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Biochemical Factors Conducive for the Development of Citrus Canker and its Management using Selected Chemicals and Plant Extracts

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ABSTRACT

Citrus belonging to *Rutaceae* family is one of the most common fruit crops in Pakistan. Citrus canker disease caused by *Xanthomonas citri* pv. *citri* is one of the important diseases that causes huge losses to citrus production. The current study was carried out to mitigate this disease by exploitation of four chemicals and plant extracts *in-vitro* at three different concentrations and in greenhouse with most efficient concentration. The laboratory and greenhouse experiments were performed through inhibition zone technique and foliar spray method, respectively. Four chemicals including streptomycin sulphate, copper oxychloride, kasugamycin and oxytetracycline with different concentrations of 1%, 2% and 3% were evaluated. Under *in-vitro* environment, streptomycin (2.7 cm) at 3% concentration after 72 hours' time interval exhibited the most significant results among all chemicals. Similarly, four plant extracts i.e. Moringa (*Moringa oleifera),* Keekar (*Acacia nilotica*), Kortuma (*Citrullus colocynthis*), and Akk (*Colotropis gigantia*) were evaluated at 25%, 35% and 45% concentrations. Most significant results (1.9 cm) were shown by Moringa extract at 45% concentration after 72 hours' time interval. Most effective Phytoextract (*Moringa oleifera)* and chemical (Streptomycin) observed *in-vitro* were selected for further evaluation in greenhouse which resulted in the reduction of disease incidence by 23% after 45 days' time interval. Current findings would provide the valuable insights for researchers and the farming community which would prove helpful in the improved management of this particular disease.

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INTRODUCTION

Citrus is the most important fruit and cash crop grown worldwide which belongs to the family *Rutaceae*. It is a highly nutritious fruit that contains 12-13% sugar, amino acid, and is the best source of vitamin C (20-25%). Being an antioxidant, it has protective ability against skin, liver and lung diseases (Tahir *et al* 2016). In terms of production, it is the second-largest fruit in the world, and Pakistan is ranked 15th among countries that produce citrus fruits. Worldwide, its production is 115 million Metric Tons (MMT) while Pakistan produces 3.6 MMT (Memon, 2017). Crop yield is affected by either abiotic factors (e.g. anaerobe, flooding, acidity, salinity, heat, drought, or nutrient deficits) or biotic factors (e.g. insect, disease or pathogen) (Li *et al.*, 2006; Li *et al.*, 2008). Abiotic and biotic stress conditions may result in

extensive loss in citrus production (Syvertsen and Hanlon, 2008). Citrus canker, which is the most harmful, common, and fatal disease caused by *Xanthomonas citri* pv. *citri* (*Xac*), is a bacterial disease that reduces citrus plant production and quality (Grade and Lad, 2018). Losses from this disease may range from 30 to 40 percent depending upon the environmental conditions (Memon, 2017). Disease incidence and severity is influenced by temperature, plant growth, source of penetration, and inoculum quantity (Efsa, 2014). Citrus is more susceptible to the *Phyllocnistis citrella* attack on citrus leaves (Hall *et al*., 2010). Citrus leaf miner (CLM) is the main citrus insect pest in all citrus-producing regions, including Bangladesh, India, Pakistan, China, Philippines, and Sri Lanka (CABI, 2017). *Xanthomonas citri* pv *citri* is gram-negative, rod-shaped bacterium which measures 1.5–2.0 × 0.5-0.75 µm having a single polar flagellum that grows exclusively aerobically (Das, 2003). Bacterial colonies have mucoid-yellow appearance because they produce the pigment xanthomonadin which travels to other plants through water air, propagating material, insect vector and transportation. Disease incidence of citrus canker increases when natural opening are favorable for the pathogen entry (Ali *et al*. 2017).

Various management practices including soil fumigation, crop rotations, tillage practices, intercropping, soil disinfestation, solarization, soil steaming, biofumigation and sanitation have been evaluated to mitigate the disease losses (Yogev *et al.*, 2009; van Bruggen *et al.*, 2016). The use of biocontrol methods and resistant types is an effective, long-lasting, and cost-effective strategy for managing citrus cankers. In order to increase public awareness of the environmental problems linked with pesticides and antibiotics, it appears necessary to demand natural compounds to manage plant diseases (Machinl *et al.* 2010; Chudasama and Poker. 2012). Plant extracts with therapeutic value have the potential to prevent a particular disease (Carotin *et al*., 2007; Sauer *et al*., 2015). Plants do not completely resist illness when biotic or abiotic substances are employed but the severity of the disease is greatly decreased in the majority of plants (Sticher *et al*., 1997). Terpenes, ketones, phenolic compounds that are found in all parts of a plant show effective control against pathogens (Burt. 2004; Golmakani and Moayyedi, 2015). Based on trends in scientific research, aqueous extracts of *Acacia nilotica*, *Citrullus colocynthis*, *Calotropis gigantea* and *Moringa oleifera* were evaluated against bacteria in this study.

Keeping in view of sustainable agriculture and green revolution, farmers and scientists always prioritize environmentally safe products to manage their crop losses. But in severe cases of the diseases, farmer is compelled to use chemicals for quick control of dispersing pathogen and to overcome the severe crop losses (Sundin *et al.*, 2016). The hypothesis of the current study is to control citrus canker through plant extracts and chemicals which may be useful and essential for effective disease management.

