



## Article

# Antibacterial Activity of *Ginkgo biloba* Extracts against *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas* spp., and *Xanthomonas vesicatoria*

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**Abstract:** Phytopathogenic bacteria represent a risk to global food production by impacting a variety of crops. The aim of this study was to characterize the contents of bioactive constituents in extracts from *Ginkgo biloba* L. leaves and fruits and test their activity against six phytopathogenic bacteria that affect horticultural crops. Gas chromatography–mass spectrometry (GC–MS) was used for the chemical profiling of the aqueous methanol extracts, and their bacteriostatic activity against *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas cichorii*, *Pseudomonas syringae* pv. *pisi*, *Pseudomonas syringae* pv. *syringae*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas vesicatoria* (formerly *Xanthomonas campestris* pv. *vesicatoria*) was determined in vitro through the agar dilution method. The protective effect of the leaf extract was tested in vivo against the most relevant bacterial pathogens based on their economic/scientific importance, i.e., *C. michiganensis* subsp. *michiganensis* and *P. syringae* pv. *pisi*, in tomato (*Solanum lycopersicum* L.) and pea (*Pisum sativum* L.) plants, respectively, under greenhouse conditions. The GC–MS characterization of *G. biloba* extracts revealed the presence of dihydro-4-hydroxy-2(3H)-furanone, 2,4-dimethyl-3-hexanol, catechol, 3-O-methyl-D-fructose, 4,6-di-O-methyl- $\alpha$ -D-galactose, methyl 2-O-methyl- $\alpha$ -D-xylofuranoside, and 3-methylmannoside. In vitro growth inhibition tests showed that, while the fruit extract had no activity, the leaf extract exhibited minimum inhibitory concentrations between 500 and 1000  $\mu\text{g mL}^{-1}$ , which may be attributed to the presence of 2,4-dimethyl-3-hexanol and catechol. In vivo tests of the leaf extract demonstrated full protection in tomato and pea plants at 1000 and 1500  $\mu\text{g mL}^{-1}$ , respectively. The results indicate that *G. biloba* leaves may be employed as a biorational source for integrated pest management in horticulture.

**Keywords:** bacterial canker; bacterial blight; bacterial speck; bacterial leaf spot; GC-MS; ginkgo; tomato; pea



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## 1. Introduction

*Ginkgo biloba* L. is a tree species belonging to the family *Ginkgoaceae* and the only living species of the Ginkgoales order. *Ginkgo biloba* trees are disease- and insect-resistant, making them exceptionally long-lived, with some specimens reportedly existing for over 2500 years. They generally reach a height of 20 to 35 m, possess an angular crown, and typically feature deep roots. The leaves are petiolated, with a fan-shaped blade and dichotomous and open venation. It is a dioecious species, where female trees produce fruit each year, while male trees do not (Figure 1). The fruits emit an unpleasant, musty odor due to the presence of butyric acid in the yellowish, fleshy part (sarcotesta), which is not consumed as it can

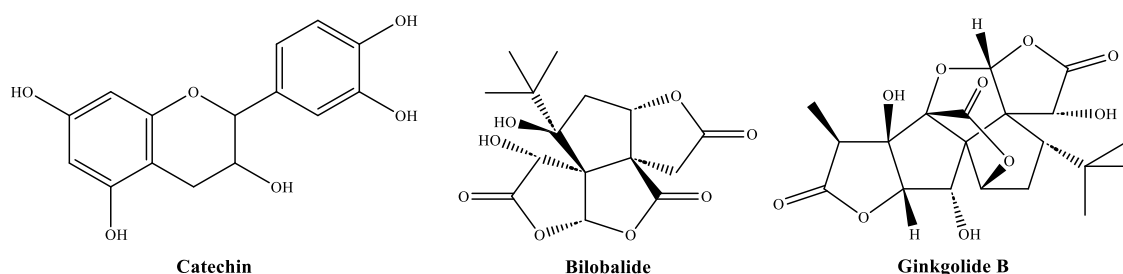
be toxic; rather, the inner, softer portion of the seed (sclerotesta)—which has a greenish color—is eaten.



**Figure 1.** (left) *Ginkgo biloba* tree bearing ripe, fruit-like sarcotestae; (center) close-up of leaves and sarcotestae; (right) fleshy seedcoat (sarcotesta) and seed (sclerotesta).

*Ginkgo biloba* is a widely used plant remedy throughout the world [1]. Its potential benefits for cardiovascular and neurodegenerative diseases have been substantiated [2,3]. Furthermore, the ginkgo nut has demonstrated potential in the prevention of neurological and cardiovascular diseases, though research in this area is limited [4].

Back in 1992, Gülz et al. initially documented the chemical composition of the waxes of *G. biloba* leaves [5], but it was not until 2008 that Lin et al. provided a more extensive report on the phytoconstituents of the aqueous methanol extract of the leaves, including forty-five glycosylated flavones and flavonols (predominantly hydroxyflavones), ten biflavones, three flavonol aglycones (myricetin, quercetin, and kaempferol), catechin, dihydroxybenzoic acid, and terpene lactones (ginkgolides and bilobalide, Figure 2) [6].



