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In Vitro Evaluation of the Efficacy of Plant Extracts, Biological Agents, and Chemical Pesticides in Controlling Xanthomonas spp Bacteria Isolated from Tomato Plants in Erbil, Kurdistan Region, Iraq

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ABSTRACT

Tomato (Solanum lycopersicum L.), coming in the second level in production and consumption over the entire world after cereals. It is an important source of essential vitamins, minerals and antioxidants Tomato provides an estimated 80% of daily intake of lycopene, folate, ascorbic acid, flavonoids, and potassium in the western diet. China, India, USA, Turkey, and Egypt are the major top producers of tomato over the entire world. According to the Ministry of Agriculture and Water Resources / Kurdistan Regional Government, total tomato production in 2021 was 248546 tons distributed over the four governorates. Xanthomonas spp. is the most significant tomato pathogen, causing losses ranging from 10% to 60% of the crop yield. Aqueous extracts of four medicinal plants, Maidenhair Fern, Turmeric, Clove, and Ginger alongside bioagents (Bacillus subtilis, Trichoderma harzianum) and commercial pesticides (Himex and Aryal Super) were evaluated for their antibacterial activity against Xanthomonas spp. isolated from infected tomato plants. Isolation, pathogenicity testing, and molecular identification confirmed the presence of X. euvesicatoria, and X. campestris. Clove extract shows strong antibacterial activity against both isolates, whereas, Ginger was moderately active against X. campestris. The bioagent, B. subtilis strongly active against the isolates, T. harzianum was ineffective. The commercial pesticide Himex was strongly effective, while Aryal Super was ineffective. Clove extract shows high inhibition rate on both X. euvesicatoria and X. campestris at 10 mg/mL. Ginger extract only moderately inhibits X. campestris at 100 mg/mL, while turmeric and maidenhair fern were not effective. B. subtilis strongly antagonistic against both bacterial species, whereas T. harzianum was ineffective. Among the chemical treatments, Himex demonstrated substantial antibacterial activity (47.37%–54.51%), while Aryal Super showed no inhibition.

The findings suggest that clove extract and Bacillus subtilis are promising eco-friendly alternatives to conventional pesticides for managing bacterial leaf spot in tomato cultivation.

***Keywords*:** Xanthomonas campestris, Xanthomonas euvesicatoria, tomato, plant extract, antibacterial activity, biological control, clove.

INTRODUCTION

Tomatoes (*Solanum lycopersicum* L.), family *Solanaceae*, is one of the most commonly grown fruits because of their nutritional, economic, and cultural significance, is it has a versatile nature since it can be cultivated under various climates, it gained a place in every because of their dense content of vitamins, minerals, and antioxidants [1]. Tomatoes have a wide range of diversity in shape, color, and size reflecting their multigenic origin and history of breeding [2]. In addition, tomatoes have a strong position in the agricultural application as an important cash crop with a relatively high production value and worth on the international market. The high demand for combating diseases that impact tomato yields, developing disease-resistant kinds, and enhancing yields are areas of its relevance [3]. *Xanthomonas* spp., induce tomato bacterial leaf spot, a disease that threatens tomato crop globally causing considerable reduce in crop yield and fruit quality. the pathogen's pathovars have already been identified as most significant tomato disease, with losses ranging from 10% to 60%. [5].

X. campestris and *X. euvesicatoria* both are tomatoes infecting bacteria, but they differ in host range and pathogenicity on tomatoes. *X. campestris* is a Gram-negative motile rod Bacteria. Previous classification had included tomato-pathogenic strains under *X. campestris pv. vesicatoria*. Present taxonomy has reassigned the strains under *X. euvesicatoria* [6][7]. *X. euvesicatoria* is the primary pathogen of tomato bacterial leaf spot. It is arguably one of the most devastating diseases of tomato crops worldwide.

