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Smilax aspera L. Leaf and Fruit Extracts as Antibacterial Agents for Crop Protection

Riccardo Fontana [1], Eva Sánchez-Hernández [2], Pablo Martín-Ramos [2,*], Jesús Martín-Gil [2] and Peggy Marconi [1,3]

[1] Department of Chemical, Pharmaceutical and Agricultural Sciences, University of Ferrara, 44121 Ferrara, Italy; fntrcr1@unife.it (R.F.); peggy.marconi@unife.it (P.M.)
[2] Department of Agricultural and Forestry Engineering, ETSIIAA, Universidad de Valladolid, 34004 Palencia, Spain; eva.sanchez.hernandez@uva.es (E.S.-H.); jesus.martin.gil@uva.es (J.M.-G.)
[3] Laboratory for the Technology of Advanced Therapy (LTTA), Technopole of Ferrara, 44121 Ferrara, Italy
* Correspondence: pmr@uva.es

Abstract: *Smilax aspera* L. (commonly known as sarsaparilla) is recognized for its composition rich in flavonoids, phenylpropanoids, steroidal saponins, stilbenoids, and tannins, exhibiting anti-inflammatory, cytotoxic, and antimicrobial properties. This study investigates the hydromethanolic extracts of its leaves and fruits through vibrational spectroscopy and gas chromatography–mass spectrometry, evaluating their potential as biorationals for safeguarding crops. Analysis of *S. aspera* leaf and fruit extracts revealed the presence of phytochemicals such as lactones and other furan derivatives. In vitro assessments against three phytopathogens—*Erwinia amylovora*, *Pseudomonas syringae* pv. *actinidiae*, and *Xanthomonas campestris* pv. *campestris*—demonstrated strong antibacterial activity, with minimum inhibitory concentration (MIC) values of 1500 $\mu g \cdot mL^{-1}$ for both extracts. Biofilm tests indicated that the leaf extract reduced biofilm formation by 78–85%, while the fruit extract led to a reduction of 73–92.5%. At a concentration of 750 $\mu g \cdot mL^{-1}$, the extracts caused a decrease in amylovoran synthesis by 41–58%. Additionally, noticeable alterations in membrane permeability were observed at MIC and MIC/2 doses. Subsequent in vivo trials conducted on *Pyrus communis* L. trees utilizing the combined aerial part extract yielded substantial protection against *E. amylovora* at a dose of 1500 $\mu g \cdot mL^{-1}$, reaching 80% wilting reduction for the leaf extract. The findings presented herein cast *S. aspera* extracts as a promising natural-based treatment against these bacterial phytopathogens.

Keywords: antibacterial; bacterial diseases; black rot disease; common smilax; fire blight; natural products

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1. Introduction

Smilax aspera L., commonly referred to as sarsaparilla, is a perennial evergreen climbing plant within the *Smilacaceae* family, a characteristic species of the Mediterranean basin. Featuring stems reaching approximately 3 m in length, this plant utilizes tendrils to affix itself to other plants for support. Growing and climbing from a rhizome, it extends numerous branches, some reaching lengths of up to 15 m, and envelops shrubs and trees with an abundance of leaves. Its semi-woody stems are adorned with multiple prickles, and its glossy heart-shaped leaves bear small prickles along their margins. The flowers are arranged in branched clusters, and the plant produces succulent berries, which are initially green, maturing into red and occasionally black. These berries, with a soft, spherical shape and measuring 7–9 mm across, each harbor up to three seeds [1].

The appeal of this plant is rooted in its historical use for medicinal purposes, particularly with regard to its rhizomes, renowned for their diaphoretic, depurative, stimulant, diuretic, and tonic properties. Traditionally, they have been incorporated into soft drinks.

Their therapeutic efficacy is ascribed to the existence of phenolic compounds and steroidal saponins within them [2,3].

Phenolic compounds, including caffeoyl shikimic acid, catechin, chlorogenic acid, isorhamnetin, kaempferol and its glycosides, quercetin, and rutin, have been identified in *S. aspera* [4]. Recent research by Kakouri et al. [3] has expanded the list to include isorhamnetin pentoside-hexoside, isoshaftoside, and luteolin glucoside. In the case of the berries, their coloration has been attributed to both their anthocyanin content [5] and carotenoids [6].

As noted above, previous studies have focused on the rhizomes of the *Smilax* genus and—to a lesser extent—on the chemical characterization of leaves and fruits [3]. However, based on the available literature, there is a research gap regarding the assessment of the antibacterial activity of *S. aspera* aerial part extracts against phytopathogens for crop protection purposes.

Specifically, three Gram-negative plant-pathogenic bacteria, namely, *Pseudomonas syringae* pv. *actinidiae* Takikawa et al. 1989 (Psa); *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson (Xcc); and *Erwinia amylovora* (Burrill 1982) Winslow et al. 1920 (EA), were selected for evaluation in this study. Since 2008, the kiwifruit industry has faced a severe threat from a pandemic outbreak of Psa [7], the causal agent of bacterial canker, while global apple and pear production is confronting a serious challenge in the form of the rapid dissemination of EA, the causal agent of fire blight [8]. In the domain of vegetable brassica crops, Xcc has emerged as a significant menace, causing black rot. These plant diseases have posed critical challenges to their respective industries, with Psa, EA, and Xcc displaying distinctive host interactions, epidemiological patterns, and control strategies. As the kiwifruit industry grapples with the intricate ecology of Psa, the apple and pear production industry seeks eco-friendly alternatives for controlling fire blight, and managers of vegetable brassica crops strive to combat black rot with limited resistance options [9–11]. This context establishes the foundation for a more profound exploration of these pressing issues, aimed at advancing our understanding of potential solutions to mitigate their impact.

The aim of this research is to investigate the phytochemical components present in the hydromethanolic extracts of *S. aspera* leaf and fruit extracts and explore their potential as natural-based treatments against the aforementioned bacterial phytopathogens—Psa, EA, and Xcc—contributing valuable insights toward sustainable crop protection strategies.

2. Material and Methods

2.1. Plant Material and Chemicals

Samples of *S. aspera* were collected in Niembro (Llanes, Asturias, Spain; 43°26′08.0″ N 4°51′30.5″ W) in November 2021. Prof. Dr. B. Herrero-Villacorta from the Agricultural and Forestry Engineering Department, ETSIIAA, Universidad de Valladolid, authenticated and identified the specimens, and the corresponding voucher specimens are stored at the herbarium of the ETSIIAA. To prepare separate composite samples of leaves and fruits, plant parts from various specimens (n = 25) were blended.

Luria–Bertani (LB) agar and LB broth were acquired from Liofilchem (Roseto degli Abruzzi, TE, Italy). Propidium iodide (CAS 25535-16-4), cetylpyridinum chloride (CAS 6004-24-6), phosphate-buffered saline (PBS), monobasic potassium phosphate (CAS 7778-77-0), potassium phosphate dibasic (CAS 7758-11-4), ammonium sulfate (CAS 7783-20-2), glycerol (CAS 56-81-5), citric acid (CAS 77-92-9), magnesium sulfate (CAS 7487-88-9), sorbitol (CAS 50-70-4), and crystal violet solution (CAS 548-62-9) were supplied by Merck KGaA (Darmstadt, Germany).

2.2. Bacterial Strains

The EA, Xcc, and Psa isolates employed in this investigation were provided by the Emilia-Romagna Phytosanitary Agency (Bologna, Italy), while control strains 30,165, 3586, and 10,604, from the Leibniz Institute DSMZ—German Collection of Microorganisms

(Brunswick, Germany), served as controls. Throughout the study, the bacteria were cultured on LB agar (30 g·L^{-1}) or in LB broth, maintaining incubation temperatures at 25/28 °C. Various inoculum concentrations were chosen based on established data and protocols, which are cited in their respective sections. For biofilm formation, a higher inoculum concentration was employed to expedite the acquisition of results and ensure the robustness of the biofilm formation process. Similarly, for the in planta experiments, a highly concentrated inoculum was also chosen. Regarding the assessment of membrane permeability and amylovoran production, a range of concentrations for propidium iodide (PI) and cetylpyridinum chloride (CPC) was explored against bacterial concentrations spanning from 10^4 to 10^7 colony-forming units (CFU). Subsequently, for standardization purposes, an inoculum concentration of 10^5 was settled upon, aligning with the existing literature and guided by the outcomes of the experiments.

2.3. Preparation of Leaf and Fruit Extracts

Dried samples of plant parts (either leaves or fruits) were mixed with a methanol/water solution (1:17 *v*/*v*). The mixture underwent heating at 50 °C for 30 min, followed by sonication using a UIP1000hdT probe-type ultrasonicator (Hielscher Ultrasonics; Teltow, Germany), and subsequent centrifugation for 15 min at 9000 rpm. The resulting supernatant was filtered through Whatman No. 1 paper and then freeze-dried to produce a solid residue. For gas chromatography–mass spectrometry (GC–MS) analysis, the freeze-dried extract was dissolved in methanol (HPLC-grade) to acquire a 5000 μg·mL^{-1} solution, with additional filtration performed thereafter.

2.4. Characterization Procedures

The vibrational spectra in the infrared region of the dried plant parts of *S. aspera* were collected by employing a Nicolet iS50 (Thermo Scientific; Waltham, MA, USA) Fourier-transform infrared (FTIR) spectrometer equipped with a diamond attenuated total reflection (ATR) system. Spectra were acquired with a 1 cm^{-1} spectral resolution covering the range of 400–4000 cm^{-1} through the co-addition of 64 scans.

The hydromethanolic extracts were subjected to analysis using GC–MS at the Research Support Services (SSTTI) at the Universidad de Alicante (Alicante, Spain). An Agilent Technologies (Santa Clara, CA, USA) model 7890A gas chromatograph coupled with a model 5975C quadrupole mass spectrometer was employed. Chromatographic conditions comprised a 1 μL injection volume; a 280 °C injector temperature, in splitless mode; and a 60 °C initial temperature, held for 2 min, followed by a ramp of 10 °C·min^{-1} up to a 300 °C, which was held for 15 min. An HP-5MS UI chromatographic column with a length of 30 m, a 0.250 mm diameter, and a 0.25 μm film was utilized for compound separation. Mass spectrometer conditions comprised a 230 °C electron impact source temperature, with the quadrupole set to 150 °C, and a 70 eV ionization energy. Identification of phytoconstituents relied on comparing their mass spectra and retention times with the National Institute of Standards and Technology and Wiley databases.

2.5. Evaluation of Antibacterial Activity

2.5.1. In Vitro Tests

Determination of the Minimum Inhibitory Concentration of Smilax aspera Extracts. To ascertain the minimum inhibitory concentration (MIC) of *S. aspera* extracts, the microdilution method, as outlined by Akhlaghi et al. [12], was utilized. Initially, EA was cultured overnight at 28 °C in LB broth with continuous shaking at 160 rpm in a MaxQ 4000 incubator (Thermo Scientific Italia, Milano, Italy). Subsequently, aliquots of *S. aspera* extract (either leaf or fruit) stock solutions were added to 120 μL of LB broth, resulting in a final concentration of 1500 μg·mL^{-1} of extract in the first well of each row within 96-well plates (Costar Corning, Corning, NY, USA). The extract was then systematically diluted with LB medium within the 96-well microplate to generate a concentration range spanning from 1500 to 62.5 μg·mL^{-1}, maintaining a total volume of 200 μL per well. Next, 10 μL of the

overnight bacterial culture was inoculated in each well, with the inoculum being standardized to a concentration of 10^4 CFU·mL^{-1}. The microplate underwent static incubation at 25 °C for 48 h. Subsequently, turbidity measurements were taken at OD_{600} using a GloMax (Promega; Madison, WI, USA) spectrophotometer. The same protocol was applied for Xcc and Psa. Data were derived from three separate experiments, each performed in triplicate for each bacterium. Streptomycin and tetracycline, added at their MIC concentrations (which were obtained from the literature), served as controls for comparison purposes.

