Research Article

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Antibacterial agents to control of pathogenic *Xanthomonas arboricola* pv. *juglandis* on walnut fruits and plants by polygodial derivatives formulations

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Abstract

BACKGROUND: Walnut bacterial blight, caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj), leads to significant yield losses in walnut production, which is a key agricultural sector in Chile. Conventional control methods based on copper have limitations owing to environmental concerns, phytotoxicity, and bacterial resistance. Plant-derived compounds such as polygodial offer a promising alternative as a result of their potent antibacterial properties. This study aimed to develop and evaluate the antimicrobial efficacy of nanoemulsions containing drimanic compounds, specifically polygodial (1) and epi-polygodial (2), as a sustainable strategy to control Xaj.

RESULTS: Both polygodial (1) and epi-polygodial (2) demonstrated significant antibacterial activity against Xaj strains *in vitro* and *in planta*. In assays with immature walnut fruits, the nanoemulsion with compound 1 at 125 ppm reduced disease severity by 40%, an efficacy level intermediate between the negative control and the commercial chemical treatment (65% reduction). The nanoformulations effectively suppressed infections on leaves and shoots, and importantly, no phytotoxic effects were observed on either fruits or plants.

CONCLUSION: Polygodial and epi-polygodial are potent antibacterial agents against Xaj, making them excellent candidates for new biopesticides. This study successfully formulated these drimanic compounds into stable nanoemulsions that effectively

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control walnut bacterial blight without causing phytotoxicity. The development of these nanoformulations represents a significant advancement towards sustainable and effective disease management in the walnut industry, offering a viable alternative to conventional copper-based bactericides.

Keywords: drimanic compounds; polygodial; epipolygodial; *Xanthomonas arboricola* pv. *Juglandis*; bacterial blight

1 INTRODUCTION

The Walnut (*Juglans regia* L.) is an economically significant nut crop cultivated in temperate climates.[1–3] According to USDA FAS data, global walnut production reached 1 991 610 tons in the 2015/2016 season, with China accounting for ≈50% of the total output[4–6] Chile ranks as the fourth-largest walnut producer globally, with the industry expanding rapidly over the past decade to >40 000 ha.[6] Walnut orchards now occupy the second-largest cultivated area in Chile, representing 12.5% of the country´s total agricultural production area (37 568 ha).[4,5] Although walnuts are grown throughout Chile, production is concentrated in the regions of Coquimbo, Valparaíso, Metropolitan, O'Higgins, Maule, Ñuble and Araucanía, with the Metropolitan region having the largest cultivated area.[4,5]

Walnut cultivation is threatened by bacterial, fungal and oomycetes pathogens that cause significant economic losses.[7–13] Effective disease control is essential for sustaining and expanding Chile´s walnut industry, as it enhances productivity, improves fruit quality, and ensures compliance with the stringent health and safety standards required by international markets.[14] The global pomology sector faces significant challenges owing to phytopathogens and pest organisms, including bacteria, fungi and viruses, which cause substantial economic losses in agricultural production.[8,14] Among these, bacterial diseases such as black walnut blight, caused by *Xanthomonas arboricola* pv. *juglandis* (Xaj), are particularly detrimental to walnut (*Juglans regia*) production, leading to yield losses of ≤50% in affected orchards.[7,9] Conventional chemical treatments have been widely used to control Xaj; however, their long-term use has led to environmental concerns, bacterial resistance, and regulatory restrictions on copper-based products.[15] As a result, the demand for sustainable and biodegradable alternatives has increased, with a focus on plant-derived bioactive compounds and nanotechnology-based delivery systems.[16,17]

Botanical nanopesticides, particularly nanoemulsions, have emerged as an innovative approach to improving the stability and bioavailability of plant-derived antimicrobial compounds.[18–20] 20 Nanoemulsions encapsulate nonpolar active ingredients within aqueous environments, allowing for enhanced solubility, controlled release and prolonged bioactivity.[21] However, their inherent instability has been a key limitation in large-scale agricultural applications. Studies have demonstrated that optimizing the oil-to-surfactant ratio plays a crucial role in achieving smaller droplet sizes and maintaining nanoemulsion stability. Excessive surfactant levels, while beneficial for stability, also may lead to phytotoxic effects, necessitating careful formulation adjustments.[12,22]

Chilean walnut production faces challenges from three major bacterial pathogens: crown gall (*Agrobacterium tumefaciens*), bacterial canker (*Pseudomonas syringae* pv. *syringae*) and bacterial blight (Xaj).[8,12,23–25] The genus *Xanthomonas* is a significant concern for a wide range of host plants, as it causes severe diseases in crops such as rice, citrus, and tomato, in addition to walnut.[10,12]

In Chile, the incidence of bacterial blight caused by Xaj has increased from north to south as a consequence of higher spring rainfall, which favors disease development and spread. Bacterial blight is one of the most recurrent infections in walnut orchards, affecting plant health, fruit quality and overall commercial yield.[8,25] The disease is particularly severe in early-flowering walnut varieties, with losses exceeding 50% in affected orchards and reaching ≤70–100% under favorable environmental conditions.[8,25] *Xanthomonas arboricola* pv. *juglandis* infects walnut trees through natural openings such as stomata, lenticels, floral stigmas, and wounds.[26] The infection may remain latent for over a year, with initial disease symptoms appearing at bud break, influenced by environmental factors such as increased moisture, relative humidity (RH) >95% and moderate temperatures.[27] The bacterium spreads via pollen, rainwater and insects, as well as from previously infected buds, catkins, twig cankers and pruning residues.[27]

The primary method for bacterial disease control in walnuts relies on copper-based compounds, including copper oxychloride and micronized copper formulations.[28] However, these chemicals pose several challenges, such as phytotoxicity, bacterial resistance, and residue accumulation on fruit and plant tissues, necessitating the development of alternative control strategies.[8–10,12,25,28] Given the economic importance of walnut production in Chile, it is crucial to find effective and environmentally sustainable solutions to prevent disease spread and mitigate financial losses. Among the most promising alternatives to conventional bactericides are drimanic compounds, a class of sesquiterpenes with bicyclic farmesane-type skeletons.[20] Drimane α, β-unsaturated dialdehydes are known for their diverse biological benefits, including antibacterial, antifungal, anti-inflammatory, cytotoxic and phytotoxic activities.[20,29]

Derita *et al.*[30] demonstrated that the biological activity of drimanic compounds, particularly polygodial (**1**), is likely to be linked to electronic properties surrounding the Δ7,8 double bond. The molecular electrostatic potential (MEP) of these compounds shown a highly positive differential zone, which may explain their potent antimicrobial effects.[31] The antibacterial activity of drimanic compound **1** has been well-documented, with reports indicating effective inhibition of both Gram-negative and -positive bacteria at minimal inhibitory concentrations (MIC) of 2–20 μg mL^{-1}.[32] Fujita and Kubo[33] further demonstrated that compound **1** also exhibits moderate antibacterial against *Bacillus subtilis* and *Staphylococcus aureus* (MIC: 100 μg mL^{-1}), as well as against *Salmonella choleraesuis* and *Escherichia coli* (MIC: 50–100 μg mL^{-1}).[30] Additionally, compound **1** effectively inhibits the *Ralstonia solanacearum* species complex (RSSC) with a MIC of 25 μg mL^{-1}.[22,34] The mode-of-action (MoA) involves membrane disruption and inhibitory of ATP synthase in bacterial cells. Furthermore, polygodial (**1**) has shown significant antibacterial activity against phytopathogens such as, *Clavibacter michiganensis* subsp. *michiganensis* and *Pseudomonas syringae* pv. *tomato*[20]

