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RESEARCH PAPERS

Antimicrobial activity of plant extracts against *Erwinia amylovora*

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Summary. Fire blight poses severe economic risks for production from economically important crops. This study assessed *in vitro* and *in vivo* antibacterial activity against *Erwinia amylovora* of non-volatile organic and aqueous extracts (from fruit peels or leaves) from pomegranate (*Punica granatum*), onion (*Allium cepa*), eucalyptus (*Eucalyptus globulus*), and garlic (*Allium sativum*). The ethanolic extract from pomegranate peel exhibited the greatest and most consistent antimicrobial activity in three *in vitro* assays (reverse agar plugs, wells, and disc diffusion). This extract also reduced disease severity during *in vivo* bioassays with immature pear fruits or detached leaves. Chemical characterization using Nuclear Magnetic Resonance spectroscopy indicated that this bioactivity was associated with the presence of compounds identified as the ellagitannins α- and β-punicalagin. These results highlight the potential of pomegranate peel extract as a potential biocontrol agent for fire blight management.

Keywords. Biocontrol, pomegranate, pome fruits, essential oils, bioactive compounds.

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INTRODUCTION

Pear (*Pyrus communis* L.) is an important *Rosaceae* crop in Algeria, ranking second after apple and amounting to 20% of national fruit production. The cultivated pear area in 2019 was 20,240 ha and production was 113,526.6 tons (MADR, 2019). *Erwinia amylovora* (Burrill) Winslow *et al.* (1920), the causal agent of fire blight, is an important bacterial pathogen affecting pome fruit trees and ornamental Maloideae hosts. This pathogen poses significant challenges for nurserymen and apple and pear producers, as it can decimate orchards within a single season (Mansfield *et al.*, 2012). This

pathogen is categorized as an A2 (and RNQP-Annex IV) quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO).

Erwinia amylovora was first detected in Algeria in 2011 in the Tipaza region (Laala *et al*., 2012), and by 2015 a total of 2,014 affected orchards (pear, medlar and apple) were detected across nine Algerian cities (Tafifet *et al*., 2020).

Effective management of fire blight requires an integrated approach, combining several cultural, chemical physical and biological control methods, to maintain *E. amylovora* below harm thresholds (Milaire, 1995). While antibiotics (e.g. streptomycin) have been widely used, concerns over resistance have led to the exploration of alternative disease management strategies (Stockwell and Duffy, 2012). Biological control has emerged as a promising eco-friendly solution, utilizing antagonistic bacteria (*Pantoea agglomerans*, *Bacillus subtilis*), as well as yeast-based biocontrol agents (e.g. *Aureobasidium pullulans*), which have demonstrated effectiveness for fire blight suppression (Johnson and Stockwell, 2000; Spinelli *et al*., 2011). Additionally, abiotic agents, including plant-derived extracts from garlic (*Allium sativum*), thyme (*Thymus vulgaris*) and pomegranate (*Punica granatum*), have shown significant antibacterial activity against *E. amylovora* (Bastas, 2020; Hernández *et al*., 2022).

Garlic (*Allium sativum*), onion (*Allium cepa*), eucalyptus (*Eucalyptus globulus*) and pomegranate (*Punica granatum*) are well known for their antibacterial activities and high contents of bioactive phenolic compounds. *Allium sativum* and *Allium cepa* contain organosulfur compounds that inhibit plant pathogenic bacteria, including *Agrobacterium tumefaciens*, *Erwinia carotovora*, *Pseudomonas syringae* and *Xanthomonas campestris* (Curtis *et al.,* 2004, Singh *et al.,* 2025). Eucalyptus (*Eucalyptus globulus*) is rich in bioactive compounds including essential oils, flavonoids, phenol acids and tannins that have antimicrobial activity against Gram-positive and Gram-negative bacteria (Amakura *et al*., 2002). Extracts from pomegranate peel, obtained by maceration, are widely used against phytopathogenic bacteria, and have shown strong antibacterial effects, especially against *Xylella fastidiosa* subsp. *pauca* (Rongai *et al.,* 2023), *Xanthomonas campestris* pv. *campestris* (Muawiya *et al.,* 2025), *Ralstonia solanacearum* and *Pectobacterium carotovorum* (Elhalag *et al.,* 2025). However, limited evidence exists regarding the effectiveness of these plant extracts against *Erwinia amylovora*, the primary pathogen responsible for fire blight.

The present study aimed to evaluate the antibacterial effects of extracts from pomegranate (*Punica granatum*), garlic (*Allium sativum*), onion (*Allium cepa*), and eucalyptus (*Eucalyptus globulus*) against *E. amylovora* isolates, through a series of *in vitro* and *in vivo* experiments. This research aimed to provide novel insights by assessing non-edible plant residues (fruit peels or leaves), to provide knowledge for potential sustainable disease management solutions, as well as addressing use of agricultural waste and supporting circular economy principles for fruit producers.

MATERIALS AND METHODS

Plant collection

Four plant species from different botanical families were selected for investigation, based on their documented bioactive properties: pomegranate fruit peel (*Punica granatum* L.), onion bulb peel (*Allium cepa* L.), garlic bulb peel (*Allium sativum* L.), and eucalyptus leaves (*Eucalyptus globulus* Labill.) (Table 1). Pomegranate fruit, onion and garlic bulbs (each approx. 5 kg) were purchased from a local market in Algiers, Algeria, during the 2021 summer growing season. Eucalyptus leaves were collected from mature trees in the botanical garden of the École Nationale Supérieure Agronomique (ENSA), Algiers. *Eucalyptus globulus* is native to Australia and introduced in Algeria. After collection, the plant material was air dried at ambient temperatures in a greenhouse for 2 weeks, protected from light and moisture. Once dried, the samples were each finely ground using an electric grinder, and were then stored in sterile, airtight containers at room temperature and protected from light until extraction.

Table 1. The plants assessed in the present study for potential extract activity against *Erwininia amylovora*.

Common name	Scientific name	Plant part used	Origin	Sampling date
Pomegranate	*Punica granatum* L.	Peel	Local market, Algiers, Algeria	2021
Garlic	*Allium sativum* L.	Peel	Local market, Algiers, Algeria	2021
Onion	*Allium cepa* L.	Peel	Local market, Algiers, Algeria	2021
Eucalyptus	*Eucalyptus globulus* Labill.	Leaves	ENSA Botanical Garden, Algiers, Algeria	2021

Preparation of plant extracts

Extractions were carried out in the chemistry laboratory at "Centre de Recherche Scientifique et Technique en Analyses Physico-Chimiques" (CRAPC) Tipaza, Algeria. Dry plant material (100 g) was subjected to hot maceration at 40 °C with continuous stirring in solvent, 100 g of dried plant material mixed with 400 mL of solvent. Three solvents with complementary physicochemical properties were selected to optimize recovery of potentially bioactive compounds across the polarity spectrum, including: ethanol (96%, CAS: 64-17-5) for compounds with intermediate polarity, ethyl acetate (99.5%, CAS: 141-78-6) for semi-polar compounds, and sterile distilled water (SDW) for polar compounds.

The initial macerations were each carried out for 60 min, and the sample solutions were filtered through filter paper (Whatman® N° 04). Each residue underwent two additional extractions of 30 min and 15 min, using the same amount of solvent and following the same procedure for each extract to ensure maximum extraction (Figure 1).

All the ethanol and ethyl acetate filtrates (organic phases) were extracted and concentrated under vacuum at 30 °C, using rotary evaporation (Dragon Lab RE100-Pro) at 100 rpm, until solid residues were obtained. The filtrates of sterile distilled water (SDW; aqueous phases) were processed to eliminate the water by lyophilization (Lyophilized Alpha 2-4 Christ LSC plus) after freezing for 24 h. This process was carried out by maintaining the product at low temperature (-53.5 °C) under a vacuum (2.50 mbar). Twelve crude solid extracts (organic and aqueous) were then preserved aseptically in sterile amber glass bottles to prevent molecular degradation from light, and were then stored in the refrigerator at 4 °C for further use (Figure 1; Table 2).

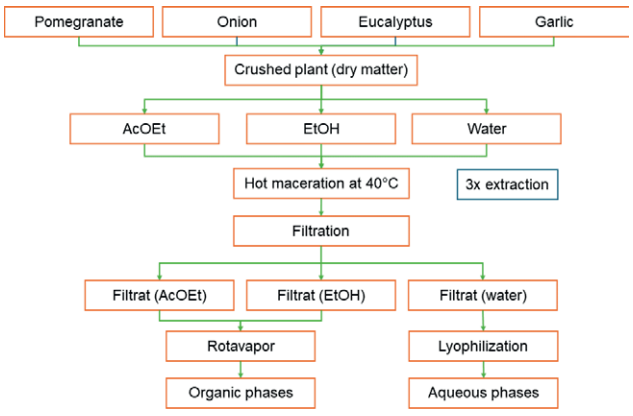

Figure 1. Schematic representation of the extraction process used to obtain potentially bioactive compounds from candidate plants.

The extract yields were calculated for each sample. Aqueous and organic extracts were then prepared by dissolving 25 mg of each resulting dry powder in 1 mL of 1% dimethyl sulfoxide (DMSO) or SDW, resulting in final concentrations of 25 mg mL^{-1} (w/v). The solutions were allowed to stand 24 h before use (Table 2).