MATERIALS AND METHODS

Sample collection

The research was conducted in 2022-2023 in the Department of Plant Pathology, Bahauddin Zakariya University, Multan. Suspected diseased samples were collected from citrus orchards of different locations (Multan and Layyah Districts). Samples of contaminated leaves were placed in plastic bags, date and location were noted. Later, they were taken to the laboratory and preserved at 4°C for further studies.

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

For the isolation of suspected pathogen, fresh infected leaves and fruits were collected. Nutrient Agar (NA) medium was used for the isolation of *X. citri* pv. *citri*. The diseased samples comprising leaves and fruits were cut into small pieces precisely with scissors and dipped in distilled water to remove the adulteration. The samples were submerged in 1 percent sodium hypochlorite for disinfection from saprophytes and after washing with distilled water the samples were placed on sterilized tissue paper for drying. Media was poured into petri plates and disease pieces were placed on the media with the help of sterilized forceps. The plates were wrapped and marked with dates and samples. The plates were incubated at 25-28°C for 48-72 hours. After 3 days, the plates were checked for bacterial growth. The diseased samples showed yellow, mucoid growth of bacteria on media (Ismail *et al.,* 2014). A loop full inoculum was picked and streaked on NA media plate in laminar air flow to attain pure isolates of bacteria (Bhure *et al.,*2019). After incubation, bacterial culture was preserved in 50% glycerol at 4°C (Fatima *et al.,* 2019).

Pathogenicity Test

Healthy plant leaves at the age of 5-6 months were

inoculated by using sterilized syringe (24 Gauge needle size) filled with 20 µl bacterial suspensions, containing 107-cfu ml^{-1} of bacteria. Suspension was injected in plants through the midrib of leaves. Sterilized water was injected in the control treatment. After 8-9 days of the application of inoculum, symptoms of the disease appeared and the bacterium (*X. citri* pv. *citri*) was re-isolated from diseased leaves. Morphological characteristics (size, shape, texture and color of colony) of re-isolated bacterium was compared with the bacterial culture that was used for inoculation.

Evaluation of Botanical Extracts against *X. citri* pv. *citri in-vitro* Conditions

Efficacy of the botanical extracts was evaluated by employing inhibition zone technique using well diffusion method (Bauer, 1966). Aqueous botanical extracts i.e., *Moringa oleifera* leaf extract, *Acacia nilotica* leaf extract, *Citrullus colocynthis* fruit extract and *Calotropis gigantea* leaf extract were tested at different concentrations to measure the inhibition zone they induce against *X. citri* pv. *citri*. The experiment was performed in completely randomized design (CRD) in Laminar Flow chamber and each treatment consists of three replications. The concentrations of extracts in treatments T1, T2, T3 and T4 are 25%, 35%, and 45%, while treatment T5 serves as the control.

Evaluation of Chemicals against *X. citri pv. citri in-vitro* Conditions

The sensitivity of the various isolates was evaluated by incorporating inhibition zone technique using the well-diffusion method. Chemicals were prepared at different concentration for their in-vitro evaluation against isolates of *X. citri* pv. *citri*. The concentrations of Streptomycin, Copper oxychloride, Kasugamycin, and Oxytetracycline in treatments T1, T2, T3, and T4 are 1%, 2%, and 3%, respectively, while treatment T5 serves as the control.

Evaluation of Moringa Extract and Streptomycin in Green-House Against *X. citri* pv. *Citri*

Six to seven months old healthy citrus plants were taken in a greenhouse and inoculated with 1×10^6 CFU bacterial inoculum. Leaves of plants were inoculated with needleless syringe by dropping 5µl per spot of bacterial suspension in abaxial surface of healthy leaves of citrus plants. 3 spots on each leaf vein side were inoculated. The plant extract and chemical which gave significant results in laboratory were sprayed on the citrus plant leaves alone and with combination. The plants were irrigated after 24 hours of these treatments and covered with polythene bags for 2 hours in order to provide artificial humid conditions. The control treatment on host rough lemon plant was conducted by inoculation with *X. citri* pv. *citri* suspension without applying the plant extract. Treatment T1 contains Streptomycin at concentrations of 3%. Treatment T2 consists of Moringa at 45% concentration. Treatment T3 combines Streptomycin and Moringa at 3% and 45% concentrations. Treatment T4 serves as the control. Data on disease-incidence was recorded after 15, 30 and 45 days of inoculation. The disease was evaluated by calculating the disease incidence percentage using the formula given.

x = % disease incidence = No. of diseased leaves/ total leaves X 100

Difference in disease incidence was evaluated by variance analysis and the difference in means was compared by the LSD.

RESULTS

The efficacy of plant extract was checked at three concentrations (25%, 35%, and 45%), after 24 hours, 48 hours, and 72 hours' time interval.

In-vitro* Evaluation of *Moringa oleifera* Extract against *Xanthomonas citri* pv. *citri

The maximum inhibition zone was observed by *M. oleifera* 1.9 cm after 72 hours followed by 1.6cm after 48 hours and 1.4cm after 24 hours at 45% concentration. The maximum inhibition zone was produced by *M. oleifera* 1.4 cm after 72 hours followed by 1.2cm after 48 hours and 1cm after 24 hours at 35% concentration. The lowest zone was observed by *M. oleifera* 0.6 cm after 24 hours followed by 0.7cm after 48 hours and 0.9cm after 24 hours at 25% concentration.

In-vitro Evaluation of *Vachellia nilotica* against *Xanthomonas citri* pv. *citri*

The maximum inhibition zone was observed by *.nilotica* 1.7cm after 72 hours followed by 1.4cm after 48 hours and 1.2cm after 24 hours at 45% concentration. Similarly, the maximum inhibition zone was produced by *V. nilotica* 1.2 cm after 72 hours followed by 1cm after 48 hours and 0.8cm after 24 hours at 35% concentration. After 72 hours the maximum inhibition zone was produced by *V. nilotica* 0.9cm followed by 0.7cm after 48 hours and 0.5cm after 24 hours at 25% concentration. In negative control no inhibition zone was recorded (Figure 2).

