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EXPLICATING BOTANICAL BACTERICIDES AS AN INTERVENTION TOOL TOWARDS CITRUS CANKER

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ABSTRACT

Recent research efforts were made to investigate botanical bactericides as an intervention tool for citrus canker. Five botanical extracts (*Eruca vesicaria*, *Eucalyptus globulus*, *Trigonella foenum-graecum*, *Nigella sativa*, and *Citrullus colocynthis*) were evaluated at three different concentrations (0.25%, 0.5%, and 0.75%) against *Xanthomonas citri* pv. *citri* under *in vitro* conditions. Among all treatments, two effective plant extracts, alone and in combinations, were assessed at three different time points (7 days, 14 days, 21 days) under greenhouse conditions. *In vitro* and greenhouse experiments were conducted under a Completely Randomized Design. Results showed that *C. colocynthis* expressed the maximum inhibition zone (3.22 mm), followed by *N. sativa* (2.82 mm), *T. foenum-graecum* (2.68 mm), *E. globulus* (1.64 mm), and *E. vesicaria* (1.31 mm) compared to the control. Effective extracts of *C. colocynthis* and *N. sativa* were assessed under greenhouse conditions, both alone and in combination, against citrus canker. The minimum disease incidence (18.74%) was exhibited by the combination of C. colocynthis and *N. sativa*, followed by *C. colocynthis* (22.59%) and *N. sativa* (23.27%) compared to the control. Based on these findings, it is recommended that the application of botanical extracts is a reliable strategy for managing citrus canker.

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INTRODUCTION

Citrus is one of the world's most imperative fruit crop and a treasure trove of active natural metabolites. Tropical and subtropical climates are favorable for its growth (Jagtap et al., 2012; Sevindik et al., 2021). Globally, citrus is cultivated on an area of 13.9 million hectares with annual production of 194.4 million tons while, Pakistan produces 2.37 million tons of citrus with area of 206.6 thousand hectares (GOP, 2023). Citrus fruits and juices have great demand due to their several beneficial health and nutritive properties as they are rich in vitamin C and folic acid and is good source of fiber. Citrus is free of fat, sodium and cholesterol while in addition, it contains potassium, calcium, thiamin, niacin, vitamin B6, phosphorus, magnesium and copper which help to reduce the risk of heart diseases and some types of cancer (Kim et al., 2004; Lestari et al., 2024).

Many diseases are reported on citrus plant such as scab, canker, tristeza virus, greening, gummosis, sooty mould, anthracnose, slow decline, and powdery mildew but among all the constraints faced by citrus crop, citrus canker is a persistent jeopardy to citrus crop (Irshad et al., 2012; Tahir et al., 2016; Fateh et al., 2017; Saeed et al., 2019). By European Union, 144 consignments of Pakistan were rejected due to this disease and resulted a significant loss (Pervaiz, 2015). Pathogens of citrus canker enter the plant through stomata or wound and reach the intracellular spaces where it discharges toxins which are responsible for hypertrophy, hyperplasia and causes yellow chlorotic halo with a sunken center. Epidermis gets raised and started to break. After fourteen days of infection, lesion starts to dry and necrotic lesions are formed. Normally management of citrus canker is done by copper based chemicals which reduce the disease but this chemical is not ecofriendly and causes some serious effects on human health. This thing enforces scientists to search out alternative to chemicals which should be environment friendly and have no or least effect on human beings (Kumar et al., 2011; Aziz et al., 2024).

The most economical, eco-friendly, and effective method for managing canker is to use resistant sources. However, under conducive environmental conditions, resistant varieties can become susceptible, and the disease can manifest in epidemic form. In such unfortunate circumstances, farmers have no option but to resort to the use of chemicals. Nevertheless, due to their health hazards and environmental pollution issues, the use of phyto-extracts is the best option, as they are eco-friendly and have minimal impact on human health.

Botanical extracts contain numerous secondary metabolites which pose no effect on growth and development of plants but express antimicrobial action (Schafer and Wink, 2009). It has been accounted for that plant extracts are extracted with different solvents and fundamental oils contains, antioxidant and bioactive compounds (Choudhury et al., 2018). Plant extracts not only exhibit antimicrobial activity but also possess antifeedant, repellent, antioxidant, galactogenic, and larvicidal activities. Therefore, the primary objective of the current research effort was to investigate the antibacterial potency of botanical extracts against *Xanthomonas citri* pv. *citri* in laboratory and greenhouse conditions.

