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Research Article

DETERMINATION OF ANTIBACTERIAL ACTIVITY OF PHYTOCHEMICALS TOWARDS *XANTHOMONAS CITRI* PV. *CITRI* CAUSING CITRUS CANKER

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ABSTRACT

Citrus canker, caused by Xanthomonas citri pv. citri, is the most destructive disease of citrus throughout the world. Synthetic chemicals have so far proved ineffective and harmful to the environment. That is why in current study, phyto-extracts were examined due to their less hazardous and eco-friendly nature. For the management of citrus canker, nine medicinal phytoextract (C. colocynthis, N. sativa, C. tamala, Z. officinale, P. nigrum, C. verum, *S. aromaticum*, E. globu, T. Graecum) were evaluated under lab condition. Maximum inhibition zone was produced by C. colocynthis (25.71mm) followed by N. sativa (21.52mm), C. tamala (18.63mm), Z. officinale (20.42mm), P. nigrum (18.99mm). C. verum (15.73mm), *S. aromaticum* (18.75mm), E. globu (15.37mm), T. Graecum (14.57mm) as compared to control under lab conditions. The most effective phytoextract (C. colocynthis, N. sativa) were evaluated under greenhouse and field condition. Minimum disease severity (12.62%) was observed when of (C. colocynthis + N. sativa) were applied in combination followed by C. colocynthis (23.23%) and N. sativa (28.31%) as compared to the control under greenhouse condition. Maximum disease incidence was noticed by N. sativa (42.18%), C. colocynthis (36.79%) and minimum disease incidence (27.40%) was observed when (C. colocynthis+ N. sativa) were applied in combinations under field condition as compared to the control and reduce the disease severity up to 50%. The results showed that C. colocynthis+ N. sativa can be implicated for the management of citrus canker.

Keyword: Citrus, *C. colocynthis*, *N. sativa*, phytoextract, managment

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1. INTRODUCTION

Citrus is among the most important fruits grown in a variety of tropical and subtropical climates around the world (Jagtap *et al*., 2012; Wali *et al*., 2013). It is a member of the *Rutaceae* family and originates from the subtropical and temperate regions of Southeast Asia (Berk, 2002). Citrus is highly valuable in our daily lives because of its high dietary value and high content of carbohydrates, amino acids, organic acids, minerals (calcium, magnesium), and vitamin C. Citrus ranked first in Pakistan's fruit industry due to its high production among all fruits (Ghafoor *et al*., 2008). In 2020, worldwide citrus production was estimated to be 194.4 million tonnes from 13.9 million hectares, whereas production in Pakistan was estimated to be 2.29 million metric tons from 206.6 thousand hectares. The global juice industry has been used to achieve 2.2 million tonnes (FAO, 2020). Citrus canker (CC) is the most serious disease affecting citrus crop productivity in Pakistan, as well as the rest of the world (Ware, 2015). Necrotic lesions on twigs, leaves, stem, and fruit, as well as leaves and fruit that turn corky with a yellow halo and a watery border after a certain time interval, are typical symptoms of citrus bacterial canker disease (Dewdney *et al*., 2016). 144 consignments of Pakistani citrus were rejected by the European Union in 2015-2016 due to the *Xcc* disease (Pervaiz *et al*., 2015). *Xcc* is a gram-negative rod-shaped bacterium with a single polar flagellum

that must be present (1.5-2.0 x 0.5-0.75). It needs a temperature of 28-30°C for successful infection, and its colony colour is yellow due to the development of "*xanthomonadin*" pigment (Afroz *et al*., 2013).

Citrus canker has been managed using a variety of management measures, including chemical, biological, cultural, and the adoption of resistant varieties. Copper-based chemicals (antibiotics and plant extracts) are strongly recommended to reduce the risk of disease spreading by pruning affected trees (Ullah *et al*., 2019). The most economical, ecofriendly and effective management of canker is the use of resistant source but under conducive environmental conditions, resistant varieties become susceptible and disease appeared in epidemic form. In this miserable condition farmers have no option except the use of chemicals but due to their health hazard effects and environmental pollution issue, use of phyto-extracts is the best option, as they are ecofriendly and have least effect on human health. That is why, in current study, different phyto-extracts were evaluated against citrus canker. Numerous secondary metabolites are present in plant extracts which have no effect on growth and development of plants but have antimicrobial properties (Schafer and Wink, 2009). It has been accounted that plant extracts are extracted with different solvents and fundamental oils contains wealthy antioxidant and bioactive compounds (Choudhary *et al*., 2019). Plant extracts not only have antimicrobial activity but also possess antifeedant, repellent, antioxidant, galoctogenic, larvicidal activities. Hence present study was done to evaluate the efficacy of different plant extracts against citrus canker caused by *Xanthomonas citri* pv. *citri*. So in current study nanoparticles were developed from silver and copper nanoparticles from eucalyptus and were evaluated against *xcc* causing citrus canker.

