Research Article

In Vitro Assessment of the Antibacterial, Antioxidant, and Anti-Inflammatory-Related Properties of Naringenin

Sherin I. Ibrahim*, Mohamed Ismail Mostafa, Fady Sayed Youssef

Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, Egypt.

Abstract | Naringenin is a citrus flavonoid with reported antioxidant, anti-inflammatory, and antimicrobial properties. This exploratory in vitro study assessed these activities. Antibacterial activity was tested against methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* by disc diffusion, and against MRSA and *Mycoplasma gallisepticum* by broth microdilution (MIC/MBC). Antioxidant activity was assessed by DPPH scavenging and using apparent catalase and superoxide dismutase (SOD) assay readouts with purified enzymes at a single concentration (25 µM), including enzyme-free blanks. COX-1 (ovine), COX-2 (human recombinant), and soybean 15-LOX inhibition were measured in cell-free assays using indomethacin (COX) and NDGA (15-LOX) as reference inhibitors. Naringenin exhibited concentration-dependent inhibition of MRSA (zones of 8.42–15.62 mm at 25–100 mg/mL), with no detectable inhibition zones against the Gram-negative bacteria under the disc diffusion conditions tested. MIC/MBC were 0.156/0.625 mg/mL for MRSA and 0.313/1.25 mg/mL for *M. gallisepticum*. DPPH scavenging reached 84.1% at 1000 µg/mL (IC_{50} 42.3 µg/mL versus 5.5 µg/mL for ascorbic acid). At 25 µM, naringenin was associated with increases of approximately 23% and 25% in the apparent catalase and SOD assay readouts, respectively. Naringenin inhibited COX-1 and COX-2 (IC_{50} 0.52 and 0.423 µM), 6–7-fold less potently than indomethacin, and inhibited 15-LOX more potently than NDGA (2.80 versus 6.08 µM). Naringenin showed in vitro putative bactericidal activity against MRSA and *M. gallisepticum* (MBC defined by no visible colonies after subculture; carry-over not excluded), but no detectable activity against the tested Gram-negative bacteria in disc diffusion under the conditions used. Naringenin exhibited DPPH radical scavenging activity, weaker COX inhibition than indomethacin, and stronger soybean 15-LOX inhibition than NDGA. The catalase and SOD findings are single-concentration readouts and do not demonstrate enzyme enhancement.

Keywords | Naringenin, Flavonoid, MRSA, *Mycoplasma gallisepticum*, Antibacterial activity, MIC, MBC, Disc diffusion, DPPH, Cyclooxygenase, Lipoxygenase, Antioxidant activity

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***Correspondence** | Sherin Ibrahim Ibrahim, Pharmacology Department, Faculty of Veterinary Medicine, Cairo University, Egypt; **Email:** dr_sheren1982@cu.edu.eg, drsheren1982@gmail.com
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INTRODUCTION

The increasing prevalence of antimicrobial resistance has reduced the effectiveness of conventional antimicrobial therapies and highlighted the need for new antimicrobial agents and complementary treatment strategies (Woo *et al*., 2023; Li *et al*., 2024; Álvarez-Martínez *et al*., 2021). However, natural antimicrobials are not inherently free from resistance development, and their efficacy requires systematic experimental evaluation (Abreu *et al*., 2012; Álvarez-Martínez *et al*., 2021).

Flavonoids are plant-derived bioactive compounds, and naringenin (4′, 5, 7-trihydroxyflavanone) is a major citrus

flavanone and the aglycone of naringin (Singh *et al*., 2023; Uçar and Göktaş, 2023). Naringenin has been investigated for antioxidant, anti-inflammatory, and antimicrobial activities, although most available evidence remains preclinical and further experimental validation is required (Cai *et al*., 2023; Uçar and Göktaş, 2023).

The antibacterial activity of naringenin has been reported against both Gram-positive and Gram-negativ bacteria, including resistant strains (Álvarez-Martínez *et al.,* 2021; Singh *et al*., 2023). In the present study, MRSA was selected as a clinically relevant resistant Gram-positive pathogen, while *Escherichia coli, Klebsiella pneumoniae,* and *Pseudomonas aeruginosa* represented clinically important Gram-negative bacteria. *M. gallisepticum* was additionally included as an important veterinary pathogen associated with respiratory disease and reduced productivity in poultry (Marouf *et al.,* 2022). The clinical importance of this pathogen is further highlighted by reports of macrolide-resistant isolates in Egypt (Ammar *et al*., 2016).

Because antibacterial activity may coexist with antioxidant and anti-inflammatory properties, complementary *in vitro* assays were used in the present study. However, the direct effects of naringenin on purified antioxidant enzyme systems remain incompletely characterized, and its activity against *M. gallisepticum*, a major cause of chronic respiratory disease in poultry, has not, to our knowledge, been reported. This study therefore aimed to evaluate the *in vitro* antibacterial activity of naringenin against clinically and veterinary relevant bacteria, including determination of the minimum inhibitory and bactericidal (mycoplasmacidal for *M. gallisepticum*) concentrations against MRSA and *M. gallisepticum* using CLSI-based and veterinary procedures, together with its antioxidant activity (DPPH radical scavenging and purified-enzyme catalase and SOD assays) and anti-inflammatory-related enzyme inhibitory activity (COX-1, COX-2, and 15-LOX inhibition assays).

MATERIALS AND METHODS

Chemicals and drugs

Naringenin [(±)-naringenin; 4′,5,7-trihydroxyflavanone; CAS No. 67604-48-2] was purchased from Sigma-Aldrich (St. Louis, MO, USA; Catalog No. N5893; purity ≥95%). A naringenin stock solution was prepared by dissolving 100 mg of the compound in 1 mL of dimethyl sulfoxide (DMSO), yielding a concentration of 100 mg/mL. The stock solution was subsequently diluted with the appropriate assay medium or solvent to prepare the required working concentrations. DMSO was used as the corresponding vehicle control where applicable. 2,2-Diphenyl-1-picrylhydrazyl (DPPH; Item No. 14805) and L-ascorbic acid (Item No. 14656) were purchased from Cayman Chemical (Ann Arbor, MI, USA); both reagents had a purity of ≥95%.

Purified catalase from bovine liver (Sigma-Aldrich/Merck, Catalog No. C40) and purified superoxide dismutase from bovine erythrocytes (Sigma-Aldrich/Merck, Catalog No. S7446) were used as the enzyme sources for the CAT and SOD assays, respectively.

The COX (ovine/human) Inhibitor Screening Assay Kit (Catalog No. 560131) and the Lipoxygenase Inhibitor Screening Assay Kit (Item No. 760700) were obtained from Cayman Chemical (Ann Arbor, MI, USA) and used according to the manufacturer's instructions. Purified 15-lipoxygenase from soybean (Glycine max; Item No. 760714) was used for the 15-LOX assay.

Antibacterial activity of naringenin against selected bacterial pathogens

Test organisms and reference guidelines

The antibacterial activity of naringenin was evaluated against methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,* and *Mycoplasma gallisepticum*. All isolates were obtained and identified at the Department of Microbiology, Faculty of Veterinary Medicine, Cairo University, Giza, Egypt. MRSA was confirmed phenotypically using a 30-µg cefoxitin disc. Disc diffusion and broth microdilution testing of the conventional bacteria were performed according to CLSI M02 and CLSI M07, respectively (CLSI, 2024a, 2024b), using the editions available at the time of testing. Susceptibility testing of *M. gallisepticum* was performed using a broth microdilution method adapted from Hannan (2000). *S. aureus* ATCC 25923 was used as the quality-control strain for disc diffusion, whereas *S. aureus* ATCC 29213, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853 were used as quality-control strains for broth microdilution; acceptable QC ranges were taken from CLSI M100 (CLSI, 2024c). Gentamicin and ciprofloxacin were tested as reference antibiotics with each of these QC strains, and the resulting MIC values fell within the CLSI M100 acceptable ranges. The ATCC quality-control strains were used for method verification only; MIC testing was not performed on *E. coli* or *P. aeruginosa* isolates because no inhibition zones were observed.

Preparation of naringenin solutions

Naringenin (100 mg) was dissolved in 1 mL of dimethyl sulfoxide (DMSO) to obtain a 100 mg/mL stock solution. For the disc-diffusion assay, working solutions of 10, 25, 50 and 100 mg/mL were prepared in DMSO. A 10-µL volume of each solution delivered 100, 250, 500, and 1,000 µg of naringenin per disc, respectively. For broth microdilution, the stock solution was diluted tenfold in the appropriate

culture medium to obtain a 10 mg/mL intermediate solution containing 10% DMSO. Twofold serial dilutions were then prepared in the same medium. After mixing 50 µL of each dilution with 50 µL of inoculum, the final naringenin concentrations ranged from 5 to 0.078 mg/mL, and the final DMSO concentration was 5% in the highest-concentration well and decreased twofold across the dilution series. Antibacterial concentrations are expressed in mg/mL (1 mg/mL = 1,000 µg/mL), DPPH concentrations in µg/mL, and the amount loaded on each disc in µg/disc.

Disc diffusion (Kirby–Bauer) assay

Three to five well-isolated colonies from overnight cultures on nutrient agar were suspended in 2 mL of sterile saline and adjusted to a 0.5 McFarland standard (1.5×10^8 CFU/mL). Within 15 min of preparation, the bacterial suspension was evenly spread over Mueller–Hinton agar plates using a sterile swab to obtain a uniform lawn. Sterile 6-mm blank discs were placed on the surface and loaded with 10 µL of each working solution, equivalent to 100, 250, 500 and 1,000 µg of naringenin per disc, and allowed to absorb the solution before incubation. Discs loaded with 10 µL of DMSO served as the negative control, and commercial discs of gentamicin (10 µg) and ciprofloxacin (5 µg) served as positive controls. Plates were incubated aerobically at 37 °C for 18–24 h. Inhibition zones were measured as the total diameter of the zone of complete inhibition, including the 6-mm disc, to the nearest 0.01 mm using a digital caliper. Zone margins were determined visually with the unaided eye. Each concentration was tested in triplicate in independent experiments, and results are expressed as the mean ± standard deviation (SD).

Minimum inhibitory concentration (MIC) for conventional bacteria

The MIC was determined by broth microdilution in cation-adjusted Mueller–Hinton broth according to CLSI. Colonies from overnight cultures on nutrient agar were suspended in saline to a 0.5 McFarland standard and diluted in broth to ≈1×10^6 CFU/mL. In 96-well plates, 50 µL of each naringenin dilution (prepared as described in Section 2.2) was mixed with 50 µL of the bacterial suspension, giving a final volume of 100 µL per well, a final inoculum of 5×10^5 CFU/mL, and final naringenin concentrations of 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 mg/mL. The final DMSO concentration was 5% (v/v) in the highest-concentration well and decreased twofold with each dilution. Each plate included a growth control (inoculum in broth), a vehicle control (5% v/v DMSO in broth with inoculum, equal to the highest DMSO concentration in the test wells), and a sterility control (broth only). Quality-control strains were tested in parallel. Plates were incubated at 37 °C for 18–24 h. The MIC was defined as the lowest concentration showing no visible growth (turbidity). Because naringenin may precipitate at high concentrations, a compound control containing naringenin and medium without bacterial inoculum was run on a separate plate to assess background color and turbidity. All assays were performed in triplicate in independent experiments.

