EFFECT OF *HELIOTROPIUM ARBAINENSE* EXTRACTS AND BIOAGENTS ON CONTROLLING BACTERIAL CANKER OF TOMATO PLANT

Nerhan A. Eid[1*], Nawal H. Mohamed [2] and Ahmed A. ElSharawy[3]

[*1]Department of Plant Protection, Desert Research Center, Cairo, Egypt

[2]Department of Medicinal and Aromatic Plants, Desert Research Center, Cairo, Egypt

[3]Department of Plant Production, Faculty of Environmental Agricultural Science, Arish University, Egypt

[*]E-mail: nerhan84@gmail.com

Many diseases in both human and plants are treated with natural compounds derived from medicinal plants and their extract. This study delves into the effects of the total alcoholic extract of *Heliotropium arbainense* and its subsequent fractions (ether, chloroform, ethyl acetate and water) were studied as potential alternatives and safe tools for combating *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) infecting tomato plants. Notably, this study showed that ethyl acetate, ether extracts of *H. arbainense* demonstrated clear antimicrobial activity against Cmm. Chromatographic techniques were employed to isolate flavonoid-free and glycoside compounds from the most potent fractions. Concurrently, rhizobacteria, specifically *Pseudomonas taiwanensis* strain Pst1 OP984768 and *Bacillus velezensis* strain Bv1 OP984765, were identified for their potential roles in promoting plant growth and acting as bioagents against bacterial wilt diseases. *In vitro* assays and greenhouse experiments were performed to assess the efficacy of these strains in controlling tomato bacterial wilt caused by Cmm. A synergistic approach, combining fresh cultures of both bacterial strains (Pst 1+ Bv1) and the ethyl acetate extract of *H. arbainense*, was assessed for its impact on disease incidence and severity. The results indicated a significant reduction in disease severity when employing this combination. Based on the findings, a blend of *H. arbainense* ethanol extract and Pst1 OP984768 strain and Bv1 OP984765 strain emerges as a promising, environmentally sustainable alternative to conventional synthetic bactericides for managing tomato bacterial wilt instigated by *C. michiganensis* subsp. *michiganensis.*

Keywords: bacterial disease, tomato, flavonoids, *Clavibacter michiganensis,* biocontrol

INTRODUCTION

With over two thousand species and hundreds of genera, the Boraginaceae family includes *Heliotropium*, a sizable genus with about 250–300 species worldwide (Fayed, 2021). This genus has a broad distribution, spanning both temperate and tropical climates across both hemispheres. Historically, *Heliotropium* species have been integral to traditional medicine, addressing ailments such as inflammations, gout, rheumatism, skin conditions, menstrual irregularities, and venomous bites (Ghori et al., 2016). Contrasting the study of singular compounds, the examination of medicinal plants and their extracts presents unique challenges. These extracts, being complex mixtures of active, semi-active, and inactive components, often target multiple pathways, and their composition can vary based on the preparation method and the specific plants utilized (Heinrich et al., 2022). The phytochemical, botanical, pharmacological, and nutritive potential of *Heliotropium* species is enormous. Among the *Heliotropium* species' active biochemical components are pyrrolizidine alkaloids, flavonoids, and terpenoids. Various extracts and active components from different *Heliotropium* species have demonstrated significant biological activities, such as antimicrobial, antiviral, antitumor, anti-inflammatory, cytotoxicity, phytotoxicity, and wound healing properties (Fayed, 2021). This study aimed to search for effective bacterial bioagents and plant extract to control tomato bacterial wilt *in vitro* and *in vivo*.

Heliotropium subulatum antioxidant, anti-cancer, and cytotoxic properties have all been investigated. From the resinous exudate of this plant species, five flavonoids were isolated and *in vitro* and *in vivo* antioxidant models were tested. Both *in vitro* and *in vivo* tests showed that tricetin has the highest antioxidant activity. Galangin was found to have the highest activity as well as the highest level of inhibition against antineoplastic. The findings indicate that triacetin has the ability to scavenge free radicals in both *in vitro* and *in vivo* models, whereas galangin could be used as an antitumor and cytotoxic agent (Singh et al., 2017).

Tomatoes, with a global production surpassing 182,000,000 tons, rank as the second most crucial crop (Al-Maawali et al., 2021 and FAOSTAT, 2022), it is also the largest vegetable crop grown in Egypt. However, disease management remains a significant challenge, affecting tomato yields worldwide. Over 200 distinct tomato diseases have been identified, with various causative agents (Jones et al., 1991). Including, canker disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is extremely subversive, it leads to rigorous remarkable damages (Sen et al., 2015). This contagious disease could prevail swiftly and cause significantly reduce productivity. With the severity of this disease and yield loss are influenced on tomato sensitivity and the ecological factors (de León et al., 2011). The optimal conditions for the disease include (25–28°C) with high moisture level

(Sharabani et al., 2014). Once established, especially in greenhouses or fields, the disease becomes challenging to manage or eradicate (Montenegro et al., 2018). Under Egyptian conditions, tomato canker has been observed on tomato crops grown in newly reclaimed regions (Abd El-Sayed, 2003). To mitigate the excessive use of pesticides in vegetable farming, several biological strategies, encompassing natural products, antibiotic production, growth promotion, and nutrient competition, have been proposed to protect plants against pathogens (Aksoy et al., 2017).

