to errors) replicate the bacterial genome at the cost of producing mutations (**Courcelle *et al.***, 2001; **Henrikus *et al.***, 2018), and their high mutation rate is a critical factor in the production of bacterial variants (mutants) with antibiotic resistance (**Cirz *et al.***, 2005; **Jaktaji & Rezaei**, 2021; **Yakimov *et al.***, 2021). Antibiotics that interfere with cell wall synthesis and DNA replication are strong inducers of the SOS response in bacteria (**Kohanski *et al.***, 2010; **Crane *et al.***, 2021). For example, β-lactam antibiotics (e.g., ampicillin) and quinolones (e.g., ciprofloxacin) induce the SOS response, producing antibiotic-resistant mutants of *E. coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* (**Cirz *et al.***, 2005; **Wang *et al.***, 2010; **Mo *et al.***, 2016; **Elfadadny *et al.***, 2024). These mutants are not induced if the bacteria have a deficiency in low-fidelity polymerase activity (**Jaktaji & Rezaei**, 2021), indicating the central role of these polymerases in inducing resistance to the above-mentioned antibiotics.

Consequently, novel therapeutic strategies that block the bacterial SOS response are required to address the challenge of antibiotic resistance (**Memar *et al.***, 2020; **Crane *et al.***, 2021; **Machuca *et al.***, 2021). The inhibition or inactivation of *recA* and *lexA* genes, which regulate the induction of the bacterial SOS response, is critical in such approaches. Thus, different compounds (e.g., p-coumaric acid, phthalocyanine tetrasulfonic acid, N(6)-(1-naphthyl)-ADP, 5-amino-1-(carbamoylmethyl)-1H-1,2,3-triazol-4-carboxamide, 3-nPBA) that inhibit the expression of these cellular targets (**Lee *et al.***, 2005; **Alam *et al.***, 2016; **Selwood *et al.***, 2018; **Chatterjee *et al.***, 2024) and binding agents (e.g., zinc, copper, mercury) or peptides that affect the function of the RecA protein (**Cline *et al.***, 2007; **Lee & Singleton**, 2004; **Bunnell *et al.***, 2017; **Yakimov *et al.***, 2017; **Ojha & Patil**, 2019) have been reported. Other authors have suggested the use of compounds (e.g., curcumin) or extracts of medicinal plants (e.g., *Cytisus austriacus*, *Chelidonium majus*, *Juglans regia*, and *Rhus glabra*) to globally inhibit the SOS response in bacteria (**Bellio *et al.***, 2014; **Mazanko *et al.***, 2020). Separately, the combined use of antibiotics and resistance-modifying agents has gained relevance in recent years as a therapeutic strategy (**Abreu *et al.***, 2012), as these agents are widely recognized for enhancing antibiotic activity against resistant bacteria and inhibiting bacterial efflux pumps (**Dias *et al.***, 2022). Despite these advances, experimental evidence supporting SOS response inhibition as an effective strategy to counteract antibiotic resistance remains scarce, and few studies have explored new molecules capable of inhibiting this pathway. In particular, whether new molecules are capable of inhibiting the SOS response and modulating the cytotoxicity of relevant antibiotics needs further investigation.

Recent studies have indicated that Colombian flora may be an important source of resistance-modifying agents (**Barrios *et al.***, 2021; **de Castro *et al.***, 2025). Among these, *Lippia origanoides* has been widely studied as a source of antimicrobial compounds (**Barreto *et al.***, 2014; **Ribeiro *et al.***, 2021), and several studies have shown that its essential oils, hydroalcoholic extracts, and main compound, pinocembrin, inhibit the SOS response triggered in *E. coli* by various mutagenic agents (**Vicuña *et al.***, 2010; **Quintero-Ruiz *et al.***, 2017; **Fuentes *et al.***, 2017; **García-Forero *et al.***, 2019).

The antibacterial mechanisms of pinocembrin have been studied across different bacterial species. Its primary effect involves modification of the cell membrane: at inhibitory concentrations, pinocembrin increases membrane permeability and can lead to leakage of cellular contents (**Wu *et al.***, 2022). Beyond its effect on the membrane, pinocembrin also affects protein synthesis, decreasing ribosomal protein expression and downregulating the NADH dehydrogenase complex, affecting overall protein synthesis and bacterial metabolism (**Rasul *et al.***, 2013; **Klančnik *et al.***, 2019). Notably, at subinhibitory concentrations, pinocembrin does not appear to alter membrane integrity (**Klančnik *et al.***, 2019); thus, its antibacterial activity may be concentration-dependent, and other mechanisms could play a major role at lower doses. However, whether pinocembrin modulates the bacterial SOS response induced by antibiotics remains unexplored.

Here, we hypothesized that the hydroalcoholic extract of *L. origanoides* and its main compound, pinocembrin, can modulate the antibacterial activity of ciprofloxacin and inhibit its ability to induce the SOS response in *E. coli*. This research is expected to provide relevant information for the development of formulations that combine ciprofloxacin and the phytochemicals, allowing for more effective control of this enteropathogen, preventing the formation of new resistant variants during therapeutic treatments, and enabling the prolonged use of broad-spectrum antibiotics such as ciprofloxacin.

