



Bioefficacy of Some Aqueous Phytoextracts Against *Clavibacter Michiganensis* Subsp. *Michiganensis* (Smith), the Cause of Bacterial Canker of Tomato

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Received: 29 February 2020 / Accepted: 16 April 2020 / Published online: 7 May 2020
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Abstract

The ability of some medicinal plants was explored to control bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*. The plants tested included *Peganum harmala*, *Allium sativum*, *Withania somnifera*, *Melia azedarach*, *Calotropis procera*, *Mentha piperita* and *Nerium oleander*. Aqueous extracts of *P. harmala* and *M. piperita* proved to be very effective under *in vitro* conditions against *Clavibacter michiganensis* subsp. *michiganensis*. In *in vivo* studies, the highest dose of dried powder of *P. harmala* and *M. piperita* kg⁻¹ of soil decreased disease severity significantly. Other yield-contributing parameters were also enhanced by the application of dried powder and no phytotoxicity was observed at given concentrations. Alkaloids, tannins, glycosides, flavonoids and saponins were detected in aqueous extracts of *P. harmala*, *W. somnifera*, *M. azedarach*, *C. procera* and *M. piperita*. Dried powders of *P. harmala* and *M. piperita* could be incorporated as an integral part in the integrated disease management programs against *Clavibacter michiganensis* subsp. *michiganensis*.

Keywords *Clavibacter michiganensis* subsp. *michiganensis* · *Peganum harmala* · *Calotropis procera* · *Withania somnifera* · *Melia azedarach* · *Mentha piperita*

Biologische Wirksamkeit einiger wässriger Phytoextrakte gegen *Clavibacter michiganensis* subsp. *michiganensis* (Smith), dem Auslöser des bakteriellen Krebses bei Tomaten

Zusammenfassung

Die Studie untersuchte die Eignung einiger Arzneipflanzen zur Bekämpfung des bakteriellen Krebses bei Tomaten, der durch *Clavibacter michiganensis* subsp. *michiganensis* verursacht wird. Zu den getesteten Pflanzen gehörten *Peganum harmala*, *Allium sativum*, *Withania somnifera*, *Melia azedarach*, *Calotropis procera*, *Mentha piperita* und *Nerium oleander*. Wässrige Extrakte von *P. harmala* und *M. piperita* erwiesen sich unter *in-vitro*-Bedingungen als sehr wirksam gegen *Clavibacter michiganensis* subsp. *michiganensis*. In *in-vivo*-Studien verringerte die höchste Dosis an getrocknetem Pulver von *P. harmala* und *M. piperita* die Krankheitsschwere signifikant. Auch andere ertragsfördernde Parameter wurden durch die Anwendung von getrocknetem Pulver verbessert, und bei den vorliegenden Konzentrationen wurde keine Phytotoxizität beobachtet. Alkaloide, Tannine, Glykoside, Flavonoide und Saponine wurden in den wässrigen Extrakten von *P. harmala*, *W. somnifera*, *M. azedarach*, *C. procera* und *M. piperita* nachgewiesen. Die getrockneten Pulver von *P. harmala* und *M. piperita* könnten als wichtiger Bestandteil in die integrierten Disease-Management-Programme gegen *Clavibacter michiganensis* subsp. *michiganensis* aufgenommen werden.

Schlüsselwörter *Clavibacter michiganensis* subsp. *michiganensis* · *Peganum harmala* · *Calotropis procera* · *Withania somnifera* · *Melia azedarach* · *Mentha piperita*

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Introduction

Tomato (*Lycopersicon esculentum* Mill.), a member of Solanaceae, is an important vegetable crop of Pakistan. The area under tomato cultivation in Pakistan is 40,238 hectares, with a total production of 566,043 tons while Khyber Pakhtunkhwa (KPK) province of Pakistan contributes an area of over 13,252 hectares with a production of 127,552 tons (Anonymous 2016). Cultivation of tomato crop has sharply increased in the country due to its growing demand. However, the demand-supply situation is still imbalanced mainly due to production constraints. Plant diseases, caused by fungi, viruses, nematodes and bacteria, are one of the major factors limiting tomato production (Agrios 2005). Among bacterial diseases, bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith), is a potentially severe disease of tomato that can occur in commercial plantings and home gardens. This infectious disease is capable of spreading rapidly resulting in devastating losses. Bacterial canker has caused huge economic losses in commercial tomato production throughout the world (Davis et al. 1984). The bacterium is aerobic, Gram-positive, non-motile, non-spore forming, curved rod (Bradbury 1986). The pathogen is seed-borne as well as plant-debris borne (Dutta et al. 2014).

Bacterial canker produces dark, necrotic lesions at the edges of older leaves. On the surface of young leaves and fruits, the bacteria multiply rapidly and produce tiny, raised, white blisters. Later, the centers of the white blisters turn brown, giving rise to “bird’s-eye” spots which is the characteristic symptom of bacterial canker. The vascular tissues of transversely cut stems of wilted plants near the nodes usually appear dark yellow to tan brown. On seedlings, initially, small, water-soaked lesions appear on stems and leaves which later turn into necrotic patches leading to stunting and wilting. On green-house-grown tomato plants, symptoms appear as chlorotic patches between veins that quickly become necrotic. On infected stems, light brown streaks or cankers appear which turn dark with the age. In many cases, plants affected by bacterial canker wilt and die (Bose et al. 2004). Warm temperatures (29–35 °C) and high relative humidity favor the disease (Shenge et al. 2010).