Typical disease symptoms include small, water-soaked marks on leaves that soon turn necrotic and yellow-haloed. Fruit lesions are made up of pale tan, scabby marks that can split as they increase in size, followed by yellowing and drop of their leaves, eventually weakening photosynthesis and fruit production. The disease is primarily spread by infected seeds, rain

splashes, irrigational water, and mechanical transmitters such as pruning equipment [5][8]. Management practices entail the use of disease-free seeds, crop rotation, sanitation, resistant varieties, and copper-based bactericides use. Resistance to copper has been reported to increase in some strains of *X. euvesicatoria* and therefore integrated disease management is essential [9]. Copper-resistant strains and the general ineffectiveness of conventional control measures make it difficult to manage *Xanthomonas* spp. [10][11].

Application of plant extracts for crop protection offers an eco-friendly and sustainable option to synthetic compounds, particularly considering the increase in demands for environmentally sustainable agricultural practices. Plant compounds have been shown to be useful in disease management to control a wide range of plant diseases, via their antimicrobial, antifungal and anti-insect properties, with reduced environmental and food crop chemical deposits [8][13][14][15]. Moreover, seed treatment using plant extracts has yielded positive results in strengthening crop resistance and germination rates, further establishing their position in agricultural systems. [16].

Material And Methods

Samples Collection

Total of 100 symptomatic samples of apparently infected tomato plants showing disease symptoms on the vegetative parts were randomly collected from fields located in different areas of Erbil province (Table1), samples were collected in early July 2024 and concluded in September 2024. They were kept in a plastic box containing ice bags after being placed in plastic bags and labeled with required information, subsequently they were transferred to the postgraduate laboratory/ Department of Plant Protection/ College of Agricultural Engineering Sciences/ Saladin University-Erbil where they were kept in the refrigerator until been processed.

Table1. Location and number of collected diseased samples

Location	Soran	Choman	Haji Omaran	Sidakan	Hiran	Khabat
No. of samples	17	20	20	12	18	13

Isolation and Purification of Bacteria

Samples were initially washed with tap water. To isolate bacteria from infected plant leaves, a small piece (5mm) was cut from the tip end of an advanced lesion disease and excised using a sterile scalpel, then were surface sterilized with 70% ethanol for 30 seconds, followed by washing three times in sterile distilled water and blotted dry on sterile tissue paper. The leave pieces were subsequently set onto nutrient agar (NA) medium and incubated in the incubator at a temperature of 26 ± 2˚C for 48h [16]. Then plates were checked for bacterial growth, the representative colonies were harvested and purified by repeated sub-culturing The pure cultures were used for pathogenicity testing in an attempt to fulfill the requirements and demonstration of Koch's postulates.

Pathogenicity Test

Pathogenicity test was performed on four-weeks-old, apparently healthy local tomato seedlings. The experiment was carried out in a plastic house at the Department of Plant Protection/ College of Agricultural Engineering Science/ University of Salahaddin/ Erbil.

Preparation of Bacterial Inoculum

Young growing colonies, cultured for 48 h. at 26 ± 2^{o}C, were suspended the in 10ml of sterile distilled water in screw capped plastic tubes. The tubes were shaken on electric shaker for 48 h. to achieve a concentration of about (10^{8} cfu /ml) the suspensions were quantitated by a spectrophotometer to be reduced to the final concentration [5].

Testing Pathogenicity

From the isolated bacteria five isolates, designated as Hiran, Kazhak, Tawkan, Konamar and sare bard, were tested for their pathogenicity according to the procedure described by [18]. Four weeks old tomato leaves (4-5 leaves per seedling) were lightly wounded on both surfaces using a sterile needle to facilitate infection, approximately 9 mL of each bacterial suspension was applied to the wounded leaves using a hand sprayer. As a control, three seedlings were sprayed with sterile distilled water. All seedlings were enclosed in polyethylene bags for 24h to maintain high relative humidity (>90%) and promote infection. The next day the plastic bags were removed, and the plants were watered daily. Symptoms were observed and recorded after 12-14 days. Ten the bacteria were reisolated from inoculated plants. The test was conducted in triplicates [18].

Colony Color Characterization and Microscopy

Preliminary identification involved macroscopic evaluation of colony characteristics, including color, texture, surface viscosity, and fluorescence under ultraviolet (UV) light. Microscopic examination was conducted to assess cell morphology and determine the Gram reaction using standard Gram staining techniques, DNA based molecular characterization of each bacterial isolate was carried out to identify them. Genomic DNA was extracted from the bacterial isolates that show positive

pathogenicity test using Beta Bayern tissue DNA preparation Kit (Beta Bayern GmbH .90453 Bayern, Germany) according to the manufacturer's guidelines as described by [13][8].