Materials and methods

Plant material and extracts

Lippia origanoides plants (**Figure 1**, left) were collected from the Chicamocha River Canyon (Los Santos, Santander, Colombia) under the permit granted by the Colombian Ministry of Environment and Sustainable Development (Contract No. 270). José Luis

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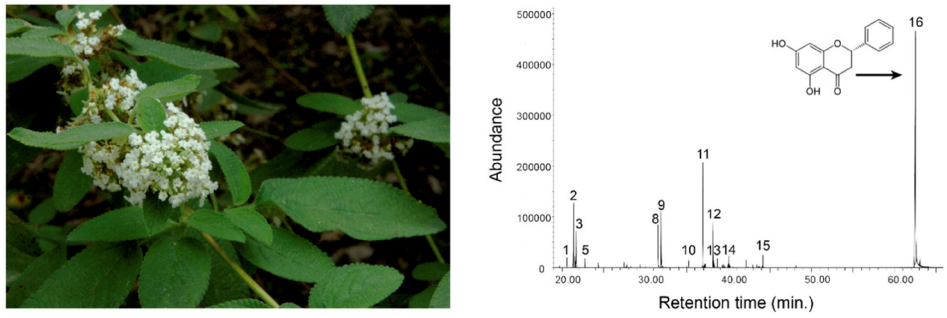

Figure 1. *Lippia origanoides* specimen and the GC-MS profile of the extract. *L. origanoides* specimen (COL560259) planted at the CENIVAM experimental complex (left). Typical GC-MS profiles of the *L. origanoides* extract obtained by supercritical fluid (CO_2) extraction (right). The major constituents of the extract were numbered according to the order of elution on the DB-5MS column.

Fernández Alonso (National University, Bogotá, Colombia) taxonomically identified *L. origanoides*, and the specimen (voucher number COL560259) was deposited in the National Herbarium of Colombia (Bogotá, Colombia). *L. origanoides* extract (LOE) was obtained by supercritical fluid extraction (SFE) as described previously (**Fuentes *et al.***, 2017). For the bioassays, the extract was dissolved in methanol (30 mg/mL), vortexed for 5 min, incubated in an ultrasonic bath for 10 min at 40°C, and centrifuged at 5000 rpm for 10 min. The supernatant (1 mL) was then filtered and stored at -80°C in an ultra-low-temperature freezer (Thermo Scientific, MA, USA). Before use, the extracted stock solutions were thawed and refrigerated at 5–8°C for 24 h (**Fuentes *et al.***, 2021).

Chemicals

High-purity gases (helium, nitrogen, hydrogen, and air) for gas chromatography were obtained from Linde S. A. (Bucaramanga, Colombia). Standard compounds (n-tetradecane and n-alkanes, C8-C25, and pinocembrin), Luria-Bertani (LB) medium, antibiotics (ampicillin, ciprofloxacin, and tetracycline), calcium carbonate, sodium dodecyl sulfate, and β-mercaptoethanol were purchased from Sigma-Aldrich Co. Inc. (Milwaukee, WI, USA). The substrates for β-galactosidase (o-nitrophenyl-β-D-galactopyranoside) and alkaline phosphatase (p-nitrophenyl phosphate), as well as LB medium, were purchased from Amresco (Solon, Ohio, USA). Sodium hydroxide was obtained from Panreac Química SLU (Barcelona, Spain). Fuming hydrochloric acid (37%), monobasic sodium phosphate monohydrate, magnesium sulfate, and potassium chloride were purchased from Merck (Darmstadt, Germany). The TRIS base was obtained from J.T. Baker (New Jersey, NJ, USA).

GC-MS analysis

Lippia origanoides extract (LOE) was chemically characterized using gas chromatography-mass spectrometry (GC-MS) analysis. Compound identification was based on chromatographic (retention times and retention indices) and mass spectroscopic (spectral interpretation and comparison with databases) criteria. Gas chromatography (GC) analyses were performed using a 6890 Plus gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with a mass selective detector MSD 5975 (Electron impact ionization, EI, 70 eV, Agilent Technologies, Palo Alto, CA, USA), a split/splitless injector (1:30 split ratio), a 7863 automatic injector, and an MS ChemStation G1701-DA data system, which included the spectral libraries WILEY, NIST, and QUADLIB 2007. A fused-silica capillary column DB-5MS (J&W Scientific, Folsom, CA, USA) of 60 m x 0.25 mm i.d., coated with 5%-phenyl poly(methylsiloxane) (0.25 µm film thickness) was used. The chromatographic conditions were as follows: The GC oven temperature was programmed from 45°C (5 min) to 150°C (2 min) at 4°C/min, then to 250°C (5 min) at 5°C/min, and finally, to 275°C (15 min)

at 10°C/min. The temperatures of the injection port, ionization chamber, and transfer line were set at 250, 230, and 285ºC, respectively. Helium (99.99%, Linde S.A., Bucaramanga, Colombia) was used as the carrier gas, with a 155 kPa column head pressure and 27 cm s^{-1} linear velocity (1 mL min^{-1}, at constant flow). A standard solution of n-alkanes (C_8-C_{25}) was used to calculate the linear retention indices (LRI) of the extract's compounds, and the LRI values were compared with those described in the database (**Babushok *et al.***, 2011). Mass spectra and reconstructed (total) ion chromatograms were obtained by automatic ion scanning of the mass spectra (m/z 30-350) at 5.1 scans s^{-1}. Chromatographic peaks were checked for homogeneity with the aid of mass chromatograms for the characteristic fragment ions and the MS ChemStation peak-purity program.

Bacterial strains and culture

We used the *E. coli* PQ37 strain [F-thr leu his-4 pyrD thi galE galK or galT lacΔU169 srl300::Tn10 rpoB rpsL uvrA rfa trp::Muc+ sfiA::Mud(Ap,lac)cts], described for the detection of genotoxic agents (**Quillardet & Hofnung**, 1985). This strain contains a gene fusion that places the lacZ gene (β-galactosidase activity) under the control of the sulA gene promoter, which is part of the SOS response and functions as a DNA damage reporter. Bacterial cultures were grown in Luria-Bertani (LB) medium supplemented with ampicillin (50 µg/mL) and tetracycline (17 µg/mL), which allowed for the verification of genotype stability. Cells were cultured in Eppendorf tubes overnight at 37°C with continuous shaking (1000 rpm) in a Thermomixer-type thermal block (Eppendorf, São Paulo, Brazil).

