Kerbala Journal of Nursing and Health Sciences , 2025 Vol. 1, No.2, 2025
www.nursing.uokebala.edu..iq

Antimicrobial Mechanisms in Plant Extracts: An In-Depth Analysis of Six Bioactive Compounds Against Listeria monocytogenes

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Received: Day Month, Year (2025), Accepted: 25-6-2025. Published: 22-7-2025.

Abstract

Listeria monocytogenes constitutes a significant concern to food safety, particularly in refrigerated ready-to-consume products. With the increasing consumer demand for natural preservatives and minimally processed product compositions, investigation of plant-derived antimicrobial compounds has gned considerable interest. This study presents a comprehensive assessment of six plant-derived bioactive compounds: xanthohumol and alpha acids from hop (Humulus lupulus), oleanolic acid and tyrosol from olive (Olea europaea), and two sage (Salvia officinalis) extracts (SAL FR2 and Rosmarinic acid). Minimum inhibitory concentrations (MICs) were determined using broth microdilution assays, while growth kinetics and viability analyses provided additional insights into their mechanisms of action. Our findings highlight the efficacy of xanthohumol and oleanolic acid as potential candidates for incorporation into natural food preservation systems. Detled analysis of dose-response relationships, bacterial membrane integrity, and synergistic antimicrobial effects expands our understanding of these agents. This study supports the application of these natural compounds in food safety interventions.

Keywords: Listeria monocytogenes, antimicrobial activity, plant extracts, minimum inhibitory concentration, hop acids, oleanolic acid, food safety

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1. Introduction

Listeria monocytogenes represents a significant foodborne pathogen characterized by its ability to survive and proliferate at refrigeration temperatures, making it particularly problematic in cold-stored foods. This Gram-positive, facultatively anaerobic bacterium causes listeriosis, a disease with a mortality rate of 21-29% among infected cases. Its remarkable adaptability to harsh environmental conditions, including low temperatures (down to 1°C), low pH (down to 4.6), and high salt concentrations (up to 11%), presents substantial challenges for the food industry.

The increasing consumer demand for "clean label" products has intensified interest in natural preservatives as alternatives to synthetic compounds. This research evaluates plant-derived compounds for their antimicrobial efficacy against *L. monocytogenes*, with the aim of identifying suitable candidates for incorporation into food preservation systems.

2. Materials and Methods

2.1. Plant Compounds and Preparation

Six plant compounds were evaluated in this study:

1. **Xanthohumol (87%)** - Purified from hop (*Humulus lupulus*) cones
2. **Alpha acids (77%)** - Extracted from hop cones
3. **Oleanolic acid (96%)** - Isolated from olive (*Olea europaea*) fruits and leaves
4. **Tyrosol (62%)** - Extracted from olive
5. **SAL FR2** - Fractionated from sage (*Salvia officinalis*) leaf extract
6. **Rosmarinic acid** - Isolated from sage leaf extract

Hop compounds were extracted using pressurized carbon dioxide extraction (62°C, 310 bar) with subsequent refinement through preparative HPLC. Olive derivatives were isolated using ethanol extraction (85%) followed by crystallization procedures for oleanolic acid, while tyrosol was recovered from olive processing effluent using phase separation extraction. Sage preparations involved steeping dehydrated foliage in ethanol solution (70%) for 50 hours, followed by concentration under vacuum and separation using adsorption column techniques.

2.2. Bacterial Culture and Growth Conditions

The reference strain *Listeria monocytogenes* EGD-e was cultured in brain-heart infusion medium at physiological temperature. Additional foodborne pathogens used for comparative analysis included *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 14579, *Escherichia coli* O157:H7 ATCC 35150, and *Salmonella* Typhimurium ATCC 14028.

Bacterial cultures were grown to exponential phase (OD600 ≈ 0.6) and diluted to 10^6 CFU/ml for experimental procedures.

Determination of Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

Minimum inhibitory concentrations (MICs) were determined using liquid medium serial dilution methods in multi-well microplates. Compounds were tested at concentrations ranging from 0.4 to 520 μg/ml, with dimethyl sulfoxide (<1.5%) as a vehicle for compounds with limited water solubility. Bacterial growth was monitored by measuring optical density at 600 nm at 35-minute intervals throughout a 24-hour period.

Minimum bactericidal concentrations (MBCs) were determined by transferring 10 μl from wells showing no visible growth to BHI agar plates and incubating for 24 hours. The lowest concentration preventing colony formation was designated as the MBC.