MATERIALS AND METHODS

Isolation, purification and identification of pathogen

Citrus leaves showing typical symptoms of canker were collected from citrus orchard and brought to Bacteriology Lab of the Department of Plant pathology, University of Agriculture Faisalabad, for isolation of bacteria. Isolation of bacteria was done through serial dilution method and was identified on the basis of biochemical and morphological characteristics (Tahir et al., 2016). Pure culture was grown in petri plate containing nutrient agar medium. Using a sterile swab, a suspension of the pure culture was spread evenly over the surface of a sterile nutrient agar plate and incubated for 24-48 hours at a temperature of 28°C for further use.

Pathogenicity test

One year old grape fruit plants were obtained from the nursery of the Institute of Horticultural Sciences, University of Agriculture Faisalabad. These plants were transplanted in pots containing field soil previously sterilized with formalin. The bacteria from the stock culture were multiplied on nutrient agar by incubating it for 48 hours at 30°C. Aqueous suspension of bacteria was prepared by adding bacterium in the water. Aqueous suspension of the bacteria having a concentration of 10^8 cells/ml was prepared by using spectrophotometer. Just before the inoculation, plants were irrigated and covered with polythene bags for 2 hour so that maximum stomata become open (Weindling, 1948). Plants were sprayed with bacterial suspension while controlled plants were sprayed with distilled water with three replications. The plants were kept under observation for two weeks in green house and symptoms, if any were recorded. Isolation of *Xanthomonas citri* pv. *citri* from diseased tissue was carried out in the same way as mentioned above and the morphological characteristics of the isolates were compared with the culture of bacteria inoculated to fulfill the requirement of Koch's postulates.

Preparation of botanical bactericides

Leaves and stems of five different plants, namely *Eruca vesicaria*, *Eucalyptus globulus*, *Trigonella foenum-graecum*, *Nigella sativa*, and *Citrullus colocynthis*, were collected from the Botanical Garden of the University of Agriculture, Faisalabad. The leaves and stems of the plants were dried in sunlight and then oven-dried at 65°C to remove the moisture. The dried leaves and stems were crushed into a fine powder using a mortar and pestle, and the powder was filtered through a muslin cloth to obtain a very fine powder. Subsequently, 5 g, 7 g, and 10 g of each plant material were dissolved in 1000 ml of distilled water.

Response of botanical extracts towards *X. citri* pv. *citri* under lab conditions

Extracts of *E. vesicaria, E. globulus, T. foenum-graecum, N. sativa* and *C. colocynthis* were prepare and three concentrations of these extracts 5, 7 and 10 g/1000 ml were prepared and were evaluated against *X. citri* pv. *citri* through inhibition zone technique. For this purpose, nutrient agar media was prepared and 1 cm circular pieces of sterilized filter paper were made with the help of sterilized scissor. Bacterial culture was spread on the whole petridish with the help of sterilized cotton swab. Then pieces of filter paper were dipped in each concentration of each phyto-extracts and placed in the center of each petridish with three replications under Completely Randomized Design (CRD) and incubated at 30°C. Inhibition zone was recorded after 24, 36 and 72 hours respectively with the help of digital vernier caliper.

Estimation of bactericidal activity of *C. colocynthis* and *N. sativa* against citrus canker under greenhouse conditions

One year old healthy grapefruit plants which were highly susceptible to *X. citri* pv. *citri* were taken from the research area, Institute of Horticultural sciences, University of Agriculture Faisalabad. These plants were transplanted into pots measuring 30×15 cm (one plant/pot) containing sterilized soil (by using formalin). Fifteen days after transplantation of citrus plants into pots, these were irrigated and covered with polythene bags for 2 hours to provide artificial condition of humidity. The aqueous suspension of the bacterium prepared from 48 hours old actively growing culture of *X. citri* pv. *citri* was inoculated with the help of a sprayer. The plants inoculated with pathogen only served as a control. Extracts of *C. colocynthis* and *N. sativa* were sprayed alone and in combination on the abaxial surface of the citrus plants. There were three replications for each treatment. Data regarding disease incidence were recorded after seven days interval for three times on grapefruit by using Croxal Scale (Croxall et al., 1952).

