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In vitro antibacterial activity of *Magnolia tamaulipana* against tomato phytopathogenic bacteria

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Abstract: The tomato (*Solanum lycopersicum* Linnaeus) is one of the most important vegetable crops in the world. Still, there are phytopathogenic bacteria that cause a decrease in the yield or can kill the plant, like *Pseudomonas syringae* pv. *tomato* (Pst), *Xanthomonas vesicatoria* (Xv), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Ralstonia solanacearum* (Rs) and *Agrobacterium tumefeciens* (At). Synthetic chemical fungicides are primarily used to control plant pathogenic bacteria, but their rapid growth makes them resistant to control. This research work is aimed at assessing the *in vitro* antibacterial activity of the ethanolic extract of *Magnolia tamaulipana* Vazquez leaves against Rs, Pst, Xv, Cmm, and At, as well as obtaining information about this plant species' chemical composition. The extract inhibited the growth of the five phytopathogenic bacteria that were tested. The growth inhibition rate ranged between 8.22 and 100%. The inhibitory concentration, $IC_{50(90)}$, required to inhibit 50 (90%) of Pst, Xv, Cmm, and At bacterial growth, was 34.71 (39.62), 23.09 (441.88), 64.75 (176.73) and 97.72 (535.48) ppm, respectively. The phytochemical analysis detected the presence of phenols, tannins, terpenes, saponins. *M. tamaulipana* ethanolic extract has antimicrobial properties and it must be considered a new control agent.

Keywords: inhibitory concentration; biological control; phytochemicals; IR-spectroscopy; *Solanum lycopersicum*

The tomato (*Solanum lycopersicum* Linnaeus; Solanaceae) is the second most important vegetable crop worldwide in economic terms, after the potato (Ali et al. 2019). In the world, there are 5.88 mil tomato hectares, with an average yield of 41.13 t/ha (FAO 2017). There are abiotic and biotic factors that reduce tomato yields (Ali et al. 2019). Among the biotic factors, phytopathogenic bacteria, such as *Pseudomonas syringae* pv. *tomato* (Pst), *Xanthomonas vesicatoria* (Xv), *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Ralstonia solanacearum* (Rs) and *Agrobacterium tumefeciens* (At), are considered causal agents

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of bacterial speck, bacterial canker, bacterial rot, and galls, respectively, affecting the production and quality of greenhouse and field-grown tomatoes (Escobar & Dandekar 2003; Balestra 2009).

Some available antibacterial agents like copper and streptomycin compounds provide excellent control over phytopathogenic bacteria, but the fast development of resistant strains limits their use. Human interest in using plants as sources of natural antimicrobial agents is increasing. Public concern regarding the risks involved in the generalised use of synthetic chemical products that can harm the environment as well as human health has led to the search for biologically active products like plant extracts and essential oils, as attractive alternatives to control microbial pathogens. The value of the antibacterial compounds present in highly-developed plants has been recognised as an important factor in disease control strategies for a long time (Soltani & Aliabadi 2013; Baştaş 2015).

Magnolia tamaulipana Vazquez (Magnoliaceae) is endemic and grows in the cloud forest of the UNESCO Biosphere Reserve "El Cielo" located in Tamaulipas, Mexico (Dieringer et al. 1999; Vázquez-G. 1999). In Mexican traditional medicine, some *Magnolia* species have been broadly used to treat cardiac, respiratory and nervous system diseases and also have antibacterial and antifungal activities. *Magnolias* spp. produce more than 255 metabolites obtained from the tissues like the bark, root, leaves, flowers and seeds; in particular magnolol and honokiol phenylpropanoids (Ramírez-Reyes et al. 2015a, b; Vásquez-Morales et al. 2015). There are few studies assessing *Magnolias* spp. extracts' effect against phytopathogenic bacteria. *Magnolia dealbata* Zucc has antimicrobial effects over *Clavibacter michiganensis* subsp. *michiganensis* (Jacobo-Salcedo et al. 2011), *Pectobacterium carotovorum* and *Pseudomonas cichorii* phytopathogenic bacteria (Ramírez-Reyes et al. 2015b); as well as *Magnolia schiedeana* (Schltdl.) over *P. carotovorum* and *P. cichorii* (Ramírez-Reyes et al. 2015a). Nevertheless, so far, the chemical composition of *M. tamaulipana* has not been determined, and its application against bacteria or phytopathogenic fungi has not reported. However, the *M. tamaulipana* ethanol extract caused inhibition in the egg-laying and food intake of *Tetranychus urticae* C.L. Koch, 1836 (Acari: Tetranychidae) (Chacón-Hernández et al. 2019). This research work is aimed at evaluating the *in vitro* antibacterial activity of the *M. tamaulipana* extract against *Pseudomonas syringae* pv. *tomato* (Pst), *X. vesicatoria* (Xv); *C. michiganensis* subsp. *michiganensis* (Cmm), *R. Solanacearum* (Rs) and *A. tumefeciens* (At), as well as to obtain information about the extract's chemical composition.