2. MATERIALS AND METHODS

2.1. Collection of Diseased Specimen:

Citrus leaves infected with Citrus canker were collected from the Research Area, Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF). These Samples were placed in the brown bag and brought to the Phytobacteriology lab for further process. Then these samples were washed with tap water and air-dried. During this procedure, safety precautions were taken.

2.2. Isolation, purification and preservation of *Xanthomonas citri* pv. *citri*

For isolation of *Xcc*, Nutrient Agar (NA) media was prepared (Glucose 2.5g, peptone 5g, Agar 15g, Beef extract 3g and 1000 mL distilled water for 1-L media) and mixed thoroughly. Then this media was autoclaved at 121^{o}C for 15 minutes by maintaining 15 PSI pressure. Then the diseased samples along with some healthy portions were cut into small pieces (2-3 mm in size). For surface disinfection, these small portions of leaves were treated with 70% ethanol and then three washing of distilled water were given. Then with the help of sterilized forceps, these samples were placed on sterilized Petri plates (contain 3 mL media). Then, these plates were wrapped and placed in an incubator (model IN-601) at 25-28^{o}C. After 24 hours, light yellow colored bacterial colonies on media plates were observed. Purification of bacteria was done by using the streaking method. By using sterilize cotton swab, a bacterial colony was picked and streaked on a new media plate. After 24 hours single colony was selected and transferred to new plates containing NA media by using the streaking method for further purification. Then for the preparation of *Xcc* 1 liter nutrient broth was prepared. For the preparation of 1-liter media, 15g nutrient broth was mixed in 1000mL distilled water in a media bottle (1L) and test tubes were autoclaved at 121^{o}C for 15 Psi for 15 min. After this 5mL nutrient broth media was poured into

five test tubes (size 10mL). Using sterilized loops, a single pure colony was selected from each isolated culture after media sterilization. These tubes were shaken for 16 hours at 28°C in a shaker (Shaker-Incubator ES 20). In a sterilized cryovial tube (3mL) add 1mL 50% glycerol and 1mL of culture broth of five isolates were poured and were kept at a temperature of -4^{o}C in a refrigerator.

2.3. **Pathogenicity test:**

A pathogenicity test was conducted to fulfill Koch's postulates. Grapefruit plants that were one year old were obtained from a nursery Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF). These plants were grown in pots (30 cm diameter) containing field soil (sandy clay) that was sterilized by soaking in a 1 percent formalin solution. To remove dust particles, these plants were sprayed with distilled water and covered with a polythene sheet for two hours to increase relative humidity. The bacteria from stock culture were multiplied on nutrient agar by incubating it for 48 hours at 28°C. The bacteria were mixed into water to make an aqueous suspension. Using a spectrophotometer the bacterial suspension was adjusted to (0.01 OD) equivalents to 10^8 colonies forming units per mL and diluted with PBS to 10^4 CFU per mL. Then the bacterial suspension was poured into a beaker. The bacterium suspension (about 2 µl) was injected into the plant leaf. Inoculation was done in the morning in the greenhouse by using the syringe method (using a syringe needleless) and some plants were kept as a control treatment injected with distilled water. Inoculated plants, showed disease symptoms observed after 7 days of inoculation whereas there were no symptoms on control treatment. The diseased sample was collected and was brought to the lab for further study. Now after inoculation and re-isolation it was confirmed *xanthomonas citri* pv *citri* is the cause of citrus canker disease.

2.4. **Evaluation of various phytoextracts in lab conditions against *Xanthomonas citri* pv. *citri* causing citrus canker**

Nine medicinal plants (Korr- *tumma*, cinnamon, black pepper, kalwanji, cloves, Taiz patta, ginger, safaida and maithy leaves were collected from chiniot bazar Faisalabad Pakistan. To remove moisture, plants were subjected to sun drying as well as an oven for 4 hours at 65^{o}C. The plant samples were ground to produce a fine powder. To make it more fine, the powder was passed through a muslin cloth. Then the supernatant was discarded and three concentrations of each medicinal plant were prepared. To prepare 3, 5 and 7% concentrations of medicinal plant, 3, 5 and 7g of each medicinal plant was added in 100 mL of distilled water separately were evaluated against *Xcc* through inhibition zone technique.

2.5. **Evaluation of various phytoextracts against *Xanthomonas citri* pv *citri* causing citrus canker**

For this purpose, Nutrient agar media (NA) was prepared and with the help of a sterilized scissor, 1 cm circular pieces of sterilized filter paper were cut and autoclaved. A sterilized cotton swab was used to disperse bacterial culture around the Petri plate in a laminar airflow chamber (RTVL-1312, Robus United Kingdom). Then pieces of sterilized filter paper were dipped into different concentrations of filtered phytoextracts (3, 5 and 7%) and placed in the center of the NA plates with *Xcc* culture. These plates were wrapped and incubated at 28 ± 2°C. The trial was designed using a Completely Randomized Design (CRD) with three replications per treatment. The control plates were treated with distilled water and inhibition zones were measured with the use of a digital Vernier caliper (500-196, Mitutoyo) after 24, 48 and 72 hours. Fisher's Least Significant Difference Test (LSD) was used to examine data from inhibition zones

