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MANAGING CITRUS CANKER: EVALUATING THE EFFECTIVENESS OF ANTIBIOTICS AND CHEMICALS FOR DISEASE CONTROL

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ABSTRACT

Citrus (family *Rutaceae*) stands as a prominent fruit crop on a global scale, bearing substantial significance. Renowned for its abundance of fiber, amino acids, antioxidants, vitamin C, and carbohydrates. However, the successful production of citrus is persistently challenged by the menacing presence of citrus canker caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), posing 5-30% yield losses in Pakistan. Current study was aimed to manage *Xcc* through antibiotics and copper-based chemicals under lab. and field conditions. *In vitro* evaluations of antibiotics showed that Enrofloxacin expressed the maximum inhibition zone (35.68mm), followed by Enco-Mix (33.50mm), Pevivet-5 (33.48mm), Kanamycin sulphate (30.41mm), Sinobiotic (30.02mm), Streptomycin sulphate (29.33mm) and Gentam-20% (28.26mm) as compared to control. Field experiments of copper-based chemicals concluded that Copper nitrate exhibited the minimum disease incidence (16.07%), followed by Copper hydrooxide (21.78%), Amistar top (22.28%), Copper oxychloride (24.37%), and Copper acetate (24.63%), but Control expressed maximum disease incidence (65.00%). The mixture of Cu $(NO_2)_3$ + Enrofloxacin under field conditions was most effective regarding management of citrus canker. Current revelation appreciated the efficacy of Cu $(NO_2)_3$ + Enrofloxacin to manage citrus canker and strongly suggested this treatment against various bacterial pathogens.

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INTRODUCTION

Citrus is an imperative fruit crop in Pakistan, contributing 34.9% to the overall fruit production (FAO, 2017). The province of Punjab, known for its favorable climate, contributes 94.8% of the total citrus production (1.9074 million tons) in the country (FAO, 2017). Citrus fruits are known for their nutritional value, being rich in nutrients such as amino acids, fiber, vitamin C, carbohydrates, and various phytochemicals like terpenes, carotenoids, flavonoids, limonoids, and alkaloids (Alexander, 2019). Pharmacological studies have provided evidence of the diverse health benefits associated with citrus fruits, including anti-inflammatory, anticancer, anthelmintic, antimicrobial, antioxidant, anti-diabetic, immunomodulatory, gastroprotective, analgesic, insect repellent, and other pharmacological effects (Al-Snafi, 2016).

However, citrus is susceptible to several diseases, including sooty mold, phytophthora root rot, tristeza complex, gummosis, citrus greening, slow decline, citrus wither tip, and citrus canker (Anjum and Javaid, 2005). Among them, citrus canker, caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), is most prevalent (Tehmina and Arshad, 2005), resulting in 5% to 30% yield losses in Pakistan (Ashraf *et al*., 2014; Hameed *et al*., 2022; Sahi *et al*., 2007). *Xanthomonas citri* subsp. *citri* is a gram-negative, rod-shaped, aerobic bacterium with a single polar flagellum measuring 0.5-0.75µm in length, primarily thriving at

temperatures of 35-39°C (Sahi *et al*., 2007).

Xanthomonas citri subsp. *citri* spreads through a combination of wind and rain within a certain distance from tree to tree (Sahi *et al*., 2007). Asian leaf miners have also been identified as facilitation factor of infection by rupturing the cuticle, providing entry points for the pathogen into the mesophyll of leaves, twigs, fruits and stems (Graham and Leite, 2004). Asian leaf miners may also act as vectors for *Xcc* dispersal (Nirvan, 1961; Sohi and Sandhu, 1968; Sinha *et al*., 1972; Cook, 1988). The pathogen can easily suspend in water and disseminate with the help of its mucilaginous coat. Rainwater typically carries the bacterium as it drips from drips down from surfaces of lesions to non-infected parts of plants or other plants (Stall *et al*., 1980).

Various strategies have been employed to manage plant diseases, such as the application of fungicides, utilization of plant defense activators, and implementation of antagonistic organisms. However, in cases where resistant varieties are unavailable and the disease reaches epidemic proportions, farmers often resort to the use of synthetic chemicals due to their rapid action and easy availability. During severe disease prevalence use of chemicals is the only option to overcome the high yield losses. Recognizing importance of chemicals, the current study was aimed to manage citrus canker by employing nine antibiotics (Enrofloxacin, Enco-Mix, Penivet-5, Kanamycin Sulphate, Sinobiotic, Streptomycin Sulphate, Gentam (20%), Benzyl Penicillin Sodium, Tylofurcin) and five copper-based chemicals (Copper nitrate, Copper hydroxide, Amistar top, Copper oxychloride, Copper acetate) under laboratory and field environments.

MATERIALS AND METHODS

Isolation, Purification, and identification

The diseased samples (*Xanthomonas citri* subsp. *citri*) of citrus were collected from the botanical garden, University of Agriculture, Faisalabad (UAF) and brought to Molecular Phyto pathological Laboratory, Department of Plant Pathology, UAF. The disease samples were rinsed through tap water, cut into small pieces, surface sterilized by dipping in 1% Sodium hypochlorite (NaOCl) for 30 seconds and then washed by using distilled water for two minutes and placed on sterile Nutrient Agar (NA) medium already poured in Petri plates and incubated at 28°C for 48 hours. The bacterial ooze on NA media were transferred to new Petri plates containing NA media through streaking method by using sterile inoculating loop and incubated at 28°C. The isolated pathogenic bacteria were purified by following the dilution plate technique on NA medium. The purified bacterium was identified on the bases of literature and following assays (Nethravathi and Yadahalli, 2016).

