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Silver Maple (*Acer saccharinum* L.) Leaf: A Potential Source of Antibacterial Compounds to Control Phytopathogenic Bacteria in Horticulture Crops

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Abstract. **In this study, a crude ethanolic extract made of silver maple (*Acer saccharinum* L.) leaves (SML) was evaluated for antibacterial activity against the phytopathogenic bacteria *Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, and *Xanthomonas fragariae*. The extract was shown to cause important inhibition zones against the three bacteria in the disc diffusion assays, revealing its antibacterial activity. The minimal inhibitory concentration (MIC) of the extract was determined thereafter for each bacterium. The extract showed the same MIC value (1.56 mg·mL^{-1}) for the three bacteria. Using a semipreparative high-performance liquid chromatography system, crude ethanolic SML extract was divided in 15 fractions and each fraction was tested for antibacterial activity against *X. fragariae* with the disc diffusion assay. Among the six fractions causing an inhibition zone, fraction 10 caused the largest inhibition. Fraction 10 was further analyzed by ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. According to quadrupole time-of-flight mass spectrometry, the main peak of fraction 10 was identified as a galloyl-bis-HHDP-glucose ($C_{41}H_{28}O_{26}$, 936.6454 g·mol^{-1}) isomer, an ellagitannin known for antibacterial activity and for stimulating plant natural defenses. The study opens new avenues of research on the valorization of SML and on the control of plant diseases caused by bacteria in organic and conventional production of horticultural crops.**

Phytopathogenic bacteria cause important economic losses in several horticultural crops (Butsenko et al., 2020; Kim et al., 2016; Nandi et al., 2018). Currently, few phytosanitary products other than copper-based pesticides are available to Canadian producers to manage diseases caused by phytopathogenic bacteria (Health Canada, 2022). The use of copper is increasingly criticized for numerous negative effects in agriculture, the environment, and human health (Gazzurelli et al., 2020; Migdał et al., 2018; Tegenaw et al., 2019). Effective, safe, and eco-friendly alternatives to copper are therefore needed for the management of plant diseases. In this respect, the exploitation of natural antimicrobial compounds present in plants is attracting growing interest of many stakeholders of the agricultural sector (Pino et al., 2013; Rguez et al., 2018).

Delisle-Houde and Tweddell (2020) recently reported the antibacterial properties of sugar maple (*Acer saccharum* Marsh.) leaf ethanolic extracts against the phytopathogenic bacteria *Pseudomonas cichorii* and *Xanthomonas campestris* pv. *vitians*. Green leaves and autumn-shed leaves displayed similar antibacterial activity (Delisle-Houde and Tweddell, 2020), indicating that antibacterial activity is unaffected by leaf senescence. Geraniin, an ellagitanin, was subsequently identified as the main antibacterial compound in sugar maple leaf (Delisle-Houde et al., 2020). Other species of the genus *Acer* were also reported for antibacterial activity or for their content in antibacterial compounds. Nikko maple (*Acer nikoense* Maxim.) was shown to contain geraniin and corilagin (Okabe et al., 2001). Cappadocian maple (*Acer cappadocicum* Gled.) leaf crude extracts prepared with different solvents revealed antibacterial activity against *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella enterica*, and *Acinetobacter baumannii* (Kausar et al., 2021). Choi et al. (2005) reported the antibacterial activity of acertannin and methyl gallate, isolated from Amur maple (*Acer ginnala* Max.), against *Staphylococcus epidermidis*, *Pseudomonas putida*, and *Salmonella typhimurium*. Recent work by Delisle-Houde and Tweddell (2020) demonstrated the efficacy of an ethanolic extract of sugar maple leaves to control lettuce (*Lactuca sativa* L.) bacterial leaf spot, caused by *X. campestris* pv. *vitians*, pointing out for the first time the possibility to exploit maple leaves to manage bacterial diseases affecting horticultural crops. To our knowledge, the antibacterial activity of silver maple (*Acer saccharinum* L.), a species often used as ornamental crop frequently encountered in the eastern United States and eastern Canada (Gabriel, 1990) generating each year residual lignocellulosic biomass, has not yet been investigated.