Plant growth-promoting rhizobacteria (PGPR) have gained recognition for their dual role in promoting plant growth and mitigating diseases. This alternative disease management approach has been adopted in various global regions (Amkraz et al., 2010). *Bacillus* spp. has effectively been used against several plant pathogenic microorganisms (Kloepper and Ryu, 2006). *Pseudomonas fluorescence* as well, has also been used for managing numerous plant pathogens, i.e., foliar diseases and soil-borne (Hashem and Abo-Elyousr, 2011). While PGPR has demonstrated significant pathogen inhibition in greenhouse settings, field results have been inconsistent (Siddiqui, 2006). A primary challenge hindering the success of these biocontrol agents (BCAs) in the field is the lack of an appropriate formulation (Kumar et al., 2014). This study evaluates the efficacy of formulated PGPR as biocontrol tools in suppressing bacterial wilt in tomato plants caused by *C. michiganensis* subsp. *michiganensis*. The potential production mechanisms, including siderophore, hydrogen cyanide (HCN), and indole acetic acid (IAA) production by the BCAs, were also investigated to determine their modes of action.

MATERIALS AND METHODS

1. Plant collection

North Sinai was the source of *Heliotropium arbainense* plants. The plant was identified using the Desert Research Center's Herbarium and a comparison to the Flora of Egypt's plant descriptions (Täckholm and Drar, 1974; Boulos, 2000 and Abd El-Ghani et al., 2017). The above ground parts of the plants were ground to a fine powder and air dried in the shade.

2. Plant Extraction and Preparation

The powder of the air-dried aerial part of the plant was extracted in a Soxhlet apparatus with 80% ethanol, dried under reduced pressure, and fractionated with ether, chloroform, ethyl acetate, and water using the remaining extract that had been dissolved in distilled water. Each subsequent

extract was fully vacuum-dried (Balbaa et al., 1981; Oliver-Bever, 1986 and Abdel-Sattar et al., 2010) (Fig. 1).

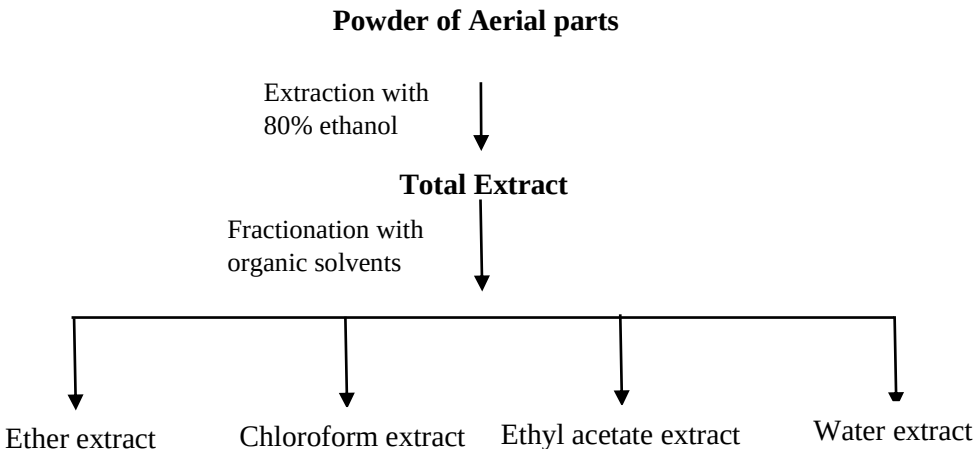

Fig. (1). Schematic diagram of an extraction process.

3. Pathogenic Bacterium

The pathogenic bacterium, Cmm, was procured from the Department of Plant Pathology at Assiut University, Egypt. This bacterium was previously identified using biochemical methods (Vos et al., 2011). It was cultured on a yeast peptone glucose agar medium (YPGA) plate at 28 ± 1°C for 5 days. For inoculation, Cmm cells from the YPG broth medium were incubated at 28±1°C for 72 h with shaking at 150 rpm. The conidial suspensions underwent centrifugation (10,000×g for 8 min), and the bacterial cells were re-suspended in tap water, adjusting the density to 2 × 108 cfu ml^{-1}.

