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Antibacterial activity of *Alternanthera philoxeroides* (Mart.) Griseb. against bacterial phytopathogens: *Erwinia carotovora, Ralstonia solanacearum* and *Xanthomonas axonopodis*

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ABSTRACT

We investigated the antibacterial activity of alligator weed (*Alternanthera philoxeroides*) organic extracts against three bacterial phytopathogens (*Erwinia carotovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis*). The extracts were prepared by soaking the dry powder of leaf, stem and root of *A. philoxeroides* into methanol, *n*-hexane, chloroform and ethyl acetate. The disk diffusion method was used to determine the antibacterial activity at 100 mg/mL extract concentration. The *n*-hexane extract of *A. philoxeroides* leaves showed the maximum inhibition zone diameter (IZD)= 28.1 mm against *R. solanacearum*, while, the corresponding value for the positive control (Penicillin) was 48 mm IZD. There was no antibacterial activity of negative control, dimethyl sulfoxide (DMSO). Gas Chromatography Mass-Spectrometry (GC-MS) analysis revealed the presence of acetic acid, 2-(2-methoxycarbonylamino-5-nitrophenylthio)-, methyl ester, at the highest concentration (31.9 %), followed by 1, 4-benzenediol, 2, 5-bis (1,1-dimethylethyl)- (15.06 %),. It was concluded that the observed biological activity in this study may be due to the presence of these compounds.

Keywords: Alligator weed, *Alternanthera philoxeroides*, antibacterial activity, bacterial phytopathogens, *Erwinia carotovora*, GC-MS, inhibition zone, leaf, organic extracts, *Ralstonia solanacearum*, root, stem, *Xanthomonas axonopodis*

INTRODUCTION

Some species of genus *Alternanthera* possess the biological activities. The alligator weed [*Alternanthera philoxeroides* (Mart.) Griseb.], (Figure 1), is used as a medicine in some countries (11). Due to the presence of numerous bioactive compounds (phaeophytin a, oleanolic acid, phytol and α-spinasterol), the alligator weed is important against bacterial invasions (5) Moreover, alligator weed also have antitumor constituents e.g. alternanthin B and N-trans-feruloyl-3, 5-dimethoxytyramine (6). Bacteria cause various diseases in plants and synthetic bactericides are used to control them. Excessive use of synthetic pesticides in agriculture have resulted in increased pollution of water, air and soil, thus damaging the environment (9). Hence, there is a need to search novel natural

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Figure 1. *Alternanthera philoxeroides* (A): single plant, (B): in population

substances to control pathogenic microbes. Some herbs act as antimicrobial agents. The aqueous and chloroform:methanolic (1:1) extracts of *A. philoxeroides* are antibacterial to *Pseudomonas aeruginosa* (15). Similarly, aqueous extracts of *A. philoxeroides* and *A. sessilis* exhibited antibacterial activity against *Bacillus pumilus, Bacillus subtilis* and *Salmonella typhi* (11). Likewise, antimicrobial activities of other species of genus *Alternanthera* have been reported against several bacterial strains. The ethanolic extracts of *A. sessilis* showed antibacterial activity against *Staphylococcus aureus* and *Staphylococcus haemolyticus.* The *A. philoxeroides* at 10, 25 and 50 µg/mL concentrations were antimicrobial against *Klebsiella pneumonia* (19). The acetone extract of *A. pungens* showed antibacterial activity against the *Mycobacterium smegmatis* inducing IZD (Inhibition zone diameter) of 18.6 mm (8). The ethanolic extract of *Alternanthera bettzickiana* exhibited antibacterial activity against *S. aureus* at concentrations of 30, 40 and 50 µg/mL inducing 11, 12 and 13 mm IZD, while, amoxicillin showed 29 mm IZD. Likewise, ethanolic extract of *A. bettzickiana* at 30, 40 and 50 µg/mL concentrations exhibited antibacterial activity against the growth of *E. coli* by creating 13, 15 and 17 mm IZD, respectively (13). Although, there are fewer reports about the antimicrobial activity of genus Alternanthera, but there is no evidence about the antibacterial activity of *A. philoxeroides* against *Erwinia carotovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis.* So, this study was aimed to investigate the antibacterial activity of *A. philoxeroides* against some bacterial phytopathogens not reported previously. Using GC-MS, we determined the chemical composition and studied the antibacterial activity of the most active fraction.