Minimum bactericidal concentration (MBC) for conventional bacteria

Following MIC determination, 10 µL aliquots were collected from wells showing no visible growth, including the MIC and higher concentrations, and subcultured onto Mueller–Hinton agar. After incubation at 37 °C for 18–24 h, plates were examined for the presence of bacterial colonies. The MBC was defined as the lowest naringenin concentration that produced no detectable growth upon subculture of a 10-µL aliquot (no ≥99.9% viable-count reduction criterion was applied, and carry-over of naringenin was not excluded; the MBC is therefore interpreted as putative). All MBC assays were performed in triplicate in independent experiments.

Antimycoplasmal activity against *Mycoplasma gallisepticum*

The isolate was recovered from stocks stored at −70 °C in PPLO broth (supplemented with 10–15% swine serum, 0.25–1% yeast extract, 1% glucose, and phenol red as the pH indicator, pH 7.8), and its viability was confirmed by growth before testing. Fresh cultures were prepared in PPLO broth before each experiment. The field isolate was used for the experimental assay, whereas *M. gallisepticum* S6 (ATCC 15302) was included for method verification. The MIC was determined by broth microdilution adapted from Hannan (2000). Twofold serial dilutions of naringenin were prepared in PPLO broth as described in Section 2.2, and an equal volume of inoculum adjusted to 10^4–10^5 color-changing units (CCU)/mL was added to each well. Final concentrations were 5, 2.5, 1.25, 0.625, 0.313, 0.156, and 0.078 mg/mL, with a final DMSO concentration of 5% (v/v) in the highest-concentration well, decreasing twofold with each dilution. Growth, sterility, and vehicle (5% v/v DMSO in PPLO broth, equal to the highest DMSO concentration in the test wells) controls, and tylosin as a reference antimicrobial, were included. Plates were sealed and incubated at 37 °C for 5 days, until the growth control showed the characteristic color change of the medium. The MIC was defined as the lowest concentration that prevented this color change. All assays were performed in triplicate in independent experiments.

For the minimum mycoplasmacidal concentration (abbreviated MBC in this paper, as for conventional bacteria), 10 µL aliquots from all wells (including the growth and vehicle controls) were subcultured onto PPLO agar without naringenin and incubated at 37 °C under

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humidified conditions (5% CO_2) for 5–7 days. Plates were examined for colony formation under a stereomicroscope. The presence or absence of colony growth was recorded by visual examination. The minimum mycoplasmacidal concentration was defined as the lowest concentration that yielded no detectable colony growth after subculture. Photographs of the subculture plates were not taken. All MBC assays were performed in triplicate in independent experiments.

In Vitro Evaluation of the Antioxidant Potential of Naringenin

DPPH Radical Scavenging Assay Chemicals

Naringenin [(±)-naringenin; 4′,5,7-trihydroxyflavanone; CAS No. 67604-48-2; Catalog No. N5893; purity ≥95%] was purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH; Item No. 14805; purity ≥95%) and L-ascorbic acid (Item No. 14656; purity ≥95%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). Analytical-grade ethanol (99%; El-Gomhouria Co., Cairo, Egypt) and dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA; purity 99.5%) were used as solvents preparation of solutions. The free radical-scavenging activity of naringenin was evaluated using the DPPH assay, with slight modifications to the methods of Blois (1958) and Shimada *et al.* (1992). A 0.1 mM DPPH solution was freshly prepared in ethanol and protected from light. A naringenin stock solution (100 mg/mL) was prepared in DMSO, diluted in ethanol to 1000 µg/mL, and then serially diluted twofold in ethanol to obtain ten sample-solution concentrations (1.953, 3.906, 7.8125, 15.625, 31.25, 62.5, 125, 250, 500, and 1000 µg/mL), with a final DMSO content of ≤1% (v/v) in the sample solutions (≤0.75% in the final reaction mixture). Ascorbic acid, used as the reference antioxidant, was dissolved directly in ethanol and serially diluted twofold to the same concentrations. Because 3 mL of sample solution was mixed with 1 mL of DPPH solution (sample: DPPH, 3:1 v/v), the final DPPH concentration in the reaction mixture was 0.025 mM, and the final concentration of each test compound was 75% of the stated sample-solution concentration. All concentrations reported in this study (Table 3 and Figure 5) refer to the nominal concentrations of the test compounds in the sample solutions before mixing with DPPH. The final concentration of each compound in the reaction mixture was 75% of the corresponding nominal sample-solution concentration.

Experimental Protocol

The reaction mixtures were thoroughly mixed and incubated at room temperature in the dark for 30 min. Absorbance was then measured at 517 nm using a UV-Vis spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) in 1 cm path-length cuvettes, with ethanol used to zero the instrument. The control (0 µg/mL) contained 1 mL of DPPH solution and 3 mL of ethanol, and was prepared under identical conditions. For naringenin, a vehicle control containing ethanol with 1% (v/v) DMSO was also included to exclude any effect of the solvent on DPPH. A sample blank for each concentration (1 mL ethanol + 3 mL sample solution, without DPPH) was included to correct for the intrinsic absorbance of the compounds. Results are expressed as the mean of three independent experiments.

The percentage of DPPH radical-scavenging activity was calculated as:

$$DPPH\ scavenging\ activity\ (\%) = [(A_{control} - (A_{sample} - A_{blank})) / A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control containing DPPH and ethanol, A_{sample} is the absorbance of the reaction mixture containing DPPH and the tested compound, and A_{blank} is the absorbance of the corresponding sample solution (sample blank) without DPPH.

The IC_{50} value, defined as the nominal sample-solution concentration required to scavenge 50% of DPPH radicals, was estimated from the mean scavenging values by linear interpolation between the two tested concentrations immediately below and above 50% scavenging.

$$IC_{50} = C_1 + [(50 - I_1) / (I_2 - I_1)] \times (C_2 - C_1)$$

Where C_1 and C_2 are the concentrations below and above 50% scavenging, respectively, and I_1 and I_2 are the corresponding scavenging percentages. IC_{50} values are reported to one decimal place and are approximate, as they are derived from two bracketing points.

Catalase (CAT) Activity

Catalase (CAT) activity was determined spectrophotometrically according to Aebi (1984), by monitoring the decrease in absorbance at 240 nm due to H_2O_2 decomposition. Purified catalase from bovine liver (Sigma-Aldrich/Merck, Catalog No. C40) was used as the enzyme source. The reaction mixture (3.0 mL final volume) contained 100 mM sodium phosphate buffer (pH 7.0), catalase (30 U per reaction, added as 0.10 mL of a 300 U/mL solution), and naringenin (25 µM) or the corresponding solvent control. Naringenin was prepared as a 100 mg/mL stock in DMSO, diluted 1:100 in ethanol (≈3.67 mM), and added to the reaction to a final concentration of 25 µM. The final solvent concentrations were approximately 0.0068% DMSO and 0.68% ethanol (v/v). Control reactions received the same volume of the DMSO–ethanol mixture without naringenin. The reaction was initiated by adding 1.0 mL of 30 mM H_2O_2 (final concentration 10 mM), and A240 was recorded every 1

s for 60 s at room temperature. The initial reaction rate was calculated from the linear portion of the absorbance decay curve between 2 and 10 s and expressed on a per-minute basis. CAT activity (U/mL of reaction mixture) was calculated as (ΔA240/min × 1000) / (ε 240 × l), where ε240 = 43.6 M^{-1} cm^{-1} and l = 1 cm. One unit corresponds to the decomposition of 1 μmol H_2O_2 per minute.

Each condition was measured in three separately prepared reaction mixtures from a common enzyme stock (technical replicates, n = 3). An enzyme-free blank containing naringenin (25 μM) and all assay reagents except catalase was run in parallel and showed no detectable change in A240 (ΔA240/min ≈ 0), indicating that naringenin did not directly decompose H_2O_2 under the assay conditions. This control does not determine the mechanism underlying the increased apparent catalase activity or exclude a direct interaction between naringenin and catalase.

Superoxide dismutase (SOD) activity

SOD activity was assessed by measuring the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT), according to Beauchamp and Fridovich (1971), with modifications to the reagent concentrations (see below). Purified bovine erythrocyte SOD (Sigma-Aldrich/Merck, Catalog No. S7446; lyophilized powder, ≥4,500 units/mg protein) was used as the enzyme source.

The reaction mixture (3.0 mL final volume) contained 50 mM sodium phosphate buffer (pH 7.8), 0.1 mM EDTA, 12 mM methionine, 50 μM NBT, and 10 μM riboflavin (the original method uses a lower riboflavin concentration, about 2 μM). Purified SOD was added as 0.05 mL of a 20 U/mL solution (1.0 U per reaction), together with naringenin (25 μM) or the corresponding vehicle control. Naringenin was prepared as a 100 mg/mL stock solution in DMSO, diluted 1:100 in ethanol (approximately 3.67 mM), and added to the reaction mixture to give a final concentration of 25 μM. The final solvent concentrations were approximately 0.0068% DMSO and 0.68% ethanol (v/v). Control reactions received the same volume of the DMSO–ethanol vehicle without naringenin.

Riboflavin was added immediately before illumination. Reaction tubes were exposed to white light for 15 min at room temperature. The spectrophotometer was zeroed using non-illuminated reaction mixtures, and absorbance at 560 nm (A560) was measured immediately after illumination.

The following illuminated reactions were prepared in parallel: (i) an enzyme-free, vehicle-only reaction, used as the reference for maximum NBT photoreduction; (ii) SOD plus vehicle (enzyme control); (iii) SOD plus naringenin (25 μM); and (iv) an enzyme-free reaction containing naringenin (25 μM), used to assess the effect of naringenin on NBT photoreduction in the absence of SOD.

The percentage inhibition of NBT photoreduction was calculated relative to the enzyme-free, vehicle-only reaction as follows:

$$\%\ NBT\ inhibition = [(A_control - A_sample) / A_control] \times 100$$

Where A_control is the A560 of the illuminated, enzyme-free, vehicle-only reaction and A_sample is the A560 of the corresponding illuminated reaction.

The enzyme-free naringenin reaction was used to assess the effect of naringenin on NBT photoreduction in the absence of SOD. This control cannot distinguish direct superoxide scavenging by naringenin from a genuine effect on SOD activity. Therefore, it was not considered a definitive correction for SOD-specific activity. Any value calculated using the enzyme-free naringenin reaction as the reference was treated as an exploratory, blank-referenced estimate, not as a definitive measure of SOD activity.

One unit (U) of SOD activity was operationally defined as the amount of enzyme causing 50% inhibition of NBT photoreduction under the specified assay conditions. The apparent SOD readout (U/mL of the added enzyme solution) was calculated as (% NBT inhibition/ 50) × (DF/ V□), where DF is the pre-assay dilution factor (DF= 1 when no dilution was applied) and V□ is the volume (mL) of the SOD-containing sample added to the reaction. This operational unit definition differs from the supplier's cytochrome c/xanthine oxidase-based unit definition. Calculations were applied only to reactions within the approximate linear inhibition range (≈20–80%).

Each condition was measured in three separately prepared reaction mixtures from a common enzyme stock (technical replicates, n = 3).