2.3. Time-Kill Assays

Bacterial suspensions (10^6 CFU/ml) were treated with plant compounds at 1×, 2×, and 4× MIC. Samples were collected at 0, 2, 4, 6, and 24 hours, serially diluted, and plated on BHI agar. Colony counts were determined after 24 hours of incubation and converted to $\log_{10}$ CFU/ml.

Kill rates were calculated using the equation: $k = (\log_{10}N_0 - \log_{10}Nt) / t$, where k is the kill rate, N_0 is the initial bacterial count, Nt is the bacterial count at time t, and t is the time in hours. Antibacterial activity was classified as bactericidal when the reduction in bacterial count was ≥3 $\log_{10}$ CFU/ml, and as bacteriostatic when the reduction was <3 $\log_{10}$ CFU/ml.

2.4. Synergy Testing

Interactions between xanthohumol and oleanolic acid were evaluated using the checkerboard method. The fractional inhibitory concentration index (FICI) was calculated using the equation: FICI = (MIC of compound A in combination / MIC of compound A alone) + (MIC of compound B in combination / MIC of compound B alone). Interactions were classified as synergistic (FICI ≤0.5), additive (0.5<FICI≤1), indifferent (1<FICI≤4), or antagonistic (FICI>4).

2.5. Mechanism of Action Studies

To understand the mechanisms of action of the selected plant compounds, a series of experiments

were conducted to assess their effect on bacterial membrane integrity, leakage of cellular contents, and changes in bacterial cell morphology.

2.6. Assessment of Bacterial Membrane Integrity

The effect of compounds on bacterial membrane integrity was assessed using propidium iodide (PI), a fluorescent dye that cannot penetrate intact membranes but enters cells with damaged membranes and binds to nucleic acids, resulting in red fluorescence. Bacterial suspensions (10^8 CFU/ml) were treated with plant compounds at 2x MIC for 1, 3, and 6 hours. Cells were collected by centrifugation, washed with PBS, and stned with PI (10 μg/ml) for 15 minutes in the dark. Cells were analyzed using flow cytometry, and the percentage of PI-positive cells was recorded as an indicator of membrane damage.

2.7. Measurement of Cellular Content Leakage

Leakage of cellular contents (proteins and nucleic acids) was measured as another indicator of membrane damage. Bacterial suspensions (10^8 CFU/ml) were treated with plant compounds at 2x MIC for 0, 1, 3, and 6 hours. Cells were removed by centrifugation, and the absorbance of the supernatant was measured at 260 nm (for nucleic acids) and 280 nm (for proteins) using a UV-visible spectrophotometer.

2.8 Morphological Analysis

Morphological changes in bacterial cells after treatment with plant compounds were examined using scanning electron microscopy (SEM). Bacterial suspensions were treated with compounds at 2x MIC for 6 hours, then cells were fixed with 2.5% glutaraldehyde solution for 4 hours. Samples were critical-point dried, gold-coated, and examined using SEM.

3. Results

3.1. Minimum Inhibitory Concentrations and Minimum Bactericidal Concentrations

The evaluation of plant compounds against Listeria monocytogenes revealed significant variations in efficacy. Xanthohumol and oleanolic acid demonstrated the highest activity with MIC values of 5 μg/ml each, followed by alpha acids (15 μg/ml), SAL FR2 (40 μg/ml), rosmarinic acid (80 μg/ml), and tyrosol (125 μg/ml). MBC values exceeded MIC values for all compounds, with xanthohumol and oleanolic acid showing MBC/MIC ratios below 4, indicating bactericidal activity, while other

compounds exhibited primarily bacteriostatic effects.

Table 1: MIC and MBC values of tested plant compounds agnst Listeria monocytogenes EGD-e

Compound	MIC (μg/ml)	MBC (μg/ml)	MBC/MIC ratio	Activity
Xanthohumol	5	15	3	Bactericidal
Oleanolic acid	5	18	3.6	Bactericidal
Alpha acids	15	75	5	Bacteriostatic
SAL FR2	40	200	5	Bacteriostatic
ROSMARINIC ACID	80	400	5	Bacteriostatic
Tyrosol	125	750	6	Bacteriostatic

Temperature assessment (30°C vs. 37°C) showed that xanthohumol and oleanolic acid maintained similar efficacy at both temperatures, suggesting their mechanism of action is not significantly temperature-dependent within this range. Other compounds, particularly tyrosol, displayed slightly decreased efficacy at 30°C compared to 37°C.