MATERIALS AND METHODS

The alligator weed was collected at flowering stage in November 2018 from village Kapurowali, Sialkot, Pakistan [32. 45° N, 74. 57° E], altitude 246 m with an annual rainfall of 950 mm. Maximum and minimum temp. in November 2018 were 16 °C and 27 °C, respectively.

Preparation of Organic Extracts of Alligator Weed

The fresh samples of alligator weed leaves, stem and roots were collected and brought to laboratory. First, these were fully washed with tap water and then with distilled

water. Then samples were dried in sunlight for 7 days. The dried samples of leaves, stem and roots of weed were powdered with pestle and mortar. The dried leaves, stem and roots @ 200 g of each were added in separate jars containing 500 mL methanol (≥ 99.9 % purity Sigma-Aldrich) for 1 week. Later on, the methanol extract was first filtered through muslin cloth and then through Whatman filter paper No.1. The filtered extract was evaporated in rotary evaporator at 45 °C to evaporate the solvent (methanol).

The methanolic extracts were fractionated by 3 organic solvents viz. *n*-hexane, chloroform and ethyl acetate. Methanolic extracts of each part (leaf, stem and root) of alligator weed were reconstituted separately in 200 mL distilled water and then added 200 mL *n*-hexane into it in a separating funnel. This mixture was incubated overnight till the formation of two layers. The upper layer of *n*-hexane was separated in the beaker and lower aqueous layer was then extracted successively with 200 mL of chloroform. After separation, the lower layer of chloroform extract was removed from the aqueous layer. The aqueous layer was then mixed with 200 mL ethyl acetate for ethyl acetate extract preparation. Similarly, the whole procedure was performed to prepare the stem and root extracts. Solvents from extracts were evaporated using rotary evaporator as mentioned above. Afterwards, the crude organic extracts were stored at 4 °C for subsequent use.

Antibacterial Assay

Bacterial cultures were obtained from the Culture Bank, University of the Punjab, Lahore, Pakistan. For bacterial growth, LB (Lysogeny Broth) medium was used. These grown cultures were kept in a refrigerator at 4 °C for subsequent use.

Preparation of control and stock solutions

The disk diffusion method was used for the antibacterial assay as per (2) with slight modifications. To prepare negative control, 166 µl DMSO (Dimethyl sulfoxide) was added into 333 µl autoclaved distilled water to make final volume of 500 µl. Similarly, to prepare positive control solutions, 50 mg penicillin (antibiotic) was mixed in 166 µl DMSO and 333 µl autoclaved distilled water to make final volume of 500 µl. Likewise, to prepare stocks solutions, 50 mg crude organic extracts (methanolic, *n*-hexane, chloroform and ethyl acetate extracts) of leaves, stem and roots were separately dissolved in 166 µl DMSO and mixed in 333 µl autoclaved distilled water to make a final volume of 500 µl.

Preparation of LB Medium for bacterial growth

LB medium was used to inoculate the bacterial species. To prepare LB medium, 1000 mL distilled water was added into the conical flask, then 5 g yeast, 10 g tryptone, 10 g NaCl and 15 g agar powder were added and mixed well to dissolve all components. Afterwards, the flask mouth was covered with aluminium foil and autoclaved for 20 minutes at 121 °C.

Antibacterial activity

Agar plates were prepared and the inoculation (streaking) of bacteria was done using sterile culture swab for uniform spread of bacterial inocula on these agar plates. Then, sterile filter paper discs (6 mm) moistened with leaves, stem, and root extracts (25 µl) of organic solvents viz. methanol, *n*-hexane, chloroform and ethyl acetate, as well as solutions of positive and negative control, were placed in the center of each agar plate.

These plates were incubated for 24 h at 30 °C in an incubator. Treatments were replicated thrice in Completely Randomized Design. After incubation, antibacterial activity was calculated by measuring the inhibition zone diameter (IZD) with scale. IZD was measured by taking mean of 2 readings taken as criss-cross pattern in each plate.

Gas Chromatography-Mass Spectrometry (GC-MS)

Constituents of *n*-hexane extract of *A. philoxeroides* leaves showing higher bioactivity were analyzed using GC-MS on Clarus 500 Mass Spectrometer whose detectable mass range was set at 35-500 m/z. Ion source and interface temperatures were 200 °C and 250 °C, respectively. Start and end times were 2.50 min and 47.14 minutes, respectively. Column oven temp. was 40 °C while injection temp. was 25 °C. Injection mode was split and flow control mode was set at 100 kPa pressure. The total flow was 13.9 mL/min while the column flow was 1.78 mL/min with a linear velocity of 48.1 cm/sec. Purge flow was kept at 3.0 mL/min and a split ratio of 5.1. The oven temperature was programmed first at 40 °C for 5 min with an increase of 5 °C min^{-1} to 80 °C, then 5 °C min^{-1} to 300 °C for 5 min. The GC-MS part of research was conducted at Thermal Energy Research Lab., National University of Sciences and Technology, Islamabad, Pakistan (1).