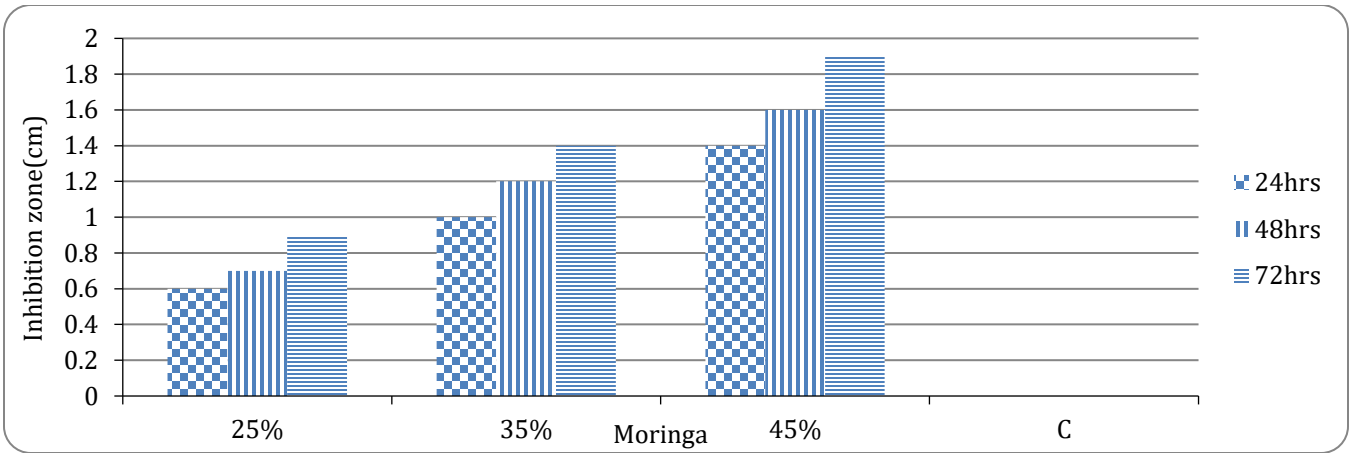

Figure 1. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by *M. oleifera.*

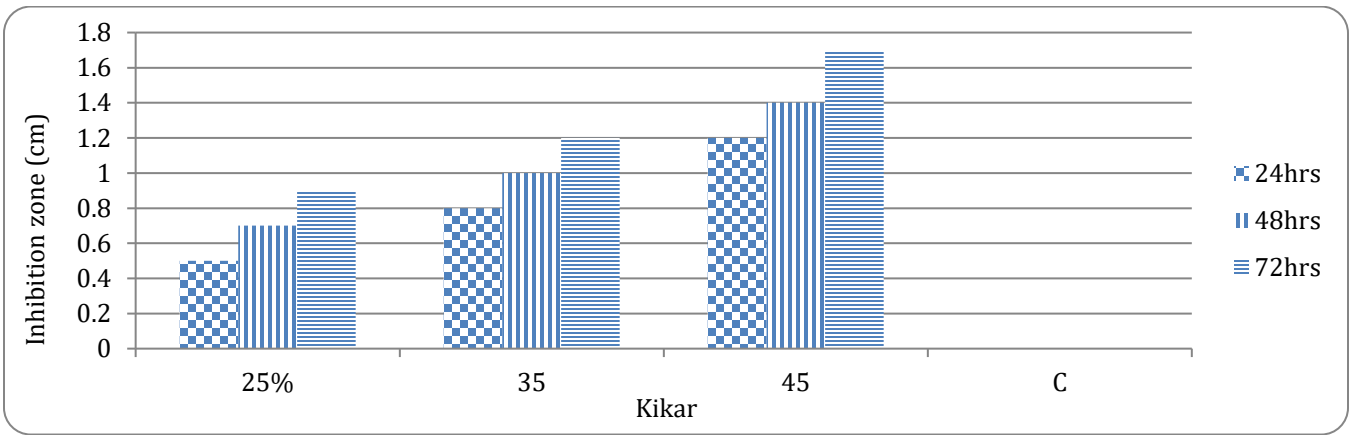

Figure 2. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by *V nilotica.*

In-vitro* Evaluation of *Citrullus colocynthis* against *Xanthomonas citri* pv. *citri

The maximum inhibition zone was produced by *C. colocynthis* 1.4cm after 72 hours followed by 1.1cm after 48 hours and 0.9cm after 24 hours at 45% concentration. Similarly, the maximum inhibition zone was produced by *C. colocynthis* 1cm after 72 hours followed by 0.8cm after 48 hours and 0.5cm after 24 hours at 35% concentration. After 72 hours the maximum inhibition zone was produced by *C. colocynthis* 0.7cm followed by 0.5cm after 48 hours and 0.3cm after 24 hours at 25% concentration. In negative control (Distilled water) no zone was recorded. (Figure 3).

