Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2, 853-862
2025
Publisher: Learning Gate
DOI: 10.55214/25768484.v9i2.4619

Phytochemicals analysis of Arum *palaestinum* extracts and their antibacterial effect against Pseudomonas *syringae pv.* tomato (Pst)

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Abstract: Arum palaestinum is a traditional medicinal wild plant indigenous to the Levant. The present study explores its phytochemical constituents and antibacterial activity in agriculture to control and inhibit the growth of Pseudomonas syringae pv. tomato (Pst). Aqueous, ethanolic, and chloroform extracts of the dried leaves, flowers, and rhizomes were prepared at a final concentration of 50 mg/mL. HPLC-MS analysis of A. palaestinum extracts revealed the presence of 13 previously characterized compounds and 2 new ones. Using the disc diffusion method and compared to ciprofloxacin and copper oxychloride that were used as controls, the aqueous extracts of the leaves, flowers, and rhizomes exhibited significant inhibition against P. syringae with high inhibition zones (IZDs). Based on the broth dilution method, the aqueous extracts possessed the greatest antimicrobial effect against P. syringae, followed by the ethanolic extracts, which showed a moderate antimicrobial effect for each part. In the field, the aqueous and ethanolic extracts controlled and eradicated the speck disease on tomato better than the chloroform extracts. Therefore, A. palaestinum can be used as an alternative to control bacterial speck disease and deserves to be further studied in organic agriculture.

Keywords: *Antibacterial activity, Arum palaestinum, LC-MS, Organic agriculture, Pseudomonas syringae, Speck disease.*

1. Introduction

Araceae is a large plant family represented by 3800 species in 118 genera spreading over a wide range of ecological habitats starting from sea level to a 3000 m altitude [1]. *Arum palaestinum* Boiss. (Black Calla Lily or Louf, *A. palaestinum*), is one of ~26 species of the *Arum* genus belonging to this family [2, 3]. It is native to Europe, Northern Africa, Western Asia, with the highest species diversity found in the Mediterranean region [4-6]. It grows up to 0.82 ft and blooms in the spring, between March and April. The plant is recognized by its dark purplish-black spadix enclosed in a reddish-brown spathe [7]. Black lily is a typical "cryptic" species, since its appendix emits mainly ethyl acetate, producing a smell of rotten fruit. In many countries, the aerial parts of *A. palaestinum* are considered ornamental plants, animal fodder and can be edible dried or after soaking in salty water. The plant is used in folk medicine to cure several chronic diseases such as stomach acidity, atherosclerosis, cancer, and diabetes [6, 8, 9].

Numerous studies have shown that *A. palaestinum* leaves contains several biologically active phytochemicals with the highest quantity of saponin, alkaloid, phenols and flavonoids [4, 6, 10].

Bacterial plants' diseases are often very difficult to eradicate. Few effective strategies are adopted to control them. Medicinal plants are among the richest bioresources of the metabolites that are currently

History: Received: 7 December 2024; Revised: 24 January 2025; Accepted: 31 January 2025; Published: 6 February 2025
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used to treat bacterial and other infections [11, 12]. Yield losses in food crops due to plant pathogenic bacteria are significant and increasing over the years. The increasing losses caused by bacterial plant pathology are explained by the emerging resistance of bacteria to the chemical agents used in plant protection. Moreover, these chemical agents harm the environment through residue accumulation leading to soil pollution and the perturbation of the soil's inner ecosystem [13]. The plant pathogenic *Pseudomonas syringae* (*P. syringae*) species is one of the most important plant pathogenic bacteria. It is divided into different pathovars based on their host range. A certain pathovar usually has a limited host range; however, the whole *P. syringae* group can cause diseases for a wide range of host plants. *P. syringae* pv. tomato (Pst) causes bacterial speck on tomato plants and is considered a highly aggressive pathogen once inside the plant [14, 15]. Among the many symptoms it causes, one can find brown-black leaf spots that are surrounded by chlorotic margin; dark superficial specks on green fruit where they can become sunken on ripe fruits, surrounded by a zone of delayed ripening. Stunting and yield loss, particularly if young plants are infected, will result due to the infection, thus reducing the market value of the speckled fruit [16, 17].