Preparation of Medicinal Plant powders and Plant Extracts

The plants were ground using an electric grinder, then the powders were sieved using flour sieve and kept in screw capped containers labeled with required information. These collected powders were used in the preparation of the extracts according to [13][19][8] with minimal adjustments.

study.

Table :2 List of the Medicinal Plants identified From Erbil and evaluated in this

No.	Scientific names	Common names
1.	*Adiantum capillus-veneris*	Maidenhair Fern
2.	*Curcuma longa*	Turmeric
3.	*Syzygium aromaticum*	Clove
4.	*Zingiber* officinale	Ginger

Preparation of the Plant Extracts

the maceration method was employed for the preparation of aqueous plant extracts. plant materials were finely ground and soaked in freshly boiled distilled water, following the protocol of [1, 19, 7] with slight modifications. The mixtures were allowed to stand for a defined period to enable extraction of the active constituents, followed by filtration through sterile gauze and Whatman No. 1 filter paper. The resulting extracts were stored at 4 °C until further use in antibacterial assays.

Assay of Antibacterial Activity of Aqueous Extracts Against Bacterial Isolates

The agar well diffusion method described by [8] [10] was used in this study to determine the antibacterial activity of each extract.

Assay of the Minimum Inhibitory Concentration (MIC) of Plant Extracts

The Minimum Inhibitory Concentration (MIC) of each aqueous plant extract was determined using a modified agar well diffusion method as described by [10]. plates were inoculated with bacterial suspensions adjusted to approximately 10 CFU/mL Wells (6 mm diameter) were punched aseptically into the agar and filled with varying concentrations of the plant extracts: 100 µL of 10 mg/mL, 50 µL of 5 mg/mL and 25 µL of 2.5 mg/mL. Streptomycin was used as a positive control, while sterile distilled water (DW) and dimethyl sulfoxide (DMSO) served as negative controls. All plates were incubated at 28 ± 2 °C for 24–48 hours. The MIC was defined as the lowest concentration of the extract that produced a measurable inhibition zone against the bacterial isolates.

Assay of Antibacterial Activity of Some Bioagents

Using the dual culture method for testing antagonistic activity of *Bacillus subtilis* and *Trichoderma harzianum* powder in accordance with the method described by [22].

Assay of Some Pesticides Antibacterial Activity

The well diffusion method described by [21] was used in this assay to determine the antifungal activity of Himex (2ml/250ml) and Metalaxy 15%+Copper-oxychloride 35% WP (ARYAL SUPER) 0.5g/250ml.

Statistical Analysis

All data were exhibited as means ± standard error (x ± SE) for three repetitions of each arranged specimen. At 95% certainty confidence level utilizing STATGRAPHICS Centurion XV Version 15.1.02. The statistical analysis was performed focusing around multifactor analysis of variance (Multifactor ANOVA). The method being used to discriminate among the means is Fisher's least significant difference (LSD) procedure. With this method, there is a 5.0% risk of calling each pair of means significantly different when the actual different equals zero (Williams et al., 2010).

Result and discussion

Isolation and identification of *Xanthomonas* bacteria

Sixty-two bacterial isolates were obtained from culturing 100 tomato samples collected from 6 different locations in Erbil province. Thirty of the samples produced round, convex, mucoid, yellow colonies typical of *Xanthomonads* spp. on NA medium (Fig1). The same colony description described by [13] [23] [24].