Anti-Biofilm Activity. Erwinia amylovora is recognized for its ability to form biofilms, a pivotal process within its pathogenic cycle. The evaluation of its impact on biofilm formation was conducted utilizing a microplate assay incorporating crystal violet, according to the procedure outlined by Wilson et al. [13]. Initially, 10^6 CFU·mL^{-1} EA suspensions were introduced into LB broth supplemented with *S. aspera* extracts (either leaf or fruit extract) at non-lethal concentrations. This mixture was placed in a 96-well U-bottom microplate and incubated at 25 °C for 72 h. After incubation, the growth media, *S. aspera* extracts, and planktonic cells were carefully removed from the microplate and rinsed with deionized water. A 1% concentration solution of crystal violet was introduced into each well, and the microplate was incubated at room temperature for 30 min. Subsequently, the dye solution was removed through multiple washes with deionized water. To enhance crystal violet solubility, decoloring solutions consisting of 90–95% ethanol (200 µL) were introduced into each well and incubated at room temperature for 15 min. The contents of the 96-well plate were transferred to a fresh and sterile microplate, and quantification of biofilm formation was performed by measuring absorbance at 570 nm using a microplate reader (Tecan-Sunrise; Tecan Italia, Cernusco sul Naviglio, MI, Italy). The same protocol was applied for Xcc and Psa. Analyses were conducted based on data obtained from three independent experiments, each conducted in triplicate for each bacterium.

Amylovoran Production Assay. Amylovoran stands as a crucial virulence determinant in the context of EA, whose pathogenicity relies on the expression of the Type III secretion system (T3SS) and the synthesis of the exopolysaccharide known as amylovoran. The CPC assay, as outlined by Bellemann et al. [14], was employed to evaluate amylovoran production. Initially, overnight cultures of EA underwent cold centrifugation, and the resulting pellets were washed with PBS. Subsequently, these pellets were diluted 1:100 in a modified Burkholderia minimal agar (MBMA) medium, consisting of 3 g of KH_2PO_4, 7 g of K_2HPO_4, 1 g of $[NH_4]_2SO_4$, 2 mL of glycerol, 0.5 g of citric acid, and 0.03 g of $MgSO_4$, supplemented with 1% sorbitol and the extracts at their MIC concentrations. The supernatants from the MBMA cultures were analyzed for their amylovoran content by incubating them with 50 µL of a 50,000 µg·mL^{-1} CPC solution per milliliter of supernatant for a duration of 10 min. The control group comprised untreated EA cells. Subsequently, turbidity measurements were taken at OD_{600} using a spectrophotometer, using a calibration curve to relate the amylovoran concentration to the absorbance values. All analyses were conducted based on data obtained from three separate experiments, each performed in triplicate.

Membrane Permeability Assay. Bacterial suspensions of EA were cultured in LB broth at 25 °C for 24 h. Subsequently, 10^5 CFU·mL^{-1} of bacteria was divided into individual Eppendorf tubes, each containing *S. aspera* extracts (either leaf or fruit extract) at the corresponding MIC and MIC/2 concentrations. The suspensions underwent incubation for durations of 180, 120, 60, and 5 min. Subsequently, they were subjected to centrifugation for 5 min at 10,000 rpm and then washed with PBS. The resulting pellet was resuspended in PI (0.5%) and incubated for 15 min, avoiding exposure to light. Each suspension sample was plated on a separate 96-well plate, and measurements were acquired using a fluorescence microplate reader (Tecan-Fluoroscan; Tecan Italia, Cernusco sul Naviglio, MI, Italy). The negative control group consisted of untreated EA cells, while the positive control group comprised EA cells treated with a 10% bleach solution. The same protocol was applied for Xcc and Psa. All analyses were based on data obtained from three separate experiments, each conducted in triplicate for each bacterium.

2.5.2. In Vivo Tests

Erwinia amylovora strains were cultured overnight in 5 mL of LB broth at a temperature of 28 °C for 24 h. Subsequently, the cultures underwent centrifugation, and the resulting bacterial pellets were resuspended in a PBS solution to achieve a bacterial concentration of 10^7 CFU·mL^{-1}. To evaluate the strains' pathogenicity toward pear seedlings, two-year-old *P. communis* variety 'San Pietro' plants were employed for greenhouse experiments. Three distinct pear trees were utilized, with each tree being subjected to infection or inoculation and subsequent treatment applied to three discrete branches. Throughout the experiments, the plants were maintained under controlled conditions, characterized by a constant temperature of 25 °C and a relative humidity of 70%, with approximately 12 h of daily sunlight exposure. For the control group, a 50 µL aliquot of EA suspension (10^7 CFU·mL^{-1}) was introduced into the shoots (15–20 cm) through scissor inoculation. In the treatment protocol, a 50 µL EA suspension was also initially inoculated onto the shoots, but seven days later, at the appearance of symptoms, *S. aspera* extracts at MIC (1500 µg·mL^{-1}) were sprayed onto each infected shoot. Disease symptoms were observed at 14 and 28 days after the introduction of EA, with manifestations on the shoots. For each shoot, both the total shoot length and the length of lesions were measured. Shoot susceptibility to fire blight was quantified using the disease index (DI), calculated as follows: DI = (length of blighted shoot/total shoot length) × 100. The experiment was conducted in triplicate shoots to ensure robustness and reproducibility of the results. Wilting areas were quantified using ImageJ software 2.9 (Fiji ImageJ for MacOS, NIH, Bethesda, MD, USA), as described by Schneider et al. [15].

2.6. Statistical Analysis

Statistical analyses were conducted using Graphpad Prism Software v.9.0.0 for MacOS (San Diego, CA, USA). The normality and homoscedasticity assumptions were evaluated using the Shapiro–Wilk test and the Bartlett test, respectively. Based on these assessments, comparisons were made by using a two-way ANOVA followed by Dunnett's post hoc test.

3. Results

3.1. Vibrational Spectroscopy

The infrared spectra of the dried leaf and fruit samples before extraction (refer to Table 1 and Figure S1) exhibited common absorption bands compatible with the presence of aromatic compounds, ketones, and alcohols containing multiple hydroxyl groups. The identified functional groups align with the chemical species detected in the extracts via GC–MS, as presented below.

Table 1. Band assignments for ATR-FTIR spectra of *S. aspera* leaf and fruit samples. Wavenumbers are expressed in cm^{-1}.

Leaf	Fruit	Assignment
3354	3378	O–H stretching of alcohols (e.g., 2,3-butanediol) and phenols
2916	2915	C–H stretching of alkanes, methyl, and methylene groups
2848	2849	C–H stretching of aldehydes
1732	1716	C=O stretching of ketones (e.g., 1-hydroxy-2-propanone and 2-hydroxy-2-cyclopenten-1-one) and aldehydes
1640	1640	C=C stretching of alkenes and aromatic compounds
1516	1521	C=C stretching and C=C–H bending vibrations of the aromatic ring (e.g., in catechol)
1472	1465	C–H bending of alkanes, methyl and methylene groups, and aldehydes
1377	1376	C–H bending of alkanes and methyl groups
1242	1246	C–O stretching vibration in esters
1161	1164	C–O–C stretching mode
1066	1066	N–O stretching frequencies in oximes
890		C–H bending in alkenes; C–H out-of-plane deformation of substituted benzene rings

3.2. Phytochemicals Identification

The *Smilax aspera* leaf extract chromatogram (Figure 1a, Table S1) includes *α*- and *β*-*D*-galactopyranoside, methyl (5.2%); cyclopropyl carbinol (3.2%); methoxy-phenyl-oxime (2.1%); and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one (1.7%), depicted in Figure 2.

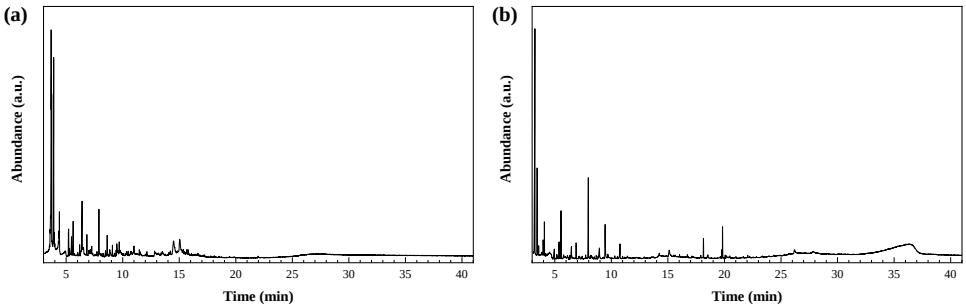

Figure 1. GC–MS chromatograms of *S. aspera* (**a**) leaf and (**b**) fruit hydromethanolic extracts.

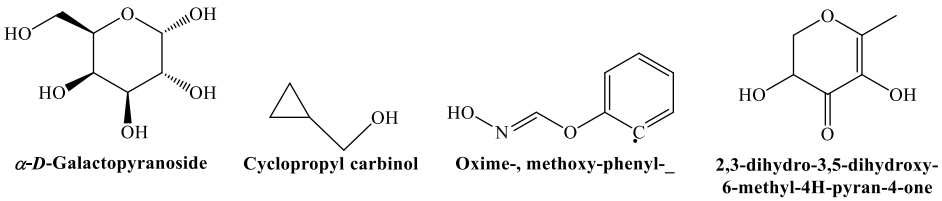

Figure 2. Structures of some of the chemical species found in *S. aspera* leaf extract.

Among the phytochemicals contained in the *S. aspera* fruit extract (Figure 1b, Table S2), three chemicals with a furan ring in their structure were found, namely, dihydro-4-hydroxy-2(3H)-furanone (or 3-hydroxy-*γ*-butyrolactone) (9.7%), 2-furan methanol (3.4%), and N-(2-furoyl)-alanine, and propyl ester (1.5%); the methyl esters of 11-octadecenoic and hexadecanoic acids (2.6% and 1.8%, respectively); *D*-fucose (1.8%); and 3,4-didehydro-proline (1.5%), as shown in Figure 3.

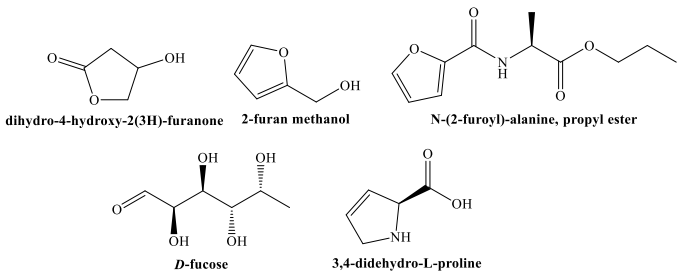

Figure 3. Structures of some of the chemical species found in *S. aspera* fruit extract.

The phytochemicals shared by the leaf and fruit extracts (Figure 4) included 1-hydroxy-2-propanone (4.4–17.4%); dialcohols such as 2,3-butanediol (2.9–3.4%) and catechol (2.3–5.5%); and lactones such as 2-hydroxy-*γ*-butyrolactone (2.1–5.3%) and 2-hydroxy-2-cyclopenten-1-one (2.1–3.8%).