2.6. Evaluation of effective plant extracts in greenhouse and field conditions:

Grapefruit plants that were one year old were obtained from a nursery, Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF). These plants were grown in pots (30 cm diameter) pots containing field soil (sandy clay) that was sterilized by 1 percent formalin solution. To remove dust particles, these plants were sprayed with distilled water and covered with a polythene sheet for two hours to increase relative humidity. *Xcc* culture was applied in plants in a greenhouse using a completely randomized design (CRD). The plant extracts that were shown to be effective were tested individually and in combination on inoculated plants in greenhouse and field conditions. After five days 3, 5 and 7 percent formulated aqueous suspension of phytoextracts were sprayed with the help of a simple hand sprayer (Pressure: 0.25-0.45MPA). After seven days, the plants were evaluated for symptom development and disease severity was recorded after 7, 14 and 21 days. Grapefruit plants that were one year old were obtained from a nursery, Institute of Horticultural Sciences (IHS), University of Agriculture Faisalabad (UAF) in a prepared field using P×P=1.0 m and R×R= 1.5 m distances in a field trial. These plants were treated with distilled water before being put in the field. Aqueous suspension of the bacteria was prepared from a 48-hour-old actively growing culture and measure the concentration with the help of a spectrophotometer @ 1×108 CFU/mL (Hitachi U-2001, model 121003). The bacterial suspension was inoculated early in the morning in the plants with the help of a syringe method and injected into the midrib of the leaf as well as veins of the lower surface of the plant leaves. After *Xcc* inoculation, phytoextracts were assessed in field trials 3, 5 and 7 % concentrations were used. Treatments were applied in plants using a Randomized completely randomized design (RCRD). Data on canker severity was recorded after 7, 14 and 21 days intervals.

2.7. Analysis of Data

Complete Randomized Design (CRD) was used for laboratory experiment, and Latin square design (LSD) was used with the probability level of 0.05

Table 1 **Medicinal plants used against *Xanthomonas citri* pv *citri* causing citrus canker**

Common Name	Scientific Name	Plant Parts Used	Active Ingredients	Mode of action
Kor tumma	*Citrullus colocynthis (L)*	Bulb	Cucurbitacin	Inhibiting cancer cell proliferation (Boykin *et al*., 2011)
Kalwanji	*Nigella sativa (L)*	Seed	Spectinomycin	Inhibiting protein synthesis (Babu *et al*., 2007)
Taiz pata	*Cinnamomum tamal(G)*	Leaves	Antihelminthic	Inhibit microtubule formation (Abo-Zaid 2020)
Ginger	*Zingiber officinale (Z)*	Root	Gingerol	inhibit pathogens that cause sore throats (Allamen *et al.,*1992)
Black pepper	*Piper nigrum(L)*	Seed	Piperin	certain antibacterialantibiotics (Allamen *et al*.,1992)
Darchini	*Cinnamomum verum(L)*	Bulb	Cinnamaldehyde	Inhibit cell division (Mata *et al*., 2015).
Cloves	*Syzygiumaromaticum(L)*	Seed	Cinnamaldehyde	Inhibit cell division (Harzallah *et al*., 2015)
Eucalyptus	*Eucalyptus globu(St)*	Leaves	EucalyptTol	Fumigant Insecticidal *Activity* (Cheng *et al*.,2009)
Methi powder	*Trigonella Foenum Graecum (L)*	Leaves	Fenugreek	Inhibit lipid enzymes (Max, 1992)

percent for average separation. Latin square design (LSD) was used with the probability level of 0.05 percent for average separation.

