Research Article

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Screening of Citrus Cultivars Against Citrus Canker and Its Allelopathic Management

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Abstract

Citrus canker is the most devastating bacterial disease. In Pakistan, where canker is endemic, cultural practices and chemical control is vital module of integrated management system. But due to non-prudent use of chemicals wicked impacts start to appear on human health and environment there was need of some alternative management which should be eco-friendly and has no adverse effect for human. Therefore, our present study was based on screening of different cultivars of citrus and allelopathic management of citrus canker. The results revealed that Cara cara navel and kinnow both performed as moderately susceptible response in field condition than all other cultivars of citrus in screening experiment. Consequently, disease incidence was observed increasing by increasing the lesion area and these cultivars can be suggested as a source of resistance against canker pathogen. In allelopathic management we observed that ethanolic extracts were more efficient than aqueous extracts and their efficacy was also increasing by increasing the concentration. Ethanolic extracts of jatropha (13.33cm) followed by amaltas (12.5cm), Arjun (11.13cm), Bougenvilla (7.21cm) have great potency against the pathogen. So these ethanolic extracts can be used as good and alternative management of citrus canker disease.

Keyword: Citrus, Response, Screening, Cultivar, Extract, Management

Introduction

Citrus is an important fruit in production and trade among other fruits and belong to family Rutacea. It is 2nd most producing and planting tree in earth planet [1]. Citrus fruit crop is great significance to Pakistan to give maximum production than other seasonal fruits [2]. It is highly nutritional fruit, containing 12-13% sugar and amino acid contents and 20-25 mg of vitamin C [3]. Studies have revealed that the components present in citrus have protective ability against skin, liver and lunges diseases because of having antioxidant potential [4-6]. This fruit subsidize to balance and healthy life style and avert from various types of human cancers and birth defects.

Approximately 137 countries covered tropical and subtropical areas predominantly under citrus cultivation [7]. Pakistan occupied 12th largest position in citrus producing countries followed by China [8]. It is remunerative crop contributing 29.55% of total fruit crops cultivated in Pakistan. Pakistan has 206,569hectares of citrus growing area among which Punjab contributed 183,210 hectares of area which gives the highest production 2,315,895 tons over all the other provinces because of its favorable environment and growing conditions [3].

But now day's citrus fruit is rescinding due to violence of many viral, fungal, nematode and bacterial diseases. Among all of them citrus canker is utmost destructive and threatening disease caused by bacteria *Xantomonas axonopodis* pv. *citri*. This is most destructive disease of many important citrus cultivars, varieties and some of citrus relatives such as Sweet orange, Grape fruit, Lemon and Mandarins. Citrus canker is believed to have originated in Asia and India in 1800s and then spread to Japan, Australia and Southeastern United States [9]. From 2015 losses due to citrus canker reached up to 30-40% [3]. Regrettably this influence increases day by day due to severe attack of Xac and premium price of exporting volume getting reduced.

Pathogen is rod shaped possess single polar flagellum and gram-negative bacterium [10]. Maximum and optimum temperature for growth is 35-39°C and 28-30°C respectively. Pathogen is motile and need water film to move from one plant to another. It became more flourish in humid conditions when stomata are fully opened [11].

Symptoms due to canker pathogen appear on the twigs, fruits and leaves. On fruit lesions are blister like develop into spongy pustules that is yellow in color. These pustules become darken to light tan and then after developing into corky brown canker. On touch this corky canker gives rough appearance [12]. Symptoms appear on leaves as water-soaked callus like lesions (2-10mm), fenced by yellow hallow and usually doesn't enduring on resistant germplasm.

As the disease intensifies, defoliation becomes the tricky problem. Fruits dropping start in mid-summer due to presence of canker in tissues or cells of plants. As severe attack causes premature fruit drop, defoliation, general tree decline, and twig die back [13]. Because once the canker is developed then there is one way to control the disease to cut down the exposed leaves and branches.