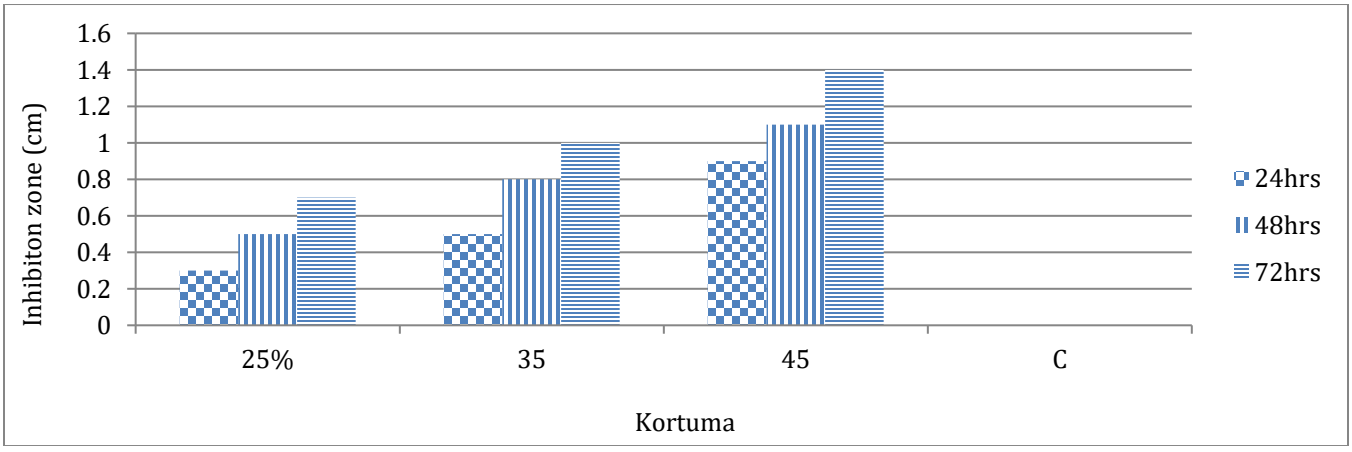

Figure 3. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by *C. colocynthis.*

In-vitro* Evaluation of *Calotropis gigantae* against *Xanthomonas citri* pv. *citri

The maximum inhibition zone was observed by *C. gigantae* 1.2cm after 72 hours followed by 1cm after 48 hours and 0.8cm after 24 hours at 45% concentration. The maximum zone was produced by *C. gigantae* 0.9cm after 72 hours followed by 0.8cm after 48 hours and 0.7cm after 24 hour at 35% concentration. After 72 hours the maximum inhibition zone was produced by *C. gigantae* 0.6cm followed by 0.5cm after 48 hours and 0.3cm after 24 hours at 25% concentration. In negative (distilled water) control no inhibition zone was recorded (Figure 4).

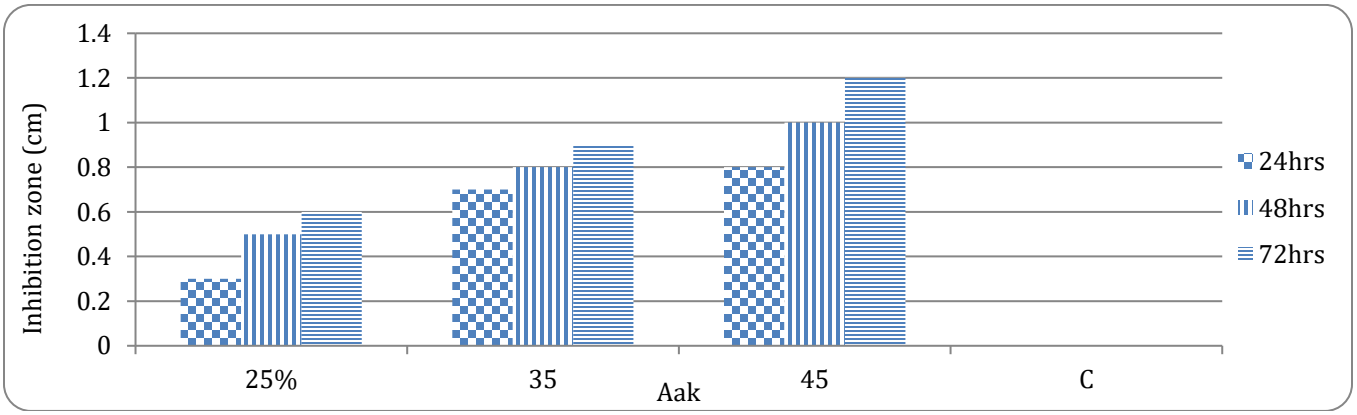

Figure 4. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by *C. gigantae.*

In vitro* Evaluation of Chemicals against *Xanthomonas citri* pv *citri

The efficacy of different chemicals was checked at three concentrations (1%, 2%, and 3%), after 24 hours, 48 hours, and 72 hours' time interval.

In-vitro* Evaluation of Streptomycin Sulphate against *Xanthomonas citri* pv. *citri

The maximum inhibition zone was produced by Streptomycin 2.7cm after 72 hours followed by 2.4cm after 48 hours and 2.0cm after 24 hours at 3% concentration. The maximum zone was observed 2.3cm after 72 hours followed by 2.0cm after 48 hours and 1.8cm after 24 hours at 2% concentration. After 72 hours maximum inhibition zone produced by Streptomycin was 2.0cm followed by 1.6cm after 48 hours and 1.4cm after 24 hours at 1% concentration (Figure 5).