3. Results

Maximum inhibition zone was produced by *C. colocynthis* (25.71) followed by *N. sativa* (21.52), *C. tamala* (18.63), *Z. officinale* (20.42), *P. nigrum* (18.99). *C. verum* (15.73), *S. aromaticum (*18.75), *E.* globu (15.37), *T. Graecum* (14.57) mm respectively as compared to control. Interaction between treatments and concentrations (T×C) showed that maximum inhibition zone (27.85) mm was produced by *C. colocynthis* at 7% (24.76 mm) at 5 % and (24.51 mm) 3 % respectively while *T. Graecum* exhibited minimum inhibition zones (12.85, 14.23, 16.62) while *N. sativa* expressed 20.64, 21.05, 22.87 *C. tamala* 17.12, 18.66, 20.11 *Z. officinale* 18.97, 20.88, 21.41 *P. nigrum* 17.67, 19.37, 19.92, *C. verum* 14.33, 15.70, 17.17, *S. aromaticum* 17.67, 17.48, 21.08, *E.* globu 13.60, 15.01, 17.52 mm inhibition zone at 3, 5 and 7% concentrations respectively in comparison to the control. Treatments and Days expressed that *T. Graecum* exhibited minimum inhibition zones (13.11, 14.61, 15.3l) mm followed by *E.* globu (14.61, 15.61, 15.9), *S. aromaticum (17.24*, 19.01, 19.99), *C. verum* (14.42, 15.67, 17.11), *P. nigrum* (17.82, 19.36, 19.78), *Z. officinale* (19.44, 20.54, 21.28), *C. tamala* (17.24, 18.71, 19.94), *N. sativa* (20.01, 21.38, 23.17) and *C. colocynthis* (24.68, 25.58, 26.85) mm respectively.Minimum disease severity (12.623) was observed when of (*C. colocynthis + N. sativa*) were applied in combination followed by *C. colocynthis* (23.230) and *N. sativa* (28.312) as compared to the control (Table 4.6 & Fig. 4.4). In treatments and concentration interaction (T × C), *C. colocynthis* expressed 21.989, 23.246 and 24.454, *N. sativa* 27.089, 28.500, 29.348 percent disease severity at 7, 5 and 3 % concentrations while minimum disease incidence was observed by the combination of both (*C. colocynthis* and *N. sativa*) 11.863, 12.652 and 13.352 % respectively at three concentrations as compared to the control Interaction between treatments and days (T×D) exhibited that *C. colocynthis* expressed 26.817, 23.269 and 19.603% disease incidence while *N. sativa* 31.760, 28.073 and 25.103 % and *C. colocynthis + N. sativa* 15.458, 12.306 and 10.104 disease incidence when applied @ 3, 5 and 7 % after seven, fourteen and twenty one days respectively as compared to the control (Table 4.8 & Fig. 4.6). Maximum disease incidence was noticed by *N. sativa* (42.180) %, *C. colocynthis* (36.790) % and minimum disease incidence (27.401) % was observed when (*C. colocynthis+ N. sativa*) were in combinationas compared to the control. Treatments and concentration interaction (T × C) *N. sativa* showed 37.100, 43.686 and 45.756 *C. colocynthis* 28.785, 38.800, 42.786 percent disease incidence at 7, 5 and 3 % concentrations while minimum disease incidence was showed by the combination of both (*C. colocynthis+ N. sativa*) 19.777, 25.334 and 37.092 respectively at three concentrations as compare to the control. Treatments and days interaction (T×D) exhibited that *N. sativa* expressed 43.089, 42.841 and 40.611 % disease incidence while *Citrullus colocynthis* (40.634, 34.996 and 34.74) 27.16, 21.11 and *C. colocynthis+ N. sativa* 29.962, 26.735 and 25.506 % disease incidence when applied @ 3, 5 and 7 % after ten, twenty and thirty days respectively as compared to the control.

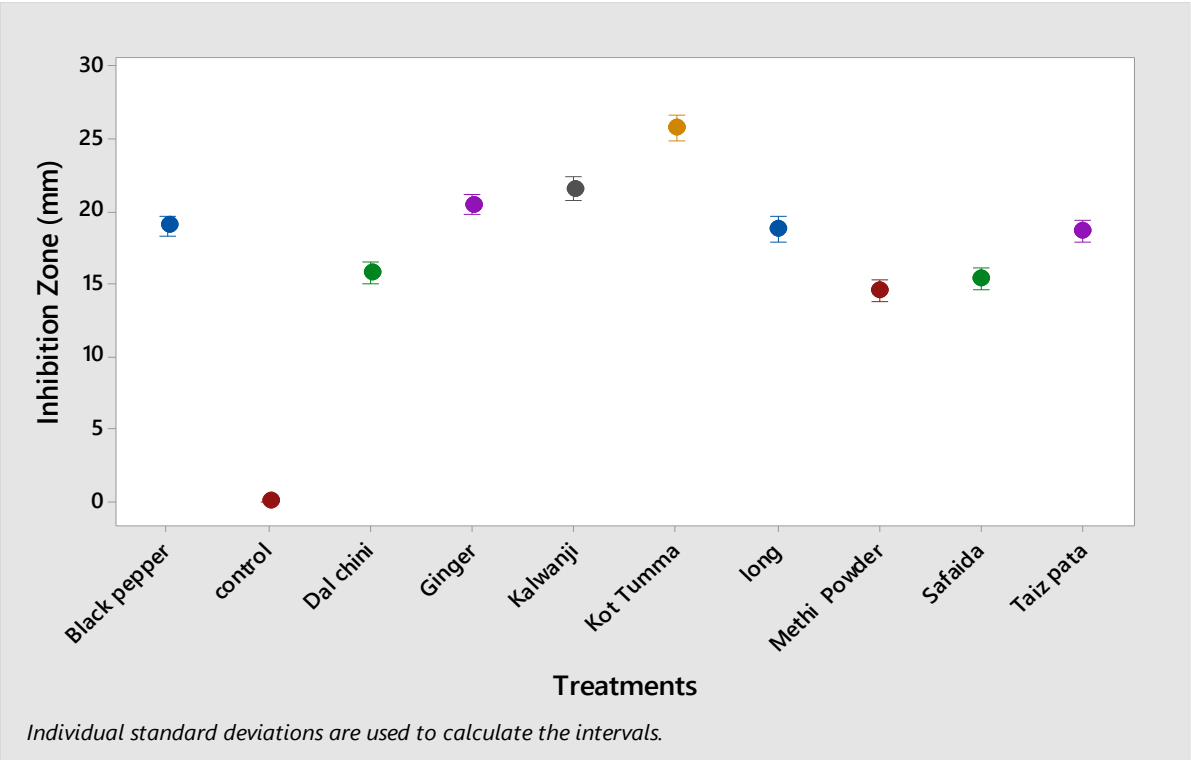

Fig. 1. Impact of different phytoextracts on the growth of *Xanthomonas citri* pv. *citri* under lab. Conditions