Antibacterial activity

The minimum inhibitory concentration (MIC), defined as the lowest concentration of a sample that inhibits microbial growth (**Andrews**, 2001), was determined by culturing *E. coli* PQ37 in the presence of different concentrations of each sample. The concentrations of the tested samples were as follows: LOE (7.8–500 µg/mL), pinocembrin (0.015–0.5 mM), ampicillin (0.21–6.73 mM), and ciprofloxacin (7.11 x 10^{-5}–4.5 x 10^{-3} mM). Bacterial cells grown overnight (12–16 h) in Eppendorf tubes were diluted by one decimal place (to an approximate cell concentration of 10^8 cells/mL) in fresh LB medium supplemented with antibiotics (see above), which contained the desired concentration of the sample being evaluated. Bacterial cells were allowed to grow for 24 h under the same culture conditions, and the entire culture was then transferred to a quartz cuvette with a 1 cm path length and 1.5 mm glass thickness (Zenith Lab, Jiangsu, China) for optical density readings at 600 nm using a Multiskan GO spectrophotometer (Thermo Scientific, Waltham, USA). Antibiotic-supplemented LB medium without bacterial inoculum was used as a blank. The positive control was always a culture grown in LB medium supplemented with ampicillin and tetracycline, without the samples under study. Three independent experimental assays were performed for each treatment group. The degree of antibacterial activity of the samples was defined according to the minimum inhibitory concentration (MIC) criteria of **Cos *et al.*** (2006). For LOE, it was defined as follows: high (MIC ≤ 100 µg/mL), moderate (MIC between 101 and 500 µg/mL), low (MIC between 501 and 1000 µg/mL), and inactive (MIC > 1000 µg/mL); for pure compounds, relevant antibacterial activity was that produced at MIC ≤ 25 µM.

Interpolating survival data in the graphical method (**Ara *et al.***, 2009), we calculated the relevant lethal concentrations (LC_{30} and LC_{50}) of LOE and pinocembrin, at which 30% and 50% of the cell population died, respectively. LC30, at which 70% of the cells survived, was considered a good measure of cell survival.

Antibiotic modulatory activity of extracts and pinocembrin

Relevant concentrations (LC_{30} and LC_{50}) of LOE (LC_{30} = 15.5 µg/mL, LC_{50} = 68.7 µg/mL) and pinocembrin (LC_{30} = 0.053 mM, LC_{50} = 0.097 mM) were selected to study their modulatory effect on the antibacterial activity of the antibiotics. The MIC of the *E. coli* PQ37 strain was determined for the co-assays (LOE + ciprofloxacin and pinocembrin +

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ciprofloxacin). In these mixtures, ciprofloxacin was analyzed within the concentration range indicated above. Bacterial cell cultures in the co-treatments were grown in Eppendorf tubes following the procedure described in the previous section. Similarly, the culture OD600nm measurement was performed as described above.

Determination of phytochemical/antibiotic interactions

For each co-treatment indicated in the previous section, we calculated the Fractional Inhibitory Concentration (FIC) index, which expresses the degree of interaction (**Hall *et al.***, 1983). The FIC is the MIC of the combined samples divided by the MIC of the drug (antibiotic) alone. For two interacting samples, A and B, the sum of the FICs ($\Sigma FIC = FIC_A + FIC_B$) was as follows: $FIC = CA/CMI_A + CB/CMI_B$, where CA and CB are the MICs of the co-assays (sample + antibiotic) and CMI_A and CMI_B are the MICs of samples A and B. The FIC index was interpreted as follows: (1) synergistic effect when $FIC \leq 0.5$; (2) additive or indifferent effect when $FIC > 0.5$ and < 1; and (3) antagonistic effect when $FIC \geq 1$ (**Konaté *et al.***, 2012). To calculate the FIC of LOE and its co-treatments, the MIC of the antibiotic was converted to extract units (µg/mL).

SOS response induction in the studied samples

The SOS response in *E. coli* was evaluated using the SOS Chromotest assay (**Quillardet & Hofnung**, 1985). A bacterial culture grown overnight was diluted (1/10) in fresh LB medium supplemented with ampicillin and tetracycline. The culture was allowed to grow in a Thermomixer heat block at 37°C and 1000 rpm until the optical density at 600 nm reached 0.4 (~10^8 cells/mL). The culture was diluted (1/10) in the same culture medium containing different concentrations of phytochemicals and antibiotics as follows: LOE (2–250 µg/mL), pinocembrin (0.002–0.500 mM), ampicillin (0.27–4.03 mM), and ciprofloxacin (7.1×10^{-5}–3.6×10^{-4} mM). Eppendorf tubes containing the samples were incubated at 8°C for 30 min to allow the incorporation of the compounds into the cells, followed by incubation for 2 h at 37°C with shaking at 1000 rpm in a Thermomixer to induce the SOS response. Each experiment included a negative control (distilled water) and a positive control (10 J/m² UVB, see below). In all cases, a minimum of three independent experiments with four internal replicates were performed.

β-galactosidase (βG) and alkaline phosphatase (AP) activities were analyzed in 96-well plates (Brand GmbH, Germany), as described by **Fuentes *et al.*** (2017). Briefly, for βG activity, cell membranes were disrupted by mixing 135 µL of buffer Z (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 0.1% SDS, and 40 mM β-mercaptoethanol, pH 7.0) with 15 µL of cell culture for 20 min at room temperature. The reaction was initiated by adding 30 µL of ONPG (4 mg mL^{-1} in 0.1 M phosphate buffer, pH 7.0). After 40 min, the enzymatic reaction was stopped by adding 100 µL Na_2CO_3 (1 M). For alkaline phosphatase (AP) activity, cell membranes were disrupted by adding 135 µL of buffer T (0.1% sodium dodecyl sulfate, 1 M Tris-HCl, pH 8.8) to 15 µL of cell culture. The enzymatic reaction was initiated by adding 30 µL of PNPP solution (4 mg mL^{-1} in buffer T). After 40 min, the reaction was stopped by adding 50 µL of HCl (2.5 M). After 5 min, 50 µL of Tris (2 M) was added to restore the color. The absorbance in the βG and AP assays was measured at $\lambda = 420$ nm using a Multiskan GO microplate reader (Thermo Scientific, MA, USA).