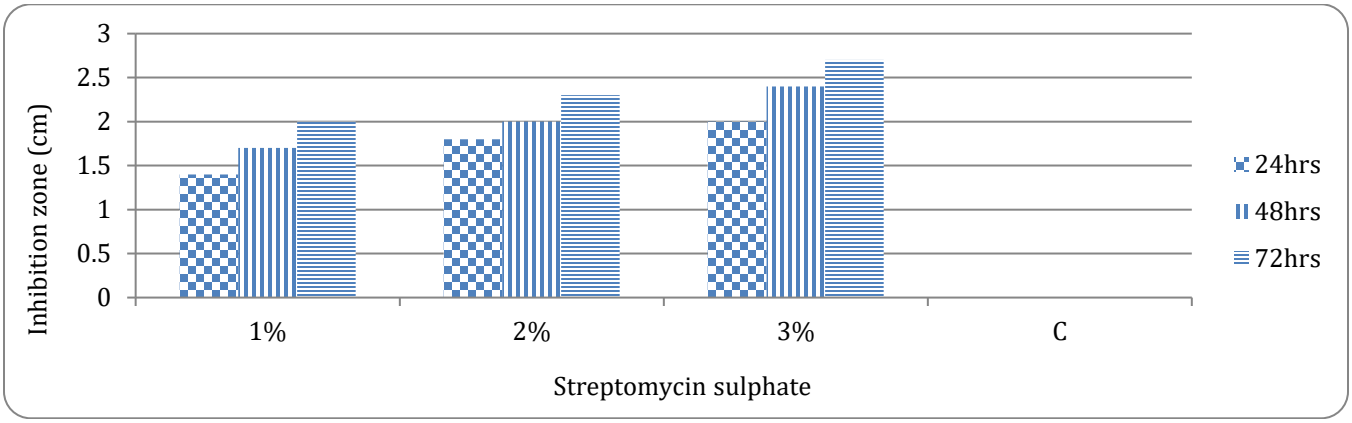

Figure 5. Impact of the interaction b/w concentration and days against *X.citri* pv *citri* by Streptomycin.

In-vitro Evaluation of Copper Oxychloride against *Xanthomonas citri* pv. *citri*

The maximum inhibition zone was produced by copper oxychloride 2.4cm after 72 hours followed by 2.1cm after 48 hours and 1.8cm after 24 hours at 3% concentration. Similarly, the maximum inhibition zone was produced 2.1cm after 72 hours followed by 1.8cm after 48 hours and 1.4cm after 24 hours at 2%

concentration. After 72 hours the maximum inhibition zone was produced by Streptomycin was 1.8cm followed by 1.5cm after 48 hours and 1.1cm after 24 hours at 1% concentration. In negative control no inhibition zone was recorded (distilled water) (Figure 6).

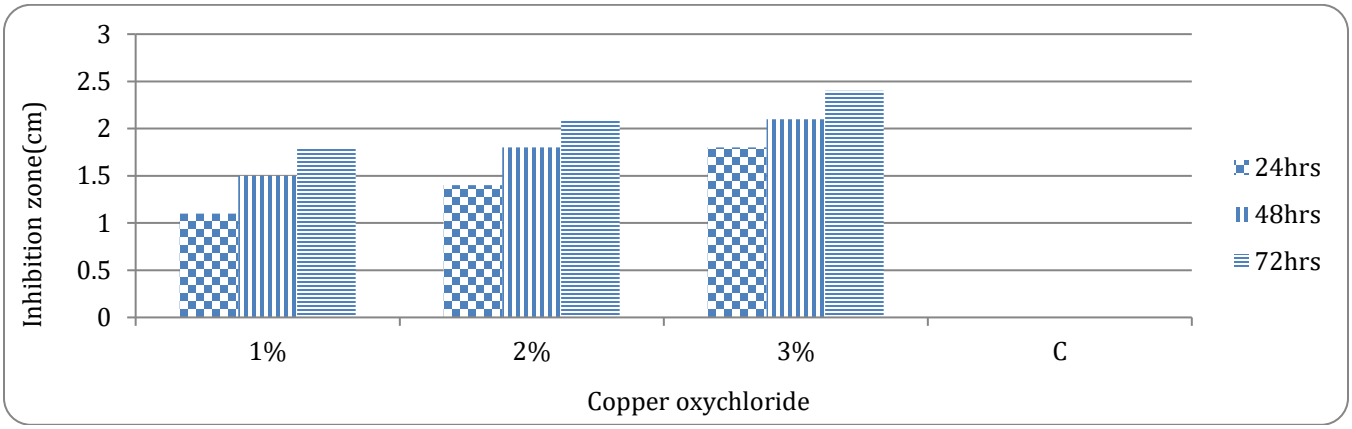

Figure 6. Impact of interaction b/w concentration and days against *X. citri* pv *citri* by Copper oxychloride.

In-vitro* Evaluation of Kasugamycin against *Xanthomonas citri* pv.*citri

The maximum inhibition zone was produced by Kasugamycin 2.2cm after 72 hours followed by 1.8cm after 48 hours and 1.6cm after 24 hours at 3% concentration. The maximum inhibition zone 1.9cm was produced after 72 hours followed by 1.5cm after 48 hours and 1.3cm after 24 hours at 2% concentration. After 72 hours the maximum inhibition zone was produced by Streptomycin was 1.6cm followed by 1.3cm after 48 hours and 1cm after 24 hours at 1% concentration (Figure 7).