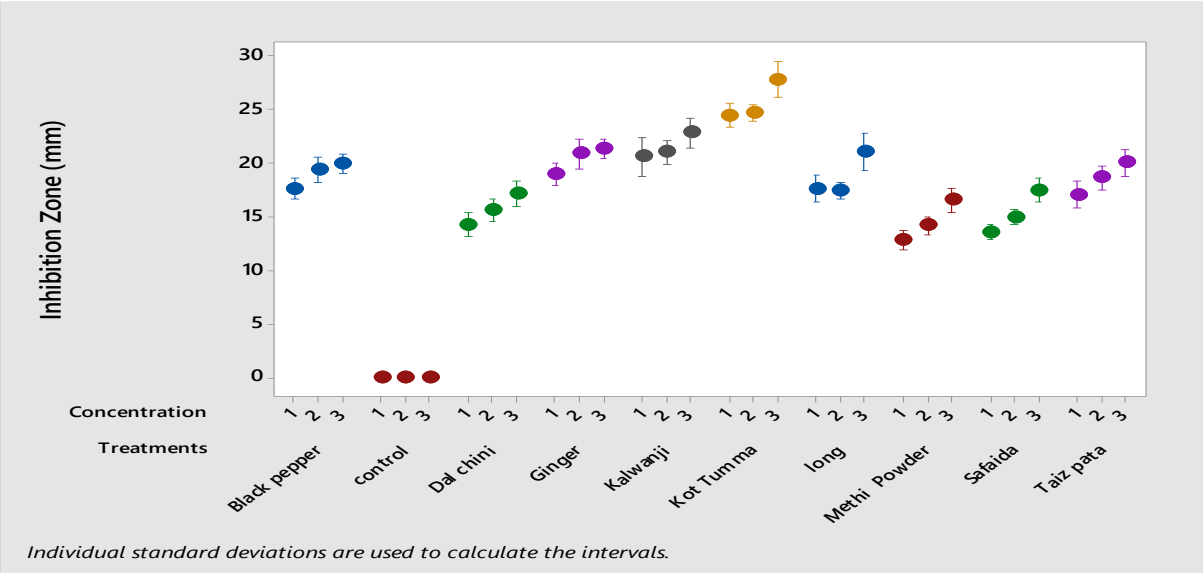

Fig.2. Impact of interaction between treatments and concentrations (T×C) against *Xanthomonas citri* pv. *citri* under lab. Conditions