In this study, an ethanolic extract made of leaves of silver maple, was shown to have antibacterial activity against three phytopathogenic bacteria (*Clavibacter michiganensis* subsp. *michiganensis*, *Pseudomonas syringae* pv. *tomato*, *Xanthomonas fragariae*) and to contain a galloyl-bis-HHDP-glucose isomer, an ellagitannin known for antibacterial activity.

Materials and methods

Bacteria. *Xanthomonas fragariae* (strain ATCC 33239), *P. syringae* pv. *tomato* (strain DC3000), and *C. michiganensis* subsp. *michiganensis* (strain *Cmm*-1375) were graciously provided by the Laboratoire d'expertise et de diagnostic en phytoprotection (MAPAQ, Québec, QC, Canada), Dr. Edel Pérez-López (Université Laval, Québec, QC, Canada), and Dr. Vicky Toussaint (Agriculture and Agri-Food Canada, Saint-Jean-sur-Richelieu, QC, Canada), respectively. The bacteria were preserved at –85 °C in 15% (w/v) glycerol (VWR International, West Chester, PA, USA). As described by Delisle-Houde et al. (2018), bacteria were cultivated in King's B (KB) liquid medium [20 g·L^{-1} of Bacto™ Proteose Peptone No. 3 (Becton, Dickinson and Company, Sparks, MD, USA), 1.5 g·L^{-1} of dibasic sodium phosphate (EM Science, Gibbstown, NJ, USA), 10 g·L^{-1} of glycerol (VWR International), 1.5 g·L^{-1} of magnesium sulfate (Fisher Scientific, Geel, Belgium)] under agitation (160 rpm) at 28 °C for 24 h and bacterial suspensions [5 × 10^5 or 1 × 10^8

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colony forming units (cfu)·mL^{-1}] were prepared using 0.5 McFarland standards in physiological saline solution (0.5% NaCl).

Preparation of the crude extract of silver maple leaves. Silver maple leaves (SML) were collected during Summer 2020 on an ornamental tree in an urban domestic garden (Québec, QC, Canada). SML were dried at room temperature (22.5 °C) and preserved at –25 °C until extraction. They were grinded mechanically (up to 2 mm) and macerated (150 g L^{-1}) under agitation in 95% ethanol for 24 h at room temperature. The extraction suspension was evaporated to dryness under reduced pressure (at a temperature of not more than 60 °C) with a rotary evaporator (BUCHI Corporation, New Castle, DE, USA). Powder was recovered with sterile ultrapure water (18.2 megohm ionic purity; Millipore Bedford, MA, USA; 10 mL), lyophilized and stored in the dark at room temperature in Mason jars.

Disc diffusion assay. Disc diffusion assays were conducted in triplicates under sterile conditions. One-hundred µL of a suspension (1×10^8 cfu·mL^{-1}) in physiological saline solution (0.5% NaCl) of either *X. fragariae*, *P. syringae* pv. *tomato* or *C. michiganensis* subsp. *michiganensis* were spread on KB solid medium [15 g·L^{-1} of agar (Hardy Diagnostics, Santa Maria, CA, USA)] in petri plates. Whatman filter paper (grade 1) discs (7 mm in diameter) soaked with 10 µL of crude ethanolic SML extract (50 mg·mL^{-1} of distilled water) were then deposited on the medium and petri plates were incubated for 48 h at 28 °C. Sterile distilled water was used as control. The diameter of the inhibition zone was rated as described in Delisle-Houde and Tweddell (2020): – (0 mm), + (<5 mm), ++ (5–10 mm), and +++ (>10 mm). Filter paper disc diameter was not considered in the diameter of the inhibition zone. The experiment was conducted twice.