This is expensive and ineffective way to manage the disease. In Pakistan, where canker is endemic, cultural practices and chemical control is vital module of integrated management system. But due to non-prudent use of chemicals wicked impacts start to appear on human health and environment. So, growers are demanding a substitute that gives executive control for canker. Control of canker with chemicals is quite problematic.

Use of resistant citrus progenies and bio control methods are more eco-friendly, long-term and economical solution for management of citrus canker. Efforts have been made little progress to defeat the bacterial pathogen using resistant cultivars. There were few reports that were believed to be effective to find some resistant citrus cultivars investigated against citrus canker. The other management of citrus canker is use of medicinal plants that is widely observed in developing countries. Because in whole life cycle of plants they face many stresses and in response produced many secondary metabolites. Metabolites of these plants are used as anti-inflammatory or anti-microbial agents. Current study has been specifically designed, to evaluate different plant extracts against citrus canker with more convenient, repeatable and accurate methods that yield worthy results in equitably short time and screening of commercial cultivar of citrus that are highly adopted by farmer in citrus growing area of Punjab, against *Xanthomonas axonopodis* pv. *citri*.

Materials and Methods

The present research work was done in the laboratory of Plant Pathology, College of Agriculture, Sargodha 2017-2018. Leaf samples infected with citrus canker were collected from the citrus orchard, College of agriculture. Infected leaf samples were placed in plastic bags, tagged with date and location and these samples were taken to the lab for further isolation and purification.

Isolation and purification

Leaves having typical canker symptoms were rinsed with tap water, surface sterilized with 1% sodium hypochlorite followed by three washings with distilled water. Cut 2mm part of lesions with some healthy part and dried on blotter paper. These pieces were slightly crushed with piston and motel. Serial dilutions of samples were made up to 10^6 concentrations and bacterial suspension placed on petri plates having NA media. Spread these drops evenly with the help of spreader. Then Plates were wrapped and incubated at 28°C in an incubator. After incubation the pure isolates were then stored at 4°C in sterilized 50% glycerol.

Identification of Pathogen

Gram staining test

In order to differentiate between gram positive and gram-negative bacteria gram staining method was performed according to Rashid and Chaudhry, (2011). Gram reaction, shape and arrangement of bacteria can be determined by gram staining method. Take a bit of *Xac* from pure culture with the help of sterilized loop and smear it on clear slide. Then slide was slightly heated to fix the bacteria. One drop of crystal violet was placed on slide for 30 seconds and then washed with sterilized water. Then add one drop of iodine for 30 seconds and washed with distilled autoclaved water. At the end placed one drop of Safranin for 30 seconds and washed with DAW. Kept the mount to dry. In order to observe the mount under 100 X microscope add one drop of Canada balsam or oil immersion on stained.

Inoculum preparation

Bacterial pure isolate was cultured in nutrient broth. Take autoclaved conical flasks. Pour nutrient broth in these conical flasks fill up to 50ml of volume. Shake these conical flasks on orbital shaker at 180 rpm for 38hrs.After 38 hrs. Media changed its color from dark to pale yellow it is the indication of multiplication of bacterial culture in nutrient broth media. Pour this media having bacterial population into autoclaved centrifuged tubes. These tubes were then centrifuged at 5000 rpm at room temperature for 12min. Definite palates were obtained by centrifugation. Suspended these palates in 0.075 M Phosphate buffer at 7.0 pH and Stored in refrigerator at 4°C temperature for future use.

Pathogenicity test

Grape fruit plants were used as indicator plants for pathogenicity test. Three plants of grape fruit were obtained from citrus nursery of department of Plant Pathology College of Agriculture, UOS. Plants were transplanted into Pots having sterilized soil, irrigated with water and covered with plastic bags for 2 hours before inoculation. Sprayed the bacterial culture adjusted to10^6cfu/ml. The inoculum was sprayed equally on all plants and again covered the plants with polythene sheets for 2 hrs. Indicator plants were kept under observation for 15 days of post inoculation.