**Figure 2.** Specific components of *Ginkgo biloba* leaf extracts reported by Lin et al. [6].

Regarding standardized *G. biloba* extract EGb 761 (a leaf extract frequently utilized as a dietary supplement), it is typically composed of flavone glycosides (24%), terpenes (6%), and other substances, such as proanthocyanidins and organic acids. Ginkgolides and bilobalide account for approximately 3.1% and 2.9% of the extract, respectively [7]. It has been suggested that some of the beneficial effects of EGb may be due to antioxidant activities, with an increasing body of evidence pointing to lipid peroxidation reactions and free radicals' participation in neuronal damage and central and peripheral vascular diseases [8,9].

*Ginkgo biloba* has been reported to possess antifungal activity by Krauze-Baranowska and Wiwart [10] and Oh et al. [11], and antibacterial activity against *Staphylococcus aureus* Rosenbach, *Bacillus thuringiensis* Berliner, *Clostridium perfringens* (Veillon and Zuber, 1898) Hauduroy et al., 1937, *Listeria monocytogenes* (Murray et al., 1926) Pirie, 1940, and *Haemophilus influenzae* (Lehmann and Neumann, 1896) Winslow et al., 1917 [12]. However, to the best of our knowledge, no prior studies have been conducted to assess the antibacterial activity of this plant species on phytopathogenic taxa.

Given the significant global economic losses caused by bacterial diseases of plants—estimated at over one billion dollars worldwide annually [13]—there is an interest in investigating biorationals based on natural products for the integrated pest management of phytopathogenic bacteria that affect horticultural crops. For instance, these products could be used to control Gram-negative bacteria such as *Pseudomonas syringae* van Hall pathovars (e.g., *Pseudomonas syringae* pv. *lisi* (Sackett 1916) Young et al. 1978 Dye & Wilkie; *Pseudomonas syringae* pv. *syringae* van Hall 1902; *Pseudomonas syringae* pv. *tomato* (Okabe 1933) Young et al. 1978), *Pseudomonas cichorii* (Swingle 1925) Stapp, 1928; and *Xanthomonas vesicatoria* (Doidge) Vauterin et al., as well as Gram-positive bacteria such as *Clavibacter michiganensis* subsp. *michiganensis* corrig. (Smith 1918) Davis et al. 1984. Among these, *P. syringae* [14] and *P. cichorii* [15] can infect a wide range of plant species, while *X. vesicatoria* (formerly *X. campestris* pv. *vesicatoria*) is the causal agent of bacterial leaf spot on tomatoes (*Solanum lycopersicum* L.) and peppers (*Capsicum annuum* L.) [16] and *C. michiganensis* subsp. *michiganensis* causes bacterial canker of tomatoes, and can also affect other solanaceous plants [17]. It should be noted that *P. syringae* pathovars and *C. michiganensis* subsp. *michiganensis* are considered among the more relevant bacterial pathogens based on their scientific/economic importance [18].

The purpose of this study was twofold: (i) to characterize the phytoconstituents of *Ginkgo biloba* L. leaf and fruit extracts and (ii) to investigate their activity against the aforementioned six phytopathogenic bacteria that affect horticultural crops.

## 2. Materials and Methods

### 2.1. Plant Materials and Reagents

*Ginkgo biloba* leaf and fruit samples were collected from 20 female trees—grown under the same environmental conditions, soil type, and agronomic factors—along the Avenida de los Monegros in Huesca City, Aragon, northeastern Spain (42°7′46.9″ N 0°24′6.3″ W) in the late days of September 2021. For drying the samples, the freeze-drying method was chosen, in agreement with Boateng and Yang [19]: samples were initially frozen at −20 °C for 24 h and then freeze-dried at −50 °C.

Eight-week-old tomato (*Solanum lycopersicum*) cv. “Optima F1” plants and fourteen-week-old pea (*Pisum sativum*) cv. “Lincoln” plants used in the in vivo experiments were supplied by Agrodepa S.L. (Palencia, Spain).

Catechol (CAS no. 120-80-9) was acquired from Thermo Fisher Scientific (Madrid, Spain). Additionally, 2,4-dimethyl-3-hexanol (CAS no. 13432-25-2), tryptic soy broth (TSB, no. CAS 8013-01-2), and tryptic soy agar (TSA, CAS no. 91079-40-2) were procured from Sigma–Aldrich Química (Madrid, Spain).

### 2.2. Bacterial Strains

*Pseudomonas cichorii* (strain CITA Pci-5), *P. syringae* pv. *lisi* (strain CITA Pspi-12), *P. syringae* pv. *syringae* (strain CITA Psy-9), *P. syringae* pv. *tomato* (strain CITA Pst-7), *X. vesicatoria* (strain CITA Xv-5), and *C. michiganensis* subsp. *michiganensis* (strain CITA Cmm-30) were supplied by the Bacteriology Lab at the Agrifood Research and Technology Center of Aragon (CITA, Zaragoza, Spain) as TSA subcultures.