The best disease control strategy to manage a hard-to-control disease, like bacterial canker, is the use of integrated disease management (IDM). Various components of IDM could include the use of resistant cultivars, pathogen-free seeds and transplants, green house disinfection, removal of infected plant debris, proper sanitation and rotation with non solanaceous plants for at least two years etc. (Gleason et al. 1993; Pradhanang et al. 2005). However, because of the complex nature of the pathogen, many of these strategies are of limited use (Hong et al. 2011). The scarcity of effective control measures and the magnitude of bacterial canker

on economical crops, necessitate the search for new control options. One of such options is the use of dried powders or extracts of medicinal plants/weeds. A number of plants/weeds have been reported (Singariya et al. 2011; Singh and Kumar 2014; Bokaeian and Saeidi 2015) to have strong antibacterial properties. These plants have secondary metabolites, such as alkaloids, flavonoids, steroids, glycosides, saponins, and tannins, which possess antimicrobial properties (Cowan 1999). The goal of this study was to explore the bacterial-canker-control potential of those medicinal plants/weeds which have a long history as folk medicines and which are easily available in large amounts year round. We evaluated seven such medicinal plants/weeds including *Withania somnifera*, *Melia azedarach*, *Peganum harmala*, *Allium sativum*, *Calotropis procera*, *Nerium oleander* and *Mentha piperita* for their ability to inhibit the *in vitro* growth of the tomato canker bacterium using aqueous plant extracts and agar well diffusion method as well as the ability of some of these plants to reduce disease severity and enhance plant parameters *in vivo*.

Keeping in view the ever-increasing demand and the economic importance of tomatoes in Pakistan, the potential threat of bacterial canker to profitable tomato crop, the lack of our resource-poor farmers to afford the high costs of disease control inputs, the possible effectiveness and the year round, free of cost, large-scale availability of these medicinal plants, we tested the potential of these for the control of bacterial canker of tomato. Both *in vitro* and *in planta* experiments were carried out. The hypothesis was that the aqueous extracts of some of these medicinal plants would be inhibitory to the *in vitro* growth of the bacterial canker pathogen and this inhibitory effect would be equally effective for the control of the disease on tomato plants grown under screen house conditions. Moreover, we hoped that the *in vivo* use of the dried powders of some of these medicinal plants, if found effective, would be very economical for our small land-holding farmers on one hand, and would eliminate or at least reduce the environment-polluting chemicals such as antibiotics or copper fungicides used to control plant bacterial diseases, on the other hand. In addition to being environment-friendly, the use of dried powder soil amendments will also deter the appearance of chemical-resistant bacteria (Cowan 1999) as well as enhance tomato yield by controlling the disease and improving the physical and chemical properties of soil. The objectives of this study were (i) to investigate the potential of the aqueous extracts of these selected plants to inhibit the *in vitro* growth of the tomato canker pathogen, and (ii) to correlate the *in vitro* bacterial growth inhibition to *in planta* disease control and plant growth parameters enhancement.

Materials and Methods

Bacterial Culture and Inoculum Preparation

Clavibacter michiganensis subsp. *michiganensis* (Cmm) was obtained from plant bacterial culture bank of the Department of Plant Pathology, the University of Agriculture (UOA), Peshawar. The bacterium was sub-cultured on nutrient agar (NA) medium (per 1 L: beef extract, 3 g; peptone, 5 g; agar, 15 g) and incubated overnight at 28°C. To confirm the purity of the culture, its morphological characteristics such as colony color, colony shapes, margins, and elevation were carefully observed. The bacterial growth was dislodged off the surface of each plate by adding a small amount of sterilized distilled water (SDW) to each plate and carefully scrapping the surface with a rubber spatula. Bacterial suspension was adjusted to 10^8 cfu/ml with the help of spectrophotometer, $OD_{600} = 0.5\text{--}0.7$ (Xiulan et al. 2010). This suspension was used for *in vitro* bacterial growth inhibition tests, pathogenicity tests and for inoculation of healthy plants during the *in planta* experiments.

Pathogenicity Tests

Pathogenicity of the bacterium was confirmed by injecting the bacterial suspension (10^8 cfu/ml, $OD_{600} = 0.5\text{--}0.7$) (Xiulan et al. 2010) into stems of four-week old tomato plants, variety Rio Grande (Din et al. 2016). Control plants were injected with sterile distilled water. The seedlings were placed in humid chamber (average RH = 80%, temperature, 25–30°C, dim light for 12 h daily) for up to four weeks. The plants were evaluated regularly for the appearance of bacterial canker disease symptoms. The bacterium was re-isolated from the infected portion of plants in sterile distilled water and was cultured on nutrient agar medium (Kleitman et al. 2009).