RESULTS

Appraisement of botanical bactericides against *X. citri* pv. *citri* under lab conditions

Among all treatments, *E. vesicaria* expressed the minimum inhibition zone (1.31 mm) followed by *Eucalyptus* (1.64 mm), *T. foenum-graecum* (2.68 mm), *N. sativa* (2.82 mm) and *C. colocynthis* (3.22 mm) as compared to control (Table 1) while in case of interaction between treatments and time, *E. vesicaria* expressed the minimum inhibition zone (0.94, 1.33, 1.64 mm) after 24, 48 and 72 hours followed by *E. globulus* (1.33, 1.64, 1.94 mm), *T. foenum-graecum* (2.28, 2.72, 3.03 mm), *N. sativa* (2.33, 2.78, 3.33 mm) and *C. colocynthis* (2.83, 3.33, 3.50 mm) after 24, 48 and 72 hours as compared to control (Table 2 and Figure 1). Similarly, in interaction between treatments and concentrations, *C. colocynthis* exhibited the maximum inhibition zone (2.55, 3.17, 3.94 mm) at 0.25, 0.50 and 0.75 % concentrations followed by *N. sativa* (1.83, 2.83, 3.78 mm), *T. foenum-graecum* (1.69, 3.17, 3.17 mm), *E. globulus* (1.11, 1.50, 2.31 mm), and *E. vesicaria* (0.92, 1.44, 1.56 mm) at 0.25, 0.50, 0.75% concentrations respectively as compared to control (Table 3 and Figure 2).

Table 1: Appraisement of botanical bactericides against *X. citri* pv. *citri* under lab conditions.

Sr #	Treatments	Common Name	Active ingredients	Inhibition zone (mm)
1	*C.colocynthis*	Bitter cucumber	Phenolics, flavonoids	3.222 a
2	*N. sativa*	Black cumin	Thymoquinone	2.814 b
3	*T. foenum-graecum*	Fenugreek	Fenugreekine,4-hydroxy isoleucine	2.675 b
4	*Eucalyptus*	Safaida	Aromadendrene	1.638 c
5	*E. vesicaria*	Tara mira	Erucic acid	1.305 d
6	Control	Distilled water		0.000 e
	LSD		0.1441	

Estimation of bactericidal activity of *Citrulus colocynthis* and *Nigella sativa* against citrus canker under greenhouse conditions

Among all treatments, *C. colocynthis* + *N. sativa* (18.75) % expressed minimum disease incidence followed by *C. colocynthis* (22.59), *N. Sativa* (23.27) as compared to control (Table 4) While in case of interaction between treatments and conc. *C. colocynthis* + *N. sativa* showed minimum disease incidence (23.33, 18.94, 14.96) at 0.25, 0.50, 0.75% conc. followed by *C. colocynthis* (26.06, 22.72, 19.00) and *N. sativa* (26.89, 23.61, 19.31) % at 0.25, 0.50 and 0.75% conc. respectively as compared to control (Table 5) Similarly interaction between treatments and days expressed that *N. sativa* express maximum disease incidence (29.11, 22.11, 18.53) % after seven, fourteen and twenty one days and *C. colocynthis* (29.28, 22.33, 16.17) and *C. colocynthis* + *N. sativa* (25.39, 17.61,13.24) % after seven, fourteen and twenty one days as compared to control (Table 6 and Figure 2

Table 2: Impact of interaction between treatments and time against *X. citri* pv. *citri* under lab conditions.

Treatments	Zone of inhibition (mm)		
	t_1 (24h)	t_2 (48h)	t_3 (72h)
E. vesicaria	0.9444 h	1.3333 g	1.6389 f
E.globulus	1.3333 g	1.6389 f	1.9444 e
T. foenum-graecum	2.2778 d	2.7222 c	3.0278 b
N.sativa	2.3333 d	2.7778 c	3.3333 a
C. colocynthis	2.8333 bc	3.3333 a	3.5000 a
Control	0.0000 i	0.0000 i	0.0000 i
LSD		0.2496	

Table 3: Impact of interaction between treatments and concentration against *X. citri* pv. *citri* under lab conditions.