Bacterial isolate, culture conditions, and inoculum preparation

Three pathogenic isolates of *E. amylovora* were used in this study: two reference isolates (CFBP 8377/EADZ8 and LMG 2024) present in the collection of the laboratory of Phytobacteriology in "École Nationale Supérieure Agronomique" (ENSA), Algiers, Algeria and a new local isolate of *E. amylovora* (ITAFEA 2022). This new isolate was obtained in 2022 from an infected pear tree sampled in a single orchard located in the Blida region, Algeria. Different samples from symptomatic plants (shoots, leaves and branches) were harvested and placed in labelled plastic bags and transported in the laboratory while keeping the samples in an ice cooler (approx. 4 °C). All the samples were washed under running tap water, surface sterilized by immersing them for 1 min in 1% (v/v) of chlorine solution, rinsing for 30 s in 70% (v/v) ethanol solution, and then washing three times (each for 1 min) in sterile distilled water to remove residual disinfectants. Each sample was then divided into small pieces (approx. 3-5 mm) with sterile scalpels, and was then incubated for 1.5 h in a sterile Phosphate-Buffered Saline solution (PBS; pH: 7.2) under constant agitation (120 rpm) at room temperature (22 to 25 °C). Serial dilutions were then made in sterile distilled water, and 50 µL of each macerate was inoculated onto King's B medium (Schaad *et al.,* 2001, Laala *et al.,* 2012) and then incubated for 3 to 5 d at 28 °C. Single colonies closest to the cultural characteristics of *E. amylovora* were then purified and refreshed three times, each on a new plate, to ensure the isolate purity. Axenic culture of *Erwinia*-like isolates were then characterized using biochemical tests, and were also identified by molecular techniques (Llop *et al*., 2000). The ITAFEA 2022 isolate, as characterized in the present study, was employed in all subsequent assay.

For preparations of inoculum, a bacterial suspension was prepared by adding a loopful of a 48 h King's B agar culture of each isolate to SDW, to reach a concentrations of approx. 10^8 CFU mL^{-1}, determined by measuring absorbance at 600 nm (~ 0.1 OD) and confirmed via serial dilution and plate counting. This was the starting concentration used for all the experiments described below.

Table 2. Plant extract codes, extraction methods, stock solution concentrations, and solvent controls for the plant extracts evaluated in the present study.

Code	Plants	Extraction solvent	Solubilizing solvent[a]	Stock conc. (mg mL^{-1})	Solvent control
P1	Pomegranate (peel)	Ethanol	1% DMSO	25	1% DMSO
P2	Pomegranate (peel)	Ethyl acetate	1% DMSO	25	1% DMSO
P3	Pomegranate (peel)	SDW	100% SDW	25	100% SDW
G1	Garlic (peel)	Ethanol	1% DMSO	25	1% DMSO
G2	Garlic (peel)	Ethylacetate	1% DMSO	25	1% DMSO
G3	Garlic (peel)	SDW	100% SDW	25	100% SDW
O1	Onion (peel)	Ethanol	1% DMSO	25	1% DMSO
O2	Onion (peel)	Ethyl acetate	1% DMSO	25	1% DMSO
O3	Onion (peel)	SDW	100% SDW	25	100% SDW
E1	Eucalyptus (leaves)	Ethanol	1% DMSO	25	1% DMSO
E2	Eucalyptus (leaves)	Ethyl acetate	1% DMSO	25	1% DMSO
E3	Eucalyptus (leaves)	SDW	100% SDW	25	100% SDW

[a]SDW: sterile distilled water , DMSO: dimethylsulfoxide.

In vitro *antibacterial assays*

Reverse agar plug diffusion assays. This technique was based on the agar plug diffusion method but applied in reverse to improve assessment of effects of plant extracts on bacterial growth (Balouiri *et al.,* 2016), with some modifications. The plant extracts (aqueous or organic extracts) were added to sterilized King's B medium, by incorporating each plant extract into 250 mL of medium to a final concentration of 25 mg mL^{-1} (w/v). The mixtures were immediately poured into Petri dishes (15 mL per dish). A 6 mm diam. agar plug containing a bacterium isolate was then inverted and transplanted in the centre of each petri dish containing King's B medium supplemented with the relevant plant extract, as well as for experimental control dishes. Streptomycin at 0.5 mg mL^{-1} was used a positive control. The dishes were then incubated at 28 °C. Four replicate dishes were used for each plant extract, solvent and *E. amylovora* isolate. The diameters of bacterium growth zones were then measured every 24 h for 7 d, using a millimeter scale ruler.

Well diffusion assays. The well diffusion technique was carried out as described by Mathabe *et al.* (2006). Bacterial suspension (50 mL) was placed in the centre of each Petri dish containing King's B medium, and was then spread evenly on the medium surface. The plates were then dried for 30 min to allow diffusion of the bacterial suspension into the medium. Two wells (6 mm diam.) were then created in each plates. One was filled with 50 µL of crude plant extract (Table 2) (organic or aqueous extracts 25 mg mL^{-1} (W/V), as a negative control, and the other well was filled with 50 µL of SDW plus streptomycin at 0.5 mg mL^{-1}, as a positive control. The plates were then incubated at 28 °C. Four replicates were performed for each plant extract, solvent and bacterial isolate. The inhibition zone diameters were then measured every 24 h for 7 d.

Disc diffusion assays. Sterilized Whatman® N°1 filter paper discs (8 mm diam.) were impregnated with different plant extracts (organic or aqueous extracts), each at 50 µL per disc, following the method described by Gormez *et al.* (2015). A 50 µL droplet of bacterial suspension was then placed in the center of each Petri plate containing King's B medium and was spread evenly on the surface. The plates were then dried for 30 min to allow diffusion of the bacterial suspension into the medium. The discs were then aseptically placed on the medium at four 4 discs per plate, and each disc was impregnated with one of the four different plant extracts, either ethanol, ethyl acetate or water extracts. Negative experimental controls were discs impregnated with SDW, and positive controls were discs containing streptomycin at 0.5 mg mL^{-1}. Four replicates were prepared for each plant extract, solvent and isolate combination. The cultures were then incubated at 28 °C, and inhibition zone diameters were measured every 24 h for 7 d.

Plant material for in-vivo *experiments*

Healthy leaves of the pear cultivar 'Santa Maria', and immature fruits of 'Dr Jules Guyot' were collected from an uninfected pear orchard at the Institut Technique de l'Arboriculture Fruitière et de la Vigne (ITAFV), Blida region, Algeria. The leaves and immature fruits, chosen for their healthy appearance and similar sizes, were stored in a refrigerator at 4 °C in airtight bags before the experiments.

Inoculation and disease control assessments on detached leaves and immature fruits

The immature fruits and detached leaves were washed with tap water, rinsed abundantly with SDW, then surface sterilized by immersion in 70% ethanol solution for 1 to 2 min, then rinsed with SDW and dried on previously sterilized absorbent paper. A 24 h bacterial culture of isolate CFBP 8377/EADZ8 was used to prepare the bacterial suspensions as described above. A loopful from the bacterial culture was transferred to a bacteriological tube containing 4.5 mL of SDW. After shaking, the bacterial suspension was adjusted to approx. CFU mL^{-1}. One wound (0.5 to 1 cm length, 0.3 cm depth) was made on each sterilized immature fruit using a sterilized scalpel. Bacterial suspension (50 µL) was then applied to each wound. As negative controls, pear fruits were each inoculated with 50 µL of SDW. Fruits were then placed in plastic boxes containing sterile Whatman paper, were sprayed each day with SDW and incubated at 25 to 26 °C. The experiment was repeated seven times. Severity of disease was assessed as indicated by extent of fruit necroses and production of exudates, using the disease index scale of Klee *et al.* (2019), with modification, where: **0** = no signs or symptoms; **1** = formation of necroses around lesions without production of exudates; **2** = less than 50% of necrosis on fruit surface with exudate production; **3** = 50% to 90% of necrosis on fruit surface with exudate production; and **4** = >90% of necrosis on fruit and exudate production.

For the inoculation of detached leaves, 50 µL of bacterial suspension was used either sprayed onto the surface of each leaf an airbrush or introduced into an incision made along the main leaf vein. As negative controls, detached leaves were inoculated with SDW using the same procedure. Each petiole of a detached leaf was introduced into 1% water agar inside glass tubes. These tubes were then incubated at 28 °C and symptoms were recorded after 2, 4, 6 and 8 days. Disease severity was estimated using a modified scale of Ruz *et al.* (2008). Progression of necrosis development was determined using the following scale, where: **0** = no necrosis; **1** = necrosis limited to the inoculation point; **2** = necrosis advancing into the leaf midrib; **3** = necrosis reaching the lateral veins; and **4** = necrosis over the whole leaf, reaching the ends of lateral veins.

Preventive and curative effects on immature fruits

Three concentrations (3, 5, or 10% w/v) of ethanolic pomegranate extract (P1) were selected to assess their *in vivo* efficacies against fire blight, and to determine if possible antibacterial activity depended on extract concentration. As the crude pomegranate extract was soluble in ethanol, so ethanol was used as the initial solvent to prepare homogeneous stock solutions. To obtain the final working solutions for *in vivo* applications, each ethanol-based stock was diluted using 1% DMSO in sterile distilled water (v/v).