Rationale for single-concentration screening

Naringenin was evaluated at one predefined concentration (25 μM) to characterize its direct effect on the readout of the purified CAT and SOD assay systems. This design represents an initial single-concentration screening comparison rather than a concentration–response or IC_{50} experiment. Reliable estimation of an IC_{50} requires measurement across a series of inhibitor concentrations spanning the response range, followed by fitting of an appropriate concentration–response model (Brooks *et al.*, 2012). The CAT and SOD results are therefore reported as apparent assay readouts under the stated assay conditions;

SOD readouts are expressed in assay-derived U/mL and should not be interpreted as evidence of increased enzyme activity.

In Vitro evaluation of the anti-inflammatory-related enzyme inhibitory activity of naringenin

COX-1 and COX-2 inhibition assay

COX-1 and COX-2 inhibition by naringenin was evaluated using the COX (ovine/human) Inhibitor Screening Assay Kit (Item No. 560131; Cayman Chemical, Ann Arbor, MI, USA), according to the manufacturer's instructions. The kit contains ovine COX-1 and human recombinant COX-2. COX-derived prostaglandin H_2 (PGH_2) was reduced with stannous chloride to a stable prostanoid product, which was quantified using the prostaglandin enzyme immunoassay (EIA) supplied with the kit.

Concentration ranges were selected to bracket the expected IC_{50} values. Indomethacin was tested at final concentrations of 0.050, 0.100, 0.250, 0.500, 1.000, and 2.000 µM for COX-1, and 0.005, 0.010, 0.025, 0.050, 0.100, and 0.250 µM for COX-2. Naringenin was tested at final concentrations of 0.050, 0.100, 0.250, 0.500, 1.000, and 2.000 µM for both isoforms. Test compounds were dissolved in DMSO; the final DMSO concentration did not exceed 1% (v/v), and the same volume of vehicle was added to the corresponding control reactions.

For each isoform, the test compound or vehicle was incubated with the enzyme, heme, and reaction buffer for 10 min at 37°C. The reaction was initiated by adding arachidonic acid and allowed to proceed for 2 min at 37°C. Enzymatic activity was then stopped by adding the kit's stannous chloride solution. The resulting COX-derived prostanoids were quantified by EIA according to the kit protocol.

Percentage inhibition was calculated relative to the corresponding uninhibited enzyme control after background correction. Each concentration was tested in three independent experiments (n = 3). IC_{50} values were estimated by nonlinear regression of percentage inhibition against the logarithm of compound concentration using GraphPad Prism and are reported as mean ± SD.

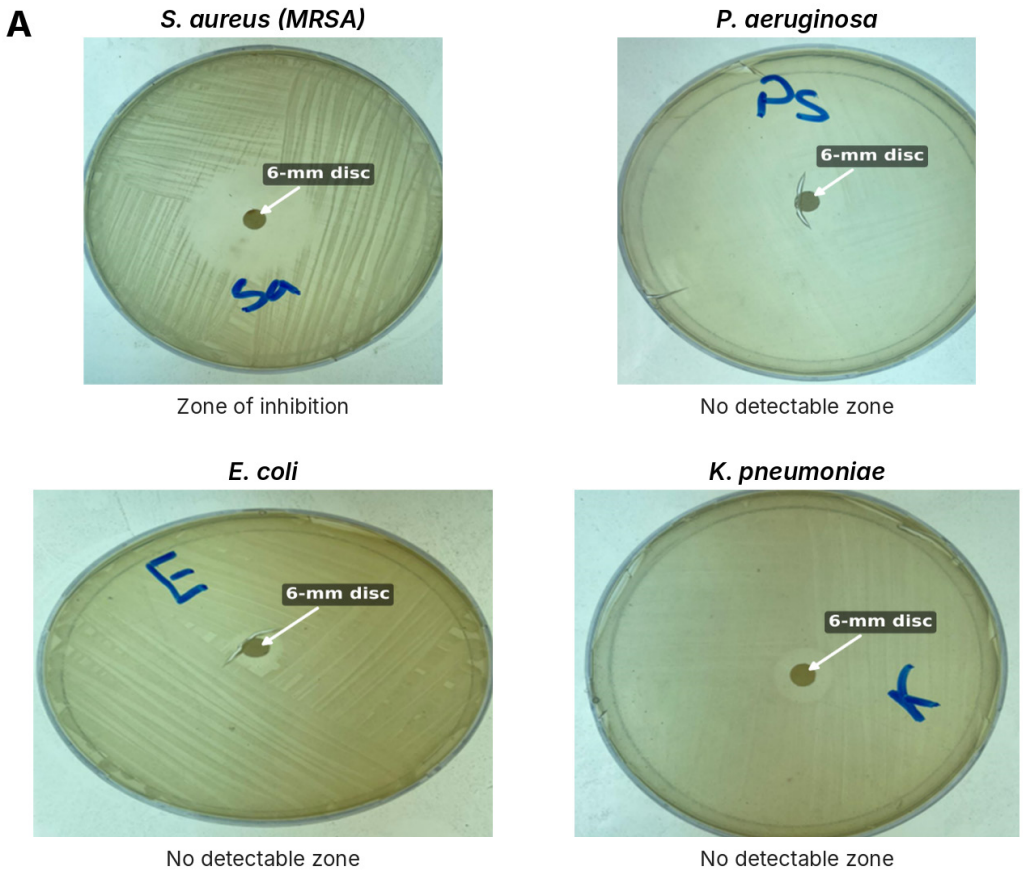
15-Lipoxygenase (15-LOX) inhibition assay

15-LOX inhibitory activity of naringenin was evaluated using the Lipoxygenase Inhibitor Screening Assay Kit (Item No. 760700; Cayman Chemical) according to the manufacturer's instructions, following a previously described kit-based approach (Omar *et al.*, 2020). The enzyme was purified soybean (Glycine max) 15-lipoxygenase (Item No. 760714), diluted 1:100 in 1× assay buffer. Naringenin was tested at final in-well concentrations of 0.01, 0.1, 1, 10, and 100 µM. Nordihydroguaiaretic acid (NDGA) was used as the reference 15-LOX inhibitor and was evaluated under identical assay conditions at final in-well concentrations of 0.1, 0.5, 1, 5, 10, and 50 µM, selected to bracket its expected IC_{50}. All concentrations are expressed as final in-well concentrations in the 110 µL reaction volume, prior to addition of the chromogen. Test compounds were dissolved in DMSO; the final DMSO concentration did not exceed 1% (v/v) in any well, and an equivalent volume of vehicle was added to the control wells.

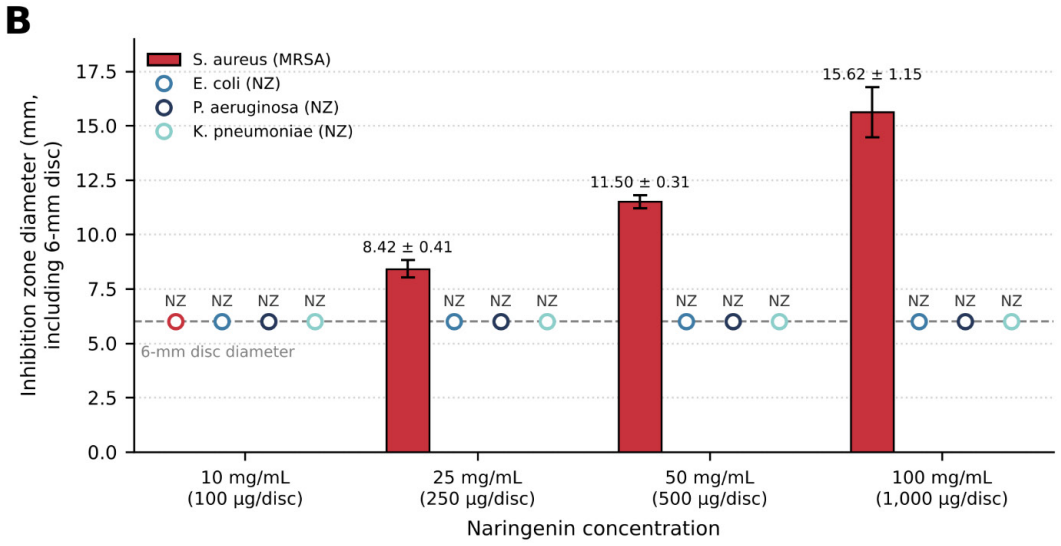
For each test well, 10 µL of naringenin or NDGA solution was mixed with 90 µL of diluted enzyme and preincubated for 5 min at room temperature. The reaction was initiated by adding 10 µL of 1 mM arachidonic acid (final concentration ≈ 91 µM in 110 µL). The plate was incubated for 10 min on an orbital shaker, after which 100 µL of freshly prepared chromogen was added, followed by shaking for 5 min. Absorbance was read at 490–500 nm. Blank, vehicle, enzyme (100% initial activity), and inhibitor-control wells were included. Percentage inhibition was calculated relative to the uninhibited enzyme control, and IC_{50} values were estimated by nonlinear regression of percentage inhibition against the logarithm of compound concentration using GraphPad Prism. Each concentration was measured in three independent experiments (n = 3).

Statistical analysis

Data are presented as mean ± SD. For the enzyme assays (CAT and SOD), n= 3 refers to technical replicates (separately prepared reaction mixtures from a common enzyme stock). For the DPPH, COX-1, COX-2, and 15-LOX assays, n = 3 refers to independent experiments. For the COX-1, COX-2, and 15-LOX concentration–response assays, an IC_{50} value was estimated separately for each independent experiment by nonlinear regression of percentage inhibition against the logarithm of compound concentration using GraphPad Prism (GraphPad Software). Comparisons between two groups were performed using an unpaired, two-tailed Welch's t-test. For CAT and SOD, which are technical replicates from a common enzyme preparation and therefore not independent, no inferential test was applied and values are reported descriptively. For the concentration–response assays, Welch's t-test was applied to the IC_{50} values obtained from individual experiments for naringenin and the corresponding reference compound; the fitted parameters from a single pooled curve were not compared. A p value < 0.05 was considered statistically significant ($p < 0.05$, $p < 0.01$, $p < 0.001$). No IC_{50} was estimated for the single-concentration CAT and SOD experiments. DPPH IC_{50} values were estimated by linear interpolation of the mean scavenging values (Section 3.1) and were not compared statistically.



Representative plates, 100 mg/mL naringenin (1,000 µg/disc), Mueller–Hinton agar, 37 °C, 18–24 h. Photographs unaltered apart from scaling; handwritten plate codes: Sa, Ps, E, K. Controls (DMSO, gentamicin, ciprofloxacin) are reported in the text and Table 1.



NZ = no detectable inhibition zone. Open circles sit at the 6-mm disc diameter (dashed line); they are not 0-mm measurements. Bars: mean ± SD, n = 3 independent experiments.

Figure 1: Antibacterial activity of naringenin against methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae)* assessed by the disc diffusion (Kirby–Bauer) method. (A) Representative plates at 100 mg/mL (1,000 µg/disc); the 6-mm disc is indicated. (B) Inhibition-zone diameters (mean ± SD, n = 3). Naringenin showed concentration-dependent activity against MRSA, with inhibition zones of 8.42 ± 0.41, 11.50 ± 0.31, and 15.62 ± 1.15 mm at 25, 50, and 100 mg/mL (250, 500, and 1,000 µg/disc), respectively; no zone was observed at 10 mg/mL (100 µg/disc). No detectable inhibition zones were observed against the Gram-negative bacteria under the disc diffusion conditions tested (NZ, no detectable zone; plotted as open circles at the 6-mm disc diameter, not as 0-mm measurements); MIC was not determined, and limited solubility and restricted agar diffusion may have affected the results. Zone diameters include the 6-mm disc.