Nanotechnology has the potential to revolutionize agricultural production by enhancing the controlled release of agrochemicals, improving nutrient utilization and increasing disease resistance in

crops.[16,35] Understanding interactions between nanoparticles and plant systems can significantly improve the efficiency of agricultural inputs while reducing their environmental impact. Encapsulation of pesticides and fertilizers in nanoparticles has been developed to minimize the excessive use of conventional agrochemicals. Studies suggest that nanoparticle formulations can reduce chemical input by 70–80%, resulting in lower costs and decreased environmental impact.[18,36] Moreover, nanopesticides delivered through nanocarriers, such as nanoemulsions, have demonstrated the ability to reduce the quantity of active ingredients required by 10–15-fold while maintaining efficacy and prolonging disease management.[21,37]

Beyond chemical control strategies, biological agents and plant extracts have been explored as promising alternatives for bacterial blight management. Mikiciński *et al.*[38] reported that essential oils such as lavender, sage, lemon balm, clove and a thyme oil-based preparation (BioZell) exhibited antibacterial activity against Xaj. However, although essential oils demonstrate notable antimicrobial potential, their stability and solubility in aqueous environments remain significant challenges for field application. The nanoemulsions developed in this study address these limitations by enhancing the bioavailability and controlled release of active compounds, offering a viable and more stable alternative to conventional essential oil applications.[12,21]

Given the challenges associated with conventional disease control methods, this study aims to evaluate the antimicrobial activity of drimanic compounds and polygodial derivatives formulated in nanoemulsions against Xaj. By integrating natural bioactive compounds with nanotechnology-based delivery systems, this research seeks to develop effective and sustainable strategies for controlling bacterial blight in walnut orchards.

2 MATERIALS AND METHODS

2.1 Chemical characterization

The synthesized compounds and natural drimanes were characterized based on physical properties such as melting point and optical rotation, and spectroscopic analyses. These latter included Fourier-transform infrared spectroscopy (FTIR), proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR), selective one-dimensional (1D) total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY), and two-dimensional (2D) ^{1}H—^{13}C heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC). Additional analyses were performed using gas chromatography-mass spectrometry (GC-MS) and high-resolution mass spectrometry (HRMS). Reaction progress was observed using thin-layer chromatography (TLC), whereas the separation and purification of synthesized compounds, were carried out using column chromatography.

2.1.1 Formulations

Nanoemulsions were prepared used drimanic compounds and bark extracts from *Drimys winteri*. A high-energy methodology was employed, utilizing high-pressure homogenization (EmulsiFlex-C3; Avestin Inc., Ottawa, Canada) to produce single-phase (O/W or W/O) or double-phase (W/O/W or O/W/O) nanoemulsions. The specific type of nanoemulsion was determined by the solubility of the active ingredient and the intended formulation type formulation (contact or systemic). The selection of surfactants and co-surfactants was guided by their hydrophilic-lipophilic balance (HLB) value, taking into account the desired MoA of the formulation.[39,40]

2.1.2 Storage stability of nanoemulsion

Fifty milliliters CAR-NE-3 were transferred into pre-sterilized centrifuge tubes and incubated at −4 °C and room temperature (RT). Hydrodynamic diameter (nm), polydispersity index (PDI) and zeta potential (ZP; mV) were measured using dynamic light scattering (DLS) equipment for 60 consecutive days after every 10 days. There were 13 runs on average for each measurement.

2.2 Nanostructure stability

The stability of the nanoemulsions was assessed by monitoring particle size, PDI and ZP *versus* time using photon correlation spectroscopy (DLS) with a Zetasizer ZS 90 analyzer (Malvern Panalytical, Malvern, UK). Nanoemulsions were monitored by DLS at three storage temperatures: 4, 25 and 37 °C. The ZP measurements were performed using specialized zeta cells (Malvern Panalytical) equipped with electrodes, under the same dilution conditions used for particle size analysis.[39,40]

2.3 Characterization of nanoemulsion formulations

The Zetasizer Nano zs (Malvern Panalytical) was used to measure the size and ZP of the nanoemulsions. This instrument operates on the photon correlation spectroscopy (PCS) principle and provides accurate size measurements in the range of 0.3 nm to 10 µm. For size analysis, 40 µL nanoemulsion sample were mixed with 1 mL HPLC-grade water in a cuvette, and the average of three readings was calculated. Specialized cuvettes were used to measure the ZP. The pH of the nonoemulsion samples was measured with a pH meter. The meter was calibrated using standad buffer solutions of pH 4 and pH 7 before the measurements.[41]

2.4 Drug loading and encapsulation efficiency

The incorporation of drimanic compounds into the nanoemulsion was quantified by centrifuging the bioactive compound-loaded nanoemulsion and separating the supernatant. The precipitate was discarded, and the concentration of the bioactive compound in the supernatant was determined via HPLC analysis.[40] The following equations were used to calculate the percentage of drug loading and encapsulation efficiency (EE):[42]

$$\text{Drug loading}\,(\%) = \left(\frac{\text{Total weight of bioactive compound}}{\text{Total weight of sample}}\right) * 100\%$$

$$\text{EE}\,(\%) = \left(\frac{\text{Actual bioactive compound loaging}}{\text{Theoretical bioactive compound loading}}\right) * 100\%$$

2.5 Biological assays

2.5.1 Biological material

The antibacterial assays were conducted in the Laboratory of Phytopathogy, Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso. Glasshouses and field trials were carried out at the Estación Experimental La Palma, Pontificia Universidad Católica de Valparaíso. Both tests were overseen by Ximena Besoain.

2.5.1.1. Walnut trees. Walnut trees aged 2 to 6 years were selected for the study. The two cultivars used were Chandler and Serr. Additional experiments were conducted in a 6-year-old commercial orchard located in the Valparaíso region. The experiments were carried out during the year 2023.

2.5.1.2. Walnut pathogen strains. The plant pathogenic bacterium isolates of Xaj (at least three isolates) were provided by the Laboratory of Phytopathogy, Escuela de Agronomía, Pontificia Universidad Católica de Valparaíso.

2.5.2 Antimicrobial efficacy analysis using plate diffusion and broth macrodilution methods

2.5.2.1. Hole-plate diffusion assay method of Hewitt and Vincent. The hole-plate diffusion assay method[43] was used to test the activity of compounds *1–19* against the Xaj. Freshly grown bacterial cultures were inoculated at a concentration of 10^7 colopny-forming units (CFU) mL^{-1} in the appropriate agar medium. Wells, 3 mm in diameter, were created using a sterile cork borer, with three to four replicates on each plate. Each well was loaded with 12 μL test compound (**1–19**); the same volume of sterile distilled water was used as a control. The plates were inoculated at 28 °C for 48 h, after which the radius of zone of growth inhibition surrounding each well was measured to evaluate antibacterial activity.