Many research tackled the chemical composition of *A. palaestinum* [4, 18-20]; however, the need for an extensive identification of its phytochemical components seems imperative. Undoubtedly, the agricultural use of this plant as a bio-bactericide has robustly prompted us to carry out the phytochemical analysis of this Lebanese wild plant as a promising antibacterial plant. For that reason, this study presents a comprehensive qualitative characterization of the phytochemical released in aqueous, ethanolic and chloroform extracts of *A. palaestinum* leaves, flowers and rhizomes using liquid chromatography–tandem mass spectrometry (LC-MS) and their activities against the *pseudomonas syringae* pv. tomato (Pst).

2. Materials and Methods

2.1. Plant Materials

Leaves, flowers and rhizomes of *A. palaestinum* were collected and identified during the spring (March–June, 2022) from the hills of South Lebanon, the plant was identified by the pharmacognosist Dr. Mohamad Khiami and can be found at the herbarium of the Lebanese University.

The leaves and flowers were washed with distilled water then dried for 20 days in the shade at room temperature. The rhizomes were washed many times to eliminate the soil particles, cut to small pieces, dried for 40 days in the shade at room temperature. All dried parts were grounded and the powder were stored at 4 °C until their use.

2.2. Preparation of the Extracts

Extracts of each part (10%) were prepared using distilled water, ethanol (99.4%) or choloroform (99.4%). Samples were placed in a shaker at 100 rounds per minute for 72 hours at room temperature, then filtered through Whatman filter papers (No.4).The extraction was repeated three times.Thereafter, the combined filtrate were then dried by rotary evaporation and stored at -20°C for later use. Dried yields of ethanolic and chloroformic extracts were dissolved in 4% DMSO and in distilled water for aqueous extracts at a final concentration of 50 mg/mL.

2.3. LC-MS Analysis

Separation and detection of the phytochemical compounds from the water, ethanol and chloroform extracts were performed on an AB Sciex X500R QTOF ESI mass spectrometer at the American Unviersity of Beirut. LC flow was split to 500 nL/min before entering the ion source. Mass spectra were acquired in centroid mode ranging from 150 to 1,000 m/z, resolution R = 30,000. A Luna Omega C18, 150 × 2.1 mm, 1.6 µm column was used, with injection volume of 1 µL. A gradient of A) H2O+ 0.1% FA (formic acid) and B) Acetonitrile+0.1% FA at a flow rate of 0.55 mL/min was used to achieve separation. Gradient conditions started at 5%B, increase to 10% B in 1 min, then to 35% B from minute 1→15, then

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619

to 50% B from minute 15→22, and finally to 80% B from minute 22→25. After a 1-min hold at 80% B, the system was re-equilibrated for 5 min with the initial conditions. UV data was acquired using a PDA (wavelength 200–800 nm ± 8 nm), MS detection was performed simultaneously.

2.4. Determination of Antibacterial Activity of A. Palaestinum Extracts Against P. Syringae Pv. Tomato (Pst)

2.4.1. Activity By Disc Diffusion Method

A preliminary check of the antibacterial acitivites of the prepared extract against *P. syringae* was performed using the disc diffusion method according to Leite, et al. [21]. *P. syringae* pv. tomato (Pst) at 1.0 x 10^5 CFU/mL was incubated in King Agar B for 24 h at 28°C. Discs (6 mm) of Whatmann filter paper (No. 1) were sterilized in an oven at 160 °C for one hour. Discs were impregnated with10 µL of the prepared extract, dried under a laminar flow sterile bench. Positive control discs containing 0.5mg of the reference bacteriocide copper oxychloride, 85%WP (Wettable Powders) (Agro Life Science Corporation, India) as (C1), or 0.5 mg Ciproloxacin (C2) (Macleods' Pharmceuticals LTD, Mumbai, India) as (C2) were used as positive controls while discs containing 10µL of 4% DMSO (C^-) were used as negative control. All Petri dishes were sealed to avoid possible evaporation of the test samples, then incubated at 28°C for 24 h. After incubation, the diameters of the bacterial growth inhibition zones were measured including the disc diameters. The antibacterial activity was expressed as the mean inhibition zone diameters, IZD (mm), and relative antimicrobial activity (RAA). The tests were performed in triplicates.