This study involved laboratory-based experiments using previously obtained microbial isolates and commercially available purified enzymes and assay reagents. No animals or human participants were involved in the study, and no biological samples were collected specifically for the purposes of this research. Therefore, ethical approval for animal or human experimentation was not required.

RESULTS

Antibacterial activity of Naringenin

Naringenin exhibited antibacterial activity against MRSA, producing inhibition zones of 8.42 ± 0.41, 11.50 ± 0.31, and 15.62 ± 1.15 mm at 25, 50, and 100 mg/mL, respectively; no inhibition zone was observed at 10 mg/mL. No detectable inhibition zones were observed against the Gram-negative bacteria (*E. coli, P. aeruginosa, and K. pneumoniae*), under the disc diffusion conditions tested, at any concentration (Table 1, Figure 1). The DMSO negative control produced no inhibition zone, and gentamicin (10 µg) and ciprofloxacin (5 µg) produced inhibition zones within the acceptable CLSI quality-control ranges for the reference strain. All quality-control strains (*S. aureus* ATCC 25923 and ATCC 29213, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC 27853) gave results within the acceptable ranges. For *M. gallisepticum*, the reference strain S6 (ATCC 15302) and tylosin gave the expected results. The growth and vehicle (5% DMSO) controls showed growth, whereas the sterility controls remained clear.

Table 1: Antimicrobial activity of naringenin against four conventional bacteria using the disc diffusion method.

Microorganism	Inhibition zone of Naringenin (mm)			
	10 mg/mL (100 µg/disc)	25 mg/mL (250 µg/disc)	50 mg/mL (500 µg/disc)	100 mg/mL (1,000 µg/disc)
MRSA	No zone	8.42 ± 0.41	11.50 ± 0.31	15.62 ± 1.15
Escherichia coli	No zone	No zone	No zone	No zone
Pseudomonas aeruginosa	No zone	No zone	No zone	No zone
Klebsiella pneumoniae	No zone	No zone	No zone	No zone

Values are expressed as mean ± SD of three independent experiments. The tested naringenin concentrations were 10, 25, 50, and 100 mg/mL, corresponding to 100, 250, 500, and 1,000 µg/disc, respectively. Inhibition-zone diameters are expressed in millimeters and include the 6-mm disc diameter. "No zone" indicates no inhibition zone beyond the edge of the disc. *M. gallisepticum* was not tested by disc diffusion; it was evaluated by broth microdilution only (Table 2).

Based on the disc diffusion results, MRSA was selected for further quantitative evaluation by broth microdilution because it showed measurable inhibition zones at more than one concentration. *M. gallisepticum*, for which disc diffusion was not performed, was evaluated directly by broth microdilution as a veterinary pathogen of interest. MIC and MBC were not determined for *E. coli*, *P. aeruginosa*, or *K. pneumoniae*, as no inhibition zones were detected at any tested concentration..

For MRSA, the MIC and MBC of naringenin were 0.156 and 0.625 mg/mL, respectively. For *M. gallisepticum*, the MIC and minimum mycoplasmacidal concentration (reported as MBC) were 0.313 and 1.25 mg/mL, respectively. The MBC/MIC ratio was 4 for both organisms, consistent with putative bactericidal activity under the assay conditions. Because MBC was defined as the absence of visible colonies after subculture of 10 µL aliquots, rather than a ≥99.9% reduction in viable counts, and antimicrobial carry-over was not excluded, this designation is provisional. MIC and MBC results are presented in Table 2 and Figures 2–4. MBC values were determined by visual examination of the subculture plates; representative images of these plates are not available.

Table 2: Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of naringenin against MRSA and *M. gallisepticum*.

Microorganism	Observed MIC (mg/mL)	Observed MBC (mg/mL)
MRSA	0.156	0.625
Escherichia coli	–	–
Pseudomonas aeruginosa	–	–
Klebsiella pneumoniae	–	–
M. gallisepticum	0.313	1.25

Note: Values are observed (modal) MIC/MBC values from three independent experiments, not means. They are the lowest concentrations that inhibited visible growth (MIC) or yielded no visible colonies after subculture of 10 µL (MBC); no ≥99.9% killing criterion or carry-over control was applied, so bactericidal activity is putative. The MBC/MIC ratio (4 for both organisms) is therefore interpreted as indicative only. For *M. gallisepticum,* MBC denotes the minimum mycoplasmacidal concentration. A dash (–) for *E. coli, P. aeruginosa, and K. pneumoniae* indicates that MIC/MBC were not determined because no inhibition zones were observed in the disc-diffusion screening. Disc diffusion was not performed for *M. gallisepticum;* this organism was evaluated directly by broth microdilution.

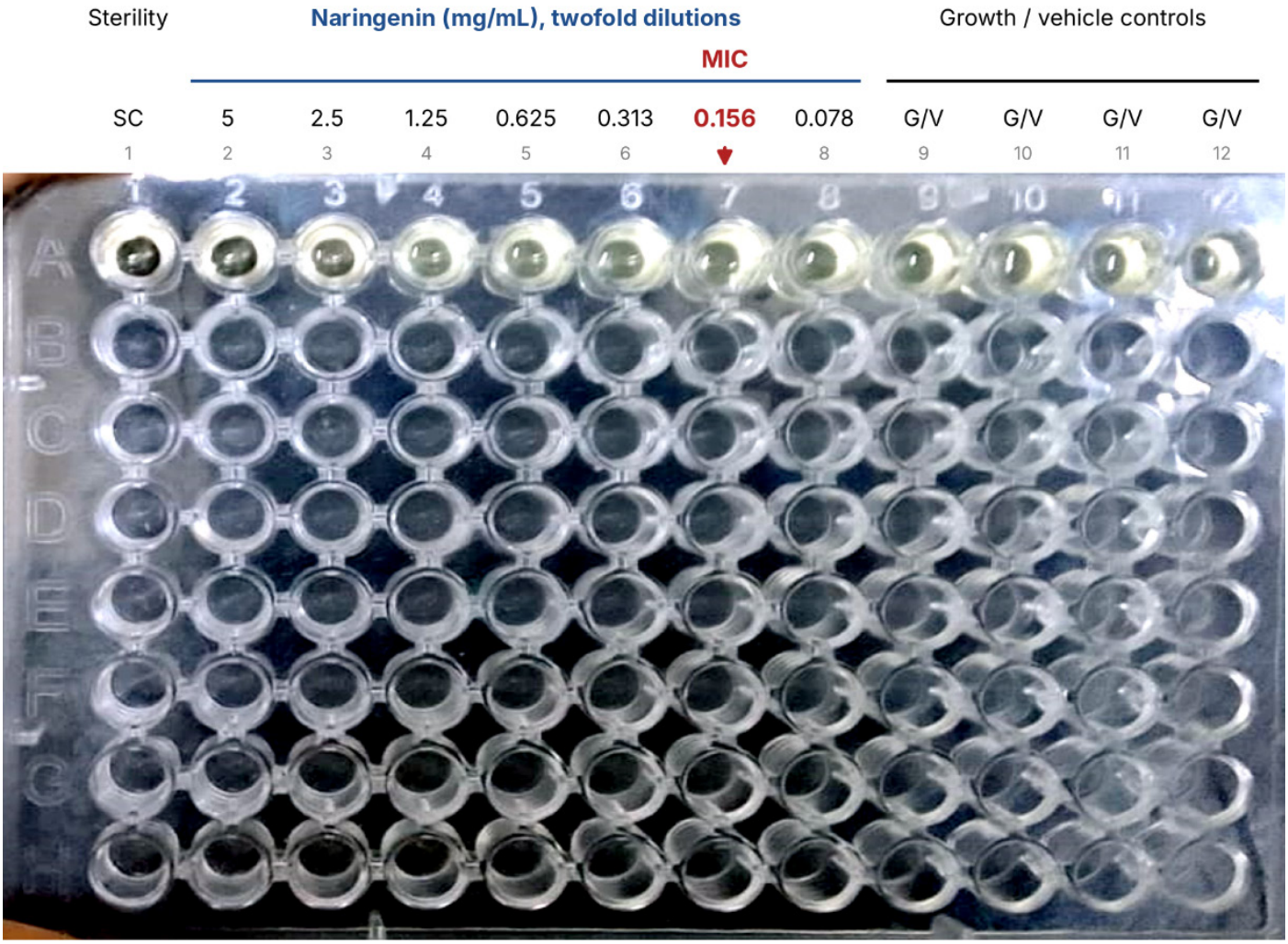
In vitro evaluation of the antioxidant potential of Naringenin

DPPH free radical-scavenging activity of Naringenin

Naringenin exhibited concentration-dependent DPPH radical-scavenging activity, increasing from 15.6% at 1.953 µg/mL to 84.1% at 1000 µg/mL (Figure 5). Ascorbic acid, the reference antioxidant, exhibited a

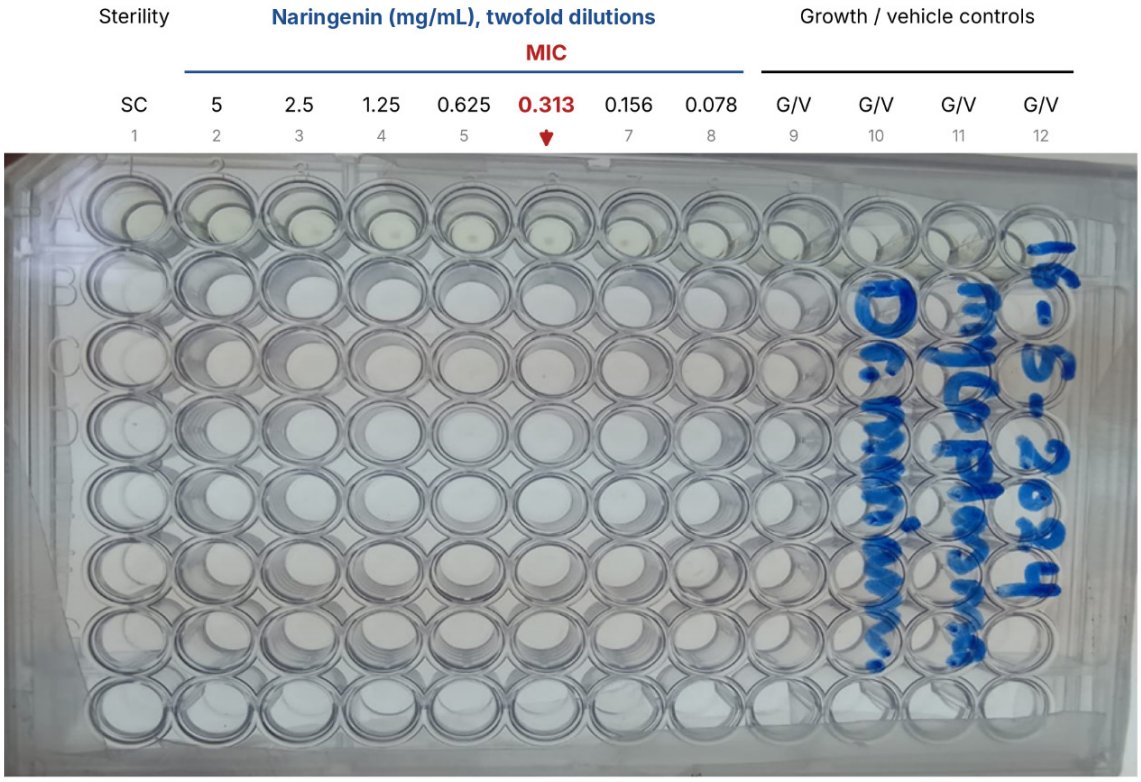
similar concentration-dependent response but consistently showed greater activity, increasing from 36.8% to 97.5% over the same range. At 31.25 µg/mL, naringenin scavenged 47.2% of the radicals and reached 55.1% at 62.5 µg/mL, so 50% scavenging fell between these two concentrations. For ascorbic acid, 50% scavenging fell between 3.906 µg/mL (46.1%) and 7.8125 µg/mL (55.9%).