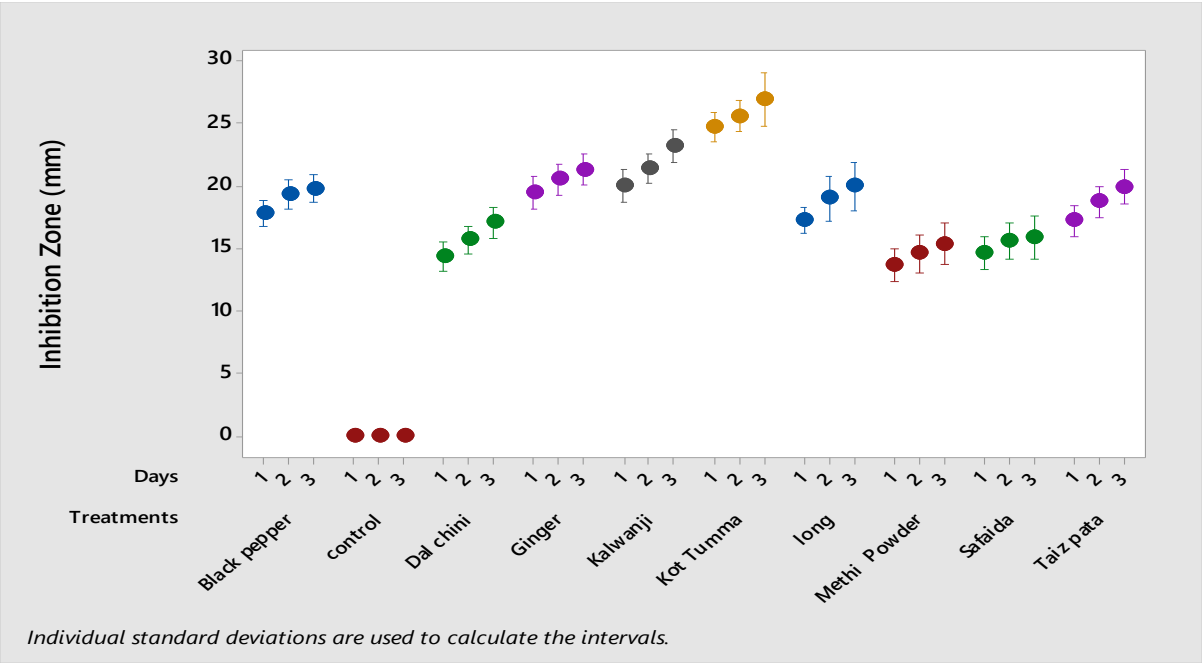

Fig. 3. Impact of interaction b/w treatments and time (TxD) on growth Xanthomonas *citri* pv. *citri* under lab. Condition

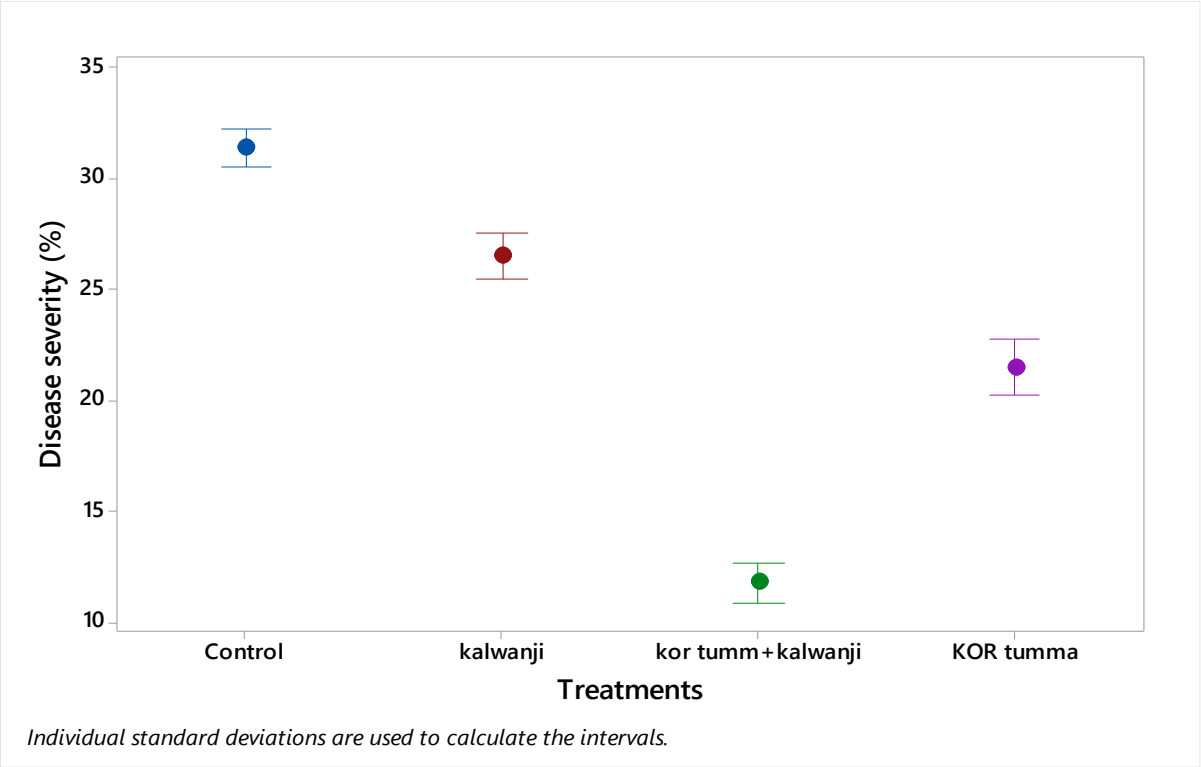

Fig. 4. Impact of plant extracts and their interaction on the development of citrus canker under greenhouse condition