Screening for resistant cultivars against citrus canker

For the evaluation of citrus cultivars against citrus canker screening experiment was establish in screen house of College of Agriculture Department of Plant Pathology in Sargodha having 28°C temperature. For screening experiment following 12 commercial varieties of citrus were selected.

Table 1: Commercial citrus cultivars

Species	Cultivars
Sweet Orange	Salustiana
Grape fruit	Shamber
Mandarins	Kinnow, Murcott and Feuterell's Early
Lime	Daesi sweet lime
Lemon	Seedless lemon
Sugar or acidless orange	Musambi and Succari
Pigmented orange	Red blood and Tarocco
Navel orange	Cara cara navel

Four Plants of one-year old age of each variety were taken. In this way total 48 plants were planted in 12 inches of pots having sterilized soil. Experiment was performed in complete randomized design. All the agronomic practices were performed to maintain the citrus plants in good health condition. Lateral shoots were cut down periodically to induce and maintain new shoots and leaves of all cultivars. After three to 4 weeks, immature leaves were grown that was sufficient for inoculation of bacteria.

Before the inoculation all the citrus plants were irrigated and properly covered with polythene sheets for 24hrs to open the maximum leave's stomata. Inoculum was provided by spray inoculation method. The bacterial culture was 1st adjusted to106cfu/ml. Spray the same bacterial inoculum uniformly on both sides of leaves until the leave's tissues were water soaked. Excess suspension was whipped off from surface of leaves.

Plants referred as a control only sprayed with sterilized distilled water. All Inoculated plants covered with plastic bags for 2 hours to create humidity and at 8, 16 and 24 days of post inoculation development of bacterial lesions were observed.

Disease assessment

Disease was assessed by calculating the percent disease incidence by given formula

Disease incidence= (No. infected leaves) / (Total number of leaves) ×100

In order to determine the response of resistance and susceptibility disease rating scale was used.

Table 2: Disease rating scale

Grades	Disease severity %	Response
0	0-0	Highly resistant
1	1-5	Resistant
3	6-10	Moderately resistant
5	11-15	Moderately susceptible
7	16-25	Susceptible
9	26 and above	Highly susceptible

Plant extracts preparation

Plant material

Fresh diseased free leaves of plants were collected from Citrus Orchard, College of Agriculture university of Sargodha to evaluate antibacterial activity of both ethanol and aqueous extracts of medicinal and aromatic plants against *Xanthomnas axonopodis* pv. *citri* through agar well diffusion method given by Irobi et al., 1994 [14].

Table 3: Plant selected for antibacterial activity test

Sr No.	Plants	Botanical names	Family
1	Jatropha	*Jatropha curcas Linn.*	Euphorbiaceae
2	Bougainvillea	*Bougainvillea spectabilis*	Nyctaginaceae
3	Amaltas	*Cassia fistula*	Fabaceae
4	Arjun	*Terminalia arjuna*	Combretaceae
5	Pot marigold	*Calendula officinalis*	Asteraceae

Plant extract preparation

For ethanolic and aqueous plant extract preparation take fresh diseased free leaves of each plant washed them with tap water, disinfect these leaves with 1% sodium hypochlorite and gave three washings with distilled water. Dry these leaves under the shade for 24hrs at room temperature. Weight 15g dried leaves from each of all above mentioned plants. The plant material was then well grinded in 100ml of ethanol solvent for ethanolic extract and distilled water for aqueous extract by using mixture grinder. Homogenate was filtered through muslin cloth and then centrifuged at room temperature for 15 min at 8000rpm and observation was made after 48 hrs. by measuring ZOI (Zone of inhibition) that indicate the absence of bacterial growth around each well.

Results

Identification of *Xanthomonas axonopodis* pv. *citri*

After 72 hours of inoculation the bacterial colony was fully grown and developed and can be identified on basis of colony morphology and microscopy. In microscope the bacterium was seen move freely bacillus and rod shaped (Fig 1a) while in colony morphology the colony of bacterium was convex i.e. expanded from the edges become mucoid and gave yellow pigmentation with odorant smell (Fig 1b). The isolate of *Xac* was gram negative bacterium because it is the property of gram negative bacteria they did not dye in purple when treated with crystal violet the cell wall retains pinkish color when the stained with safranin and this pinkish color washed away when treated with ethanol (Fig 1c).