4. Isolation of Antagonistic Bacteria

Twelve bacterial isolates, intended as BCAs, were derived from the rhizosphere and endosphere of tomato, potato, and eggplant plants cultivated in a field. The King's B medium (KB) slopes (20 g proteose peptone, 1.5 g K_2HPO_4, 1.5 g $Mg_2SO_4.7H_2O$, and 20 g agar) was used to maintain the BCAs.

5. Biocontrol Activity of BCAs *In Vitro*

The antagonistic activity of the twelve BCAs against Cmm was assessed *in vitro*. Cmm cell suspension was cultivated using KB medium and left until dried, then 9 mm wells were filled with 0.05 ml of the isolated BCAs. Plates were incubated at 27±1°C for two days, post which the inhibition zones (cm) were measured. Four replicates of each treatment were used in each of the two experiments. In addition, their morphological and cultural

biochemical techniques, the majority of bacterial isolates that demonstrated in strikgly clear antagonistic ability against the Cmm were chosen and identified (Schaad et al., 1988 and Bergey, 1994).

6. Bacterial Identification and Characterization

Genomic DNA was extracted from the two bacterial isolates (Pst 1 and Bv1) and amplified using 16s rRNA. Sequences were compared with type strains in the GenBank database at the National Centre for Biotechnology Information (NCBI). Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA) program's neighbor-joining method. For bacterial characterization, bioMérieux, Marcy-l'Étoile, France, used API 50CH/B and API ZYM.

7. Quantitative Antibacterial Activity Assay by Minimum Inhibitory Concentration (MIC) and Total Activity of Plant Extracts

The minimum inhibitory concentration (MIC) of the plant extracts against *C. michiganensis* subsp. *michiganensis* was assessed using a sensitive serial dilution microplate method (Eloff, 1998). *Heliotropium subulatum* extracts were prepared at 10 mg/ml and 100 µl were added to the first well of a 96-well microtiter plate and serially diluted 1:1 with water. Bacterial cultures (100 µl) were added to each well. Gentamicin was used as positive control and five solvents were used as solvents control. The microplates were incubated overnight at 37°C in 100% relative humidity, 40 µl of 0.2 mg/ml INT (p-iodonitrotetrazolium violet, Sigma®) were dissolved in hot water, and added to the microplate wells and incubated at 37°C for 2 h.

8. Biocontrol Activity of Plant Extract and Bacteria *In Vivo* Plant Material and Inoculation

Tomato (*Solanum lycopersicum*) plants were cultivated in a greenhouse at 25±5°C and 50±5% relative humidity with a 16/8 h (light/dark). Seeds of tomato were cultivated in pots (10 cm, diameter) containing a peat moss 50% and perlite 50% mix. *C. michiganensis* subsp. *michiganensis*. For the inoculation of Cmm in tomato plants at the 5-6 leaves stage (Werner et al., 2002), 4-holes (1.5 cm apart from each other, 5 cm depth) were prepared in all tested plants, including controls, the mixture (20 ml/plant, 1:1, v/v) of Cmm suspension (10^9 CFU/g) was injected into the four-holes (1 ml/hole) (Jang et al., 2022). Treatments were T1: ethyl acetate extract of *H. arbainense*, T2: Pst 1+ Bv1, T3: ethyl acetate extract of *H. arbainense* + Pst 1+ Bv1 and T4: control. After 31 days, the aerial part weight of the plants was recorded. The experiment was performed with three replicates with eight plants for each treatment. Disease symptoms were categorized and scored based on leaf wilting percentages as follows: for, the severity of the inoculated plants was recorded at 21 days post-inoculation (dpi) following five categories: 0 = no symptoms; 1= 0–25% leaf wilting; 2= 26–50% leaf wilting; 3= 51–75% leaf wilting; 4= 76–100% leaf wilting; and 5= dead (Mohd Nadzir et al., 2019).

9. Antioxidant Enzyme Assay

Ten days post-inoculation, tomato leaves were collected and processed for the antioxidant enzyme assay by a mixer mill (Retsch, MM200, Haan, Germany). After homogenizing the sampled leaves in 250 L of a 50 mM potassium phosphate buffer containing 1 mM EDTA for the catalase assay, the supernatant was collected and centrifuged at 10,000 rpm for 15 min at 4°C, and the activity was determined by a catalase assay kit (Cayman, Item No. 70700, Ann Arbor, MI, USA). and the glutathione peroxidase activity was evaluated using a glutathione peroxidase assay kit (Cayman, Item No. 703102, Ann Arbour, MI, USA). Leaves were homogenized in 1 ml of homogenization buffer containing 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 10 mM DTT. Peroxidase activity was measured following the manufacturer's guidelines. (Abcam, ab155895, Cambridge, UK). Total protein content was estimated using the Bradford method (Bradford, 1976) . The experiment was replicated twice with three replicates for each treatment.

