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Evaluation of the antibacterial activity *in vitro* and in the greenhouse of the extracts of *Salvia amarissima* against *Clavibacter michiganensis* subsp. *michiganensis*

Evaluación de la actividad antibacteriana *in vitro* y en invernadero de extractos de *Salvia amarissima* contra *Clavibacter michiganensis* subsp. *michiganensis*

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ABSTRACT

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), stands as one of the most devastating threats for tomato cultivation. This study aimed to investigate the antimicrobial efficacy of the ethyl acetate fraction obtained from the acetone-soluble extract of *Salvia amarissima* leaves and flowers (EC-SA AcOEt), along with the diterpenoids amarissinina A (STJ-3) and amarissinina C (STJ-1), as potential biorational treatments for *Cmm* control, in comparison to a traditional chemical control. Through *in vitro* inhibition assays, it was observed that the diterpenoid STJ-1 from *S. amarissima* showed the highest antimicrobial activity among all the treatments, at a concentration of 25 µg/mL. Under greenhouse conditions, treatment with 25 µg/mL of STJ-1 from *S. amarissima* reduced in 30 % the incidence and 42 % the average severity index of bacterial canker in tomato plants in comparison to the positive control. The results suggest that *S. amarissima extracts*, particularly STJ-1, represent a promising biorational alternative for *Cmm* control in tomato crops. These extracts exhibit superior efficacy compared to traditional chemical products, offering a sustainable and effective solution in the fight against this disease.

KEY WORDS: Bacterial cancer, tomato, bioprospecting, cancer herb, bacterial control

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RESUMEN

El chancro bacteriano causado por *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), representa una de las amenazas más devastadoras para el cultivo de tomate. En este estudio se investigó la eficacia antimicrobiana de la fracción de acetato de etilo obtenida a partir del extracto soluble en acetona de hojas y flores *de Salvia amarissima* (EC-SA AcOEt), así como de los diterpenoides amarissinina A (STJ-3) y amarissinina C (STJ-1), como posibles tratamientos biorracionales para el control de *Cmm*, comparándolos con un control químico tradicional. Mediante ensayos de inhibición *in vitro*, se observó que el diterpenoide STJ-1 de *S. amarissima* mostró la mayor actividad antimicrobiana de todos los tratamientos, a una concentración de 25 µg/mL. En condiciones de invernadero, el tratamiento con 25 µg/mL de STJ-1 de *S. amarissima* redujo en un 30 % la incidencia y un 42 % el índice promedio de la severidad del cáncer bacteriano en plantas de tomate respecto al testigo positivo. Los resultados indican que los extractos de *S. amarissima*, en particular el STJ-1, representan una alternativa biorracional prometedora para el control de *Cmm* en los cultivos de tomate. Estos extractos exhiben una eficacia superior a la proporcionada por los productos químicos tradicionales, ofreciendo una solución sostenible y efectiva en la lucha contra esta enfermedad.

PALABRAS CLAVE: Cáncer bacteriano, tomate, bioprospección, hierba del cáncer, control bacteriano

Introduction

Tomato (*Solanum lycopersicum*) stands as one of the most consumed vegetables globally, playing a pivotal role in the Mexican agri-food sector (SADER, 2020). However, a persistent and highly damaging disease afflicting this crop is bacterial canker, attributed to the bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). Classified as an international quarantine pathogen (EFSA, 2014; EPPO, 2016), the severity of this disease underscores its potential impact on agriculture worldwide, leading to substantial economic losses by compromising both the quantity and quality of tomatoes (Gartemann *et al.,* 2003; Eichenlaub & Gartemann, 2011; Sen *et al.,* 2015; Nandi *et al.,* 2018). For instance, a study by Borboa-Flores *et al.* (2009) estimated annual economic losses of approximately US$40 million in Mexico due to this disease. Moreover, recent reports from tomato growers in Michigan, USA, have indicated losses amounting to up to US$300,000 (Peritore-Galve *et al.,* 2021).

Characteristic symptoms of bacterial canker encompass fruit damage, commonly referred to as bird's eye spots, as well as wilting and plant death, marginal chlorosis of leaves, and the

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formation of lesions on the canker stem (Bae *et al.,* 2015). These symptoms arise from *Cmm* infection, which obstructs the xylem, impeding plant water transport. The bacterium spreads through seeds (Werner *et al.,* 2002; Nandi *et al.,* 2018; Peritore-Galve *et al.,* 2021) and can also be transmitted via seedlings, tools, contaminated soil, and water (Tancos *et al.,* 2013), along with contact between fluids from healthy and diseased plants (Sharabani *et al.,* 2013).

Greenhouse production systems have been observed to be particularly prone to more severe symptoms and losses due to environmental conditions and continuous farming practices that favor pathogen proliferation (Martinez-Castro *et al.,* 2018; Yuqing *et al.,* 2018).

Currently, various strategies have been explored to combat bacterial canker, with the foremost approach revolving around the use of healthy seeds devoid of *Cmm* contamination (de León *et al.,* 2011). Chemical control methods primarily involve the application of antibiotics and cupric compounds, including copper oxychloride, oxide, or hydroxide (de León *et al.,* 2008; Milijašević *et al.,* 2009). Additionally, biological control methods (Nandi *et al.,* 2018), utilization of plant extracts (Stefanova Nalimova *et al.,* 2005; Siddique *et al.,* 2020), and bacteriocins (Mirzaee *et al.,* 2021) have been investigated. However, these treatments exhibit limited efficacy due to the considerable variation in pathogen aggressiveness and virulence (Croce *et al.,* 2016; Wassermann *et al.,* 2020), coupled with the lack of commercially available antibacterial compounds that address diverse environmental and toxicological concerns.