Medicinal Plants and Extracts Preparation

Green leaves and succulent branches of the seven medicinal plants/weeds (30–60 plants per species) viz *W. somnifera*, *M. azedarach*, *P. harmala*, *A. sativum*, *C. procera*, *N. oleander* and *M. piperita* were collected from different regions of Peshawar and Darra Adam Khel, F.R Kohat, Khyber Pakhtunkhwa province of Pakistan, authenticated by a weed botanist of the Department of Weed Science, UOA, Peshawar, Pakistan and specimen were deposited in the university herbarium. The plant materials of each species were separately pooled together, washed under tap water, cut into small pieces and then shade-dried for about six weeks. The brittle-dried cut-pieces of each plant species separately ground to make very fine powder (2 mm mesh) (Kumar et al. 2011). The powders of all plant species were

separately stored at 4°C in air tight plastic containers (Oguz et al. 2013). In order to prepare aqueous extracts, 10 g powder (10% w/v) of each plant species was separately mixed with sterilized distilled water (SDW) and the volume was adjusted to 100 ml (Frey and Mayers 2010). The suspension of each plant species was soaked for 48 h, and filtered through Whatman filter (20 µm). The solid material was discarded; the filtrate was saved and used for making different dilutions (75%, 50%, and 25%).

In Vitro Studies

To test the ability of different dilutions of the aqueous extracts prepared from the finely ground powders of different plant species to inhibit the *in vitro* growth of Cmm, a paper disc diffusion method was used (Bauer et al. 1966). For this purpose, a bacterial lawn was prepared by pouring 100 µl of bacterial suspension (10^8 cfu/ml) and spreading it uniformly on the surface of nutrient agar (NA) plates. Sterilized paper discs (6 mm) were moistened with 20 µl of each plant extract dilution and air dried. Negative control paper discs were moistened with 20 µl of SDW and positive control paper discs with 20 µl of Terramycin (200 ppm). Each NA plate received 5 paper discs; three discs for the three dilutions, one disc for negative and one for positive control. The plates were incubated at $28^\circ\text{C} \pm 2$ for 24 h and the resulting inhibition zones were measured across the discs with transparent plastic ruler. The experiment was repeated two times using five replications arranged in completely randomized design. The results obtained were analyzed in comparison with the standard antibiotic and the first five most effective plant extracts were selected for *in vivo* study.

Detection of Secondary Metabolites in Aqueous Extracts of Plants

The aqueous extracts (10% w/v, prepared as described before) of those plants that performed better in *in vitro* growth inhibition tests against Cmm, were tested for the presence of major anti-bacterial plant secondary metabolites in them. To detect alkaloids, 1.5 ml of 1% HCl was mixed with 2 ml of aqueous extract. The mixture was heated in water bath for a few minutes and then 6 drops of Mayer's reagent were added to it. The appearance of orange-colored precipitate indicated the presence of alkaloids (Rasool et al. 2010). For the detection of flavonoids, a few drops of lead acetate and 2 ml of the plant aqueous extract were mixed together. Formation of yellow precipitate indicated the presence of flavonoids (Salhan et al. 2011). For the existence of tannins and saponins in the aqueous extract of the plants, the protocol of Saidulu et al. (2014) was followed. Briefly, a few drops of ferric chloride were mixed with 5 ml of aqueous extract; the appearance of black precipitate was considered

to be indicative of tannin. Saponin was detected by adding 2 ml of the plant extract to 5 ml of distilled water, agitating the mixture for 3 min, resulting in persistent frothing. To show the presence of glycosides in the plant aqueous extracts, protocol of Sachin et al. (2011) was followed.

In Vivo Studies

Based upon *in vitro* results, five plants/weeds viz *P. harmala*, *W. somnifera*, *M. azedarach*, *C. procera*, and *M. piperita* were selected for further evaluation. Pot experiments were conducted in screen house to test the efficiency of different doses (i.e., 10, 20, 30 and 40 g kg⁻¹ of dry potted soil) of finely ground dried powders of the selected medicinal plants on the control of tomato canker. Twenty five-days old tomato transplants (cv Rio Grande) were transplanted to 15 cm-diameter earthen pots containing two kg steam-sterilized (100°C for 6h) soil pre-mixed with the different doses of plant powder (Ramesh et al. 2009). For plant inoculation, bacteria suspension was prepared as mentioned before. Five days post-transplantation, all plants were inoculated by clipping the lowest leaf with scissor dipped in bacterial suspension as well as spraying plants with the bacterial suspension (Rashmi et al. 2012). For comparison, standard antibiotic terramycin (100 ppm) was used (sprayed 4 times at two-week's interval, on inoculated plants) as a positive control. Inoculated potted plants without antibiotic or plant extract powder acted as negative control. The experiment was performed using completely randomized design (CRD) with five replications. The potted plants were watered as needed. The experiment was terminated 90 days after transplanting and data were recorded on disease severity, and yield-related parameters such as plant height (cm), weight of fresh plant (g), number of shoots plant⁻¹, tomato yield plant⁻¹ (g), root weight (g), and dry plant weight (g).

Disease Severity (%) and Data Analysis

The five-category disease rating scale of Wai et al. (2013), with slight modifications (Din et al. 2016) was used for recording disease severity data. Using the equation, $DS\% = 100 \sum n / 4N$ (Bdliya and Dahiru 2006), the disease severity (%) for each treatment was then computed (DS% = disease severity (%), $\sum n$ = sum of all ratings of the scale, 4 is the highest category on the rating scale and N = sample size). Severity data were recorded four times for each treatment at two weeks interval.