Minimum inhibitory concentrations. Minimum inhibitory concentrations (MICs) were determined using flat-bottom 96-well microplates (Sarstedt AG & Co., Nümbrecht, Germany) as described by Delisle-Houde et al. (2018). *Xanthomonas fragariae*, *P. syringae* pv. *tomato*, or *C. michiganensis* subsp. *michiganensis* (5×10^5 cfu) were suspended in 100 µL of KB liquid medium containing different concentrations of crude ethanolic SML extract previously dissolved in ultrapure water. Microplates were incubated at 28 °C for 24 h and after the incubation period, 10 µL of sterile 2,3,5-triphenyl-2H-tetrazolium chloride (1 mg·mL^{-1}; Ward's Science, Rochester, NY, USA) was added to each well. The lowest concentration at which no metabolic activity was observed (absence of red coloration in the well) corresponded to the MIC. Each concentration was tested in three replicates. The experiment was conducted twice.

Fractioning of the crude ethanolic SML extract by semipreparative high-performance liquid chromatography. Freeze-dried powder of ethanolic SML extract was dissolved in ultrapure water (50 mg mL^{-1}) and divided in 15 fractions using a semipreparative high-performance liquid chromatography (HPLC) system (Waters Corporation, Milford, MA, USA) and a fraction collector (Waters Corporation); ultraviolet detector was monitored at wavelength 280 nm. The extract was separated on a 5-µm XTerra MS C18 OBD column (19 mm × 100 mm; Waters Corporation). Mobile phases [100% ultrapure water and 100% HPLC-grade acetonitrile] and elution gradient were set as described in Delisle-Houde et al. (2020). Flow rate was 20 mL·min^{-1} at room temperature. Three injections of 1 mL (50 mg·mL^{-1}) of SML ethanolic extract was performed. Each of the 15 fractions corresponded to 4 min and run time was 60 min. Fractions were dissolved in a minimum of sterile ultrapure water (30 µL), and their antibacterial activity was determined against *X. fragariae* with disc diffusion assay.

Ultra-performance liquid chromatography/quadrupole time-of-flight mass spectrometry. The fractions causing the largest inhibition zones against *X. fragariae* in the disc diffusion assays were analyzed as described in Delisle-Houde et al. (2020). A Waters Acquity ultra-performance liquid chromatography (UPLC) system (Waters Corporation) equipped with a binary high-pressure pump, an automatic sample injector, a ultraviolet detector, and a column manager was used. Separation was carried out on a 1.7-µm Acquity UPLC BEH C18 column (2.1 mm × 100 mm; Waters Corporation) at a flow rate of 0.4 mL·min^{-1} and a temperature of 40 °C. Solvent A (0.2% aqueous acetic acid) and solvent B (acetonitrile) were used as mobile phase. A linear gradient elution was run as follows: 0–4 min, 2% B in A; 4–6 min, 2% to 90% B in A; 6–6.1 min, 90% to 2% B in A; 6.1–10 min, 2% B in A. The injection volume was 2.5 µL (5 mg·mL^{-1}), and ultraviolet wavelength was monitored at 280 nm. MS analysis was performed on a Micromass quadrupole time-of-flight mass spectrometry (Q-TOF) micro (Waters Corporation) equipped with an electrospray ionization (ESI; Waters Corporation) source. MS data were collected and processed by Masslynx software (Waters Corporation) under the negative ion mode. The ESI source conditions were applied as follows: full scan data acquisition was performed from *m/z* 100 to 1500, the capillary voltage was 1800 V in negative mode, cone voltage was set at 70 V, source temperature at 120 °C, and desolvation temperature at 250 °C. Nitrogen and argon were used as the cone and collision gases, respectively. The cone and desolvation gas flow rates were 50 L·h^{-1} and 350 L·h^{-1}, respectively. Compounds were identified using KEGG COMPOUND (www.kegg.jp) libraries.