Statistical Analysis

For statistical analysis, ANOVA ($P \leq 0.05$), was performed by using statistical software Minitab 19 followed by Fisher's LSD test.

RESULTS AND DISCUSSION

The organic solvent extracts of *A. philoxeroides* leaves suppressed the growth of phytopathogenic bacterial species. Chemical constituents of the most active fraction were identified through Gas Chromatography-Mass Spectrometry (GC-MS) analysis.

***A. philoxeroides* leaves**

Methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* leaves were antibacterial against *E. carotovora* as evident from the inhibition zone diameter (IZD) of 19.55, 22, 20 and 21 mm. While, penicillin, used as a positive control, exhibited 42 mm IZD. Similarly, methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* leaves showed IZD of 21.13, 28, 19.08 and 26 mm against *Ralstonia solanacearum*. While penicillin showed 48 mm inhibition. Similarly, methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* leaves showed IZD of 19.80, 21.5, 16.16 and 18.63 mm against *Xanthomonas axonopodis*. While penicillin showed 46 mm IZD. DMSO solution kept as negative control did not inhibit the growth of all test bacterial species (Figure 2). In another study, methanolic extract of *Cornus macrophylla* at 100 mg/mL extract concentration exhibited a 21.5, 36.3, 25.3, and 23.7 mm IZD; *n*-hexane showed a 33, 40, 32.8 and 28.7 mm IZD; chloroform showed a 18.8, 29, 22.3 and 21.6 mm IZD; and ethyl acetate showed a 23.5, 30.2, 30 and 22.3 mm IZD against *E. carotovora*, *Pseudomonas syringae*, *R. solanacearum*, and *X. axonopodis*, respectively (1). In the present study, leaf extract proved more effective than to stem and root extracts. Similar results were reported by (20) where leaf extract of *Pistacua atlantica*

subsp. *kurdica* effectively inhibited the growth of *Staphylococcus aureus* than bark and stem extracts. This leaf extract caused the largest inhibition zone of 7.5 mm.

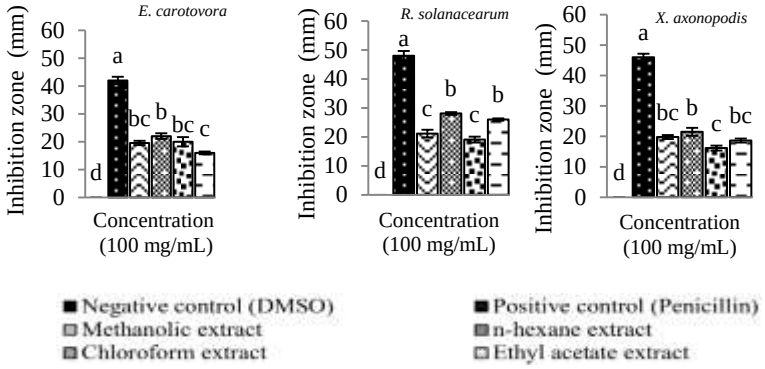


Figure 2. Inhibitory effects of *Alternanthera philoxeroides* leaves organic solvent extracts (Methanolic, *n*-hexane, chloroform and ethyl acetate) on growth of *Erwinia carotovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis*. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ as determined by Fisher's LSD test. Vertical bars show standard error of means of three replicates.

***A. philoxeroides* stem**

Methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* stem also showed antibacterial activity against *E. carotovora* by creating 16.14, 18.73, 14.8 and 15.66 mm IZD. While, penicillin showed 42 mm IZD. Likewise, methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* stem showed IZD of 14.52, 15.93, 19.73 and 18 mm against *R. solanacearum.* While, penicillin showed 48 mm inhibition. Similarly, methanolic, *n*-hexane, chloroform and ethyl acetate extracts of *A. philoxeroides* stem showed IZD of 15.03, 14.06, 17.6 and 18.03 mm against *X. axonopodis.* While, penicillin showed 46 mm IZD and DMSO did not inhibit the growth of any bacterial pathogen (Figure 3). In another study, *n*-hexane stem extract of *Amaranthus viridis* showed 13.83, 9.83, 13.83 and 10.67 mm IZD, against *P. syringae*, *R. solanacearum*, *E. carotovora* and *X. axonopodis*, respectively. While the chloroform stem extract of *A. viridis* showed 13.18, 12.5, 13.17 and 14.17 mm inhibition, against *P. syringae, R. solanacearum, E. carotovora* and *X. axonopodis*, respectively (2).