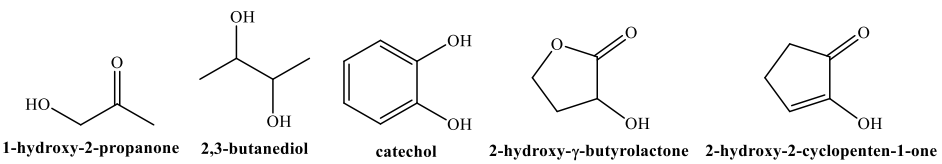

Figure 4. Chemical structures of some of the chemical species identified in both *S. aspera* leaf and fruit extracts.

3.3. Antibacterial Activity

3.3.1. In Vitro Antibacterial Activity

The MIC assessment of the *S. aspera* extracts against phytopathogens was carried out using a microplate assay (Figure 5). The analysis indicated that the *S. aspera* extracts at lower concentrations, ranging from 500 to 125 µg·mL^{-1}, did not display any efficacy. In contrast, the tested extracts exhibited a complete inhibition of bacterial growth at 1500 µg·mL^{-1}. Further testing of these concentrations in an agar matrix inoculated with bacteria confirmed the absence of detectable growth, providing evidence that the MIC and Minimum Bactericidal Concentration (MBC) values aligned.

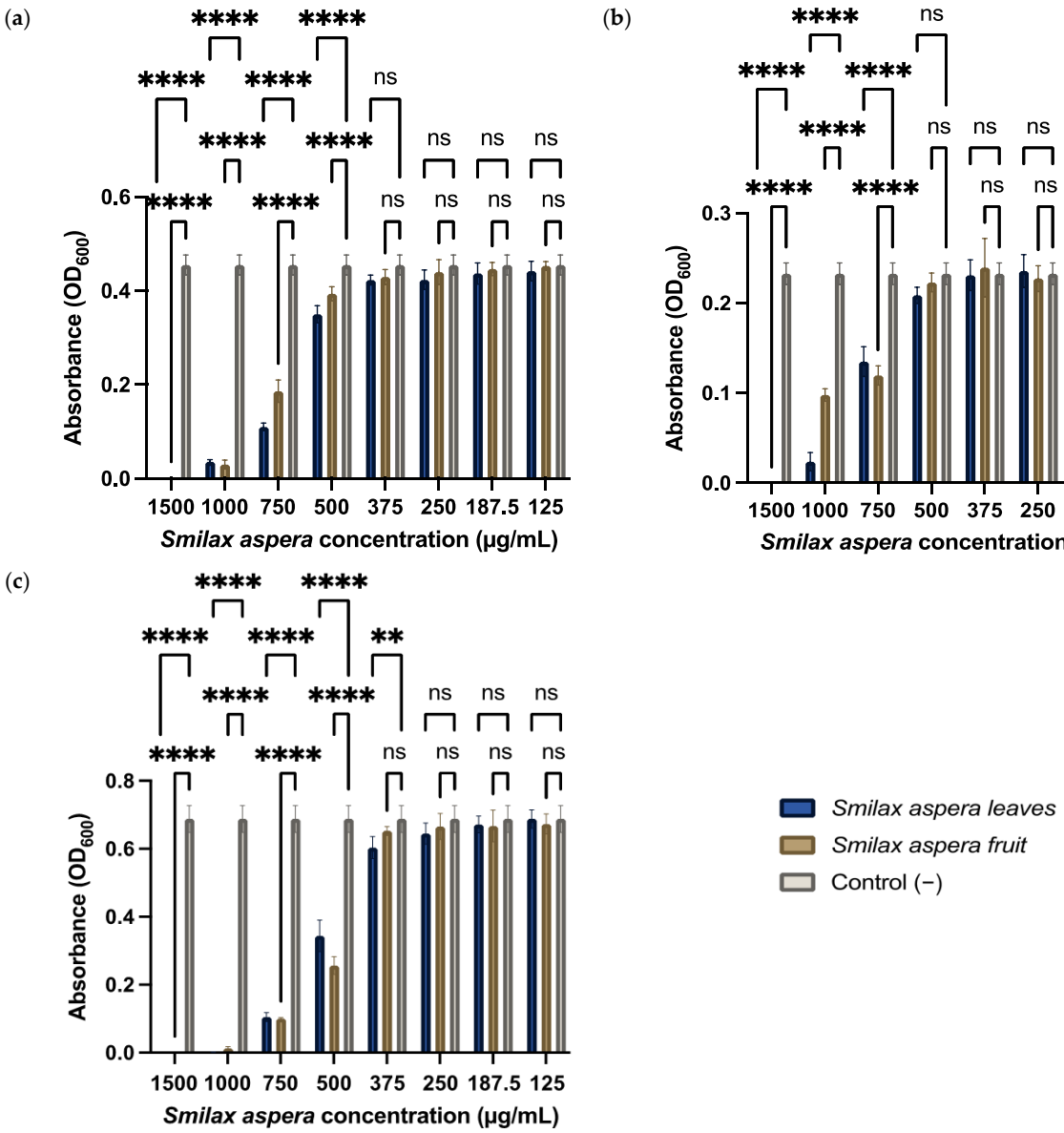

Figure 5. Antimicrobial activity of *S. aspera* extracts against (**a**) *Erwinia amylovora*; (**b**) *Pseudomonas syringae* pv. *actinidiae*; and (**c**) *Xanthomonas campestris* pv. *campestris*. The presented data reflect the mean ± SD of three independent experiments, each conducted in triplicate. The levels of significance are indicated as follows: ns $p \geq 0.1$; ** $p < 0.01$; **** $p \leq 0.0001$.

A comparison of the antibacterial activity of the *S. aspera* fruit and leaf extracts with that of antibiotics (Table 2) revealed that the extracts were less effective against the bacteria under study.

Table 2. Antimicrobial susceptibility (OD_{600}) of the three phytopathogens to conventional antibiotics.

Pathogen	Streptomycin	Tetracycline	*Smilax aspera* Leaf Extract	*Smilax aspera* Fruit Extract
Erwinia amylovora	0.000 ± 0.000	0.000 ± 0.000	0.014 ± 0.002	0.018 ± 0.003
Pseudomonas syringae pv. *actinidiae*	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.000	0.003 ± 0.001
Xanthomonas campestris pv. *campestris*	0.000 ± 0.000	0.000 ± 0.000	0.001 ± 0.001	0.001 ± 0.001

3.3.2. Anti-Biofilm Activity

Biofilm formation is a bacterial survival strategy wherein colonies enhance intercellular communication mechanisms under stressful conditions to create a more resistant and cohesive community. In the context of fire blight, black rot, and Psa canker pathogenesis, evaluating anti-biofilm properties is crucial. *Smilax aspera* leaf and fruit extracts were added at concentrations both at and below their MICs to bacterial suspensions. Subsequently, biofilm formation was quantified using spectrophotometer readings. Figure 6 depicts a significant decrease in biofilm formation when compared to the control. The leaf extracts reduced biofilm formation by 85%, 78%, and 82% when used at their MIC concentrations against Xcc, EA, and Psa, respectively. This effect was slightly stronger with the fruit extract, reaching a reduction in biofilm formation of 92.5% against Xcc, 73% against EA, and 86.5% against Psa. This observation can primarily be attributed to the phenolic compounds present in the leaves and fruits, which are believed to act as anti-biofilm agents.

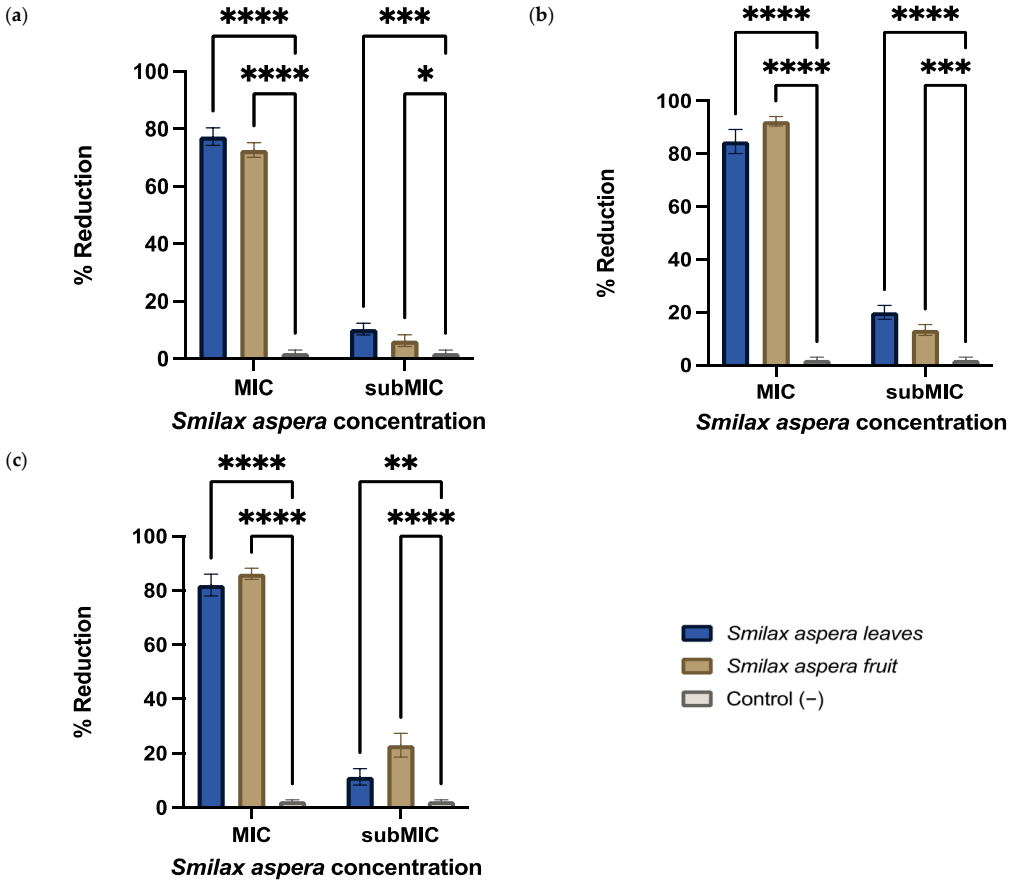

Figure 6. Percentage of in vitro biofilm reduction assay on (**a**) *E. amylovora*; (**b**) *P. syringae* pv. *actinidiae*; and (**c**) *X. campestris* pv. *campestris* compared to the untreated control. The presented data reflect the mean ± SD of three independent experiments, each conducted in triplicate, with values expressed as percentages. The levels of significance are indicated as follows: * $p < 0.1$; ** $p < 0.01$; *** $p < 0.001$; **** $p \leq 0.0001$.

3.3.3. Amylovoran Production

In the pursuit of a more profound understanding of how exposure to the two *S. aspera* extracts affects virulence, this study investigated their impact on amylovoran production. The extracts from leaves and fruits used at a subMIC concentration, 750 $\mu g \cdot mL^{-1}$, resulted in a reduction in amylovoran synthesis by 41% and 58%, respectively, as depicted in Figure 7. The virulence of EA relies on amylovoran production, and these results suggest that the phytoconstituents may target this crucial virulence factor, aligning with the previously observed effects.