10. Isolation and Purification of Flavonoid Compounds from Active Extracts

The previously prepared successive fractions were subjected to chromatographic screening. The separation was carried out using Whatmann paper No. 1 and solvent systems (a) BAW (butanol:acetic acid:water 4:1:5, v/v/v), solvent systems (b) acetic acid:water (15:85, v/v), and solvent systems (c) through the TLC technique using the ethyl acetate:methanol: water (30: 5: 4, v/v/v) system (Mabry et al., 2012)**.**

11. Isolation and Purification of Flavonoid of Compounds from Ethyl Acetate Extract

To isolate compounds from the ethyl acetate extract, a silica gel column was used. The process started with chloroform, followed by ethyl acetate and finally methanol, with a gradual increase in polarity. After collection, the fractions were concentrated under vacuum and subjected to TLC using system (c), then subjected to preparative TLC using the same system. The bands were visualized under UV, scratched and eluted with methanol. After that, the eluted bands were dried and finally purified using a Sephadex LH 20 column (Al-Jaber, 2011).

12. Isolation and Purification of Flavonoid of Compounds from Ethereal Extract

Compounds were isolated from the ethereal extract using a silica gel column. The column was first washed with ether, then with chloroform, followed by ethyl acetate and finally methanol. The polarity was gradually increased during the process. The collected fractions were concentrated under vacuum and placed to TLC by system (c). Preparative TLC was then performed using the same system. The bands have been visualized under UV

light, scratched and eluted using methanol. The eluted bands have been dried and purified using a Sephadex LH 20 column (Nawal and Abeer, 2010).

13. Statistical Analysis

The completely randomized design (CRD) method was used to analyze the data. It was also quantified as standard error (SE). Several parameters' means were compared using the SPSS statistical analysis software version 16's procedures. Duncan's multiple range test and one-way ANOVA were used to estimate the mean separation, and differences were deemed statistically remarkable (p<0.05) (Duncan, 1955).

RESULTS AND DISCUSSION

1. Isolation of Antagonistic Bacteria

Twelve bacterial isolates have been isolated from rhizosphere and endosphere of healthy plants (tomato, potato and eggplant) cultivated in Al-Arish area, North Sinai. Data in Table (1) represent the hosts and locations of heterotrophic bacterial isolates which were obtained from the examined plants. The obtained isolates belonged to two microbial groups according to morphological characteristics and gram reaction (short and long rod). Microbes were associated with every plant tissue and, in combination with the plant form the holobiont (Backer et al., 2018 and Compant et al., 2019).

2. Biocontrol Activity of BCAs *in Vitro*

A total of 12 bacterial isolates comprising of 6 isolates of fluorescent *Pseudomonas* (short rods) and 6 isolates of long rods were assessed *in vitro,* for their antagonistic effects against) *C. michiganensis subsp. michiganensis* using the agar. The antagonistic effect of selected isolates against *C. michiganensis subsp. michiganensis* based on the inhibition zone was recorded. Bacteria that displayed remarkable inhibition activity were considered as antagonists and selected for further investigations. There corresponding data of inhibition zone diameter ranged from 5 to 19 mm with Pst 63 and Pst 1, respectively (Table 2). Among the collected antagonist isolates, fourteen isolates (Pst 1 and Bv1) recorded high significant antagonistic activity against *C. michiganensis subsp. Michiganensis*. These obtained results could be supported in light of the findings of Ahmed et al. (2022), who stated that *Bacillus* strains isolated from tobacco plant, displayed potent antibacterial prowess, with inhibition zones measuring around 1.5 to 2.86 cm against *Ralstonia solanacearum*. It is worth noting that rhizobacteria, which bolster plant growth, not only enhance plant vitality but also serve as formidable adversaries to certain phytopathogens. Species like *Pseudomonas* sp., *Bacillus* sp., and *Streptomyces* sp. have been documented for their efficacy in curbing bacterial wilt (He et al., 2021).

Table (1). Hosts of twelve Bacterial isolates from Egypt.

Bacterial code	shape	Host
Pst 32	Short rod	Tomato
Pst 1	Short rod	Tomato
Pst 42	Short rod	Tomato
Pst 5	Short rod	Tomato
Pst 63	Short rod	Potato
Pst 18	Short rod	Potato
Bv1	Long rod	Potato
Bv14	Long rod	Potato
Bv17	Long rod	Eggplant
Bv12	Long rod	Eggplant
Bv19	Long rod	Eggplant
Bv 66	Long rod	Eggplant

Table (2). Antibacterial activity of BCAs against *Clavibacter michiganensis* subsp. *michiganensis* expressed as zone of inhibition (mm).