Pathogenicity test

One year old disease-free citrus plants were collected from Nursery of Institute of Horticultural Sciences, UAF. The seedlings were transposed in pots containing soil and farmyard manure (2:1). Under natural conditions, the seedlings were inoculated after 7-10 days of transplanting through pin pricking technique. The seedlings were observed after a one-week interval for the appearance of the visual canker symptoms. The bacteria were re-isolated from leaves of inoculated citrus seedlings and cultured on NA media. After purification, further microscopic and biochemical tests were performed to confirm the pathogen (Nethravathi and Yadahalli, 2016).

Pectinolytic activity test of *Xcc*

Xcc was cultivated on Hankins's media and incubated at 32°C. Pectinolytic activity of *Xcc* was confirmed on the base of a clear zone appearance around bacterial.

Trehalose, maltose, sorbitol, sucrose and lactose test:

Xcc was cultured on King's media B in test tubes containing trehalose, maltose, sorbitol, sucrose and lactose and placed for incubation at 32 °C for 48 hours. *Xcc* was confirmed on the bases of changes in color and production of gas.

H_2S Production by *Xcc*

The bacterial isolates were placed on MacConkey broth and left for incubation at 35°C for 48 hours. After 48 hours, *Xcc* was confirmed by appearance of black color of lead acetate paper and rotten egg smell due to the production of H_2S gas.

Indole production test

Indole production test were employed to check the capacity of organism to split indole from the amino acid tryptophane by the intracellular enzyme tryptophanase formed by *Xcc*. *Xanthomonas citri* subsp. *citri* culture were added to the broth and placed for incubation at 32°C for 48 hours. Then, Kovac's reagents were added to the medium and *Xcc* was identified after the appearance of cherry red color in the medium.

Utilization of Citrate by *Xcc*

Xcc culture were inoculated on Simmon's Citrate Agar plates and placed in the incubator for 48 hours at 32°C. Citrate positive results were confirmed on the bases of color changes from green to blue.

α-methyle D glucoside test

Xcc culture grown on Kings Medium B were relocated to α-methyle D glucoside medium and incubated for 48 hours at 32°C. *Xcc* was recognized by the appearance of pink color of the *Xcc* colony.

In vitro* evaluation of antibiotics against *Xcc

Different antibiotics including Enrofloxacin, Enco-Mix, Pevivet-5, Kanamycin sulphate, Sinobiotic, Streptomycin sulphate and Gentam-20% with three different concentrations (300, 500, and 700ppm) were evaluated under Complete Randomized Design (CRD) in lab. conditions by using disk plate method. The disks of 10mm diameter were put in already prepared concentrations of 300, 500, and 700ppm in distilled water, separately and then placed on NA media plates containing fully grown *Xcc*. The data were recorded after 24, 48 and 72 hours.

***In vivo* evaluation of copper-based chemicals against citrus canker:**

One-year old citrus plants were collected from Nursery of Institute of Horticultural Sciences, UAF and placed in pots at Department of Plant Pathology, UAF. Five chemicals including Copper nitrate, Copper hydroxide, Amistar top, Copper oxychloride and Copper acetate with three concentrations (0.5, 0.75, 1.00%) were evaluated against citrus canker under field conditions by using foliar spray. The disease incidence was recorded after 5, 10, and 15 days of treatments' application.

Evaluation of copper-based chemicals and antibiotics against citrus canker under field condition

Most effective antibiotic in laboratory experiments and copper-based chemical in field trials were examined against citrus alone and in combination. Treatments were applied through foliar spray method after one week of inoculation except control. Disease incidence was recorded after 5, 10 and 15 days of treatments' application.

RESULTS

Pathogenicity test

The pathogen was confirmed by following Koch's postulates. Symptoms observed on leaves, morphology on NA medium, and microscopic identification confirmed the disease was citrus canker caused by *Xanthomonas citri* subsp. *citri*.

Biochemical analysis

A clear zone was formed around the bacterial growth in Hankins's media indicating the pectin degradation due to secretion of pectate lyase by the bacterium thus confirmed the isolated bacterium as *Xcc* in Pectinolytic activity test. Acid was produced by trehalose, maltose, sucrose and lactose while sorbitol was not consumed on King's media B and the change in color and production of gas indicated positive results. The rotten egg smell in MacConkey broth confirmed the release of H_2S that also indicated positive results for *Xcc*. Similarly, red color appeared in indole production test after addition of Kovac's reagents also confirmed the positive result. The production of canker and visible change in color of Simon's citrate agar media from green to blue Simon's citrate agar plates indicated the results were positive. α-methyle D glucoside was fermented by culture of bacterium which was recognize by pink result that indicated the positive result (Table 1).