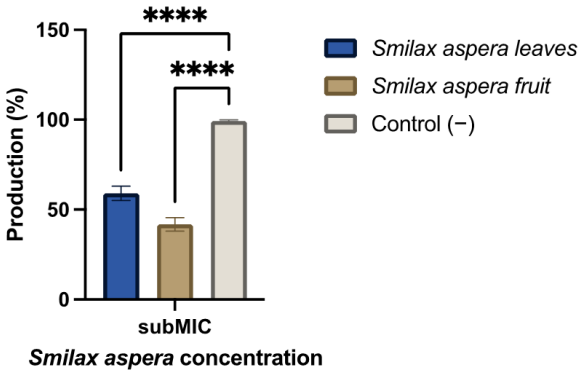

Figure 7. Percentage of amylovoran production in *E. amylovora* in the presence of *S. aspera* leaf and fruit extracts compared to the negative control. The presented data reflect the mean ± SD of three independent experiments, each conducted in triplicate, with values expressed as percentages. The level of significance is indicated as follows: **** $p < 0.001$.

3.3.4. Permeability Alteration

This assay was designed to determine whether extract concentrations known for their antibacterial properties could impact bacterial membrane permeability. Diverse concentrations of each extract were assessed on bacterial suspensions at various time points. To assess membrane changes, PI—a fluorescent intercalating agent—was introduced into bacterial suspensions of EA, Xcc, and Psa. PI cannot permeate intact membranes but becomes detectable when membrane integrity and permeability are compromised, as this agent enters the bacterial cell and intercalates with DNA bases.

As anticipated, upon reaching the MIC of the *S. aspera* extracts, noticeable alterations in membrane permeability were observed, akin to the positive control involving bacteria treated with a 10% bleach solution (Figure 8). However, even at reduced concentrations, such as MIC/2 (750 $\mu g \cdot mL^{-1}$), a discernible augmentation in membrane permeability to PI was evident. Specifically, the 1/2 MIC concentration resulted in 28%, 34%, and 21% increases in PI uptake for EA, Xcc, and Psa, respectively. This suggests that even at reduced extract concentrations, membrane integrity and permeability were compromised.

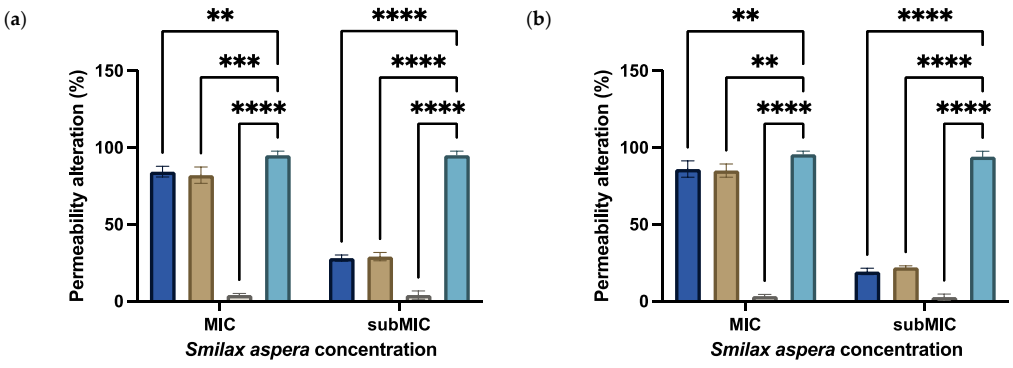

Figure 8. *Cont.*

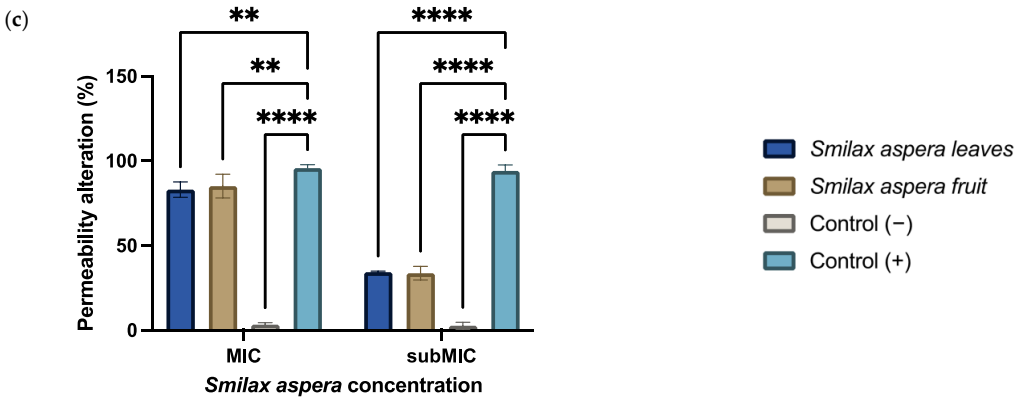

Figure 8. In vitro membrane permeability assay on (**a**) *E. amylovora*; (**b**) *P. syringae* pv. *actinidiae*; and (**c**) *X. campestris* pv. *campestris*, assessed according to PI intake compared to the untreated control. The presented data reflect the mean ± SD of three independent experiments, with three replicates each and values expressed as percentages. The levels of significance are indicated as follows: ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3.4. In Vivo Antibacterial Activity

To elucidate the efficacy of *S. aspera* extracts against EA, a preliminary investigation was conducted to assess the antibacterial impact within a plant system. This experimental approach provides insights into the dynamic interaction between the bacterium and plant cells, presenting a more realistic perspective.

Given that EA is the causative agent of fire blight in apple and pear trees, we assessed the antibacterial properties of *S. aspera* extracts on pear leaves previously inoculated with this bacterium. Shoot blight initiates at the tips of growing shoots and rapidly progresses down into older parts of the twig. Initially, blighted twigs appear water-soaked and subsequently turn dark brown or black. As affected shoots wilt, the twigs bend at the growth point, resembling a shepherd's crook or an inverted "J". Blighted leaves may persist on dead branches throughout the summer but fall during periods of high humidity. Under warm and humid conditions, infected shoots may exude droplets of creamy white bacteria.

The control pear tree branches, depicted in Figure 9a–c, exhibited progressive symptoms of fire blight over a span of 10 days post-inoculation. Initially, at 7 days post-inoculation (Figure 9b), the branches displayed the first signs of infection, with some areas devoid of leaves and exhibiting brown spots. By day 10 post-inoculation (Figure 9c), almost all the leaves had dropped, and characteristic wilting with dark-brown hooks was evident on some branches. Additionally, visible exudate production was observed on individual branches (Figure 9d), indicative of advanced disease progression.

In contrast, the branches treated with *S. aspera* leaf extract, as shown in Figure 10a–c, exhibited a significant reduction in disease severity. Two days post-inoculation (Figure 10a), no symptoms of fire blight were apparent. Seven days post-treatment with *S. aspera* leaf extract (Figure 10b), few necrotic areas were observed on some leaves, indicating partial efficacy of the treatment. However, by day 10 post-treatment (Figure 10c), the severity of necrotic areas was reduced by 80% compared to that of the control, with no signs of exudate production or hook wilting detected.

Similarly, treatment with *S. aspera* fruit extract, illustrated in Figure 11a–c, also led to a reduction in disease severity. While necrotic areas were observed on some leaves 7 days post-treatment (Figure 11b), indicative of partial efficacy, by day 10 post-treatment (Figure 11c), the severity of necrotic areas was reduced by 45% compared to the control, with no signs of exudate production or hook wilting observed. These findings highlight *S. aspera* extracts' potential as a promising treatment option for managing fire blight in apple trees, particularly when derived from *S. aspera* leaves.

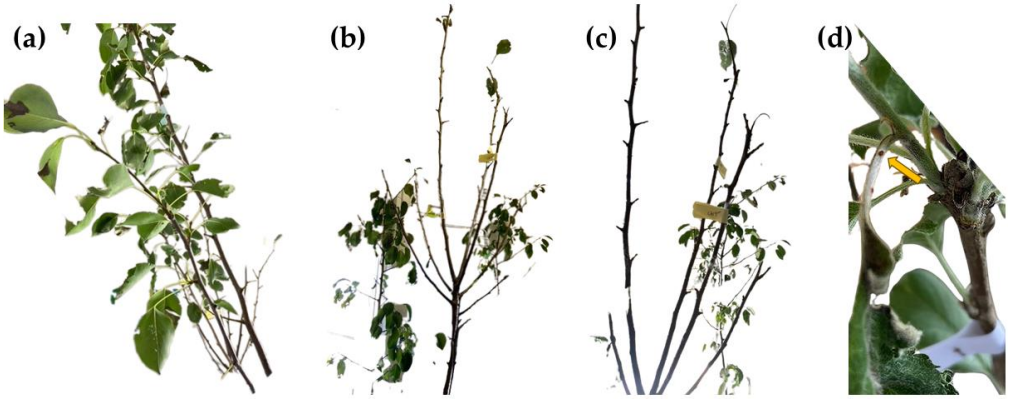

Figure 9. In planta assay with *E. amylovora* inoculation without subsequent treatment (positive control): (**a**) a control pear tree branch pre-inoculation, (**b**) a control pear tree branch 7 days post-inoculation, (**c**) a control pear tree branch 10 days after inoculation, and (**d**) zoomed-in view of exudate production.

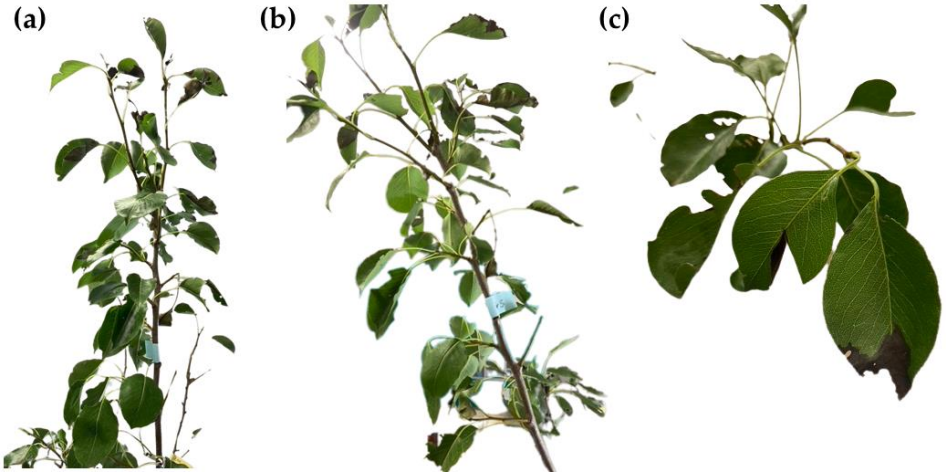

Figure 10. Results of an in planta assay depicting the effect of the *S. aspera* leaf extract against *E. amylovora*: (**a**) a pear tree branch 2 days post-inoculation, (**b**) a pear tree branch 7 days after treatment, and (**c**) a pear tree branch 10 days after treatment with a focus on individual leaves.

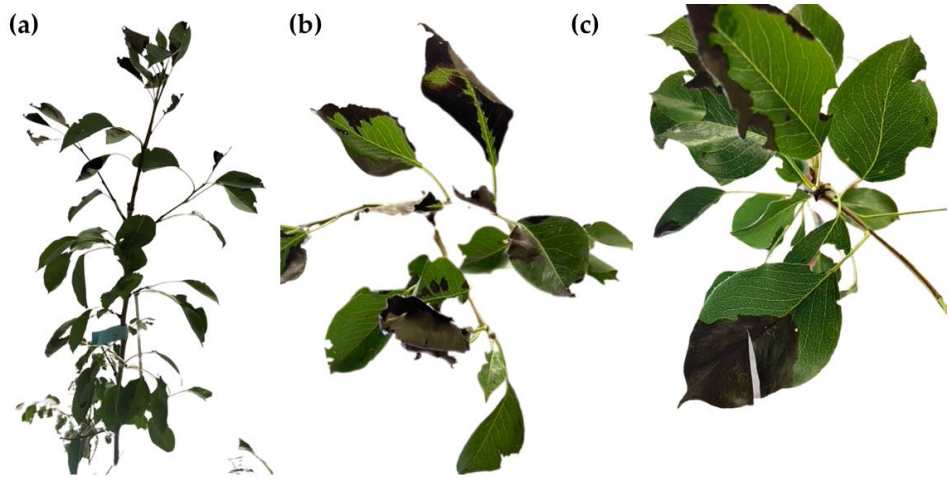

Figure 11. In planta assay illustrating the effect of the *S. aspera* fruit extract against *E. amylovora*: (**a**) a pear tree branch 2 days post-inoculation, (**b**) a pear tree branch 7 days after treatment, and (**c**) a pear tree branch 10 days after treatment with a specific focus on individual leaves.