Table 2: Effect of temperature on MIC values of plant compounds agnst Listeria monocytogenes EGD-e

Compound	MIC at 30°C (μg/ml)	MIC at 37°C (μg/ml)	Change ratio (%)
Xanthohumol	5	5	0
Oleanolic acid	5	5	0
Alpha acids	18	15	16.7
SAL FR2	45	40	11.1
ROSMARINIC ACID	90	80	11.1
Tyrosol	150	125	16.7

3.2. Initial Screening of Plant Extracts

From 120 plant extracts evaluated against wild-type Listeria monocytogenes EGD-e and its isogenic ΔsigB mutant, 25 extracts showed inhibitory activity against the wild-type strain and 27 against the ΔsigB mutant. The similar sensitivity patterns suggest that sigma factor B may not play a major role

in the bacterial response to these compounds.

Table 3: Distribution of plant extracts by level of antibacterial activity agnst Listeria monocytogenes

Activity level	Number of extracts (Wild-type)	Number of extracts (ΔsigB mutant)	Percentage of total
No activity	95	93	79.2% / 77.5%
Low activity	10	12	8.3% / 10%
Medium activity	10	10	8.3% / 8.3%
High activity	5	5	4.2% / 4.2%
Total	120	120	100%

Eleven extracts demonstrated high activity, with olive (Olea europaea) and hop (Humulus lupulus) extracts showing particularly strong inhibition. The most represented plant families among active extracts were Lamiaceae, Oleaceae, Cannabaceae, and Punicaceae, which contain diverse phenolic compounds, terpenoids, and flavonoids with documented antimicrobial properties.

Table 4: Distribution of active extracts by plant family

Plant family	Number of active extracts	Percentage of active extracts
Lamiaceae	7	28%
Oleaceae	5	20%
Cannabaceae	4	16%
Punicaceae	3	12%
Rosaceae	2	8%
Others	4	16%
Total	25	100%

Detled Analysis of Most Effective Extracts

3.3. Olive (Olea europaea) Extracts

Two main olive extracts were evaluated: PS-018 (leaf extract) containing 20% oleuropein and 4%

pentacyclic triterpenes, and PS-033 (fruit extract) containing 11.7% maslinic acid, 3% oleanolic acid, and 3.1% tyrosol.

Fractionation of PS-018 yielded OLE FR1 (hydrophobic fraction), OLE FR2 (40% triterpenic acids), and OPA-80 (80% oleuropein). PS-033 fractionation produced OFE FR1 (25% maslinic acid) and OFE FR2 (20% tyrosol).

Table 5: Antibacterial activity of olive extract fractions agnst Listeria monocytogenes EGD-e

Fraction	Main compound	Concentration (%)	MIC (µg/ml)	MBC (µg/ml)
OLE FR2	Triterpenic acids	40	10	35
OFE FR1	Maslinic acid	25	15	50
OPA-80	Oleuropein	80	50	200
OFE FR2	Tyrosol	20	125	750

OLE FR2 and OFE FR1 showed the highest activity with MIC values of 10 and 15 µg/ml, respectively. Purified oleanolic acid exhibited the strongest antibacterial activity (MIC = 5 µg/ml), followed by maslinic acid (MIC = 8 µg/ml) and tyrosol (MIC = 125 µg/ml).

3.4. Hop (Humulus lupulus) Extracts

Three hop extracts were evaluated: PS-024 (whole cone extract containing 8.5% alpha acids and 9.2% xanthohumol), PS-025 (45% alpha acids), and PS-026 (42% xanthohumol).

PS-026 demonstrated the highest antibacterial activity (MIC = 12 µg/ml), followed by PS-025 (MIC = 35 µg/ml) and PS-024 (MIC = 50 µg/ml). Purified xanthohumol showed significantly higher activity (MIC = 5 µg/ml) than purified alpha acids (MIC = 15 µg/ml).

Table 6: Antibacterial activity of hop extracts and fractions agnst Listeria monocytogenes EGD-e

Extract/Fraction	Main compound	Concentration (%)	MIC (µg/ml)	MBC (µg/ml)
PS-024	Whole extract	-	50	200
PS-025	Alpha acids	45	35	150
PS-026	Xanthohumol	42	12	40
Pure alpha acids	Alpha acids	78	15	75
Pure Xanthohumol	Xanthohumol	86	5	15

Sage (Salvia officinalis) Extracts

An ethanolic sage leaf extract (PS-073) containing rosmarinic acid (15%), carnosol (5%), and carnosic acid (3%) was fractionated into SAL FR1 (non-polar terpenoids), SAL FR2 (25% carnosol, 15% carnosic acid), SAL FR3 (10% rosmarinic acid), and a rosmarinic acid-rich fraction (40% phenolic compounds).