Results

Antibacterial activity of the crude ethanolic SML extract. The results of the disc diffusion assays (Expt. 1) are presented in Table 1. Crude ethanolic SML extract caused an inhibition zone for the three bacteria tested (Table 1). *Xanthomonas fragariae* appeared slightly more sensitive (diameter of the inhibition zone >10 mm) than *P. syringae* pv. *tomato* and *C. michiganensis* subsp. *michiganensis* (inhibition zone diameter of 5–10 mm) (Table 1). The control showed no inhibition zone. The results were the same in Expt. 2. The MIC of the extract was 1.56 mg·mL^{-1} for the three bacteria in both Expts. 1 (Table 1) and 2.

Fractioning of the crude extract by semipreparative HPLC. Crude ethanolic SML extract was divided in 15 fractions using a semipreparative HPLC system (Fig. 1). Each fraction was tested for antibacterial activity against *X. fragariae* using the disc diffusion assay. Among the 15 fractions (vials 1–15), six (vials 9–14) caused an inhibition zone (Table 2). Fraction 10 (vial 10) caused the largest inhibition zone (diameter of the inhibition zone >10 mm) followed by fractions 12 (vial 12) and 13 (vial 13) (inhibition zone diameter of 5–10 mm), and fractions 9 (vial 9), 11 (vial 11), and 14 (vial 14) (diameter of the inhibition zone <5 mm) (Table 2). The largest inhibition zone (Table 2) corresponded to the peak showing the highest relative abundance on the chromatogram (Fig. 1).

Identification of compounds by UPLC/Q-TOF-MS. The fraction 10 was further analyzed by UPLC/Q-TOF-MS (Figs. 2 and 3).

Table 1. Antibacterial activity of crude ethanolic silver maple leaves extract against phytopathogenic bacteria as determined by the diameter of the inhibition zones (disc diffusion assay) and the minimal inhibitory concentrations (MICs).

Phytopathogenic bacteria	Inhibition zone[i]	MIC[ii] (mg·mL^{-1})
Clavibacter michiganensis subsp. *michiganensis* (strain *Cmm*-1375)	++,[iii] ++,[iv] ++[v]	1.56 ± 0.0
Pseudomonas syringae pv. *tomato* (strain DC3000)	++, ++, ++	1.56 ± 0.0
Xanthomonas fragariae (strain ATCC 33239)	+++, +++, +++	1.56 ± 0.0

[i] Inhibition zone diameter of 5–10 mm (++) and >10 mm (+++).
[ii] Each value represents the mean of three replicates ± standard deviation.
[iii] Replicate 1.
[iv] Replicate 2.
[v] Replicate 3.