4. Discussion

4.1. Phytochemical Profile

Regarding the identified phytoconstituents, caution is warranted as the samples were collected from a single area. Discrepancies in the phytochemical profile and bioactivity may result from differences in the extraction process, individual genotype-dependent factors, location-specific intra-varietal variations, and seasonal fluctuations. Additionally, the potential presence of distinct chemotypes arising from minor genetic and epigenetic changes should be considered.

Regarding the phytochemicals exclusively identified in the leaf extract, methoxyphenyl-oxime and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one were also reported in the extracts of *Rubia tinctorum* L., known for its potent antimicrobial properties [16]. Among the chemical compounds found only in the fruit extract, dihydro-4-hydroxy-2(3H)-furanone was previously detected in *Urtica dioica* L., *Equisetum arvense* L. [17], and *Ginkgo biloba* L. [18] extracts. It is also a major constituent of *Crocus sativus* L. (corresponding to a content of 22%) and has demonstrated antifungal activity against various fungi (including *Aspergillus fumigatus* Fresen., *Cryptococcus neoformans* (Sanfelice) Vuill., *Pyricularia oryzae* Cavara, and *Trichophyton rubrum* (Castell.) Sabour.) [19]. As for 2-furanmethanol (or 2-hydroxymethyl-furan), a phytochemical related to 5-(hydroxymethyl)furan-3-carboxylic acid, it was previously found in *Paederia foetida* L. [20] and showed significant antifungal activity against fungi of the genus *Aspergillus*.

Concerning the chemical species shared by both extracts, 1-hydroxy-2-propanone, a primary alcohol substituent on acetone, was previously found in *G. biloba* [18]. 2,3-Butanediol, a short diol, is easily produced by different native bacteria from sugars or lignocellulosic biomasses. Catechol (or 1,2-dihydroxybenzene) is a ubiquitous phytochemical common to several plants including *Allium sativum* L. [21], *R. tinctorum* [16], *Quercus ilex* L. [22], *Sambucus nigra* L. [23], and *Euphorbia serrata* L. [24]. Both 2-hydroxy-γ-butyrolactone (position isomer of 3-hydroxy-γ-butyrolactone) and 2-hydroxy-2-cyclopenten-1-one were also identified in *R. tinctorum* [16]. N-(2-furoyl)-alanine, propyl ester may act as a precursor for most of the compounds identified in this analysis.

The examination of the phytochemicals identified in the *S. aspera* leaf and fruit extracts suggests the metabolism of parent compounds during the extraction procedure, with 1-(3,6,6-trimethyl-1,6,7,7a-tetrahydrocyclopenta[c]pyran-1-yl)ethenone being the most probable precursor for pyrans and 5-phenyl-2-furoyl-alanine, propyl ester fulfilling this role for furans (Figure 12).

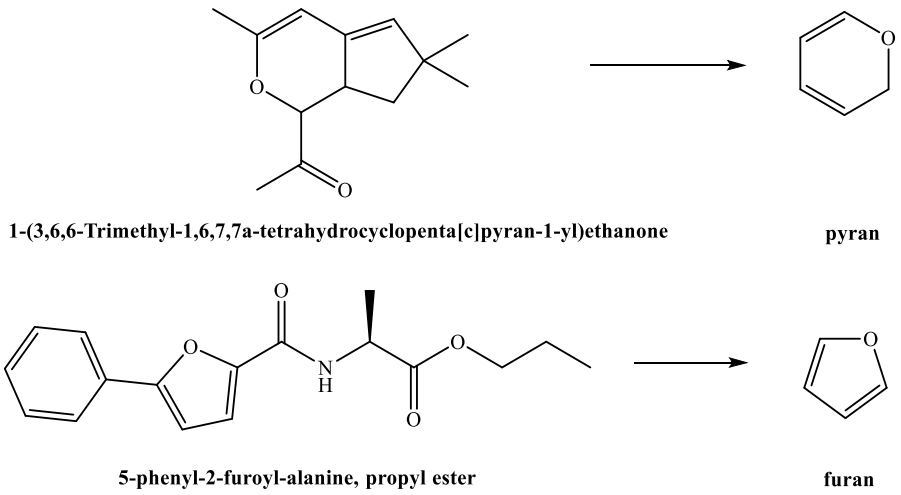

Figure 12. Suggested pyran and furan precursors.

Nonetheless, it is widely acknowledged that furans may also be formed directly through the dehydration of cellulose or fructose, resulting in anhydrosugar intermediates

like levoglucosan and levoglucosenone. Subsequently, at 250 °C, these intermediates can be converted into furans, including furanone and dihydro-hydroxy-2(3H)-furanone, as well as acetaldehyde and glycolaldehyde [25,26]. Examination of their potential energy surfaces indicates that furans represent the minima in Gibbs free energy (ΔG) within the product mixture. Hence, it cannot be discounted that furans might be generated through coupling reactions of the oxygenated organic molecules present in the pyrolysis vapors, which are formed during the extraction and chromatography procedures (Figure 13).

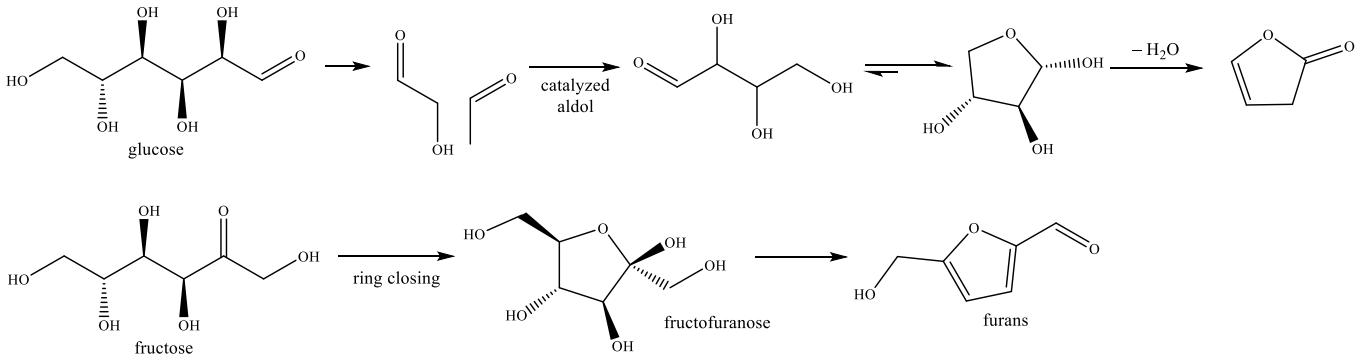

Figure 13. Proposed pathways for the transformation of carbohydrate-derived products into furans.

4.2. On the Antibacterial Activity

4.2.1. Mechanism of Action

The specific mode of action of *S. aspera* extracts was not studied herein, but an additive or synergistic behavior is expected due to the presence of several compounds with well-established antimicrobial action, as discussed above.

As in the case of polyphenols from *S. china* [27], the most probable mechanism of action of polyphenols would involve the alteration of the bacterial cell wall and damage to the cell membrane, inducing the leakage of cell contents, ultimately resulting in bacterial decomposition and death.

Regarding non-phenolic constituents, monosaccharide derivatives are recognized for their extensive range of biological effects on both Gram-negative and Gram-positive organisms, demonstrating efficacy against bacteria such as *Escherichia coli* (Migula 1895) Castellani & Chalmers 1919; *Bacillus subtilis* G; *Salmonella typhimurium* (Loeffler 1892) Castellani & Chalmers 1919; and *Staphylococcus aureus* Rosenbach 1884 [28–30]. Specifically, the chromatographic analysis of *S. aspera* leaf extract identified the presence of *α*- and *β*-*D*-galactopyranoside. Recent research by Hosen et al. [31] investigated the antimicrobial activity of methyl *β*-*D*-galactopyranoside (MGP) and its analogs and derivatives. MGP and its esters were evaluated for their physicochemical and pharmacokinetic properties through a comprehensive computational study involving thermodynamics, molecular dynamics, and molecular docking, revealing efficient binding to key targets, such as CTX-M-15 extended-spectrum *β*-lactamase from *E. coli* (PDB:4HBT). Moreover, other derivatives [32] have demonstrated in vitro antibacterial potential against *Bacillus cereus* Frankland & Frankland 1887, *B. subtilis*, *E. coli*, *S. typhimurium*, and *Pseudomonas aeruginosa* (Schroeter 1872) Migula 1900.

However, within the context of prospective agricultural applications, further in-depth analyses are necessary to validate these hypotheses and explore other potential mechanisms, such as abnormal intracellular oxidative stress, integration into genomic DNA, or restriction of the production of extracellular polymers [33].

4.2.2. Activity of *S. aspera* and Other *Smilax* spp. Extracts

Regarding the *Smilax* species under investigation, *S. aspera*, there are few studies on its antimicrobial activity, with contradictory results. Gyawali et al. [34] prepared a methanolic extract of leaves and bark that exhibited high effectiveness against *S. typhimurium* and

Pseudomonas spp. but only at concentrations as high as 4, 6, and 8%. Moreover, the extract showed low activity against *E. coli*, *Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887, and *S. aureus*. In turn, Mohammad Sawalha [35] found that ethanolic extracts of *S. aspera* fruits exhibited an inhibition zone of 20 mm against *S. aureus* and *Candida albicans* (C.P. Robin) Berkhout but showed no activity against *E. coli*. In comparison, the aqueous extraction only inhibited *C. albicans*, with an inhibition zone of 13 mm. Abbasołu and Türköz [36] prepared an extract from *S. aspera* fruits using chloroform and ethanol and tested it against bacteria such as *E. coli*, *P. aeruginosa*, *Streptococcus faecalis* Andrewes & Horder 1906, and *S. aureus* and fungi including *C. albicans*, *Candida parapsilosis* (Ashford) Langeron & Talice, and *Candida pseudotropicalis* (Castell.) Basgal (=*Kluyveromyces marxianus* (E.C. Hansen) Van der Walt), with inhibition values of 6300 $\mu g \cdot mL^{-1}$, higher than those reported herein. Higher antimicrobial activity was reported for steroidal saponins from *S. aspera* roots, which showed antifungal activity against *C. albicans*, *Candida glabrata* (H.W. Anderson) S.A. Mey. & Yarrow (=*Nakaseomyces glabratus* (H.W. Anderson) Sugita & Takashima), and *Candida tropicalis* (Castell.) Berkhout, with MIC values in the range of 25–50 $\mu g \cdot mL^{-1}$ [37], results similar to those obtained for spirostanol saponins from *Smilax medica* Bott. roots, with inhibition ranging between 12.5 and 50 $\mu g \cdot mL^{-1}$ [38], and in line with the biological properties of steroidal saponins sourced from other species of the *Smilax* genus reported by Tian et al. [39].