Immature pomegranate fruits were prepared and wounded, as described above. Fifty µL of each concentration of plant extract was applied to each lesion. After 24 h, in a second step, 50 µL of bacterial suspension 1×10^8 CFU mL^{-1} of isolate CFBP 8377/EADZ8 was introduced and applied to each lesion site previously inoculated with the plant extract as a preventive treatment. The same procedure was followed for the curative effect test, but this started with application of 50 µL of bacterial suspension to each lesion. After 24 h incubation, 50 µL of plant extract was applied to the same lesion. Sterile distilled water (SDW) and 1% (v/v) dimethyl sulfoxide (DMSO) served as negative experimental controls (T-) for uninoculated plants. Plants inoculated with the bacterium and treated with either SDW or DMSO served as positive controls (T+). The inoculated fruits were then placed in a glass box containing moistened paper to maintain humidity, and the boxes were incubated at 28 °C for 7 to 9 d. Disease severity was assessed as described for the pathogenicity test (above). For each extract concentration, the curative and preventive assays were repeated seven times.

Preventive and curative effect on detached leaves

For assessing preventive effects on detached leaves, the surfaces of all detached leaves were separately sprayed once with the plant extracts at concentrations of 3, 5, or 10% w/v, 1 d before inoculation with the *E. amylovora* isolate (CFBP 8377/EADZ8). A 50 µL bacterial suspension was used for each inoculation, as a foliar spray applied with an airbrush sprayer.

For assessing curative effects, 50 µL of bacterial suspension of isolate CFBP 8377/EADZ8 was inoculated into each leaf principal vein incision. On the following day, 1 mL of plant extract was applied was applied to each detached leaf, using an airbrush sprayer. Each petiole of a detached leaf was then introduced into 1% water agar inside a glass tube. Sterile distilled water (SDW) and 1% DMSO served as negative controls, while leaves inoculated with the bacterium and treated with either DMSO or SDW served as positive controls. The tubes were then incubated at 28 °C, and developing symptoms were recorded after 2, 4, 6 and 8 d. These operations

were repeated seven times for each concentration. Severity of disease was assessed as described above.

Fractionation and bioactivity-guided isolation of pomegranate peel extract

To isolate and identify the bioactive compounds responsible for antibacterial activity, a stepwise liquid-liquid extraction was carried out on the ethanolic pomegranate peel extract (P1), followed by antibacterial screening and chromatographic separation of the most active fraction. The ethanolic extract (P1) was placed in a separatory funnel and then subjected to a liquid-liquid separation using solvents with increasing polarity: chloroform, ethyl acetate, and n-butanol, using the method described by Cherfia *et al.* (2017). This approach exploited specificity and polarity of each organic solvent to selectively extract different classes of phytochemicals (Figure 2). The first extraction step involved chloroform, which extracts triterpenoid compounds. The procedure for this phase, was as follows: each solution was extracted (20 mL of pomegranate ethanolic extract with 500 mL of SDW), and the extraction solvent (100 mL of chloroform), were introduced into a funnel. After stirring, the mixture was left to settle for at least 20 min until two phases are obtained: an aqueous (upper) phase and an organic or chloroform (lower) phase. This procedure was repeated three times. The chloroform extract was then dried at 35 °C under vacuum using a rotary evaporator (DRAGON LAB RE100-Pro).

The aqueous phase obtained from the chloroform extraction was subsequently extracted with ethyl acetate, following the same protocol. The organic phase obtained was evaporated at 35 °C on a rotary evaporator (DRAGON LAB RE100-Pro), which allowed for extraction of monoglycosides and partial extraction of di- glycosides.

The aqueous phase from the ethyl acetate phase, and the organic phase, were then subjected to n-butanol extractions, again following the above procedure. The organic phase was evaporated at 60 °C using the rotary evaporator. This phase makes it possible to extract the remaining di- and tri-glycosides. The extraction was repeated three times, and the extracts of each phase were stored until used for the antibacterial activity tests.

The most active fraction (n-butanol) was further subjected to gel column chromatography to isolate individual bioactive compounds. A gradient of solvents with increasing polarity was used to elute compounds. Coloured bands were collected in test tubes and stored for further analyses (Table 3).

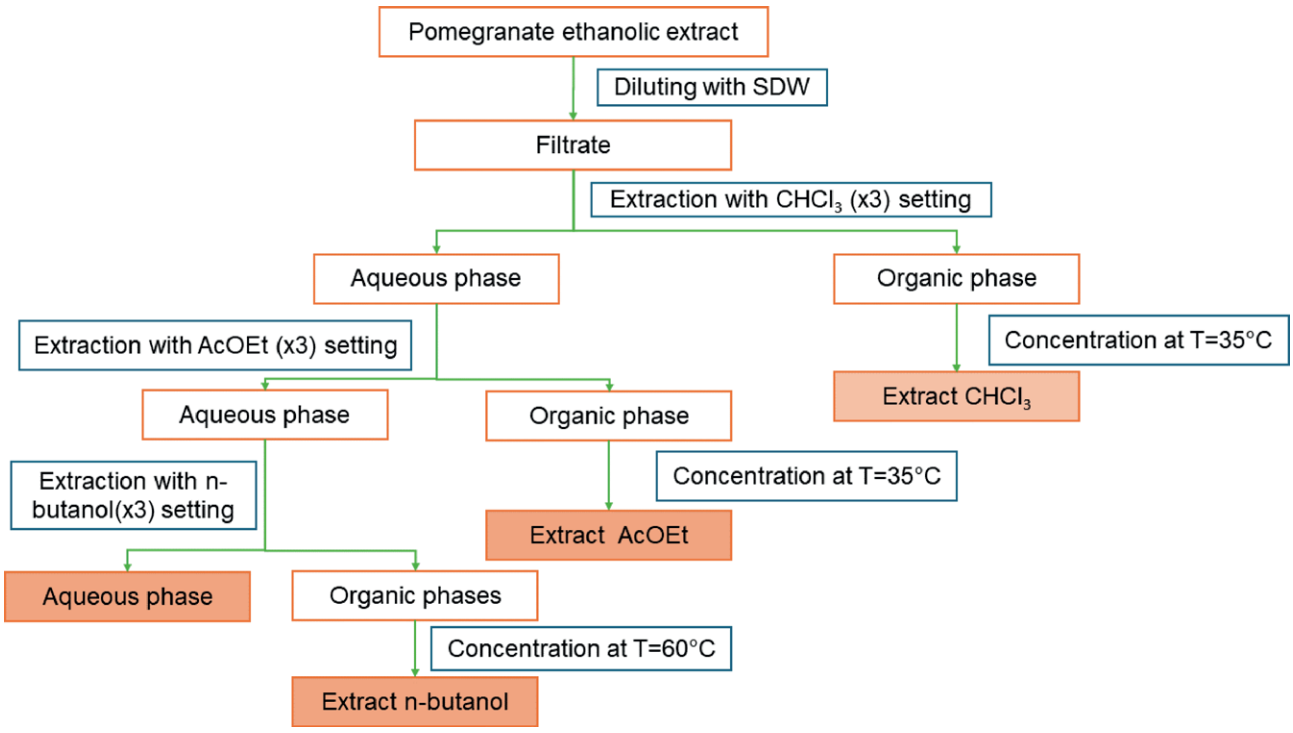

Figure 2. Schematic representation of the extraction process used to obtain potentially bioactive compounds from pomegranate plants, that were extracted from the plants using steriled distilled water (SDW), n-butanol, ethyl acetate (AcOEt) or chloroform ($CHCl_3$).

Table 3. Fractionation protocol used in the present study that applied different solvent mixtures in silica gel column chromatography for extracting potentially active compounds from plant tissues.

Solvent mixture	Fractions
Hexane	Fraction 01
Hexane-Ethyl Acetate (95:5; v/v)	Fraction 01
Hexane-Ethyl Acetate (90:10; v/v)	Fraction 02,03
Hexane-Ethyl Acetate (85:15; v/v)	Fraction 04,05,06
Hexane-Ethyl Acetate (80:20; v/v)	Fraction 07,08
Hexane-Ethyl Acetate (70:30; v/v)	Fraction 08,09,10
Hexane-Ethyl Acetate (60:40; v/v)	Fraction 10,11,12,13
Hexane-Ethyl Acetate (50:50; v/v)	Fraction 13
Hexane-Ethyl Acetate (40:60 ; v/v)	Fraction 13,14
Hexane-Ethyl Acetate (30:70; v/v)	Fraction 14,15
Ethyl Acetate	Fraction 15,16
Ethyl Acetate-Methanol (90:10; v/v)	Fraction 16,17
Ethyl Acetate-Methanol (75:25; v/v)	Fraction 17,18
Ethyl Acetate-Methanol (50:50; v/v)	Fraction 18,19,20
Methanol	Fraction 20
Methanol-Acetic Acid (75:25; v/v)	Fraction 21
SDW (Sterile Distilled Water)	Fraction 22

Assessment of preventive effects of pomegranate peel extract fractions on detached leaves

The well diffusion assay procedure (described above) was used to determine the ability of the liquid/liquid extracts and the obtained fractions of the pomegranate n-butanol extract (the most effective) to inhibit the growth of isolate CFBP 8377/EADZ8. Additionally, the preventive effects on immature fruits and detached leaves were assessed using the protocols described above. Four replicates for each phase (aqueous or organic) were used in the experiments.