2.5.2.2. In vitro tests to evaluate the bactericidal activity of drimanic and polygodial derivatives. The antibacterial activity of the drimanic and polygodial derivatives was assessed using broth macrodilution methods as described previously.[44,45] All tests were performed in duplicate. In brief, serial dilutions of Xaj were prepared in 1 mL yeast–peptone–glucose broth (YPGB). Antimicrobial substances dissolved in DMSO (5 mg mL^{-1}) were added to YPGB at final concentrations of 1, 2, 4, 8, 16, 32, 64, 128 and 256 μg mL^{-1}. Sterile water and DMSO were included as negative controls. The bacterial inocula were prepared from an overnight culture and adjusting to a concentration to 1–5 × 10^6 CFU mL^{-1}. Ten microliters of each dilution were inoculated into YPGB and incubated at 0, 24, 48 and 72 h, with two replications per dilution. The minimal inhibitory concentrations (MIC) was defined as the lowest concentration the compound that inhibited visible growth, whereas the minimal bactericidal concentrations (MBC) was determined as the lowest concentrations that reduced bacterial viability by 99.9%. Both parameters were evaluated after 72 h of incubation on a rotatory shaker at 28 °C.

2.5.3 Assays with vegetal material

2.5.3.1. Effect of the formulations on walnut fruits inoculated with Xanthomonas arboricola *pv.* juglandis. The experiments were conducted using asymptomatic immature walnut fruits (*J. regia* cv. Chandler) at phenophase Gf + 75. The methodology was adapted from Martins *et al.*[46] Fruits were surface-disinfected in a 70% ethanol solution for 30 s, rinsed with sterile distilled water, and allowed to air-dry at RT. They were then immersed in a bacterial suspension of Xaj prepared to an optical density at 600 nm (OD_{600}) = 0.3 for 30 min with gentle agitation. After inoculation, the fruits were placed in disinfected plastic boxes lined with absorbent paper. The boxes were covered and kept at RT under natural light for 24 h. The following day, different formulations were applied using a sprayer: (1) sterile water (control) and (2) compounds **1–5** at a concentration of 31, 62.5, 125 or 250 ppm. Five fruits were used per treatment. A treatment without inoculation or formulation was included as a negative control. To evaluate the results, a severity scale (Figure 4) was established based on that described by Solar *et al.*[47] The severity of fruit damage was calculated using the modified Towsend–Heuberger formula (1943):

$$\text{Severity}\,(\%) = \Sigma\frac{(n\times v)}{i\times N}*100$$

where n = number of fruits in each severity class, v = value of the severity class (0,1,2,3,4), i = highest severity class (4) and N = total number of fruits (5).

2.5.3.2. Evaluation of antagonistic agents in glasshouse and field conditions. Inoculation assays were prepared using the Xaj strain grown on YPGA. Suspensions were adjusted to ≈10^7 CFU mL^{-1} for glasshouse experiments and 10^8 CFU mL^{-1} for field treatments. To promote water congestion, plants were covered overnight with polyethylene bags.

In glasshouse experiments, 2-year-old walnut-trees were used with four replicates per treatment. Five treatments with the antagonist agent were conducted simultaneously. The inoculum was sprayed twice, at a 20-day interval, simulating two infecting rains. Treatments included polygodial derivatives, 'Agrygent Plus' (80 ppm), and distilled water as controls. For each replicate, 400 leaves (4 × 100) were sampled at fruit harvest. On both leaves and fruits, bacterial spots were counted, and disease incidence and severity were calculated.[48]

In field experiment, 2-year-old trees were randomized for either three or four replications, with plants sprayed with the antagonist 24 h before inoculation. Controls included 'Agrygent Plus' (80 ppm) and water. Disease development and sampling were monitored for ≤3 weeks' postinoculation. Sampling involved 180 leaves (60 per replicate) or 720 leaves (180 per replicate), depending on the treatment. In commercial orchards, 6-year-old trees were treated five times with the antagonist from late May to early July, approximately every 10–14 days. Control plants were sprayed with distilled water. Sampling included fruits and 100 leaves (4 × 25) per replicate.

For all samples, a 0-to-5 severity scale was applied, corresponding to the affected leaf area, where 0 = 0%, 1 = 1%, 2 = 3%, 3 = 6%, 4 = 12% and 5 = ≥ 24%.[48]

Disease severity (S) calculated for each plant was calculated using the formula:

$$\text{Disease severity}\,(S) = \left(\frac{\Sigma \text{In}}{N\times 5}\right)*100$$

where I_n = severity index for each leaf, N = total number of leaves per plant and 5 = the maximum severity index value in the scale. All data were statistically analyzed using ANOVA with Tukey´s honestly significant difference (HSD) test ($P \leq 0.05$).

3 RESULTS

3.1 Chemistry

Drimanic compounds **1**–**7** (Figure 1) were successfully synthesized and characterized, as described by Derita *et al.*[30] The synthesis process of each compound is detailed in the referenced study. Figure 1 shows the chemical structures of drimanic sesquiterpenes evaluated for their antibacterial activity against Xaj. Among these, polygodial (**1**) is a dialdehyde recognized for its potent antimicrobial properties. Drimenol (**2**) and drimenal (**3**) possess hydroxyl and aldehyde functional groups, respectively, which may influence their bioactivity. Drimendiol (**4**), containing two hydroxyl groups, and 1β-Acetoxy-Polygodial (**5**), an acetylated derivative of polygodial, may exhibit variations in activity owing to their structural

modifications. Additionally, compounds (**6**) and (**7**), identified as 9α-Hydroxy-Polygodial and 9β-Hydroxy-Polygodial, feature hydroxyl substitutions at the C-9 position, which could impact their biological function.

3.2 Storage stability of nanoemulsion

Table 1 presents the particle size characteristics, PDI and ZP of 10% oil-in-water nanoemulsions containing compounds **1** and **2**, formulated with mixed lipid phases. The results indicate that formulations T1, T2 and T3, prepared with a mixture of oleic acid (OA) and linoleic acid (LA), exhibited significantly smaller particle sizes (ranging from 10.0 to 12.81 nm) compared to the commercial formulation (T4), which had a considerably larger particle size of 282 nm.

The PDI values indicate that formulations T1, T2 and T3 had relatively low polydispersity, suggesting a more uniform size distribution, whereas the commercial formulation (T4) exhibited a significantly higher PDI (1.6), indicating greater heterogeneity in droplet size.

Regarding ZP, formulations T1, T2 and T3 displayed values close to neutral (ranging from 0.16 to 0.75 mV), whereas the commercial formulation showed a lower ZP value (0.13 mV), which may suggest lower electrostatic stability. These results highlight the potential of the OA:LA-based formulations to maintain stable nanoemulsions with smaller and more uniform particles compared to the commercial formulation.

3.3 Nanostructure stability

The stability of polygodial nanoemulsions formulated with Tween 80 and Triton X-100 was evaluated over a 56-week period at 25 °C. The assessment included measurements of particle size, PDI and overall structural integrity of the formulations.

Figure 2(A) shows the stability of the polygodial Tween 80 nanoemulsion. Panel (a) presents the Z-average particle size over time, indicating fluctuations in the early weeks, followed by a gradual increase in size as the storage period progressed. Panel (b) compares the particle size distribution at 2 weeks and 54 weeks, highlighting slight aggregation and size increase over time.

Figure 2(B) illustrates the stability of the polygodial Triton X-100 nanoemulsion. In panel (a), the Z-average particle size remains more stable throughout the 56 weeks compared to Tween 80, suggesting better long-term stability. Panel (b) compares the particle size distribution at 2 weeks and 54 weeks, showing minimal changes, indicating that this formulation maintained a more uniform size distribution over time.