Table 1. Biochemical Assay of *Xanthomonas citri* subsp. *Citri.*

Sr	Biochemical Tests	*X. citri* subsp. *citri*
1	Pectinolytic activity	+
2	Utilization of trehalose, maltose, sorbitol, sucrose and lactose	+
3	H_2S production	+
4	Indole production	+
5	Utilization of citrate	+
6	α-methyle D glucoside	+

***In vitro* evaluation of different antibiotics against *Xanthomonas citri* subsp. *citri*.**

Among all treatments, Enrofloxacin expressed the maximum inhibition zone (35.68mm), followed by Enco-Mix (33.50mm), Pevivet-5 (33.48mm), Kanamycin sulphate (30.41mm), Sinobiotic (30.02mm), Streptomycin sulphate (29.33mm) and Gentam-20% (28.26mm) as compared to control under *in vitro* conditions (Table 2, Figure 1).

Table 2. Mean inhibition zone (mm) exhibited by different antibiotics at different concentrations and time periods in the growth of *Xanthomonas citri* subsp. *Citri.*

Sr.	Treatment	Inhibition Zone (mm)
1	Enrofloxacin	35.68a
2	Enco-Mix	33.50b
3	Penivet-5	33.48c
4	Kanamycin Sulphate	30.41c
5	Sinobiotic	30.02d
6	Streptomycin Sulphate	29.33e
7	Gentam (20%)	28.26f
8	Benzyl Penicillin Sodium	0
9	Tylofurcin	0
10	Control	0
	LSD	0.3276

The interaction between treatment and concentrations (Tr×C) exhibited that Enrofloxacin expressed maximum inhibition zone at concentrations of 700ppm(36.15mm), 500ppm (36.04mm), and 300ppm(34.85mm) followed by Enco-mix (31.01, 33.43 and 36.07mm), Penivet-5 (29.88, 30.57 and 30.99mm), Kanamycin sulphate (30.81, 28.53 and 31.91mm), Sinobiotic (29.61, 30.65 and 29.79mm), Streptomycin sulphate (26.47, 30.58 and 30.95mm), and Gentam-20% (27.56, 28.87 and 28.37mm) as compared to control (Table 3, Figure 2 and 3).

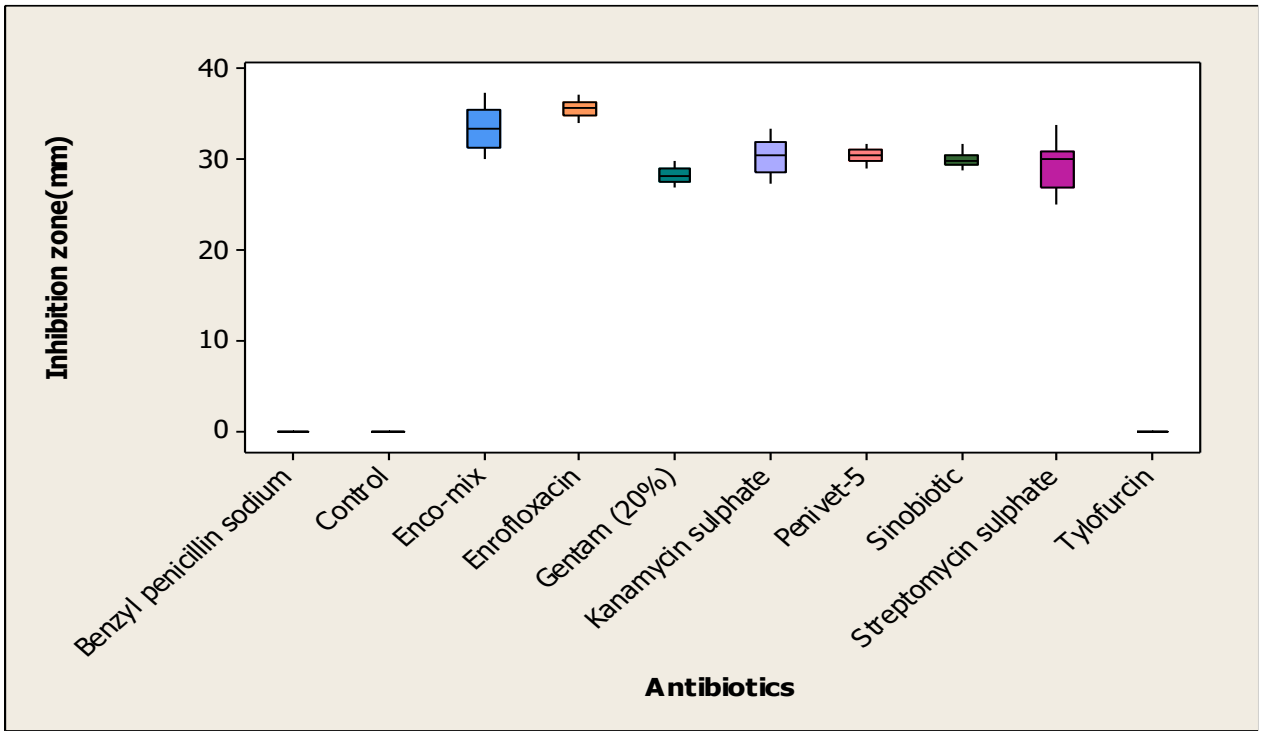

Figure 1. Mean inhibition zone (mm) exhibited by different antibiotics at different concentrations and time periods in the growth of *Xanthomonas citri* subsp. Citri.