Data recorded for plant growth characters such as plant height, root length, plant fresh and dry weights etc., and yield were considered as dependent variable. Treatments such as different medicinal plant extracts and their doses were considered as independent variables. The data

recorded on different parameters were subjected to analysis of variance, using Statistix (Campbell and Madden 1990). To differentiate treatment means of different dependent variables, Fisher's protected least significance difference (LSD) test at $p=0.05$ was used (Gomez and Gomez 1984).

Results

Morphology and Pathogenicity of *C. michiganensis* Subsp. *michiganensis*

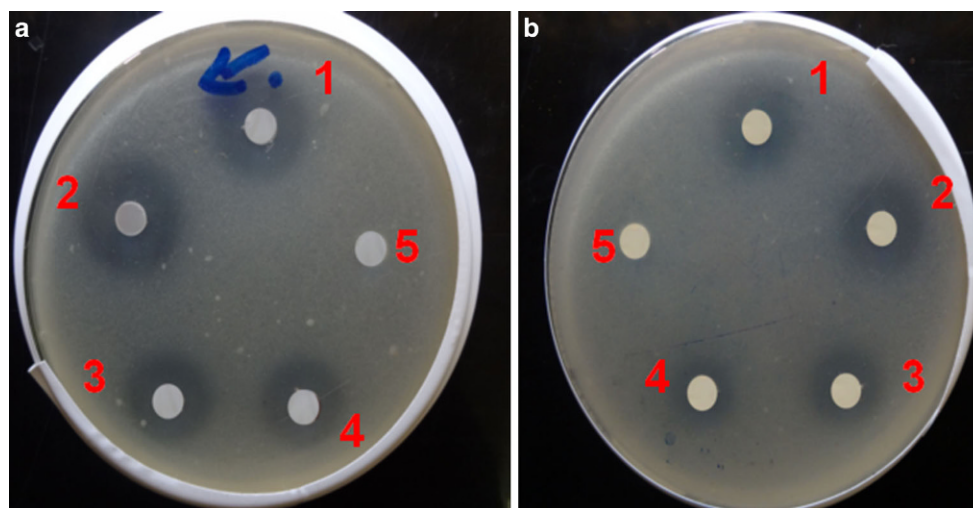
To verify that the preserved culture was pure and fully pathogenic, its morphological characteristics were checked by growing the bacterium on nutrient agar (NA) for 48 h at 28°C and its pathogenicity was confirmed by inoculating it to one-month-old tomato seedlings. The pathogen produced convex, mucoid and yellow colonies on the medium.

Table 1 Effect of aqueous extracts of some medicinal plant species on the *in vitro* growth inhibition of *Clavibacter michiganensis* subsp. *michiganensis*, 24 h after incubation at 30°C

Medicinal plants	Concentration (%)	Zones of inhibition (mm)	% decrease over Terramycin
<i>Melia azedarach</i>	100	12.00cd	48.58
	75	10.80defg	43.72
	50	8.80hijk	35.40
<i>Peganum harmala</i>	100	14.40b	58.30
	75	13.00bc	52.63
	50	10.00fghi	40.48
<i>Nerium oleander</i>	100	9.40ghijk	38.06
	75	8.40jk	34.00
	50	6.40l	25.91
<i>Calotropis procera</i>	100	11.40def	46.15
	75	9.80ghij	39.68
	50	8.60ijk	34.82
<i>Allium sativum</i>	100	9.20hijk	37.25
	75	8.40jk	34.00
	50	8.00k	32.39
<i>Withania somnifera</i>	100	11.800cd	47.77
	75	10.20efgh	41.30
	50	9.00hijk	36.44
<i>Mentha piperita</i>	100	13.00bc	52.64
	75	11.60cde	46.96
	50	9.40ghijk	38.06
Control (water)	N/A	0.00n	N/A
Terramycin	200ppm	24.70a	N/A
LSD	N/A	1.49	N/A

Means with different letters within a column are significantly ($p \leq 0.05$) different. Each value is a mean of five replicates. The experiment was repeated twice with similar results

Fig. 1 *In vitro* growth inhibition zones (mm) of *Clavibacter michiganensis* subsp. *michiganensis* produced by aqueous extracts of *P. harmela* (a), and *M. piperita* (b), using paper disc method. 1 = Terramycin, 2 = 100%, 3 = 75%, 4 = 50% and 5 = sterilized distilled water



The inoculated tomato seedlings produced disease symptoms 10 days after inoculation. Initial symptoms appeared on lower leaves; later the stem showed splitting and wilting symptoms. Wilting symptoms on one side of seedlings indicated xylem infection. The bacterium was re-isolated from the infected plant samples to confirm Koch postulates.

In Vitro Antibacterial Assay of the Phytoextracts

Aqueous extracts (100%, 75%, and 50% concentrated) of *P. harmala*, *M. piperita*, *A. sativum*, *W. somnifera*, *M. azedarach*, *C. procera* and *N. oleander* decreased the *in vitro* growth of Cmm. Among the different plants tested, the undiluted water extract of *P. harmala* showed the maximum anti-bacterial activity by producing 14.40 mm zone of inhibition (ZI) followed by that of *M. piperita* (13.00 mm ZI), and 75% concentrated extract of *P. harmala* (13.00 mm ZI), (Table 1, Fig. 1). The anti-bacterial activities of the undiluted aqueous extracts of *M. azedarach* (12.00 mm ZI) and *W. somnifera* (11.80 mm ZI) were at par with each other. Surprisingly, *A. sativum* exhibited the minimum antibacterial activity (9.20 mm ZI) which was at par with that of *N. oleander* (9.40 mm ZI). The positive control, terramycin (200 ppm), exhibited the highest antibacterial activity (24.70 mm ZI) while negative control produced no ZI. In comparison with the standard antibiotic, terramycin

(200 ppm), the undiluted water extracts of *P. harmala* and *M. piperita* were 58.30% and 52.64%, respectively, as effective as the antibiotic.