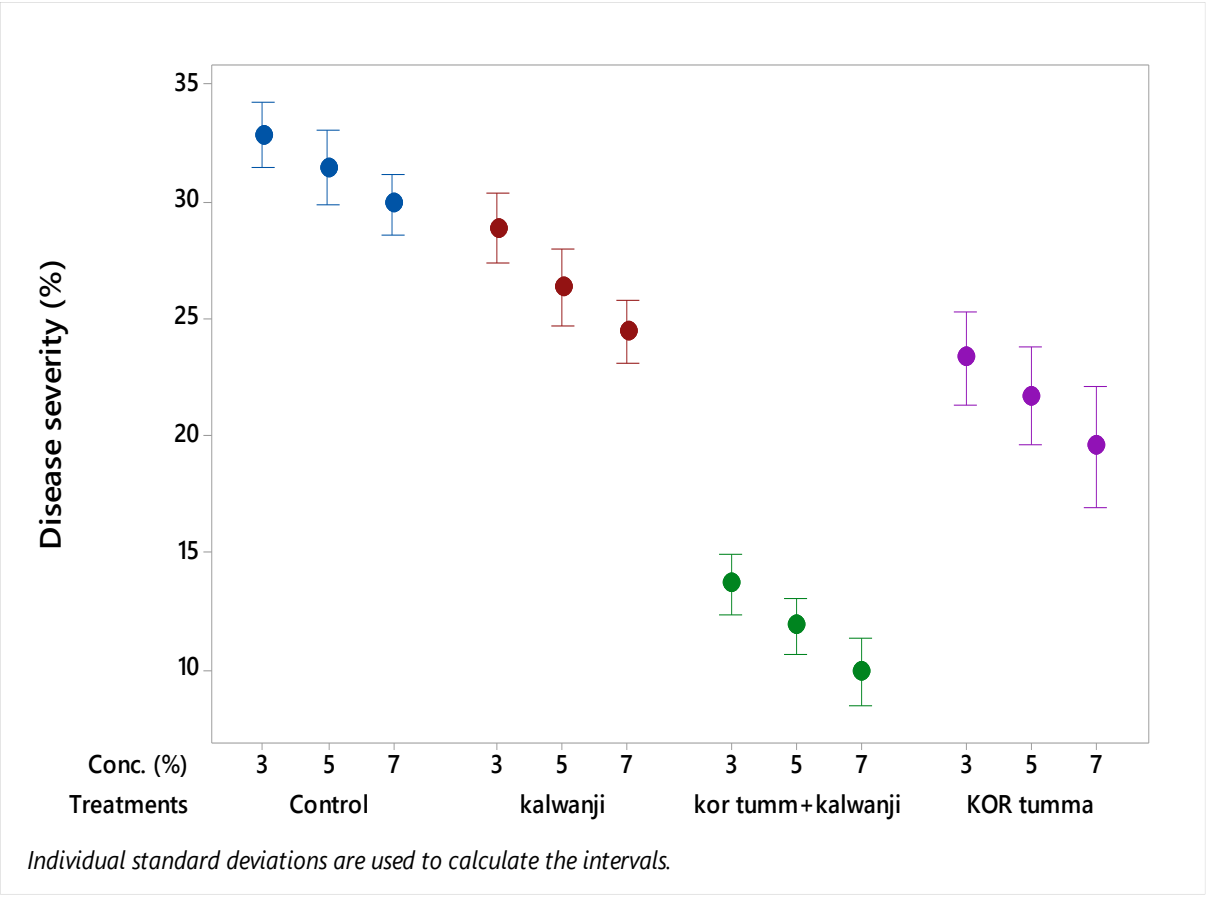

Fig.5. Impact of interaction between treatments and concentrations (T×C) against citrus canker under greenhouse condition