Figure 3 displays the PDI of both formulations over the 56-week period. Tween 80 nanoemulsions exhibited higher PDI variability, suggesting increased heterogeneity and particle size distribution fluctuations. By contrast, Triton X-100 nanoemulsions maintained a more stable and lower PDI, indicating better colloidal stability. These results suggest that Triton X-100 is a more effective surfactant for maintaining nanoemulsion stability over extended storage periods.

The initial variability in particle size observed during the first 10 weeks, particularly in the Tween 80 formulation [Figure 2(A)], can be attributed to the system reaching a state of quasi-equilibrium. Immediately following high-energy homogenization, the nanoemulsion is in a thermodynamically unstable state. During this initial maturation period, rapid kinetic processes such as surfactant rearrangement at the oil–water interface and the dissolution of the smallest, most unstable droplets via Ostwald ripening are dominant. These dynamic adjustments lead to fluctuations in the measured Z-average particle size. Once this initial equilibration phase is complete, the system enters a slower, more predictable aging process, reflected by the more stable degradation trend observed after ≈10 weeks. The higher PDI of the Tween 80 formulation during this period (Figure 3) further supports this interpretation, indicating a more heterogeneous population compared to the Triton X-100 system, which exhibited much greater initial stability as shown in Figure 2(B).

3.3.1 *Encapsulation efficiency of fungicides in nanoparticles*

The total amount of encapsulated drimane, defined as 100%, was determined following its complete extraction from the nanoparticles. This was achieved by dissolving the nanoparticle samples in acetone. The resulting solution was then filtered through a 0.22-µm Millipore membrane, and the fungicide concentration was quantified via HPLC, as described previously.

The EE was measured to evaluate the interaction between the nanocarriers and the encapsulated compounds, **1** and **2**. The formulations, prepared with Triton X-100 and Tween 80, are well-suited for delivering hydrophobic compounds such as drimanes 1 and 2. Owing to their low aqueous solubility, these compounds

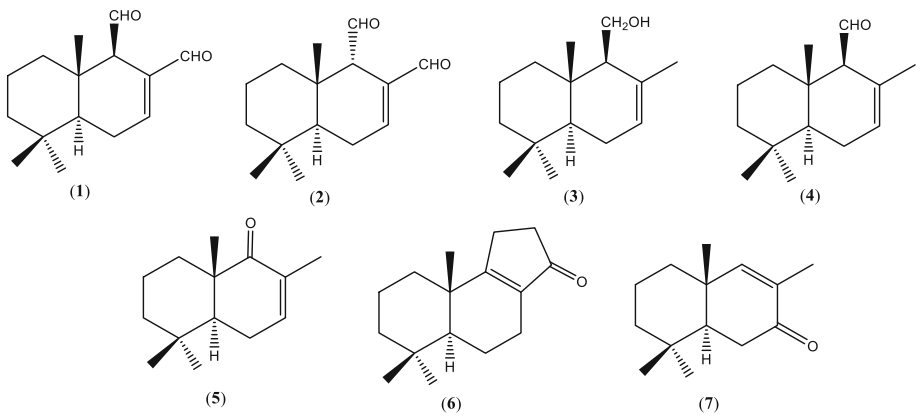

Figure 1. Chemical structures of drimanic compounds (**1**–**7**) evaluated for activity against *Xanthomonas arboricola* pv. *juglandis*. The figure presents the chemical structures of drimanic sesquiterpenes tested for antibacterial activity. Polygodial (**1**) is a dialdehyde with strong antimicrobial properties. Drimenol (**2**), drimenal (**3**) and drimendiol (**4**) contain hydroxyl and aldehyde functional groups, whereas 1β-acetoxy-polygodial (**5**) is an acetylated derivative. Compounds **6** and **7** (9α- and 9β-hydroxy-polygodial) feature hydroxyl modifications at C-9, potentially affecting their biological activity.

Table 1. Particle size characteristics of 10% oil-in-water nanoemulsions containing compounds **1** and **2**, formulated with mixed lipid phases

Oil type formulation	Particle size (nm)	PDI	ZP (mV)
OA:LA (T1)	10.0 ± 0.03	0.257	0.19 ± 0.02
OA:LA (T2)	11.93 ± 0.3	0.348	0.16 ± 0.01
OA:LA (T3)	12.81 ± 0.06	0.447	0.75 ± 0.31
Commercial formulation: (T4)	282 ± 11.30	1.6	0.13 ± 0.01

exhibit a high affinity for the lipophilic core of the nanocarriers. Consequently, both drimanes demonstrated a high encapsulation efficiency (>99%) and remarkable stability, with the payload being retained for >210 days. These results indicate a strong affinity between the fungicides and the nanocarriers. These findings are consistent with previous reports, such as that by Asrar *et al.*,[49] who reported encapsulation efficiencies between 95.2% and 98.3% for antimicrobial agents in polymeric nanospheres, which also exhibited a slow-release profile.

3.3.2 *Antibacterial activity*

The MIC and MBC results for the drimanic compounds are summarized in Figs 4 and 5, and Table 2. Among the seven evaluated compounds, polygodial (**1**) exhibited the strongest antibacterial activity against Xaj, with MIC and MBC values as low as 31 μg mL^{-1} for certain strains. Compound **2** displayed the highest *in vitro* antibacterial potency overall, followed by compound **4**, which showed moderate effectiveness.

By contrast, compounds **5**–**7** exhibited low antibacterial activity. Notably, compounds **3** and **4** were less active against the tested strains when evaluated on fruits. These results indicate that the structural characteristics of compounds **1** and **2** contribute to their enhanced antibacterial efficacy, making them promising candidates for further development.

Figures 4 and 5 illustrate the inhibitory effects of nanoformulated compounds **1** and **2** on the growth of Xaj strains 639 and 644 over time.

Figure 4 shows that the nanoformulation of compound **1** exerted a strong bactericidal effect at a concentration of 250 ppm for both *Xanthomonas* strains after 24 h. Strain 644 exhibited greater sensitivity, with inhibition observed at concentrations as low as 62.5 ppm. These results suggest that compound **1**, when formulated as a nanoemulsion, retains significant antibacterial activity, particularly against strain 644. The triplicate assays confirmed the reproducibility of these findings.

Figure 5 presents the inhibitory effect of the nanoformulation of compound **2**, which demonstrated bactericidal activity at concentrations of 250, 125, 62.5 and 31 ppm for both *Xanthomonas* strains. Notably, strain 639 showed higher sensitivity to compound **2** at the initial time point (time 0). This suggests that compound **2**, even at lower concentrations, is highly effective in suppressing bacterial growth. The triplicate assays validated these results, confirming their reliability.

These findings highlight the potential of nanoemulsified drimanic compounds as alternative antibacterial agents for controlling Xaj, with compound **2** showing a broader inhibitory range at lower concentrations.

3.4 Biological assays of fruits

The impact of different treatments on the severity of Xaj infection in immature walnut fruits was assessed using the damage index (DI) (Figures 6–8).