The IC_{50} values were estimated by linear interpolation between the two concentrations bracketing 50% scavenging (Table 3; the calculations are given in the table footnote). Based on nominal sample-solution concentrations, the IC_{50} of naringenin was 42.3 µg/mL, compared with 5.5 µg/mL for ascorbic acid, indicating that naringenin was approximately 7.8-fold less potent than the reference antioxidant. Because the sample solution and DPPH reagent were mixed at a 3:1 (v/v) ratio, the final IC_{50} values in the reaction mixture were 31.7 µg/mL for naringenin and 4.1 µg/mL for ascorbic acid. All concentrations are expressed as nominal sample-solution concentrations, and values represent the mean of three independent experiments.



Photograph of the plate (row A) with contrast adjusted uniformly across the entire image; no region was altered. Annotations are digital and placed outside the photograph. SC, sterility control (well 1); wells 2–8, naringenin 5–0.078 mg/mL; G/V, growth and vehicle (5% DMSO) controls (wells 9–12). MIC = 0.156 mg/mL (well 7). MBC (0.625 mg/mL) comes from subculture results not shown and is not assessable from this photograph.

Figure 2: Determination of the MIC and MBC of naringenin against MRSA by broth microdilution. Well 1 is the sterility control; wells 2–8 contain twofold serial dilutions of naringenin (5 to 0.078 mg/mL) inoculated with MRSA; wells 9–12 are the growth and vehicle (5% DMSO) controls, all showing visible growth. The MIC was the lowest concentration with no visible growth (well 7, 0.156 mg/mL). The compound control (naringenin in broth without inoculum) was run on a separate plate. The MBC (0.625 mg/mL, well 5) was determined by subculture onto Mueller–Hinton agar (data not shown); this MBC is an operational endpoint from subculture and is not demonstrated by the photograph. The photograph is illustrative only; MIC endpoints were read visually on the plate, and contrast in the photograph is limited. Contrast was adjusted uniformly across the entire image, and digital labels are placed outside the image. The image is from one of three independent experiments; the same MIC (0.156 mg/mL) was obtained in all three.



Unaltered photograph of the plate (row A); annotations are digital and placed outside the photograph. Handwritten marking on the plate (date, organism, "dilutions") is original laboratory labeling. SC, sterility control (well 1); wells 2–8, naringenin 5–0.078 mg/mL; G/V, growth and vehicle (5% DMSO) controls (wells 9–12). MIC = 0.313 mg/mL (well 6). MBC is not assessable from this photograph.

Figure 3: Determination of the MIC of naringenin against *M. gallisepticum* by broth microdilution. Well 1 is the sterility control; wells 2–8 contain twofold serial dilutions of naringenin (5 to 0.078 mg/mL) inoculated with *M. gallisepticum*; wells 9–12 are the growth and vehicle (5% DMSO) controls. The MIC was the lowest concentration preventing the characteristic color change of the PPLO broth (well 6, 0.313 mg/mL). The minimum mycoplasmacidal concentration (MBC, 1.25 mg/mL, well 4) was determined by subculture onto PPLO agar and visual examination of colony growth (images not shown); this value is an operational endpoint from subculture and cannot be confirmed visually from the photograph. The photograph is unaltered and illustrative only (MIC endpoints were read visually on the plate); digital labels are placed outside the image, and the handwriting on the plate is original laboratory labeling. The image is from one of three independent experiments; the same MIC (0.313 mg/mL) was obtained in all three.

Table 3: DPPH radical-scavenging IC_{50} values of naringenin and ascorbic acid, based on nominal sample-solution concentrations and estimated by linear interpolation.

Compound	Bracketing points (µg/mL → % scavenging)	IC_{50} (µg/mL; nominal, interpolated)
Naringenin	31.25 → 47.2%; 62.5 → 55.1%	42.3
Ascorbic acid	3.906→ 46.1%; 7.8125→55.9%	5.5

IC_{50} values are based on nominal sample-solution concentrations before mixing with DPPH and were estimated by linear interpolation between the two bracketing concentrations (approximate values). The corresponding final IC_{50} values in the reaction mixture (75% of nominal; sample solution:DPPH = 3:1, v/v) are 31.7 µg/mL for naringenin and 4.1 µg/mL for ascorbic acid. IC_{50} was calculated by linear interpolation between the two concentrations bracketing 50% scavenging: $IC_{50} = C_1 + [(50 - I_1)/(I_2 - I_1)] \times (C_2 - C_1)$. Naringenin: 31.25 + [(50 – 47.2)/ (55.1 – 47.2)] × (62.5 – 31.25) = 42.3 µg/mL. Ascorbic acid: 3.906 + [(50 – 46.1)/ (55.9 – 46.1)] × (7.8125 – 3.906)= 5.5 µg/mL.

Superoxide dismutase (SOD) and catalase (CAT) activities

The effects of naringenin on catalase and SOD assay readouts were evaluated using purified enzymes (Table 4 and Figure 6). Because commercial purified enzymes were used rather than cells or tissues, these experiments reflect changes in assay readouts in the presence of naringenin and do not indicate induction of an endogenous antioxidant response.

At the single screening concentration of 25 µM, naringenin increased the apparent catalase activity to 12.95 ± 0.21 U/mL, compared with 10.50 ± 0.17 U/mL in the control (+23.3%). Enzyme-free naringenin blanks showed no detectable change in A240 (ΔA240/min ≈ 0), indicating that naringenin did not directly decompose H_2O_2 under these conditions. However, the mechanism underlying this increase was not investigated, and compound–enzyme interactions, solvent effects, and other analytical effects

were not excluded. The approximately 23% difference is therefore not interpreted as confirmed activation of catalase.

Table 4: Effect of naringenin (25 µM) on the apparent readouts of purified SOD and catalase assays.

Parameter	Naringenin (25 µM)	Control (vehicle)
Apparent SOD assay readout, uncorrected (U/mL of added enzyme solution) [a]	28.01 ± 0.14	22.50 ± 0.30
Apparent SOD assay readout, blank-referenced exploratory estimate (U/mL of added enzyme solution) [b]	≈ 26	22.50
Apparent catalase activity (U/mL of reaction mixture) [c]	12.95 ± 0.21	10.50 ± 0.17

Values are mean ± SD (n = 3 technical replicates). Control = vehicle (0.0068% DMSO and 0.68% ethanol, v/v). Descriptive values only; no inferential test was applied because the replicates are technical. [a] Calculated against the vehicle-only, enzyme-free reaction (A_control); includes the direct effect of naringenin on NBT photoreduction. [b]Calculated against the enzyme-free naringenin blank, which was ≈14% lower than the vehicle blank; an exploratory estimate from mean values, without SD; not a definitive correction for SOD-specific activity. [a],[b] U/mL of the added enzyme solution. [c] U/mL of the reaction mixture; therefore SOD and CAT readouts come from separate assays with different unit definitions and are not directly comparable.

Table 5: COX-1 (ovine) and COX-2 (human recombinant) concentration series and IC_{50} values (n = 3).

Assay	Concentration series (µM)	IC_{50} (µM), mean ± SD	p-value vs indomethacin
Naringenin – COX-1	0.050, 0.100, 0.250, 0.500, 1.000, 2.000	0.520 ± 0.030	0.0014
Naringenin – COX-2	0.050, 0.100, 0.250, 0.500, 1.000, 2.000	0.423 ± 0.042	0.0042
Indomethacin – COX-1	0.050, 0.100, 0.250, 0.500, 1.000, 2.000	0.071 ± 0.002	—
Indomethacin – COX-2	0.005, 0.010, 0.025, 0.050, 0.100, 0.250	0.069 ± 0.005	—

Values are mean ± SD. IC_{50}, half-maximal inhibitory concentration of COX-1 or COX-2 activity, determined from the concentration series shown for each compound. n= 3 independent experiments per compound and isoform. p-values are for the comparison of each naringenin IC_{50} against the indomethacin IC_{50} for the same enzyme isoform (COX-1 or COX-2), using a two-tailed Welch's unpaired t-test on the experiment-level IC_{50} values; p-values are given to four decimal places. —, not applicable (reference compound).