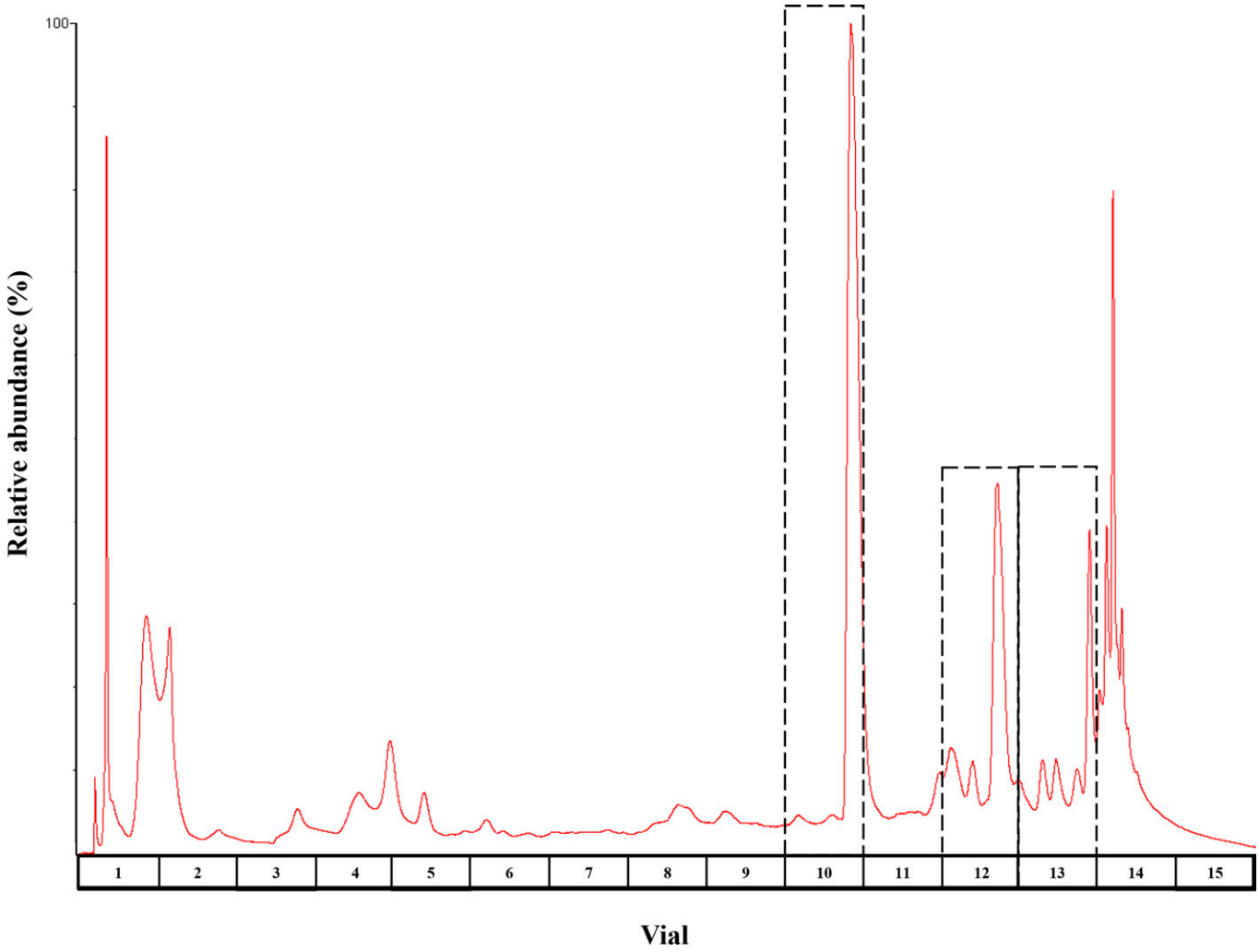

Fig. 1. Representative high-performance liquid chromatography (HPLC)–ultraviolet chromatogram of the crude ethanolic silver maple leaves extract divided in 15 fractions (vials 1–15; retention time = 4 min/vial) by semipreparative HPLC system (5-µm XTerra MS C18 OBD column; 19 mm × 100 mm; Waters Corporation). Fractions causing the largest inhibition zones against *Xanthomonas fragariae* (strain ATCC 33239) are in the boxes.

Table 2. Antibacterial activity of the fractions of crude ethanolic silver maple leaves extract obtained by semipreparative high-performance liquid chromatography

Fraction (vial)	Inhibition zone[i]
1	–,[ii] –,[iii] –[iv]
2	–, –, –
3	–, –, –
4	–, –, –
5	–, –, –
6	–, –, –
7	–, –, –
8	–, –, –
9	+, +, +
10	+++, +++, +++
11	+, +, +
12	++, ++, ++
13	++, ++, ++
14	+, +, +
15	–, –, –

[i] Antibacterial activity was determined using the disc diffusion assay with *Xanthomonas fragariae* (strain ATCC 33239) where no inhibition zone (–), inhibition zone diameter <5 mm (+), inhibition zone diameter of 5–10 mm (++), and inhibition zone diameter >10 mm (+++).
[ii] Replicate 1.
[iii] Replicate 2.
[iv] Replicate 3.