βG and AP activities were calculated using the following relationship: enzyme units = $(1000 \times A420)/t$, where A420 is the optical density at $\lambda = 420$ nm, and t is the incubation time (min) with the substrate (ONPG or PNPP). The ratio of βG units to AP units ($R = \beta G/AP$) reflects sulA gene induction, even when protein synthesis is inhibited. The genotoxicity criterion used was the SOS induction factor (I), which represents the normalized sulA gene induction data for each treatment (LOE, pinocembrin, and antibiotics). Therefore, this was considered an indirect measure of primary DNA damage (genotoxicity) induced by the treatments. This parameter was calculated as $I = Rt/Rnt$, where t and nt correspond to the treated and untreated cells, respectively. Samples were classified as non-genotoxic if $I < 1.5$, inconclusive if I was between 1.5 and 2.0, and genotoxic if I was > 2.0, and a clear SOS concentration-response relationship was observed.

SOS response induction by UVB radiation

For comparison, UVB radiation was used as a well-known inducer of the SOS response (**Fuentes *et al.***, 2017). Cultures (0.5 mL) with an optical density (OD600nm) of 0.4 (~10^8 cells/mL) were distributed in 5 cm diameter Petri dishes for irradiation during the assays. The cells were irradiated in the dark using a BS-02 UVA/UVB irradiation chamber equipped with a UV-MAT radiation controller (Opsytec Dr Groebel, Ettlingen, Baden-Württemberg, Germany). This radiation controller continuously measures the irradiance, calculates the irradiation dose, and stops irradiation after the target dose is reached. The dose range studied was between 5 and 180 J/m². The irradiated cultures were transferred back into Eppendorf tubes and incubated for 2 h at 37°C with shaking at 1000 rpm in a thermomixer to induce the SOS response. Each experiment included a negative control (distilled water). β-galactosidase (βG) and alkaline phosphatase (AP) assays, enzyme activity calculation, and the SOS induction factor (I) were performed as previously described. In all cases, a minimum of three independent experiments with four internal replicates were conducted.

SOS response inhibition in Escherichia coli *by phytochemicals*

The inhibition of the SOS response in *E. coli* PQ37 was studied using the co-incubation procedure described previously (**Fuentes *et al.***, 2006). Cells were treated simultaneously with the phytochemicals (LOE or pinocembrin) at the concentrations indicated above and with the SOS inducer (antibiotic or UVB radiation). The concentrations of the SOS inducers used were as follows: ciprofloxacin (9.1 x 10^{-3} mM) and UVB radiation (80 J/m²). After exposure to SOS inducers, the cells were cultured for 2 h at 37°C with shaking at 1000 rpm in a Thermomixer (Eppendorf, São Paulo, Brazil). β-galactosidase (βG) and alkaline phosphatase (AP) assays, enzyme activity calculations, and SOS induction factor (I) were performed as previously described. An SOS inhibitory effect was considered to be demonstrated when a significant reduction in the SOS induction factor (I) by the phytochemical was observed. In all cases, a minimum of three independent experiments with four internal replicates were performed.

Statistical analysis

Average values for OD600nm, MIC, and I, and their corresponding standard errors were calculated. Data normality was assessed using the Shapiro-Wilk test, and homogeneity using the Fmax test. As the treatment data did not always follow a normal distribution, we used the Kruskal-Wallis test for independent samples, and to evaluate the dose-response relationship in the SOS induction experiments, we used Pearson's product-moment correlation analysis. In all analyses, a p-value of < 0.05 was considered significant. Statistical analyses and data plots were performed using the stats and ggplot2 programs, respectively, on the R platform version 4.3.3 (**R Core Team**, 2024).

Results

GC-MS analysis

The main compounds present in the *L. origanoides* extract were determined using GC-MS and are shown in **Figure 1** (right). The GC-MS profile of the *L. origanoides* extract showed 16 peaks, including those with GC relative area < 1%. The extract composition (GC relative area ≥ 1% in parenthesis) was as follows: 1 – α-phellandrene (1.0%). 2 – *ρ*-cymene (6.2%). 3 – limonene (1.0%). 5 – 1,8-cineole (3.5%). 8 – thymol (4.6%). 9 – carvacrol (6.3%). 10 – α-copaene (1.0%). 11 – *trans*-β-caryophyllene (10.9%). 12 – **α**-humulene (4.6%). 13 – γ-muurolene (1.0%). 14 – δ-cadinene (1.3%). 15 – Not identified compound (1.3%). 16 – Pinocembrin (54.9%). The major constituents of the *L. origanoides* extract were the following: pinocembrin (54.9%), trans-β-caryophyllene (10.9%), carvacrol (6.3%), p-cymene (6.2%), thymol (4.6%), and 1,8-cineole (3.5%). These results confirmed pinocembrin as the predominant compound in LOE, validating its selection for individual evaluation.