Bacterial code	Zone of inhibition (mm)
Pst 32	6ij
Pst 1	19a
Pst 42	8gh
Pst 5	6ij
Pst 63	5j
Pst 18	9fg
Bv1	17b
Bv14	10ef
Bv17	12cd
Bv12	13c
Bv19	11de
Bv 66	7 hi

3. Bacterial Identification and Characterization

The most effective antagonistic bacteria have been pinpointed using the 16Sr DNA amplification and sequencing techniques facilitated using Sigma Scientific services. The 16S rDNA sequences of these isolates have been archived in NCBI/GenBank database, bearing the accession numbers: strain Pst1 OP984768 and strain Bv1 OP984765. A phylogenetic tree was meticulously crafted through the comparative analysis of the 16S rRNA genes, utilizing multiple algorithms present in the CLC free workbench, version 4.5.1. Based on the analysis, it was discerned that Pst1 OP984768 strain and Bv1 OP984765 strain were exhibited a 99% match with the 16S rDNA sequences of the Pst 1 and Bv1 isolates, respectively (Badran et al., 2023).

4. Quantitative Antibacterial Activity Assay by Minimum Inhibitory Concentration (MIC) and Total Activity

The serial microdilution outcomes were meticulously analyzed by the Analysis of Variance (ANOVA) single factor statistical approach. The findings highlighted significant variances in the sensitivities of the tested microorganisms to the different extracts, with a significance level of $p < 0.01$. As detailed in Table (3), the MICs spanned from 0.02±0.00 to 0.52±0.15 mg/ml. The average MIC values, indicative of microbial susceptibility to the extracts, ranged between 0.09 and 0.34 mg/ml for *H. arbainense* water extract and *H. arbainense* ethyl aetate extract, respectively. A notable statistical difference ($\rho < 0.05$) was observed in the mean MIC values of the extracts against the bacterial pathogen. The ethyl acetate extract of *H. arbainense* stood out with a mean MIC of 0.34 mg/ml, closely followed by the ethereal extract at 0.26 mg/ml. Conversely, the water extract of *H. arbainense* exhibited the least activity, registering at 0.09 mg/ml. According to Eloff et al. (2007), acetone showcased a MIC of 51% against six fungi, with dimethylsulphoxide trailing at 45%, methanol at 32%, and ethanol at 30%. The antioxidant and antibacterial properties of 13 South African plant extracts were identified by Adamu et al. (2014). Additionally, they discovered that *Maesa lanceolata* extracts outperformed other plant extracts in their ability to combat four nosocomial bacteria.

Table (3). Minimal Inhibitory Concentration (MIC) (mg /ml) and Total Antibacterial Activity (TAA) (ml /g) of the five *Heliotropium arbainense* extracts with different solvents against *Clavibacter michiganensis* subsp. *michiganensis.*

***Heliotropium arbainense* extracts**	***Clavibacter michiganensis* subsp. *michiganensis***	
	MIC (mg/ml)	**TAA (ml/g)**
H. arbainense total extract	0.1800c	142.47d
H. arbainense $CHCl_3$ extract	0.1500d	651.63a
H. arbainense ethrteal extract	0.2600b	493.28b
H. arbainense ethyl aetate extract	0.3400a	91.52e
H. arbainense water extract	0.0900e	250.27c
Gentamicin (+ control)	0.0003g	NAf
solv.1 (- control)	0.0600e	NAf
solv.2 (- control)	0.0200g	NAf
solv.3 (- control)	0.0400f	NAf
solv.4 (- control)	0.0300fg	NAf
solv.5 (- control)	0.0700e	NAf

NA not applicable

The total antibacterial activity (TAA) is gauged by MIC in ml/g and the extraction yield in mg/g of plant material (Eloff, 2004). TAA essentially quantifies the volume of water or solvent that, when combined with 1 g of the

extract, will still inhibit the growth of the pathogen (Eloff, 2004). The effectiveness of the extracts against C. *michiganensis subsp. Michiganensis* differed in a statistically significant way ($p = 0.01$). The antibacterial assay revealed higher antibacterial activities for *H. arbainense* (chloroform and ethereal extracts), with TAA values of 651.63 and 493.28 ml/g, respectively (Table 3). The efficacy, represented as total activity in ml/g, aids in selecting plant species, while the MIC and TAA values serve as instrumental pharmacological metrics to gauge the potency (in mg/ml) of plant extracts, facilitating the isolation of bioactive compounds (Eloff, 2004).