### 2.3. Extract Preparation

The preparation of the leaf, sarcotesta, and sclerotesta extracts was carried out according to the methodology described in [20]; the powdered sample was digested in a methanol/water (1:1, v/v) solution for 2 h, sonicated for 10 min in pulsed mode using a model UIP1000hdT probe-type ultrasonicator (20 kHz, 1000 W; Hielscher Ultrasonics, Teltow, Germany), and allowed to stand for 24 h. The solution was then centrifuged at 9000 rpm for 15 min, and the supernatant was filtered using Whatman No. 1 paper. Samples of the extracts were freeze-dried to obtain the solid residue. For gas chromatography–mass spectrometry (GC–MS) analyses, 25 mg of the freeze-dried extracts were dissolved in 5 mL of methanol (HPLC grade) to yield a 5 mg·mL<sup>−1</sup> solution, which was filtered before use.

#### 2.4. Extract Characterization

The extracts were analyzed using GC–MS at the Research Support Services at the University of Alicante (Alicante, Spain). The equipment utilized was a model 7890A gas chromatograph coupled to a model 5975C quadrupole mass spectrometer (Agilent Technologies; Santa Clara, CA, USA). The conditions for chromatography included 3 injections per vial, 1  $\mu\text{L}$  injection volume, an injector temperature of 280  $^{\circ}\text{C}$ , and an initial oven temperature of 60  $^{\circ}\text{C}$ . The temperature then increased at a rate of 10  $^{\circ}\text{C}\cdot\text{min}^{-1}$  to a final temperature of 300  $^{\circ}\text{C}$ , which was held for 15 min. The chromatographic column employed was an Agilent Technologies HP-5MS UI 30 m in length, 0.250 mm in diameter, and with a 0.25  $\mu\text{m}$  film. The temperatures of the electron impact source of the mass spectrometer and of the quadrupole were set at 230 and 150  $^{\circ}\text{C}$ , respectively; ionization energy was set at 70 eV. For calibration, test mixture 2 for apolar capillary columns according to Grob and PFTBA tuning standards were used. Component identification was based on a comparison of their mass spectra and retention time to those of authentic compounds, and by computer matching using the National Institute of Standards and Technology (NIST11) database.

The infrared spectrum of the dried powdered leaves was acquired using a Nicolet iS50 Fourier-transform infrared spectrometer (Thermo Scientific; Waltham, MA, USA) with a diamond attenuated total reflectance system. The spectra were collected in the 400–4000  $\text{cm}^{-1}$  range, with a 1  $\text{cm}^{-1}$  spectral resolution, by co-adding 64 scans.

#### 2.5. In Vitro Antibacterial Activity Assessment

The bacteriostatic activity was assessed according to the Clinical and Laboratory Standards Institute (CLSI) standard M07–11 [21], using the agar dilution method to determine the minimum inhibitory concentrations (MICs). Colonies of each species were incubated at 28  $^{\circ}\text{C}$  for 24 h in TSB. Serial dilutions of the cell suspension were then performed, beginning from a concentration of  $10^8$   $\text{CFU}\cdot\text{mL}^{-1}$ , to obtain a final inoculum of  $10^4$   $\text{CFU}\cdot\text{mL}^{-1}$ . The bacterial suspensions were then applied to the surface of the TSA plates to which the treatments had previously been added at concentrations between 62.5 and 1500  $\mu\text{g}\cdot\text{mL}^{-1}$ . The plates were incubated at 28  $^{\circ}\text{C}$  for 24 h, and readings were taken after 24 h. MICs were determined as the lowest concentrations of the bioactive products at which no bacterial growth was visible. All experiments were conducted in triplicate, with 3 plates per treatment/concentration. Statistical analyses of antibacterial activity data were conducted using IBM (Armonk, NY, USA) SPSS Statistics v.25.

For comparison, the MIC values of five antibiotics commonly used in clinical practice, viz. ampicillin (AM), benzylpenicillin or penicillin G (PG), ciprofloxacin (CI), gentamicin (GM), and tetracycline (TC), were determined using ETEST<sup>®</sup> strips.

#### 2.6. In Vivo Experiments on Tomato and Pea Plants under Greenhouse Conditions

Tomato plants cv. “Optima F1” were subjected to experiments in accordance with the guidelines in [22], with a few minor modifications. In a greenhouse cabin of biological containment level 2 (NCB2), tomato plants were grown in multi-pots (4  $\times$  4 cm per pot) containing sterilized peat as substrate, at  $20 \pm 2$   $^{\circ}\text{C}$  day/ $17 \pm 2$   $^{\circ}\text{C}$  night, with supplementary lighting to give a 12 h day. The last irrigation was carried out 48 h before inoculation, at which time the relative humidity was set at 100%. The *G. biloba* leaf extract was applied through spraying (3 mL per plant) at two concentrations (MIC and MIC  $\times$  2, i.e., 500 and 1000  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively, taking into consideration that some of the treatment may be lost due to evaporation, runoff, degradation, etc., thus necessitating a higher concentration to achieve the same effect as in vitro); control plants were sprayed with water. After two hours, an isolated colony of *C. michiganensis* subsp. *michiganensis* was inoculated to the node of the first true leaf with the stem, with the aid of a sterile entomological pin. The plants were then covered with polyethylene bags for five days, and the next irrigation was carried out 24 h after inoculation. Throughout the experiment, the plants were irrigated three times a week. For the pea assay, the experiments were carried out according to the guidelines in [23]. Pea plants were cultivated under the aforementioned greenhouse