Table 3. Mean inhibition zone (mm) exhibited by different antibiotics at different concentrations in the growth of *Xanthomonas citri* subsp. *citri*

Treatment (T)	Inhibition Zone (mm)		
	Conc. (ppm)		
	C1(300)	C2(500)	C3(700)
Enrofloxacin	34.85e	36.15a	36.04a
Enco-Mix	31.01e	33.43c	36.07a
Penivet-5	29.88f	30.57e	30.99e
Kanamycin Sulphate	30.81e	28.53g	31.91d
Sinobiotic	29.61f	30.65e	29.79f
Streptomycin Sulphate	26.47i	30.58e	30.95e
Gentam (20%)	27.56h	28.87g	28.37g
Benzyl Penicillin Sodium	0.00j	0.00j	0.00j
Tylofurcin	0.00j	0.00j	0.00j
Control	0.00j	0.00j	0.00j
LSD		0.5674	

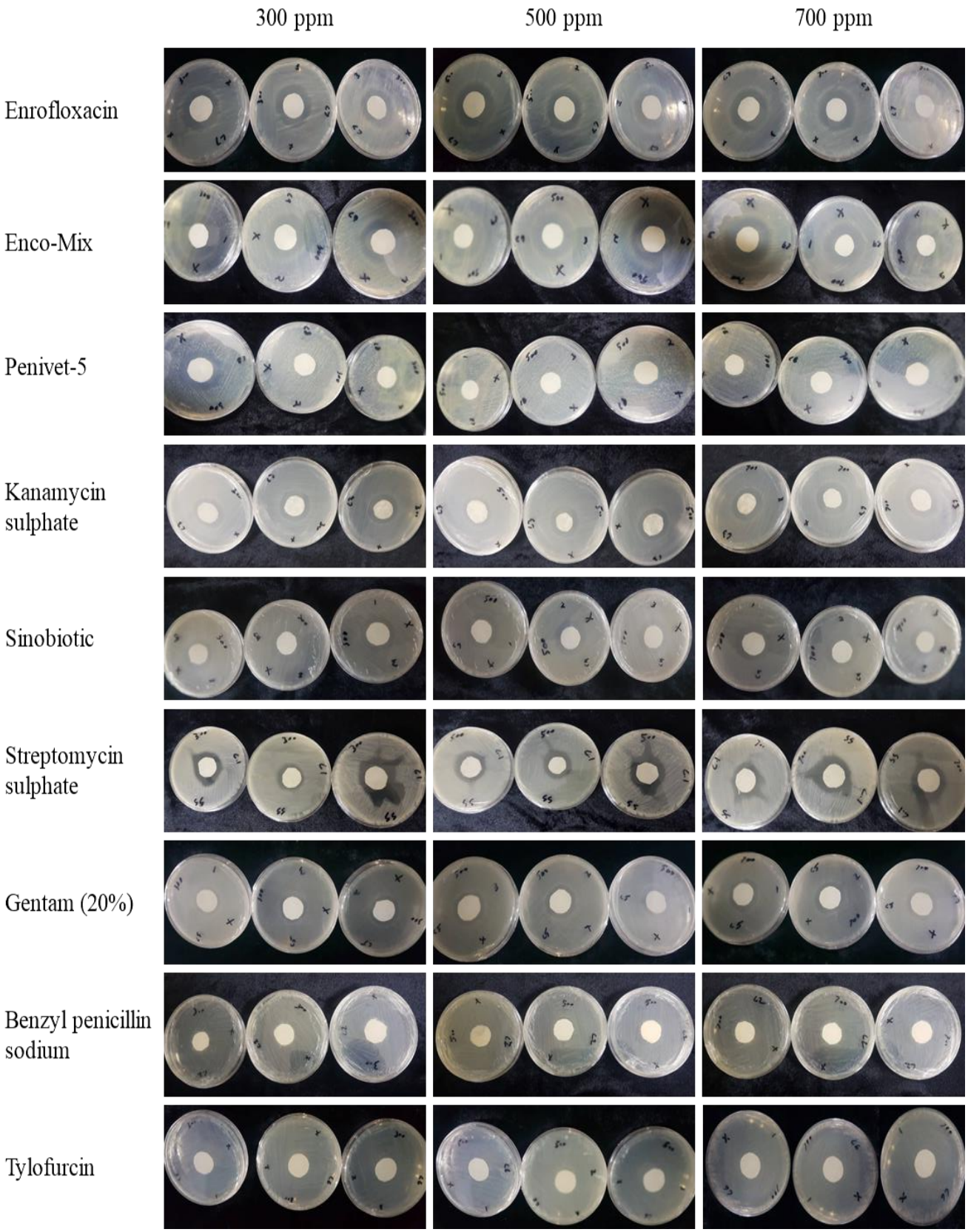

Figure 2. Inhibition zone (mm) exhibited by different antibiotics with three replications at three concentrations (300, 500, 700ppm) against growth of *Xanthomonas citri* subsp. Citri.