Figure 6 presents the severity scale used to evaluate walnut fruits subjected to different treatments. The scale classifies disease severity from 0 (no symptoms) to 4 (very strong infection),

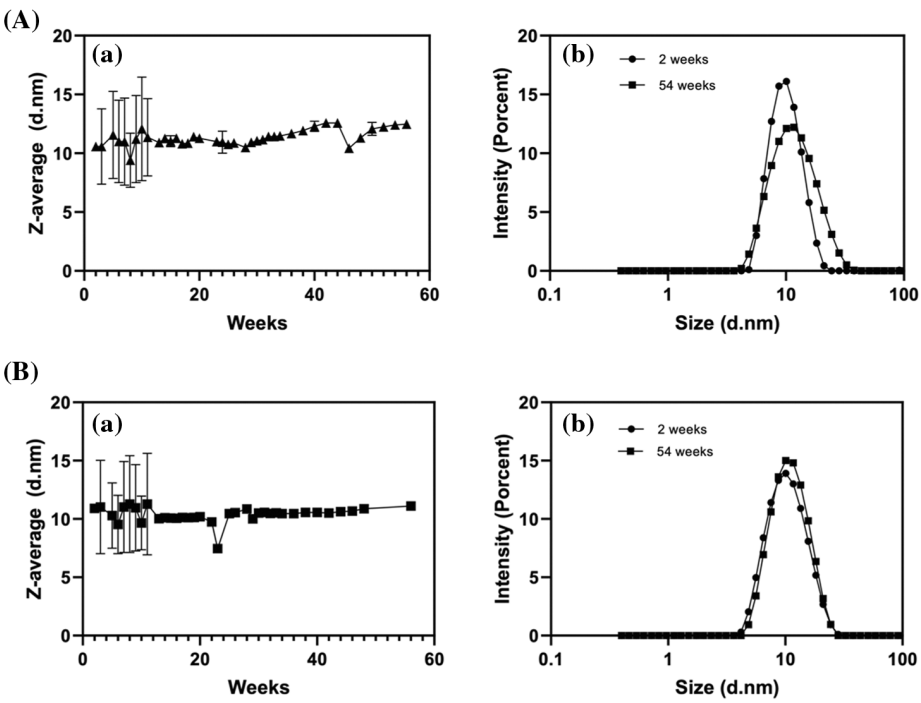

Figure 2. Stability evaluation of polygodial Tween 80 nanoemulsion (A) and polygodial Triton X-100 nanoemulsion (B). (a) Stability assessment conducted at 25 °C, with measurements performed in triplicate over a 56-week period. (b) Size comparison of the nanoemulsions at 2 weeks and 54 weeks.

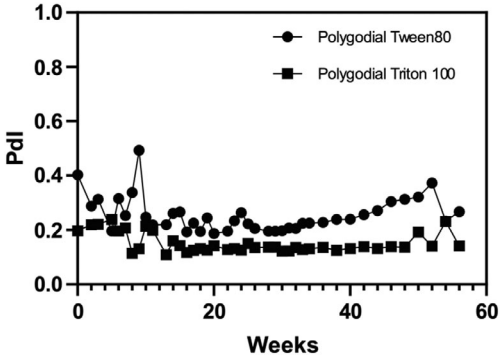

Figure 3. Polydispersity Index (PDI) of polygodial nanoemulsions with Tween 80 and Triton X-100 over 56 weeks. The PDI of polygodial nanoemulsions was monitored to assess stability. Tween 80 nanoemulsions showed greater variability over time, indicating increased heterogeneity, whereas Triton X-100 nanoemulsions maintained a more stable PDI, suggesting better formulation stability.

based on the presence and intensity of necrotic lesions. This visual reference standardizes the evaluation of disease progression in response to the applied formulations. Figure 7 shows the visual comparison of immature walnut fruits after treatment application. Treatments T2 (polygodial) and T4 (Agrygent Plus) significantly reduced the symptoms of bacterial blight compared to the positive control (T0), which exhibited the highest level of disease severity. Additionally, treatment T4 was significantly more effective than treatment T3, indicating a higher level of disease suppression. The negative control (T00), which was not inoculated, showed no visible symptoms, confirming the reliability of the assay. Figure 8 presents a quantitative analysis of disease severity reduction across treatments at 125 ppm. Treatments containing polygodial (T2) and the commercial product Agrygent Plus (T4) resulted in the highest reductions in disease severity. Specifically, Agrygent Plus (T4) achieved the greatest reduction (65%), followed by polygodial (40%) and canelo oil (30%). By contrast, compounds **3**, **5** and **6** exhibited only moderate reductions (10–25%), whereas compound **7** showed phytotoxicity, indicating a potential negative effect on fruit health.

Table 3 summarizes the percentage reduction in disease severity for compounds **1**–**7** in nanoemulsion formulations, as evaluated in walnut fruits. Agrygent Plus (65%) demonstrated the highest efficacy, followed by polygodial (40%) and canelo oil (30%). Compounds **2**, **3**, **4**, **5** and **6** showed varying degrees of disease reduction, with compound **6** being the least effective (10%). Notably, compound **7** exhibited phytotoxic effects, suggesting potential toxicity to walnut fruits.

Overall, these results demonstrate that polygodial-based formulations (T2) and Agrygent Plus (T4) effectively reduced the severity of bacterial blight in walnut fruits, with T4 exhibiting the most