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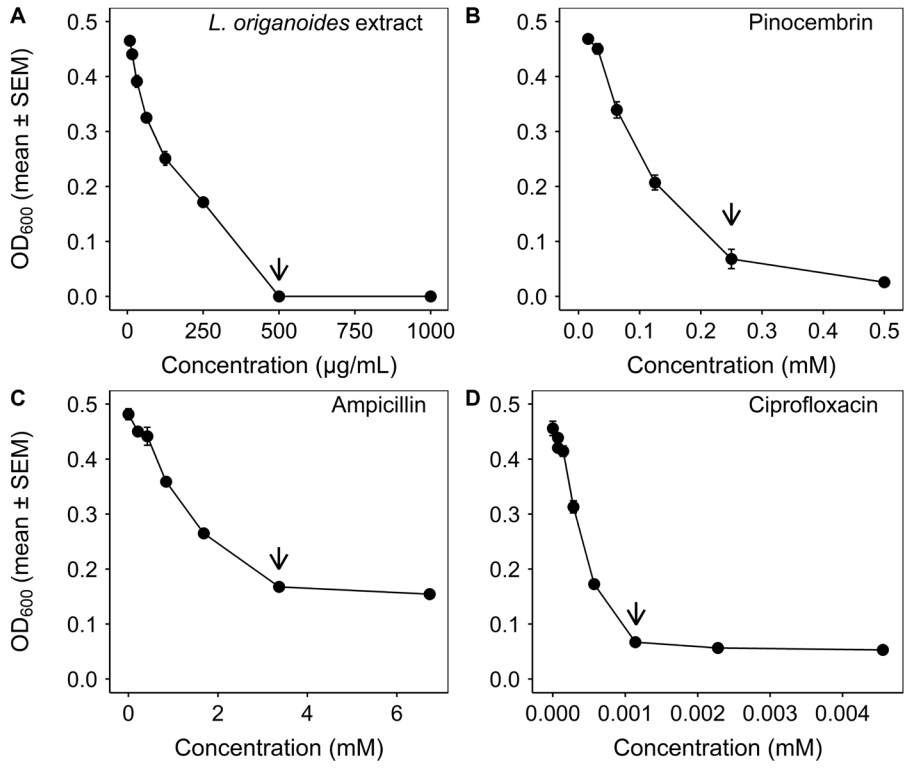

Figure 2. Dose-response curves of *Escherichia coli* PQ37 exposed to phytochemicals and antibiotics. The figure represents the growth inhibition curves of *E. coli* PQ37 at different concentrations of the tested samples: **(A)** LOE, **(B)** pinocembrin, **(C)** ampicillin, and **(D)** ciprofloxacin. The MIC for each compound is indicated by an arrow. Optical density values at 600 nm are presented with their standard errors (n = 3).

Antibacterial activity

The antibacterial activity of LOE, pinocembrin, ciprofloxacin, and ampicillin was first determined to establish reference MIC values for the following co-treatment assays. **Figure 2** shows the concentration-inhibition relationship of bacterial growth following exposure of the *E. coli* PQ37 strain to the studied phytochemicals and antibiotics. The MICs of the phytochemicals and antibiotics were as follows: LOE (500 µg/mL); pinocembrin (0.25 mM); ampicillin (3.36 mM); and ciprofloxacin (0.0011 mM). Thus, the degree of cytotoxicity of these samples was (**Cos *et al.***, 2006): LOE – moderate antibacterial activity (MIC between 101 and 500 µg/mL), pinocembrin – irrelevant antibacterial activity (MIC > 25 µM), ampicillin – irrelevant antibacterial activity (MIC > 25 µM), and ciprofloxacin – relevant antibacterial activity (MIC ≤ 25 µM). These results show a moderate inhibitory capacity on bacterial growth for the phytochemicals and antibiotics studied.

Notably, the MIC of ciprofloxacin was approximately 3000 times lower than that of ampicillin. The dose-response curve for ampicillin not only showed consistently high MIC values (MIC = 3.36 mM), but this value was also observed at relatively higher optical density values (OD600nm ≈ 0.16), indicating a baseline level of bacterial growth under these conditions, which may be associated with β-lactamase production, encoded within the (sfiA::Mud (Ap, lac) cts) genotype of the *E. coli* PQ37 strain. Given these results, ampicillin was excluded from subsequent trials.

Antibiotic modulatory activity of extracts and pinocembrin

To evaluate whether LOE and pinocembrin could modulate ciprofloxacin antibacterial activity, the LC_{50} and LC_{30} lethal concentrations for the phytochemicals were calculated by

interpolating OD600nm data, using the graphical method (**Ara *et al.***, 2009). The following results were obtained: LOE (LC_{50} = 68.7 µg/mL, LC_{30} = 15.5 µg/mL), and pinocembrin (LC_{50} = 0.097 mM, LC_{30} = 0.053 mM). These sublethal concentrations (< MIC) were used in co-assays to evaluate the modulatory capacity of ciprofloxacin's activity (**Table 1**).

At their respective LC_{30} concentrations, LOE (LC_{30} = 15.5 µg/mL) and pinocembrin (LC_{30} = 0.053 mM) did not alter the MIC of ciprofloxacin. Furthermore, these co-assays (No. 1 and 3) showed FIC values = 1, indicating a slight antagonistic effect between the components of the mixtures. At its LC_{50} concentration, LOE (LC_{50} = 68.7 µg/mL) halved the MIC of the antibiotic ciprofloxacin. In this case, the interaction showed an FIC value of ≈ 0.51, indicating an additive or neutral effect. Finally, at its LC_{50} (LC_{50} = 0.097 mM), pinocembrin reduced the MIC of ciprofloxacin by 3.6 times. For this interaction, an FIC of approximately 0.26 was observed, indicating synergism between the components of the mixture. These results clearly indicate the potential of pinocembrin as an antibiotic activity modifier.