Detection of Plant Secondary Metabolites in Their Aqueous Extracts

To determine if the presence or absence of a certain secondary metabolite in the aqueous extract of a plant was correlated to its ability to inhibit the *in vitro* growth of Cmm, the aqueous extracts of the selected plants (*P. harmala*, *M. piperita*, *W. somnifera*, *M. azedarach* and *C. procera*) were analyzed for the presence of major plant metabolites such as alkaloids, tannins, saponins, flavonoids and glycosides. The results revealed that the aqueous extracts of all plants had moderate amounts of alkaloids, tannins, saponins, flavonoids, and glycosides (Table 2). However, the extracts of some plants were found to have higher amounts of some metabolites. For example, *P. harmala* had more alkaloids and saponins than the other plants. Likewise, *M. piperita* had more alkaloids and tannins.

Table 2 Detection of common secondary metabolites in aqueous extracts of medicinal plant species

Plant aqueous extracts	Secondary metabolites				
	Alkaloids	Tannins	Saponins	Flavonoids	Glycosides
<i>Peganum harmala</i>	++	+	++	+	+
<i>Mentha piperita</i>	++	++	+	+	+
<i>Withania somnifera</i>	+	+	+	+	+
<i>Melia azedarach</i>	+	+	+	+	+
<i>Calotropis procera</i>	+	+	+	+	+

+ Moderate, ++ High. The experiment was repeated once with similar results

Table 3 Effect of dried powders of medicinal plants on severity (%) of bacterial canker of tomato 15-, 30-, 45- and 60-days post-inoculation

Medicinal plants	Doses (g kg ⁻¹ soil)	14 days post-inoculation	28 days post-inoculation	42 days post-inoculation	56 days post-inoculation
<i>Peganum harmala</i>	10	26.53 hi	33.23 fg	35.13 gh	39.00 g
	20	21.73 ijk	22.73 j	23.90 l	24.73 i
	30	18.10 lm	13.66 l	12.70 o	11.76 kl
	40	13.13 m	12.63 l	11.30 p	10.33 l
<i>Mentha piperita</i>	10	30.13 efg	32.26 g	37.66 fg	45.96 e
	20	25.40 ghij	26.53 i	27.43 jk	28.56 h
	30	20.26 jkl	17.03 k	15.66 n	13.83 k
	40	18.03 lm	13.86 l	12.23 op	11.40 kl
<i>Melia azedarach</i>	10	34.80 cde	38.33 de	40.83 e	48.86 de
	20	32.10 def	33.00 g	35.00 h	38.83 g
	30	29.13 fgh	28.23 hi	25.93 kl	25.16 i
	40	24.76 ghij	19.40 k	18.50 m	19.30 j
<i>Calotropis procera</i>	10	38.56 bc	42.86 c	49.26 c	54.26 c
	20	36.33 cd	36.16 ef	41.36 e	46.46 e
	30	32.46 def	33.40 fg	39.90 ef	42.56 f
	40	22.26 ijk	27.96 i	25.40 kl	24.13 i
<i>Withania somnifera</i>	10	43.767 b	54.50 b	62.66 b	64.30 b
	20	39.13 bc	39.80 cd	51.73 c	55.00 c
	30	36.00 cd	37.30 de	44.86 d	50.10 d
	40	33.70 cdef	31.10 gh	32.36 i	29.63 h
Terramycin	200 ppm	3.33 n	1.00 m	0.00 q	0.00 n
Inoculated control	N/A	61.333 a	66.40 a	76.80 a	85.36 a
LSD value	N/A	5.52	3.11	2.59	3.37

Means with different letters within a column are significantly ($p \leq 0.05$) different. Each value is a mean of five replicates. The experiment was repeated once with similar results

In Vivo Studies

Effect of Medicinal Plant's Powder on Disease Severity

To find out whether the *in vitro* anti-bacterial activity of the medicinal plants could hold true under *in vivo* conditions, four doses (10, 20, 30 and 40 g kg⁻¹ of soil) of the dried powder of each plant species (except *A. sativum* and *N. oleander* whose aqueous extract exhibited very little *in vitro* activity) were tested for the control of bacterial canker of tomato. In comparison to un-treated control plants, all four doses of the dried powders of the five medicinal plants tested significantly ($p < 0.05$) reduced disease severity at 14-, 28-, 42-, and 56-days post-inoculation (Table 3). In comparison to Terramycin (200 ppm)-treated plants at 56-days post-inoculation, however, only those plants treated with the higher doses (40 g kg⁻¹ soil and 30 g kg⁻¹ soil) of *P. harmala* and *M. piperita* performed better. Plants treated with 40 g kg⁻¹ soil of the dried powders of *P. harmala* and *M. piperita* at 56-days post-inoculation had 10.33% and 11.40%, respectively, more disease severity than the plants treated with terramycin (200 ppm). The disease severity levels of plants treated with 40 g kg⁻¹ soil of *M. piperita*, 30 g

kg⁻¹ soil of *M. piperita* and 30 g kg⁻¹ soil of *P. harmala* were statistically at par with each other.