Recently, global attention has shifted towards employing plant products as chemotherapeutic agents for plant protection. Botanical pesticides are gaining traction, with several plant products being embraced worldwide as environmentally friendly alternatives. Natural compounds such as essential oils, phenolic compounds, saponins, flavonoids, terpenoids, steroids, fatty acids, alkaloids, and others isolated from medicinal and aromatic plants have a long history of demonstrating bioactivity against various pathogens (Hernandez-Diaz *et al.,* 2001; Nostro & Papalia, 2012). Over 550 diterpenoids have been documented from different *Salvia* species (Kabouche & Kabouche, 2008), many of which exhibit a broad spectrum of intriguing biological activities and fulfill ecological roles, including antimicrobial, anticancer, antiviral, antioxidant, and anti-inflammatory functions (Jassbi *et al.,* 2017; González-Chávez *et al.,* 2018). The exploration of botanicals derived from *Salvia amarissima* has garnered significant attention due to the presence of pharmacologically active molecules such as flavonoids, phenols, terpenoids, and steroids (Jassbi *et al.,* 2006; 2016).

Numerous investigations on various *Salvia* species have underscored their potent antibacterial activity, with examples cited below. Both crude extracts and bioactive compounds isolated from these plants have been utilized for comparison with positive controls. Plant secondary metabolites, including phenolic compounds, terpenes, and alkaloids, selectively exhibit antibacterial action against diverse microorganisms through various mechanisms, such as protein or adhesin binding, enzymatic inhibition, substrate deprivation, and membrane disruption (Cowan, 1999). While much of the research has focused on human pathogens like *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*, antibacterial activities have been reported in compounds isolated from species such as *Salvia reptans* and *S. greggii* against *Bacillus cereus*, *Micrococcus luteus* (Martínez-Vázquez *et al.,* 1998), and *Bacillus subtilis* (Kawahara *et al.,* 2004).

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Additionally, it has been noted that extracts of different polarities from *Salvia sessei* were effective against *Staphylococcus haemolyticus*, *S. hominis*, and *Enterococcus faecalis* (Gómez-Rivera *et al.,* 2018).

However, there remains limited research on the utilization of plant extracts as an alternative for controlling phytopathogenic bacteria in integrated crop management, particularly in the context of tomatoes. This represents a viable alternative with reduced environmental impact. A study by Stefanova Nalimova *et al.* (2005) discovered methanolic extracts from plants exhibit antimicrobial activity against phytopathogenic bacteria, specifically of the genus *Xanthomonas*, under *in vitro* conditions. Siddique *et al.* (2020) discovered in plant aqueous phytoextracts activity against the bacteria Clavibacter. Therefore, the objective of this study was to assess the antimicrobial activity of the ethyl acetate fraction of *Salvia amarissima*, derived from the acetone-soluble extract (EC-SA AcOEt), and two diterpenoids: amarissinin A (STJ-3) and C (STJ-1), through *in vitro* and greenhouse assays, focusing on their efficacy against *Clavibacter michiganensis* subsp. *michiganensis (Cmm).*

Material and Methods

Isolation and purification of chemical constituents from *Salvia amarissima*

The ethyl acetate fraction, derived from the acetone-soluble extract of *Salvia amarissima* leaves and flowers (EC-SA AcOEt), along with the diterpenoids amarissinin A (STJ-3) and amarissinin C (STJ-1), were utilized in this study, having been previously prepared and characterized according to Fragoso-Serrano *et al.* (2019). In summary, dried and ground leaves and flowers (3.5 kg) of *S. amarissima* underwent extraction through maceration with acetone (20 L) across three stages with independent solvent replacement. The resultant extract was filtered, and the solvent was distilled under reduced pressure, yielding 190 g of extract. This extract was suspended in hexane (1 L) and subjected to liquid-liquid extraction with a MeOH/H_2O 4:1 mixture across three stages with independent solvent replacement (3 × 1 L). The hydroalcoholic phase underwent distillation under reduced pressure to remove methanol (MeOH), followed by the addition of 1 L of water to the resulting suspension, which was then subjected to liquid-liquid extraction with ethyl acetate (AcOEt, 1 L × 3). The organic phase was dried with anhydrous Na_2SO_4, filtered, and the solvent was distilled under reduced pressure, resulting in 50 g of residue (SMSPA AcOEt). A 46.3 g sample of the EtOAc fraction underwent purification through silica gel column chromatography (60 G, 4.5 cm i.d. × 15.0 cm h, frs. 500 mL), eluting with hexane/EtOAc and EtOAc/acetone mixtures in ascending polarity. From the obtained fractions, amarissinin C (STJ-1) (3.1 g, obtained with hexane/EtOAc 4:1) and amarissinin A (STJ-3) (1.5 g, obtained with hexane/EtOAc 7:3) were isolated and purified. The compounds were identified through comparison with authentic samples of each substance via thin-layer chromatography and through comparison of their 1H and 13C nuclear magnetic resonance spectra (Figs. S1-S4) (Bautista *et al.,* 2016).

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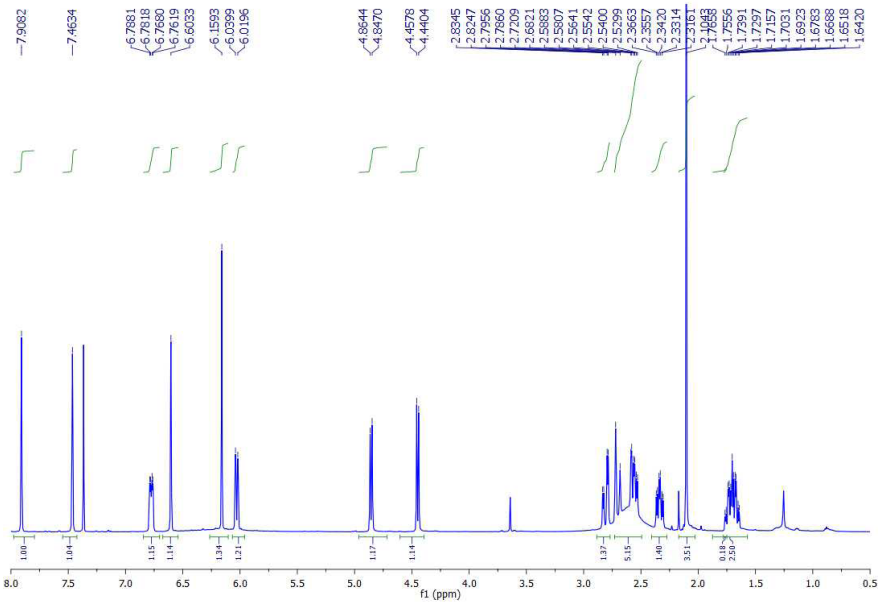

Figure S1. [1] H NMR spectrum of compound STJ-3 (amarissinin A) in $CDCl_3$ + DMSO-d_6 at 500 MHz.