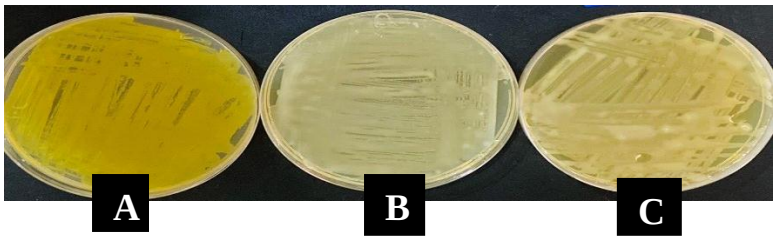


Fig1. Isolated bacteria showing morphological characteristics

Pathogenicity of Xanthomonas

The results (Table3, fig 2) showed that bacteria isolated from the leaves were highly virulent and induced the formation of specific bacterial spots symptoms on the leaves of inoculated tomato seedlings, the symptoms appeared after 12-14 days. Initially, small, circular and yellowish or light brown in color spots were observed on the surface of inoculated leaves. Subsequently, the spots enlarged, faint dark brown, and water soaked then become numerous and covered the whole plant. Uninoculated control plants show no symptoms

Table3. Results of pathogenicity test of the bacteria isolated from different locations

location	Hiran	Kazhak	Tawkan	Konamar	Sarebard
Result	Positive	Negative	Positive	Positive	Positive

The same symptoms were also mentioned by [13][25][26] [27]. This satisfied the first postulate, demonstrating that the suspected pathogen is consistently associated with the disease.

(A) control-ve. (B) Hiran. (C) Sare bard. (D) Tawkan. (E) Kunamar.

Figure 2. *In vivo* pathogenicity effects of selected bacterial isolates on tomato seedlings. (A) Negative control (sterile distilled water); (B) Isolate Hiran; (C) Isolate Sare Bard; (D) Isolate Tawkan; (E) Isolate Kunamar.

DNA Based Molecular Identification of Bacterial Isolates

Genomic DNA Extraction

The figure (3) shows the results of gel electrophoresis of bacterial DNA extracted from the five pathogenic isolates.

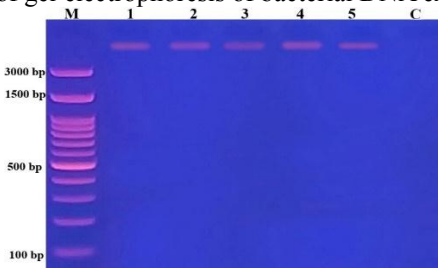


Fig3. The electrophoresis of bacterial DNA extracted from the pathogenic bacteria.

PCR amplification of partial genes

The figure (4) shows the PCR produces of the amplified extracted bacterial DNA of the five pathogenic isolates.

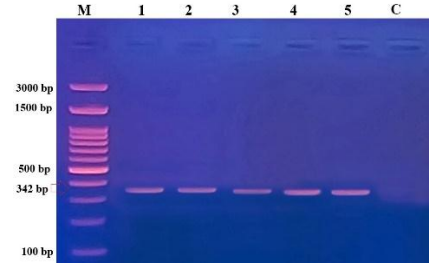


Fig4. PCR amplification of partial 16S rRNA gene from bacteria, wells include M; Ladder (3000-100 bp), lane1-5; gene bands with the size of 372 bp amplified and C indicate negative control without the band.

Molecular Identification of Genus

The sequence of partial genes was aligned by the BLAST program from GenBank (*http://blast.ncbi.nlm.nih.gov/*) and was used to compare our amplified sequences with stored sequences of other species. The results from the BLAST (Table 4) indicated that the highest query sequence was 100% identical.

Table 4. Percentage distribution of isolated bacteria species into same bacteria according to stored blast of GenBank/ NCBI of partial 16S rRNA gene.

Samples Accession Number	Query Cover %	Identic Number %	GenBank Accession Number	GenBank Identification
	100	100	PP103252	
PV167688	100	100	MG654644	*X. euvesicatoria*
PV167692	100	100	CP166448	
	100	100	NR_104965	
	100	100	CP155997	
PV167689	100	100	CP066968	*X. campestris*
PV167690	100	100	CP156002	
	100	100	OR626095	
	100	100	GQ461739	
PV167691	100	100	AY288081	*X. perforans*
	100	100	CP167822	
	100	100	CP018475	

Phylogenetic inferences

MEGA 11 program of Phylogenetic analysis based on 16S rRNA nucleotide sequence (Figure5), revealed grouping of four investigated species of *Xanthomonas* sp. on expected lines. From sequence divergence similarity data and phylogeny constructed, it was revealed that species belonging to respective genera were close to each other. The five samples of bacteria species grouped each cluster with high similarity to the same bacteria stored in GenBank.