MATERIAL AND METHODS

Pathogens. *Pseudomonas syringae* pv. *tomato* (Pst), *X. vesicatoria* (Xv); *C. michiganensis* subsp. *michiganensis* (Cmm), *R. Solanacearum* (Rs) and *A. tumefeciens* (At) came from the bacteria collection of the "Universidad Autónoma Agraria Antonio Narro" (UAAAN) Agricultural Parasitology Division (DPA) Biofungicides Laboratory (LB). We conditioned the bacteria by replanting them in cross-open grooves, in order to obtain pure cultures on Nutrient agar (BD Bioxon, model 210400). The identification used the morphological and physiological traits established by Schaad et al. (2001).

Preparation of plant material and extracts. *M. tamaulipana* leaves were collected in May 2017 at the BRC. The uncontaminated leaves of *M. tamaulipana* (i.e., without external agents and physical contaminants) were exposed to sunlight to remove their moisture (Abiodun et al. 2017). The dry leaf samples were ground into a fine powder by an electric mill (Cuisinart DBM-8, USA). The powder of *M. tamaulipana* (150 g) was mixed with 96% ethanol (500 mL) to prepare the extract that was kept under constant stirring on Thermo Scientific™ Cimarec™ Digital Stirring Hotplates for three days at room temperature (27 °C), in amber bottles covered with aluminium (Moreno-Limón et al. 2011). Subsequently, the solution was filtered using Whatman No. 4 paper. The solvent was removed from the extract by passing it through a vacuum system on a rotary evaporator at 60 °C for two hours (Yamato Scientific America Inc., Model-RE301, Japan), and the rest of the solvent was dried in an oven chamber (Shel-lab Model 1535, Sheldon Manufacturing Inc., USA) for 72 hours. Finally, the extract was recovered in solid form to prepare 2 000 µg/mL of a stock solution for the antibacterial activity tests and the phytochemical composition analysis (Moreno-Limón et al. 2011; Jasso De Rodríguez et al. 2015; Chacón-Hernández et al. 2019).

Antibacterial activity tests by micro-plates dilution technique. Micro-plates with 96 wells were used in the assay (Ultra CruzTM plates, USA). Each well filled with 100 µL of the nutrient broth

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(BD Bioxon, model 210300, Mexico) supplemented with 2,3,5-triphenyltetrazolium chloride (TTC, tetrazolium red, Sigma T-8877, USA). 100 µL of the *M. tamaulipana* ethanolic extract was added to assess the different concentrations, starting with the 4th column of the micro-plate (1 000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 3.9 ppm). Finally, 100 µL of the plant pathogens were added (Pst, Xv, Cmm, Rs, and At) according to the test concentrations, as well as a positive control in column two (Nutrient agar and the pathogen), an ethanol control to rule out its effect on the inhibition was placed in column three, a nutrient agar with TTC only was placed in column one. The plant pathogens had been previously transferred to the nutrient stock, adjusted at a concentration of 10^{-8} bacterial colony forming units (UFC) UFC/mL. After the inoculation, the micro-plates were covered with a lid and incubated at 28 °C during the night. The micro-plates were read at 540 nm in the photometric spectrum (Thermo Scientific™ Multiskan™ GO) and the results were recorded using Thermo Scientific™ SkanIt™ software. We replicated the assay three times and the inhibition values were calculated after obtaining the inhibition percentage, with the following formula:

$$\text{Inhibition}(\%) = \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{sample}}} \times 100 \quad (1)$$

where: A_{control} – the absorbance of the control sample in the micro-plate; A_{sample} – the absorbance assessing the *M. tamaulipana* ethanolic extract activity.