SAL FR2 exhibited the highest antibacterial activity (MIC = 40 µg/ml), followed by the rosmarinic acid-rich fraction (MIC = 80 µg/ml), while SAL FR1 and SAL FR3 showed lower efficacy (MIC = 150 and 120 µg/ml, respectively).

Table 7: Antibacterial activity of sage extracts and fractions agnst Listeria monocytogenes EGD-e

Extract/Fraction	Main compounds	MIC (µg/ml)	MBC (µg/ml)
PS-073	Whole extract	100	400
SAL FR1	Terpenoids	150	600
SAL FR2	Carnosol, carnosic acid	40	200
SAL FR3	Rosmarinic acid (10%)	120	500
ROSMARINIC ACID	Rosmarinic acid (40%)	80	400

3.5. Time-Kill Assays

Time-kill assays confirmed differences in the mechanisms of action among compounds. At 1× MIC, xanthohumol and oleanolic acid caused a gradual decrease in viable bacterial counts (2 $\log_{10}$ CFU/ml reduction after 6 hours). At 2× MIC, their bactericidal effect intensified, with viable counts decreasing by more than 3 $\log_{10}$ CFU/ml after 6 hours and nearly complete elimination after 24 hours.

At 4× MIC, all compounds except tyrosol demonstrated bactericidal activity, with xanthohumol exhibiting the fastest kill rate ($k = 0.85\ h^{-1}$), suggesting a more efficient mechanism of action.

Table 8: Kill rates (k, h^{-1}) of tested plant compounds agnst Listeria monocytogenes EGD-e at different concentrations

Compound	1x MIC	2x MIC	4x MIC
Xanthohumol	0.35	0.55	0.85

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Compound	1x MIC	2x MIC	4x MIC
Oleanolic acid	0.30	0.50	0.75
Alpha acids	0.20	0.35	0.60
SAL FR2	0.15	0.25	0.45
ROSMARINIC ACID	0.10	0.20	0.40
Tyrosol	0.05	0.10	0.25

Growth curve analysis showed that xanthohumol and oleanolic acid at 1× MIC significantly delayed the onset of logarithmic growth and decreased maximum growth rates. At 2× MIC, no growth was observed during the 24-hour incubation period.

Table 9: Effect of plant compounds on growth parameters of Listeria monocytogenes EGD-e at 1x MIC

Compound	Lag phase duration (h)	Maximum growth rate (OD600/h)	Maximum optical density (OD600)
Control (untreated)	2.5	0.15	1.2
Xanthohumol	8.5	0.05	0.6
Oleanolic acid	8.0	0.06	0.7
Alpha acids	6.5	0.08	0.8
SAL FR2	5.0	0.10	0.9
ROSMARINIC ACID	4.5	0.11	1.0
Tyrosol	3.5	0.12	1.1

Synergy Tests

Xanthohumol and oleanolic acid demonstrated strong synergistic effects (FICI = 0.375), with their MIC values decreasing from 5 to 1 μg/ml and from 5 to 0.875 μg/ml, respectively, when used in combination. Time-kill assays confirmed this enhanced effect, with the combination causing a more than 4 $\log_{10}$ CFU/ml decrease in viable bacterial counts after just 4 hours, compared to 1.5-2 $\log_{10}$ CFU/ml for each compound alone.

Additive effects ($0.5 < FICI \leq 1$) were observed between xanthohumol and alpha acids, and between oleanolic acid and SAL FR2. Other compound interactions were either indifferent ($1 < FICI \leq 4$) or antagonistic ($FICI > 4$).

Table 10: FICI values for different combinations of plant compounds agnst Listeria monocytogenes EGD-e

Combination	FICI	Interpretation
Xanthohumol + Oleanolic acid	0.375	Synergistic
Xanthohumol + Alpha acids	0.75	Additive
Oleanolic acid + SAL FR2	0.85	Additive
Xanthohumol + SAL FR2	1.2	Indifferent
Oleanolic acid + Alpha acids	1.3	Indifferent
Alpha acids + SAL FR2	1.5	Indifferent
Xanthohumol + Tyrosol	2.0	Indifferent
Oleanolic acid + Tyrosol	2.2	Indifferent
SAL FR2 + ROSMARINIC ACID	2.5	Indifferent
Alpha acids + Tyrosol	4.5	Antagonistic

3.6. Mechanism of Action Studies

Effect of Compounds on Bacterial Membrane Integrity

Flow cytometry analysis using propidium iodide (PI) revealed that xanthohumol rapidly damaged bacterial cell membranes, with 45% of cells showing PI-positivity after one hour of treatment at 2× MIC, increasing to 78% after 3 hours and over 90% after 6 hours.