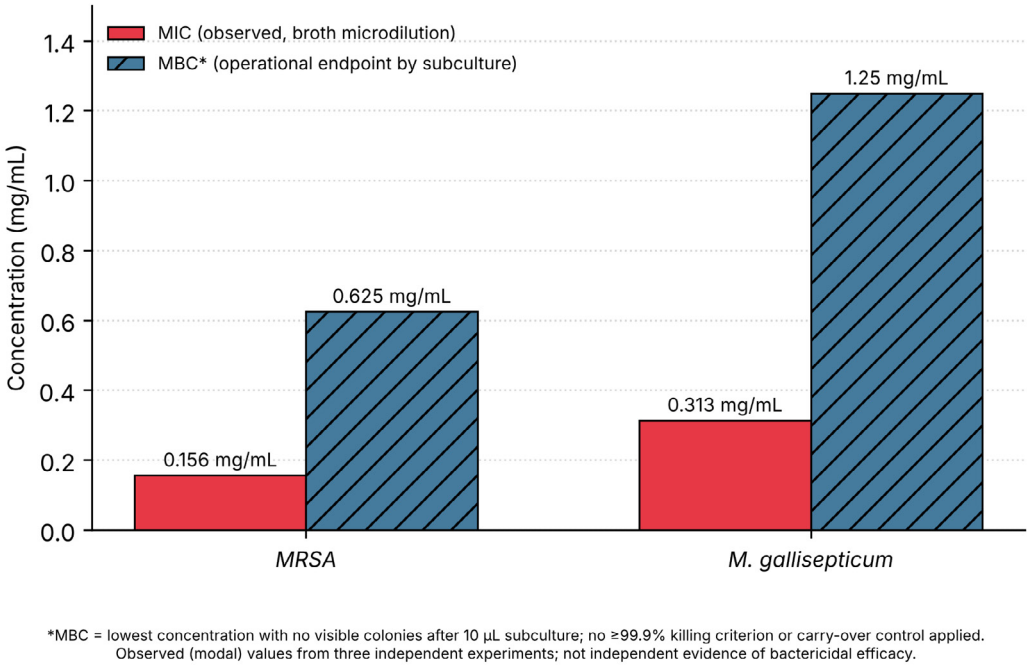
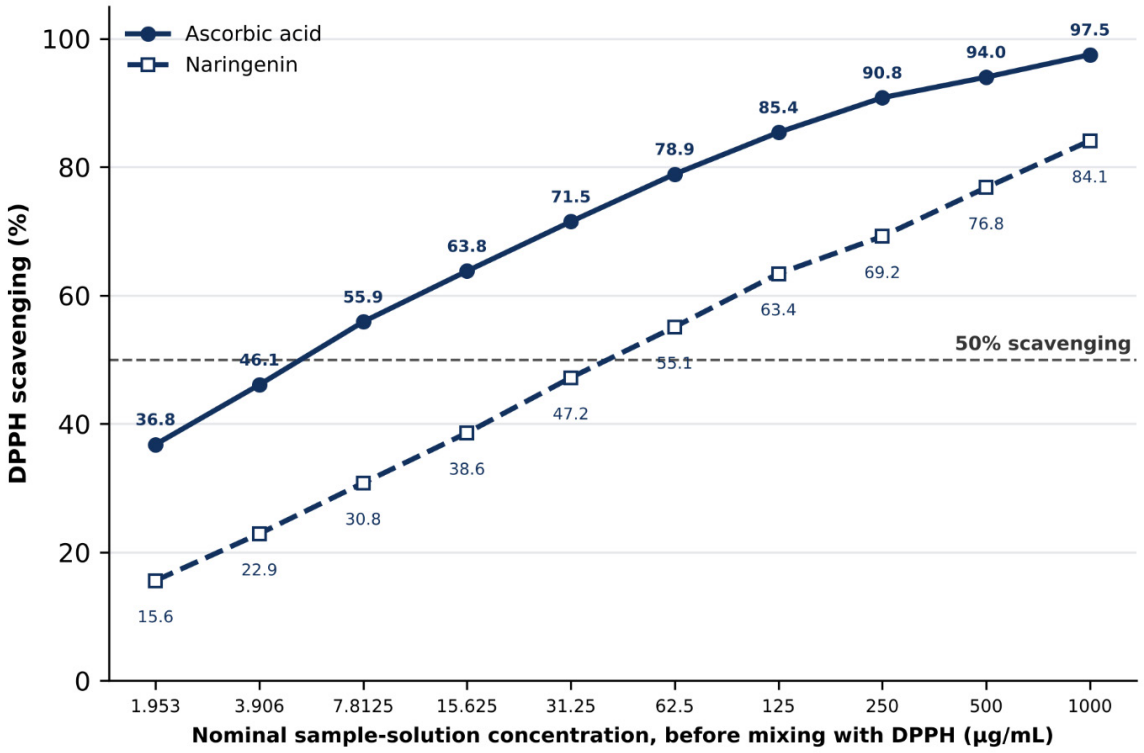


Figure 4: MIC and MBC values of naringenin against MRSA and *M. gallisepticum* determined by broth microdilution. Naringenin showed putative bactericidal activity under the assay conditions against both organisms (MBC/MIC ratio = 4): MRSA (MIC 0.156 mg/mL; MBC 0.625 mg/mL) and *M. gallisepticum* (MIC 0.313 mg/mL; minimum mycoplasmacidal concentration 1.25 mg/mL). MIC/MBC were not determined for *E. coli, P. aeruginosa, and K. pneumoniae* because no inhibition zones were observed. MBC values are operational endpoints determined by subculture (no ≥99.9% killing criterion applied), so the bars do not independently verify bactericidal efficacy. Values (mg/mL) are the modal values from three independent experiments.

Table 6: IC_{50} values of naringenin and nordihydroguaiaretic acid (NDGA) against soybean 15-LOX (assay-specific comparison).

Assay	Concentration series (μM)	IC_{50} (μM), mean ± SD
Naringenin – soybean 15-LOX	0.01, 0.1, 1, 10, 100	2.80 ± 0.02
NDGA – soybean 15-LOX	0.1, 0.5, 1, 5, 10, 50	6.08 ± 0.18

Data are presented as mean ± SD of three independent experiments (n= 3). Naringenin versus NDGA: p = 0.0009 (two-tailed Welch's unpaired t-test on experiment-level IC_{50} values, n = 3 per group). The enzyme is soybean (Glycine max) 15-LOX, not a mammalian enzyme, and the comparison with NDGA is specific to this assay and its conditions. p-values are given to four decimal places. IC_{50} values were estimated by nonlinear regression of percentage inhibition against the logarithm of compound concentration. Concentrations are final in-well concentrations. 15-LOX, 15-lipoxygenase.



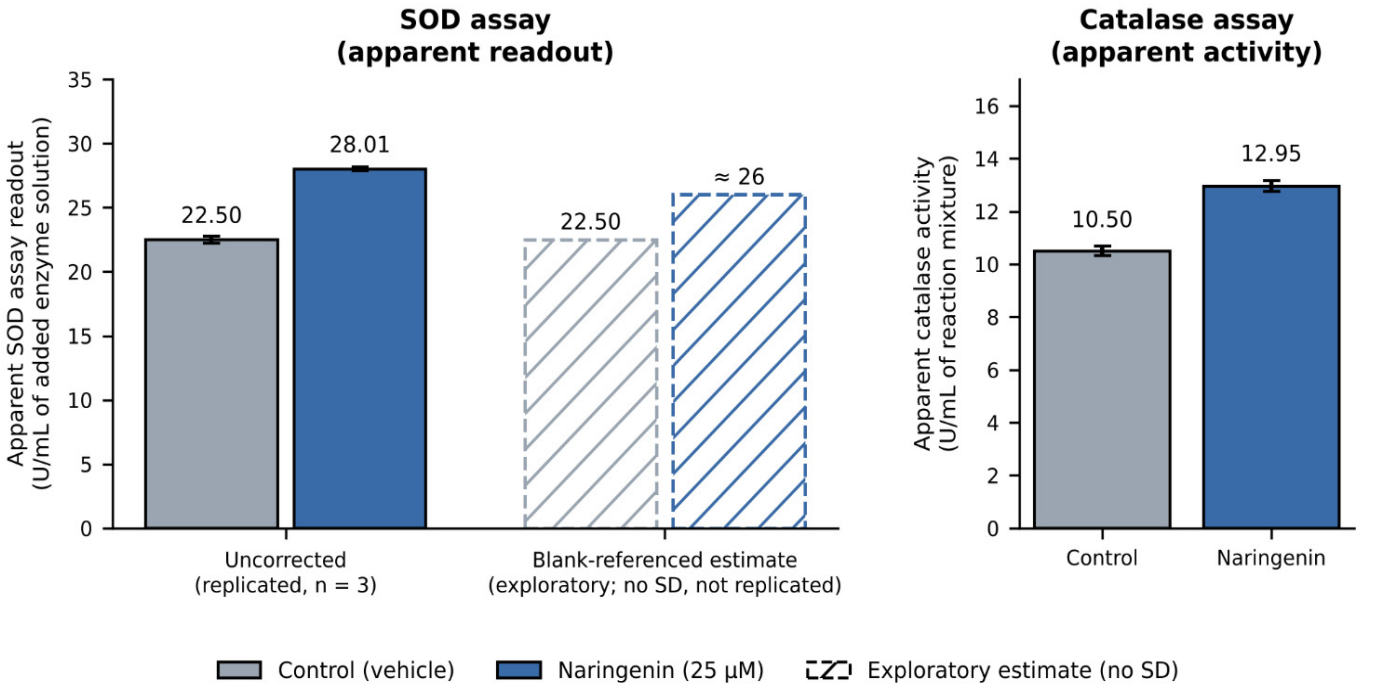
Mean of three independent experiments; error bars (SD) are smaller than the symbols. Final in-reaction concentrations are 75% of nominal.

Figure 5: Concentration-dependent DPPH radical-scavenging activity of naringenin and ascorbic acid (1.953-1000 μg/mL, sample-solution concentration). Values are the mean of three independent experiments; error bars (SD) are smaller than the symbol size. The dashed horizontal line indicates 50% scavenging. Concentrations represent nominal sample-solution concentrations before mixing with DPPH. The corresponding final IC_{50} values in the reaction mixture were 31.7 μg/mL for naringenin and 4.1 μg/mL for ascorbic acid.

The apparent SOD readout was 28.01 ± 0.14 U/mL with naringenin, compared with 22.50 ± 0.30 U/mL in the control (+24.5%), corresponding to approximately 70% and 56% inhibition of NBT photoreduction, respectively. However, the absorbance at 560 nm in the enzyme-free reaction containing naringenin was approximately 14% lower than that of the vehicle control, indicating that naringenin reduced NBT photoreduction independently of the enzyme. When inhibition was calculated against the naringenin blank, the estimated apparent SOD activity was approximately 26 U/mL, and the estimated increase relative to the control decreased to approximately 16%.

Therefore, the increase in the apparent SOD readout cannot be unambiguously attributed to enhanced SOD enzymatic activity. The enzyme-free naringenin blank cannot distinguish among direct superoxide scavenging by naringenin, assay interference, and a genuine effect on SOD activity.

These findings are single-concentration screening results from technical replicates (mean ± SD, n = 3) and do not establish a dose–response relationship or an IC_{50}. Together with the DPPH radical-scavenging activity, the apparent SOD readout is consistent with the radical-scavenging properties of naringenin. Neither result establishes enhancement of enzymatic activity.



SOD and catalase use different assay-derived units; bar heights must not be compared between panels. Naringenin reduced NBT photoreduction by ≈14% in enzyme-free reactions, so the SOD increase may reflect scavenging or assay interference. Neither panel demonstrates enzyme activation.

Figure 6: Effect of naringenin (25 µM) on the apparent readouts of purified superoxide dismutase (SOD) and catalase (CAT) assays. Important: naringenin reduced NBT photoreduction by approximately 14% in enzyme-free reactions, so the higher apparent SOD assay readout may reflect superoxide scavenging or assay interference rather than enhanced enzyme activity; neither panel demonstrates enzyme activation. Bars show mean ± SD (n = 3 technical replicates from a common enzyme stock). Each reaction contained 30 U catalase (CAT assay) or 1.0 U SOD (SOD assay). Control reactions received the vehicle (0.0068% DMSO and 0.68% ethanol, v/v). For SOD, solid bars show apparent activity calculated against the vehicle-only, enzyme-free reaction (uncorrected), and hatched bars show the value calculated against the enzyme-free naringenin blank (≈14% lower than the vehicle blank), estimated from mean values without SD. SOD activity is expressed as U/mL of the added enzyme solution and CAT activity as U/mL of the reaction mixture, so the two are not directly comparable. No inferential statistics were applied because the replicates are technical. These single-concentration screening data reflect changes in assay readout with purified enzymes and do not demonstrate enhancement of enzymatic activity; the SOD increase may partly reflect superoxide scavenging or assay interference by naringenin.