The chromatogram showed one major peak at a retention time of 6.9 min (Fig. 2). According to quadrupole time-of-flight mass spectrometry, the main peak of fraction 10 was a galloyl-bis-HHDP-glucose ($C_{41}H_{28}O_{26}$, 936.6454 g·mol^{-1}) isomer. The *m/z* value observed for galloyl-bis-HHDP-glucose in negative ion mode was 935.1973 (Fig. 3). The presence of methylquercetin (*m/z* = 315.0757) was also noted (Fig. 3). In addition, fractions 12 and 13 were analyzed by UPLC/Q-TOF-MS and quercetin-O-hexoside, quercetin-O-pentoside, pentagalloylglucoside, and methylellagic acid-pentose were identified (data not shown).

Discussion

Many compounds extracted from wild and cultivated plants showed toxicity against fungi or bacteria (Copping and Duke, 2007; Dayan et al., 2009; Manici et al., 1997; Pino et al., 2013). These compounds represent an interesting alternative to copper-based pesticides that are extensively used for the control of bacterial plant diseases.

In this study, crude ethanolic SML extract was investigated for antibacterial activity against three phytopathogenic bacteria *X. fragariae*, *P. syringae* pv. *tomato*, and *C. michiganensis* subsp. *michiganensis* using disc diffusion assay, a method commonly used to evaluate the antimicrobial activity of plant extracts (Balouiri et al., 2016). The extract was shown to cause important inhibition zones against the three bacteria revealing its antibacterial activity. The MICs of the extract were thereafter determined. The MIC was 1.56 mg·mL^{-1} for the three bacteria. It is interesting to note that Gram-negative bacteria (*P. syringae* pv. *tomato* and *X. fragariae*) and the Gram-positive bacterium (*C. michiganensis* subsp. *michiganensis*) showed the same sensitivity to the extract on the basis of the MIC values. The toxicity of the SML extract against the tested bacteria is similar to that of sugar maple leaves extract against *X. campestris* pv. *vitians* (MIC of 1.56 mg·mL^{-1}) reported by Delisle-Houde et al. (2020); sugar maple leaves extract was also shown to control lettuce bacterial leaf spot caused by *X. campestris* pv. *vitians* (Delisle-Houde and Tweddell, 2020).

Crude extract of SML was thereafter fractionated by HPLC. Each fraction was tested