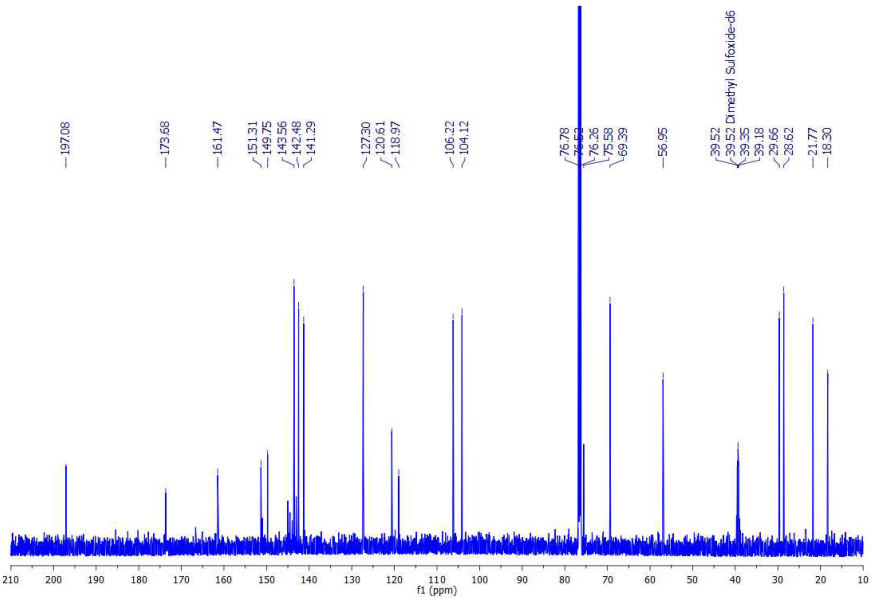

Figure S2. [13]C NMR spectrum of compound STJ-3 (amarissinin A) in $CDCl_3$ + DMSO-d_6 at 500 MHz.

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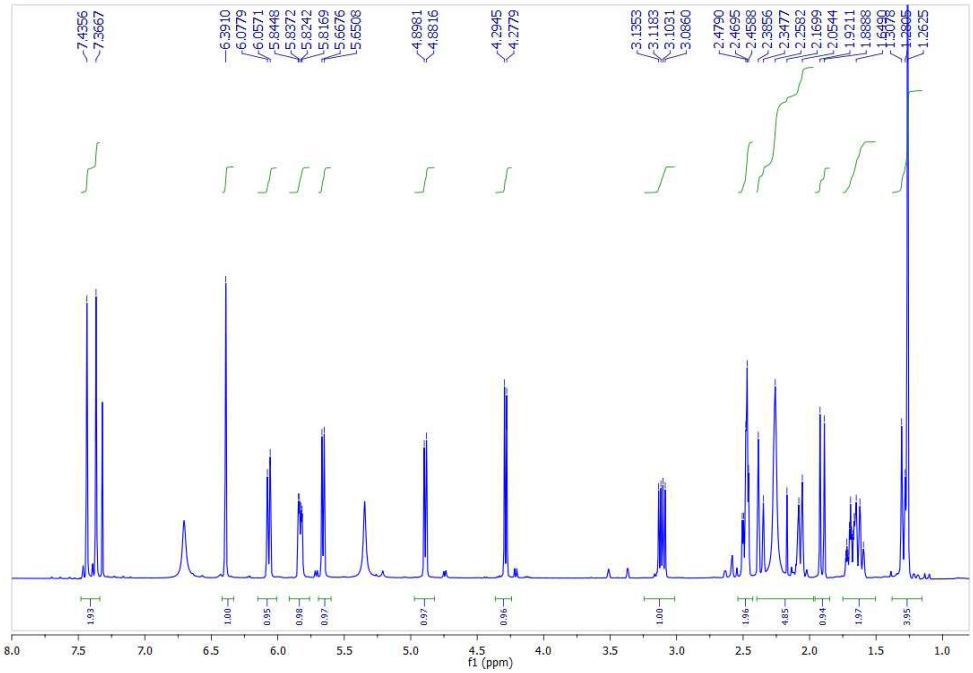

Figure S3. [1] H NMR spectrum of compound STJ-3 (amarissinin C) in $CDCl_3$ + DMSO-d_6 at 500 MHz.