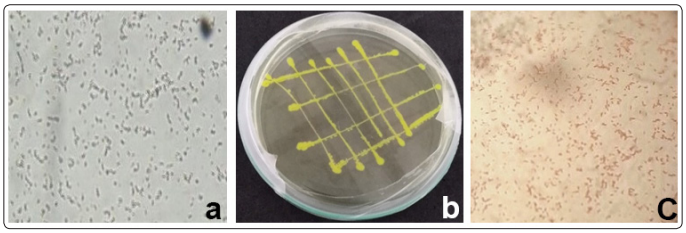

Figure 1: **(a)** Colony morphology **(b)** microscopy of *Xanthomonas axonopodis* pv. Citri **(c)** Gram staining of *Xanthomonas axonopodis* pv. Citri

Pathogenicity test

The virulence or pathogenicity of isolate was tested by Koch's postulates. Plants that were selected for pathogenicity test were covered with polythene bags for 2 hrs before and after inoculum were sprayed. After 15 dpi results showed that the isolate was virulent in nature and showed pathogenicity through lesion formation and canker symptoms on leaves of citrus plants. For further clarification when bacterium was re-isolated from diseased tissue and carried the procedure of isolation and purification it showed same colony character of original bacteria and was pathogenic bacteria (Fig 2a, 2b)

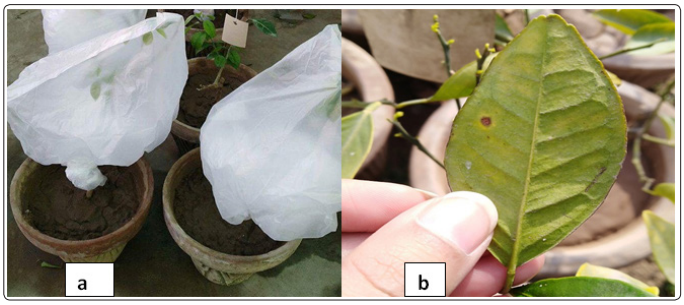

Figure 2: (a) Indicator plants of citrus covered with polythene bags (b) Lesion formation of isolate on indicator plants

Screening of citrus cultivars

Results clearly showed that cara cara novel (Navel orange) showed the (MS) moderately susceptible response followed by kinnow (Mandarins) while Murcott, Daesi sweet lime, Seedless lemon and Torocco showed the (S) susceptible response among all the cultivars (Table 4).

Table 4: Level of susceptibility/resistance against citrus canker presented by citrus cultivars/varieties

Sr.#	Cultivars	Ratings	Responses
1	**Sweet Orange** Salustiana	9	HS
2	**Grape fruit** Shamber	9	HS
	Mandarins		
3	Kinnow	5	MS
4	Murcott	7	S
5	Feuterell's Early	9	HS
	Lime		
6	Daesi sweet lime	7	S
	Lemon		
7	Seedless lemon	7	S
	Sugar or acidless orange		
8	Musambi	9	HS
9	Succari	9	HS
	Pigmented orange		
10	Red blood	9	HS
11	Tarocco Navel orange	7	S
12	Cara cara navel	5	MS

HS= highly susceptible: MS=moderately susceptible: S= Susceptible

In case of disease incidence we observed that at 8 dpi Feutrell's early showed 14.9% disease incidence rate followed by Tarocco and Succari that exhibited 16% and 26% disease incidence respectively (Fig 3b). At 16 dpi Musambi showed 39% disease incidence than that of Murcott. Salustiana displayed maximum disease incidence that is 72% while kinnow showed least disease incidence that is 25% at 24 dpi. In salustiyana at 8th dpi the average lesion numbers were 1.2 and its disease incidence was 51.01% as the average number of lesion was increasing from 1.2 to 2.2 then its disease incidence was also increased from 51.01 to 60%. This significant relation is extremely observed in all cultivar of citrus (Fig 3a).