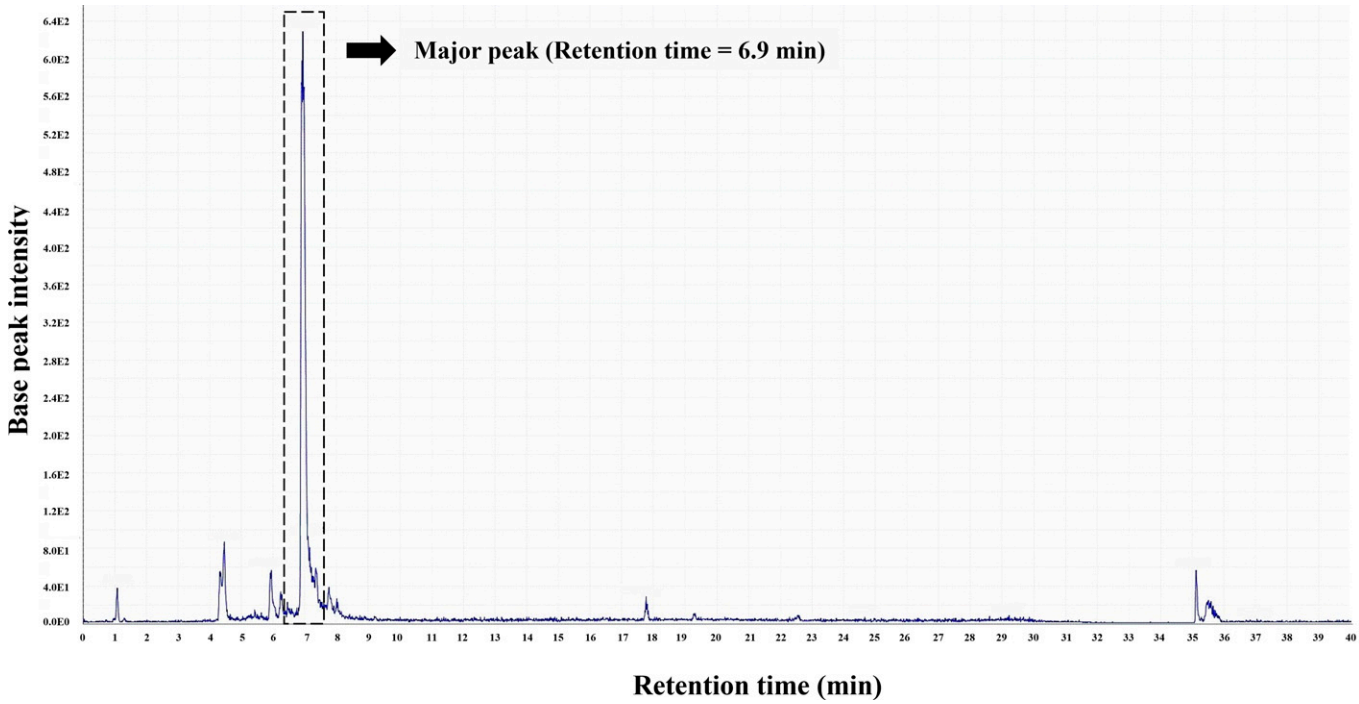

Fig. 2. Representative ultra-performance liquid chromatography (UPLC)-ultraviolet chromatogram of fraction 10 by Waters Acquity UPLC system (1.7 μm Acquity UPLC BEH C18 column; 2.1 mm × 100 mm; Waters Corporation). The main compound present in fraction 10 is in the box.

for antibacterial activity using disc diffusion assay with *X. fragariae*. According to the inhibition zone diameter, the strongest antibacterial activities were observed in fractions 10, 12, and 13 where galloyl-bis-HHDP-glucose, quercetin-O-hexoside, quercetin-O-pentoside, pentagalloylglucoside, methylellagic acid-pentose, and methylquercetin were identified as the main compounds. Tannins (including galloyl-bis-HHDP-glucose, pentagalloylglucoside, and methylellagic acid-pentose) and flavonoids (such as methylquercetin, quercetin-O-hexoside, and quercetin-O-pentoside) are classes of compounds known for their antimicrobial activity (Ncube et al., 2008; Scalbert, 1991). Galloyl-bis-HHDP-glucose was among the most abundant phenolic compounds in some extracts of *Cytinus hypocistis* L. and *Hippophae rhamnoides* L. with antimicrobial activity (Radenkovs et al., 2018; Silva et al., 2020). Pentagalloylglucoside was one of the two major phenolic compounds identified in an extract of male flowers of *Castanea sativa* with antimicrobial activity (Alaya et al., 2021). The present work strongly suggests that galloyl-bis-HHDP-glucose, quercetin-O-hexoside, quercetin-O-pentoside, pentagalloylglucoside, methylquercetin, and methylellagic acid-pentose are the main antibacterial compounds present in the SML, galloyl-bis-HHDP-glucose being the most abundant. Future work will be conducted to purify these compounds from SML to further characterize their antibacterial activity.