Evaluation of different copper-based chemicals against citrus canker under field conditions

Among all treatments, Copper Nitrate expressed the minimum disease incidence (16.07%), followed by Copper hydrooxide (21.78%), Amistar top (22.28%), Copper oxychloride (24.37%), Copper acetate (24.63%), but Control expressed maximum disease incidence (65.00%) against citrus canker under field conditions (Table 4, Figure 4). Interaction between treatment and their different concentrations (Tr×C), expressed that Copper nitrate showed minimum disease incidence (19.11, 16.44 and 12.67%), followed by Copper hydrooxide (24.11, 22.11 and 19.11%), Amistar top (23.89, 22.28 and 20.67%), Copper oxychloride (26.56, 24.22 and 22.33%), Copper acetate (26.61, 24.67 and 22.61%), at different concentrations i.e. 0.5, 0.75 and 1% respectively, but Control expressed maximum disease incidence (65.00%) (Table 5, Figure 5). The interaction between treatments and their sprays (Tr×S) expressed that Copper nitrate exhibited minimum disease incidence (21.33, 15.44 and 11.44%), followed by Copper hydroxide (28.11, 21.33 and 15.89%), Amistar top (27.61, 21.22 and 18.00%), Copper oxychloride (30.78, 24.11 and 18.22%), Copper acetate (31.78, 23.56 and 18.56%), after different sprays i.e., S1, S2 and S3 respectively, but Control expressed maximum disease incidence (65.00%) (Table 6).

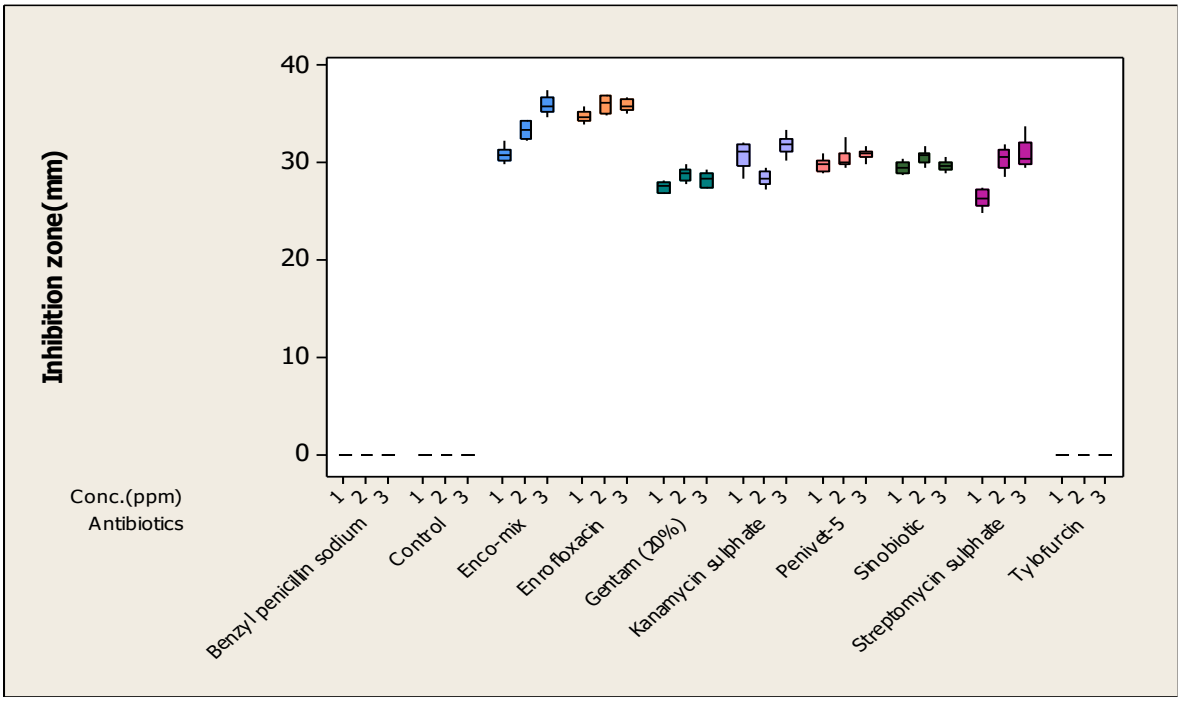

Figure 3. Mean inhibition zone (mm) exhibited by different antibiotics at different concentrations in the growth of *Xanthomonas citri* subsp. Citri.

Table 4. Effect of different copper-based chemicals (at different concentration and number of sprays) on the mean disease incidence (percent) of citrus canker under field conditions.

Sr.	Treatments	Chemical Formulae	Disease incidence (%)
1	Copper nitrate	$Cu(NO_3)_2$	16.07e
2	Copper hydroxide	$Cu(OH)_2$	21.78d
3	Amistar top	$C_{22}H_{17}N_3O_5 + C_{19}H_{17}Cl_2N_3O_3$	22.28c
4	Copper oxychloride	$Cu_2(OH)_3Cl$	24.37b
5	Copper acetate	$Cu(CH_3COO)_2$	24.63b
6	Control	Distilled water (H_2O)	65.00a
	LSD	0.3609	

Table 5. Effect of different copper-based chemicals at different concentrations on the mean disease incidence (percent) of citrus canker under field conditions.