Evaluation of the phytochemical composition. The *M. tamaulipana* ethanolic extract was analysed to conduct the phytochemicals' qualitative detection tests, including the carbohydrates (Molisch's test); the reducing sugars (Fehling and Benedict's tests); the alkaloids (Dragendorff and Sonneschain's tests); flavonoids (Shinoda's test and NaOH test); the cyanogenic glycosides (Gringnard's test); the saponins (foam test); the sterols and terpenes (Liberman-Burchard's test); the tannins {$FeCl_3$, $K_3[Fe(CN)_6]$ and gelatine tests}; the quinones (Börntraguer's test); the purins (HCl tests); the polysaccharides (Lugol's test); the soluble starch (KOH and H_2SO_4 tests); as well as the carotenoids (H_2SO_4 and $FeCl_3$ tests) (Sahgal et al. 2009; Usman et al. 2009).

Infrared spectroscopy (IR). The *M. tamaulipana* ethanolic extract samples do not require any special treatment in order for them to be analysed. The sieved fine powder was analysed under a spectrophotometer FT-IR Nicolet™ iS™10, coupled to a Smart iTR-ATR conditioned with a diamond tip, GX00 model, at ambient temperature. The analysis encompassed a range of 600–4 000 cm with 100 scans per analysis. Every scanning was repeated three times (Jasso De Rodríguez et al. 2015).

Statistical analysis. The inhibition percentages of the bacterial growth were analysed by an ANOVA, using Tukey's HSD mean comparison test (SAS, version 2002). We used a probit analysis to estimate the inhibitory concentration [$IC_{50(90)}$], including the CI_{95} values (Finney 1971).

RESULTS

Antibacterial activity (effect). The ethanolic extract concentrations obtained from the *M. tamaulipana* leaves showed significant differences over the growth of *P. syringae* pv. *tomato* ($F = 5339.64$; $df = 8, 18$; $P < 0.0001$), *X. vesicatoria* ($F = 470.75$; $df = 8, 18$; $P < 0.0001$), *C. michiganensis* subsp. *michiganensis* ($F = 168864$; $df = 8, 18$; $P < 0.0001$), *R. solanacearum* ($F = 578.16$; $df = 8, 18$; $P < 0.0001$) and *A. tumefeciens* ($F = 3247.48$; $df = 8, 18$; $P < 0.0001$). Significant differences were observed among the phytopathogenic bacteria at every concentration (1 000 ppm: $F = 45.20$; $df = 4, 10$; $P < 0.0001$; 500 ppm: $F = 134.57$; $df = 4, 10$; $P < 0.0001$; 250 ppm, $F = 2140.45$; $df = 4, 10$; $P < 0.0001$; 125 pm: $F = 1\,064.94$; $df = 4, 10$; $P < 0.0001$; 62.5 ppm: $F = 3094.51$; $df = 4, 10$; $P < 0.0001$; 31.2 ppm: $F = 905.86$; $df = 4, 10$; $P < 0.0001$; 15.62 ppm, $F = 752.58$; $df = 4, 10$; $P < 0.0001$; 7.8 ppm, $F = 586.50$; $df = 4, 10$; $P < 0.0001$; 3.9 ppm: $F = 2\,014.45$; $df = 4, 10$; $P < 0.0001$). The extract was more efficient over Rs than over At, Xv, Pst and Cmm, since only 31.2 ppm was required to inhibit 100% of the bacterial growth (Table 1).

The inhibitory concentration $IC_{50(90)}$ required to inhibit 50 (90%) of the bacterial growth was lower for Xv, followed by Pst (Table 2). We could not calculate the $IC_{50(90)}$ for Rs, because a larger number of bioassays with lower concentrations are required to do the calculation. However, if only 3.9 ppm were required to inhibit 61.91% of the bacterial growth, the $IC_{50(90)}$ of the extract should be lower for Rs than concentration required for Xv ($IC_{50} = 23.09$ ppm).