The relative antimicrobial activity was calculated as follows:

RAA = [(inhibition zone diameter mean of active plant)^2/ (inhibition zone diameter mean of reference antibiotic)^2] × 100

2.4.2. Determination of Minimum Inhibitory And Bactericidal Concentrations

The minimal inhibitory concentration (MIC) values were determined by broth dilution assay [22]. MIC and minimum bactericidal concentrations (MBC) of the *A. palaestinum* extracts were determined in nutrient broth using the standard dilution technique. Negative control (tube containing extract and growth medium without inoculum) and positive control (tube containing growth medium inoculated with bacteria) were used. In test tubes, 0.5mL of each diluted extracts with different concentration (5,10,15,20,25,30,35,40,45 and 50 mg/mL) were added to 10 mL nutrient broth inoculated with *P. syringae* at 10^5 CFU/mL. All tubes were incubated at 28°C for 48 hours. MIC values were taken as the lowest concentration of extracts that produced no visible bacterial growth when compared with the control tubes. In order to evaluate MBC, 100 µL of each MIC tubes and next tubes with higher concentration of extracts was placed on nutrient agar and incubated at 28°C for 48 hr. The lowest concentration which no bacterial growth observed on nutrient agar plates was considered as MBC. The tests were conducted in triplicates.

2.4.3. Antimicrobial Activity of A. Palaestinum Extracts Against Tomatoes' Bacterial Speck Disease

The plant extracts were evaluated in vivo against bacterial specks in tomatoes. Three tomato plants (8 weeks old) kept inside a greenhouse with 65% relative humidity at 28°C were used for each treatment.

The plants were sprayed with 50 mL of *P. syringae* suspension at 10^5 CFU/mL. Three days after inoculation, plants were treated with either sterile distilled water (SDW) as negative control, copper oxichloride at 2.5mg/mL and ciproloxacin at 0.5mg/mL dissolved in water as positive controls ($C_1$and C_2, respectively) or *A. palaestinum* extracts (45 mg/mL). After 5 days, 3 leaflets were randomly picked from every tomato plants and the number of specks in each was counted. The means and standard deviations were then caculated.

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619

2.5. Statistical Analysis

Statistical analyses were performed by Microsoft Excel 2019 to calculate the means and standard deviation (SD).

3. Results

3.1. Identification of Chemical Components of A. Palaestinum Extracts by LC-MS

HPLC-MS analysis was based on the peak area percentages, retention time, molecular formula and molecular weight. Thirteen compounds were detected and present in the analysis of aqueous, ethanolic and chloroform fractions of different parts of *A. palaestinum* (Table 1). It revealed the presence of one chemical compounds in aqueous leaves and flowers' fractions and 3 in aqueous rhizomes' fraction, nine other chemical compounds in ethanolic leaves' extract where 5 were identified in the ethanolic flower extracts and 4 in the ethanolic rhizomes' extract. In the chloroform extracts, 5 compounds were identified in the leaves and flowers each, while 4 were in the rhizomes. N-Octadecylbenzylamine was common in all aqueous fractions and the two chemical compounds 10,12-pentacosadiynamine and Eicosenamide were found in aqueous rhizomes' fraction. Palmitamide, Linolenyl alcohol, Octadecanamide and Geranylcitronellol were common in all ethanolic and chloroformic fractions. Pyritiduim, Cyclopiamine A and Ethylpheophorbide A were in the ethanolic leaves' fraction, Piptamine was common in the ethanolic leaves and flowers and chloroform leaves fractions. Two additional compounds were detected in the ethanolic leaves and chloroformic flowers fractions but were not identified.