In Vitro Evaluation of the Anti-Inflammatory-Related Enzyme Inhibitory Activity of Naringenin

COX and 15-LOX Inhibition

Naringenin showed IC_{50} values of 0.520 ± 0.030 µM for COX-1 and 0.423 ± 0.042 µM for COX-2, giving a descriptive COX-1/COX-2 ratio of approximately 1.23. The values indicate comparable inhibitory potency against both isoforms, although the IC_{50} was numerically lower for COX-2; this difference is described descriptively and was not assessed for statistical significance. Both values were higher than the corresponding indomethacin IC_{50} values (0.071 ± 0.002 µM and 0.069 ± 0.005 µM, respectively), indicating that naringenin was less potent than the reference NSAID against both isoforms ($p = 0.0014$ for COX-1 and $p = 0.0042$ for COX-2, Welch's t-test on experiment-level IC_{50} estimates; Table 5 and Figure 7).

In contrast, for 15-LOX, the IC_{50} of naringenin was 2.80±0.02 µM compared with 6.08±0.18 µM for nordihydroguaiaretic acid (NDGA) ($p = 0.0009$), indicating approximately 2.2-fold greater potency of naringenin than the reference at this target (Table 6 and Figure 8).

DISCUSSION

Naringenin exhibited concentration-dependent antibacterial activity against MRSA, as demonstrated by increasing inhibition-zone diameters in disc diffusion, and showed putative bactericidal activity in broth microdilution. It was also active against *M. gallisepticum*.

An MBC/MIC ratio of ≤ 4 is consistent with putative bactericidal rather than bacteriostatic activity under the assay conditions, pending confirmation by time-kill assays or viable-count determination with carry-over controls (e.g., dilution or membrane-filtration neutralization); naringenin may therefore merit further evaluation as a

botanical alternative or adjunct to conventional agents, although the effective concentrations were relatively high. In contrast, no inhibition zones were observed for the Gram-negative species (*Escherichia coli*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*) at any concentration tested, up to 100 mg/mL (1,000 µg/disc). The growth and vehicle (5% DMSO) controls showed growth, so the inhibition observed is attributable to naringenin rather than to the solvent.

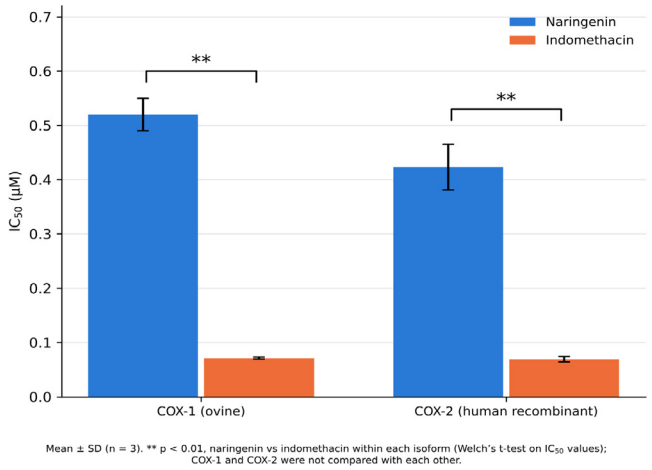


Figure 7: Inhibitory activity of naringenin and indomethacin against cyclooxygenase isoforms. IC_{50} values (µM) of naringenin (blue) and indomethacin (orange) against COX-1 (ovine) and COX-2 (human recombinant), determined using the COX (ovine/human) Inhibitor Screening Assay Kit. IC_{50} values were estimated by nonlinear regression of percentage inhibition against the logarithm of compound concentration. Data are presented as mean ± SD of three independent experiments (n = 3); error bars represent SD. Statistical significance of naringenin versus indomethacin within each isoform (COX-1 and COX-2) was assessed using an unpaired, two-tailed Welch's t-test applied to the IC_{50} values from the independent experiments (**p < 0.01). Comparisons are between naringenin and indomethacin within each enzyme isoform, not between COX-1 and COX-2.

Direct comparison of the present MIC and MBC values against MRSA with earlier reports is limited by differences in strains, compound form, solvent, and test methodology. Céliz *et al.* (2011), using agar dilution and direct-contact assays, found that naringenin, naringin, and prunin were inactive against *S. aureus* at 0.25 mmol/L (about 0.07 mg/mL), a concentration below the MIC observed here (0.156 mg/mL, about 0.57 mmol/L). In the same study, prunin alkyl esters with 10–12 carbon chains reduced S. aureus viability by about 6 log units and were inactive against the Gram-negative strains tested, which is compatible with the absence of detectable inhibition zones against the Gram-negative bacteria in disc diffusion here. Lather *et al.* (2020) also reported antimicrobial and preservative activity for synthetic naringenin derivatives designed as glucosamine-6-phosphate synthase inhibitors, supporting the potential of the naringenin scaffold as a basis for developing antimicrobial compounds.

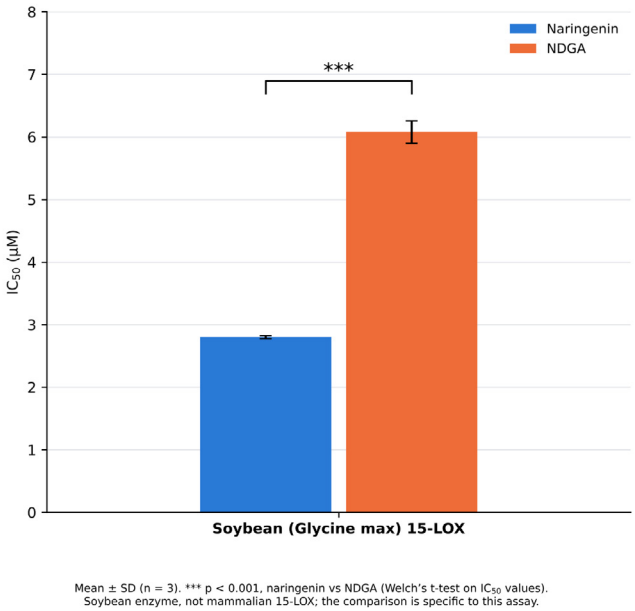


Figure 8: Inhibitory activity of naringenin and nordihydroguaiaretic acid (NDGA) against soybean 15-lipoxygenase (15-LOX). IC_{50} values (µM) of naringenin (blue) and NDGA (orange) were determined using the Lipoxygenase Inhibitor Screening Assay Kit. Data are presented as mean ± SD of three independent experiments (n = 3). Statistical significance versus NDGA was assessed using an unpaired, two-tailed Welch's t-test applied to the IC_{50} values from the independent experiments (***p < 0.001).

The activity against *M. gallisepticum* appears not to have been reported previously, to our knowledge, and data on flavonoid activity against mycoplasmas are limited. This statement is based on a PubMed search (accessed 13 October 2026; query: naringenin AND (*Mycoplasma* OR *Gallisepticum*); no date or language limits), which returned two records, neither of which evaluated the direct antimicrobial activity of naringenin against *M. gallisepticum* (one examined naringenin in a model of *M. pneumoniae*-induced airway remodeling; Lin *et al.*, 2018). The search was not repeated in Scopus or Web of Science. The MIC and MBC values indicate that naringenin inhibits this organism and is putatively mycoplasmacidal in vitro, although only at high concentrations. The underlying mechanism was not investigated.

The structural features of naringenin, specifically the 4′,5,7-trihydroxyflavanone backbone, may contribute to its antimicrobial activity. Previously proposed modes of action for naringenin and related flavonoids include four mechanisms. First, membrane disruption, in

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which naringenin inserts into the phospholipid bilayer, increasing permeability and causing leakage of ions and cellular contents. Second, inhibition of nucleic acid synthesis via intercalation and topoisomerase inhibition, as demonstrated by Mori *et al*. (1987) and Ohemeng *et al*. (1993). Third, biofilm suppression through quorum sensing interference and downregulation of virulence factors. Fourth, inhibition of fatty acid synthesis by targeting the FAS II pathway, which is essential for bacterial membrane biogenesis. These mechanisms were not examined in the present study.

The MIC of naringenin against MRSA (156 µg/mL) is moderate compared with conventional antibiotics, and the effective concentrations were relatively high. This value is much lower than the solution concentration that failed to produce a zone in disc diffusion (10 mg/mL, equivalent to 100 µg/disc), and zones appeared only at 25 mg/mL (250 µg/disc) and above. This difference is consistent with poor diffusion of a poorly soluble compound from the disc through agar. A similar explanation was proposed by Ng›uni *et al*. (2015) for the absence of detectable inhibition in disc diffusion assays. For this reason, broth microdilution is the more informative assay for naringenin.

The MIC for *M. gallisepticum* was higher than that for MRSA, so the two organisms should not be considered equally susceptible. Mycoplasmas lack a cell wall, and their membranes contain sterols, which may influence their susceptibility to membrane-active compounds. This possibility was not investigated.

The absence of inhibition zones for the Gram-negative species is consistent with the barrier function of the outer membrane, in which lipopolysaccharide and restrictive porins can limit the entry of hydrophobic compounds such as naringenin. This explanation was not tested here. Because MIC values were not determined for these organisms, the results show only that no inhibition zones formed under the disc diffusion conditions used, and poor diffusion from the disc may also contribute. Additive effects of naringenin with conventional antibiotics against multidrug-resistant *S. aureus* have been reported in broth microdilution (Ng'uni *et al*., 2015). Testing combinations with membrane-permeabilizing agents or efflux pump inhibitors against Gram-negative bacteria would be a logical next step.

M. gallisepticum causes chronic respiratory disease in poultry, with substantial economic losses. Current treatment approaches commonly involve antimicrobial agents such as tylosin and fluoroquinolones, and increasing resistance has been reported (Ammar *et al*., 2016). The putative bactericidal activity observed here suggests that naringenin may have potential as an alternative or complementary agent, but the effective in vitro concentrations were relatively high, and no conclusions about clinical use can be drawn without pharmacokinetic, safety, and *in vivo* efficacy data.

This study has several limitations, including its exclusively in vitro design and the use of a limited number of bacterial isolates. MIC endpoints were read visually. MBC values were determined by visual examination of the subculture plates, and representative images of these plates were not available. MIC and MBC were not determined for the Gram-negative bacteria because no inhibition zones were observed in the disc diffusion screening, which may underestimate activity because of the poor diffusion of naringenin in agar. Naringenin has poor aqueous solubility and the concentrations tested exceed it, so precipitation may have affected visual MIC readings; a compound control was run on a separate plate. The MBC was defined as no growth after subculture of 10 µL aliquots rather than by a ≥99.9% killing criterion, and carry-over of naringenin onto the agar was not excluded. Residual compound may therefore have suppressed colony recovery, and the bactericidal activity is described as putative. Only one isolate of each species was tested. The active concentrations are high, and cytotoxicity was not assessed.

Future investigations should focus on: (1) *in vivo* efficacy in animal models of MRSA infection and mycoplasmal respiratory disease; (2) combination therapy with conventional antibiotics to assess synergistic potential; (3) mechanistic studies using transcriptomics and proteomics to identify molecular targets; and (4) formulation development to enhance bioavailability and stability, as naringenin has limited aqueous solubility.

The current findings indicate that naringenin showed radical-scavenging and enzyme-inhibitory activity across several cell-free *in vitro* test systems. Naringenin exhibited concentration-dependent DPPH radical-scavenging potential, attaining 84.1% inhibition at 1000 µg/mL, which was accompanied by increased apparent catalase and SOD readouts in purified-enzyme assays (these do not establish enhancement of enzymatic activity). Furthermore, naringenin inhibited COX-1 and COX-2 (less potently than indomethacin) and soybean 15-LOX (more potently than NDGA). This multi-target pattern is in line with the pharmacological profile described for naringenin, in which antioxidant and anti-inflammatory effects are reported to coexist (Hernández-Aquino and Muriel, 2018; Uçar and Göktaş, 2023).