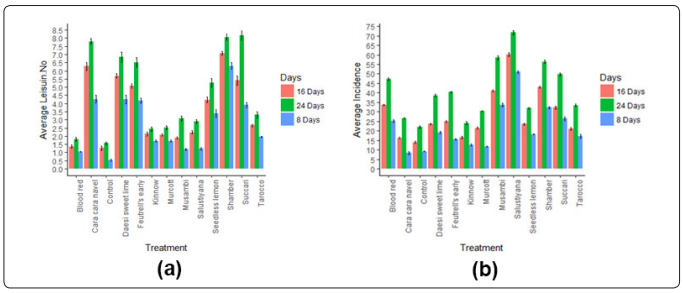

Figure 3: Screening of citrus germplasm against Xanthomonas axonopodispv. *citri* average lesion number (a) disease incidence (b)

Evaluation of ethanolic plants extracts against *Xanthomonas axonopodispv. citri.*

Ethanolic extracts of five different medicinal plants were assessed using well diffusion method against *Xanthomonas axonopodis* pv. *citri*. Results explicitly showed that they have aptitude to reduce the growth of Xac when applied at three different concentrations Fig 4. Ethanolic extract of Jatropha showed highest zone of inhibition at 15% which was 13.33cm followed by Amaltas (12.5cm) and Arjum (11.33cm) while least ZOI was observed in Arjun (0.52cm) followed by pot meri gold (0.56cm), Bougenvilla (0.82cm), Amaltas (6.83cm) and Jatropha (7.66cm) when applied at 5% (Fig 5) and increase to some extend when concentration increased to 10% (Table 2). Ethanolic extract showed significant response and zone of inhibition was increasing by increasing the concentrations of extracts and the same trend (Fig 7).

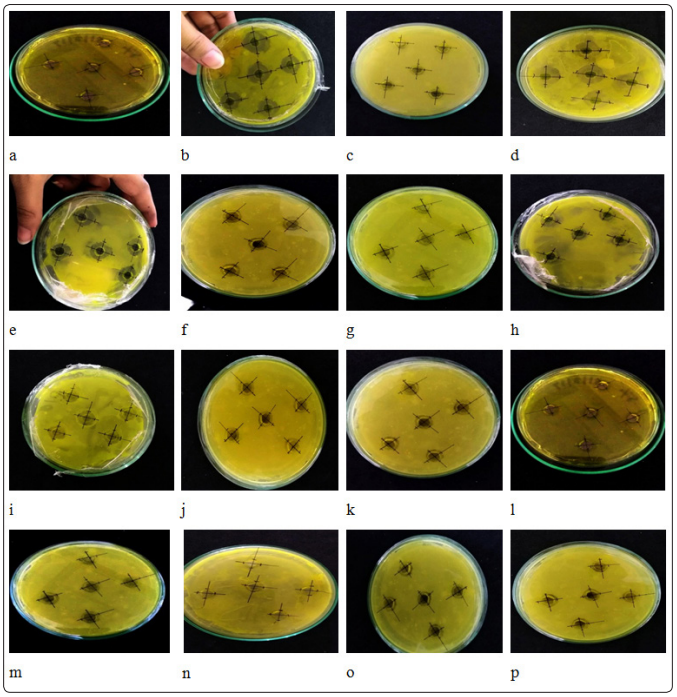

Figure 4: Efficacy of ethanolic plant extracts at 10% concentration (a=Jatropha, b=Arjun, c= Pot marigold, d=Amaltas, e=Bougenvilla), 15% concentration (f=Arjun, g=Pot meri gold h=Amaltas, i=Jatropha, j=Bougenvilla) and 5 % concentration (k=Jatropha, l=Pot merigold, m=Arjun, n=Bougenvilla, o=Amaltas) and p=control against *Xanthomonas axonopodis* pv. *citri* under laboratory condition.