Treatment (T)	Disease incidence (%)		
	Concentration (%)		
	C1(0.5)	C2(0.75)	C3(1.00)
Copper nitrate	19.11g	16.44h	12.67i
Copper hydroxide	24.11cd	22.11e	19.11g
Amistar Top	23.89d	22.28e	20.67f
Copper oxychloride	26.56b	24.22cd	22.33e
Copper acetate	26.61b	24.67c	22.61e
Control	65.00a	65.00a	65.00a
LSD		0.3609	

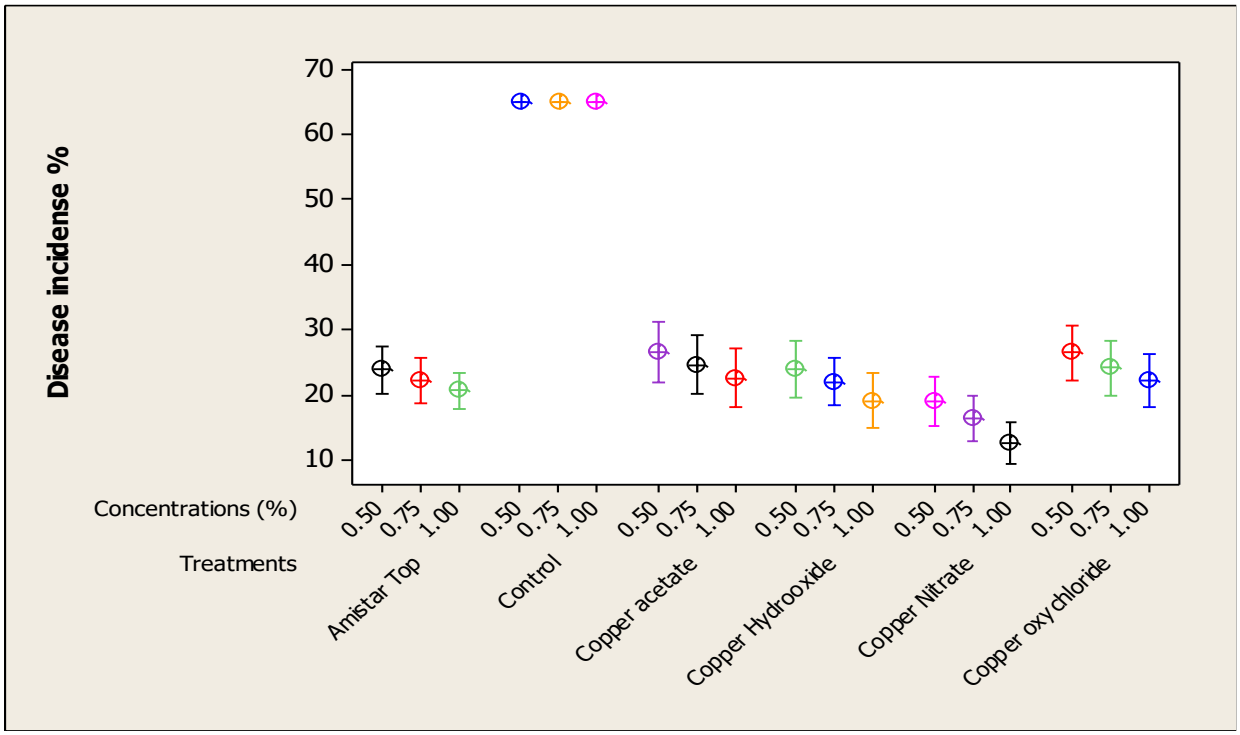

Figure 5. Effect of different copper-based chemicals at different concentrations on the mean disease incidence (percent) of citrus canker under field conditions.

Table 6: Effect of different copper-based chemicals on the mean disease incidence (percent) of citrus canker, recorded after different number of sprays under field conditions.

Treatment (T)	Disease incidence (%)		
	Spray (S)		
	S1	S2	S3
Copper nitrate	21.33f	15.44h	11.44i
Copper hydroxide	28.11d	21.33f	15.89h
Amistar top	27.61d	21.22f	18.00g
Copper oxychloride	30.78c	24.11e	18.22g
Copper acetate	31.78b	23.56e	18.56g
Control	65.00a	65.00a	65.00a
LSD		0.3609	

Evaluation of chemicals, antibiotics alone and in combination against Citrus Canker

Among all treatments, Enrofloxacin + Cu $(NO_2)_3$ expressed minimum disease incidence (18.22%), followed by Enrofloxacin (21.28%), Cu $(NO_2)_3$ (22.28%) as compared to control under field conditions (Table 7, Figure 6). The interaction between treatments and days Tr×D that indicates Enrofloxacin+ Cu $(NO_2)_3$ expressed minimum disease incidence (20.17, 18.67 and 15.83%) on D1, D2 and D3 respectively followed by Enrofloxacin (24.50, 22.50 and 16.83%) and Cu $(NO_2)_3$ (26.83, 22.17 and 17.67%) as compared to control against Citrus canker under field conditions (Table 8, Figure 7).

Table 7. Mean disease incidence (percent) of citrus canker disease after application of different antibiotics (alone and in combination) under field conditions.

Sr.	Treatments	Disease Incidence (%)
1	Cu $(NO_2)_3$ + Enrofloxacin	18.22d
2	Enrofloxacin	21.28c
3	Cu $(NO_2)_3$	22.28b
4	Control	40.98a
	LSD	0.6249