In the DPPH assay, naringenin scavenged radicals in a concentration-dependent manner, reaching 84.1% at 1000

μg/mL compared with 97.5% for ascorbic acid, with IC_{50} values of 42.3 and 5.5 μg/mL, respectively. Naringenin therefore showed moderate activity, about 7.8-fold lower than the reference antioxidant. These results agree with previous reports on the free radical-scavenging capacity of naringenin (Cavia-Saiz *et al.*, 2010; Rashmi *et al.,* 2018). Chemically, naringenin is a 4′,5,7-trihydroxyflavanone whose phenolic hydroxyl groups can donate hydrogen atoms or electrons to DPPH, with the 4′-OH group considered an important determinant of radical stabilization (Chen *et al.*, 2023). However, naringenin lacks the B-ring catechol group and the C2=C3 double bond that enhance the activity of other flavonoids, which likely explains its lower potency than ascorbic acid, a strong hydrogen and electron donor. Because IC_{50} values depend on assay conditions, particularly the DPPH concentration (0.025 mM here), comparisons with other studies should be made with caution. Finally, DPPH is a cell-free chemical assay that does not reflect the absorption, metabolism, or intracellular activity of naringenin, and the IC_{50} values were estimated by linear interpolation between two concentrations, so they should be regarded as approximate. Confirmation in complementary antioxidant assays and cell-based models is warranted.

The catalase and SOD experiments used commercial purified enzymes and therefore assessed changes in assay readouts in the presence of naringenin; they do not indicate induction of an endogenous antioxidant response. At the single tested concentration of 25 μM, naringenin increased the apparent catalase activity (12.95 ± 0.21 vs. 10.50 ± 0.17 U/mL). The enzyme-free blank showed no detectable direct decomposition of H_2O_2 by naringenin under the assay conditions. However, this single-concentration experiment does not establish a concentration–response relationship or identify the mechanism underlying the increased apparent readout. The effect could reflect enhanced catalase activity, protection of catalase from inactivation, or a direct interaction between naringenin and the enzyme, including allosteric modulation; these possibilities were not distinguished in the present study.

The apparent SOD readout also increased in the presence of naringenin (28.01 ± 0.14 vs. 22.50 ± 0.30 U/mL), corresponding to approximately 70% and 56% inhibition of NBT photoreduction, respectively. However, naringenin reduced NBT photoreduction by approximately 14% in the enzyme-free reaction. The exploratory blank-referenced estimate was approximately 26 U/mL. Thus, the higher apparent SOD readout cannot be unambiguously attributed to enhanced SOD enzymatic activity and may partly reflect direct superoxide scavenging by naringenin or other assay-related effects. This interpretation is consistent with the radical-scavenging activity observed in the DPPH assay, but does not establish the mechanism of the SOD readout.

SOD and catalase act sequentially in antioxidant defense: SOD converts superoxide to hydrogen peroxide and oxygen, while catalase decomposes hydrogen peroxide to water and oxygen. However, the single-concentration, purified-enzyme design used here cannot establish coordinated activity or an endogenous antioxidant response. Further concentration–response and mechanistic studies are needed to distinguish these possibilities. Previous studies reported that naringenin restored SOD and catalase activities in animal models, including cadmium-induced hepatotoxicity (Renugadevi and Prabu, 2010) and streptozotocin-treated animals (Rashmi *et al.,* 2018). Those findings come from *in vivo* systems in which naringenin protects against an oxidative insult and endogenous enzymes are preserved or upregulated, which differs from the direct purified-enzyme assays used here; the present results therefore neither confirm nor contradict them. Reviews describe broader antioxidant mechanisms, including preservation of glutathione, attenuation of lipid peroxidation, and modulation of redox-sensitive signaling (Hernández-Aquino and Muriel, 2018; Uçar and Göktaş, 2023). In the present study, only direct radical scavenging (DPPH) is clearly supported; effects on endogenous enzymatic defenses would need to be tested in cell- or tissue-based models.

The relationship between oxidative stress and inflammation is relevant to the present findings because the two processes can reinforce one another. Reactive oxygen species may activate redox-sensitive pathways such as NF-κB and MAPKs, increasing the expression of inflammatory mediators, while activated inflammatory cells can generate additional reactive oxygen and nitrogen species. Previous work has shown that naringenin can interfere with several components of this network. Hämäläinen *et al.* (2007) reported inhibition of NF-κB activation, iNOS expression, and nitric oxide production in activated macrophages. Park *et al.* (2012) observed suppression of NF-κB and MAPK signaling together with reductions in nitric oxide, prostaglandin E2, COX-2, TNF-α, IL-1β, and MCP-1 in LPS-stimulated microglial cells. Liu *et al.* (2016) also described an AMPK-ATF3-dependent mechanism that negatively regulates TLR4 signaling. These cellular effects act largely through signaling and gene expression, whereas the present enzyme assays measured direct inhibition: naringenin inhibited COX-1 and COX-2 less potently than indomethacin and 15-LOX more potently than NDGA. Together, these reports provide a plausible context for the radical-scavenging and anti-inflammatory-related enzyme inhibitory activities observed here, although the present in vitro data do not establish these mechanisms.

Cellular studies provide supporting evidence that naringenin can influence the cyclooxygenase pathway: Raso *et al.* (2001) reported reduced PGE_2 release and COX-2 expression in activated macrophages, and Park *et al.* (2012) likewise observed suppression of COX-2 expression and PGE_2 production.

Taken together, the direct enzyme-inhibition data indicate that naringenin is a weaker COX-1/COX-2 inhibitor than indomethacin but a more potent 15-LOX inhibitor than NDGA under the present assay conditions. Indomethacin showed similar IC_{50} values for COX-1 and COX-2 here (0.071 and 0.069 μM), whereas preferential COX-1 inhibition is often reported; this may reflect the 10-min preincubation at 37 ˚C used in the assay, since indomethacin is a time-dependent COX inhibitor, but this was not examined. However, the sub-micromolar COX IC_{50} values obtained here differ markedly from an earlier cell-free study in which naringenin did not inhibit COX-1 or COX-2 at concentrations up to 100 μM (Willenberg *et al.*, 2015). Possible explanations include differences in assay format, enzyme source, preincubation conditions, solvent effects, and flavonoid aggregation or interference with the immunoassay readout; these were not examined, and the COX results should be confirmed with an orthogonal method before firm conclusions are drawn. The 15-LOX inhibition data nonetheless warrant consideration of assay parameters and formulation variables. Holanda *et al.* (2023) demonstrated that although naringenin-containing preparations inhibit 15-LOX, the apparent inhibitory efficacy can shift significantly when naringenin is delivered via a nanostructured vehicle. This is relevant given naringenin's poor aqueous solubility and extensive metabolic biotransformation, both of which strongly influence free drug concentrations and systemic exposure (Uçar and Göktaş, 2023). Direct comparisons of absolute 15-LOX IC_{50} values across studies are therefore only meaningful when experimental parameters, including enzyme origin, substrate kinetics, solvent matrix, and formulation state, are closely aligned. In this regard, it should be noted that the 15-LOX assay employed the soybean enzyme, which is a widely used screening model but is not identical to human lipoxygenases; the findings should therefore be confirmed using human 5-LOX and 15-LOX isoforms.

Viewed as a whole, the cell-free data show direct radical scavenging and inhibition of COX and 15-LOX, whereas the catalase and SOD readouts remain inconclusive. Cell-based and animal studies have described effects of naringenin on NF-κB, MAPK, and TLR4-related signaling, but these pathways were not examined here (Hernández-Aquino and Muriel, 2018; Liu *et al.*, 2016; Uçar and Göktaş, 2023).

Several limitations of this study must be acknowledged. First, because the experiments were conducted exclusively *in vitro,* they do not account for critical *in vivo* pharmacokinetic factors, including drug absorption, hepatic metabolism, tissue distribution, immune network interactions, and systemic clearance. Second, naringenin's poor aqueous solubility and modest oral bioavailability may limit the free tissue concentrations attainable *in vivo* via conventional delivery routes (Uçar and Göktaş, 2023). Third, while the DPPH assay demonstrates chemical radical neutralization, it cannot alone confirm intracellular antioxidant capacity. Fourth, further confirmation of anti-inflammatory efficacy in cells or tissues through specific cellular markers such as pro-inflammatory cytokines, eicosanoid levels, or transcription factor activation would strengthen these conclusions. Fifth, indomethacin potency at COX-2 was higher than commonly reported, and its COX-1 IC_{50} lies close to the lowest concentration tested; the COX IC_{50} values should therefore be interpreted relative to the reference compound under the present assay conditions.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, this exploratory *in vitro* study characterized the antibacterial, antioxidant, and anti-inflammatory-related enzyme inhibitory activities of naringenin. Naringenin showed preliminary antibacterial activity against MRSA and antimycoplasmal activity against *M. gallisepticum*. No detectable inhibition of the tested Gram-negative bacteria was observed in the disc-diffusion assay under the experimental conditions used; MIC values were not determined for these organisms DPPH radical-scavenging activity was observed under the cell-free assay conditions. In the purified-enzyme assays, naringenin increased the apparent catalase and SOD readouts at a single concentration (25 μM). However, naringenin also reduced NBT photoreduction in the enzyme-free blank, and these results do not establish enhanced enzymatic activity.

Naringenin was less potent than indomethacin against COX-1 and COX-2, but more potent than NDGA in the soybean 15-LOX assay under the conditions tested. Because the 15-LOX assay used a soybean enzyme, this result should not be assumed to predict activity against mammalian 15-LOX.

These findings are hypothesis-generating. Future studies should include concentration–response testing of the CAT and SOD readouts with biological replicates; cell- or tissue-based models to assess effects on endogenous antioxidant defenses; MIC testing of the Gram-negative

bacteria; cytotoxicity and selectivity assessment relative to antibacterial concentrations; and confirmation of 15-LOX inhibition using a mammalian enzyme. If these preliminary antibacterial findings are supported by such studies, further work could evaluate the pharmacokinetics, bioavailability, safety, and efficacy of naringenin in relevant *in vivo* models.

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NOVELTY STATEMENT

To the best of our knowledge, this is the first report evaluating naringenin against *M. gallisepticum*, an important veterinary pathogen responsible for chronic respiratory disease and significant production losses in poultry. In addition, this study provides a comparative *in vitro* assessment of naringenin's antibacterial, antioxidant, and anti-inflammatory-related enzyme inhibitory activities within a single experimental framework, using standardized CLSI and veterinary testing procedures, and directly benchmarks its enzymatic inhibitory potency against established reference compounds (ascorbic acid, indomethacin, and NDGA). This statement is based on a PubMed search (accessed 13 October 2026; query: naringenin AND (mycoplasma OR gallisepticum); no date or language limits).

AUTHOR'S CONTRIBUTION

MIM: conceptualization, writing review and editing, supervision. SII: Methodology, investigation, formal analysis, writing original draft. FSY: methodology, investigation, data curation. All authors read and approved the final manuscript.

Data availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Generative AI and AI-assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest, financial or otherwise, related to the publication of this manuscript.

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