conditions in  $4 \times 4$  cm pots that contained a mixture of sterilized peat and sand (2:1). The *G. biloba* leaf extract was sprayed at two concentrations (MIC and  $\text{MIC} \times 2$ , i.e., 750 and  $1500 \mu\text{g}\cdot\text{mL}^{-1}$ , respectively) onto treated plants, and control plants were sprayed with water. Two hours after treatment, an isolated colony of *P. syringae* pv. *pisi* was inoculated using a sterile entomological pin, by pricking the back of the stem at the two youngest nodes of the plant, at its junction with the stipula. Inoculation of the leaflets or stipules took place at the same time as that of the stems, with 4–8 pricks in the youngest leaflets and stipules. The irrigation regime was similar to that described above for tomato plants.

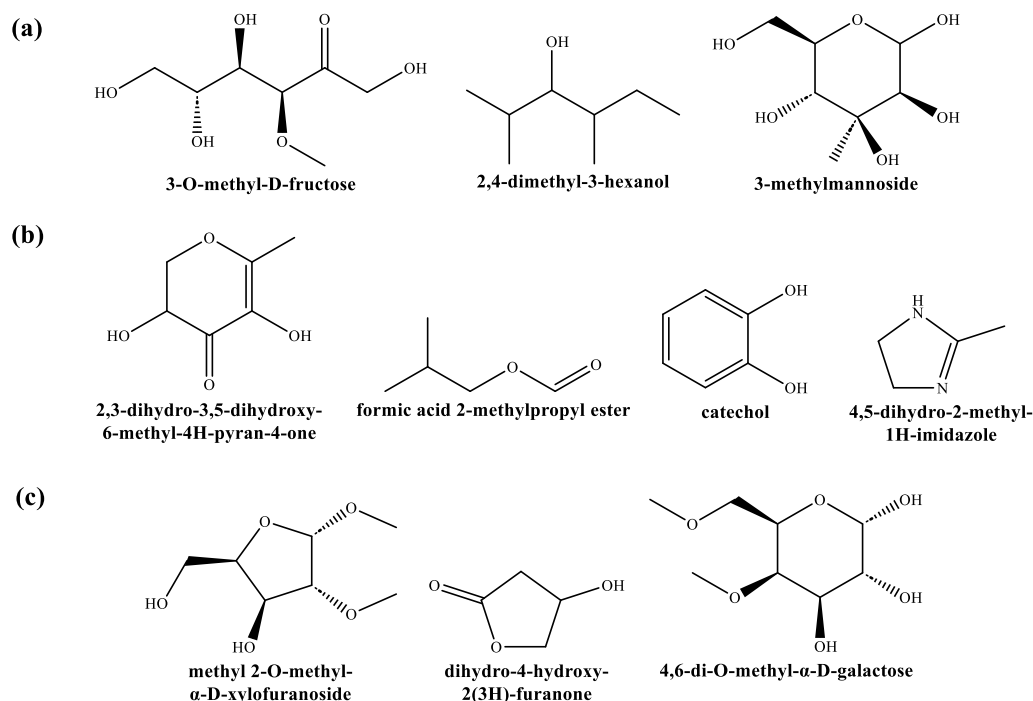
In both experiments, five plants per treatment (negative control, positive control, MIC, and  $\text{MIC} \times 2$ ) were used, with two independent replicates. In regard to the tomato plant trials, the first replicate began on 31 January 2023 and concluded on 10 February 2023; the second replicate began on 3 February 2023, ending on 13 February 2023. Regarding the pea plant trials, the first replicate started on 27 January 2023, concluding on 6 February 2023. The second replicate started on 30 January 2023, ending on 9 February 2023. Concerning the experimental layout, a completely randomized design was used, where each experimental unit (plant) was randomly assigned to one of the treatments.

Disease incidence was evaluated after ten days of inoculation, using the following formula:  $I (\%) = (\text{Number of plants with symptoms} / \text{Total number of plants assayed}) \times 100$ .

### 3. Results

#### 3.1. Extract Phytoconstituents Elucidation by GC–MS

In the GC–MS analysis of the leaf extract (Figure S1, Table S1, Figure 3a), the main identified compounds were: 3-*O*-methyl-*D*-fructose (18.1%); 2,4-dimethyl-3-hexanol (18.1%); acetic acid (16.2%); 3-methylmannoside (9.8%); acetic acid, 4-methylphenyl ester (4.4%); catechol (3.3%); 1-hydroxy-2-propanone (2.4%); and dihydro-4-hydroxy-2(3H)-furanone or  $\beta$ -hydroxy- $\gamma$ -butyrolactone (1.2%).



**Figure 3.** Chemical structures of several of the main constituents of (a) *G. biloba* leaf; (b) sarcotesta; and (c) sclerotesta extracts.