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Table 1.
LC–MS analysis of the aqueous, ethanolic, and chloroform fractions of *A. palaestinum.*

Fractions	m/z (Da)	Retention time (min)	Molecular formula	Identification
Aqueous leaves' extract	360.3607	17.325	$C_{25}H_{45}N$*	*N-Octadecylbenzylamine*
Aqueous flowers' extract	360.3607	17.459	$C_{25}H_{45}N$*	*N-Octadecylbenzylamine*
Aqueous rhizomes' extract	326.3770	13.546	$C_{22}H_{47}N$	*10,12-pentacosadiynamine*
	360.3612	17.464	$C_{25}H_{45}N$*	*N-Octadecylbenzylamine*
	310.3101	15.437	$C_{20}H_{39}NO$	Eicosenamide
	438.2379	6.287	$C_{26}H_{27}N_7$	Pyritidium
	468.2482	6.470	$C_{26}H_{33}N_3O_5$	Cyclopiamine A
	256.2628	13.663	$C_{16}H_{33}NO$**	Palmitamide
	282.2784	13.896	$C_{18}H_{32}O$**	Linolenyl alcohol
	593.2741	14.303	$C_{35}H_{63}N_4O_5$	Not found in database
	284.2941	15.116	$C_{18}H_{37}NO$**	Octadecanamide
Ethanolic leaves' extract Ethanolic leaves' extract	310.3095	15.208	$C_{20}H_{36}O$**	Geranylcitronellol
	332.3303	15.300	$C_{23}H_{41}N$***	Piptamine
	621.3056	15.861	$C_{37}H_{40}N_4O_5$	Ethyl pheophorbide A
	256.2625	13.673	$C_{16}H_{33}NO$**	Palmitamide
	282.2778	13.892	$C_{18}H_{32}O$**	Linolenyl alcohol
	332.3311	14.834	$C_{23}H_{41}N$***	Piptamine
	284.2939	15.126	$C_{18}H_{37}NO$**	Octadecanamide
Ethanolic flowers' extract	310.3095	15.235	$C_{20}H_{36}O$**	Geranylcitronellol
	256.2624	13.651	$C_{16}H_{33}NO$**	Palmitamide
	282.2786	13.899	$C_{18}H_{32}O$**	Linolenyl alcohol
	284.2944	15.119	$C_{18}H_{37}NO$**	Octadecanamide
Ethanolic rhizomes" extract	310.3101	15.216	$C_{20}H_{36}O$**	Geranylcitronellol
Chloroformic leaves' extract	256.2623	13.665	$C_{16}H_{33}NO$**	Palmitamide
	282.2783	13.889	$C_{18}H_{32}O$**	Linolenyl alcohol
	284.2941	15.111	$C_{18}H_{37}NO$**	Octadecanamide
	310.3097	15.221	$C_{20}H_{36}O$**	Geranylcitronellol
	332.3302	13.345	$C_{23}H_{41}N$***	Piptamine
	537.1024	8.872	$C_{26}H_{21}N_2O_9P$	Not found in database
	256.2628	13.663	$C_{16}H_{33}NO$**	Hexadecanamide
	282.2784	13.895	$C_{18}H_{32}O$**	Linolenyl alcohol
Chloroformic flowers' extract	284.2937	15.112	$C_{18}H_{37}NO$**	Octadecanamide
	310.3099	15.206	$C_{20}H_{36}O$**	Geranylcitronellol
	256.2628	13.664	$C_{16}H_{33}NO$**	Hexadecanamide
	282.2781	13.899	$C_{18}H_{32}O$**	Linolenyl alcohol
	284.2941	15.135	$C_{18}H_{37}NO$**	Octadecanamide
Chloroformic rhizomes'extract	310.3099	15.227	$C_{20}H_{36}O$**	Geranylcitronellol

Note: * Mean common peaks in the aqueous leaves, flowers and rhizomes fractions. ** Mean common peaks in all ethanolic and chloroform fractions, while *** mean common peaks in ethanolic leaves, ethanolic flowers, and chloroform leaves extracts fractions.