^{1}H Nuclear Magnetic Resonance (NMR) characterization of pomegranate peel extract

3-(Trimethylsilyl)-2,2,3,3-tetradeutero-propionic acid sodium salt (TSP-d_4, CAS N. 24493-21-8, 99%D: Armar Chemicals), hydrochloric acid (HCl, 37%w, CAS N. 7647-01-0; ≥99.5%: Sigma-Aldrich), sodium oxalate (Na_2C_2O4, CAS N. 62-76-0; ≥99.5%: Sigma-Aldrich), sodium azide (NaN_3, CAS N. 26628-22-8; ≥99.5%: Sigma-Aldrich), and deuterium oxide (D_2O, CAS. N. 7789-20-0, 99.86% D: Eurisotop) were used for sample preparation. Whatman syringe membrane filters (PTFE, pore size 0.2 µm, diam. 47 mm) were provided by Sigma-Aldrich. NMR tubes (Wilmad WG-1000-7) were provided by ATS Life SciencesThe buffer was prepared according to the procedure of Musio *et al.* (2024).

For sample preparation, 3 mL of oxalate buffer solution (pH 4.2) was added to 300 mg of finely ground pomegranate. The mixture was then shaken for 2 min at 2500 rpm in a VORTEX shaker, and then sonicated at room temperature at 40 kHz for 5 min. The resulting mixture was centrifuged for 10 min at 4700 × g, and the supernatant was filtered off using a syringe filter (PTFE, porosity = 0.2 µm, diam. = 47 mm). A 540 µL aliquot of the filtrate (was then mixed with 60 µL of TSP/D_2O, and the resulting solution was transferred into an NMR tube.

For NMR measurement, a Bruker Avance 400 MHz spectrometer equipped with a 5 mm inverse probe and a BACS autosampler was used, to perform the 1D ^{1}H NOESY NMR experiment. This was implemented with a selective pre-saturation step to remove the residual water signal. The NMR experiment was conducted at 298.1 ± 0.1 K. The following acquisition parameters were optimized and adopted to record the 1D ^{1}H NOESY measurement: pulse program = noesygppr1d; size of fid (TD) = 128 K data points; spectral width (SW) = 20 ppm; transmitter offset = 4.70 ppm; dummy scans (ds) = 4; number of scans (ns) = 64; acquisition time = 8.12 s; mixing time (d8) = 0.01 s; recycle delay (d1) = 30 s. Before the analysis, calibration of the 90◦ pulse on the sample solution was performed.

Statistical analyses

Statistical analyses were carried out using R software (version 4.4.2) and RStudio (version 2025.05.0). For the *in vitro* data, differences in mean parameters were assessed using the non-parametric Kruskal-Wallis test followed by Dunn's test with Bonferroni correction (packages: FSA, rcompanion). For the *in vivo* data, one-way ANOVA followed by Tukey's HSD test determined mean differences between treatments (package: multcomp). The analysis of preventive effects of pomegranate peel extract fractions on detached leaves was conducted using an unpaired two- tailed T-test, along with the Kolmogorov-Smirnov test, comparing treated and untreated samples. All data was managed and visualized using the tidyverse suite, with ggh4x for enhanced plot details. All assessments were carried out in four replicates *in vitro* and seven replicates *in vivo*.

RESULTS

In vitro *antibacterial assays*

Results from the reverse agar plug assay are summarized in Figure 3. A low bar indicates greater antibacte-

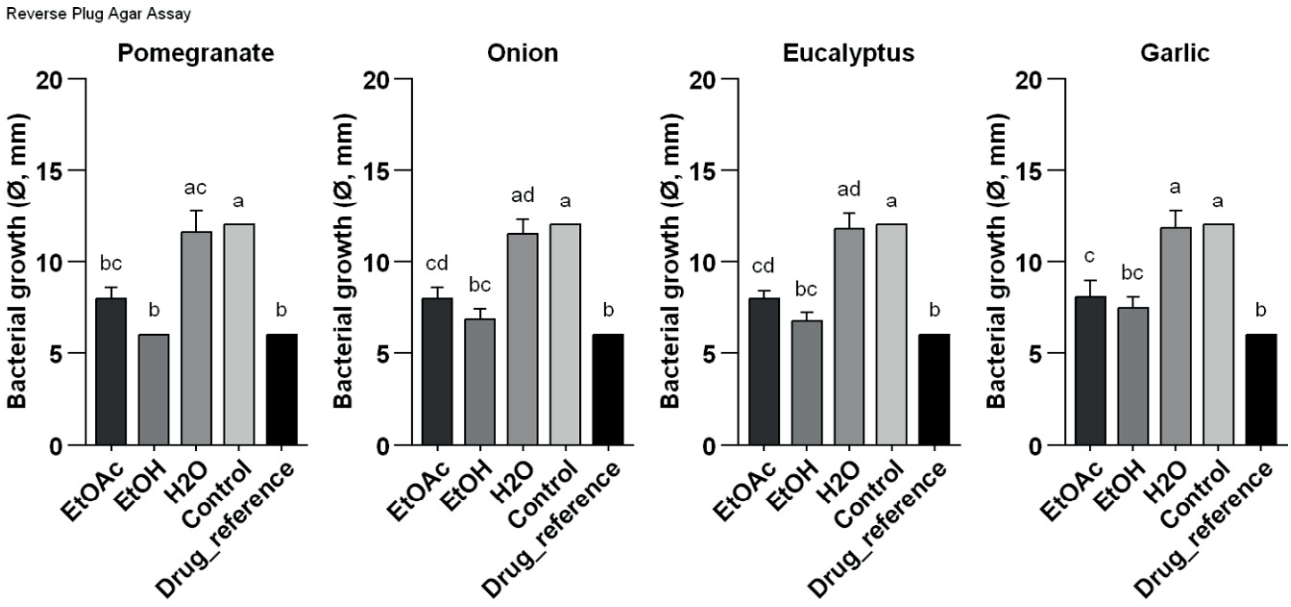

Figure 3. Mean *Erwinia amylovora* colony diameters (mm) resulting from treatments with organic (ethanol or ethyl acetate) or aqueous (water) extracts from pomegranate, onion, eucalyptus or garlic on the growth of CFBP 8377/EADZ8, LMG 2024 strains and ITAFAE 2022 isolate, using reverse agar plug assay. Error bars represent ± standard deviations), while mean accompanied by different letters are different at $P < 0.05$). "Control" values are from water (H_2O) controls and "drug reference" values are from streptomycin at 0.5 mg mL^{-1}.

rial activity against *E. amylovora*. All ethanolic (EtOH) and ethyl acetate (EtOAc) plant extracts reduced bacterial growth, compared to the experimental controls. Conversely the water plant extracts were not effective in reducing bacterial growth compared to the controls. The EtOH extract from pomegranate (P1) together with the streptomycin reference, were the most inhibitory, showing the least bacterial growth (6 mm), followed by the EtOAc extract (P2, 7–8 mm). Onion, eucalyptus and garlic EtOH extracts (O1, E1 and G1) gave the least bacterial growth compared with their corresponding EtOAc extracts. The water plant extract of all plants (P3, O3, G3 and E3) did not significantly reduce bacterial growth.

The well diffusion assays showed significant inhibition (halo diameters), for all the plant extracts indicating antibacterial activity ($P < 0.05$) as showed in Figure 4. The EtOH extract of pomegranate (P1) was the most inhibitory, producing the largest inhibition zones (approx. 20-21 mm) and similar to the pesticide reference, followed by the EtOAc extract (P2), while the water plant extract (H_2O) (P3) showed less inhibition. For onion and eucalyptus EtOH and EtOAc extract fractions had the largest inhibition halos, compared to the H_2O extracts which had less inhibition. The garlic extracts (EtOAc, EtOH, and H_2O) gave similar and moderate inhibition halos, generally approx. 12–13 mm in diameter.

The disk diffusion assays gave larger diameter inhibition zones than the well diffusion assays, indicating greater antibacterial activity. Only pomegranate and eucalyptus extracts, particularly their EtOH and EtOAc extracts, significantly inhibited the bacterial growth. The EtOH extract from pomegranate (P1) was the most inhibitory of the bacterium (zone diam. 16–17 mm), followed by the EtOAc extract (P2). The onion and garlic extracts all gave very limited to no inhibition halos. The water plant extract (H_2O) (P3, O3, G3, E3) did not give significant reductions in bacterial growth compared to the controls in this assay (Figure 5).

Pomegranate ethanolic extract effects on artificially inoculated detached pomegranate leaves and immature fruits

Efficacy of pomegranate extract (P1) for controlling *E. amylovora* infections was evaluated on detached pear leaves and immature fruits, from preventive and curative treatments (Figure 6 and Figure 7). Disease severity indices (DSI) were recorded and compared across different extract concentrations (3, 5, or 10%), alongside negative and positive experimental controls. All the extract solutions were prepared using 1% DMSO solutions as a co-solvent. At this concentration, DMSO had no significant antibacterial effect, so the observed bioactivity was attributable to the plant extract.