SOS response induction by studied samples and UVB radiation

To identify the level of genotoxicity of the inducers and rule out genotoxic activity of the phytochemical compounds, the individual capacity to induce the SOS response of LOE, pinocembrin, ampicillin, ciprofloxacin, and UVB radiation was evaluated. Only two agents, ciprofloxacin and UVB radiation, induced the SOS response in *E. coli* PQ37 (**Figure 3**). Compared to the negative control (untreated), the antibiotic ciprofloxacin exceeded the genotoxicity threshold (I > 2.0) at a concentration of 7.1×10^{-5} mM, significantly increasing the induction of the SOS response ($r = 0.84$, $p < 0.05$) starting at a concentration of 4.9×10^{-4} mM and reaching a maximum (I = 7.5 ± 0.9) at a concentration of 9.1×10^{-3} mM (**Figure 3A**). Remarkably, the MIC (0.0011 mM) of ciprofloxacin fell within the concentration range that induced the SOS response in this strain. Similarly, UVB radiation produced a significant increase in the SOS response ($r = 0.95$, $p < 0.05$), reaching a maximum (I = 12.7 ± 7.9) at a dose of 100 J/m² (**Figure 3B**). A reduction in I values was observed at these concentrations and doses, possibly associated with the increased toxicity of these agents. In contrast, LOE, pinocembrin, and ampicillin did not induce the

Table 1. Minimum Inhibitory Concentration (MIC) and Fractional Inhibitory Concentration (FIC) values for the co-assays studied

Co-assays	MIC			FIC			Concept
	Phytoc.	CIP	Co-assays	FIC_A	FIC_B	ΣFIC	
No. 1 (LOE (15.5 µg/mL) + CIP)	500 µg/mL	0.48 µg/mL	0.48 µg/mL	0.00096	1	1.00096	Antagonist
No. 2 (E (68.7 µg/mL) + CIP)	500 µg/mL	0.48 µg/mL	0.24 µg/mL	0.00048	0.5	0.50048	Indifferent
No. 3 (PIN (0.053 mM) + CIP)	0.25 mM	0.0011 mM	0.0011 mM	0.0045	1	1.00450	Antagonist
No. 4 (PIN (0.097 mM) + CIP)	0.25 mM	0.0011 mM	0.00028 mM	0.00113	0.25	0.25113	Synergist

Note. The table represents the reduction in the MIC of ciprofloxacin when combined in co-assays with phytochemicals at two different concentrations (LC_{30} and LC_{50}, respectively). MIC values are presented for each co-assay and each antibiotic. CIP: ciprofloxacin, PIN: pinocembrin. FICA = MIC of co-assay/MIC of the evaluated compound (LOE or pinocembrin). FICB = MIC of co-assay/MIC of antibiotics. $\Sigma FIC = FIC_A + FIC_B$. FIC values were interpreted as follows: Synergistic effect (FIC ≤ 0.5); additive or neutral effect (FIC between 0.5 and 1); antagonistic effect (FIC > 1).

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SOS response in the test strain (**Table S1,** https://raccefyn.co/index.php/raccefyn/article/view/4124/5405). The results showed that the phytochemicals LOE and pinocembrin are harmless to the *E. coli* PQ37 strain, indicating their potential use as inhibitors of the SOS response in this model. As expected, the production of the β-lactamase enzyme by this strain eliminates the antibiotic ampicillin from the cell, even at cytotoxic concentrations.

SOS response inhibition in Escherichia coli *by phytochemicals*

We studied the ability of LOE and pinocembrin to inhibit the SOS response induced by ciprofloxacin and UVB radiation. **Figure 4A** shows the inhibitory effect of LOE on the SOS response produced in *E. coli* PQ37 by ciprofloxacin (9.1 x 10^{-3} mM) and UVB radiation (80 J/m2); this response shows a clear relationship with the LOE concentration in both cases ($r = -0.85$, $r = -0.91$, $p < 0.05$, respectively). The inhibitory effect was significant ($p \leq 0.05$) starting at 170 µg/mL of LOE against ciprofloxacin and at 62.5 µg/mL against UVB radiation (**Table S1,** https://raccefyn.co/index.php/raccefyn/article/view/4124/5405).

Similarly, **Figure 4B** shows the inhibitory effect of pinocembrin on the SOS response produced in *E. coli* PQ37 by ciprofloxacin (9.1 x 10^{-3} mM) and UVB radiation (80 J/m²). This response showed a clear relationship with the pinocembrin concentration in both cases

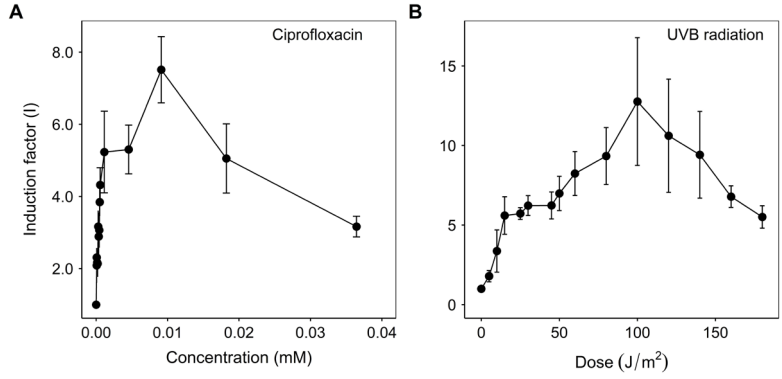

Figure 3. Induction of the SOS response by ciprofloxacin and UVB radiation. The figure shows the induction of the SOS response in *Escherichia coli* by the inducing agents **(A)** ciprofloxacin, and **(B)** UVB radiation. The average values of I and their standard errors are presented (n = 3).