***A. philoxeroides* root**

The methanolic, *n*-hexane, chloroform and ethyl acetate extracts of *A. philoxeroides* root also showed antibacterial activity in the form of inhibition zone diameter (IZD) of 13, 15, 13.4 and 9.5 mm against *E. carotovora.* While penicillin showed 42 mm IZD. Then, methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* root showed IZD of 10, 14.6, 9 and 8 mm against *R. solanacearum.* While, penicillin showed 48 mm inhibition. Similarly, methanolic, *n*-hexane, chloroform and ethyl acetate extract of *A. philoxeroides* root showed IZD of 11, 12.55, 14 and 8 mm against *X. axonopodis.* While, penicillin showed 46 mm IZD. The effect of DMSO was invisible in all cases (Figure 4).

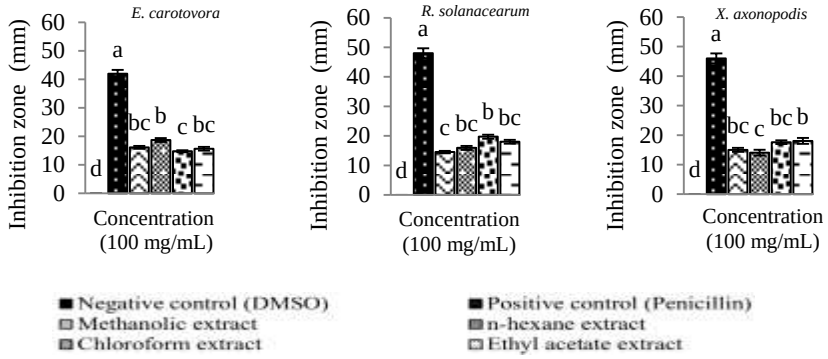


Figure 3**.** Inhibitory effects of *Alternanthera philoxeroides* stem organic solvent extracts (Methanolic, *n*-hexane, chloroform and ethyl acetate) on growth of *Erwinia carotovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis*. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ as determined by Fisher's LSD test. Vertical bars show standard error of means of three replicates.

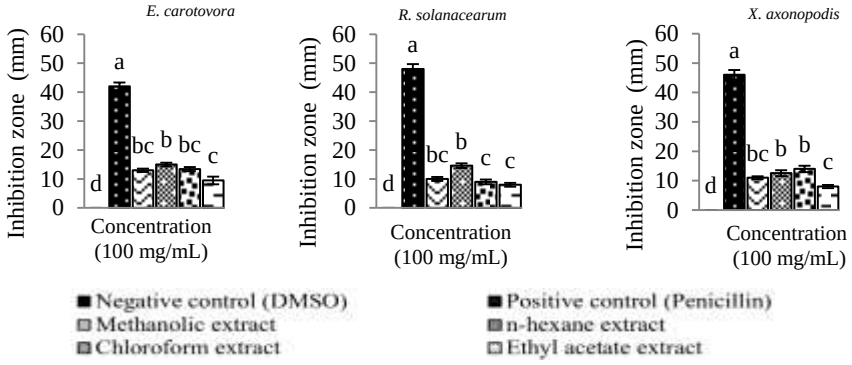
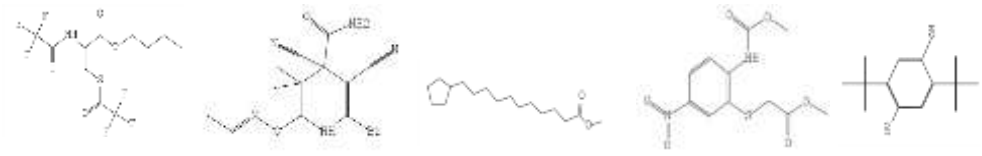


Figure 4**.** Inhibitory effects of *Alternanthera philoxeroides* roots organic solvent extracts (Methanolic, *n*-hexane, chloroform and ethyl acetate) on growth of *Erwinia carotovora*, *Ralstonia solanacearum* and *Xanthomonas axonopodis*. Bars topped by the same letter do not differ significantly at $P \leq 0.05$ as determined by Fisher's LSD test. Vertical bars show standard error of means of three replicates.