Concerning the antimicrobial efficacy of extracts from other species of the *Smilax* genus, a variety of solvents have been explored, but most studies have tested their activity against human pathogens, providing very few examples of activity tests against phytopathogens. As in the case of *S. aspera*, the results are not consistent from one study to another, but alcoholic extraction media are generally associated with the best results, as discussed below. It is worth noting that, given the diversity of plant parts chosen for the extraction (leaves, fruits, rhizomes, etc.) and the variety of extraction media, antimicrobial-activity-testing methods, assayed concentrations, and pathogens studied, direct efficacy comparisons are not applicable.

Concerning leaf extracts and fruit extracts like the ones studied herein, crude extracts derived from the aerial parts of *Smilax larvata* Griseb., including chloroform, ethyl acetate, hydroalcoholic, and *n*-hexane extracts, displayed no antimicrobial effects against *S. aureus*, *Staphylococcus epidermidis* (Winslow & Winslow 1908) Evans 1916, *E. coli*, *P. aeruginosa*, *Agrobacterium tumefaciens* H, and *C. albicans*. Only the ethanolic extract demonstrated a modest antifungal response, comparable to that of ketoconazole, against *C. albicans* [40].

Seo et al. [41] compared the antimicrobial activity of a leaf extract from *Smilax china* L. using DMSO, methanol, ethanol, acetone, and water as solvents. The DMSO extraction did not inhibit any of the studied pathogens: *E. coli* exhibited resistance to all five solvents; *Listeria monocytogenes* B showed greater susceptibility to the methanol extract (IZ = 11.7 mm), and *S. aureus* showed greater susceptibility to the ethanol extract (IZ = 10.7 mm), while *S. typhimurium* showed greater susceptibility to the aqueous extract (IZ = 11.8 mm). Additionally, polyphenols from *S. china* effectively inhibited bacteria such as *B. subtilis*, *E. coli*, *L. monocytogenes*, *S. typhimurium*, and *S. aureus*, with inhibition levels varying from 195.31 to 781.25 $\mu g \cdot mL^{-1}$ [27].

Ethanolic and methanolic extracts of *Smilax glabra* Roxb. and *Smilax corbularia* Kunth inhibited the growth of bacteria isolated from the mouth at 31,250–500,000 $\mu g \cdot mL^{-1}$; however, their aqueous extracts showed no activity [42]. The hexane leaf extract from *Smilax macrophylla* Griseb. displayed strong inhibitory effects against *Alternaria alternata* (Fr.) Keissl., *Ganoderma lucidum* (Curtis) P. Karst., *Pasteurella multocida* A, *E. coli*, *B. subtilis*, and *S. aureus*, with inhibition values ranging from 30,400 to 53,100 $\mu g \cdot mL^{-1}$. In contrast, its methanol extract demonstrated no activity against *A. alternata*, *E. coli*, and *S. aureus* [43]. The ethanolic leaf extract from *Smilax perfoliata* Lour. resulted in inhibition zones ranging from 10 to 13 mm against *B. subtilis*, *Proteus mirabilis* B, *B. cereus*, *S. typhimurium*, *P. aeruginosa*, *S. epidermidis*, and *C. albicans*; however, no inhibitory activity was observed against *S. aureus* and *E. coli* [44].

The ethanolic extract derived from the aerial parts of *Smilax campestris* Griseb. and its hexane and dichloromethane fractions exhibited high activity against *C. albicans* spp. and *Cryptococcus gattii* (Vanbreus. & Takashio) Kwon-Chung & Boekhout, with MIC values $\leq$ 2000 $\mu g \cdot mL^{-1}$. Conversely, the butanol and hydroalcoholic fractions demonstrated inactivity against all the studied yeasts [45].

Dhanya Shree et al. [46] explored the susceptibility of bacteria (*B. cereus*, *S. typhimurium*, *E. coli*, and *Shigella flexneri* Castellani & Chalmers 1919) and fungi (*Aspergillus niger* Tiegh. and *Bipolaris* sp.) to leaf and fruit methanolic extracts from *Smilax zeylanica* L. Unlike the results reported herein, in which both extracts showed similar effectiveness, in their study, the leaf extract demonstrated more prominent antibacterial activity when compared to the fruit extract, and both extracts proved effective against the tested fungi.

Rajbhandari and Paneru [47] evaluated the leaf extract of *Smilax ovalifolia* Roxb. ex D.Don in different solvents, namely, dichloromethane, ethyl acetate, or methanol, against *E. coli* and *S. aureus*, resulting in inhibition zones of approximately 16–19 mm for *E. coli* and 15–17 mm for *S. aureus*.

The in vitro assessment of ethylacetate, hexane, and methanol extracts from *Smilax kraussiana* Meisn. leaves was conducted by Hamid and Aiyelaagbe [48] against a panel of human-pathogenic microorganisms, encompassing six bacteria and six fungi. The findings revealed significant inhibitory effects on the growth of the twelve tested organisms. Particularly, the hexane and methanol extracts exhibited pronounced inhibition against *B. subtilis* and *S. aureus* (Gram-positive) within the concentration range of 25,000 to 200,000 $\mu g \cdot mL^{-1}$, surpassing the inhibitory potency of the ethylacetate extract. Conversely, all the extracts demonstrated comparatively lower antibacterial activity against Gram-negative bacteria, including *E. coli*, *K. pneumoniae*, *P. aeruginosa*, and *Salmonella typhi* (Schroeter 1886) Warren & Scott 1930. Notably, the three extracts displayed strong antifungal properties against *A. niger*, *C. albicans*, *Epidermophyton floccosum* (Harz) Langeron & Miloch, *Penicillum notatum* Westling (=*Penicillium chrysogenum* Thom), *Rhizopus stolonifer* (Ehrenb.) Vuill., and *Tricophyton rubrum* (Castell.) Sabour.

It should be noted that the previously mentioned significant variations in MIC values found in the literature are also applicable to root extracts: Joo et al. [49] reported that *S. china* root extracts exhibited strong antimicrobial activity (500 $\mu g \cdot mL^{-1}$) against *Cutibacterium acnes* (Gilchrist 1900) Scholz & Kilian 2016, a bacterium associated with acne. The ethanolic extract from *Smilax glabra* Roxb. rhizomes, along with the ethyl acetate and *n*-butanol fractions, demonstrated considerable activity against *S. aureus* (50 $\mu g \cdot mL^{-1}$), while water and ethyl acetate fractions exhibited activity against *C. albicans* and *S. aureus*, showcasing MIC values of 200 $\mu g \cdot mL^{-1}$ [50]. Nonetheless, according to McMurray et al. [51], the aqueous extract exhibited low activity, inhibiting *L. monocytogenes*, *Salmonella enteritidis* (Gaertner 1888) Castellani & Chalmers 1919, and *E. coli* within the range of 31,250–250,000 $\mu g \cdot mL^{-1}$. Cáceres et al. [52] investigated the antimicrobial, larvicidal, leishmanicidal, and schisonticidal activities of an ethanolic extract from *Smilax domingensis* Willd. rhizomes. The authors highlighted the results obtained for *B. subtilis*, *Mycobacterium smegmatis* (Trevisan 1889) Lehmann & Neumann 1899, *P. aeruginosa*, *S. aureus*, and *Sporothrix schenckii* Hektoen & C.F. Perkins, with MICs of 250 $\mu g \cdot mL^{-1}$, and reported a MIC value of 500 $\mu g \cdot mL^{-1}$ for *S. typhimurium*, *C. neoformans*, and *Trichophyton mentagrophytes* (C.P. Robin) R. Blanch.

4.2.3. Comparison with Synthetic Antimicrobials

In this study, bactericides from the aminoglycoside group (streptomycin) and polycyclic derivatives of naphthracenecarboxamides (tetracycline) were selected for comparison purposes, as they were initially the primary treatments employed to manage the bacterial infections under investigation.

The antibacterial activity of *S. aspera* fruit and leaf extracts was found to be lower than that of the two conventional antibiotics against all the bacterial strains under study. It is important to note, however, that the improper usage of the aforementioned conven-

tional antibiotics has contributed to the emergence of resistant strains, necessitating the exploration of other broad-spectrum control techniques [33]. In fact, higher doses than those assayed in this study for the extracts (1500 $\mu g \cdot mL^{-1}$) have been reported for conventional bactericidal products. For example, streptomycin resistance was observed in some isolated *E. amylovora* strains from Ohio, with inhibition values spanning from 500 to 2500 $\mu g \cdot mL^{-1}$ [53]. Comparable findings were reported in California by Förster et al. [54], with MICs ranging from 0.5 to >2000 $\mu g \cdot mL^{-1}$.

Regarding streptomycin activity against *P. syringae* pv. *actinidiae*, Cameron and Sarojini [55] compiled results from 19 strains, showing inhibition values in the range of 3.5 to >2000 $\mu g \cdot mL^{-1}$. Lee et al. [56] studied 10 strains with inhibition between 10 and 500 $\mu g \cdot mL^{-1}$. For *X. campestris* pv. *campestris*, the authors observed susceptibility to ampicillin, penicillin, and tetracycline at concentrations $\leq$ 6.25 $\mu g \cdot mL^{-1}$; carbenicillin, cephalothin, gentamicin, and kanamycin at 25.80 $\mu g \cdot mL^{-1}$; and bacitracin, neomycin, and streptomycin at >400, 160, and 640 $\mu g \cdot mL^{-1}$, respectively [57]. These values are in line with those of other pathovars of *X. campestris*, which could be inhibited by amoxicillin, cephalexin, chloramphenicol, penicillin G, streptomycin sulfate, or tetracycline. Specifically, tetracycline inhibited *X. campestris* pv. *musacearum* at 20 $\mu g \cdot mL^{-1}$ [58] and *X. campestris* pv. *vesicatoria* at doses ranging from 0.24 $\mu g \cdot mL^{-1}$ [59] to 156 $\mu g \cdot mL^{-1}$ [60].

Despite their lower efficacy, the presence of multiple antimicrobial compounds with different and concurrent modes of action in the extracts could be an advantage over conventional bactericidal compounds, potentially preventing the development of resistant strains. Therefore, *S. aspera* extracts may play a role in combating drug-resistant bacteria or diminish antibiotic reliance, possibly via a synergistic combination with these antibiotics, as has also been suggested for *S. china* [61].

4.2.4. Comparison with Other Products for Pear Tree Protection

Regarding the comparison of in planta results, in [62], the application of hydroethanolic extracts of *Thymus vulgaris* L., *Rhus coriaria* L., and *Eucalyptus globulus* Labill. Reached efficacies of 61.72%, 46.52%, and 29.94%, respectively, against *E. amylovora* on three-year-old 'Royal Gala' apple trees at concentrations of 20% (200,000 $\mu g \cdot mL^{-1}$, i.e., two orders of magnitude higher than those evaluated in this study). In [63], three extracts of *Moringa oleifera* Lam., namely, methanolic extract (MIC = 1000 $\mu g \cdot mL^{-1}$), hydroalcoholic extract (MIC = 1000 $\mu g \cdot mL^{-1}$), and hydroalcoholic extract with maltodextrins (MIC = 1000 $\mu g \cdot mL^{-1}$), were applied to two-year-old apple trees of the 'Gala' cultivar. The maltodextrin extract demonstrated the highest effectiveness, resulting in an 80% reduction in wilting compared to the control. Likewise, the methanolic and hydroalcoholic extracts showed a reduction in the infected area by 65% and 71%, respectively (slightly lower than that obtained for *S. aspera* hydromethanolic leaf extract but at a lower application dose).