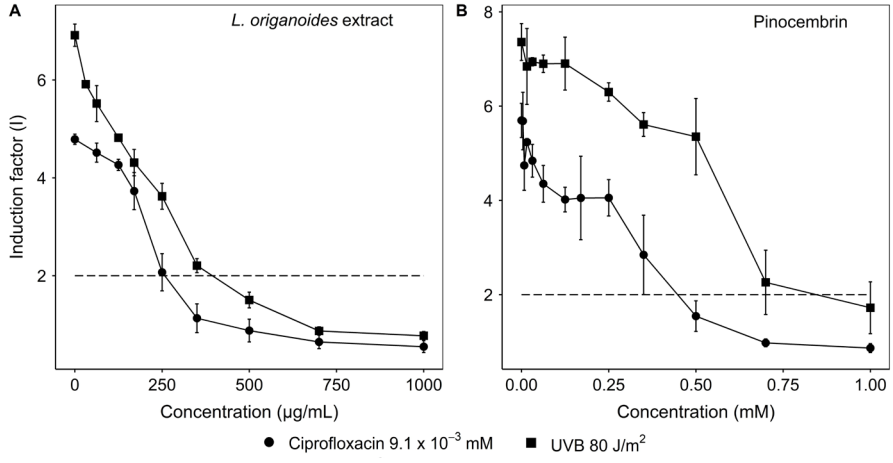

Figure 4. SOS response induction factor for co-treatments with phytochemicals. The figure represents the effect of *Lippia origanoides* extract on the SOS response induced by two different agents. Average I values and their standard errors are presented (n = 3). The dashed line denotes the induction factor above which an inducer is considered genotoxic.

($r = -0.87$, $r = -0.90$, $p < 0.05$, respectively). In this case, the inhibitory effect was significant ($p \leq 0.05$), starting at a pinocembrin concentration of 0.031 mM against ciprofloxacin and 0.35 mM against UVB radiation (**Table S1,** https://raccefyn.co/index.php/raccefyn/article/view/4124/5405). The similarities in concentration-dependent responses in both figures suggest that pinocembrin is largely responsible for the inhibitory effect of LOE on the bacterial SOS response.

Discussion

The relative amounts of pinocembrin, trans-β-caryophyllene, and carvacrol, the main compounds found in the GC-MS analysis, were consistent with previous studies (**Fuentes *et al.***, 2021). As initially mentioned, pinocembrin was the major component, constituting over fifty percent of the LOE mass and could therefore play a significant role in the cytotoxic and inhibitory activity of the extract.

According to established cytotoxic criteria (**Cos *et al.***, 2006), LOE exhibited moderate antibacterial activity (MIC = 500 µg/mL), consistent with a previous finding (**de Castro *et al.***, 2025). This moderate activity may be associated with its predominant component, as detailed in **Figure 1**. Although pinocembrin did not show relevant antibacterial activity against *E. coli* (MIC = 250 µM > 25 µM), this value was comparable in magnitude to that previously observed against *Campylobacter jejuni* (**Klančnik *et al.***, 2019). The interaction between pinocembrin and other molecules present in LOE could explain the moderate cytotoxicity observed in the extract as a whole. Pinocembrin has been shown to modify cell membrane permeability, the oxidation-reduction balance, and ribosomal protein expression within the cell (**Klančnik *et al.***, 2019; **Wu *et al.***, 2022), processes associated with cell death. Other authors who studied the essential oils of *L. origanoides* (**Barreto *et al.***, 2014; **Martínez *et al.***, 2021) found moderate activity (450–750 µg/mL) against *E. coli* and low activity (1000 µg/mL) against *Staphylococcus aureus*, attributing bioactivity to terpenes such as thymol and carvacrol. The antibacterial results obtained here for LOE and pinocembrin experiments are consistent with those observed for other flavonoids (**Górniak *et al.***, 2019), meaning that flavonoid-rich extracts often act through moderate mechanisms.

Additionally, pinocembrin exerted a synergistic effect on ciprofloxacin activity, potentiating it by approximately fourfold, a noteworthy finding. Synergistic effects on ciprofloxacin activity have also been described for extracts of *Lawsonia inermis*, *Punica granatum*, *Arachis hypogaea*, and *Sesamum indicum* against pathogenic bacterial species. These extracts have been shown to reduce the MIC of the antibiotic by half (**Shareef *et al.***, 2020). Another flavonoid, biochanin A, has also demonstrated synergistic activity with ciprofloxacin in resistant isolates of *S. aureus* (**Liu *et al.***, 2011). This effect was not observed in the LOE-ciprofloxacin co-treatments; on the contrary, LOE showed a clear antagonistic effect, possibly related to other components of the extract, which may interfere with antibiotic uptake.

Results from the SOS Chromotest indicated that ciprofloxacin is a strong inducer of the SOS response in *E. coli*, with induction kinetics consistent with those observed with UVB radiation, used as a mutagenic positive control. Ciprofloxacin is known to cause DNA breaks by affecting DNA gyrase and topoisomerase activity, resulting in SOS-mediated mutagenesis in both in vitro (**Mamber *et al.***, 1993; **Cirz *et al.***, 2005; **Wang *et al.***, 2010) and in vivo (**Cirz *et al.***, 2005; **Crane *et al.***, 2021) models. In this study, ciprofloxacin was found to induce the bacterial SOS response at a concentration of 7.11×10^{-5} mM, 15 times lower than its MIC. This concentration was considerably lower than previously reported values (**Wang *et al.***, 2010), indicating possibly strain-specific differences in DNA repair capacity or sensitivity. As expected, UVB radiation also proved to be a potent SOS inducer in *E. coli*, consistent with its widely acknowledged capacity to generate photoproducts that interfere with DNA replication (**Quillardet & Hofnung**, 1985; **Ikehata & Ono**, 2011). Accordingly, both agents provide a basis for evaluating the SOS-inhibitory activity of the tested compounds.