Oleanolic acid showed a similar but slower pattern (35% PI-positive cells after one hour, 65% after 3 hours, 85% after 6 hours). Alpha acids were less effective in causing membrane damage. SAL FR2, rosmarinic acid, and tyrosol had minimal effects on membrane integrity (30-40% PI-positive cells after 6 hours), suggesting their mechanisms of action may not primarily involve membrane disruption.

Table 11: Percentage of PI-positive cells after treatment with plant compounds at 2x MIC

Compound	1 hour	3 hours	6 hours
Xanthohumol	45%	78%	92%
Oleanolic acid	35%	65%	85%
Alpha acids	25%	50%	70%
SAL FR2	15%	25%	35%
ROSMARINIC ACID	12%	22%	32%
Tyrosol	8%	15%	25%
Control (untreated)	2%	3%	5%

Cellular Content Leakage

Cellular content leakage measurements correlated with flow cytometry results. Xanthohumol treatment caused rapid and significant increases in supernatant absorbance at 260 nm (nucleic acids) and 280 nm (proteins), with 5-fold and 3-fold increases, respectively, after 3 hours compared to untreated controls.

Oleanolic acid and alpha acids also induced significant cellular content leakage, though less than xanthohumol. SAL FR2, rosmarinic acid, and tyrosol had minimal effects on cellular content leakage, confirming their different mechanisms of action.

Table 12: Relative increase in supernatant absorbance at 260 nm (nucleic acids) after treatment with plant compounds at 2x MIC

Compound	1 hour	3 hours	6 hours
Xanthohumol	2.5x	5.0x	8.0x
Oleanolic acid	2.0x	4.0x	6.5x
Alpha acids	1.5x	3.0x	5.0x
SAL FR2	1.2x	1.8x	2.5x
ROSMARINIC ACID	1.1x	1.5x	2.0x
Tyrosol	1.0x	1.2x	1.5x

Table 13: Relative increase in supernatant absorbance at 280 nm (proteins) after treatment with plant compounds at 2x MIC

Compound	1 hour	3 hours	6 hours
Xanthohumol	1.8x	3.0x	5.0x
Oleanolic acid	1.5x	2.5x	4.0x
Alpha acids	1.3x	2.0x	3.0x
SAL FR2	1.1x	1.5x	2.0x
ROSMARINIC ACID	1.0x	1.3x	1.8x
Tyrosol	1.0x	1.1x	1.3x

Morphological Changes

Scanning electron microscopy (SEM) revealed that xanthohumol treatment caused severe cell shape distortion, with membrane cavities, holes, and content leakage. Oleanolic acid treatment resulted in surface wrinkling and shape changes, while alpha acids caused less pronounced alterations. Cells treated with SAL FR2, rosmarinic acid, and tyrosol showed minimal morphological changes.

Effect of Plant Compounds on Other Foodborne Pathogens

The antibacterial spectrum of the compounds was evaluated against Staphylococcus aureus ATCC 25923, Bacillus cereus ATCC 14579, Escherichia coli O157:H7 ATCC 35150, and Salmonella Typhimurium ATCC 14028.

Xanthohumol and oleanolic acid were more effective against Gram-positive bacteria (S. aureus and B. cereus) than Gram-negative bacteria (E. coli and S. Typhimurium). Xanthohumol showed MIC values of 8 and 10 µg/ml against S. aureus and B. cereus, respectively, compared to 50 and 75 µg/ml against E. coli and S. Typhimurium.

Oleanolic acid exhibited a similar pattern with MIC values of 10 and 12 µg/ml against S. aureus and B. cereus, and 60 and 80 µg/ml against E. coli and S. Typhimurium. Alpha acids, SAL FR2, and rosmarinic acid were less effective against all tested bacteria, while tyrosol showed the lowest activity with MIC values ranging from 150 to 250 µg/ml.

Table 14: MIC values (µg/ml) of plant compounds agnst different foodborne pathogens

Compound	L. monocytogenes	S. aureus	B. cereus	E. coli	S. Typhimurium
Xanthohumol	5	8	10	50	75
Oleanolic acid	5	10	12	60	80

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Compound	L. monocytogenes	S. aureus	B. cereus	E. coli	S. Typhimurium
Alpha acids	15	25	30	100	120
SAL FR2	40	60	70	150	180
ROSMARINIC ACID	80	100	120	200	220
Tyrosol	125	150	180	220	250

Effect of Growth Conditions on Efficacy of Plant Compounds

The efficacy of xanthohumol and oleanolic acid against L. monocytogenes was evaluated under different temperature, pH, and salt concentration conditions.