Effect of Medicinal Plant's Powder on Yield and Growth Parameters of Tomato

The general trend of the effect of dried powders of the medicinal plants tested on tomato yield and plant growth characters was almost similar to that observed in case of disease severity. All doses of the dried powders of all tested plants (except 20 g kg⁻¹ soil of *W. somnifera* for yield, 10 g kg⁻¹ soil of *W. somnifera* for yield and plant height, and 10 g kg⁻¹ soil of *C. procera* for plant height) significantly ($p < 0.05$) enhanced yield, fresh and dry weights per plant and plant height as compared to untreated inoculated control plants (Table 4). The higher dose (40 g kg⁻¹ soil) of the dried powder of *P. harmala* enhanced dry weight, fresh weight, yield, and height per plant by 75.37%, 75.70%, 73.11%, and 92.57%, respectively, of the antibiotic, terramycin (200 ppm). This was followed by the higher dose (40 g kg⁻¹ soil) of *M. piperita*. The corresponding per plant enhancements in dry weight, fresh weight, yield, and height

Table 4 Effect of dried powders of medicinal plants on yield and other plant growth parameters of tomato plants inoculated with *Clavibacter michiganensis* subsp. *michiganensis*

Medicinal plants	Doses g kg ⁻¹ soil	Dry weight (g)	Fresh weight (g)	Yield (g)	Height (cm)
<i>Peganum harmala</i>	10	37.96 j	124.00 l	251.53 ijk	61.50 hijk
	20	44.93 h	149.00 h	352.10 efg	82.83 bcde
	30	61.56 d	185.43 e	418.27 cd	93.16 abc
	40	67.10 c	201.53 c	452.47 c	96.00 a
<i>Mentha piperita</i>	10	34.00 k	101.73 n	235.13 jk	61.00 hijk
	20	43.20 hi	132.00 k	318.20 gh	65.83 ghij
	30	53.90 ef	164.10 f	384.53 def	82.50 cde
	40	65.90 c	196.57 d	401.83 cde	95.16 ab
<i>Melia azedarach</i>	10	30.16 lm	91.73 o	234.87 jk	53.93 jkl
	20	38.43 j	114.10 m	284.47 hij	65.00 ghij
	30	44.90 h	136.77 j	301.10 ghi	73.46 efgh
	40	51.83 f	155.37 g	334.47 fgh	83.16 bcde
<i>Calotropis procera</i>	10	28.10 m	83.06 p	217.47 kl	49.33 klm
	20	33.90 k	100.97 n	234.73 jk	56.33 ijkl
	30	41.86 i	124.47 l	285.67 hij	66.66 fghi
	40	48.93 g	145.90 hi	318.97 gh	78.83 def
<i>Withania somnifera</i>	10	24.43 n	71.46 q	167.97 lm	48.00 lm
	20	31.03 l	93.80 o	168.10 lm	51.33 kl
	30	36.40 jk	111.33 m	234.40 jk	58.43 ijkl
	40	34.23 k	136.77 j	284.77 hij	66.30 fghij
Terramycin	200 ppm	89.03 a	266.23 a	618.87 a	103.70 a
Inoculated control	N/A	16.93 o	50.66 r	153.50 m	37.00 m
LSD	N/A	2.75	3.30	63.31	12.58

Means with different letters within a column are significantly ($p \leq 0.05$) different. Each value is a mean of five replicates. The experiment was repeated once with similar results

were 74.01%, 73.83%, 64.94% and 91.76%, respectively, of those obtained by the antibiotic terramycin (200 ppm).

Discussion

Organic amendment of soil using different plants/weeds for the control of plant diseases is an active research domain. In addition to being environment friendly, plant products are effective against many plant pathogens, both soil-borne and phylloplane-borne. Different formulations such as dried powders, green manures (Naz et al. 2015a, 2015b), foliar sprays or soil drenches with aqueous extracts (Deberdt et al. 2012) have been reported to have successfully controlled different biotic diseases. This practice is expected to replace or reduce the amount of different chemicals used for the control of plant diseases, resulting in the reduction of environmental pollution and human health hazards. In our current soil organic amendment research, we explored the plant-disease control bioefficacy of some abundantly and free-of cost available plant species, which are well-known locally for their use as folk medicine. These plants included: (i) *W. somnifera* is a medium-sized Solanaceous shrub hav-

ing bioactive plant substances. Organic extracts of leaves of this plant were found to have high antibacterial activity against some human's and plant's bacterial pathogens (Singariya et al. 2012). (ii) *M. azedarach*, a member of the family Meliaceae, is one of the most useful traditional medicinal plants (Biswas et al. 2002). Methanol extracts of its seeds, fruits, leaves as well as flowers are very effective against some plant bacterial pathogens (Viqar et al. 2008; Neycee et al. 2012). Leaves of *M. azedarach* were found to have all major plant secondary metabolites, i.e. alkaloids, saponins, tannins, phenols, glycosides, steroids and flavonoids (Fazil et al. 2012). (iii) *P. harmala* is an important medicinal plant which is used in global folk medicine (Sharaf et al. 1997; Jumaa 2005). It has good antibacterial and antifungal properties (Lala et al. 2004). (iv) *C. procera* is a salt tolerant, small compact, desert shrub which is well known for its traditional medicinal properties (Murti et al. 2010). (v) *M. piperita* is a perennial medicinal plant containing 0.5–4% volatile oil (Dew and Evans 1984). Volatile oil, aqueous extracts as well as organic extracts of *M. piperita* have been reported (Saeed et al. 2006; Bupesh et al. 2007; Al-sum and Al-Arfaj 2013) to have good antibacterial properties. The presence of many of these bioac-

tive plant substances was confirmed by our test results. Our tests, however, are not analytical and show only rough estimates of the substances under investigation.