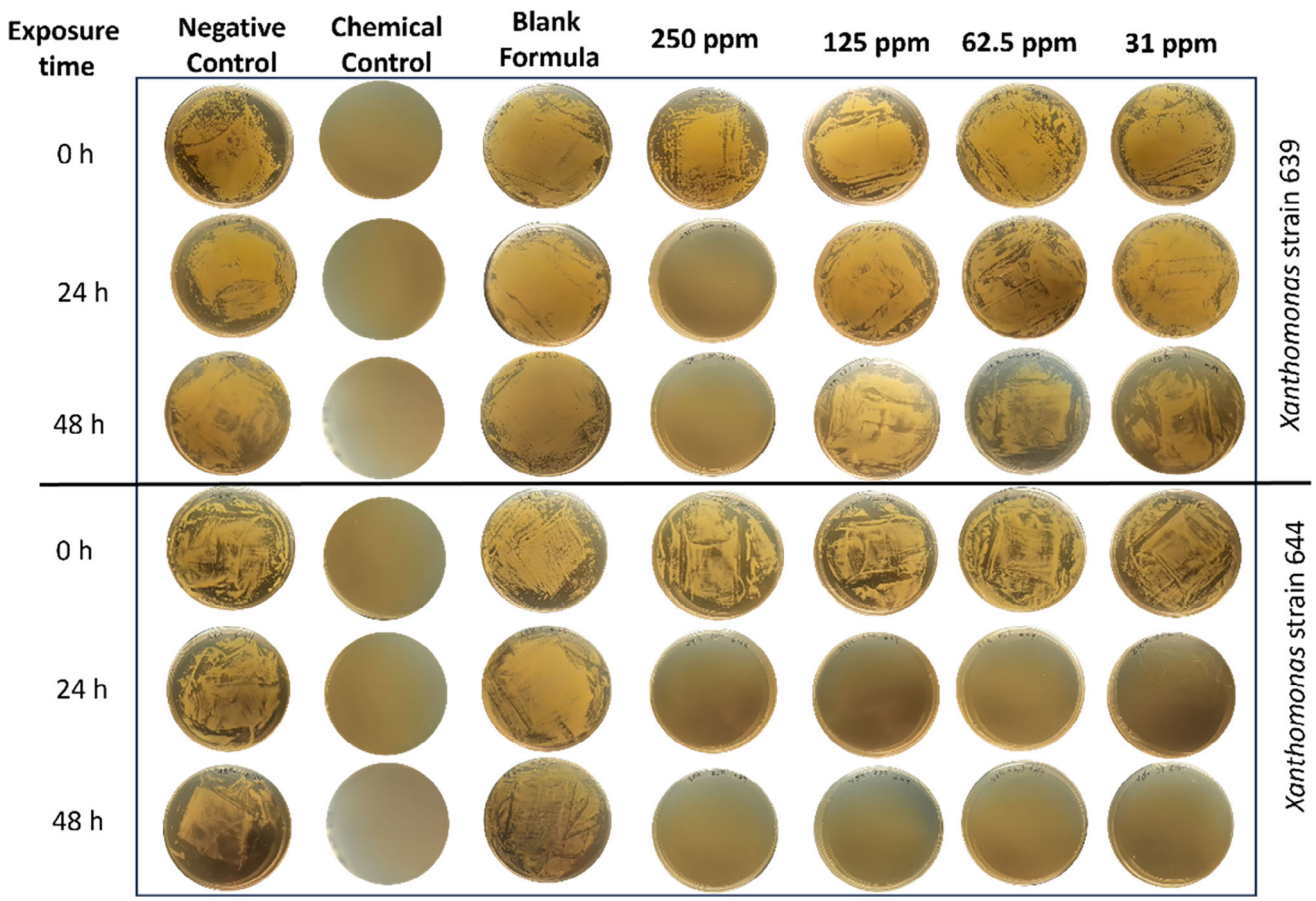

Figure 4. Inhibitory effect of the nanoformulation of compound **1** on the growth of *Xanthomonas* strains 639 and 644 over time. The nanoformulation of compound **1** exhibited a bactericidal effect at a concentration of 250 ppm for both *Xanthomonas* strains after 24 h. Strain 644 showed greater sensitivity to the compound, with inhibition observed at concentrations as low as 62.5 ppm. All assays were performed in triplicate, and a representative plate is displayed in the figure.

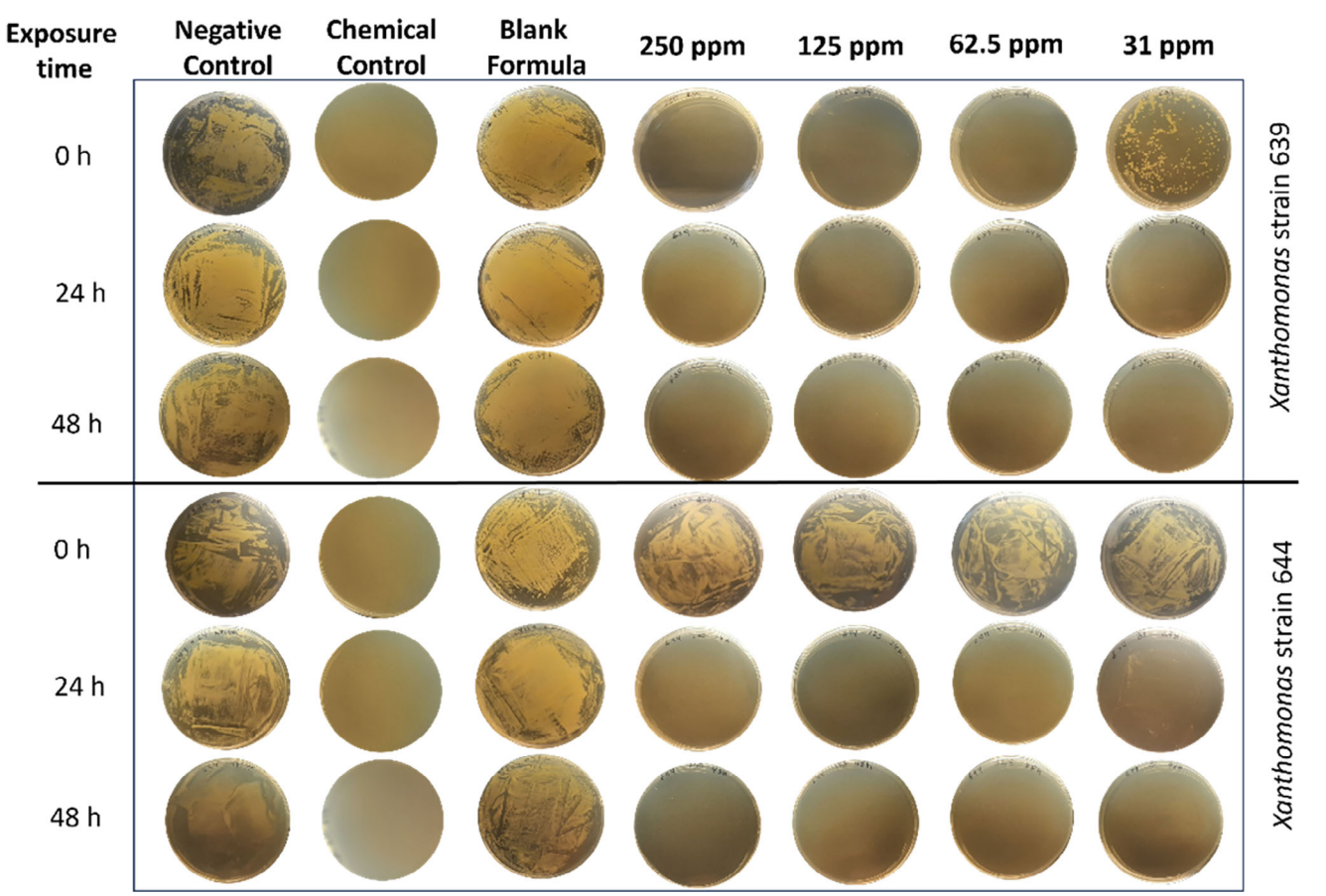

Figure 5. Inhibitory effect of nanoformulation of compound **2** on the growth of *Xanthomonas* strains 639 and 644 over time. The nanoformulation of compound **2** exhibited a bactericidal effect at concentration of 250, 125, 62.5 and 31 ppm for both *Xanthomonas* strains. Strain 639 demonstrated greater sensitivity to compound **2** at time 0. All assays were conducted in triplicate, with a representative plate shown in the figure.

pronounced effect. These findings support the potential of natural drimanic compounds as alternative biocontrol agents for managing Xaj.

3.5 Biological assays of plants

The assay was conducted in a glasshouse at the Phytopathology Laboratory under controlled conditions at a temperature of 28 °C. A total of 20 walnut plants (*J. regia* cv. Chandler), ≈2 m in height and grown in 50-L pots, were used.

In the glasshouse trial, treatments T2 (polygodial) and T4 (Agrygent Plus) maintained plants in better condition compared to T1, T3 and the positive control T0 (Fig. 9). This suggests a protective effect against Xaj infection.