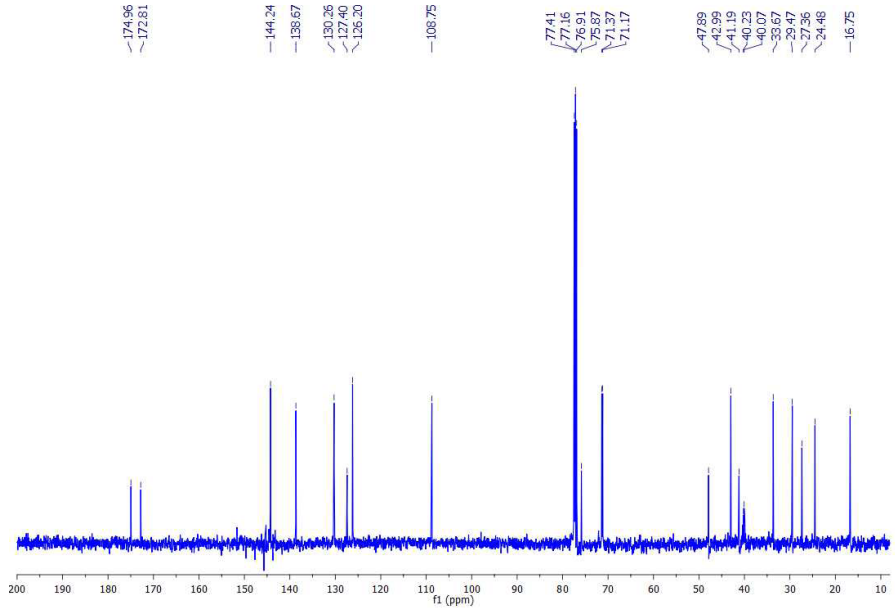

Figure S4. [13]C NMR spectrum of compound STJ-3 (amarissinin C) in $CDCl_3$ + DMSO-d_6 at 500 MHz.

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The AcOEt fraction (EC-SA AcOEt) and the diterpenoids amarissinin A (STJ-3) and C (STJ-1) (Table 1) were dissolved in water:acetone 85:15 vehicle to achieve initial concentrations of 10 mg/mL and were subsequently employed in *in vitro* and greenhouse assays.

Bacterial culture of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)

The bacterial strain *Cmm* AcR42, obtained from cultures of *S. lycopersicum* in Penjamo, Guanajuato, Mexico, and characterized in the Biología Molecular de Plantas (Plant Molecular Biology) laboratory at IPICYT, was employed.

For the preparation of the *Cmm* inoculum, a pure culture was grown for 48 hours in an 802 liquid medium containing polypeptone (1 g/L), yeast extract (2 g/L), and magnesium sulfate (0.92 g/L). The culture was adjusted to an absorbance of 0.2 (approximately 1 x 10^5 CFU/mL) at a wavelength of 600 nm, using sterile deionized water.

Determination of the *in vitro* Minimum Bactericidal Concentration (MBC) of *S. amarissima* extract

The 802 broth dilution method was employed using the total ethyl acetate extract (EC-SA AcOEt) and the two diterpenoids of *S. amarissima* (STJ-1 and STJ-3). Five different dilutions were tested: 100, 50, 25, 12.5, and 6.25 µg/mL. Cultures were prepared in five tubes, each containing 1 mL of sterile 802 broth for each treatment, and five replicates were conducted for each concentration. After 24 hours, 100 µL samples were extracted from the tubes, inoculated in Petri dishes with 802 agar, and incubated at 28°C for 24 and 48 hours, following the method outlined by Horna *et al.* (2005). Subsequently, the bactericidal effect of the extracts was evaluated by counting Colony-Forming Units per milliliter (CFU/mL), as proposed by Toribio *et al.* (2004).

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Table 1. Description and concentrations of treatments of *S. amarissima* extracts and controls to determine *in vitro* antimicrobial activity against *Clavibacter michiganensis* subsp. *michiganensis*.

Treat	Name	Final Concentration	Description
T1	EC-SA AcOEt	100 µg /mL	From a stock of 10 mg/mL previously diluted in water: acetone (85:15), each of the concentrations was adjusted with the crude extract of S. *amarissima* (EC-SA).
T2		50 µg /mL	
T3		25 µg /mL	
T4		12.5 µg /mL	
T5		6.25 µg /mL	
T6	Diterpenoid STJ-1	100 µg /mL	From a stock of 10 mg/mL previously diluted in water: acetone (85:15), each of the concentrations was adjusted with the fraction of S. *amarissima* (STJ-1).
T7		50 µg /mL	
T8		25 µg /mL	
T9		12.5 µg /mL	
T10		6.25 µg /mL	
T11	Diterpernoid STJ-3	100 µg /mL	From a stock of 10 mg/mL previously diluted in water: acetone (85:15), each concentration was adjusted with the crude extract of S. *amarissima* (STJ-3).
T12		50 µg /mL	
T13		25 µg /mL	
T14		12.5 µg /mL	
T15		6.25 µg /mL	
T16	Positive Control of chemical treatment (TQ)	3 g/L	Copper oxychloride 3 g/L was used following the recommendations of the manufacturer for the control of *Cmm*. It is one of the most widely used agrochemicals against *Cmm*.
T17	Solvent Control (TS)	85:15 Water: acetone	Water and acetone were added at a ratio of 85:15 and the bacterial suspension to ensure that the solvent did not affect the growth of the bacteria and that any bactericidal action was due to the effects of the plant compounds.
T18	Growth Positive Control (T+)	*Cmm* 1×10^5 CFU/mL	Culture medium and bacterial suspension were added. Growth of *Cmm* expected.
T19	Growth Negative Control (T-)	No *Cmm*	Only 100 µL of 802 culture medium was added. No growth of microorganisms is expected.

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Greenhouse bioprospecting test with tomato plants

Tomato plants (*Solanum lycopersicum* var. Ailsa Craig) at 38 days after germination (dag) were utilized, with seeds provided by the University of Nottingham. Seed germination involved surface disinfection using a 5 % sodium hypochlorite solution, followed by sowing in individual pots containing commercial soil mix (Sunshine Mix #6, Sun Grow Horticulture, Vancouver, BC, CA). The pots were then placed in a growth chamber at 25 °C, with a light-dark cycle of 16 hours light and 8 hours dark. Subsequently, the plants were transferred to a high-tech greenhouse with separate areas designated for challenges with microorganisms, maintaining a controlled temperature of 28 °C and humidity of 60 %. A total of seven treatments with 10 replicates were implemented for incidence and severity trials in the greenhouse. These treatments were as follows: T1 - crude extract of *S. amarissima* (EC-SA AcOEt) at 50 µg/mL, T2 and T3 - two diterpenoids (STJ-1 and STJ-3) at 25 µg/mL each, T4 - chemical control using 3 g/L copper oxychloride, T5 - solvent control consisting of water:acetone in a ratio of 85:15, T6 - Positive Control involving 100 µL of *Cmm* bacterial suspension adjusted to 1 x 10^5 CFU/mL, and finally, T7 - Negative Control represented by syringe inoculation with 0.1M $MgCl_2$ without *Cmm*.