Evaluation of aqueous plants extracts against *Xanthomonas axonopodis*pv. *citri.*

Aqueous extracts were applied to evaluate their efficacy against citrus canker pathogen. Results showed that there was considerable difference in their efficacy when aqueous extract of medicinal plants were practiced against *Xanthomonas axonopodis*pv. *Citri*. Jatropha plant extract significantly inhibit the growth of the pathogen 1.46cm followed by 1.41cm by Amaltas at 15% concentration while at 10% concentration the zone of inhibition reduced in the same trend as the aqueous extract performed. (Table 5)

Graphical explanation represents that among ethanolic and aqueous treatments ethanolic extracts of medicinal plants exhibited high performance than the aqueous extracts (Fig 6). Plant extracts of jatropha performed well and give remarkable zone of inhibition than the other followed by Amaltas, Bougenvilla in both ethanolic and aqueous extracts respectively while least ZOI was observed in pot marigold in case of ethanolic extracts and Arjun in case of aqueous extract (Fig 5).

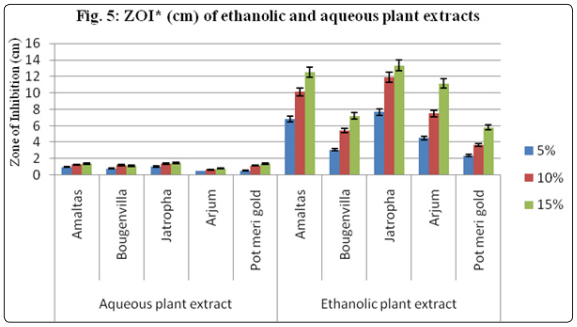

Table 5: Zone of inhibition in cm of five different ethanolic plants extract treatments after 48 hrs. of application against *Xanthomonas axonopodis* pv. *citri*

Sr. #	Treatments	Ethanolic plant extract concentrations			Aqueous plant extract concentrations		
		5%	10%	15%	5%	10%	15%
1	Jatropha	7.66	11.9	13.3	1.04	1.37	1.46
2	Amaltas	6.83	10.1	12.5	0.99	1.27	1.41
3	Arjun	4.52	7.5	11.13	0.52	0.66	0.80
4	Bougenvilla	3.1	5.42	7.21	0.82	1.21	1.12
5	Pot meri gold	2.4	3.66	5.80	0.56	1.14	1.41

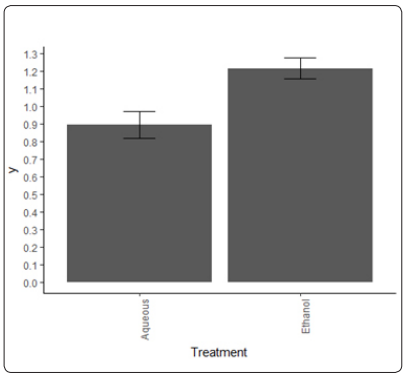

Figure 6: Ethanolic extracts displayed high performance than the aqueous extracts. X-axis represents treatments and Y-axis represents their efficacy

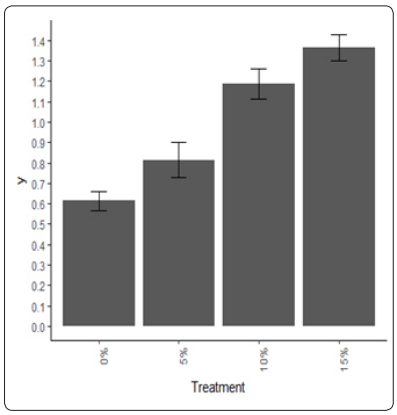

Figure 7: Comparison between different doses of treatments. X-axis represents doses of treatments and Y-axis represents their performance

Discussion

Xanthomonas axonopodis pv. *citri* is potential threat to citrus industry throughout worldwide. Under favorable condition once disease become develop it is difficult to manage disease with conventional mode. Allelopathic management and genetic resistant is eco-friendly and long-lasting solution to canker disease. Among them screening is the short-term solution for relative resistant and susceptibility of citrus germplasm. Since 1980's citrus cultivars were evaluated for their resistance and susceptibility against citrus canker [15]. The citrus genotype that was well-known for their resistant to citrus canker is now become susceptible.