In the detached leaf assay under preventive treatment (Figure 7), application of pomegranate extract (P1)

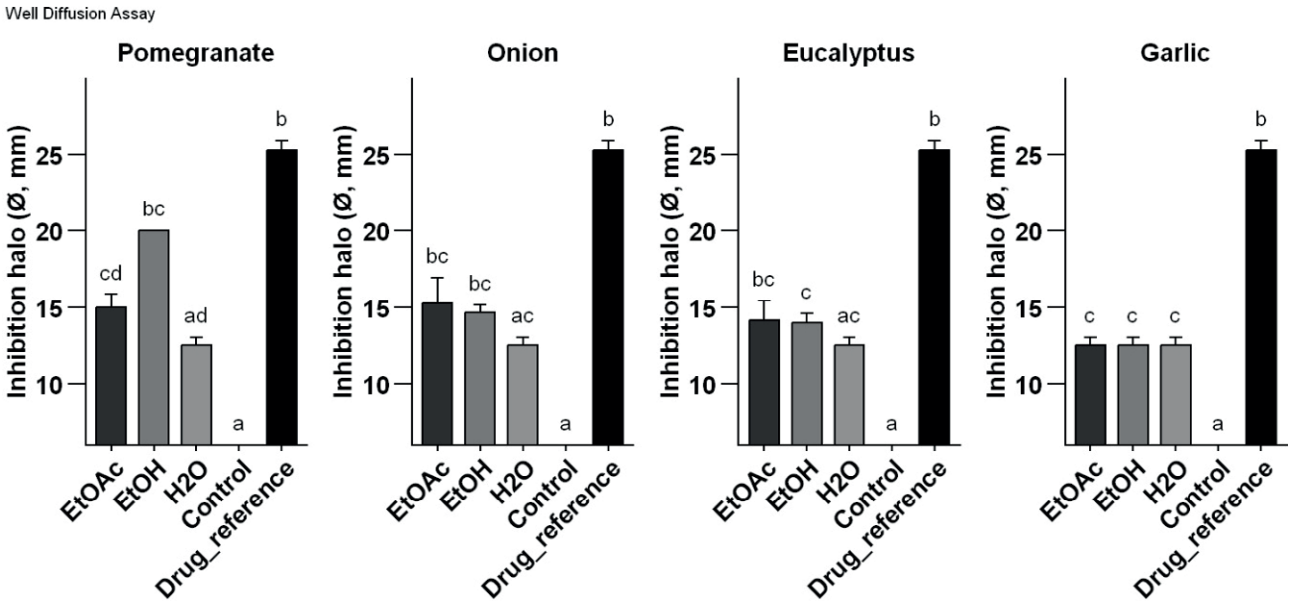

Figure 4. Antibacterial effect of organic (Ethanol and ethyl acetate) and aqueous (H_2O) extracts from four plants (pomegranate, onion, eucalyptus and garlic) on the growth of CFBP 8377/EADZ8, LMG 2024 strains and ITAFAE 2022 isolate, using well diffusion assay. Error bars represent ± SD (Standard Deviation), while values followed by different letters are significantly different. ($P < 0.05$). Control and drug reference represents the negative (SDW) and positive control (streptomycin 0.5 mg mL^{-1}) respectively.

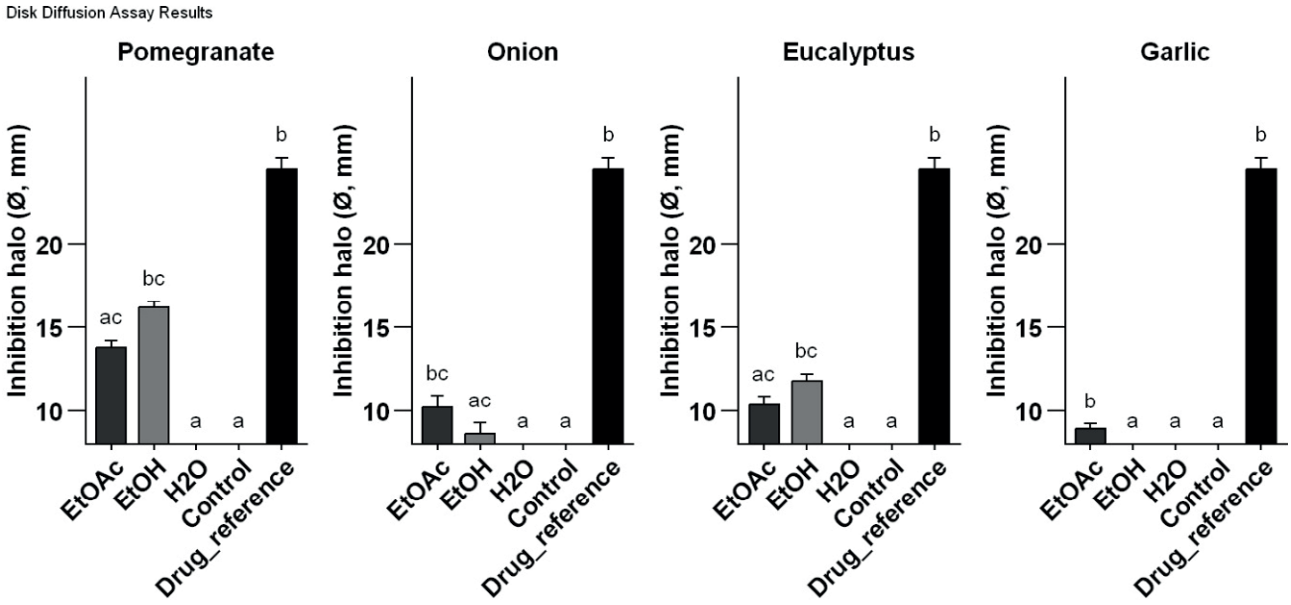

Figure 5. Antibacterial effect of organic (Ethanol and ethyl acetate) and aqueous (H_2O) extracts from four plants (pomegranate, onion, eucalyptus and garlic) on the growth of CFBP 8377/EADZ8, LMG 2024 strains and ITAFAE 2022 isolate, using disc diffusion assay. Error bars represent ± SD (Standard Deviation), while values followed by different letters are significantly different. ($P < 0.05$). Control and drug reference represents the negative (SDW) and positive control (streptomycin 0.5 mg mL^{-1}) respectively.

at 10% gave near-complete suppression of disease symptoms, with DSI values comparable to those observed for the negative control. Similarly, the 5% concentration gave low DSI values, that were not different ($P > 0.05$) from the 10% and negative control treatments (Figure 8B). In contrast, the untreated positive control gave sig-

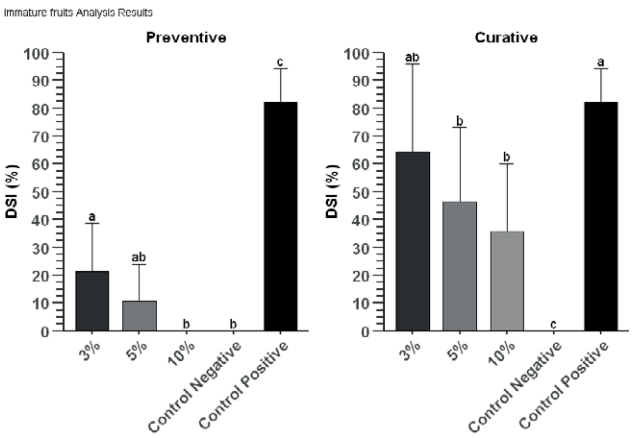

Figure 6. Mean fire blight severity indices on immature pear fruits treated with three ethanolic extract concentrations of pomegranate peel. A: disease incidence (DSI) after preventive treatments. B: DSI after curative treatments. Error bars indicate ± standard deviations of means. Mean accompanied by different letters are significantly different. ($P < 0.05$).

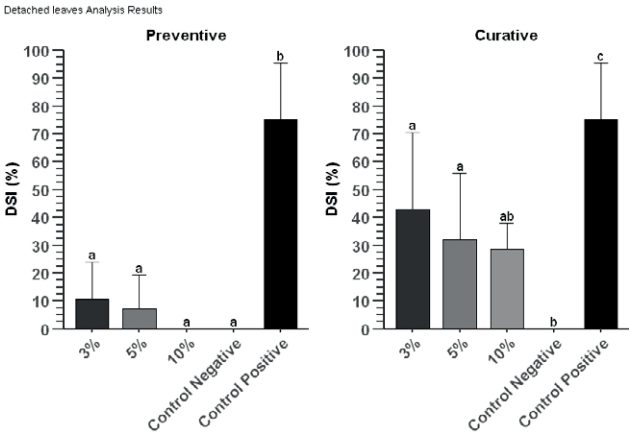

Figure 7. Mean fire blight severity indices on detached pear leaves treated with three ethanolic extract concentrations of pomegranate peel. A: disease incidence (DSI) after preventive treatments. B: DSI after curative treatments. Error bars indicate ± standard deviations of means. Mean accompanied by the same letter are not significantly different (values with the same letter have $P > 0.05$).

Figure 8. Effects of pomegranate ethanol extract (P1) on the incidence of fire blight of pear after the extract treatments were applied to immature fruits (A), or detached leaves (B).

nificantly greater DSI, confirming the effectiveness of the extract for preventing infections, particularly at high concentrations.