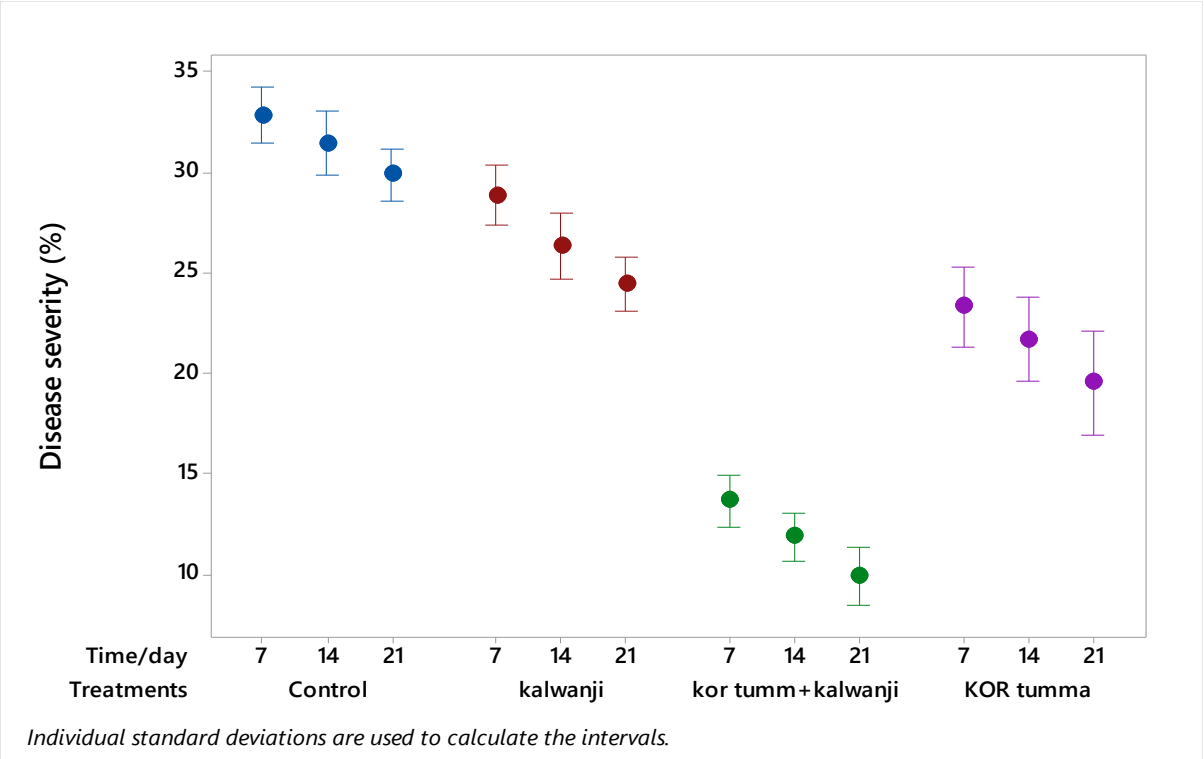

Fig.6. Impact of interaction between treatments and days (T×D) against citrus canker under greenhouse conditions

4. Discussion

Citrus fruit is the most valuable cash and natural product produced in all over the world. Tropical and subtropical environment is ideal for its development. The use of synthetic chemicals is efficient but harmful to the environment. Continuous use of chemicals causes resistance in pathogen (Waqas *et al*., 2018). That is why in current study plant extract were evaluated towards citrus canker as they contain antimicrobial compounds which are less toxic, ecofriendly, safe and easily biodegradable (Atiq *et al*., 2018). In the contemporary study, nine plant extracts (*P. nigrum*, *Z. officinale*, *C. tamala*, *C.* verum, *S. aromaticum*, *E. globu*, *T Graecum*, *N. sativa* and *C. colocynthis*) were evaluated in the lab conditions towards *Xcc* causing citrus canker. Among these extracts *N. sativa* and *C. colocynthis* produced maximum inhibition zone and are also effective under greenhouse and field conditions against citrus canker alone and in combination. *N. sativa* contains various antimicrobial compounds like Thymoquinone (TQ), ampicillin, ciprofloxacin, Protein, glucose, separated amino acids, tannins, phenolics, saponins, flavanoids, flavone glucosides, alkaloids, terpenoids, anthranol, steroids, cucurbitacins, saponarin and cardic glycoloids; among these, the most common one is Thymoquinone (TQ) (Al-Snafi, 2016). Which damages the external membrane of bacterial cell wall by disturbing its structure at specific binding sites and also affect multiple targeted sites and cause reduction in cytoplasmic pH which results in cell wall disruption. Antimicrobial compounds of *N. sativa* they disrupt the structure of bacterial spores and inhibit enzyme synthesis. They also change the shape and size of the cell which leads to death of bacteria (Parsad *et al*., 2019). *C. Colocynthis* (L.) have great potential as antimicrobial compounds against bacteria and are used in the management of various plant diseases. Cucurbitacin is the most active compound of *colocynthis* which degrade the bacterial cell wall, extracellular proteins, inhibit DNA gyrase, Cell membranes degrade, prevent dNTPs from binding and soluble proteins which results in death of bacteria (Othman *et al*., 2019). Results of the current study are supported by the finding of (Bhagwat *et al*., 2018), who investigated the antibacterial efficacy of different phytoextracts against *Xcc* and exposed that these phytoextracts are eco-friendly, less toxic and more effective. *N. sativa* and *C. colocynthis* were found to be most effective plant extract against bacterial canker in the field as well as in greenhouse condition. Outcomes of the present study are also supported by (Shricharan *et al*., 2014) who evaluated extracts of (*P. hysterophorou, C. colocynthis, Aristolochia indica, N. sativa* and *E. hirta*). Among these the *C. colocynthis* and *N. sativa* are most effective under greenhouse and field conditions. Similar results were also reported by (Islam *et al*., 2014) who studied five plant extracts (*Hibiscus subdariffa, Psidium guajava, Punica granatum, Spondias pinnata and Tamarindus indica*) under greenhouse and field condition and concluded that *Tamarindus indica* efficiently reduce the incidence of citrus nker up to 50% . Similarly (Tahir *et al*., 2014) evaluated ten plant extracts (*Allium sativum L., Allium cepa L., Azadirachta indica, Capsicum Annum, Calotropisgi gantea, Dalbrgia sissoo, Eucalyptus camelduensis, Gardenia florida, Melia azedarach, Zingiber officinalis*) against *Xcc* through inhibition zone technique and are significant results. Various antimicrobial compounds are present in plants therefore it is the need of the time to use and evaluate the maximum potential of these plant extracts for making them as a part of different integrated disease management strategies. This strategy will also help in reducing the environmental hazards and their toxic effects on human beings.

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6. Author's Contributions:

HRM conducted the research trials and wrote manuscript. Conceive the idea and planning of research work was by MA and NAR. UA and SA helps in statistical analysis, MU helped in conducting research. AN and MA read the manuscript and gave their possible comments and corrections to finalize the manuscript. MFU and SI correct the references according to journal formatting.

7. Conflict of interests:

Authors do not have any conflict of interest.

8. References

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