Figure 9 illustrates the effects of different treatments on Chandler walnut plants inoculated with Xaj. The treatments included canelo oil (T1), polygodial (T2) and product 3 (T3), each applied at 125 ppm. These were compared to the inoculated positive control (T0) and the conventional commercial product Agrygent Plus

Table 2. MIC and MBC (μg mL^{-1}) values of the drimane series against *Xanthomonas arboricola* pv. *juglandis* strains

	X. arboricola pv. *juglandis 639*		*X. arboricola* pv. *juglandis 644*		*X. arboricola* pv. *juglandis* sp	
Compound	MIC	MBC	MIC	MBC	MIC	MBC
1	250	250	31	31	31	31
2	31	31	31	31	31	31
3	250	>250	250	250	250	250
4	250	250	62.5	250	125	125
5	125	125	125	125	125	125
6	>250	>250	>250	>250	>250	>250
7	>250	>250	>250	>250	>250	>250
Blank formula	>250	>250	>250	>250	>250	>250
AGRYGENT PLUS	16	16	16	16	16	16

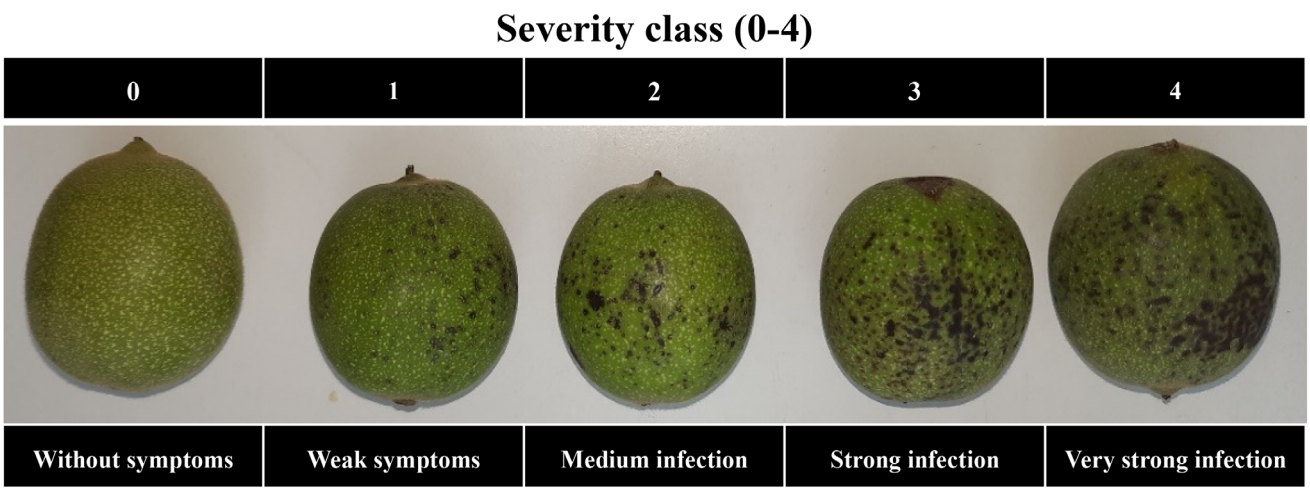

Figure 6. Severity scale used to evaluate walnut fruits from different treatments. The figure displays the severity classification (0–4) used to assess the degree of infection in walnut fruits treated with different formulations. The scale ranges from 0 (no symptoms) to 4 (very strong infection), with increasing levels of disease severity characterized by the presence and intensity of necrotic lesions. This visual reference was utilized to standardize the evaluation of disease progression in response to the applied treatments.

(T4). After 24 h of treatment application, plants were inoculated with Xaj using 125 mL bacterial suspension at a concentration of 1×10^8 CFU mL^{-1}. To facilitate infection, the plants were covered with plastic sheeting for 24 h.

The DI analysis (Fig. 10) revealed that treatment T4 (Agrygent Plus) was statistically different from T0, T1 and T3, confirming its superior efficacy in controlling bacterial blight. These results align with those presented in Fig. 7. Regarding the polygodial-based treatment (T2), its control effect was intermediate, as it did not significantly differ from either T4 or the other treatments. This suggests that a higher concentration of polygodial could improve its effectiveness in controlling Xaj. No phytotoxicity was observed in any of the treatments, indicating their safety for use in walnut plants (Fig. 6). Figure 10 presents the DI in walnut plants subjected

Figure 7. Immature walnut fruits of the Chandler variety affected by *Xanthomonas arborícola* pv. *juglandis*. The effect of the products Poltimox (T1), polygodial (T2) and product 3 (T3) at 125 ppm compared to the noninoculated negative control (T00), the inoculated positive control (T0) and a conventional commercial product Agrygent Plus (T4).

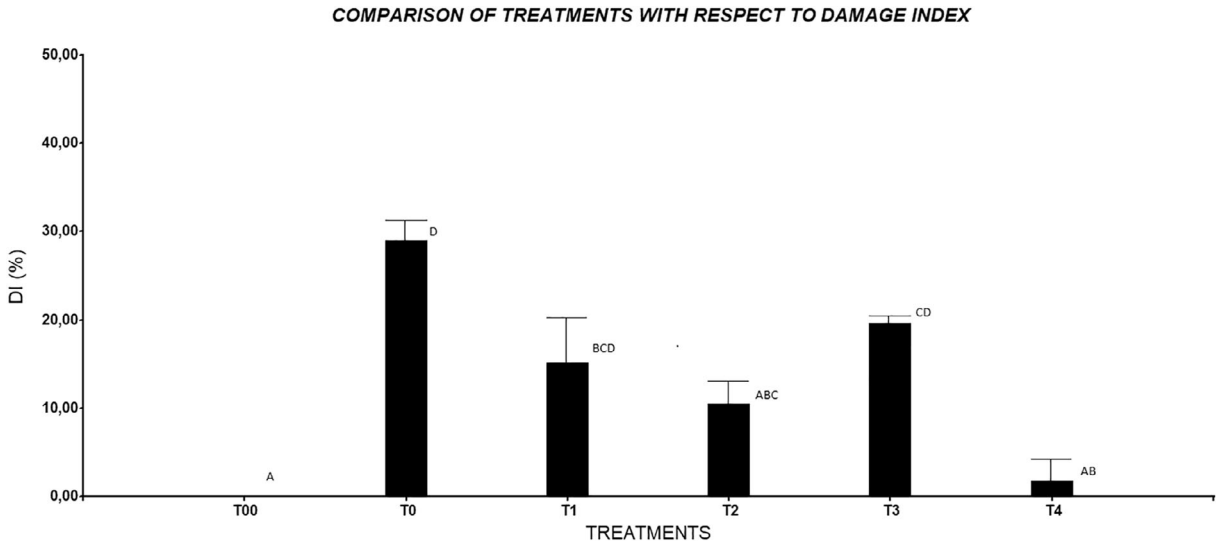

Figure 8. Effect of treatments at 125 ppm on immature fruits of walnut plants (*Juglans regia* cv. Chandler) inoculated with *Xanthomonas arboricola* pv. *juglandis*. The damage index (DI) for walnut fruits under different treatments. T4 (Agrygent Plus) had the lowest DI, indicating the highest efficacy, whereas T2 (polygodial) and T1 provided moderate disease suppression. T3 showed lower effectiveness, and T0 (inoculated control) exhibited the highest disease severity. The noninoculated control (T00) remained symptom-free. Means with the same letter are not significantly different ($P > 0.05$).

to different treatments. T4 (Agrygent Plus) showed the lowest DI, confirming its strong antibacterial effect. Treatments T1, T2 and T3 exhibited moderate disease suppression, whereas the untreated control (T0) had the highest disease severity, similar to T1 and T3. Treatments labeled with the same letter are statistically not significantly different ($P > 0.05$).

4 DISCUSSION

The results of this study demonstrate that nanoemulsions formulated with drimanic compounds, particularly polygodial (**1**) and epi-polygodial (**2**), are effective against Xaj both *in vitro* and in plant material. The stability and biological activity of these formulations can be interpreted based on their physicochemical properties and their interaction with the pathogen.