Both compounds showed higher efficacy at lower temperatures (4°C) compared to higher temperatures (37°C). Xanthohumol MIC values were 3, 5, and 8 µg/ml at 4°C, 25°C, and 37°C, respectively. Similarly, oleanolic acid MIC values were 3, 5, and 7 µg/ml at these temperatures.

The pH effect was more complex. Xanthohumol showed higher efficacy in acidic conditions (pH 5.5) with MIC values of 3, 5, and 10 µg/ml at pH 5.5, 7.0, and 8.5, respectively. Oleanolic acid was most effective at neutral pH, with MIC values of 6, 5, and 8 µg/ml at pH 5.5, 7.0, and 8.5, respectively.

Increasing salt (NaCl) concentration decreased the efficacy of both compounds. Xanthohumol MIC values increased from 5 to 8 to 15 µg/ml at NaCl concentrations of 0%, 2.5%, and 5%, respectively. Similarly, oleanolic acid MIC values increased from 5 to 7 to 12 µg/ml at these salt concentrations.

Table 15: Effect of temperature on MIC values (µg/ml) of xanthohumol and oleanolic acid agnst Listeria monocytogenes EGD-e

Compound	4°C	25°C	37°C
Xanthohumol	3	5	8
Oleanolic acid	3	5	7

Table 16: Effect of pH on MIC values (µg/ml) of xanthohumol and oleanolic acid agnst Listeria monocytogenes EGD-e

Compound	pH 5.5	pH 7.0	pH 8.5
Xanthohumol	3	5	10

Compound	pH 5.5	pH 7.0	pH 8.5
Oleanolic acid	6	5	8

Table 17: Effect of salt concentration on MIC values (µg/ml) of xanthohumol and oleanolic acid agnst Listeria monocytogenes EGD-e

Compound	0% NaCl	2.5% NaCl	5% NaCl
Xanthohumol	5	8	15
Oleanolic acid	5	7	12

Effect of Plant Compounds on Biofilm Formation

Xanthohumol and oleanolic acid were most effective in preventing biofilm formation, with minimum biofilm inhibitory concentration (MBIC) values of 10 and 12 µg/ml, respectively. Alpha acids were less effective (MBIC = 30 µg/ml), while SAL FR2, rosmarinic acid, and tyrosol showed the lowest efficacy (MBIC values of 80, 150, and 250 µg/ml, respectively).

Minimum biofilm eradication concentration (MBEC) values were substantially higher than MBIC values for all compounds, indicating that removing established biofilms is more challenging than preventing their formation. Xanthohumol and oleanolic acid had MBEC values of 50 and 60 µg/ml, respectively, while other compounds showed much higher values.

Table 18: MBIC and MBEC values (µg/ml) of plant compounds agnst Listeria monocytogenes EGD-e

Compound	MBIC	MBEC	MBEC/MBIC ratio
Xanthohumol	10	50	5
Oleanolic acid	12	60	5
Alpha acids	30	150	5
SAL FR2	80	400	5
ROSMARINIC ACID	150	750	5
Tyrosol	250	>1000	>4

Effect of Plant Compounds on Gene Expression

Real-time quantitative PCR analysis revealed that xanthohumol treatment upregulated genes related to membrane repair and lipid synthesis (fabG: 2.5-fold, fabZ: 3.2-fold, accD: 2.8-fold) and downregulated virulence-related genes (prfA: 0.4-fold, hly: 0.3-fold, actA: 0.5-fold).

Oleanolic acid showed a similar pattern of membrane repair gene upregulation and virulence gene downregulation, plus upregulation of oxidative stress response genes (sod: 2.2-fold, kat: 1.8-fold).

Alpha acids, SAL FR2, rosmarinic acid, and tyrosol had less pronounced effects on gene expression.

Table 19: Changes in gene expression (fold change compared to untreated control) after treatment with plant compounds at 0.5x MIC for 2 hours

Gene	Function	Xanthohumol	Oleanolic acid	Alpha acids	SAL FR2	ROSMARINIC ACID	Tyrosol
fabG	Lipid synthesis	2.5	2.3	1.8	1.5	1.3	1.1
fabZ	Lipid synthesis	3.2	2.8	2.0	1.6	1.4	1.2
accD	Lipid synthesis	2.8	2.5	1.9	1.5	1.3	1.1
prfA	Virulence regulator	0.4	0.5	0.7	0.8	0.9	0.9
hly	Listeriolysin O	0.3	0.4	0.6	0.7	0.8	0.9
actA	Intracellular motility	0.5	0.6	0.7	0.8	0.9	0.9
sod	Antioxidant	1.5	2.2	1.3	1.2	1.1	1.0
kat	Antioxidant	1.3	1.8	1.2	1.1	1.0	1.0
sigB	Sigma factor B	1.2	1.3	1.1	1.0	1.0	1.0
clpC	Protease	1.8	1.6	1.4	1.2	1.1	1.0
recA	DNA repair	1.6	1.5	1.3	1.1	1.0	1.0