Our *in vitro* results indicated that the aqueous extracts of *P. harmela* and *M. piperita*, *M. azedarach*, *W. somnifera* and *C. procera* were highly effective in inhibiting the growth of Cmm, the causal agent of bacterial canker of tomato. These results suggest the presence of anti-microbial secondary metabolites in these plants. Our results are similar to those of Sarpeleh et al. (2009) and Amel et al. (2012). Sarpeleh et al. (2009) chopped fresh *P. harmela* into small pieces and buried the plant material into bacteria- and fungi-infested soil two-three months prior to plantation. They found that the buried plants or plant parts slowly released water-soluble anti-microbial substances which killed pathogens present in soil. Moreover, some of these water soluble substances were absorbed by plant roots resulting in killing of pathogens present inside host plant tissue. The use of finely ground dried powder of *P. harmela*, in case of our studies, confirms the good results for this species. Amel et al. (2012) found *P. harmela* to be effective even against antibiotic-resistant bacteria and some other microbes. Under *in vitro* studies, it effectively restricted the growth of *Candida albicans* and *Lactobacillus*. Four important alkaloids including harmine, marmaline, harmalol and peganin were found in *P. harmela*. Harmine has been reported to have strong antibacterial and antifungal activities (Amel et al. 2012). Using *in vitro* agar well diffusion assay, Bupesh et al. (2007) found that aqueous as well as organic extracts of *M. piperita* leaves had strong antibacterial activity against a range of pathogenic bacteria. Saeed et al. (2006) tested the antibacterial activity of *M. piperita* against eleven human pathogenic bacteria, using disc diffusion method. They reported that the essential of peppermint exhibited the highest activity (11.78 mm mean ZI) followed by its juice (10.41 mm mean ZI). Similarly, 1:1, 1:5, 1:10 and even 1:20 dilutions of aqueous extracts of *Mentha* species were found to be effective against six human pathogenic bacteria (Al-sum and Al-Arfaj 2013). Water extracts of *M. azedarach* have been reported to enhance the defense system of cucumber against *Meloidogyne incognita* (Cavoski et al. 2012). The researchers found that the activities of catalase and peroxidase enzymes involved in host H₂O₂ detoxification were reduced, suggesting enhanced defense system of the host plant. Soil organic amendment with dried powder of *Withania somnifera* and *W. coagulans* had a significant effect on improving plant growth characters *in vivo*, reducing bacterial load g⁻¹ soil and affecting bacterial wilt disease severity by decreasing the AUDPC values (Khan et al. 2019a; Najeeb et al. 2019). Saponins and steroids were also reported to be present in significant amounts in leaves of *W. somnifera* (Panchal et al. 2016). Finely ground powder of *C. procera*, added to soil (40 g kg⁻¹soil) was found to be

about 60% as effective as the antibiotic in reducing bacterial wilt (BW) severity and as effective as the antibiotic in enhancing yield and yield parameters (Din et al. 2016). The researchers concluded that the dried powder of this plant had the potential to become an effective component of the integrated disease management against BW of tomato.

Contrary to the results of some other researchers (Jeyaseelan et al. 2010; Al-Obaidi 2014), however, the aqueous of *A. sativum* and *N. oleander*, according to our findings, did not perform as good as those of the other plants that we tested. Jeyaseelan et al. (2010) reported that both, organic solvent-extracts and water extracts of *A. sativum* bulbs inhibited the growth of plant pathogenic bacteria such as *Xanthomonas axonopodis* and *Ralstonia solanacearum* under *in vitro* conditions. This difference in results might be explained on the basis of formulation of the medicinal plant used, type of the solvent used, concentration of the extract used, and different nature of the bacterial pathogens involved. For example, we used finely ground dried powder of garlic to prepare its aqueous extract, whereas the other researchers used fresh garlic bulbs for preparing water extracts. Fresh garlic bulbs have some volatile compounds which are not present in the stored dried powder. Similarly, ethyl acetate-extracts of garlic were found to be twice as effective as aqueous extracts against bacterial pathogens (Jeyaseelan et al. 2010) indicating more solubility of anti-microbial chemicals of garlic in organic solvents. Likewise, Al-Obedi (2014) found that the aqueous extract of *N. oleander* significantly reduced the *in vitro* growth of *Staphylococcus aureus* and *Escherichia coli*. As little concentrations as 25 mg ml⁻¹ produced growth inhibition zones of 22 mm and 19 mm, respectively, of the two bacteria. In this case, the difference in results might be due to different concentrations used and different nature of the pathogens.