Pathogen growth, plant inoculation, and treatment application

For challenges with *Cmm*, the inoculum was adjusted to an absorbance of 0.2 (approximately 1 x 10^5 CFU) at a wavelength of 600 nm. Infection was initiated by injecting 0.5 mL of the inoculum using an insulin needle into the stem between the root and the first true leaf, 40 days after germination (dag) of tomato plants.

The first application of the treatments was carried out preventively and through drenching, with a volume of 50 mL applied directly to the substrate when the plants were 38 dag. Subsequently, three more applications of the treatments were administered every 15 days until fruit harvest (90 dag). These treatments were applied via spraying, and the progression of the disease in the plants was monitored by collecting data at 55 and 90 dag.

Monitoring and measurement of agronomic data in tomato plants

In all treatments, plant height from the base of the soil to the tip of the main apex, stem diameter (1 cm above the cotyledons), and number of flower clusters were evaluated; these measurements were made 55 days after germination (dag). Yield was evaluated based on fruit weight per treatment and data for this category were obtained at 90 dag.

Assessment of incidence and severity

Two evaluations of tomato plants after *Cmm* infection were conducted. The first assessment occurred when the plants were 55 dag, and the second evaluation was performed at 90 dag.

In each experimental unit, the percentage of affected or incidence (I) (Equation I) and the

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Average Severity Index (ASI) (Equation II) were calculated using the methodologies proposed by Arauz (1998) and Couto *et al.* (2007), respectively.

The formulas for calculating these indices are as follows:

$$I = \frac{Total\ of\ diseased\ plants}{Total\ of\ sampled\ plants} \times 100 \qquad \text{I}$$

$$ASI = \frac{(Severity\ grade\ X\ Frecuency)}{Total\ of\ evaluated\ plants} \times 100 \qquad \text{II}$$

In this study, incidence refers to the proportion of diseased plants in relation to the total number of plants evaluated and is reported as a percentage. Quantitative data on leaf damage associated with *Cmm* bacteria was obtained by counting the number of damaged branches per plant. The average severity index (ASI) measures the frequency with which different categories of damage are observed in relation to the total number of diseased plants. For this particular study, five severity categories were utilized, which were based on the percentage of foliage affected by the phytopathogen *Cmm* (Table 2).

Table 2. Severity categories for the evaluation of damage caused by *Clavibacter michiganensis* subsp. *michiganensis* on tomato plants.

Severity category	Affected foliage (%)
I	1-20
II	21-40
III	41-60
IV	61-80
V	81-100

Statistical analysis for *in vitro* and greenhouse assays

The data from the *in vitro* assays were analyzed using a completely randomized experimental design comprising 19 treatments (Table 1) with 5 replicates. Each repetition corresponded to one Petri dish per evaluation. An analysis of variance and a Tukey mean comparison test ($p > 0.05$)

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were conducted based on the results obtained from the CFU/mL counts at 24 and 48 hours. The statistical analysis was carried out using the Minitab v2017 software.

For the greenhouse bioprospecting trials, data on agronomic variables such as plant height, stem diameter, number of flower clusters, and yield were analyzed. Additionally, incidence, foliar damage, and severity data were collected on tomato plants using a completely randomized experimental design consisting of 7 treatments with 10 replicates. Each replicate represented one tomato plant evaluated. An ANOVA was performed, followed by a mean comparison test using Tukey's method (*p-value > 0.05*). These analyses were conducted using the Minitab v2017 statistical program. The results were based on evaluations conducted at 55 and 90 dag.

Results and Discussion

Determination of the *in vitro* Minimum Bactericidal Concentration (MBC*)* of *S. amarissima* extract.

Table 3 describes the results of the *in vitro* antimicrobial activity of the AcOEt fraction of *Salvia amarissima* (EC-SA AcOEt) and two isolated diterpenoids (STJ-1 and STJ-3) at different concentrations ranging from 100 to 6.25 µg/mL, as well as the controls; chemical, solvent, positive and a negative control through the measurement of the concentration of colony forming units (CFU/mL) of *Clavibacter michiganensis* subsp. *michiganensis (Cmm)* and at different time intervals (24 and 48 h).

Table 3. Effect of *Salvia amarissima* crude extract (EC-SA AcOEt) and fractions STJ-1 and STJ-3 on the growth of *Clavibacter michiganensis* subsp. *michiganensis.*

No.	Treatment	Final Concentration	*Cmm* (CFU/mL) x 10^7 24 h	*Cmm* (CFU/mL) x 10^7 48 h
T1		100 µg /mL	171.60 ± 3.66 ^{d}	255.20 ± 2.47 ^{d}
T2		50 µg /mL	77.00 ± 2.02 ^{ef}	155.60 ± 2.50 ^{e}
T3	EC-SA AcOEt	25 µg /mL	67.80 ± 3.94 ^{ef}	143.00 ± 2.48 ^{e}
T4		12.5 µg /mL	51.40 ± 2.76 ^{fg}	129.20 ± 3.48 ^{efg}
T5		6.25 µg /mL	41.40 ± 3.14 ^{fg}	121.80 ± 2.55 ^{efg}

Average plus standard error, equal letters, no significant differences by Tukey's test (*p > 0.05).*

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Continuation

Table 3. Effect of *Salvia amarissima* crude extract (EC-SA AcOEt) and fractions STJ-1 and STJ-3 on the growth of *Clavibacter michiganensis* subsp. *michiganensis.*