Treatments	Zone of inhibition (mm)		
	C_1 (0.25%)	C_2 (0.50%)	C_3 (0.75%)
E. vesicaria	0.9167 i	1.4444 h	1.5556 gh
E.globulus	1.1111 i	1.5000 gh	2.3056 e
T. foenum-graecum	1.6944 fg	3.1667 b	3.1667 b
N.sativa	1.8333 f	2.8333 c	3.7778 a
C. colocynthis	2.5556 d	3.1667 b	3.9444 a
Control (dis. water)	0.0000 j	0.0000 j	0.0000 j
LSD		0.2496	

Table 4: Impact of interaction between treatments and their concentrations against citrus canker under greenhouse conditions.

Sr #	Treatments	Conc. (%)	Disease incidence (%)
1	*C. colocynthis + N. sativa*	0.25% + 0.25%	18.745 d
2	*C. colocynthis*	0.25%	22.593 c
3	*N. sativa*	0.25%	23.269 b
4	Control (dis. water)		54.000 a
	LSD	0.009	

Table 5: Effect of interaction between treatments and their concentrations against citrus canker under greenhouse conditions

Treatments	Disease incidence (%)		
	C_1 (0.25%)	C_2 (0.50%)	C_3 (0.75%)
C. colocynthis + N. sativa	22.333 d	18.944 e	14.958 f
C. colocynthis	26.056 b	22.722 cd	19.000 e
N. sativa	26.889 b	23.611 c	19.306 e
Control (dis. water)	54.000 a	54.000 a	54.000 a
LSD		0.0034	