5. Biocontrol Activity of Plant Extract and Bacteria *In Vivo*

5.1 Disease suppressive activity

Among the four treatments, T3: *H. arbainense* ethyl acetate extract + Pst 1+ Bv1 significantly ($p < 0.05$) suppressed disease incidence compared to control T4 (Table 4). The percentage of plant wilting was 90.62% in control group, while the prevalence of disease in plants treated with ethyl aetate extract of *H. arbainense* + Pst 1+ Bv1 was only 20.28% (Table 4). In the present study, it was found that the combination of ethyl aetate extract of *H. arbainense* with the mixture of fresh culture of the two studied aantagonistic bacteria (Pst 1+ Bv1) significantly reduced tomato wilt caused by Cmm. In plant assay, T3: ethyl aetate extract of *H. arbainense*+ Pst 1+ Bv1 significantly reduced disease incidence and severity in the pot assay. Jang et al. (2022) also found that tomato wilt caused by Cmm could be reduced by using water extracts from *Bacillus* strains H8-1 and K203.

Table (4). Disease incidence (%), severity, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato plants.

Treatments	Disease incidence (%)	Disease severity (0-5)
T1	50.93b	2.4b
T2	40.68c	2.1c
T3	20.28d	1.5d
T4	90.62a	3.9a

5.2 Antioxidant enzyme activity in tomato plants

Under non-inoculated conditions, the combination of *H. arbainense* plant extract (ethyl aetate extract) with a fresh culture of two bacteria (Pst 1+ Bv1) showed a propensity to elevate the levels of antioxidant enzymes. These enzymes included peroxidase (pox), glutathione peroxidase (Gpox), and catalase (CAT) when juxtaposed with the control (Table 5). Under conditions inoculated with Cmm, Peroxidase activity in the Pst 1+ Bv1 with *H. arbainense* ethyl aetate extract was recorded as 1.5 m/min/mg protein. The activity of glutathione peroxidase was noted at 0.6 m/min//mg protein, which

was significantly ($p < 0.05$) elevated compared to the control plants. The CAT activity was marked at 580 m/min/mg protein in T3 treatment Pst 1+ Bv1 with *H. arbainense* ethyl aetate extract, showcasing a notable difference from the control (Table 5).

Table (5). Activity of antioxidant enzymes such as superoxide dismutase, peroxidase, glutathione peroxidase and catalase after inoculation of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm).

Treatments	Peroxidase (pox)	Glutathione peroxidase (Gpox)	Catalase (CAT)
T1	0.9c	0.2c	412c
T2	1.1b	0.3b	463b
T3	1.5a	0.6a	580a
T4	0.4d	0.1d	214d

Jang et al. (2022) observed that water extracts of *Bacillus* sp. H8-1 and *Bacillus* sp. K203 played a role in bolstering plant-defense mechanisms. leading to an uptick in antioxidant enzyme levels in plants inoculated with Cmm. This observation aligns with other studies where the introduction of DL--aminobutyric acid or acibenzolar-S-methyl amplified the activity of reactive oxygen species (ROS)-scavenging antioxidant systems, including peroxidase, phenylalanine ammonia-lyase, and glutathione peroxidase (Baysal et al., 2003; Soylua et al., 2003 and Baysal et al., 2005). Consequently, these water extract treatments might enhance plant defenses against Cmm infections by augmenting antioxidant enzyme activity. This suggests that these extracts could potentially fortify plant defense mechanisms against Cmm through pathways dependent on salicylic acid, thereby mitigating tomato wilt. The PI2 gene, encoding a proteolytic enzyme inhibitor and acting as a jasmonic-acid-dependent marker gene, can be activated by wounding (Takishita et al., 2018). During a Cmm infection, the enzyme responsible for ethylene synthesis, 1-aminocyclopropane-1-carboxylic acid (ACC)-oxidase, is triggered. The ethylene production instigated by Cmm in host plants might play a pivotal role in the progression of the disease (Cantarel et al., 2009 and Savidor et al., 2012). In plants treated with water extract, there was a marked decline in the expression of the ACO gene, which encodes 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase, mirroring the suppression of the disease.

6. Isolated Compounds from Ethyl Acetate and Ethereal Extracts

H1 and H4 were isolated from ethereal extract and (H2, H3 and H5 were isolated from ethyl acetate extract.

6.1. H_1= Apigenin

Compound H_1 was yellow crystals, R_f value in System a= 0.79 and System b =0.14, the UV $\lambda_{max,}$ nm (Table 6) showed the following:

1. Band I at 336 nm and band II at 267 nm pointed out that the compound may be flavones in nature.
2. Bathochromic shift in band I with NaOMe with an increase of intensity indicated the presence of free OH group at position 4'.
3. Bathochromic shift in band I with addition of NaOAc indicated the presence of free OH group at position 7.
4. Addition of H_3BO_3 gave no changes to the shift, which obtained by NaOAc indicating the absence of orthodihydroxy groups at B-ring.
5. Bathochromic shift in band I with addition of $AlCl_3$ indicated the presence of free OH group at position 5.
6. No shift in band I in $AlCl_3$ spectrum after the addition of HCl, which indicated the absence of orthodihydroxy groups.