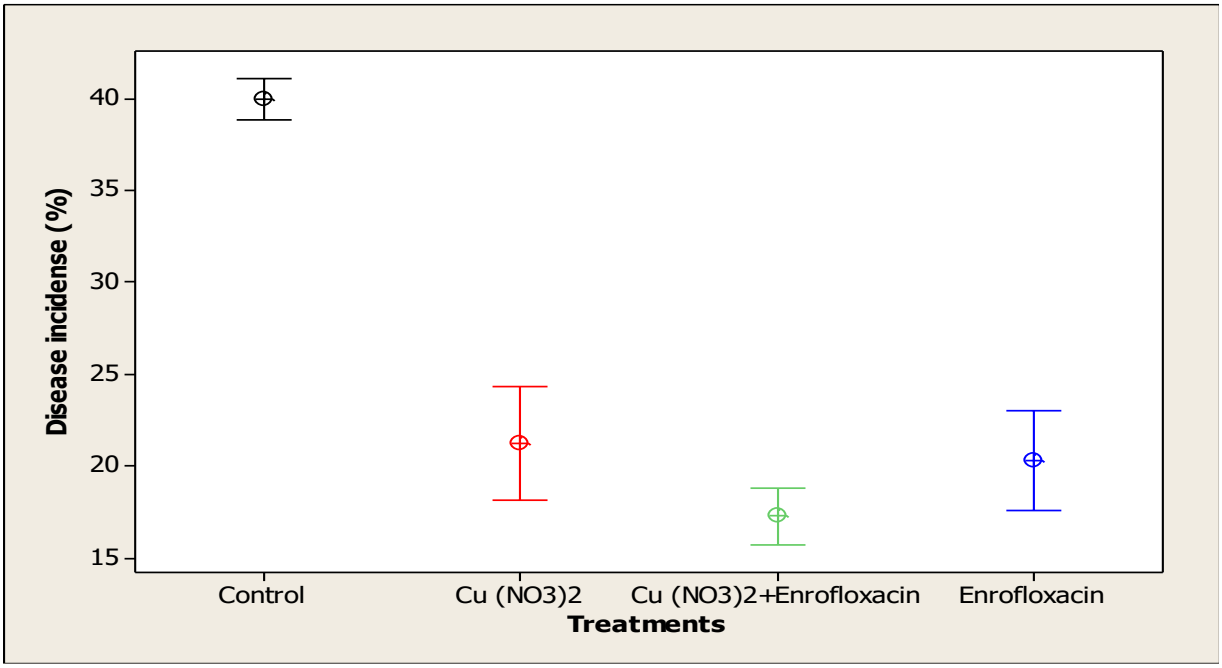

Figure 6. Mean disease incidence (percent) of citrus canker disease after application of different antibiotics (alone and in combination) under field conditions.

Table 8. Mean disease incidence (percent) of citrus canker disease at different intervals (days) after application of different antibiotics (alone and in combination) under field conditions

Treatments	Disease incidence (%)		
	Days (D)		
	D1	D2	D3
Cu $(NO_2)_3$ + Enrofloxacin	20.17g	18.67h	15.83j
Enrofloxacin	24.50e	22.50f	16.83ij
Cu $(NO_2)_3$	26.83d	22.17f	17.67hi
Control	39.47c	40.87b	42.60a
LSD		1.2498	

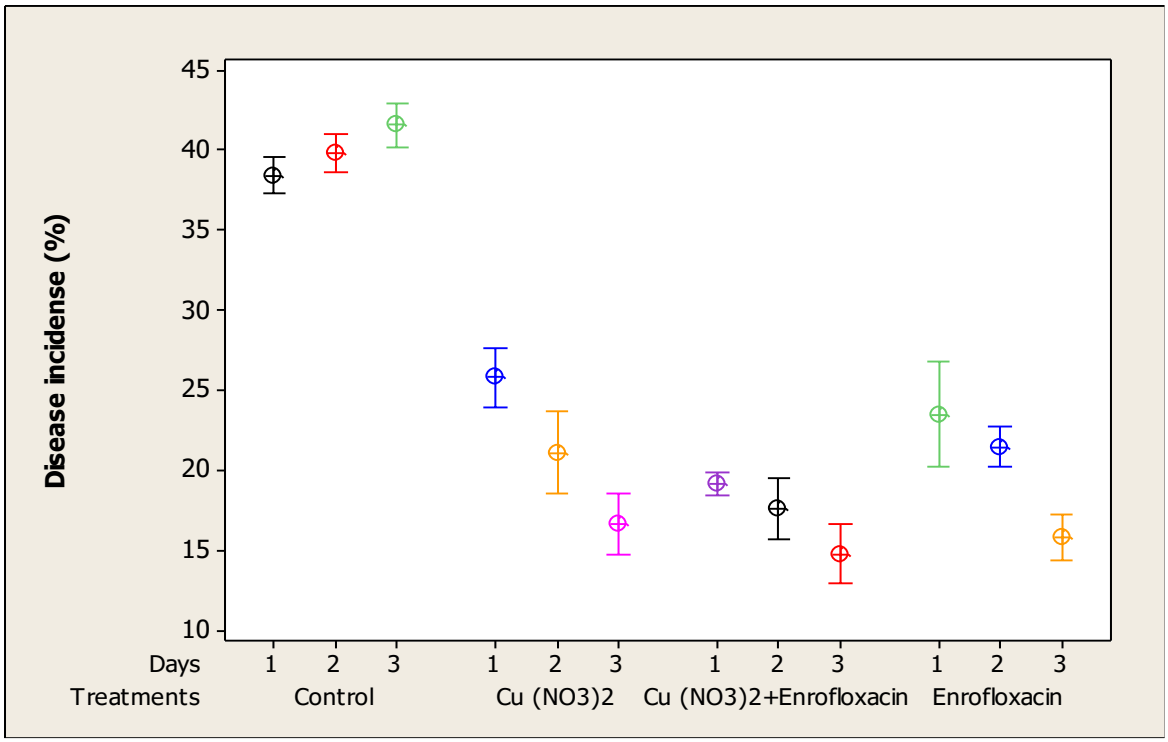

Figure 7. Mean disease incidence (percent) of citrus canker disease at different intervals (days) after application of different antibiotics (alone and in combination) under field conditions.