Phytochemical composition. The results obtained from the phytochemical analysis of the ethanolic extract of the *M. tamaulipana* leaves appear

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Table 1. Inhibition percentages at different concentrations of the *Magnolia tamaulipana* ethanolic extract against tomato phytopathogenic bacteria

Phytopathogenic bacteria	1 000 ppm	500 ppm	250 ppm	125 ppm	62.5 ppm	31.2 ppm	15.62 ppm	7.8 ppm	3.9 ppm
Pseudomonas syringae pv. tomato	96.28 ± 0.94^{aB}	92.69 ± 1.04^{bC}	89.17 ± 0.21^{cC}	89.17 ± 0.21^{cC}	76.38 ± 0.94^{dB}	34.10 ± 0.54^{eC}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{fC}
Xanthomonas vesicatoria	91.19 ± 0.25^{aC}	80.93 ± 0.77^{bD}	79.69 ± 0.31^{bD}	76.63 ± 0.41^{bD}	76.51 ± 0.10^{bB}	64.15 ± 2.63^{cB}	29.75 ± 2.85^{dB}	10.01 ± 2.49^{eB}	0.00 ± 0.00^{fC}
Clavibacter michiganensis subsp. *michiganensis*	96.89 ± 0.08^{aB}	95.56 ± 0.29^{bB}	94.42 ± 0.01^{cB}	94.1 ± 0.07^{cB}	31.58 ± 0.02^{dD}	6.76 ± 0.15^{eE}	0.00 ± 0.00^{fD}	0.00 ± 0.00^{fC}	0.00 ± 0.00^{fC}
Ralstonia solanacearum	100 ± 0.00^{aA}	100 ± 0.00^{aA}	100 ± 0.00^{aA}	100 ± 0.00^{aA}	100 ± 0.00^{aA}	100 ± 0.00^{aA}	88.98 ± 0.78^{bA}	71.91 ± 1.14^{cA}	61.91 ± 1.18^{dA}
Agrobacterium tumefeciens	95.46 ± 0.40^{aB}	94.36 ± 0.37^{aBC}	78.8 ± 0.23^{bD}	49.57 ± 1.29^{cE}	45.65 ± 0.55^{dC}	27.13 ± 0.29^{eD}	16.47 ± 0.49^{fC}	9.54 ± 0.60^{gB}	8.22 ± 0.64^{gB}

*means (±SE) within the rows and columns followed by the different lowercase and uppercase letters are significantly different ($P \leq 0.05$; ANOVA and Tukey's HSD)

in Table 3. The ethanolic extract showed the presence of flavonoids, tannins, sterols and terpenes, saponins, carotenoids, carbohydrates, purines, free reducing sugars, soluble starch and quinones. Alkaloids did not show up in the extract.

Infrared spectroscopy (RI). Figure 1 shows Fourier's transform infrared spectroscopy of *M. tamaulipana*, with a strong and clear presence of peaks in different regions; like at 3 309.0 cm, due to the OH, 2 960–2 868 cm due to the phenolic group stretching vibration, as well as to the C-H, probably a branched alkane, 1 604.3–1 488.8 cm due to the yielding at the stretching band of the C-C ring, 1 510.0–1 456.1 cm due to the O-H's flexural vibration. The spectroscopy has a peak at 1 364.6 cm, probably due to an isopropyl group. On the other hand, three peaks at 1 264.1, 1 036.3, 1 024.1 cm were obtained, suggesting the presence of phenolic compounds, together with band 3 309.9 cm.

DISCUSSION

This research work showed that the ethanolic extract of *M. tamaulipana* leaves had inhibitory effects over the growth of phytopathogenic bacteria, Pst, Xv, Cmm, At, and Rs. The inhibition rate ranged between 8.22 and 100%. The inhibition of the bacterial growth increased as the extract concentrations were augmented. Several species of *Magnolia,* like *M. officinalis*, *M. grandiflora*, *M. liliflora*, *M. obovata* and *M. dealbata* have antibacterial and antifungal properties. The ethanolic extract of the spring flowers from *M. dealbata* inhibited the growth of *P. carotovorum* (inhibition zone 14.95 ± 0.76 mm), but the extract did not have any effect over *P. cichorii* (Ramírez-Reyes et al. 2015b). The same authors did not find any effect in the bark extract from the adult trees, poly-follicles, seeds and bark from the young trees collected during the spring, summer and fall. While the ethanolic extract obtained from the spring flowers of *M. schiedeana* had an inhibitory effect over *P. carotovorum,* the extract did not have any effect over *P. cichorii*. Furthermore, the ethanolic extract obtained from the poly-follicles, seeds, bark and leaves of the adult and young trees did not affect the growth of both strains (Ramírez-Reyes et al. 2015a).