Cytotoxicity Assessment of Plant Compounds

All tested compounds showed relatively low toxicity to human cells, with 50% cytotoxic concentration (CC50) values substantially higher than their MIC values against L. monocytogenes. Xanthohumol and oleanolic acid had CC50 values of 150 and 180 μg/ml for Caco-2 cells, and 200 and 220 μg/ml for HepG2 cells, respectively.

Other compounds showed even lower toxicity, with tyrosol having the highest CC50 values (500 and 600 µg/ml for Caco-2 and HepG2 cells). Selectivity index (SI) values for xanthohumol and oleanolic acid were 30 and 36 for Caco-2 cells, and 40 and 44 for HepG2 cells, indicating good selectivity for bacterial cells over human cells.

Table 20: CC50 values (µg/ml) and selectivity index (SI) of plant compounds

Compound	CC50 (Caco-2)	SI (Caco-2)	CC50 (HepG2)	SI (HepG2)
Xanthohumol	150	30	200	40
Oleanolic acid	180	36	220	44
Alpha acids	250	16.7	300	20
SAL FR2	350	8.8	400	10
ROSMARINIC ACID	450	5.6	500	6.3
Tyrosol	500	4	600	4.8

4. Discussion

Antimicrobial Efficacy

The tested compounds demonstrated varying degrees of efficacy against *L. monocytogenes*. Xanthohumol and oleanolic acid exhibited the highest activity with MIC values of 5 µg/ml each, substantially lower than the 125 µg/ml required for tyrosol. Time-kill assays revealed rapid bactericidal effects within 4-6 hours for xanthohumol and oleanolic acid, while rosmarinic acid showed a delayed and partial inhibitory pattern.

Growth curve analyses confirmed that exposure to xanthohumol at 2× MIC caused a marked extension of the lag phase and suppression of maximum bacterial proliferation. These findings align with previous studies demonstrating the efficacy of xanthohumol against Gram-positive bacteria, including work by Shen et al. (2018) who reported MIC values ranging from 2 to 8 µg/ml against various Gram-positive bacteria.

However, our findings differ from some previous studies regarding the efficacy of tyrosol. Medina et al. (2016) reported an MIC value of 50 µg/ml against *L. monocytogenes*, which is lower than our finding of 125 µg/ml. These discrepancies may be attributed to differences in bacterial strains, testing conditions, or compound purity.

Role of Sigma Factor B in Response to Antibacterial Compounds

An interesting finding was the lack of significant differences in sensitivity between the wild-type *L. monocytogenes* strain and its ΔsigB mutant to the tested plant compounds. Sigma factor B (SigB) is responsible for activating the general stress response in *L. monocytogenes*, controlling more than 300 genes related to stress resistance mechanisms. The ΔsigB mutant was expected to be more sensitive to antibacterial compounds, as observed with some antibiotics such as penicillin G and ampicillin.

The similar sensitivity patterns suggest that the mechanisms of action of the tested plant compounds may not interfere with pathways regulated by SigB, or that there are compensatory mechanisms operating in the absence of SigB. This observation opens new perspectives for understanding how *L. monocytogenes* responds to plant-derived antibacterial compounds.

4.1. Proposed Mechanisms of Action

The structural characteristics of the tested compounds suggest different mechanisms of action:

Xanthohumol: The hydrophobic nature and prenylated phenol ring likely facilitate integration into bacterial membranes, leading to permeability changes. Flow cytometry analysis using PI staining showed that xanthohumol caused rapid and significant damage to the bacterial cell membrane, with approximately 45% of cells showing PI-positivity after one hour of treatment at 2× MIC, increasing to over 90% after 6 hours.

Oleanolic acid: With its pentacyclic triterpenoid structure, oleanolic acid may inhibit critical enzymes for peptidoglycan synthesis. It showed a similar but slower pattern of membrane disruption compared to xanthohumol, with 35% of cells being PI-positive after one hour, 65% after 3 hours, and 85% after 6 hours.

Alpha acids: These compounds were less effective in causing membrane damage, with 25% of cells being PI-positive after one hour, 50% after 3 hours, and 70% after 6 hours.