DISCUSSION

Citrus canker, caused by one of the most damaging subfamilies of the bacterial phytopathogen *Xanthomonas citri* subsp. *citri* (Xcc), is a serious threat to the world's most valuable citrus fruit crop. Symptoms of a severe infection include leaf loss, early fruit drop, dieback, severe fruit blemishes or discoloration and a decline in fruit quality (Ali *et al*., 2023). Present experiments were designed to examine the efficacy of different antibiotics and copper-based chemicals. On the basis of average data Enrofloxacin proved to be the best one which inhibited the growth of *Xcc* in laboratory by 35.68mm (Inhibition zone) followed by Enco-Mix, Penivet-5, Kanamycin sulphate, Sinobiotic, Streptomycin sulphate, Gentam-20% but Benzyl penicillin sodium and Tylofurcin gave no result. Earlier studies have reported the efficacy of a number of antibiotics against *Xcc* but the efficacy of Enrofloxacin has never been reported by any researcher. Outcomes of present study was supported by the findings of Jaskani who studied the effect of different antibiotics for controlling citrus canker and concluded that kanamycin sulphate expressed the higher inhibition zone followed by Streptomycin Sulphate and Lincomycin against *Xanthomonas citri* subsp. *citri* (Jaskani *et al*., 2021). Already, nine antibiotics against citrus canker caused by *Xanthomonas citri* subsp. *citri* revealed Levofloxacin was most significant at 700 ppm concentration (Hameed *et al*., 2022). Another study supported our finding under *in vitro* circumstances and concluded streptomycin sulphate and bromopol (each @ 100, 250, and 500 ppm) were the most efficient antibiotics against *Xanthomonas citri* subsp. *citri* (Kharat *et al*., 2020). Enrofloxacin involves in inhibiting the bacterial DNA gyrase and topoisomerase IV enzymes, which are essential for the replication, transcription, and repair of bacterial DNA (Bush *et al*., 2020). Enrofloxacin interferes with the activity of DNA gyrase, an enzyme found in bacteria that is responsible for introducing negative supercoils into the bacterial DNA molecule. By inhibiting DNA gyrase, enrofloxacin prevents the relaxation of supercoiled DNA during replication and transcription, leading to the inhibition of bacterial DNA synthesis (Bush *et al*., 2020).

Among copper-based chemicals, Copper Nitrate proved to be the best one which controls the disease 16.07% (disease incidence), followed by copper hydroxide, Amistar top, Copper oxychloride and Copper acetate. The consequences of the current study were presented

by Malik who came to the conclusion that the bordeaux mixture was best for eradicating the canker on leaves and fruit. Another therapy that successfully reduced the occurrence of disease on leaves and fruit was copper hydroxide (Malik *et al*., 2020). Canker incidence was significantly lower in citrus plants that were treated by copper oxychloride (Rehman *et al*., 2020). Mean data of combined treatments (antibiotic + copper-based chemical) field experiments exhibited minimum disease incidence (18.22%) in case of Enrofloxacin+ Cu $(NO_2)_3$, followed by Enrofloxacin (21.28%) and Cu $(NO_2)_3$ (22.28%). Earlier studies have supported our experiments regarding the efficacy of copper-based chemicals and antibiotics alone and combined but Enrofloxacin with Cu $(NO_2)_3$ combined has never been reported by any researcher. Present findings depict by Rehman who ecaluated the combination of antibiotics and chemicals. The treatments were streptomycin+ copper oxychloride followed by kasugamycin + copper oxychloride and copper oxychloride + validamycin. The minimum disease severity was found by the combination of copper nitrate + streptomycin (Sharif *et al*., 2021).

Copper ions have the ability to disrupt the integrity and function of bacterial cell membranes (Shams *et al*., 2020). They can interact with the phospholipids in the cell membrane, causing structural damage and increased permeability. This disruption compromises the bacterial cell's ability to maintain proper osmotic balance and essential cellular processes, eventually leading to cell death. Moreover, it can directly interact with bacterial DNA, leading to DNA strand breaks and damage (Li *et al*., 2019). This interference with the DNA structure and stability disrupts bacterial replication and transcription processes, preventing the bacteria from proliferating and surviving.

CONCLUSION

Enrofloxacin and copper nitrate exhibited remarkable efficacy among nine antibiotics and six copper-based compounds, respectively against *Xanthomonas citri* subsp. *citri* under *in vitro* conditions. The combination of Cu $(NO_2)_3$ and Enrofloxacin was revealed as the most effective antibacterial agent among all evaluated treatments against citrus canker under field conditions. Cu $(NO_2)_3$ + Enrofloxacin would be a successful amendment to manage citrus canker and other bacterial pathogens.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

CONTRIBUTION OF AUTHORS

Hamza Shahbaz conducted research and wrote original manuscript; Muhammad Atiq conceived the idea and edited manuscript; Nasir Ahmad Rajput member supervisory committee; Ahsan khan member supervisory committee.

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