Under curative treatment conditions (Figure 7), effectiveness of the pomegranate extract against bacterial infections was somewhat reduced. Nevertheless, the 10% extract reduced disease severity, with DSI values significantly less than those of the positive control and similar to the negative control. Lower concentrations (3 and 5%) showed moderate efficacy, with DSI levels greater than from the 10% treatment but still less than those from the untreated controls (Figure 8B).

The results from the immature fruit assays mirrored the trends observed in leaves. From the preventive treatment (Figures 6 and 8A), the 10% pomegranate extract gave the greatest protective effects, maintaining DSI values close to zero and equivalent to negative controls. The 5% extract treatment gave intermediate efficacy, while the 3% treatment was less effective. The positive controls again gave significantly greater disease severity, indicating the susceptibility of untreated pomegranate fruits to *E. amylovora*.

From the curative treatments on fruits (Figures 6 and 8A), the 5 and 10% extracts gave the greatest reductions in disease severity. The 3% concentration also reduced the DSI compared to the untreated control, but the reduction was not significantly different ($P > 0.05$) from the positive control. The protective effect was clear-

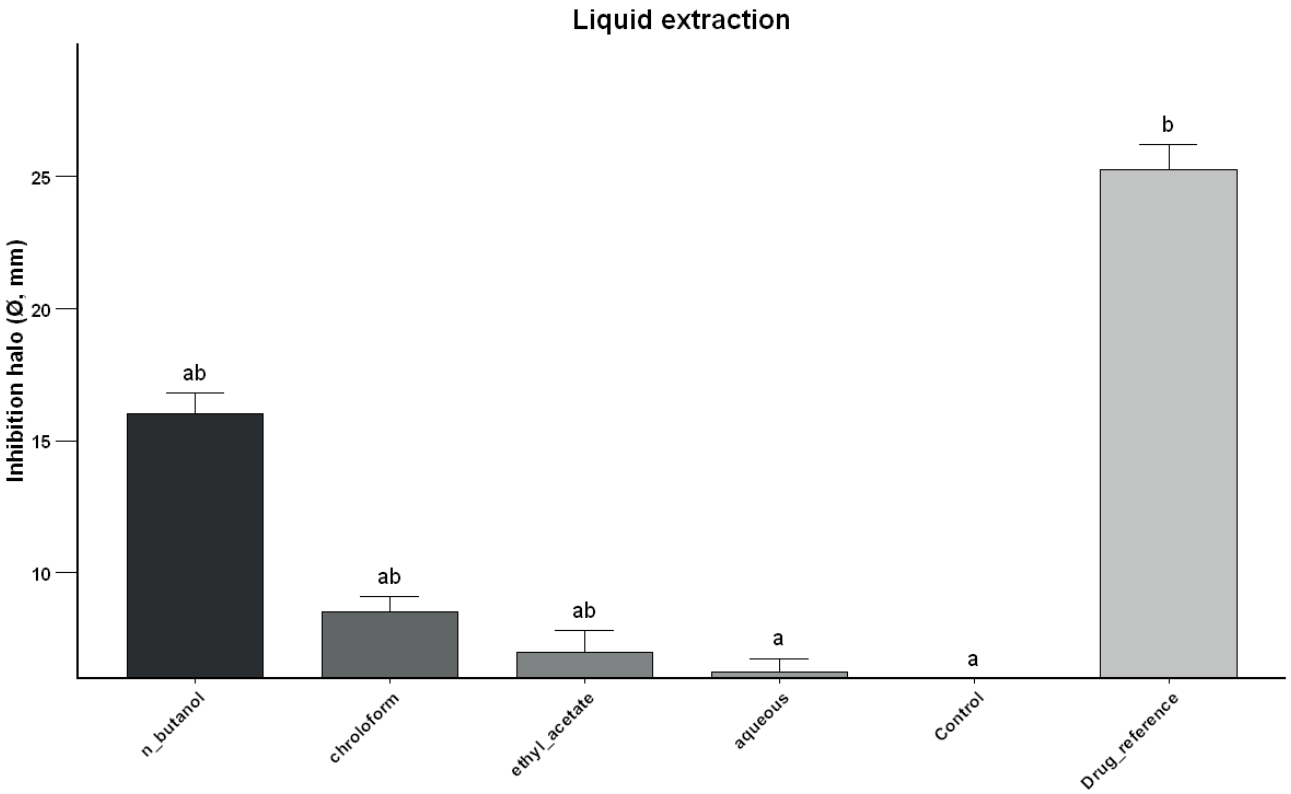

Figure 9. Mean diameters of inhibition halos of *Erwinia amylovora* CFBP 8377/EADZ8 in well diffusion assay, for extracts obtained using different solvents (n-butanol, chloroform, ethyl acetate, or aqueous fraction) applied to pomegranate peel. Error bars indicate standard deviations. Means accompanied by the same letter are not significantly different ($P > 0.05$). The control treatment was sterile distilled water, and the drug reference was streptomycin at 0.5 mg mL^{-1}.

ly more evident at the higher concentrations of pomegranate extract.

Antibacterial activities of solvent and chromatographic fractions

In this experiment, a stepwise liquid-liquid extraction was carried out on ethanolic extracts of pomegranate peel to isolate compounds with antibacterial activity. Figure 9 illustrates the effectiveness of each solvent for extracting antibacterial agents, based on resulting *E. amylovora* growth inhibition. Among the chloroform, ethyl acetate or n-butanol solvents tested, n-butanol extract was the most antibacterial, as demonstrated by the largest mean inhibition zone diameter (16 ± 0.40 mm). In contrast, the chloroform and ethyl acetate fractions produced smaller inhibition zones of (means = 8.50 ± 0.28 mm for chloroform and 7.0 ± 0.40 mm for ethyl acetate), indicating the presence of some bioactive compounds, though with lower potency or concentrations (Figure 9). To assess its protective effect *in vivo*, the n-butanol extract was tested as preventive treatment against fire blight on detached leaves and immature fruits. Figure 9 shows that the untreated group had high DSI severity on detached leaves and immature fruits, indicating that *E. amylovora* caused severe symptoms. In contrast, the treated group had low DSI values in detached leaves and immature fruits, confirming protection against the pathogen. These results indicate that application of n-butanol extract in advance protected the plant tissues, probably by inhibiting early stages of bacterial infection or colonization. Therefore, n-butanol extract could be an effective agent to prevent fire blight (Figure 10).

The most active n-butanol fraction was subjected to further separation via column chromatography. Figure 11 shows a range of antibacterial activities across the various eluted fractions. While most of the fractions had minimal effects, fractions F18, F19, F20 and F22 had strong antibacterial activity, with F22 showing the greatest inhibition (mean inhibition zone diameter = 21 ± 1 mm), comparable to those from the crude n-butanol extract (mean = 20 ±1 mm), and close to the positive

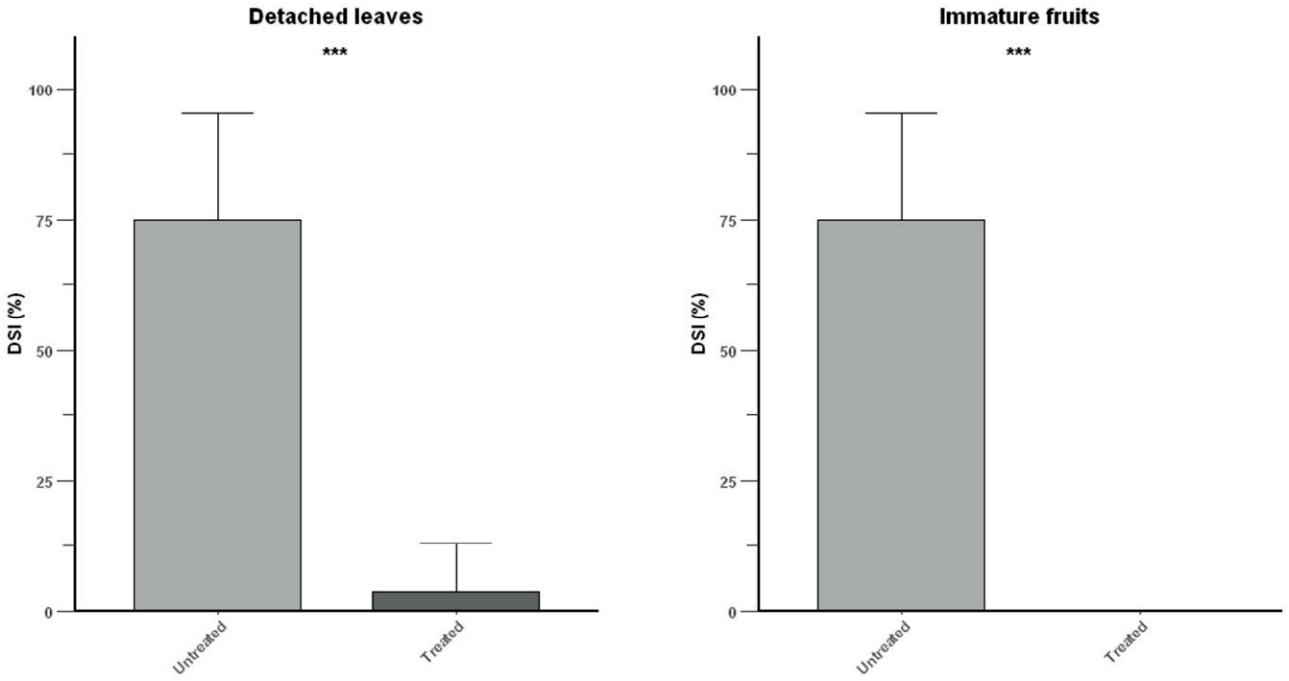

Figure 10. Preventive effect of n-butanol extract on fire blight severity on pear species. A: disease severity incidence (DSI) on detached leaves of pear. B: DSI on immature fruits of pear. Error bars indicate ± standard deviations of means. Asterisk means significantly different ($P < 0.001$).

controls (mean = 25 ± 1 mm). This indicates successful isolation and concentration of the most bioactive components within specific chromatographic fractions, which will be useful for further identification and characterization of the active compounds (Figure 12).