5. Conclusions

Gas chromatography–mass spectrometry analyses of *S. aspera* leaf and fruit hydromethanolic extracts revealed the shared presence of phytoconstituents such as 1-hydroxy-2-propanone, 2,3-butanediol, catechol, 2-hydroxy-γ-butyrolactone, and 2-hydroxy-2-cyclopenten-1-one. Both extracts demonstrated high antibacterial activity against three relevant phytopathogens under in vitro conditions, inhibiting *P. syringae* pv. *actinidiae*, *X. campestris* pv. *campestris*, and *Erwinia amylovora* at a dose of 1500 $\mu g \cdot mL^{-1}$. The permeability assays showed noticeable alterations in membrane permeability both at the MIC and MIC/2. At the MIC, the leaf extracts reduced biofilm formation by 85%, 78%, and 82% against Xcc, EA, and Psa, respectively, while the fruit extract achieved reductions of 92.5%, 73%, and 86.5%, respectively. Furthermore, when used at a dose of MIC/2, the leaf and fruit extracts resulted in a reduction in amylovoran synthesis by 41% and 58%, respectively. Subsequent in planta testing against EA demonstrated that the leaf extract (at the MIC) exhibited the highest

effectiveness, resulting in an 80% reduction in wilting area, while the fruit extract led to a 45% reduction in wilting 10 days after inoculation. The reported results suggest that *S. aspera* extracts may hold promise with respect to combatting drug-resistant bacteria or lessening the need for antibiotics in agricultural settings when used in conjunction with antibiotics.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agronomy14020383/s1. Figure S1: ATR-FTIR spectra of *S. aspera* leaves and fruits prior to extraction; Table S1: Phytochemicals identified in *S. aspera* hydromethanolic leaf extract; Table S2: Phytochemicals identified in *S. aspera* hydromethanolic flower extract.

Author Contributions: Conceptualization, J.M.-G. and P.M.; methodology, P.M.-R., J.M.-G. and P.M.; validation, P.M.-R. and P.M.; formal analysis, R.F., E.S.-H. and P.M.-R.; investigation, R.F., E.S.-H., P.M.-R., J.M.-G. and P.M.; resources, J.M.-G. and P.M.; writing—original draft preparation, R.F., E.S.-H., P.M.-R. and J.M.-G.; writing—review and editing, R.F., E.S.-H. and P.M.-R.; visualization, R.F. and E.S.-H.; supervision, P.M.-R. and P.M.; project administration, P.M.-R. and J.M.-G.; funding acquisition, P.M.-R. and J.M.-G. All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The data supporting the findings of this study are available within the article and its Supplementary Materials.

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References

1. Bhatta, S.; Joshi, L.R.; Kunwar, R.M.; Bussmann, R.W.; Paniagua-Zambrana, N.Y. *Smilax aspera* L., *Smilax ovalifolia* Roxb. Smilacaceae. In *Ethnobotany of the Himalayas*; Kunwar, R.M., Sher, H., Bussmann, R.W., Eds.; Springer International Publishing: Cham, Switzerland, 2021; pp. 1869–1879. [CrossRef]
2. Quattrocchi, U. *CRC World Dictionary of Medicinal and Poisonous Plants: Common Names, Scientific Names, Eponyms, Synonyms, and Etymology*; CRC: Boca Raton, FL, USA, 2012; p. 3960.
3. Kakouri, E.; Hatziagapiou, K.; Kanakis, C.; Nikola, O.; Lambrou, G.I.; Trigas, P.; Kanaka-Gantenbein, C.; Tarantilis, P.A. Cytotoxic and antioxidant activity of a chemically characterized extract of *Smilax aspera* leaves and stems. *Appl. Sci.* **2023**, *13*, 4784. [CrossRef]
4. Salas-Coronado, R.; Hernández-Carlos, B.; Llaguno-Guilberto, J.; Santos-Sánchez Norma, F. Phenolic compounds in genus *Smilax* (Sarsaparilla). In *Phenolic Compounds: Natural Sources, Importance, and Applications*; Marcos, S.-H., Mariana, P.-T., Maria del Rosario, G.-M., Eds.; IntechOpen: Rijeka, Croatia, 2017; pp. 233–260. [CrossRef]
5. Longo, L.; Vasapollo, G. Extraction and identification of anthocyanins from *Smilax aspera* L. berries. *Food Chem.* **2006**, *94*, 226–231. [CrossRef]
6. Delgado-Pelayo, R.; Hornero-Méndez, D. Identification and quantitative analysis of carotenoids and their esters from sarsaparilla (*Smilax aspera* L.) berries. *J. Agric. Food. Chem.* **2012**, *60*, 8225–8232. [CrossRef] [PubMed]
7. Santos, M.G.; Nunes da Silva, M.; Vasconcelos, M.W.; Carvalho, S.M.P. Scientific and technological advances in the development of sustainable disease management tools: A case study on kiwifruit bacterial canker. *Front. Plant Sci.* **2024**, *14*, 1306420. [CrossRef]
8. Piqué, N.; Miñana-Galbis, D.; Merino, S.; Tomás, J. Virulence factors of *Erwinia amylovora*: A review. *Int. J. Mol. Sci.* **2015**, *16*, 12836–12854. [CrossRef]
9. Donati, I.; Cellini, A.; Sangiorgio, D.; Vanneste, J.L.; Scortichini, M.; Balestra, G.M.; Spinelli, F. *Pseudomonas syringae* pv. actinidiae: Ecology, infection dynamics and disease epidemiology. *Microb. Ecol.* **2020**, *80*, 81–102. [CrossRef]
10. Pedroncelli, A.; Puopolo, G. This tree is on fire: A review on the ecology of *Erwinia amylovora*, the causal agent of fire blight disease. *J. Plant Pathol.* **2023**, *in press*. [CrossRef]

11. Vicente, J.G.; Holub, E.B. *Xanthomonas campestris* pv. campestris (cause of black rot of crucifers) in the genomic era is still a worldwide threat to brassica crops. *Mol. Plant Pathol.* **2013**, *14*, 2–18. [CrossRef]
12. Akhlaghi, M.; Tarighi, S.; Taheri, P. Effects of plant essential oils on growth and virulence factors of *Erwinia amylovora*. *J. Plant Pathol.* **2019**, *102*, 409–419. [CrossRef]
13. Wilson, C.; Lukowicz, R.; Merchant, S.; Valquier-Flynn, H.; Caballero, J.; Sandoval, J.; Okuom, M.; Huber, C.; Brooks, T.D.; Wilson, E.; et al. Quantitative and qualitative assessment methods for biofilm growth: A mini-review. *Res. Rev. J. Eng. Technol.* **2017**, *6*, 1–25.
14. Bellemann, P.; Bereswill, S.; Berger, S.; Geider, K. Visualization of capsule formation by *Erwinia amylovora* and assays to determine amylovoran synthesis. *Int. J. Biol. Macromol.* **1994**, *16*, 290–296. [CrossRef]
15. Schneider, C.A.; Rasband, W.S.; Eliceiri, K.W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **2012**, *9*, 671. [CrossRef]
16. Langa-Lomba, N.; Sánchez-Hernández, E.; Buzón-Durán, L.; González-García, V.; Casanova-Gascón, J.; Martín-Gil, J.; Martín-Ramos, P. Activity of anthracenediones and flavoring phenols in hydromethanolic extracts of *Rubia tinctorum* against grapevine phytopathogenic fungi. *Plants* **2021**, *10*, 1527. [CrossRef]
17. Langa-Lomba, N.; Buzón-Durán, L.; Martín-Ramos, P.; Casanova-Gascón, J.; Martín-Gil, J.; Sánchez-Hernández, E.; González-García, V. Assessment of conjugate complexes of chitosan and *Urtica dioica* or *Equisetum arvense* extracts for the control of grapevine trunk pathogens. *Agronomy* **2021**, *11*, 976. [CrossRef]
18. Sánchez-Hernández, E.; González-García, V.; Palacio-Bielsa, A.; Lorenzo-Vidal, B.; Buzón-Durán, L.; Martín-Gil, J.; Martín-Ramos, P. Antibacterial activity of *Ginkgo biloba* extracts against *Clavibacter michiganensis* subsp. michiganensis, Pseudomonas spp., and Xanthomonas vesicatoria. *Horticulturae* **2023**, *9*, 461. [CrossRef]
19. Zheng, C.-J.; Li, L.; Ma, W.-H.; Han, T.; Qin, L.-P. Chemical constituents and bioactivities of the liposoluble fraction from different medicinal parts of *Crocus sativus*. *Pharm. Biol.* **2011**, *49*, 756–763. [CrossRef] [PubMed]
20. Wang, L.; Jiang, Y.; Han, T.; Zheng, C.; Qin, L. A phytochemical, pharmacological and clinical profile of *Paederia foetida* and *P. scandens*. *Nat. Prod. Commun.* **2014**, *9*, 879–886. [CrossRef] [PubMed]
21. Teixeira, A.; Sánchez-Hernández, E.; Noversa, J.; Cunha, A.; Cortez, I.; Marques, G.; Martín-Ramos, P.; Oliveira, R. Antifungal activity of plant waste extracts against phytopathogenic fungi: *Allium sativum* peels extract as a promising product targeting the fungal plasma membrane and cell wall. *Horticulturae* **2023**, *9*, 136. [CrossRef]
22. Sánchez-Hernández, E.; Balduque-Gil, J.; Barriuso-Vargas, J.J.; Casanova-Gascón, J.; González-García, V.; Cuchí-Oterino, J.A.; Lorenzo-Vidal, B.; Martín-Gil, J.; Martín-Ramos, P. Holm oak (*Quercus ilex* subsp. ballota (Desf.) Samp.) bark aqueous ammonia extract for the control of invasive forest pathogens. *Int. J. Mol. Sci.* **2022**, *23*, 11882. [CrossRef]
23. Sánchez-Hernández, E.; Balduque-Gil, J.; González-García, V.; Barriuso-Vargas, J.J.; Casanova-Gascón, J.; Martín-Gil, J.; Martín-Ramos, P. Phytochemical profiling of *Sambucus nigra* L. flower and leaf extracts and their antimicrobial potential against almond tree pathogens. *Int. J. Mol. Sci.* **2023**, *24*, 1154. [CrossRef] [PubMed]
24. Sánchez-Hernández, E.; González-García, V.; Palacio-Bielsa, A.; Casanova-Gascón, J.; Navas-Gracia, L.M.; Martín-Gil, J.; Martín-Ramos, P. Phytochemical constituents and antimicrobial activity of *Euphorbia serrata* L. extracts for *Borago officinalis* L. crop protection. *Horticulturae* **2023**, *9*, 652. [CrossRef]
25. Kundu, C.; Biswas, S.; Thomas, B.S.; Appadoo, D.; Duan, A.; Bhattacharya, S. Evolution of functional group of lignocellulosic biomass and its delignified form during thermal conversion using synchrotron-based THz and laboratory-based in-situ DRIFT spectroscopy. *Fuel* **2023**, *348*, 128579. [CrossRef]
26. Kim, S.; Evans, T.J.; Mukarakate, C.; Bu, L.; Beckham, G.T.; Nimlos, M.R.; Paton, R.S.; Robichaud, D.J. Furan production from glycoaldehyde over HZSM-5. *ACS Sustain. Chem. Eng.* **2016**, *4*, 2615–2623. [CrossRef]
27. Xu, M.; Xue, H.; Li, X.; Zhao, Y.; Lin, L.; Yang, L.; Zheng, G. Chemical composition, antibacterial properties, and mechanism of *Smilax china* L. polyphenols. *Appl. Microbiol. Biotechnol.* **2019**, *103*, 9013–9022. [CrossRef]
28. Islam, M.; ArÏFuzzaman, A.; Rahman, M.; Rahman, M.A.; Kawsar, S.M.A.K. Novel methyl 4,6-*O*-benzylidene-*a*-*D*-glucopyranoside derivatives: Synthesis, structural characterization and evaluation of antibacterial activities. *Hacettepe J. Biol. Chem.* **2019**, *47*, 153–164. [CrossRef]
29. Kawsar, S.M.A. Regioselective synthesis, characterization, and antimicrobial activities of some new monosaccharide derivatives. *Sci. Pharm.* **2014**, *82*, 1–20. [CrossRef]
30. Misbah, M.M.; Ferdous, J.; Bulbul, M.Z.; Chowdhury, T.S.; Dey, S.; Hasan, I.; Kawsar, S.M. Evaluation of MIC, MBC, MFC and anticancer activities of acylated methyl *β*-*D*-galactopyranoside esters. *Int. J. Biosci.* **2020**, *16*, 299–309.
31. Hosen, M.A.; El Bakri, Y.; Rehman, H.M.; Hashem, H.E.; Saki, M.; Kawsar, S.M.A. A computational investigation of galactopyranoside esters as antimicrobial agents through antiviral, molecular docking, molecular dynamics, pharmacokinetics, and bioactivity prediction. *J. Biomol. Struct. Dyn.* **2023**, *42*, 1015–1030. [CrossRef] [PubMed]
32. Ahmmed, F.; Islam, A.U.; Mukhrish, Y.E.; Bakri, Y.E.; Ahmad, S.; Ozeki, Y.; Kawsar, S.M.A. Efficient antibacterial/antifungal activities: Synthesis, molecular docking, molecular dynamics, pharmacokinetic, and binding free energy of galactopyranoside derivatives. *Molecules* **2022**, *28*, 219. [CrossRef] [PubMed]
33. Han, Q.; Feng, L.; Zhang, Y.; Zhang, R.; Wang, G.; Zhang, Y. Effect of juglone against *Pseudomonas syringae* pv *actinidiae* planktonic growth and biofilm formation. *Molecules* **2021**, *26*, 7580. [CrossRef] [PubMed]
34. Gyawali, R.; Dahal, B.; Gautam, R.; Shrestha, S.; Joshi, S.; Luitel, A.; Khanal, D. Phytochemical studies traditional medicinal plants of Nepal and their formulations. *Int. J. Biol. Pharm. Allied Sci.* **2014**, *3*, 189–203.