Our results agree with findings of (4) in which they concluded that *Urospermum picroides* inhibited the growth of *E. carotovora* to 7-8 mm. The *Phytolacca dioica* showed antibacterial activity in terms of formation of 11.66 mm inhibition zone against the growth of *R. solanacearum* (12). Similarly, *Taraxacum officinale* showed antibacterial activity in terms of 17.2 mm zone of inhibition against *X. axonopodis* (17). There are reports of several plants showing antibacterial activity against these bacterial phytopathogens but, there is no evidence of antibacterial activity of alligator weed against these bacterial pathogens. As extraction procedures greatly influence the types of metabolites to be extracted so, in addition to leaves, stem and roots of alligator weed were also explored in this study, using different organic extracts viz. methanolic, *n*-hexane, chloroform and ethyl acetate.

Gas Chromatography-Mass Spectrometry (GC-MS)

In total, 25 compounds were identified in *n*-hexane extract of *A. philoxeroides* leaves (Table 1). The retention time (RT), name of the compounds, peak area (%), molecular weights and their molecular formulae are shown in Table 1. Out of 25 compounds, 6 compounds were dominant: Acetic acid, 2-(2-methoxycarbonylamino-5-nitrophenylthio)-, methyl ester (31.92 %), 1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)- (15.06 %), 4-Pyridinecarboxamide, 6-bromo-4,5-dicyano-1,2,3,4-tetrahydro-3,3-dimethyl-2-[[(1methylethyamino]oxy] (8.53 %), L-Cysteine, N-(trifluoroacetyl)-, butyl ester, trifluoroacetate (ester) (6.59 %), Cyclopentaneundecanoic acid, methyl ester (5.4 %) and 3-Bromo-N-(2-thiazolyl) benzamide (3.49 %). These compounds are depicted in Figure 5.



L-Cysteine, N-(trifluoroacetyl)-, butyl ester, trifluoroacetate (ester)	4-Pyridine carboxamide, 6-bromo-4,5-dicyano-1,2,3,4-tetrahydro-3,3-dimethyl-2-[[(1-methyl ethylidene)amino]oxy]	Cyclopentaneundecan oic acid, methyl ester	Acetic acid, 2-(2-methoxycarbonyl amino-5- nitro phenylthio)-, methyl ester	1,4-Benzenediol, 2,5-bis (1,1-di methylethyl)-

Figure 5. Compounds identified from GC-MS analysis of *n*-hexane extract of *Alternanthera philoxeroides* leaves at > 5 % concentrations.

Out of identified compounds in the *n*-hexane extract of *A. philoxeroides* leaves in this study, some compounds were reported first time but some compounds were reported earlier in literature. The compound e.g. 1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl) identified in ethyl acetate extract from marine actinomycetes suppressed the growth of multidrug resistant bacteria e.g. *Acinetobacter baumannii, S. aureus*, and *P. aeruginosa* (21). Similarly, Cyclopentaneundecanoic acid, methyl ester, isolated from isopropanol extract of *Carica papaya* was antibacterial against *S. aureus, Streptococcus faecalis, Escherichia coli* and *Proteus mirabilis.* In disc diffusion assay, inhibition zone of 14.33, 11.39, 12.98, and 12.37 mm was formed against *S. aureus, S. faecalis, E. coli* and *P. mirabilis*, respectively (7).

No literature was available about the *n*-hexane extract of *A. philoxeroides* leaves, but, GC-MS analysis of 2 organic extracts viz. ethanol and methanol have been described. There were 12 compounds in ethanol extract of aerial parts (flowers, stem and leaves) of *A. philoxeroides* GC-MS analysis (14). Our results differed from earlier findings either because they used ethanol extract or due to different conditions used in GC-MS. Similarly, 12 compounds were also obtained from the methanolic extract of *A. philoxeroides* leaves (10). These 12 compounds were different from 12 compounds in ethanol extract of *A. philoxeroides* (14) and with *n*-hexane extract of *A. philoxeroides* leaves (present study). Esters and phenolics have antimicrobial activity (3,16,18). Plant habitat, environmental

Table 1. Compounds identified in GC-MS analysis of *n*-hexane extract of *Alternanthera philoxeroides* leaves