^{1}H Nuclear Magnetic Resonance characterization of pomegranate peel extract

The spectrum 1D ^{1}H NOESY of pomegranate extract (Figure 13) shows presence of several natural compounds including: leucine, alanine, malic acid, succinic acid, citric acid, glucose and fructose. In the aromatic region many signals were detected, including eight well-resolved signals between 6.69 ppm and 7.04 ppm ascribed to aromatic protons of α and β punicalagin, according with data reported by Kraszni *et al.* (2013).

DISCUSSION

This study has highlighted the significant antibacterial potential of selected bioactive agents, including plant-derived extracts from the Algerian plants *Punica granatum* (pomegranate peel), *Eucalyptus globulus* (eucalyptus leaves), *Allium cepa* (onion peel) and *Allium sativum* (garlic peel) against *Erwinia amylovora*, the causative agent of fire blight. This study was carried out to determine potential effectiveness of these plant extracts as components of sustainable fire blight management. The results indicated that organic solvent extracts, particularly ethanol (EtOH) and ethyl acetate (EtOAc), had stronger antibacterial effects than aqueous (H_2O) extracts, regardless of the assay method used. This was probably due to solvent/solute polarity interactions, because bioactive compounds such as phenolics, flavonoids, tannins and organosulfur compounds are moderately polar or slightly hydrophobic and are poorly extracted by water. Polar solvents (including water) are generally ineffective for solubilizing phenolic compounds and terpenoids, which are more readily extracted with mid-polar solvents such as ethanol and ethyl acetate (Tripathi *et al.,* 2025, França *et al.,* 2026). This emphasizes the need for appropriate extraction protocols when evaluating antimicrobial potential of plant materials. These results align with previous studies attributing the antimicrobial efficacy of pomegranate peel to its high content of polyphenolic compounds, such as gallotannins, ellagitannins, anthocyanins, and various phenolic acids (Peršurić *et al.,* 2020; Eghbali *et al.,* 2021; Pangallo *et al.,* 2021).

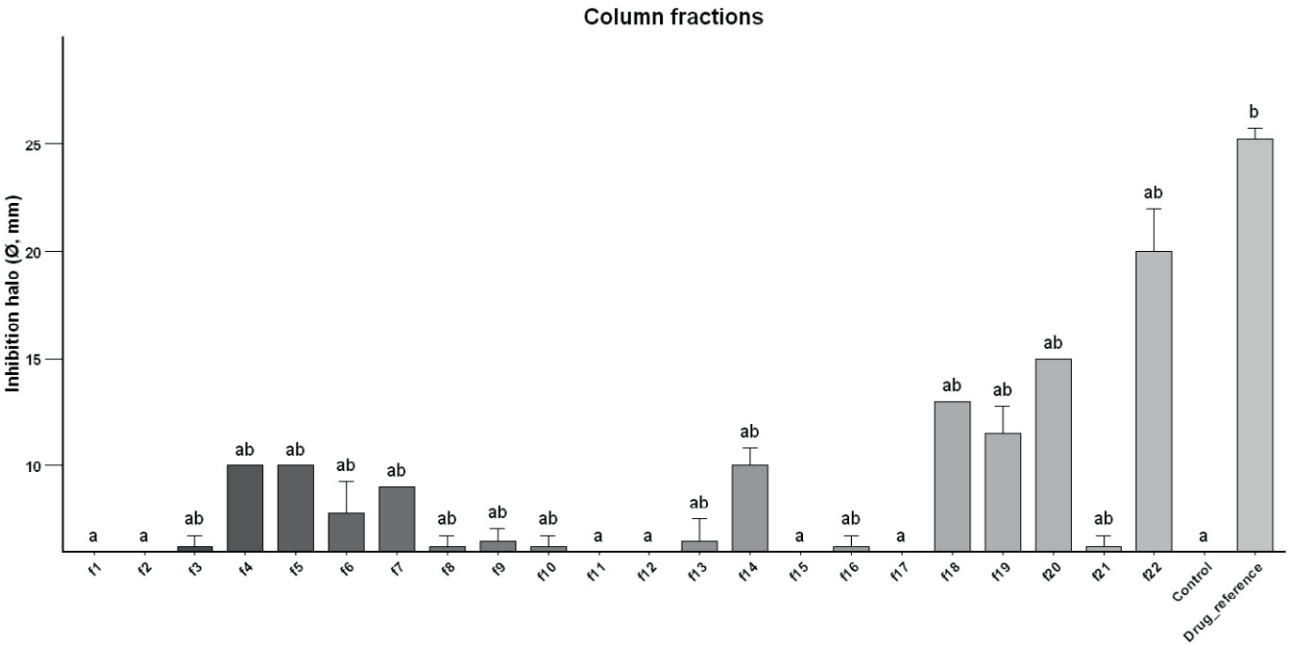

Figure 11. Mean diameters of inhibition halos of *Erwinia amylovora* CFBP 8377/EADZ8 in well diffusion assay, of 22 fractions of the pomegranate n-butanol extract on the growth *in-vitro*. Error bars indicate standard deviations. Means accompanied by the same letter are not significantly different ($P > 0.05$). The control treatment was sterile distilled water, and the drug reference was streptomycin at 0.5 mg mL^{-1}.

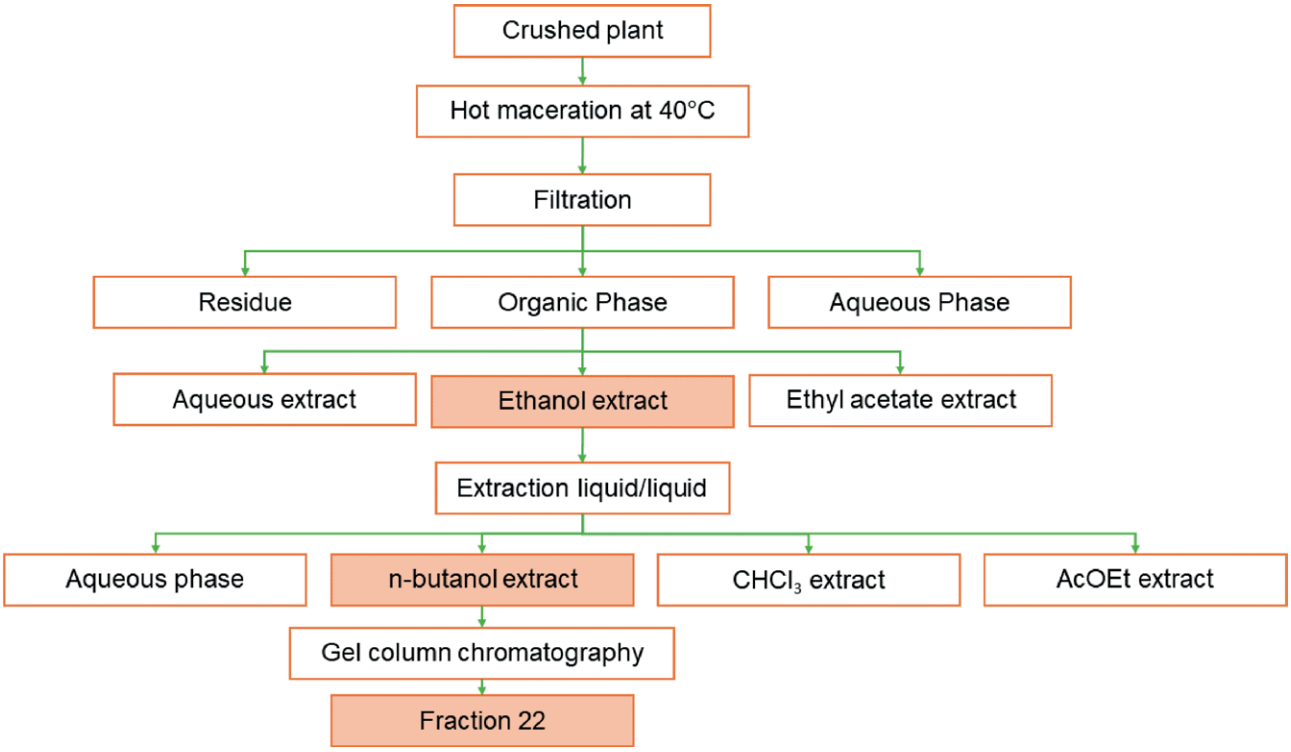

Figure 12. A schematic illustrating all the steps involved in the assessment of antibacterial activity of plant extracts against *Erwinia amylovora*.