^{1}H-NMR (DMSO-d6) (Table 7) coincided with that of apigenin. From the UV analysis, compound H_1 may be apigenin. The ^{1}H-NMR spectral analysis was compared with published data which revealed that the compound was apigenin.

6.2. H_2= Apigenin-7- *O*-β -glucoside

Compound H_2 was yellow crystals, R_f value in System a= 0.54 and System b=0.50, U V $\lambda_{max,}$ nm (Table 6) was coinciding with that of apigenin except no bathochromic shift obtained with NaOAc in band II which suggest the occupation of position 7. The ^{1}H-NMR showed the presence of glucose sugar at position 7.

^{1}H-NMR (DMSO-d6) (Table 7) coincided with that of apigenin-7-O-glucoside. From the R_f value, colour reaction, UV spectral data and ^{1}H-NMR spectral analysis; compound E_2 was confirmed as apigenin-7-O-glucoside.

6.3. H_3= Apigenin-7- *O*-β- galactoside

Compound H_3 was yellow crystals, R_f-value in System a= 0.50 and System b= 0.47. UV Spectral data $\lambda_{max,}$ nm (Table 6) was coinciding with that of Apigenin except no bathochromic shift obtained with NaOAc in band II which suggest the occupation of position 7. This compound when subjected to complete acid hydrolysis gave apigenin as aglycone and galactose as sugar moiety.

^{1}H-NMR (DMSO-d6) (Table 7), coincided with that of apigenin-7-galactoside. From R_f value, UV spectral data, ^{1}H NMR and complete acid hydrolysis; compound H_3 was apigenin-7-galactoside.

6.4. H_4= Luteolin

Compound H_4 was yellow crystals, R_f value in System a= 0.75 and System b =0.10. UV Spectral data $\lambda_{max,}$ nm (Table 6) showed the following:

1. Band I at 349 nm and band II at 253 nm indicated that the compound may be flavones in nature.

2. Bathochromic shift of band I with NaOMe with an increase of intensity indicated the presence of free OH group at position 4'.
3.NO Bathochromic shift in band II with addition of NaOAc pointed out the absence of free OH group at position 7.
4. Hypsochromic shift in band I with addition of NaOAc/ H_3BO_3 indicated the presence of orthodihydroxy groups at B-ring.
5. Bathochromic shift in band I with addition of $AlCl_3$ indicated the presence of free OH group at position 5 and/or orthodihydroxy group.
6. Hypsochromic shift in band I in $AlCl_3$ spectrum after the addition of HCl indicated the presence of orthodihydroxy groups.

^{1}H-NMR (DMSO-d6) Table (7), coincided with that of luteolin. From R_f value, UV spectral data and ^{1}H NMR; compound H_4 was luteolin free.

6.5. H_5= Luteolin 7,3′-di-*O*-β-glucoside

Compound H_5 was yellow crystals, R_f value in System a = 0.75 and System b =0.55. UV Spectral data $\lambda_{max,}$ nm (Table 6), was coinciding with that of luteolin except no bathochromic shift obtained with NaOAc in band II which suggest the occupation of position 7 and Addition of H_3BO_3 gave no changes to the shift, which obtained by NaOAc indicating the absence of orthodihydroxy groups at B-ring.

^{1}H-NMR (DMSO-d6) (Table 7), coincided with that of luteolin-3',7 diglucoside. From R_f value, UV spectral data and ^{1}H NMR; compound H_5 was luteolin-3',7 diglucoside.

Table (6). UV spectral data of isolated flavonoids.

Comp. no	**MeOH**	**NaOMe**	**NaOAc**	**H_3BO_3**	**$AlCl_3$**	**HCl**
H_1	267, 296, 336	275, 324, 393	274, 301, 376	368, 302, 338	276, 301, 348, 384	276, 229, 340, 381
H_2	266, 334	240, 272, 385	252, 263, 350	266, 335	274, 300, 382	275, 340, 380
H_3	269, 282, 337	282, 356, 406	271, 322, 337	269, 332, 340	273, 359, 373	274, 359
H_4	242, 253, 349	266, 329, 401	269, 326, 384	259, 370	274, 328, 426	275, 355, 358
H_5	268, 346, 351	274, 323, 379	270, 354	268, 322, 350	268, 304, 349	272, 303, 350

Table (7): ^{1}H-NMR spectral data of isolated flavonoids.