3.2. Antimicrobial Activity of A. Palaestinum

The antimicrobial activities of the leaves (L), flowers (F) and rhizomes (R) of *A. palaestinum* extracts against the phytobacterium *P. syringae.* using the disk diffusion method is shown in Figure 1 and Table 2. DMSO, the vehicle used to suspend the dried extract that was used as a negative control had no affect on the bacerial growth.

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619

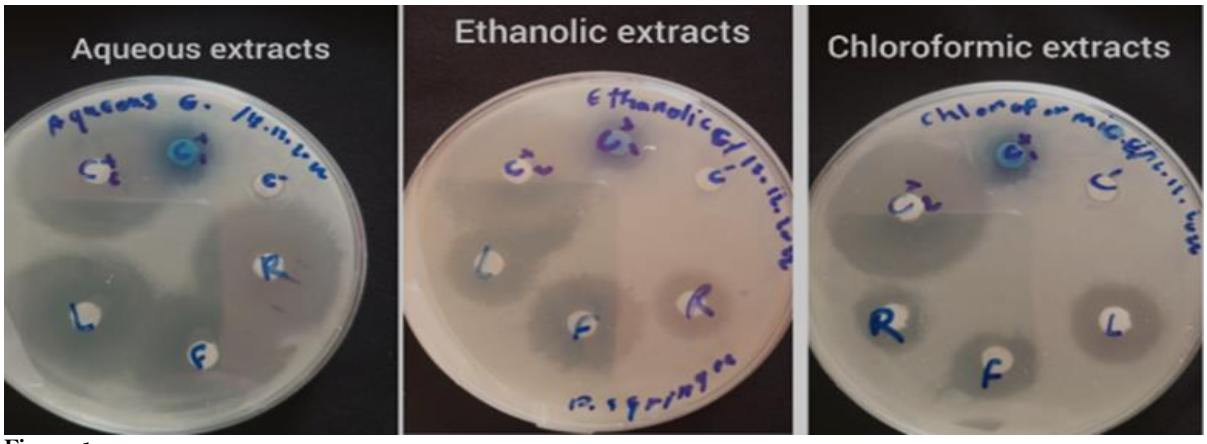

Figure 1.
Representative Figure of Agar Diffusion Assay of *A. palaestinum* Extracts against *P. syringae.* L: Leaves extract, F: Flowers extract, R: Rhizomes extract, C1:copper oxichloride , C2 :Ciproloxacin and C- :DMSO.

As seen in Figure 1, the aqueous extracts showed the highest antimicorbial activity and was confirmed by the calculated relative antimicrobial activities RAA1 and RAA2 (Table 2). The aqueous extracts yielded a high RAA2 (≥70) with IZD between 26.6 and 28.3mm. This is followed by the ethanolic extracts and chloroform leaves extracts with moderate RAA2 (≥30) and IZD ranging between 17.3 and 20.6mm against *P. syringae.* The chloroform flowers and rhizomes extracts showed low RAA2 (< 30) and IZD between 15.6 and 15.3mm, respectively. On the other hand, all *A. palaestinum* extracts showed high RAA1 activity against *P. syringae.*

Table 2.
Inhibition zones diameters (IZD) and relative antimicrobial activities to C1 (RAA1) and to C2 (RAA2) of *A. palaestinum* Extracts against *P. syringae.*

***A. palaestinum* extracts' type**	**IZD±SD (mm)**	**RAA1**	**RAA2**
Aqueous leaves	28.3±0.57	408.6	83.3
Aqueous flowers	27.6±0.57	388.6	79.2
Aqueous rhizomes	26.6±0.57	361	73.6
Ethanolic leaves	20.6±0.57	216	44.1
Ethanolic flowers	19.6±0.57	196	39.9
Ethanolic rhizomes	17.6±0.57	158	32.2
Chloroformic leaves	17.3±0.57	152.6	31.14
Chloroformic flowers	16±1	130.6	26.6
Chloroformic rhizomes	15.3±0.57	119.4	24.3
C_1	14±0	100	20.3
C_2	31±0	490	100