No.	Treatment	Concentration	*Cmm* (CFU/mL) x 10^7 24 h	*Cmm* (CFU/mL) x 10^7 48 h
T6		100 µg /mL	127.80 ± 3.72 ^{e}	170.80 ± 1.93 ^{d}
T7		50 µg /mL	56.60 ± 1.36 ^{g}	97.20 ± 1.85 ^{ef}
T8	Diterpenoid STJ-1	25 µg /mL	4.40 ± 0.81 ^{h}	39.20 ± 2.98 ^{h}
T9		12.5 µg /mL	5.00 ± 1.30 ^{h}	38.80 ± 2.92 ^{h}
T10		6.25 µg /mL	6.20 ± 0.86 ^{h}	38.40 ± 2.37 ^{h}
T11		100 µg /mL	133.40 ± 2.83 ^{e}	174.20 ± 4.45 ^{d}
T12		50 µg /mL	64.60 ± 2.33 ^{g}	95.40 ± 1.63 ^{e}
T13	Diterpenoid STJ-3	25 µg /mL	15.00 ± 1.41 ^{h}	48.00 ± 1.14 ^{fgh}
T14		12.5 µg /mL	8.60 ± 0.67 ^{h}	40.60 ± 2.52 ^{gh}
T15		6.25 µg /mL	7.20 ± 1.46 ^{h}	40.60 ± 2.11 ^{gh}
T16	Chemical Control (TQ)	3 g/L	233.4 ± 11.17 ^{c}	342.0 ± 24.69 ^{c}
T17	Solvent Control (TS)	85:15	294.4 ± 32.98 ^{b}	434.60 ± 9.19 b
T18	Positive Control (T+)	*Cmm* +	356.2 ± 14.01 ^{a}	485.60 ± 5.98 ^{a}
T19	Negative Control (T-)	*Cmm* -	0 ± 0 ^{i}	0 ± 0 ^{h}

Average plus standard error, equal letters, no significant differences by Tukey's test ($p > 0.05$).

At 24 h of incubation, the best antimicrobial effect was that of treatment T8: STJ-1 at a concentration of 25 µg /mL, showing the lowest bacterial growth of 4.40 x 10^7 CFU/mL of *Cmm*. Treatments T9 and T10 corresponding to the diterpenoid STJ-1 at 12.5 µg /mL and 6.25 µg /mL, also controlled the bacteria to a lesser degree than T8. Next in effectiveness followed treatments T13-T15, which were statistically equal and showed differences from the controls, and were better than the chemical control (T16= 233.4 x 10^7 CFU/mL), thus recommending the use of STJ-1 and 3 fractions at 25 µg/mL to control *Cmm* growth (Table 3). These results are related to the work of Bozov *et al.* (2020) who isolated a new neoclerodane diterpenoid and two fur-clerodane diterpenoids from the aerial parts of *Camedrio aquatica* (*Teucrium scordium, Lamiaceae*) and tested their antibacterial and antifungal activity against various pathogens, finding similar activities against bacterial species such as *Staphylococcus aureus, Staphylococcus pyogenes, L. monocytogenes, Escherichia coli* and *Salmonella abony* with Minimum Inhibitory Concentration (MIC) values ranging

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from 250 to 500 µg /mL (Bozov *et al.,* 2020). On the other hand, Fozia *et al.* (2021), isolated two new diterpenes from Horehound (*Ballota pseudodictamnus*, *Lamiacea*e); ballodolyl acids A and B, and were tested for their antibacterial activity against *E. coli* and *Salmonella typhi*. Finding that at a concentration of 30 µg/mL, both compounds exhibit more potent antibacterial properties in the Zone of Inhibition (ZOI) ranging between 11-13 and 11-12 mm, respectively, against *E. coli* and *S. typhi* strains (Fozia *et al.,* 2021).

Regarding the ethyl acetate extract of *Salvia amarissima* (EC-SA AcOEt) (T1-T5), it was observed that the most effective concentration for inhibiting the growth of *Cmm* was 6.25 µg/mL, resulting in 41.40 x 10^7 CFU/mL (T5). Although no significant differences were noted between concentrations T2-T5, it was concluded that applying this compound within the concentration range of 6.25 to 50 µg/mL is effective for controlling the phytopathogen *Cmm*. Fragoso-Serrano *et al.* (2019) identified a diterpene called amarisolide in the chemical composition of this extract, which corresponds to a neoclerodane glycoside and may be responsible for the observed antimicrobial activity. However, previous studies by López-Ferrer *et al.* (2010) demonstrated that hexanoic and methanolic extracts of *S. amarissima* did not exhibit antimicrobial activity against several bacterial species, a finding that could be related to the results reported by Horiuchi *et al.* (2007). The latter study suggested that compounds such as carnosol and carnosic acid, present in extracts of common sage, have a synergistic action that weakly inhibits the growth of Gram-positive bacteria while not affecting Gram-negative bacteria due to the presence of an external membrane that hinders their entry.

At 48 hours of incubation, a similar inhibition behavior against *Cmm* was observed compared to the results at 24 hours. The most effective treatments were T10 and T15 for STJ-1 and STJ-3, respectively, with CFU/mL counts of 38.40 x 10^7 and 40.60 x 10^7. For EC-SA, the most effective treatment was T5 at a concentration of 6.25 µg/mL. The Chemical Control (T16) exhibited a population of 342 x 10^7 CFU/mL, which, although statistically lower than the positive control, was less efficient than the treatments with EC-SA AcOEt extracts and fractions STJ-1 and STJ-3. The best inhibition results against *Cmm* were obtained with treatments T5, T10, and T15, showing more effective data than the Solvent Control at both exposure times. This suggests that the observed antimicrobial activity is not solely attributable to the solvent used in the preparation of the extract. The Positive Control (T18) exhibited the highest *Cmm* population (485.60 x 10^7 CFU/mL) compared to all treatments and controls after 48 hours. It is noteworthy that the negative control (T-) showed no bacterial growth at both time intervals, indicating the absence of *Cmm* in this treatment.