35. Mohammad Sawalha, M.K. Phytochemical screening of wild *Ephedra foeminea* and *Smilax aspera* fruits and their in-vitro pharmacological activities. M.Sc. Thesis, Al-Quds University, Jerusalem, Israel, 2018.
36. Abbasołu, U.; Türköz, S. Antimicrobial activities of saponin extracts from some indigenous plants of Turkey. *Int. J. Pharmacogn.* **2008**, *33*, 293–296. [CrossRef]
37. Belhouchet, Z.; Sautour, M.; Miyamoto, T.; Lacaille-Dubois, M.-A. Steroidal saponins from the roots of *Smilax aspera* subsp. *mauritanica*. *Chem. Pharm. Bull.* **2008**, *56*, 1324–1327. [CrossRef] [PubMed]
38. Sautour, M.; Miyamoto, T.; Lacaille-Dubois, M.-A. Steroidal saponins from *Smilax medica* and their antifungal activity. *J. Nat. Prod.* **2005**, *68*, 1489–1493. [CrossRef] [PubMed]
39. Tian, L.-W.; Zhang, Z.; Long, H.-L.; Zhang, Y.-J. Steroidal saponins from the genus *Smilax* and their biological activities. *Nat. Prod. Bioprospect.* **2017**, *7*, 283–298. [CrossRef] [PubMed]
40. Konopatzki Hirota, B.C.; da Silva Paula de Oliveira, C.; Merino, F.; dos Santos Verdam, M.; Da Silva, C.; Murakami, F.; Miguel, O.; Miguel, M. Larvicide and antifungal activities of sarsaparilla (*Smilax larvata*) extracts. *Int. J. Pharm. Pharm. Sci.* **2015**, *7*, 308–311.
41. Seo, H.-K.; Lee, J.-H.; Kim, H.-S.; Lee, C.-K.; Lee, S.-C. Antioxidant and antimicrobial activities of *Smilax china* L. leaf extracts. *Food Sci. Biotechnol.* **2012**, *21*, 1723–1727. [CrossRef]
42. Jeeno, P.; Tongban, S.; Yana, P.; Wongta, A.; Sutan, K.; Yadoung, S.; Hongsibsong, S. Tentative identification of phytochemicals from *Smilax glabra* and *Smilax corbularia* extracts by LC-QTOF/MS and their bioactive potential. *Plants* **2022**, *11*, 2089. [CrossRef] [PubMed]
43. Zubair, M.; Rizwan, K.; Rashid, U.; Saeed, R.; Saeed, A.A.; Rasool, N.; Riaz, M. GC/MS profiling, *in vitro* antioxidant, antimicrobial and haemolytic activities of *Smilax macrophylla* leaves. *Arab. J. Chem.* **2017**, *10*, S1460–S1468. [CrossRef]
44. Borkataky, M. Antimicrobial and antioxidant activity of *Smilax perfoliata* Lour. *Der. Pharm. Lett.* **2014**, *6*, 246–250.
45. Morais, M.I.; Pinto, M.E.A.; Araújo, S.G.; Castro, A.H.F.; Duarte-Almeida, J.M.; Rosa, L.H.; Rosa, C.A.; Johann, S.; Lima, L.A.R.d.S. Antioxidant and antifungal activities of *Smilax campestris* Griseb. (*Smilacaceae*). *Nat. Prod. Res.* **2014**, *28*, 1275–1279. [CrossRef]
46. Dhanya Shree, V.S.; Arbin, A.; Saema Noorain, G.K.; Sahana, B.K.; Prashith Kekuda, T.R. Preliminary phytochemical analysis, antimicrobial and antioxidant activity of *Smilax zeylanica* L. (*Smilacaceae*). *J. Drug Deliv. Ther.* **2018**, *8*, 237–243. [CrossRef]
47. Rajbhandari, M.; Paneru, D.P. Phytochemical analysis and antimicrobial activity of *Smilax ovalifolia* Roxb. Ex D. Don. *Nepal J. Sci. Technol.* **2020**, *19*, 89–96. [CrossRef]
48. Hamid, A.; Aiyelaagbe, O. The screening of phytoconstituents, antibacterial and antifungal properties of *Smilax kraussiana* leaves. *Pharm. Sin.* **2011**, *2*, 267–273.
49. Joo, J.-H.; Han, M.-H.; Kim, J.-I.; Kim, J.-E.; Jung, K.-H.; Oh, H.S.; Chung, Y.S.; An, H.J.; Lee, J.D.; Moon, G.-S.; et al. Antimicrobial activity of *Smilax china* L. root extracts against the acne-causing bacterium, *Cutibacterium acnes*, and its active compounds. *Molecules* **2022**, *27*, 8331. [CrossRef]
50. Xu, S.; Shang, M.-Y.; Liu, G.-X.; Xu, F.; Wang, X.; Shou, C.-C.; Cai, S.-Q. Chemical constituents from the rhizomes of *Smilax glabra* and their antimicrobial activity. *Molecules* **2013**, *18*, 5265–5287. [CrossRef] [PubMed]
51. McMurray, R.L.; Ball, M.E.E.; Tunney, M.M.; Corcionivoschi, N.; Situ, C. Antibacterial activity of four plant extracts extracted from traditional chinese medicinal plants against *Listeria monocytogenes*, *Escherichia coli*, and *Salmonella enterica* subsp. enterica serovar Enteritidis. *Microorganisms* **2020**, *8*, 962. [CrossRef]
52. Cáceres, A.; Cruz, S.M.; Martínez, V.; Gaitán, I.; Santizo, A.; Gattuso, S.; Gattuso, M. Ethnobotanical, pharmacognostical, pharmacological and phytochemical studies on *Smilax domingensis* in Guatemala. *Rev. Bras. Farmacogn.* **2012**, *22*, 239–248. [CrossRef]
53. Jimenez Madrid, A.M.; Ivey, M.L.L. An overview of streptomycin resistance in *Erwinia amylovora* from Ohio apple orchards. *Plant Health Prog.* **2023**, *24*, 56–61. [CrossRef]
54. Förster, H.; McGhee, G.C.; Sundin, G.W.; Adaskaveg, J.E. Characterization of streptomycin resistance in isolates of *Erwinia amylovora* in California. *Phytopathology* **2015**, *105*, 1302–1310. [CrossRef]
55. Cameron, A.; Sarojini, V. *Pseudomonas syringae* pv. actinidiae: Chemical control, resistance mechanisms and possible alternatives. *Plant Pathol.* **2013**, *63*, 1–11. [CrossRef]
56. Lee, J.-H.; Kim, J.-H.; Kim, G.-H.; Jung, J.-S.; Hur, J.-S.; Koh, Y.-J. Comparative analysis of Korean and Japanese strains of *Pseudomonas syringae* pv. actinidiae causing bacterial canker of kiwifruit. *Plant Pathol. J.* **2005**, *21*, 119–126. [CrossRef]
57. Chang, C.; Donaldson, R.; Crowley, M.; Pinnow, D. A new semiselective medium for the isolation of *Xanthomonas campestris* pv. campestris from crucifer seeds. *Phytopathology* **1991**, *81*, 449–453. [CrossRef]
58. Yemata, G.; Fetene, M. In vitro antibacterial activity of some antibiotics against *Xanthomonas campestris* pv. musacearum: Ensete ventricosum (Welw.) Cheesman pathogen. *J. Phytopathol. Pest Manag.* **2017**, *4*, 48–57.
59. Kim, J.Y.; Kim, J.Y.; Park, J.Y.; Kim, J.-S.; Seo, M.-K.; Shin, M.-K.; Kim, J.-H. Synergistic bactericidal effects of carvone and β-lactams against *Xanthomonas campestris* pv. *vesicatoria*. *Appl. Biol. Chem.* **2023**, *66*, 44. [CrossRef]
60. Kavitha, K.S.; Satish, S. Evaluation of antimicrobial and antioxidant activities from *Toona ciliata* Roemer. *J. Anal. Sci. Technol.* **2013**, *4*, 23. [CrossRef]
61. Wang, M.; Bai, Q.-X.; Zheng, X.-X.; Hu, W.-J.; Wang, S.; Tang, H.-P.; Yu, A.-Q.; Yang, B.-Y.; Kuang, H.-X. *Smilax china* L.: A review of its botany, ethnopharmacology, phytochemistry, pharmacological activities, actual and potential applications. *J. Ethnopharmacol.* **2024**, *318*, 116992. [CrossRef]

62. Bastas, K.K. Effects of some plant extracts against *Erwinia amylovora* by the induced resistance on apple in vitro and in vivo conditions. In Proceedings of the 6th International Conference on Sustainable Agriculture and Environment (ICSAE-6), Konya, Turkey, 3–5 October 2019; pp. 504–509.
63. Fontana, R.; Macchi, G.; Caproni, A.; Sicurella, M.; Buratto, M.; Salvatori, F.; Pappadà, M.; Manfredini, S.; Baldisserotto, A.; Marconi, P. Control of *Erwinia amylovora* growth by *Moringa oleifera* leaf extracts: In vitro and in planta effects. *Plants* **2022**, *11*, 957. [CrossRef]