Note: SD: Standard deviation

3.2. MIC and MBC of the Active Plant Extracts

The minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of *A. palaestinum* extracts against *P. syringae* were determined (Table 3). The phytopathogen was sensitivite to the extracts with MIC and MBC ranging between 10-35 mg/mL and 10-40 mg/mL respectively. These results are in concordance with the inhibitory activities obtained. The aqueous leaves' extract exhibited by far the strongest bactericidal effects on *P. syringae* with an MIC and MBC of 10 mg/mL. As the aqueous flowers and rhizomes extracts, MIC of 10mg/mL and a MBC of 15mg/mL were obtained for both.

The MIC and MBC of the ethanolic leaves extract were higher (25mg/mL) while the MIC and MBC of the ethanolic flowers and rhizomes extracts were the same, 25 mg/mL and 30 mg/mL

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619

respecetively. The chloroformic extracts showed the higher values of MIC ranging between 30-35 mg/mL and MBC ranging between 30-40mg/mL.

Table 3.
MIC and MBC of *Arum palaestinum* extracts against *Pseudomonas syringae.*

***A. palaestinum* extracts**	**MIC±SD (mg/mL)**	**MBC±SD (mg/mL)**
Aqueous leaves	10±0	10±0
Aqueous flowers	10±0	15±5
Aqueous rhizomes	10±0	15±0
Ethanolic leaves	25±5	25±5
Ethanolic flowers	25±0	30±0
Ethanolic rhizomes	25±5	30±0
Chloroformic leaves	30±5	30±0
Chloroformic flowers	35±0	40±0
Chloroformic rhizomes	30±5	35±0

Note: SD: Standard deviation.

3.4. Activity of A. Palaestinum Extracts Against Tomatoe's Bacterial Speck Disease in Vivo

The activity of the different extracts compared to the bacteriocide copper oxychloride (C1) and the antibiotic ciprofloxacin (C2) against tomatoes' bacterial speck is shown in Table 4. In the field, the effect of *A. palaestinum* extracts on speck disease was noticeable.

The aqueous, ethanolic and chloroform leaves' extracts were the most powerful as an antibacterial where an obtained average of <1 speck/leaflet, while treatment with the chloroform flowers and rhizomes extracts showed means of 9.6 and 10.2 specks/leaflet, respectively. The negative control recorded the highest number of specks of 30.8 specks per leaflet. The positive controls C1 and C2 recorded 4.7 and 0 respectively.

Table 4.
The effects of *A. palaestinum* extracts on the bacterial speck disease in the field. C-: negative control (distilled water), C1: positive control 1 (copper oxychloride), C2: positive control 2(Ciprofloxacin).

***A. palaestinum* extracts**	**Mean of number speck/leaflet ±SD**
Aqueous leaves	0.33±0.50
Aqueous flowers	0.33±0.50
Aqueous rhizomes	0.55±0.52
Ethanolic leaves	0.55±0.52
Ethanolic flowers	0.66±0.50
Ethanolic rhizomes	0.66±0.50
Chloroform leaves	0.88±0.33
Chloroform flowers	9.66±1.30
Chloroform rhizomes	10.22±2.12
C1	4.77±0.83
C2	0.0±0.0
C-	30.88±2.34

Note: SD: Standard deviation.