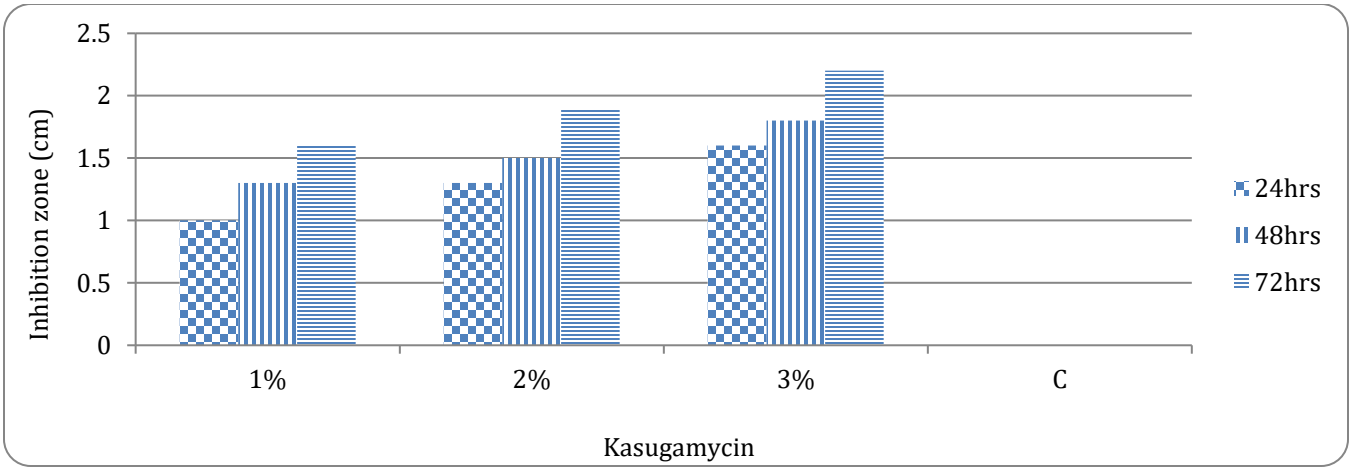

Figure 7. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by Kasugamycin.

In-vitro* Evaluation of Oxytetracycline against *Xanthomonas citri* pv.*citri

The maximum inhibition zone was produced by oxytetracycline 1.8cm after 72 hours followed by 1.5cm after 48 hours and 1.3cm after 24 hours at 3% concentration. Similarly, the maximum inhibition zone was produced 1.5cm after 72 hours followed by 1.2cm after 48 hours and 0.9cm after 24 hours at 2% concentration. After 72 hours the maximum inhibition zone was produced by Streptomycin was 1.3cm followed by 1cm after 48 hours and 0.7cm after 24 hours at 1% concentration. In negative control no inhibition zone was recorded (distilled water) (Figure 8).

Evaluation of Chemical and Plant Extract against *Xanthomonas citri* pv *citri* in Greenhouse

In laboratory conditions, *Moringa oleifera* and streptomycin sulphate showed the most effective results against *Xcc,* so these were evaluated in greenhouse alone

and with combination to check their efficacy against citrus canker disease. For this purpose, six month susceptible plant of citrus were taken in the greenhouse, the leaves of these plants were artificially inoculated through syringe method by using20 µl (local isolates) bacterial suspension (pure culture), contained 10^7cells/ml. The chemical, plants extract, and their combination was applied through spray method (two treatments) with three replication under complete randomized design (CRD), Control plants were sprayed with distilled water only.

Evaluation of *Moringa oleifera* against *Xanthomonas citri* pv.*citri* under Greenhouse Conditions

The result showed that minimum disease incidence (49%, 35% and 28%) was observed at 45% concentration after fifteen, thirty and forty-five days. The disease incidence (68%, 58% and 42%) at 35% concentration was observed after fifteen, thirty and forty-five days. Similarly, the disease incidence (76%, 62% and 52%) was observed after fifteen, thirty and forty-five days' time intervals at 25% concentration. The maximum disease incidence (85%, 72% and 60%) was observed in control where only distilled water was sprayed for comparison (Figure 9).

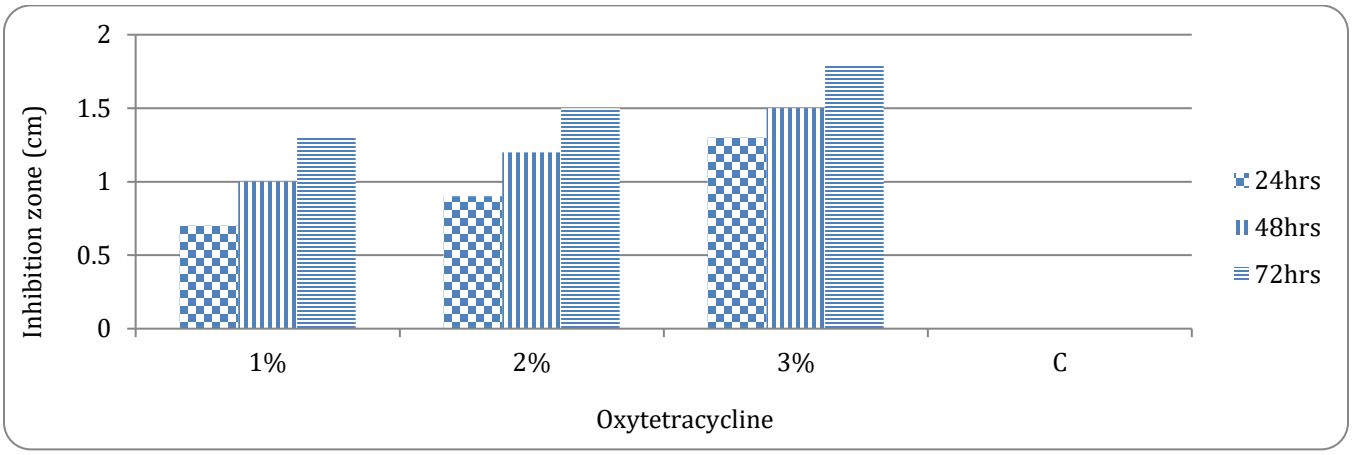

Figure 8. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by oxytetracycline.