Regarding the sarcotesta extract chromatogram (Figure S2, Table S2, Figure 3b), the following phytoconstituents were detected: acetic acid (11.9%); 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one and related compounds (8.7%); formic acid 2-methylpropyl

ester and related compounds (5.4%); 1-hepten-4-ol (4.5%); catechol (4.1%); dihydroxyacetone (3.9%); 5-hydroxymethylfurfural (3.6%); 1-hydroxy-2-propanone (3.3%); 2-hydroxy-2-cyclopenten-1-one (3.1%); 4,6-di-O-methyl- $\alpha$ -D-galactose (1.6%); 4,5-dihydro-2-methyl-1H-imidazole (1.3%); 2,4-dihydroxy-2,5-dimethyl-3(2H)-furan-3-one (1.3%); 2,5-dimethyl-4-hydroxy-3(2H)-furanone (1.0%);  $\alpha$ -methyl mannofuranoside (1.3%); and azelaic acid (1.1%).

Lastly, GC–MS analysis of the sclerotesta extract (Figure S3, Table S3, Figure 3c) yielded the following main constituents: methyl 2-O-methyl- $\alpha$ -D-xylofuranoside (19.1%); dihydro-4-hydroxy-2(3H)-furanone (11.7%); 4,6-di-O-methyl- $\alpha$ -D-galactose (8.4%); catechol (7.9%); 3-methylmannoside (6.3%); 3-O-methyl-fructose (6.1%); tetrahydro-2-methylthiophene (3%); and 2-furanmethanol (2.5%).

### 3.2. Leaves Vibrational Spectra

*Ginkgo biloba* leaves showed absorption bands at 3309, 2915, 2849, 1731, 1712, 1630, 1605, 1515, 1465, 1371, 1243, 1166, 1104, 1020, and 832  $\text{cm}^{-1}$ . The intense band at 3309  $\text{cm}^{-1}$  was attributed to OH stretching, while the one at 2915  $\text{cm}^{-1}$  was associated with methylene ( $\text{CH}_2$ ) stretching, and 2849  $\text{cm}^{-1}$  with symmetric vibration of  $\text{CH}_2$  groups. The bands at 1731 and 1712  $\text{cm}^{-1}$  were likely due to C = O stretching in unconjugated ketones, esters (cutin), and aldehydes (hemicelluloses); the one at 1605  $\text{cm}^{-1}$ , to C = C aromatic skeletal vibrations (syringyl units); and the one at 1515  $\text{cm}^{-1}$ , to aromatic skeletal vibrations (guaiacyl rings). The band at 1020  $\text{cm}^{-1}$  can be ascribed to the C–O stretching of secondary alcohols (in cellulose and lignin), despite the absence of the cellulose bands at 1032  $\text{cm}^{-1}$  and 1050  $\text{cm}^{-1}$  (assigned by [24] to the C–O stretching of secondary and primary alcohols, respectively). Other band assignments were: 1630  $\text{cm}^{-1}$  to carbonyl groups of unsaturated acids; 1465  $\text{cm}^{-1}$  to  $\text{CH}_2$  in-plane bending; 1371  $\text{cm}^{-1}$  to  $\text{CH}_2$  wagging in lipids and  $\beta$ -1,3 glucans; 1243  $\text{cm}^{-1}$  to cutin and other polysaccharides; 1166 and 1104  $\text{cm}^{-1}$  to asymmetric and symmetric C–O–C in esters (cutin, cellulose, pectin); and 832  $\text{cm}^{-1}$  to C–C or C–N ring stretching or to C–H, O–H, and C = O deformation.

### 3.3. Antibacterial Activity of the Extracts

The results of the bacterial growth inhibition tests of the *G. biloba* extracts against *C. michiganensis*, *P. cichorii*, the three pathovars of *P. syringae*, and *X. vesicatoria* are summarized in Table 1. The hydromethanolic fruit (sarcotesta and sclerotesta) extracts were the least effective, with MIC values above 1500  $\mu\text{g}\cdot\text{mL}^{-1}$ . The leaf extract showed higher activity, with MIC values ranging from 500 to 1000  $\mu\text{g}\cdot\text{mL}^{-1}$ . Table 2 presents the results of the bacteriostatic activity tests for two of the extract phytoconstituents (2,4-dimethyl-3-hexanol and catechol).

**Table 1.** Minimum inhibitory concentrations (expressed in  $\mu\text{g}\cdot\text{mL}^{-1}$ ) of *G. biloba* extracts against the horticultural pathogens.

Bacteria	Leaf Extract	Sarcotesta Extract	Sclerotesta Extract
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	500	n.a.	n.a.
<i>Pseudomonas cichorii</i>	750	n.a.	n.a.
<i>Pseudomonas syringae</i> pv. <i>pisi</i>	750	n.a.	n.a.
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	750	n.a.	n.a.
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	750	n.a.	n.a.
<i>Xanthomonas vesicatoria</i>	1000	n.a.	n.a.

n.a.: no activity detected at the highest assayed dose.

For comparison purposes, the MIC values of the five antibiotics commonly used in clinical practice (namely, benzylpenicillin, ampicillin, gentamicin, ciprofloxacin, and tetracycline) are provided in Table 3.

**Table 2.** Minimum inhibitory concentrations (expressed in  $\mu\text{g}\cdot\text{mL}^{-1}$ ) of two *G. biloba* extract phytoconstituents against the horticultural pathogens.