Sr.#	R.T. (min)	Name of Compound	Molecular Formula	Molecular Weight	Peak Area %
1	**4.015**	**L-Cysteine, N-(trifluoroacetyl)-, butyl ester, trifluoroacetate (ester)**	**$C_{11}H_{13}F_6NO_4S$**	**369**	**6.59**
2	**4.43**	**4-Pyridinecarboxamide, 6-bromo-4,5-dicyano-1,2,3,4-tetrahydro-3,3-dimethyl-2-[[(1-methylethylidene)amino]oxy]**	**$C_{13}H_{16}BrN_5O_2$**	**353**	**8.53**
3	4.840	3-Methyl-4-(phenylthio)-2-prop-2-enyl-2,5-dihydrothiophene 1,1-dioxide	$C_{14}H_{16}O_2S_2$	280	2.33
4	10.955	5-Nitro-1,3-benzothiazol-2-amine	$C_7H_5N_3O_2S$	195	1.16
5	11.460	Isoshyobunone	$C_{15}H_{24}O$	220	1.16
6	12.895	Bromperidol	$C_{21}H_{23}BrFNO_2$	419	1.94
7	17.020	Fucoxanthin	$C_{42}H_{58}O_6$	658	1.55
8	17.065	2-Chloro-5-(1,2,4-triazol-4-yl)aniline	$C_8H_7ClN_4$	194	1.55
9	17.650	Thiamphenicol	$C_{12}H_{15}Cl_2NO_5S$	355	1.16
10	17.890	Kinetin	$C_{10}H_9N_5O$	215	1.55
11	19.390	1-(2-Nitrophenyl)piperazine	$C_{10}H_{13}N_3O_2$	207	1.16
12	21.260	3-Chloro-4-(1,2,4-triazol-4-yl)benzoic acid	$C_9H_6ClN_3O_2$	223	0.78
13	23.385	3-Bromo-N-(2-thiazolyl) benzamide	$C_{10}H_7BrN_2OS$	282	3.49
14	24.890	1-(4-Methylphenyl)-1H,2H,3H,4H,9H-pyrido[3,4-b]indole-3-carboxylic acid	$C_{19}H_{18}N_2O_2$	306	1.94
15	26.230	Isocalamendiol	$C_{15}H_{26}O_2$	238	3.1
16	**29.595**	**Cyclopentaneundecanoic acid, methyl ester**	**$C_{17}H_{32}O_2$**	**268**	**5.4**
17	30.635	Dihydrosteviobiside	$C_{32}H_{52}O_{13}$	644	2.32
18	31.845	Lysergic Acid	$C_{16}H_{16}N_2O_2$	268	1.94
19	35.050	Thiocolchicine	$C_{22}H_{25}NO_5S$	415	1.16
20	35.445	4-Bromo-5-nitro-1H-imidazole	$C_3H_2BrN_3O_2$	191	0.77
21	38.620	10-Octadecenal	$C_{18}H_{34}O$	266	1.55
22	38.655	Aristolochic acid	$C_{17}H_{11}NO_7$	341	0.39
23	39.110	Chalcone	$C_{15}H_{12}O$	208	1.5
24	**41.635**	**Acetic acid, 2-(2-methoxycarbonylamino-5-nitrophenylthio)-, methyl ester**	**$C_{11}H_{12}N_2O_6S$**	**300**	**31.92**
25	**43.800**	**1,4-Benzenediol, 2,5-bis(1,1-dimethylethyl)-**	**$C_{14}H_{22}O_2$**	**222**	**15.06**
		Total Peak Area			**100**

Note: Compounds highlighted in bold were detected in higher (>5%) concentrations.

conditions, stage of growth, extraction procedures and GC-MS conditions/parameters e.g. type of GC-MS, greatly influence the identified compounds.

CONCLUSIONS

The *n*-hexane fraction of *A. philoxeroides* leaves showed higher antibacterial activity than stem and root fractions. *n*-hexane extract of *A. philoxeroides* leaves showed antibacterial activity as inhibition zone diameter (IZD) of 22, 28 and 21.5 mm against *E. carotovora*, *R. solanacearum* and *X. axonopodis*, respectively. On the other hand, the penicillin used as reference compound created IZD of 42, 48 and 46 mm, respectively. GC-MS analysis revealed that acetic acid, 2-(2-methoxycarbonylamino-5-nitrophenylthio)-, methyl ester and 1, 4-Benzenediol, 2, 5-bis (1,1-dimethylethyl)- were

present at higher concentrations. It was concluded that the antibacterial activity observed in this study may be due to the presence of these compounds.

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