The observed lower disease severity, enhanced yield and yield-contributing plant growth characters of the inoculated tomato plants treated with 40 g/kg soil of *P. harmela*, *M. piperita* and the other medicinal plants could be based upon many factors. One possibility is the presence of various anti-microbial compounds in the finely ground dried powders of the medicinal plants added to soil. Our results indicated that all the major anti-microbial compounds (i.e., alkaloids, tannins, saponins, flavonoids, and glycosides) were present in the aqueous extracts prepared from dried powders of *P. harmela*, *M. piperita*, *M. azedarach*, *W. somnifera* and *C. procera*. These antimicrobial compounds have diverse mechanisms of action against pathogens. For example, alkaloids inhibit vital enzymes, such as topoisomerase. Others damage DNA by intercalating into it (Tanaka et al. 2006). Similarly, some tannins directly kill bacteria by damaging their cell membranes (Mainasara et al. 2012). Other tannins bind to bacterial adhesins, re-

sulting in the prevention of bacterial attachment to host cells (Wang 2014). Steroidal saponins damage cell membrane by disabling its sterol component, resulting in the death of bacterial cell (Wang et al. 2000). The phenolic compounds, flavonoids, produced in response to pathogen's attack, coagulate bacterial proteins and enzymes of vital pathways (Al-Obaidi 2014). The anti-microbial action of these bioactive plant compounds is, however, dose- and pathogen-contact dependent (Regnault-Roger et al. 2005; Naz et al. 2015b; Khan et al. 2019b). In our studies, the doses and amounts in soil amended with plant powder are most likely much lower than when pure substances are considered. Furthermore, there is no true contact between the pathogen in the plant and these substances in soil; unless some of them are directly absorbed by plant roots and go inside plant tissue to kill the pathogen as reported by Sarpeleh et al. (2009).

The possible activation of innate defense mechanism(s) of plants by natural elicitor compounds present in the dried powders of the medicinal plants (Walters et al. 2005) is another major fascinating scenario responsible for the observed lower disease severity and enhancement of yield and yield-contributing factors of the treated plants. Some of the components of the activated plant defense include reactive oxygen species or ROS-mediated oxidative burst, strengthening of cell walls, synthesis of anti-microbial compounds, changes in the levels of defense-related enzymes and priming of host cells etc. (Daayf et al. 2000; Kuc 2006; Cavoski et al. 2012). A coffee-leaf extract formulation, when sprayed on plants, induced defense genes in tomato against the bacterial pathogen *Xanthomonas vesicatoria* (Medeiros et al. 2009). By soil drenching potato plants with aqueous extracts *Hibiscus sabdariffa*, *Punica granatum* and *Eucalyptus globulus*, Hassan et al. (2009) demonstrated the elicitation of systemic resistance in potato against BW. Pre-inoculation foliar application of leaf extracts of *Datura metel* induced systemic resistance in rice plants, as evident from the accumulation of pathogenesis-related proteins and other defense-related compounds, resulting in reduced incidence of sheath blight and bacterial blight disease of rice under greenhouse conditions (Kagale et al. 2004). Interestingly enough, Graham and Myers (2011) discovered that the application of SAR (systemic acquired resistance) activators in the form of soil drench was more effective and the effect was more prolonged than their foliar sprays to control citrus canker bacterium, a non-soil-borne pathogen of citrus. The longevity of the activity of soil-applied SAR activators was attributed to their gradual release, uptake by the plant roots, and translocation to the actively growing shoots (Sur and Strok 2003; Graham and Myers 2011). Moreover, these researchers also concluded that soil-applied SAR inducers could deter the appearance of copper resistance in bacteria. Other researchers (Graham

and Leite 2007; Francis et al. 2009) also recommended soil application of SAR inducers to control citrus canker.

Our *in vivo* results of the higher doses (40g/kg soil) of the dried powders of *P. hamela*, and *M. piperita* are very encouraging. The finely ground powders of these plants added to soil proved to be about 80% as effective as the antibiotic in terms of reducing the disease severity, and 73% as effective as the antibiotic in enhancing yield and yield-contributing plant growth characters. This suggests that the dried powders of these plants or their extracts could be used as an effective component of the integrated disease management against bacterial canker of tomato or possibly other plant diseases. Taking advantage of the small-scale agricultural systems prevalent in Pakistan, dried powders of these medicinal plants could be directly applied to the root-zones of tomato plants with practically no increase in the input costs. Additionally, these plants are evergreen, available in large amounts free of cost which make them an attractive disease control option. Moreover, infested seed-bed soils could be treated with dried powders or green manures of these plants to make them disease-free which in turn could be used to produce disease-free tomato transplants. Many anti-microbial plant's secondary metabolites are more soluble in organic solvents than in water (Al-Obaidi 2014). Further research is needed for the evaluation of different organic solvents for the extraction of these anti-microbial metabolites, elucidation of chemical structures in case of discovery of new compounds, and testing the disease-control properties of these natural compounds against other plant pathogens. Moreover, the disease-control ability of these medicinal plants could even be further enhanced. For example, more complete physical disruption of the plant tissue by producing and using very fine powders versus coarse powders; mulching of the powder on the surface of soil versus mixing in soil; addition of small amount of some chemical to the powders etc. Other possible future research could include the identification and purification of the possible SAR inducer(s) present in the dried powders of these plants.

Conflict of interest M. Siddique, N. Din, M. Ahmad, A. Ali, I. Naz, S.S. Alam and N. Ullah declare that they have no competing interests.

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