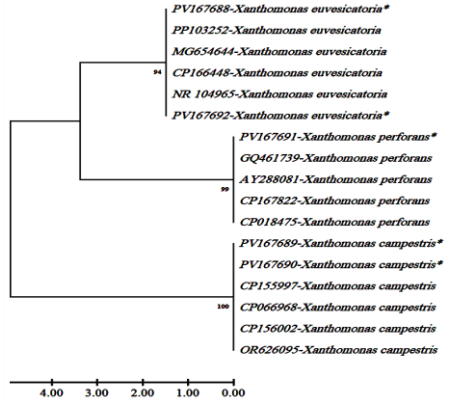


Fig5. Employing Neighbor joining of Mega 11 program, shows phylogenetic positioning of the five bacterial samples (*) with similar GenBank sequences of 16S rRNA that are available in GenBank

Assay of Antibacterial Activity of Aqueous Extracts Against *Xanthomonas euvesicatoria*

In vitro assay results (Figure 6) demonstrated a statistically significant inhibitory effect ($P < 0.05$) of some extracts on the growth of *Xanthomonas euvesicatoria*. However, there was notable variability in the degree of inhibition among the different plant extracts, which may be attributed to factors such as the specific plant species used, the nature of the solvent, or the susceptibility of the pathogen.

Among the tested plant extracts, only Clove exhibited antibacterial activity against *X. euvesicatoria*, whereas Maidenhair Fern, Turmeric, and Ginger were not effective. These findings are in agreement with previous studies by [28][29] who reported similar patterns of extract activity. Clove extract demonstrated percent of inhibition across all tested concentrations: 100mg/ml (34.59%), 50%, 30.20% and 25mg/ml (25.65%). These results support the findings of [8], who confirmed the significant antibacterial potential of Clove extract against *X. euvesicatoria*.

In the case of biological agents, only *B. subtilis* exhibited antibacterial activity, achieving (42.55%) inhibition of bacterial growth, in contrast, *T. harzianum* showed no inhibitory effect, aligning with results reported by [30].

While in the case of chemicall pesticides, Aryal Super showed no antibacterial activity, whereas Himex exhibited substantial inhibitory activity, inhibiting (54.51%) of the bacterial growth. These results are consistent with those reported by [31].

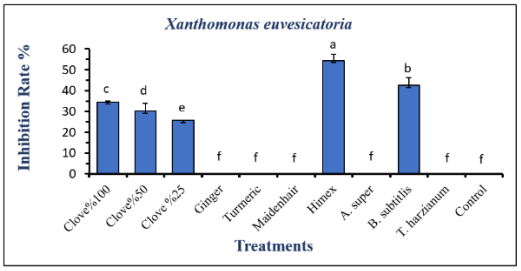
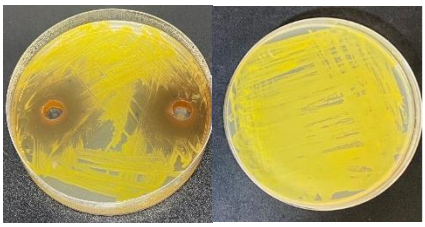


Fig6. In vitro antibacterial activity and MIC of plant extracts, pesticides and bioagents against *X. euvesicatoria*



(A) (B)

Fig7. Testing antibacterial activity against *X. euvesicatoria*. A: Clove extract, B: Negative control (DW)

Assay of Antibacterial Activity of Aqueous Extracts Against *Xanthomonas campestris*

The in vitro assay results (Figure 8) revealed a statistically significant inhibitory effect ($P < 0.05$) of certain extracts on the growth of *Xanthomonas campestris*. However, the antibacterial efficacy varied among the different extracts, which may be attributed to differences in the plant species, phytochemical constituent, or the sensitivity of the target pathogen.