A key factor influencing the long-term performance of the nanoemulsions is their structural stability. The presence of aldehydic functional groups in the drimanic terpenoid scaffold of compounds **1** and **2** contributes to their partial solubility in aqueous environments, which can affect the overall stability and aggregation behavior of the nanoemulsion during storage. As observed in the long-term stability assays, these interactions can lead to phenomena such as Ostwald ripening, particularly at lower temperatures, resulting in increased droplet size and reduced colloidal stability. Despite these formulation challenges, the developed nanoemulsions maintained a high encapsulation efficiency (>99%) and demonstrated potent antibacterial efficacy, highlighting their potential as a viable delivery system for these bioactive compounds.

Table 3. % severity reduction for compounds **1**–**7** in nanoemulsion evaluated in walnut fruits

Compounds	% Severity reduction
Canelo oil	30
1	40
2	35
3	15
4	25
5	15
6	10
7	phytotoxic
Agrygent Plus	65

The strong antibacterial activity observed in the *in vitro* assays is consistent with the known MoAs of polygodial. One proposed MoA for its potent effect is the interaction of its dialdehyde groups with primary amine residues on the bacterial cell surface, forming pyrrole structures that disrupt critical microbial functions. This hypothesis is supported by previous findings where the antifungal activity of polygodial against *Saccharomyces cerevisiae* was inhibited by compounds containing primary amines. Given that the microbial cell membrane is the primary target of polygodial, it is plausible that this MoA extends to its bactericidal action against Xaj, offering a basis for developing novel antimicrobial agents with enhanced surface activity.

The effectiveness of these plant-derived nanoformulations is consistent with recent advances in nanopesticides for crop protection. For example, a recent study by Cruz *et al.*[50] demonstrated that nanoemulsions containing *Cinnamomum verum* essential oil effectively inhibited *Xanthomonas citri* subsp. *citri* in citrus fruits, highlighting advantages such as improved stability, controlled release and enhanced tissue penetration compared to conventional applications. Likewise, in the present study, the polygodial-based nanoemulsion (T2) significantly reduced Xaj infection in both walnut fruits and plants under controlled conditions. This enhanced antimicrobial activity is likely to be attributable to the increased solubility and prolonged retention of the active compounds on plant surfaces, a key benefit of nano-delivery systems. Furthermore, the absence of phytotoxic effects in our trials, a finding also reported by Cruz *et al.*,[50] underscores

Figure 9. Chandler variety walnut plants affected by *Xanthomonas arboricola* pv. *juglandis*. The effects of canelo oil (T1), polygodial (T2) and product 3 (T3), each at 125 ppm, are compared to the inoculated positive control (T0) and a conventional commercial product, Agrygent Plus (T4).

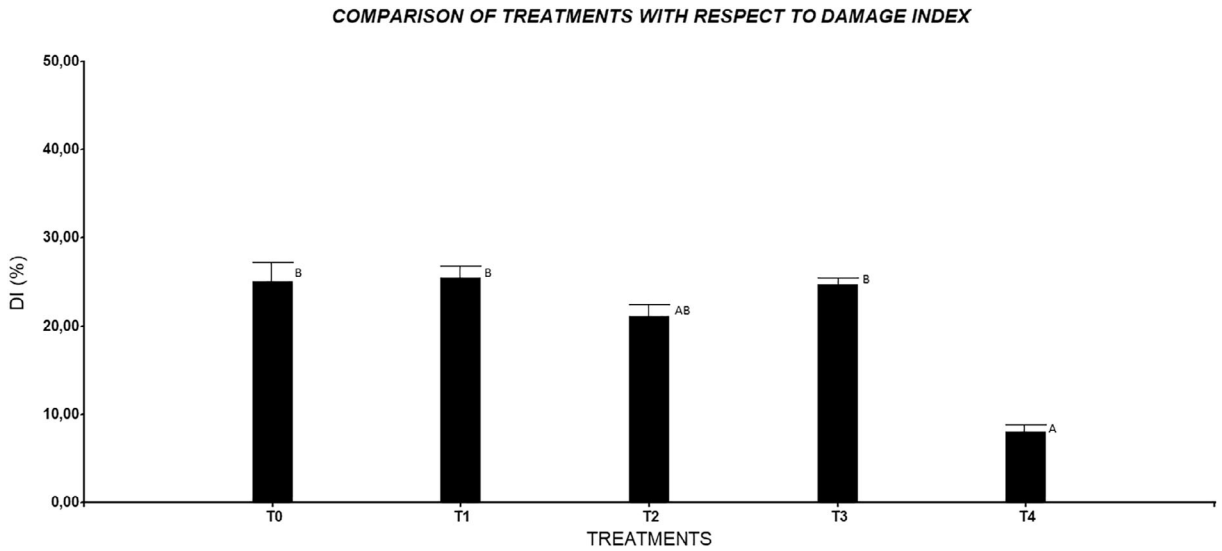

Figure 10. Effect of treatments on *Juglans regia* cv. Chandler plants inoculated with *Xanthomonas arboricola* pv. juglandis. The damage index (DI) in walnut plants subjected to different treatments. Treatment T4 (Agrygent Plus) exhibited the lowest DI, indicating the highest efficacy in controlling bacterial blight. Treatments T1, T2 and T3 showed moderate disease suppression, whereas the untreated inoculated control (T0) had a high DI, similar to those of T1 and T3. Treatments labeled with the same letter are not significantly different ($P > 0.05$).

the safety and feasibility of using nanoemulsified botanical compounds as sustainable treatments in agriculture.

5 CONCLUSIONS

This study clearly demonstrates the antibacterial activity of polygodial (**1**) and epi-polygodial (**2**) against Xaj, a pathogen responsible for severe economic losses in walnut production. Notably, the epimer epi-polygodial (**2**) exhibited a strong MBC, indicating that stereochemistry at C-9α plays a crucial role in enhancing its antibacterial efficacy against bacterial blight. The antimicrobial properties of these drimanic compounds position them as promising candidates for natural antibiotic development.

Furthermore, this study represents the first report of natural and hemisynthetic drimane-based nanoemulsions formulated against bacterial blight, both *in vitro* and under field-mimicking

conditions (*in planta* and in fruit assays). The findings highlight the feasibility of formulating scalable nanoemulsions for agricultural application, incorporating drimanic compounds while maintaining physical stability. Various lipid phases, including LAC, LA, OA and LA:OA, were evaluated, with stable nanoemulsions successfully formulated using Tween 80 as a surfactant. The results indicate an inverse correlation between the maximum loading capacity of drimanic compounds and the molecular weight of the lipid phase, following the trend: OA > LA > LA:OA.

The nanoemulsions developed in this study demonstrated superior physical stability and antimicrobial efficacy, offering a sustainable alternative to conventional bactericides. These findings pave the way for future research to refine nanoemulsion formulations, optimize field application strategies, and assess their long-term impact on disease management and crop health in commercial walnut orchards.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS STATEMENT

The authors declare that ethics approval for this research is not necessary since no human or animal related samples were used.

AUTHOR CONTRIBUTIONS

E.W. contributed to the purification of natural compounds from the bark of *Drimys winterii*. A.O. contributes to the assays on *in vitro* Xaj strains and nanoformulations. I.M. supervised the whole study. G.B. and I.A. performed the isolation and synthesis of all compounds. L.E. and I.M. collected the spectroscopic data. P.G. and V.M contributed strains of bacteria. I.M. conceived and designed the biologic experiments. R.P. performed the biologic experiments. A.M., M.C., D.C.-N. and I.M. collaborated in the discussion and interpretation of the results. I.M., M.C. D.C.-N and A.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript. M.V, X.B and Y.O they performed and monitored fruit assays with XAJ strains and assays on walnut plant in greenhouses conditions.

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