Evaluation of agronomic variables in tomato plants during greenhouse bioprospecting trials

Table 4 summarizes the results obtained 55 days after germination (dag) of the plants and after the second application of the *S. amarissima* extract and diterpenoid treatments. Despite the most effective concentrations identified in previous trials being 25 µg/mL or lower, a concentration of 50 µg/mL was included in the greenhouse trials. This decision was made considering that treatments tend to be less effective under these conditions due to the complexity of factors

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involved. Significant differences in agronomic characteristics among the treatments were observed. Plants treated with EC-SA AcOEt (50 µg/mL) (T1) exhibited the greatest average height (43.26 cm), followed by plants treated with Diterpenoid STJ-3 (25 µg/mL) (T3) and Negative Control (T-) (42.48 cm). These three conditions showed statistically significant differences among themselves. Conversely, plants under the Positive Control (T+) treatment had the lowest average height (28.71 cm). Notably, by this time, a high incidence and severity associated with bacterial canker were observed, and approximately 70 % of the plants in this treatment had already died, preventing them from reaching the flowering and fruiting stage.

Table 4. Agronomic characteristics of tomato plants against infection by *C. michiganensis* subsp. *michiganensis* at 55 days after germination (55 dag).

Treatment	Plant height (cm)	Stem diameter (cm)	No. of flower clusters	Yield fruit weight (g) (90 dag)
T1: EC-SA AcOEt (50 µg /mL)	43.26 [a]	0.80 [b]	3.58 [c]	113.99 [d]
T2: Diterpenoid STJ-1 (25 µg /mL)	40.12 [e]	0.78 [c]	4.45 [a]	125.18 [a]
T3: Diterpenoid STJ-3 (25 µg /mL)	41.15 [c]	0.76 [d]	4.05 [b]	120.33 [c]
T4: Solvent Control (TS)	39.43 [f]	0.74 [f]	1.04 [f]	0 [f]
T5: Chemical Control (TQ)	40.68 [d]	0.76 [e]	2.36 [e]	102.61 [e]
T6: Positive Control (T+)	28.71 [g]	0.61 [g]	0 [g]	0 [g]
T7: Negative Control (T-)	42.48 [b]	0.83 [a]	3.48 [d]	123.48 [b]

Averages with the same letter in the same column are statistically equal. Tukey mean differences ($p > 0.05$).

In terms of stem diameter, plants treated with EC-SA AcOEt (T1) and Diterpenoid STJ-1 (T2) exhibited larger diameters on average (0.80 cm and 0.78 cm, respectively), while those under the Positive Control (T+) had the smallest diameter on average (0.61 cm). The highest average number of flower clusters was observed in plants treated with Diterpenoid STJ-1 (T2) (4.45 flower clusters), followed by those treated with Diterpenoid STJ-3 (T3) and EC-SA AcOEt (T1). Notably, an acceleration in flowering time was observed in the EC-SA AcOEt treatment (T1), with the first flowers appearing on day 43-45 dag. In contrast, plants under the Solvent Control (TS) treatment exhibited the lowest number of flower clusters (1.04 clusters), and a value of 0 was recorded for T6 due to plants already dying from *Cmm* infection.

Fruit yield results obtained at 90 dag were highest in plants treated with Diterpenoid STJ-1 (T2) (125.18 g), followed by those treated with Negative Control (T-) (123.48 g) and EC-SA AcOEt (T1) (113.99 g). These differences were statistically significant among themselves. Additionally, the Chemical Control (TQ) also exhibited a fruit yield (102.61 g).

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Treatments with EC-SA AcOEt (T1) and Diterpenoid STJ-1 (T2) demonstrated positive impacts on tomato plant growth and yield, characterized by increased stem heights and diameters, as well as a higher number of flower clusters and fruit yield. These results suggest that these treatments could be considered potential strategies to improve tomato yield under *Cmm*-infected conditions at 75 dag.

Analysis of incidence and severity assessment in tomato plants

Table 5 summarizes key data related to the incidence (percentage of affected plants), leaf damage (number of dead branches per plant), and severity (degree of affectation in affected plants) of bacterial canker symptoms at two critical moments of plant development (at 55 and 90 days after germination, respectively), corresponding to the second and fourth application of the treatments.

According to Table 5, treatment T1: EC-SA AcOEt (50 µg/mL) exhibited an initial incidence of 50 % at 55 days after germination (dag), which increased to 70 % at 90 dag. During the same period, the average severity index (ASI %) showed a significant increase from 40.6 % to 67.6 %. Treatment T2: Diterpenoid STJ-1 (25 µg/mL) displayed an incidence of 40 % at 55 dag, rising to 80 % at 90 dag, with the ASI % increasing from 37.8 % to 59.4 %. Similarly, T3: Diterpenoid STJ-3 (25 µg/mL) showed a similar pattern, with an incidence of 40 % at 55 dag and an increase to 70 % at 90 dag, while the ASI % increased from 42.7 % to 66.8 %.

In contrast, the T4:TS treatment showed high incidence and severity at both evaluation times, reaching 70 % incidence at 55 dag and increasing to 90 % at 90 dag. The ASI % also notably increased from 75.3 % to 93.6 %, indicating ineffectiveness in preventing or controlling the disease. The solvent control did not significantly differ from the positive control, suggesting the active compounds of *S. amarissima*, present in the STJ-1 fraction, effectively controlled the spread of the phytopathogen *Cmm*.

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Table 5. Evaluation of the incidence, leaf damage, and severity of symptoms associated with bacterial cancer in tomato plants (*S. lycopersicum*).