Among the tested plant extracts, only Clove and Ginger demonstrated antibacterial activity against *X. campestris*. Extracts of Maidenhair Fern and Turmeric were not effect. Clove extract showed strong antibacterial (inhibition) activity across all tested concentrations: 100mg/ml (35.10%), 50mg/m (35.49%), and 25mg/ml (32.16%), indicating potent activity even at lower concentrations. These results are consistent with the findings of [8], who reported significant antibacterial properties of Clove extract against *X. campestris*.

Ginger extract showed moderate activity, 1oomg/ml (23.69%), however, it exhibited no inhibitory effect at 50mg/ml and 25mg/ml concentrations, corroborating previous findings by [32].

As for the biocontrol agents, *Trichoderma harzianum* and *Bacillus subtilisin*, only *B. subtilis* showed antibacterial activity of (44.43%) inhibition. In the contrary, *T. harzianum* showed no inhibitory effect, which aligns with the findings supported by [33] who also find the same results when testing these bioagent against *Xanthomonas campestris*.

Two chemical pesticides Himex and Aryal Super were evaluated for their efficacy against *X. campestris*. Aryal Super exhibited no antibacterial activity, whereas Himex demonstrated a substantial inhibitory effect, controlling 47.37% of bacterial growth, consistent with the results of [33].

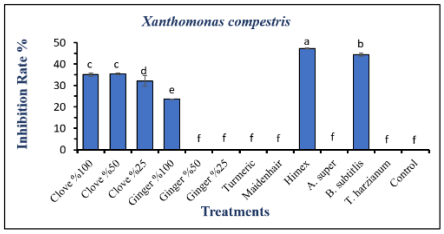
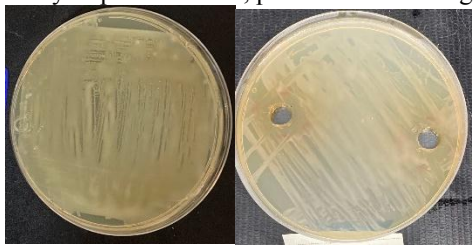


Fig8. In vitro antibacterial activity of plant extracts, pesticides and bioagents against *X. campestris*



A B

Fig9. The effect of clove extract on the growth of X. campestris bacteria:
A: Negative control, B: Antibacterial activity

Conclusions and recommendations

Among the evaluated treatments, Clove extract (Syzygium aromaticum) showed significant antibacterial activity against both X. euvesicatoria and X. campestris, even at lower concentrations, making it the most effective plant-based extract tested. Ginger (Zingiber officinale) demonstrated moderate activity but was only effective against X. campestris, while Maidenhair Fern (Adiantum capillus-veneris) and Turmeric (Curcuma longa) showed no inhibitory effect on either pathogen.

Regarding biological control agents, Bacillus subtilis exhibited strong antagonistic activity, supporting its use as a reliable bioagent in integrated disease management programs. In contrast, Trichoderma harzianum did not display any antibacterial effect, indicating its limited applicability against these bacterial pathogens.

For chemical control, Himex showed considerable antibacterial efficacy, while Aryal Super was completely ineffective, suggesting possible resistance development or formulation limitations.

In summary, the findings underline the potential of clove extract and Bacillus subtilis as eco-friendly, sustainable alternatives to conventional bactericides for managing bacterial leaf spot disease in tomatoes

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تقييم مقارن في المختبر للتأثيرات المضادة للبكتيريا لمستخلصات نباتية مختارة ضد بكتيريا Xanthomonas campestris و Xanthomonas euvesicatoria المعزولة من نباتات الطماطم في محافظة أربيل,إقليم كردستان,العراق كركوك