Bacteria	2,4-Dimethyl-3-Hexanol	Catechol
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	375	187.5
<i>Pseudomonas cichorii</i>	750	500
<i>Pseudomonas syringae</i> pv. <i>pisi</i>	750	375
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	750	500
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	500	500
<i>Xanthomonas vesicatoria</i>	250	187.5

**Table 3.** Minimum inhibitory concentrations (expressed in  $\mu\text{g}\cdot\text{mL}^{-1}$ ) of conventional antibiotics (for clinical use) against the horticultural pathogens.

Bacteria	PG	AM	GM	CI	TC
<i>Clavibacter michiganensis</i> subsp. <i>michiganensis</i>	0.125	0.094	0.38	0.38	0.19
<i>Pseudomonas cichorii</i>	$\geq 32$	$\geq 256$	3	6	24
<i>Pseudomonas syringae</i> pv. <i>pisi</i>	$\geq 32$	48	1	0.25	1
<i>Pseudomonas syringae</i> pv. <i>syringae</i>	$\geq 32$	48	0.25	0.047	1
<i>Pseudomonas syringae</i> pv. <i>tomato</i>	$\geq 32$	16	0.125	0.047	0.75
<i>Xanthomonas vesicatoria</i>	$\geq 32$	$\geq 256$	0.75	0.125	1

PG: penicillin G; AM: ampicillin; GM: gentamicin; CI: ciprofloxacin; TC: tetracycline.

### 3.4. Tomato Plant Protection against *C. michiganensis* subsp. *michiganensis*

Given that *G. biloba* leaf extract was the most active in the in vitro tests, it was further tested as a protective treatment against bacterial canker in tomato plants cv. “Optima F1” (Figure 4). In the positive control (*C. michiganensis* subsp. *michiganensis* artificially inoculated on plants treated with bidistilled water only), all plants exhibited a typical vascular discoloration as brown streaks on the stem (Figure 4b). Plants treated with the extract at a concentration equal to the MIC ( $500 \mu\text{g}\cdot\text{mL}^{-1}$ ) showed an incidence of 80%, although the stem discoloration was less pronounced compared to the positive control. On the other hand, plants treated with the highest concentration (twice the MIC, i.e.,  $1000 \mu\text{g}\cdot\text{mL}^{-1}$ ) did not show symptoms of stem decay or phytotoxicity, appearing entirely similar to those of the negative control.

**Figure 4.** Symptoms of bacterial canker produced by *C. michiganensis* subsp. *michiganensis* in plants of tomato cv. “Optima F1” after 10 days of inoculation: (a) negative control; (b) positive control; (c) *G. biloba* leaf extract at  $500 \mu\text{g}\cdot\text{mL}^{-1}$ ; and (d) *G. biloba* leaf extract at  $1000 \mu\text{g}\cdot\text{mL}^{-1}$ .

### 3.5. Pea Plant Protection against *P. syringae* pv. *pisi*

Figure 5 shows the results obtained for pea plants cv. “Lincoln” treated with different concentrations of *G. biloba* leaf extract, particularly MIC and MIC  $\times$  2 (i.e., 750 and 1500  $\mu\text{g}\cdot\text{mL}^{-1}$ , respectively). The positive control showed a high susceptibility to bacterial blight, with an incidence of 100% of plants showing the characteristic brown and papery leaflet lesions. The plants treated with the extract at 750  $\mu\text{g}\cdot\text{mL}^{-1}$  exhibited a relatively lower susceptibility, with an incidence of 60% (i.e., 6 plants out of the 10 tested showed symptoms, albeit with less virulence than that of the positive control). In order to provide full protection, the dose had to be increased up to 1500  $\mu\text{g}\cdot\text{mL}^{-1}$ . As in the case of tomato plants, no phytotoxicity symptoms were observed, with no visual differences between the negative control and the MIC  $\times$  2 treated plants.



**Figure 5.** Symptoms of bacterial blight produced by *P. syringae* pv. *pisi* in plants of pea cv. “Lincoln” after 10 days of inoculation: (a) negative control; (b) positive control; (c) *G. biloba* leaf extract at 750  $\mu\text{g}\cdot\text{mL}^{-1}$ ; and (d) *G. biloba* leaf extract at 1500  $\mu\text{g}\cdot\text{mL}^{-1}$ .

## 4. Discussion

### 4.1. On the Identified Phytochemicals and Their Antimicrobial Activity

The main phytochemicals identified in *G. biloba* hydromethanolic extracts (Figure 3) were: dihydro-4-hydroxy-2(3H)-furanone (present in both leaf and sclerotesta extracts); 2,4-dimethyl-3-hexanol; catechol (present in the three extracts); 1-hydroxy-2-propanone; 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; acetic acid (identified both in leaf and sarcotesta extracts); formic acid 2-methylpropyl ester; 1-hepten-4-ol; 3-*O*-methyl-*D*-fructose (present in both leaf and sclerotesta extracts); 4,6-di-*O*-methyl- $\alpha$ -*D*-galactose; 2-*O*-methyl- $\alpha$ -*D*-xylofuranoside; and 3-methylmannoside (present in both leaf and sclerotesta extracts). Their occurrence in other extracts, together with available information on their antimicrobial activity, is summarized below.