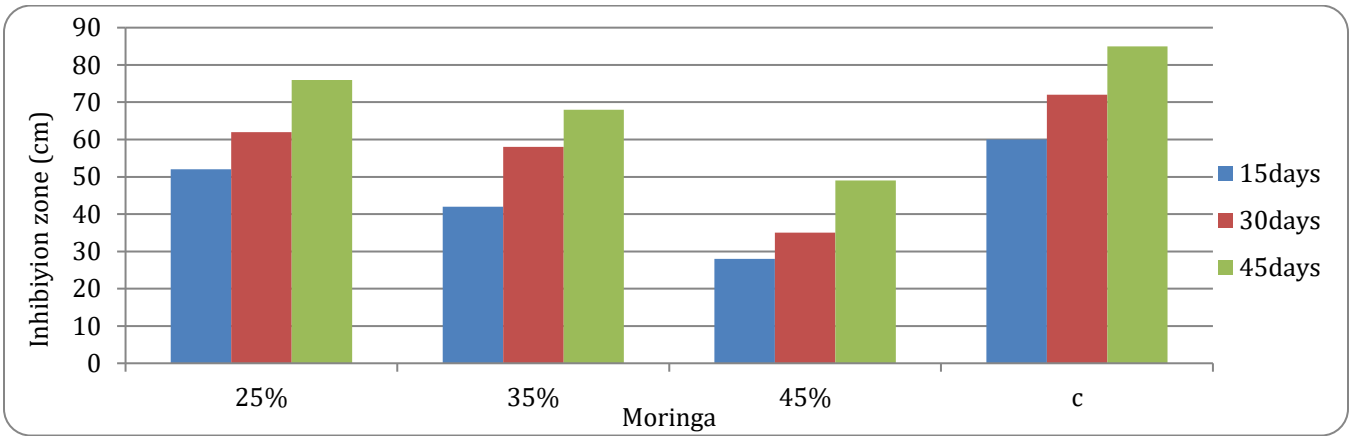

Figure 9. Impact of the interaction between concentration and days against *X. citri* pv *citri* by *Moringa oleifera* in greenhouse conditions.

Evaluation of Streptomycine Sulphate against *Xanthomonas citri* pv.*citri* under Greenhouse Condition

The result showed that minimum disease incidence (30%, 24% and 20%) was observed at 8% concentration after fifteen, thirty and forty-five days. The disease incidence (36%, 25% and 22%) at 6% concentration was observed after fifteen, thirty and forty-five days. Similarly, the disease incidence (42%, 36% and 24%) was observed after fifteen, thirty and forty-five days' time interval at 4% concentration. The maximum disease incidence (85%, 72% and 60%) was observed in

control where only distilled water was sprayed for comparison (Figure 10).

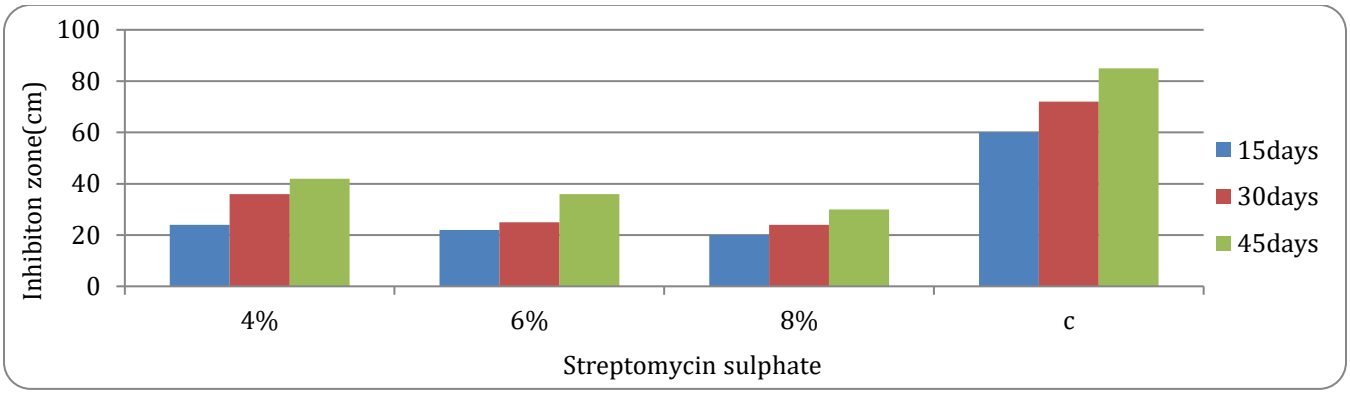

Figure 10. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by Streptomycin sulphate in greenhouse conditions.

Evaluation of Streptomycine sulphate+ *Moringa oleifera* against *Xanthomonas citri* pv. *citri* under Greenhouse Condition

The combine combined efficacy of *Moringa oleifera* and Streptomycine sulphate was checked against citrus canker disease. The result showed that minimum disease incidence (23%, 18% and 13%) was observed at 16+8% concentration after fifteen, thirty and forty-five days' time intervals. The disease incidence (28%, 23% and 15%) at 12+6% concentration was observed after fifteen, thirty and forty-five days' time intervals. Similarly, the disease incidence (36%, 30% and 21%) was observed after fifteen, thirty and forty-five days' time intervals at 8+4% concentrations. The maximum disease incidence (85%, 72% and 60%) was observed in control where only distilled water was sprayed for comparison (Figure 11).