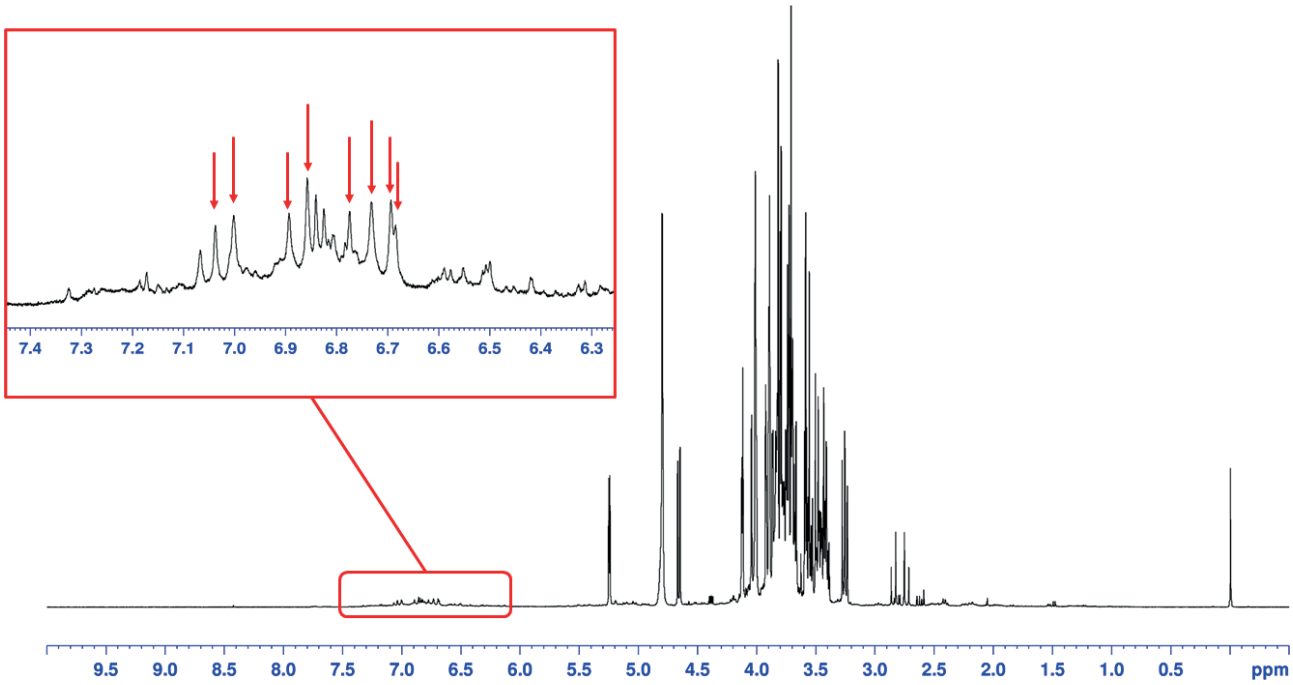

Figure 13. Spectrum 1D 1H-NOESY in H2O/D2O (90/10) at pH 4.2 for dry pomegranate peel. The red arrows indicate signals ascribed to aromatic protons of α and β punicalagin.

Among the tested extracts, the ethanol extract of pomegranate peel was the most effective against *E. amylovora*, showing potent *in vitro* and *in vivo* antibacterial activity. In the well diffusion assay, all extracts from pomegranate, including the aqueous extract, produced measurable inhibition zones, though the ethanol extract was the most inhibitory. This indicates that under certain conditions, water-extractable compounds may exert mild activities, potentially due to diffusion-related effects or synergistic interactions not detectable in other assay formats (reverse agar plug or disc diffusion) (Wang *et al.,* 2011; Sajjad *et al.,* 2015; Khaleel *et al.,* 2016). *In vivo* tests further confirmed the high potential of the pomegranate EtOH extract. Preventive treatment with a 10% extract concentration (ethanol-based stock extract in 1% DMSO solution) on detached pear leaves and immature fruits reduced the DSI, approaching values observed in the negative experimental controls. This indicates prophylactic potential for pomegranate peel extract, likely due to early inhibition of bacterial colonization. Conversely, curative applications of the extract were less effective, highlighting a limited ability to control established infections, as has been reported with other plant-based antimicrobials (Doolotkeldieva & Bobusheva, 2016; Fontana *et al.,* 2022).

Eucalyptus leaf extracts also demonstrated moderate antibacterial activity against *E. amylovora*, particularly the EtOH and EtOAc fractions. This is likely attributable to their contents of essential oils and polyphenols, including eucalyptol and other antimicrobial terpenoids (Bastas, 2020; Hernández-Escamilla *et al.,* 2021; Pinto *et al.,* 2023). However, the inhibition levels were consistently lower than those observed for pomegranate extracts, indicating either lower concentrations or reduced efficacy of active compounds. Onion and garlic extracts, although traditionally recognized for their antimicrobial properties, exhibited limited effectiveness in the present study. Minor inhibition observed in the well diffusion assay may have been due to volatile components or residual solvent effects. These results suggest that the active compounds could be present in insufficient concentrations in non-edible plant parts, or may require alternative extraction strategies for activity against *E. amylovora* (Bastas, 2020).

Bioactivity-guided fractionation of the pomegranate EtOH extract, using liquid-liquid partitioning and column chromatography, gave identification of particularly active fractions, especially the n-butanol phase and chromatographic fractions F18, F19, F20, and F22. These results indicate potential enrichment of potent compounds, possibly including di- and tri-glycosides or high-molecular-weight polyphenols (Karimi *et al.,* 2020). These results open a promising avenue for the isolation and structural elucidation of novel antibacterial agents.

α- and β-punicalagin are two essential ellagitannin compounds found in pomegranate peel extracts (PPEs), that contribute strong inhibitory effects of pomegranate extracts in crop protection. Their antimicrobial effects are due to several mechanisms, including disruption of microbial membranes, inhibition of essential microbial enzymes, metal ion chelation, and interference with quorum-sensing and biofilm formation (Salim *et al.,* 2025). The inhibitory impacts against *E. amylovora* are boosted by the synergistic action of these isomers, either with each other or with other polyphenols such as ellagic acid and flavonoids (Rongai *et al*., 2019; Gosset-Erard *et al.,* 2021; Salim *et al.,* 2025). Although punicalagin is polar and water-soluble, the ethanolic extract exhibited greater antibacterial activity than the aqueous extract. This may be due to the greater chemical stability of phenolic metabolites in ethanol, while aqueous conditions can promote rapid degradation of bioactive compounds. Punicalagin can also undergo spontaneous hydrolysis to form ellagic acid, which may further conjugate or polymerize into complex ellagitannins, potentially affecting its bioactivity (Larrosa *et al.,* 2006). Future studies will include direct NMR and structural analyses of the bioactive *n*-butanol fraction and its most active fractions (F18, F19, F20, and F22), to confirm the molecular determinants of their antibacterial activity.

In summary, the results from the present study demonstrate the strong antibacterial activity of pomegranate peel extracts, particularly in ethanol-based preparations. The strong preventive effects observed, combined with identification of the active fractions, indicate potential for development of pomegranate-derived products as effective, natural biocontrol agents against *E. amylovora*. The present study also reinforces the importance of extraction method optimization, and highlights the potential of plant wastes within sustainable agricultural practices. Future research will focus on the structural characterization of the bioactive *n*-butanol fraction and its most active sub-fractions, to firmly establish the chemical basis of the observed antibacterial activity.

CONCLUSIONS

Development of sustainable, effective, eco-friendly compounds for containing and counteracting development of pathogens is an important goal in phytopathological research, aiming to identify natural molecules or compounds that are effective for plant disease management. There are several reported research activities on extracts and/or compounds of plant origins that are effective in counteracting pathogens and pests that have subsequently been used in integrated pest management programs. The present study is the first comprehensive comparative screening of Algerian plant waste against locally isolated and reference *E. amylovora* isolates, and has shown that ethanol extracts from pomegranate peels have potential as antibacterial agents against *E. amylovora*, the causal agent of fire blight. Among the plant-based extracts tested, including those from eucalyptus, onion, and garlic, the pomegranate extract had greatest efficacy, particularly when applied preventively at 10% concentration. Results from *in vitro* assays and *in vivo* experiments emphasize the potential for Algerian plant waste as sustainable resources for plant disease management. The observed antibacterial effects are probably due to presence of phenolic compounds, especially hydrolysable tannins, which are abundant in pomegranate peel. The bioactive compound recovery process involved biological and pathogenicity testing, bioactivity-guided fractionation, and gel column chromatography, allowing for the isolation and evaluation of the most potent fractions. Further identification and structural characterization of the active compounds are necessary to elucidate mechanisms of action. Future studies should also address the stability, phytotoxicity, and formulation potential of these extracts for uses under field conditions. Increased understanding of possible synergistic or antagonistic interactions among extract components could inform development of effective combination treatments or standardization strategies. Considering the limited curative options currently available for fire blight, these approaches are promising and potentially sustainable alternatives for integrated pest management and to advance circular economy principles.

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