Dihydro-4-hydroxy-2(3H)-furanone was previously identified in *Equisetum arvense* L. and *Urtica dioica* L. extracts [25]. The presence of this phytochemical in *G. biloba* hydromethanolic extracts may be the result of the hydrolysis/breaking of ginkgolide and bilobalide components (Figure 2) [26]. Dihydro-4-hydroxy-2(3H)-furanone is a major constituent in the extract of *Crocus sativus* L. (22% content), which exhibited antifungal activity against *Pyricularia oryzae* Cavara, *Cryptococcus neoformans* (Sanfelice) Vuill., *Trichophyton rubrum* (Castell.) Sabour., and *Aspergillus fumigatus* Fresen. [27]. 2,4-dimethyl-3-hexanol, identified in leaf extracts of *Silene uniflora* Roth and *Pentstemon microphylla* (Roxb.) Wight & Arn. [28], has been shown to possess considerable biocontrol activity against *Aspergillus flavus* Link and the generation of aflatoxin in stored rice grains [29,30]. In the activity assays presented here (Table 2), 2,4-dimethyl-3-hexanol yielded MIC values in the 250 to 750  $\mu\text{g}\cdot\text{mL}^{-1}$  range.



The presence of catechol should be attributed to the rupture of catechin (a constituent identified by Lin et al. [6] in the *G. biloba* leaves aqueous methanol extract) (Figure 2). Catechol had previously been identified in the extracts of *Taxus baccata* L. [31], *Allium sativum* L. [32], *Sambucus nigra* L. [33], and *Quercus ilex* L. [34], with concentrations ranging from 1.6 to 6.4% (compared to 3.3–7.9% in the extracts reported herein). Catechol oxidizes to melanoid pigments that are derivatives of benzoquinone, which is reported to have antimicrobial properties, slowing the decay of damaged fruits and other parts of plants. Catechol has been found to have antifungal activity against phytopathogenic taxa such as *Penicillium italicum* Wehmer and *Fusarium oxysporum* Schlecht. [35]. In our assays, catechol showed MIC values ranging from 187.5 to 500  $\mu\text{g}\cdot\text{mL}^{-1}$  (Table 2).

1-hydroxy-2-propanone is an alpha-hydroxy ketone found, for instance, in *T. baccata* leaf aqueous ammonia extracts [31]. Hydroxyacetone has also been detected, but not quantified, in several different edible plants, such as black cabbages, pulses, mentha, onion family vegetables, and rape. Additionally, it was identified (alongside acetic acid) as a major constituent of bioactive metabolites from tomato endophytic fungi, which had antibacterial activity against *X. vesicatoria* [36]. Furthermore, asymmetric reduction of hydroxyacetone leads to (*R*)-1,2-propanediol, a building block for antibacterial agents [37], and methylglyoxal—produced non-enzymatically from dihydroxyacetone—exhibits high non-peroxide antibacterial activity [38].

2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one was identified in *Hibiscus syriacus* L. [39], *S. nigra* [33], *Punica granatum* L. [40], and *T. baccata* [31]. In terms of antifungal activity, it is effective against *Phytophthora megasperma* Drechsler, *Verticillium dahliae* Kleb., and *Diaporthe amygdali* (Delacr.) Udayanga, Crous & K.D. Hyde, with MIC values between 375 and 750  $\mu\text{g}\cdot\text{mL}^{-1}$  [33].

The presence of acetic acid in contents higher than those detected herein has been reported, for instance, in *Artocarpus altilis* (Parkinson) Fosberg 1941 methanolic extracts (34.7%) [41]. In the particular case of *G. biloba*, its presence has been documented in ginkgo vinegar (obtained from the ginkgo seed coat by fermentation) [42]. Research has proven that it has good antibacterial activity against *Pseudomonas aeruginosa* (J.Schröter) Migula [43] at 1660  $\mu\text{g}\cdot\text{mL}^{-1}$ . Concerning formic acid 2-methylpropyl ester (or isobutyl formate), it belongs to a family of esters which provide a sweet, floral, and fruity odor and flavor to foods and beverages, without any known antimicrobial properties.

1-hepten-4-ol was detected in leaf and bark extracts of *Moringa concanensis* Nimmo [44] and is a volatile constituent of *Litchi chinensis* Sonn. [45] and *Aspalathus linearis* (Burm.f.) R.Dahlgren [46]. It was tested against two barley fungal pathogens, *Fusarium culmorum* (Wm.G.Sm.) Sacc. and *Cochliobolus sativus* (S.Ito & Kurib.) Drechsler ex Dastur, but demonstrated low activity [47].

3-*O*-methyl-*D*-fructose has been found to be present in *Ichnocarpus frutescens* (L.) W.T.Aiton [48] and in *T. baccata* [31], and reports have indicated its antibacterial activity [49]; 4,6-di-methyl- $\alpha$ -*D*-galactose has been found in the Siberian ginseng *Eleutherococcus senticosus* (Rupr. & Maxim.) Maxim. [50]; and 2-*O*-methyl- $\alpha$ -*D*-xylofuranoside has been reported in *Alternanthera sessilis* (L.) DC. [51] and *T. baccata* [31]. Additionally, 3-methylmannoside has been found in *Psidium guajava* L. [52]. None of these polysaccharides have been identified as bactericides. Nonanedioic acid (azelaic acid), however, has been shown to have an inhibitory effect on common dermatophytes, such as *Scopulariopsis brevicaulis* (Sacc.) Brainier, *Candida glabrata* (H.W.Anderson) S.A.Mey. & Yarrow, *Candida albicans* (C.P.Robin) Berkhout, and *Pityrosporum ovale* (Bizz.) Castell. & Chalm. [53].