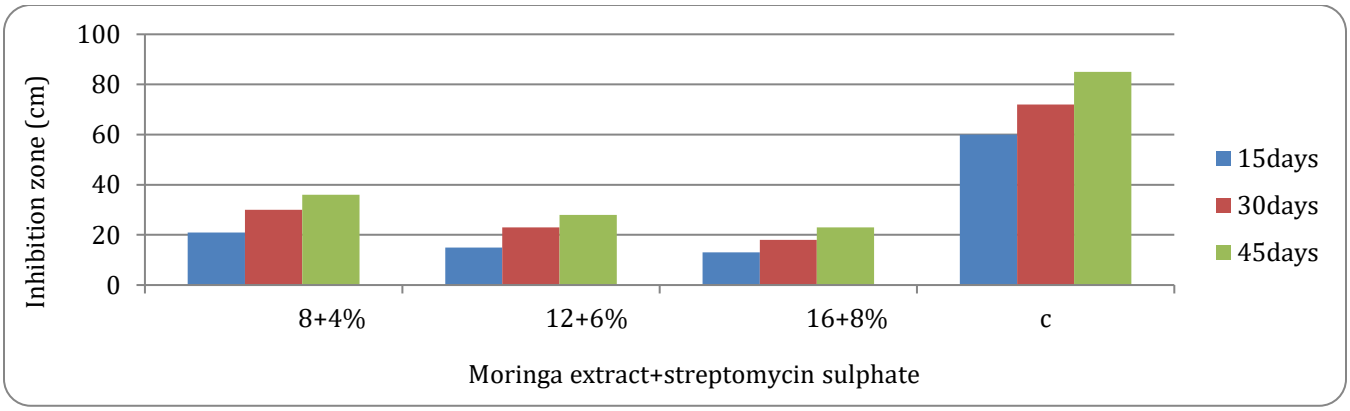

Figure 11. Impact of the interaction b/w concentration and days against *X. citri* pv *citri* by *Moringa oleifera*+Streptomycin sulphate in greenhouse conditions.

DISCUSSION

Citrus canker disease is found all over the world and has great economic importance. This disease has caused trouble in the citrus industry to which different management procedures are being applied to control *X. citri* pv. *citri.* The market value of the product has declined due to the infestation caused by bacteria in the citrus-growing nurseries and plantations. The current findings are consistent with the researchers' earlier study. Abhang (2015) gathered the diseased acid lime samples exhibiting canker signs and procured seven distinct strains of *X. citri* pv. *citri*, which were cultured on NA for additional study. Our pathogenicity test results demonstrated the ability of the *X. citri* pv. *citri* test strains to generate canker disease after two weeks of inoculation. Similar results were obtained by Lin

(2008), who examined the pathogenicity of *X. citri* pv. *citri* on four distinct Citrus species. The pathogenicity results also align with those reported by Mustafa (2015), who used spray inoculation of suspension on two-year-old citrus to validate the pathogenicity of *X. citri* pv. *citri.* Citrus canker being an economical issue is essential to be managed through ecological tactics. Regarding this, the current study was carried out in the laboratory and under greenhouse conditions that potentially controlled the disease to a certain extent by utilizing four chemicals and plant extracts. The plant extracts are gaining importance in the current era because of their ecological nature and are being used against various destructive to plant pathogens. Many researchers have described the utilization of plant by-products with antimicrobial capabilities against numerous phytopathogenic bacteria and fungi (Falcón-Piñeiro *et al.*, 2023). The present research aimed to determine how efficient plant extracts against *X. citri* pv. *citri.* Our laboratory results are parallel with Rasool and Jahanbakhsh (2011), Kavitha *et al.* (2013), Chawech *et al.* (2015) and Sadiq *et al.* (2017). It is evident from previous studies that plant extracts can inhibit the growth of bacteria by causing damage to the structure and function of cell membrane and cell wall. (Guo *et al.*, 2021). *A. nilotica* has the potential to cause destruction of the cell membrane of pathogen (Sadiq *et al.*, 2017). The presence of rutin compound in extracts acts as type II topoisomerase inhibitor (Araruna *et al.*, 2012). The main reason of causing antibacterial activity is the abundance of polyphenolic compounds in plant extracts which act by altering the permeability of bacterial membrane. This alteration causes a reduction in the synthesis of ATP and aborts all the functions such as less selectivity and movement toward harmful compounds that depend on ATP Later, polyphenolic compounds enter the cytoplasm causing denaturation of the enzymes that are involved in replication and quorum sensing (Fontana *et al.*, 2022). Peptide Mop2 in moringa extract has the ability to cause damage to the cellular membrane (Wang *et al.*, 2023). When plant diseases proliferate severely, the use of chemicals becomes the last option to optimize the yield losses and inputs due to high potential of disease management. Copper oxychloride causes cell death by causing damage to DNA (Iwase *et al.*, 2014). Kasugamycin causes interference in the reaction of the 30S subunit of the mRNA and ribosome (Schluenzen *et al.*, 2006). Tetracycline in oxytetracycline causes inhibition of bacterial growth either by destroying the membrane or by interfering with the synthesis of protein (Schnappinger and Hillen, 1996). Streptomycin in streptomycin sulphate causes interference with the synthesis of protein of bacteria in the ribosome (Singh *et al.*, 2020).

It is concluded that the management of citrus canker disease is done properly by using chemicals and plant extracts timely, epidemiological forecasting models and by the control of insect vector.

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