C. no.	H2`	H6`	H3`	H5`	H8	H3	H6	H1```	H1```	R.S.P.	OCH_3
H_1	7.9d. 7.5Hz	7.9d. 7.5Hz	6.9d. 7.5Hz	6.9d. 7.5Hz	6.5d. 2.5Hz	6.75s	6.1d. 2.5Hz	-	-	-	-
H_2	7.95d. 7.5Hz	7.95d. 7.5Hz	6.95d. 7.5Hz	6.95d. 7.5Hz	6.8d. 2.5Hz	6.75s	6.2d. 2.5Hz	5.1d. 7.5Hz gl	-	3.1-3.9m	-
H_3	7.8d. 7.5Hz	7.8d. 7.5Hz	7.2d. 7.5Hz	7.2d. 7.5Hz	6.9d. 2.5Hz	6.78s	6.4d. 2.5Hz	5.2d. 7Hz gal	-	3.5-4m	-
H_4	7.6d. 2.5Hz	7.45dd 7.5, 2.5Hz	-	6.92d. 7.5Hz	6.8d. 2.5Hz	6.75s	6.45d. 2.5Hz	-	-	-	-
H5	7.42d. 1.7Hz	7.44dd, J = 2.1, 8.3 Hz	-	6.90 (d, J = 8.3 Hz)	6.75 (s)	6.79 s	6.44 d, 2.1Hz	5.08 (d, J = 7.5 Hz)gl	5.01 (d, J = 7.6 Hz) gl	3.5-3.8m	-

CONCLUSION

The combined application of *Pseudomonas taiwanensis* strain Pst1 OP984768 and *Bacillus velezensis* strain Bv1 OP984765 and the ethyl acetate extract of *H. arbainense* has proven effective in controlling Cmm-induced tomato wilt and canker disease both *in vitro* and in greenhouse settings. This synergy suggests that combination of *H. arbainense* ethyl aetate extract mixed with *Pseudomonas taiwanensis* strain Pst1 OP984768 and *Bacillus velezensis* strain Bv1 OP984765 can be used as a safe and environmentally friendly method of controlling bacterial wilt disease in tomato crops. The results advocate for the broader adoption of this combined treatment approach.

Given that flavonoids, a prominent group of phenolic compounds derived from plants, are present in the ethyl acetate and ether extracts, they can play a pivotal role in plant disease control. These compounds present a sustainable and eco-friendly solution for managing bacterial wilt disease in tomatoes. Future research should explore the potential of Pst1 and Bv1 combined with *H. arbainense* against a broader range of pathogens and on different host plants, aiming to integrate this innovative approach into eco-friendly crop management strategies.

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تأثير مستخلصات نبات الإرهبة والعوامل الحيوية على مكافحه مرض القرحة البكتيرية لنبات الطماطم

نرهان عبد السلام عيد[1]*، نوال هداية محمد[2]، أحمد عبد العليم شعراوي[3]
قسم وقاية النبات، مركز بحوث الصحراء، القاهرة، مصر
قسم النباتات الطبية والعطرية، مركز بحوث الصحراء، المطرية، القاهرة. مصر
قسم الانتاج النباتي، كليه العلوم الزراعية البيئية، جامعة العريش، العريش، مصر

يتم علاج العديد من الأمراض التي تصيب الإنسان والنبات بالمركبات الطبيعية المشتقة من النباتات الطبية ومستخلصاتها. تتعمق هذه الدراسة في تأثيرات المستخلص الكحولي الكلي لنبات *Heliotropium arbainense* وجزيئاته اللاحقة (الإيثر والكلوروفورم وأسيتات الإيثيل والماء) كأداة بديلة وآمنة محتملة لمكافحة *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) يصيب نباتات الطماطم. والجدير بالذكر أن هذه الدراسة أظهرت أن مستخلصات أسيتات الإيثيل والأثير من نبات *H. arbainense* أظهرت نشاطًا واضحًا مضادًا للميكروبات ضد Cmm. تم استخدام التقنيات الكروماتوغرافية لعزل المركبات الخالية من الفلافونويد والجليكوسيدات من الأجزاء الأكثر فعالية. في الوقت نفسه، تم تحديد الريزوبكتيريا، وتحديدًا سلالة *Pseudomonas taiwanensis* Pst 1 984768OP سلالة *Bacillus velezensis* 1Bv، 984765OP، لأدوارها المحتملة في تعزيز نمو النبات والعمل كعوامل حيوية ضد أمراض الذبول البكتيرية. تم إجراء اختبارات معملية وتجارب في الصوبة لتقييم مدى فعالية هذه السلالات البكتيرية في مكافحة الذبول البكتيري وتم تقييم الفاعلية لكل من السلالات البكتيرية (Bv1+Pst) ومستخلص خلات الايثيل *Heliotropium arbainense* لتأثيرة على حدوث المرض وشدته، أشارت النتائج إلى انخفاض كبير في شدة المرض عند استخدام هذا المزيج، بناءً على النتائج، يظهر مزيج من مستخلص إيثانول *H. arbainense* وسلالة 1Pst 984768OP وسلالة 1Bv 984765OP كبديل واعد ومستدام بيئيًا لمبيدات البكتيريا الاصطناعية التقليدية لإدارة ذبول بكتيريا الطماطم الناجم عن *C. michiganensis*.