On the other hand, the ethanolic seed extract of *M. dealbata* has an antimicrobial activity (inhibition zone > 10 mm) against *C. michiganensis*, *P. aeruginosa*, *A. baumannii*, *A. lwoffii*, *Candida albicans*,

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Table 2. Inhibitory concentrations of the *Magnolia tamaulipana* ethanolic extract against tomato phytopathogenic bacteria

Phytopathogenic bacteria	IC_{50} (CI_{95})	IC_{90} (CI_{95})	β	± SE	χ^2	Pr > χ^2
Pseudomonas syringae pv. tomato	34.71 (15.60–64.06)	239.62 (136.29–431.07)	2.61	0.30	74.45	< 0.0001
Xanthomonas vesicatoria	23.09 (14.93–33.87)	441.88 (280.48–781.36)	1.71	0.14	138.4	< 0.0001
Clavibacter michiganensis subsp. *michiganensis*	64.75 (55.01–75.12)	176.73 (147.78–221.41)	5.03	0.44	130.3	< 0.0001
Ralstonia solanacearum	–	–	–	–	–	–
Agrobacterium tumefeciens	97.72 (74.28–121.54)	535.48 (414.63–756.38)	2.97	0.31	93.53	< 0.0001

$IC_{50(90)}$ – inhibitory concentration in ppm, causing 50 (90%) inhibition of bacterial growth

Table 3. Qualitative phytochemical analysis of the *Magnolia tamaulipana* extracts

Phytochemicals	Ethanol extract		
Alkaloids	Dragendorff: –		Sonneschain: –
Carbohydrates	Molisch's: +		
Carotenoids	+		
Coumarins	–		
Flavonoids	flavonol: flavonol-xanthone		NaOH: flavanone
Free reducing sugars	Fehling's: +		Benedict: +
Glycosides	Gringnard: –		
Polysaccharides	lugol: –		
Purines	HCI: +		
Quinones	NH_4OH: –	H_2SO_4: anthraquinone	Börntraguer: benzoquinone
Saponins	foam: +		
Sterols and Terpenes	Liberman-Burchard: sterol		Rosenthaler: –
Soluble starch	KOH and H_2SO_4: +		gelatine: +
Tannins	$FeCI_3$: catechol		$K_3[Fe(CN)_6]$: phenols

+ indicates presence; – indicates absence

C. tropicalis and *Trichosporon belgeii* (Jacobo-Salcedo et al. 2011). Just like other *Magnolia* spp., *M. tamaulipana* also has an antibacterial activity. However, the distinctive results from different species might depend, to a great extent, on the parts of the plant used to make the extracts, the phenological stage of the sampling material, as well as the season in which the plant material was collected. According to the results obtained from this research work and other studies, *Magnolia* species are a source of new antimicrobial agents.

The genus *Magnolia* is a rich source of several biologically active compounds (Watanabe et al. 2002). The phytochemical analysis' results indicate that saponins, tannins, flavonoids, and terpenes were present in the ethanolic extract of the *M. tamaulipana* leaves (Table 3). The results confirm the presence of phenolic derivatives and flavonoids that are essential for deploying free radical scavenging abilities (Devatha et al. 2016). Alkaloids, coumarins, glycosides, and polysaccharides were absent in the *M. tamaulipana* leaves. Although, the dicotyledonous Magnoliaceae family is well known for producing alkaloids, and so is the genus *Magnolia*, from the phytochemical data available to date, it is evident that not all species of this genus are capable of producing alkaloids and coumarins (Watanabe et al. 2002). Devatha et al. (2016) reported the absence of alkaloids, terpenoids, and flavonoids in the extract of *M. champaca* leaves. Watanabe et al. (2002) reported that out of 37 *Magnolia* spp., only *M. grandiflora* has alkaloids, coumarins, flavonoids, lignans, neolignans, phenylpropanes, terpenoids, and other secondary metabolites. From this, we can infer that *Magnolias* are capable of showing biological activities of different intensity, against diverse phytopathogenic microorganisms, with the help of extracts obtained from different tissues and different solvent polarities.