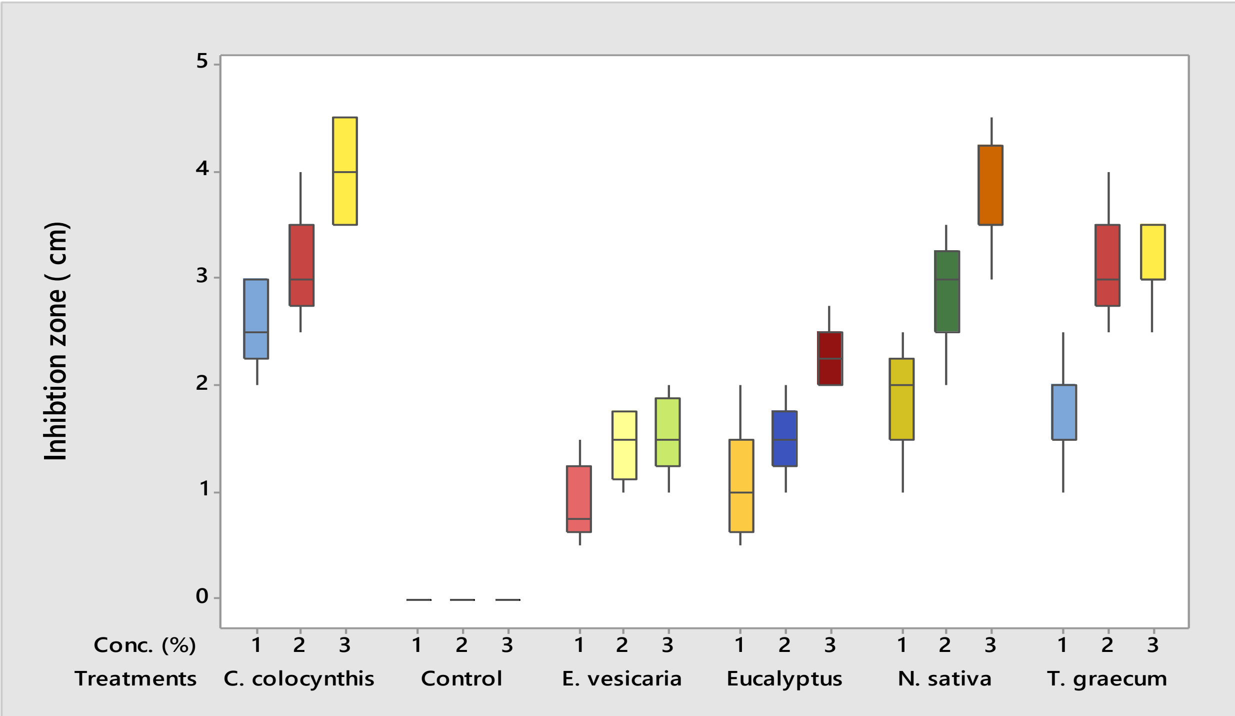

Figure 1: Phyto-extracts of five different plants were evaluated at three concentrations (5 g, 7 g, 10 g/Liter) under lab conditions using inhibition zone technique. All plant extracts significantly reduced bacterial growth.

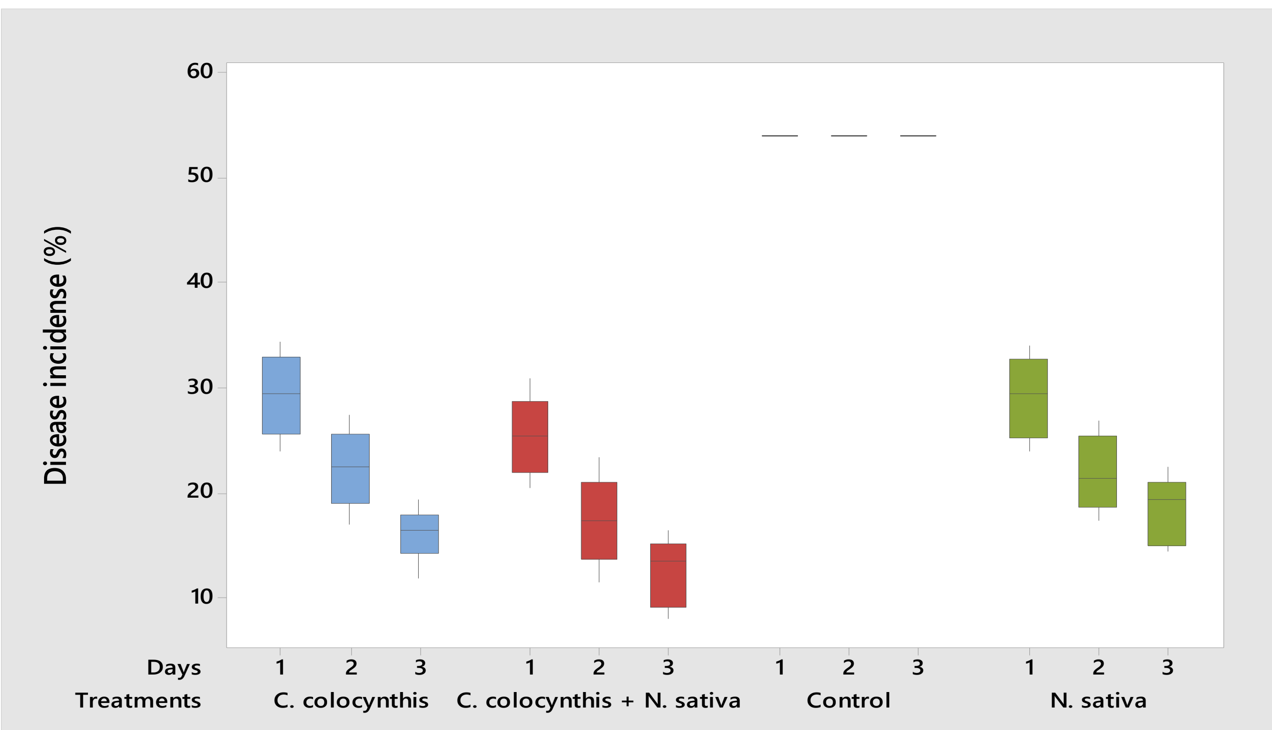

Figure 2: Two plant extracts alone and in combinations at three different times (7 days, 14Days, 21days) were evaluated under green-house conditions. All treatments significantly reduced disease incidence of citrus canker on treated plants. The minimum disease incidence was exhibited by the combination *C. colocynthis + N. sativa* after 21 days of application. While the bars indicate standard deviation.