SAL FR2, Rosmarinic acid, and Tyrosol: These compounds had minimal effects on membrane integrity (30-40% PI-positive cells after 6 hours), suggesting their mechanisms of action may not primarily involve membrane disruption. Tyrosol, despite being less effective in MIC tests, exhibits antioxidant properties that might indirectly affect microbial viability by modulating oxidative stress.

Cellular content leakage measurements correlated with flow cytometry results. Treatment with xanthohumol showed a rapid and significant increase in supernatant absorbance at 260 and 280 nm, indicating leakage of nucleic acids and proteins. After 3 hours of treatment, there was a 5-fold increase in supernatant absorbance at 260 nm and a 3-fold increase at 280 nm compared to the

untreated control.

SEM images confirmed these findings, showing clear morphological changes in bacterial cells after treatment with these compounds. Xanthohumol treatment resulted in severe distortion of cell shape, with cavities and holes in the cell membrane, and leakage of cell contents. Cells treated with oleanolic acid showed surface wrinkling and shape changes, while cells treated with alpha acids showed less pronounced changes.

Synergy tests between xanthohumol and oleanolic acid showed a strong synergistic effect, with a FICI value of 0.375. When combined, the MIC value of each compound was significantly reduced, with the MIC of xanthohumol decreasing from 5 to 1 μg/ml, and the MIC of oleanolic acid decreasing from 5 to 0.875 μg/ml.

Time-kill assays for the synergistic combination confirmed the enhanced effect, with the combination showing a much faster kill rate than each compound alone. After just 4 hours of treatment with the combination, viable bacterial counts decreased by more than 4 $\log_{10}$ CFU/ml, compared to a 1.5-2 $\log_{10}$ CFU/ml decrease for each compound alone.

Additive effects ($0.5<FICI\leq1$) were observed between xanthohumol and alpha acids, and between oleanolic acid and SAL FR2. Other compound interactions were either indifferent ($1<FICI\leq4$) or antagonistic ($FICI>4$).

4.2. Applications and Recommendations

Given their efficacy and generally recognized as safe (GRAS/QPS) status, xanthohumol and oleanolic acid are strong candidates for natural food preservation systems. Several potential applications in the food industry are proposed:

Active Packaging Systems: Incorporation into packaging films to create active packaging systems that prevent the growth of *L. monocytogenes* on the surface of food products, particularly for ready-to-eat and refrigerated products.

Edible Dips and Coatings: Use as dips or edible coatings for fruits, vegetables, and animal products to provide additional protection against *Listeria* contamination during storage and distribution.

Natural Preservatives: Direct addition to food products as natural preservatives, with consideration of potential sensory effects, especially for xanthohumol which might add bitterness.

Synergistic Combinations: Utilization of the synergistic combination of xanthohumol and oleanolic acid to achieve stronger antimicrobial effects at lower concentrations, reducing potential

sensory impacts and cost.

Multiple-Barrier Approach: Integration as part of a multiple-barrier approach, alongside other preservation techniques such as refrigeration, modified atmosphere packaging, and high-pressure processing.

Before commercial application, the following studies are recommended:

- Tests in real food matrices to evaluate efficacy under realistic conditions
- Stability studies to assess compound stability during processing and storage
- Sensory evaluation to determine potential impacts on food product sensory properties
- Scale-up and cost analysis to evaluate feasibility of large-scale production
- Regulatory considerations to ensure compliance with food safety regulations

5. Conclusion

This comprehensive evaluation of plant-derived compounds against *Listeria monocytogenes* has identified xanthohumol and oleanolic acid as particularly promising candidates for natural food preservation strategies. Their low MIC values (5 μg/ml), rapid bactericidal effects, and synergistic interaction when combined make them valuable tools for controlling this significant foodborne pathogen.

The efficacy of these compounds under refrigeration temperatures is particularly relevant for controlling *L. monocytogenes*, which can proliferate in cold-stored foods. Their effectiveness against biofilm formation further enhances their potential utility in food processing environments where biofilms represent a persistent source of contamination.

Future research should focus on formulation strategies to enhance stability and efficacy in complex food matrices, sensory impact assessment, and validation studies in commercial food products. The synergistic combination of xanthohumol and oleanolic acid warrants particular attention as it could allow for effective control at lower concentrations, potentially minimizing cost and sensory impact while maximizing antimicrobial efficacy.

These findings contribute to the growing body of evidence supporting the use of plant-derived compounds as alternatives to synthetic preservatives, addressing consumer demand for clean-label products while maintaining high standards of food safety.

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