Fourier-transform infrared spectroscopy (FTIR) is a high-resolution analytical tool that allows us to identify chemical compounds and elucidate their structural compounds. FTIR is a quick non-destructive test to detect fingerprints in herbal extracts or powders. FTIR spectroscopy is not capable of

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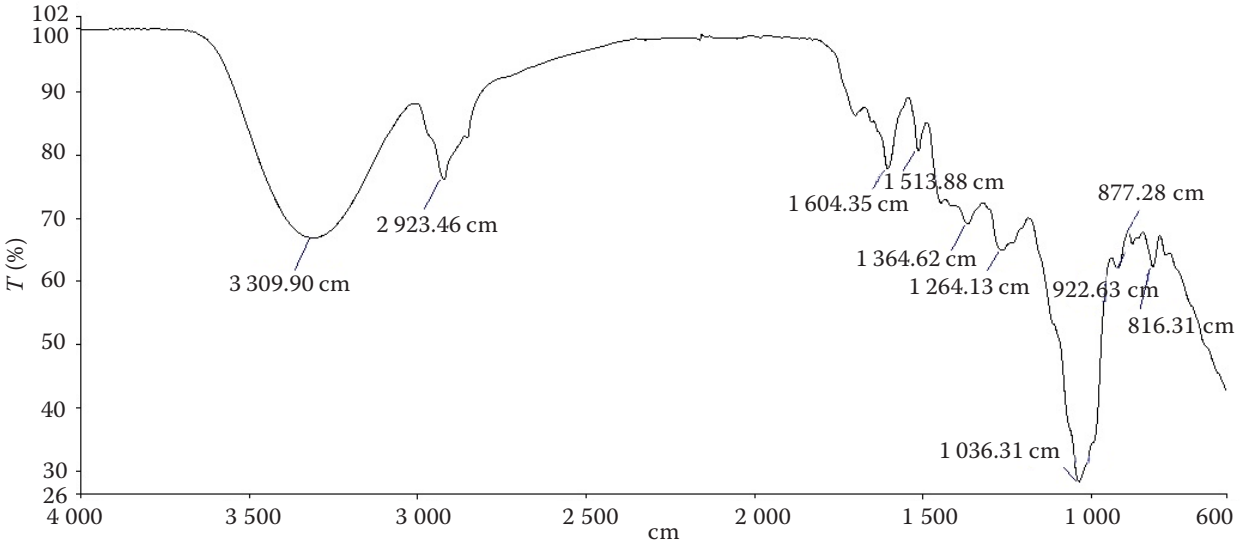

Figure 1. The infrared spectrum of the ethanol extract of the *Magnolia tamaulipana* leaves
T (%) – percentage of transmittance; cm = wavenumbers

determining the main compound of the test extract accurately, but it shows the most abundant chemical compounds (Altemimi et al. 2017). Ribeiro-Da Luz (2006) carried out a study of the total attenuated reflectance spectroscopy on leaves, as a tool for ecological and botanical studies. They observed the different phenological stages of several plant species, including *M. grandiflora*, which shows bands similar to the bands found in *M. tamaulipana*. The authors established a relationship between the peak at 1 050 cm (our result is 1 036.3 cm) and the presence of the CH_2, C=O and C-O groups, usually produced by the waxes and cutin in the leaves, linked to the abundance of amorphous silica on the leaf's surface. Wax and cutin, the vegetable cuticle, contain terpenoids and flavonoids, which have antifungal activities (Ziv et al. 2018).

The limited options of products for bacterial disease control, resistance, and the need to preserve the environmental and human health, demand nonharmful ecological alternatives. The possibility of detecting natural substances with a bactericidal effect can expand the variety of products that we can use against phytopathogenic bacteria for crop protection. The extract showed an *in vitro* inhibitory effect over the tomato phytopathogenic species, *P. syringae* pv. *tomato*, *X. vesicatoria*, *C. michiganensis* subsp. *michiganensis*, *R. solanacearum*, and *A. tumefeciens*. This could be due to the presence of the phytochemicals detected in the qualitative assay, as well as in the infrared spectroscopy analysis, where we confirmed the presence of some functional groups from families of compounds such as, carbohydrates, carotenoids, reducing sugars, flavonoids, purines, quinones, saponins, sterols, soluble starch, tannins, and phenols. According to our results, the ethanolic extract of *M. tamaulipana* has antibacterial properties and can become a new control agent. These results are sufficient grounds for future greenhouse or open-field trials that will help determine their effect over diseases that affect other crops.

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