Treatment	55 dag (2nd application of treatments)			90 dag (4th application of treatments)		
	Incidence (diseased plants %)	Foliar damage (No. of damaged branches)	Average Severity Index (ASI %)	Incidence (diseased plants %)	Foliar damage (No. of damaged branches)	Average Severity Index (ASI %)
T1: EC-SA AcOEt (50 µg /mL)	50 %	46.0 ^{c}	40.6 ^{e}	60 %	70 $_{a}$	67.6 ^{d}
T2: Diterpenoid STJ-1 (25 µg /mL)	40 %	28.3 ^{a}	37.8 ^{f}	70 %	86.6 ^{b}	59.4 ^{e}
T3: Diterpenoid STJ-3 (25 µg /mL)	40 %	37.5 ^{b}	42.7 ^{d}	60 %	73.4 ^{a}	66.8 ^{d}
T4: Solvent Control (TS)	70 %	71.3 ^{e}	75.3 ^{b}	90 %	96.8 ^{c}	93.6 ^{b}
T5: Chemical Control (TQ)	60 %	63.4 ^{d}	55.2 ^{c}	80 %	85.1 ^{b}	85.4 ^{c}
T6: Positive Control (T+)	70 %	80.6 ^{f}	84.7 ^{a}	100 %	98.8 ^{c}	100 ^{a}
T7: Negative Control (T-)	0 %	0 ^{g}	0 ^{g}	0 %	0 ^{d}	0 ^{f}

Averages with the same letter in the same column are statistically equal. Tukey mean differences ($p > 0.05$).

For T5: Chemical Control (TQ), an incidence of 60 % was observed at 55 dag, increasing to 80 % at 90 dag, accompanied by an ASI % increase from 55.2 % to 85.4 %, indicating limited effect of copper application for disease control. Treatment T6: Positive control (T+) exhibited 100 % incidence and severity at both evaluation times, indicating severe and constant infection. T7: Negative control (T-) showed no incidence or severity at any evaluation time.

The results highlight the effectiveness of treatments T1 (EC-SA AcOEt) (Figure 1A) and T2 (Diterpenoid STJ-1) (Figure 1B) in reducing the incidence and severity of bacterial canker in tomato plants, surpassing even the commercial chemical control (TQ) and the positive control (T+). These findings support the promising prospect of using *S. amarissima* extracts and diterpenoids as natural and effective alternatives for *Cmm* control in agriculture.

The data from Table 5 and Figure 1 provide a clear understanding of the effects of different treatments on the phenotype of tomato plants during evaluation against the phytopathogen *Cmm*. The treatments with the crude extract, fraction STJ-1, and STJ-3 of *Salvia amarissima* (T1, T2,

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and T3) demonstrated a positive effect on controlling the incidence and severity of bacterial canker in tomato plants. Conversely, treatments TS (T4) and T5 (TQ) were ineffective in controlling the disease. Likewise, T+ (T6) treatment demonstrated that the bacteria-causing bacterial canker was able to induce the highest incidence and severity of symptoms in tomatoes.

Figure 1. Phenotype of tomato plants (*S. lycopersicum*) of 55 dag during the first evaluation of the different treatments used against *Cmm*.

A) Total extract of *S. amarissima* T1: EC-SA AcOEt (50 µg/mL), B) STJ-1 diterpene (25 µg/mL), C) STJ-3 diterpene (25 µg/mL). Triplicates are shown in each treatment.

Conclusions

The *in vitro* assay indicates that the three extracts from *S. amarissima* have *in vitro* antimicrobial activity against the tomato phytopathogen *Cmm*, which suggests that they could have potential as antimicrobial agents for the control of this bacterium in crops, even with better results than commercial control; this may be due to the different types of terpenes and phenols that this botanical species possesses and that have been reported in various works as compounds with polyphenolic structure that promote cell lysis (Monroy *et al.,* 2007). This study is the first to report the activity of *S. amarissima* extract against *Cmm* in tomato plants; however, the ability of *Salvia officinalis* to control different plant diseases has been tested, showing effectiveness against late blight of potato (Dorn *et al.,* 2007) and downy mildew of cucumber, onion, and lettuce (Schmitt *et al.,* 2008; Nowak *et al.,* 2009).

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Specifically, the application of the EC-SA AcOEt fraction and the isolated diterpenoids STJ-1 and STJ-3 from *S. amarissima* showed significant inhibition of *Cmm* growth under *in vitro* conditions. The most effective treatments were observed at concentrations of 6.25, 25, and 6.25 µg/mL, respectively, after 24 hours of incubation. Treatment T10 (STJ-1 at 6.25 µg/mL) exhibiting the best inhibition of bacterial growth at 48 hours.

In terms of agronomic results, the most effective treatment for tomato plant growth and yield was T1: EC-SA AcOEt at 50 µg/mL, which achieved a growth of 43.26 cm height, while T2: Diterpenoid STJ-1 at 25 µg/mL resulted in a yield of 125.18 g per plant. These findings indicate that Diterpenoid STJ-1 from *S. amarissima* shows promising potential to inhibit pathogen growth and improve yield.

Additionally, the T2: Diterpenoid STJ-1 treatment at 25 µg/mL also showed a positive effect by reducing incidence and severity to 70 % and 59.4 %, respectively, at 90 days after germination, compared to T6: Positive control (T+), which showed 100 % incidence and severity during the same evaluation period. This supports the potential of these extracts in the control of this disease in the tomato crop.

Overall, the diterpenoid STJ-1 from *S. amarissima* emerges as a promising natural alternative for controlling bacterial diseases in tomato crops, surpassing traditional chemical control methods in terms of efficacy. These findings contribute to the field of bioprospecting and suggest new possibilities for developing sustainable and environmentally friendly solutions for crop protection against pathogens.

Author contribution

Conceptualization of the work, I.G. López-Muraira, A.G. Alpuche-Solís; Development of methodology, E.M. Rodríguez-González; Photographs, E.M. Rodríguez-González; Data analysis, E.M. Rodríguez-González, I.G. López-Muraira, A.G. Alpuche-Solís; Writing and manuscript preparation, E.M. Rodríguez-González, I.G. López-Muraira, A.G. Alpuche-Solís; Drafting, revising and editing, H. Flores-Martínez, H. Silos-Espino, I. Andrade-González, V.S. Farías-Cervantes, I.G. López-Muraira, A.G. Alpuche-Solís; Fund acquisition of, A.G. Alpuche-Solís.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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