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الخلاصة

الطماطم ***(Solanum lycopersicum L.)*** هي واحدة من أهم المحاصيل الاقتصادية وتأتي في المستوى الثاني في الإنتاج والاستهلاك في جميع أنحاء العالم بعد الحبوب. تعتبر الطماطم مصدرًا مهمًا للعديد من الفيتامينات والمعادن ومضادات الأكسدة الأساسية إلى جانب منتجاتها المشتقة، تعد الطماطم واحدة من المصادر الغذائية الرئيسية للكاروتينات حيث توفر ما يقدر بنحو 80٪ من المدخول اليومي من الليكوبين وحمض الفوليك وحمض الأسكوربيك والفلافونويد والبوتاسيوم في النظام الغذائي الغربي. يبلغ إجمالي كمية الطماطم المنتجة عالميًا 337.16 مليون طن. تعد الصين والهند والولايات المتحدة وتركيا ومصر من أكبر المنتجين الرئيسيين للطماطم في جميع أنحاء العالم. وفقًا لوزارة الزراعة والموارد المائية / حكومة إقليم كردستان العراق (بيانات غير منشورة) بلغ إجمالي كمية الطماطم المنتجة في عام 2021 248546 طنًا. تأتي محافظة أربيل في المقدمة بكمية إنتاج تبلغ 105371 طنًا. تليها السليمانية، ودهوك، وكرميان، بإجمالي إنتاج بلغ 94332 و46628 و2215 طنًا على التوالي. وقد حُددت بكتيريا زانثوموناس ***spp.*** كأهم مرض يصيب الطماطم، حيث تراوحت خسائرها بين 10% و60% من إجمالي غلة المحصول. في هذه الدراسة، قُيّمت المستخلصات المائية لأربعة نباتات طبية، هي: سرخس كزبرة البئر ***(Adiantum capillus-veneris)*** ، والكركم ***(Curcuma longa)*** ، والقرنفل ***(Syzygium aromaticum)***، والزنجبيل ***(Zingiber officinale)*** ، إلى جانب عوامل بيولوجية ***Bacillus subtilis*** و ***Trichoderma harzianum***ومبيدان تجاريان ***Himex***و ***(Aryal Super***، وذلك لفعاليتها المضادة للبكتيريا ضد بكتيريا زانثوموناس ***spp.*** المعزولة من نباتات الطماطم المصابة في محافظة أربيل. أكدت العزلة واختبار الإمراضية والتحديد الجزيئي ***(*** تسلسل جينات الرنا الريبوسومي 16 ***S***والتحليل التطوري ***(***وجود ***X. euvesicatoria*** و ***X. campestris.***أظهرت النتائج أن مستخلص القرنفل يتميز بنشاط مضاد للبكتيريا قوي ضد ***Xanthomonas euvesicatoria*** و***Xanthomonas campestris***، بينما كان الزنجبيل فعالاً بشكل معتدل فقط ضد ***X. campestris.*** وُجد أن العامل الحيوي، ***Bacillus subtilis***، أظهر نشاطًا مضادًا قويًا ضد العزلات، بينما لم يكن ***Trichoderma harzianum*** فعالًا. أظهر اختبار مبيدَين تجاريَّين أن ***Himex*** أظهر نشاطًا جيدًا، بينما لم يُظهر ***Aryal Super*** أي نشاط مضاد للبكتيريا. أظهر مستخلص القرنفل أعلى نشاط مضاد للبكتيريا ضد كلٍّ من ***X. euvesicatoria*** (تثبيط بنسبة 34.50% عند 10 ملغ/مل) و ***X. campestris (35.10%).***أظهر مستخلص الزنجبيل تثبيطًا متوسطًا ضد بكتيريا ***X. campestris*** فقط (23.69% عند 100 ملغ/مل) ، بينما لم يُظهر الكركم وسرخس كزبرة البخور أي نشاط مضاد للبكتيريا. أظهرت بكتيريا ***B. subtilis*** تأثيرات مضادة قوية (42.55%-44.43%) ضد كلا النوعين من البكتيريا، بينما لم تُظهر بكتيريا ***T. harzianum*** أي فعالية. من بين المعالجات الكيميائية، أظهر مبيد ***Himex*** نشاطًا مضادًا للبكتيريا قويًا (47.37%-54.51%)، بينما لم يُظهر مبيد ***Aryal Super*** أي تثبيط.

تشير النتائج إلى أن مستخلص القرنفل وبكتيريا ***Bacillus subtilis*** بدائل صديقة للبيئة واعدة للمبيدات التقليدية في مكافحة تبقع الأوراق البكتيري في زراعة الطماطم.

الكلمات المفتاحية: مستخلص نباتي، طماطة، نشاط مضاد للبكتريا، المكافحة الحيوية، ***Xanthomonas campestris, Xanthomonas euvesicatoria***.