#### 4.2. On the Antimicrobial Activity of *G. biloba* Extracts

In agricultural systems, a study on the antifungal action of *G. biloba* outer seedcoat extracts against rice sheath blight (which is caused by *Rhizoctonia solani* J.G. Kuhn) reported significant inhibition at 500  $\mu\text{g}\cdot\text{mL}^{-1}$  [11]. Regarding antibacterial activity, crude *G. biloba* extracts were tested by Ražná et al. [12] against human and veterinary pathogens, including Gram-positive bacteria such as *B. thuringiensis*, *C. perfringens*, *H. influenzae*, *L.*

*monocytogenes*, and *S. aureus*, and Gram-negative bacteria such as *Klebsiella pneumoniae* (Schroeter, 1886) Trevisan, 1887, *Escherichia coli* (Migula, 1895) Castellani and Chalmers, 1919, *Salmonella enterica* (ex Kauffmann and Edwards, 1952) Le Minor and Popoff, 1987, *Shigella sonnei* (Levine, 1920) Weldin, 1927, and *Yersinia enterocolitica* (Schleifstein and Coleman, 1939) Frederiksen, 1964. Among these species, *S. aureus*, *E. coli*, *K. pneumoniae*, and *Y. enterocolitica* were the most susceptible, with the best results being obtained for *S. aureus* (MIC<sub>50</sub> = 64.2 and MIC<sub>90</sub> = 72.2 µg·mL<sup>-1</sup>) and *Y. enterocolitica* (MIC<sub>50</sub> = 85.3 and MIC<sub>90</sub> = 99.6 µg·mL<sup>-1</sup>). These outcomes were consistent with the ones reported herein, in which greater activity was observed against Gram-positive *C. michiganensis* subsp. *michiganensis* bacteria (MIC = 500 µg·mL<sup>-1</sup>) than against Gram-negative *P. syringae*, *P. cichorii*, and *X. vesicatoria* bacteria (whose MIC values ranged from 750 to 1000 µg·mL<sup>-1</sup>). However, the MIC values for our extracts were higher (i.e., their activity was lower) than those reported by Ražná et al. [12].

#### 4.3. Comparison with Conventional Antibiotics

Upon comparison of the activity of the extract with those of gentamicin, tetracycline, and ciprofloxacin—representative of aminoglycosides, tetracyclines, and quinolones classes, respectively, that are commonly used in plant production in some countries outside of the European Union, as well as to treat human and animal diseases [54]—the efficacy of the conventional antibiotics was orders of magnitude higher (with MIC values in the 0.047 to 24 µg·mL<sup>-1</sup> range, depending on the pathogen). Concerning the other two antibiotics tested, viz. penicillin G and ampicillin, both were very effective against the Gram-positive *C. michiganensis* subsp. *michiganensis*; however, resistance was observed for all the Gram-negative bacteria, given that some strains of *P. syringae* produce β-lactamases [55] (e.g., some reports have indicated that 58% of *P. syringae* pv. *syringae* strains are resistant to ampicillin [56]) and *X. vesicatoria* does too [57]. It is noteworthy that the utilization of conventional antibiotics in agriculture (including tetracyclines, aminoglycosides, β-lactams, lincosamides, macrolides, pleuromutilins, and sulphonamides) may involve risks related to the emergence of resistance. Consequently, while the effectiveness of the natural extracts is not as high, they might be seen as a promising alternative to meet the requirements of the Global Action Plan on Antimicrobial Resistance set out by the World Health Organization (WHO).

## 5. Conclusions

Bacteriostatic activity was only detected for the *G. biloba* leaf extract (with MIC values ranging from 500 to 1000 µg mL<sup>-1</sup> depending on the bacterial strain), in which 3-O-methyl-D-fructose and 2,4-dimethyl-3-hexanol were the main constituents. In vivo tests on tomato and pea plants artificially inoculated with *C. michiganensis* subsp. *michiganensis* and *P. syringae* pv. *pisi*, respectively, demonstrated the potential of *G. biloba* leaf extract as a protective treatment, although higher concentrations than the in vitro-determined MICs were required (1000 and 1500 µg·mL<sup>-1</sup>, respectively). The reported findings suggest an alternative valorization pathway for *G. biloba* leaves as biorationals for crop protection in addition to their use as dietary supplements.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae9040461/s1>, Figure S1. GC-MS chromatogram of *G. biloba* leaf hydromethanolic extract; Figure S2. GC-MS chromatogram of *G. biloba* sarcotesta hydromethanolic extract; Figure S3. GC-MS chromatogram of *G. biloba* sclerotesta hydromethanolic extract; Table S1. Constituents of *G. biloba* leaf hydromethanolic extract; Table S2. Constituents of *G. biloba* sarcotesta hydromethanolic extract; Table S3. Constituents of *G. biloba* sclerotesta hydromethanolic extract.

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