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Both LOE and pinocembrin inhibited the bacterial SOS response induced by the fluoroquinolone ciprofloxacin and UVB radiation, supporting the initial hypothesis that these compounds can interfere with the SOS pathway. The combined treatment with LOE and ciprofloxacin (9.1 x 10^{-3} mM) showed inhibition starting at 170 µg/mL of LOE, while the combined treatment with LOE and UVB radiation (80 J/m²) showed inhibition starting at 62.5 µg/mL of LOE. The observed variation in effective concentrations (170 µg/mL and 62.5 µg/mL) may reflect the distinct potency and nature of the DNA damage caused by each inducer. The combined treatment with pinocembrin and ciprofloxacin (9.1 x 10^{-3} mM) showed inhibition starting at 0.031 mM pinocembrin, while the combined treatment with pinocembrin and UVB radiation (80 J/m²) showed inhibition starting at a pinocembrin concentration of 0.350 mM. As indicated in previous studies using a lower UVB dose (10 J/m²), significant SOS inhibition was achieved with a pinocembrin concentration of 0.015 mM (**Fuentes *et al.***, 2017; **García-Forero *et al.***, 2019). These differing results suggest that the effective pinocembrin concentration responds to the level of damage: at the higher UVB dose used here, a correspondingly higher concentration is required for effective suppression.

These results are like previous reports on SOS inhibitors by natural compounds. Curcumin has been shown to significantly reduce the SOS response induced by sub-inhibitory concentrations of levofloxacin without affecting bacterial survival (**Bellio *et al.***, 2014), similar to the non-cytotoxic effect observed here for pinocembrin. More recently, olivetol, a lichen-derived metabolite, was shown to reduce the expression of core SOS genes, including *lexA*, *recA*, and *sulA* (**Şener & Gökalsın**, 2026). However, unlike pinocembrin, olivetol reduced the ciprofloxacin MIC by half in one of the tested strains, a modest potentiating effect compared to pinocembrin, but a similar inhibition of the SOS signal itself. This contrast suggests that the magnitude of SOS inhibition varies depending on the strain tested and can be related to antibiotic potentiation but is not proportional to the antibacterial effect; in this case, pinocembrin combines both properties more effectively than these other natural SOS inhibitors previously studied.

Although molecular targets have been identified for both curcumin and olivetol, the present study did not directly assess gene targets, and the effect is therefore interpreted as a global inactivation of the SOS pathway rather than a specific interaction. In addition, some observations argue against this inactivation being a consequence of modified membrane permeability or antibiotic uptake. Cytotoxicity did not decrease with increasing pinocembrin concentrations (**Table S1,** https://raccefyn.co/index.php/raccefyn/article/view/4124/5405), as would be expected from membrane damage. Moreover, pinocembrin demonstrated reduced SOS signal at sub-inhibitory concentrations, previously shown not to affect membrane permeability (**Klančnik *et al.***, 2019). Lastly, pinocembrin reduced the SOS response induced by UVB radiation, whose effect does not depend on cellular uptake or membrane permeability. Together, these findings indicate that pinocembrin acts on a common target for both inducers, through a mechanism distinct from its cytotoxic activity, potentially involving the previously reported effects on protein synthesis, or a more specific interaction with components of the SOS response such as the RecA-LexA system, as observed in the aforementioned studies. Clarifying these mechanisms will require further studies, such as quantification of SOS gene expression under pinocembrin treatment.

These results support our central hypothesis: pinocembrin potentiates the ciprofloxacin activity while simultaneously suppressing the SOS response, at concentrations below those required for direct antibacterial activity. Consequently, SOS suppression by this compound could limit the emergence of previously described ciprofloxacin-resistant variants during treatment (**Cirz *et al.***, 2005; **Alam *et al.***, 2016; **Crane *et al.***, 2021) and increase antibiotic susceptibility (**Mo *et al.***, 2016). These results highlight pinocembrin's potential as a SOS-response modulator and reinforce the concept that targeting this pathway represents a complementary strategy against the global rise of antibiotic resistance, as such an approach not only enhances antibacterial efficacy but could also mitigate the risk of resistance development.

Further in vitro and in vivo studies are needed to identify optimal pinocembrin concentrations for utilization, with particular emphasis on pharmacokinetic and pharmacodynamic parameters in complex scenarios, such as clinical and veterinary practice, as well as in environments prone to rapid resistance spread, including the animal production industry. Future work should also validate these findings across additional strains, particularly those of clinical relevance, to address the limitations associated with using a single reporter strain and to directly confirm the compound's ability to limit the development of mutations. Overall, the results presented here provide a preliminary basis supporting the potential of these compounds, whose application as a novel antimicrobial tool will require further experimental validation.

Conclusions

The LOE and pinocembrin compounds alone do not exhibit relevant antibacterial activity against *E. coli*. In combination with the antibiotic ciprofloxacin, pinocembrin demonstrated a discernible synergistic effect on antibiotic activity. Furthermore, this compound proved to be a potent inhibitor of the bacterial SOS response induced by ciprofloxacin, even at lower concentrations than those that produce synergistic antibiotic activity. In this regard, pinocembrin is, preliminarily, a promising molecule for the development of combination therapies aimed at enhancing antibiotic activity, modulating the bacterial SOS response, and potentially the mechanisms that generate bacterial variants resistant to ciprofloxacin. Finally, this study establishes an important precedent for future research in complex systems to validate and enhance antibiotic activity and modulate antibiotic resistance.

Supplementary information

See the supplementary information in https://raccefyn.co/index.php/raccefyn/article/view/4124/5405

Author contributions

ASM and JLF: conceptualization; EES and JLF: funding acquisition; ASM: methodology; ASM, EES, and JLF: formal analysis; ASM: preparation and writing of the original draft; EES and JLF: writing, review, and editing; JLF: project administration. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare there are no conflicts of interest.

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