4. Discussion

Arum is a genus of about 26 species of flowering plants in the family Araceae, native to different parts of the globe with the highest species diversity in the Mediterranean region. Around 180 phytochemicals were identified in the leaves of *A. palaestinum*, mainly flavonoids (quercetin dihexoside, vitexin-O-glucoside,…), phenolic acids (caffeoyl-hexose, pyrocatechol,…) , terpenoids (euphopubescenol, masilinic acid,…), alkaloids, iridoids and amino acids along with unknown compounds [4, 23]. The LC-MS analysis used in this study revealed for the first time the presence of 13 new compounds (N-Octadecylbenzylamine; 10,12-pentacosadiynamine; Eicosenamide; Pyritiduim; Cyclopiamine A;

Edelweiss Applied Science and Technology
ISSN: 2576-8484
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Palmitamide; Linolenyl alcohol; Octadecanamide; Geranylcitronellol; Piptamine; Ethyl pheophorbide A and two unknown compounds) in the tested samples of *A. palaestinum*.

Moreover, the LC-MS analysis revealed the presence of chemical components with different biological functions. Three important chemical compounds common in ethanolic and chloroformic extracts of leaves, flowers and rhizomes of *A. palaestinum* have been discovered, Linolenyl alcohol is described as an antibacterial agent [24] Palmitamide for tissue regeneration preparation [25] and Geranylcitronellol which is an antileishmanial agent [26]. Three other important chemical components of ethanolic leaves' extract have been detected, Pyritidium acts as antiprotozoal agent and has antiviral properties [27] Cyclopiamine A is a oxindole alkaloid with antitumor and antibacterial properties [28], and Ethyl pheophorbide A has significant anti-proliferative effects in several human cancer cell lines [29] and broad virucidal activities [30]. Furthermore, Piptamine present in the ethanolic leaves' and flowers' extracts and chloroformic leaves' extract also acts as an antibiotic [31] .

The antibacterial activity assessment of *A. palaestinum* in vitro demonstrated that the aqueous and ethanolic extracts of all parts and chloroformic leaves extract had a significant antibacterial activity against *P. syringae*. Chloroformic flowers and rhizomes extracts further revealed an inhibitory effect on *P. syringae*. In addition, all extracts showed an inhibitory effect on growth, spread and infection of speck disease on the tomato plants in the field experiment. This results is compatible and related with the LC-MS analysis results, which showed the presence of antibacterial compounds in ethanolic and chloroformic extracts of leaves, flowers and rhizomes of *A.palaestinum* . Our result is compatible with previous studies reported about the antibacterial effects of *Arum* subspecies , where it was demonstrated that the crude *A. palaestinum* flowers aqueous extracts have antibacterial activity against the Gram-positive bacteria *Staphylococcus aureus*, and *Enterococcus faecium*; and the Gram-negative bacteria *Klebsiella pneumoniae*, *Proteus vulgaris* , *Escherichia coli*,and *Pseudomonas aeruginosa* Dwikat, et al. [32]. Jaber, et al. [33] demonstrate that the water and methanol extracts of *Arum hygrophilum* leaves also had significant antibacterial activity against *P. aeruginosa*. Also, leaves and berries of *Arum maculatum* were observed antibacterial activities against *P. aeruginosa* [34].

The significant antibacterial effect of *A. palaestinum* against *P. syringae* reveals a promising hope in their control in organic agriculture like others plant extracts demonstrated by Simonetti, et al. [35] to prevent the bacterial canker caused by *p.syringae* and use in this disease management.

5. Conclusion

This study is considered as a preliminary investigation for future development of naturally occurring bacteriocide to be used in organic agriculture. *A. palaestinum* extracts revealed promising results to eradicate *P. syringae*. The plant is rich in phytochemicals worth to be studied in controlling agriculture pests and other agricultural investigations.

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

Authors' Contributions:

Jamilah Borjac conceived and designed research. Samaher Ghaith conducted experiments and analyzed the data and wrote the manuscript. Dany Abi Shahine conducted HPLC analyses. Jamilah Borjac, Michel Afram and Antoine Abou Fayad reviewed the manuscript. All authors read and approved the final manuscript.

Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619

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Edelweiss Applied Science and Technology
ISSN: 2576-8484
Vol. 9, No. 2: 853-862, 2025
DOI: 10.55214/25768484.v9i2.4619