Experiment on screening of citrus cultivar by Burhan et al., 2007 of selected 26 was performed. In his experiment he suggested that variety of pigmented orange i-e Tarocco and varieties of mandarin Murcott and Feutrell's early showed resistant to citrus canker which was similar to our results [16]. Under same field conditions variety of grape fruit shamber displayed susceptibility against *Xanromonas axonopodis* pv. *citri*. In present evaluation when these accessions were artificially inoculated with *Xanthomonas aonopodis* pv. *Citri* we have found that all genotypes exhibited typical canker symptoms. This means these accessions are not of active resistant to canker disease. This asymptomatic behavior may be due to difference in environmental conditions or insufficient attack of pathogen. Our results matched with the Mustafa et al., 2015 who screened 15 commercial citrus cultivars against *Xac. axonopodis* pv. They observed that among artificially inoculated citrus genotypes Blood red, Musambi and Shamber were highly susceptible varieties and their defense system was not activated to inhibit the development of canker pathogen. Sweet lime and feutrell's early were susceptible to disease development [17].

Alternative control strategies like use of plants extracts is another way to manage the disease. Green plants have good reservoir of active molecules that have great potency against bacterial growth [18]. Our present study gives direction to identify and search new source of biomolecules that have efficient antimicrobial activity and can be exploited for product development. It was drive from the results that ethanol extract were observed more efficient than that of aqueous extracts. Jatropha performed well and give remarkable zone of inhibition than the other ethanolic extracts by inhibit the growth of the pathogen by making upto 13.33cm inhibition zone followed by Amaltas (12.5cm) while least inhibition zone was observed in potmeri gold (5.80cm) even highest concentration at 15%. Our results are verified by work of Umamaheswari et al., 2008; Fawad et al., 2012 those evaluated antibacterial activity of different solvent plant extracts of against different gram-negative bacteria [19,20]. He demonstrated that ethanolic extract of showed 8mm, 12mm and 14mm of zone of inhibition against gram negative bacteria. The same observation was also made by Annad, 2011; Fawad et al., 2012 who exhibited almost same results when he demonstrated that bougenvilla displayed 13.1mm of zone of inhibition when applied against pathogenic bacteria [20].

Amaltas extract was also having good antimicrobial activity against different plant pathogens as described by Neelam, 2016. In case of aqueous extracts same findings have been documented by Gena et al., 2006 who tested different aqueous plant extracts against *Xanthomonas* and suggested that amaltas did not show any significant ZOI against bacteria at 10% concentration while bougenvilla and Pot meri gold exhibited to some extent made inhibition zone [21,22].

Conclusion

Citrus canker is bacterial disease caused by *Xanthomonas axonopodis* pv. *citri*. once the canker is developed then there is one way to control the disease to cut down the exposed leaves and branches. In Pakistan, where canker is endemic, cultural practices and chemical control is vital module of integrated management system. But due to non-prudent use of chemicals wicked impacts start to appear on human health and environment. Control of canker with chemicals is quite problematic. As the world fluctuating to organic farming, our present study based on screening of resistant citrus progenies and allelopathic management of canker pathogen. As far these are more eco-friendly and long-term solution for management of citrus canker disease. In allelopathic management five different ethanolic and aqueous extracts of different medicinal plants at different concentrations were evaluated against *Xanthomonas axonopodis* pv. Citri under laboratory conditions [23]. All the treatments showed remarkable potency against Xac. Therefore, it is concluded that ethanolic extracts have great potency against the disease for both quality and quantity of the citrus production in Pakistan [24,25].

Acknowledgement

Dr. Muhammad Usman Ghazanfar conceived the idea and facilitated, guided and supervised the experiment. Ms Anbreen Fatima executed the field visits and took experimental data according to the instructions of Dr. Salman Ahmad while Mr. Waqas Raza analyze the experimental data and finalized the manuscript.

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