Table 6: Effect of interaction between treatments and days against citrus canker under greenhouse conditions.

Treatment	Disease incidence (%)		
	D_1 (7 days)	D_2 (14 days)	D_3 (21 days)
C. colocynthis + N. sativa	25.389 c	17.611 e	13.236 g
C. colocynthis	29.278 b	22.333 d	16.167 f
N. sativa	29.111 b	22.111 d	18.583 e
Control (dis. water)	54.000 a	54.000 a	54.000 a
LSD	0.0341		

DISCUSSION

Citrus canker is a leading and hardly manageable threat to citriculture. Commonly, plant diseases are managed by synthetic chemicals but injudicious use of these chemicals is responsible for environmental pollution which is also toxic to human beings and animals. Unremitting and undue use of these chemicals towards *X. citri* pv. *citri* creating resistance in pathogen which make it difficult to manage citrus canker. The only safe and eco-friendly way to manage this disease is the use of botanical extracts due to presence of antimicrobial substances in the plants (Atiq et al., 2023). Natural compounds found in plants are said to have gigantic remedial potential in plants since they can be utilized as an antimicrobial agent with no symptoms that are frequently connected with synthetic microbes (Ashfaq et al., 2013). The incessant utilization of synthetic substances is neither practical nor environment friendly. While chemical control is straightforward, direct, and quick, and assists with taking care of disease issues but proceeded with reliance on pesticides has demonstrated to be unacceptable, and in reality has prompted significant degradation and causes environmental pollution. Intervention of citrus canker through plant extracts with anti-microbial properties have assisted to keep away the issues, as it is an environmentally suitable procedure to limit the utilization of synthetic compounds (Atiq et al., 2018). So, in current study five plant extracts were investigated against *X. citri* pv. *citri* causing citrus canker. Among all tested botanical extracts, *E. vesicaria* exhibited the minimum inhibition zone under lab conditions while, the minimum disease incidence was expressed by combination of *C. colocynthis* and *N. sativa* under greenhouse conditions. Present findings were supported by the work of Shricharan and Sivabalan (2020), who investigated extracts of two plant i.e. *P. hysterophorous* and *E. hirta* against *X. axonopodis* pv.*citri.* Similar results were also reported by (Leksomboon et al., 2001). Vallejo et al. (2019), also determined that seed and peel extracts of grape pomace also have antimicrobial activity against *X. axonopodis* pv *citri.*

Thymoquinone (TQ) is one of the most active constituents of *N. sativa* and has different beneficial properties i.e antimicrobial affect against *Staphylococcus aureus*, *Shigella flexneri, Escherichia coli, Salmonella typhimurium and Pseudomonas aeruginosa (*Forouzanfar et al., 2014). Dried extracts of these plants contain natural elicitor compounds that act as systemic acquired resistance (SAR) activators, triggering the plant's inactive defense systems to combat pathogens. Furthermore, the botanical extracts influence antioxidant enzyme activities (POD, SOD, CAT), hydrogen peroxide, total phenols, and protein contents, all of which play critical roles in the plant's defense mechanisms. Additionally, the presence of phytohormones in the botanical extracts influences various physiological processes within the plant (Mitra and Paul, 2017).

CONCLUSION

Present exploration has drawn conclusion that *C. colocynthis* is the most fruitful treatment among all tested botanical extracts. *Eruca vesicaria* expressed the minimum inhibition zone followed by *Eucalyptus globus*, *Trigonella foenum-graecum*, *Nigella sativa* and *Citrullus colocynthis* as compared to control. Minimum disease incidence was observed when phyto-extracts of *C. colocynthis* and *N. sativa* applied in integration against citrus canker.

FUTURE IMPLICATIONS OF RECENT REVELATIONS FOR GROWERS

Growers can cope with citrus canker, a formidable challenge to citrus industry by using currently investigated fruitful management tool.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTIONS

NA conducted research trials and wrote original manuscript; MA conceived idea and supervised research work; NAR finalized the manuscript; AMA reviewed and edited the manuscript; GAK and AN helped in research trials; AA helped in the manuscript write-up; MMJ performed the statistical analysis; AJ prepared the tables and graphs and AA helped in data collection.

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