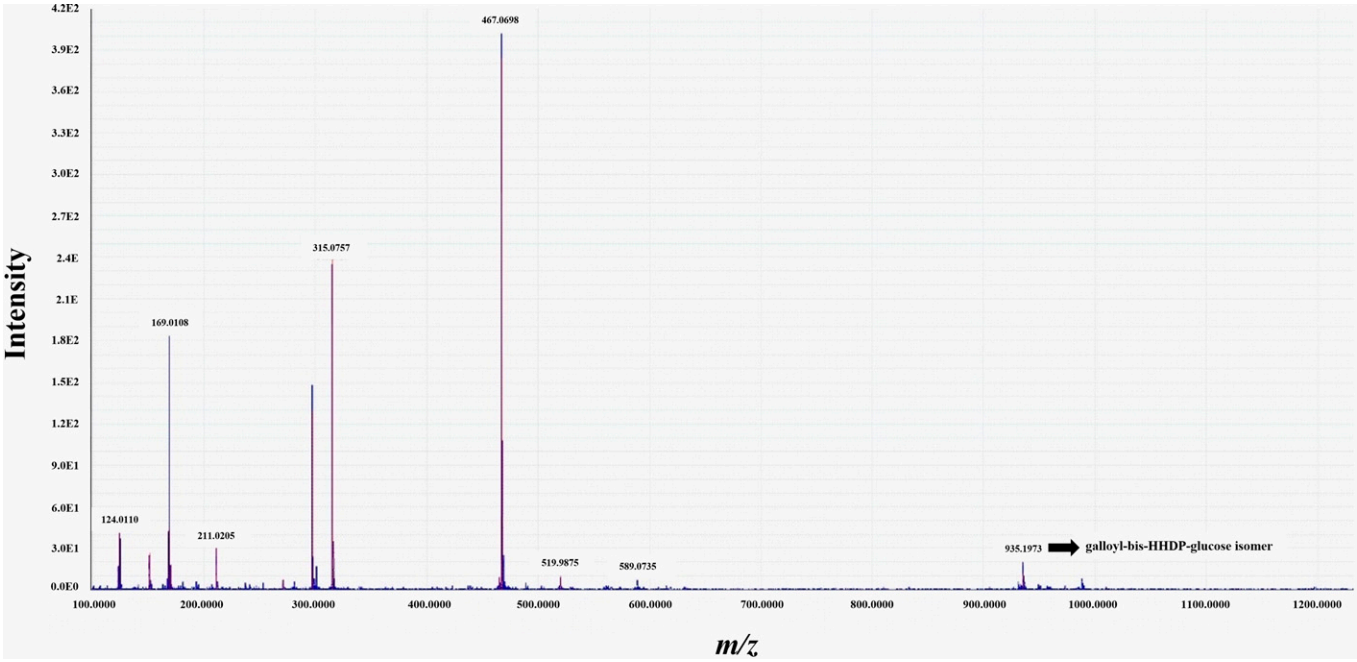

Fig. 3. Identification of the major antibacterial compound present in fraction 10. Mass spectrum (*m/z* 100–1250) obtained by Micromass Q-TOF micro equipped with an electrospray ionization (ESI) source in negative ion mode.

Chemicals are known to repress plant pathogens by direct toxic effect and/or by activation of plant natural defenses (Trotel-Aziz et al., 2006; Youssef et al., 2014). Phenolic-rich extracts from red maple (*Acer rubrum* L.) were recently reported to induce plant defense mechanisms in *Nicotiana tabacum* L. leaves; pentagallates were identified as the main compounds involved in the stimulation of plant defense mechanisms (Peghaire et al., 2020). More recently, Grellet-Bournonville et al. (2021) reported that galloyl-bis-HHDP-glucose in methanolic extracts of strawberry (*Fragaria* × *ananassa* Duchesne ex Rozier) leaves induced defense response in *Arabidopsis thaliana* (L.) Heynh. against *Pseudomonas viridiflava*. The presence of galloyl-bis-HHDP-glucose in SML extract is of particular interest in the perspective of an eventual utilisation of the extract for the control of plant diseases caused by phytopathogenic bacteria.

This study reports for the first time 1) the antibacterial activity of an ethanolic extract made of SML against phytopathogenic bacteria and 2) the presence in SML of a galloyl-bis-HHDP-glucose isomer, an ellagitannin known for antibacterial activity and for stimulating plant natural defenses, opening new avenues of research on the valorization of SML and on the control of plant diseases caused by bacteria in organic and conventional production of horticultural crops. Future work will be conducted with SML at different development stages and with autumn-shed leaves to determine the effect of leaf senescence on both antibacterial activity and galloyl-bis-HHDP-glucose content.

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