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Eco-Friendly Cinnamaldehyde Based Emulsion for Phytopathogenic Bacterial Growth Inhibitor

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Authors' contributions

This work was carried out in collaboration between both authors. Authors designed the study and wrote the protocol, performed the study, wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The formulation plays an essential role in achieving the successful delivery and biological activity of any plant protection products. This study aimed to develop a cinnamaldehyde water-based formulation (oil-in-water emulsion) via a high-shear stirring emulsification method. Cinnamaldehyde emulsion was successfully prepared and characterized using different physicochemical parameters (emulsion stability, persistent foaming, accelerated storage at 54℃ for 2 weeks, and stability at 0℃ for one week, as well as pH, surface tension, flash point, viscosity, and particle size distribution). Also, the antibacterial activity was verified *in vitro* against some important phytopathogenic bacteria; *Erwinia amylovora*, *Pectobacterium aroidearum*, *Pseudomonas aeruginosa*, and *Ralstonia solanacearum* using well diffusion method. In addition, the minimum inhibition concentration (MIC) was determined by the twofold dilution method. The results revealed that the prepared formulation showed good storage stability, exhibited non-Newtonian shear-thinning behavior and promising antibacterial activity. The inhibition zones against the tested phytopathogenic bacteria were ranged

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from 10.3 mm to 52.0 mm. MICs of the prepared formulation were 15.63, 31.25, 62.5, and 15.63 µl/ml against *Erwinia amylovora*, *Pectobacterium aroidearum*, *Pseudomonas aeruginosa* and *Ralstonia solanacearum*, respectively. Our results provide an environmentally friendly formulation with promising activity to control the agricultural crop disease.

Keywords: Cinnamaldehyde; an oil-in-water emulsion; formulation; phytopathogenic bacteria.

1. INTRODUCTION

Nowadays, plant phytopathogenic bacteria are responsible for huge economic losses in crop yield and the quality of plant production worldwide [1-3], collectively, they cause losses of over $1 billion dollars worldwide every year to the food production sector [4,5]. Avoiding or mitigating crop losses due to plant diseases is an important consideration in plant production. The rapid and effective control of the plant diseases in crop cultivation is generally achieved using synthetic pesticides, which became essential to maximize agricultural productivity. Despite their beneficial role in agriculture, pesticides have negative environmental effects on the other living on-target organisms [3,6,7]. Therefore, there has been a growing interest in research concerning alternative natural pesticides and antimicrobial active compounds with less harmful effects to control plant pathogenic diseases, such as essential oils (EOs) [8]. EOs could be used as alternatives for chemical pesticides as they are naturally biodegradable products, with antimicrobial properties, low mammalian toxicity, and low environmental impact [2,9-11]. The individual constituents of EOs are also considered as a promising natural antimicrobial agent against plant pathogens [12].

Cinnamaldehyde, which represents ~ 65% of the composition of *Cinnamomum sp*. EO [13,14] is an example of a natural protectant against plant pathogens. This compound is generally recognized as safe (GRAS) by the United States Food and Drug Administration (FDA), and Flavour and Extract Manufacturer's Association (FEMA), and has been granted A status (may be used in foodstuffs) by the Council of Europe [15-17].Cinnamaldehyde is active against pathogenic bacteria, fungi, and viruses [18], besides other activities related to human health like anti-inflammatory, and antioxidant potentials. Therefore, it is widely used in the food, drug, and cosmetic industries [19]. However, EOs and their individual constituents are generally unstable in the presence of light, heat, oxygen, and humidity. Also, they are highly volatile and present poor water solubility which required using organic solvents for their dilution and delivery [20-22]. Hence, a suitable intervention is necessary to overcome these challenges like using the emulsion technology in which water is used as a preparation medium and a delivery system.

An emulsion is a thermodynamically unstable system consisting of at least two immiscible liquids or phases, one of which is dispersed in the form of droplets in the other [23-25]. Emulsions are categorized into two types: oil-in-water (O/W) and water-in-oil (W/O) emulsions [26]. Oil-in-water (O/W) emulsions have many important industrial applications covering different aspects in food [27], pharmaceuticals [28], petrochemicals [29], cosmetics [30] and agrochemicals [23,31]. Agrochemical oil-in-water emulsion (EW) is a formulation in which water-insoluble liquid pesticides or organic solvents containing dissolved solid pesticides are dispersed as small droplets in water to form oil-in-water emulsions by adding suitable surfactants and energy [32]. Oil-in-water emulsion greatly reduces the concentration of surfactants and/or organic solvents compared with microemulsions (ME, the concentration of surfactants in ME is usually approximately ≥ 20%) and conventional emulsifiable concentrate (EC, which uses a large amount of solvent) [33,34]; Oil-in-water emulsions also improves the delivery and penetration efficacy of bioactive ingredients as pesticide delivery systems. This formulation is generally considered as a safe and environmentally friendly water-based pesticide formulation [35].

The present study was designed to prepare cinnamaldehyde-in-water emulsion for evaluation as a potential eco-friendly bactericide. Physiochemical properties, stability, and antibacterial activity against some important plant phytopathogenic bacteria will be also studied.

2. MATERIALS AND METHODS

2.1 Chemicals

Magnesium sulfate heptahydrate and cinnamaldehyde were purchased from Sigma

Aldrich (St. Louis, MO, USA), and Polyoxy-35-castor oil (Cremophor-EL, non-ionic surfactant) was purchased from Sigma-Merck (Germany). Agar, glycerol, dipotassium phosphate, sodium phosphate dibasic dodecahydrate, sodium phosphate monobasic dehydrate, propylene glycol, and sodium hydroxide were purchased from ADWIC, El Nasr Company, Egypt. Calcium carbonate was purchased from Sigma- Aldrich, Germany. Magnesium oxide and methyl red were purchased from Qualikems Fine Chemicals. India. Ammonia solution was purchased from Prolabo. Proteose peptone was purchased from Techno pharmchem, India. Water was obtained from Milli-Q water purification system (Millipore, LABCONCO Corporation, USA). All other chemicals used were of analytical reagent grade.

2.2 Phytopathogenic Bacterial Strains

The bacterial isolates used in this study were obtained from Bacterial Disease Research Department. Plant Pathology Research Institute, Agricultural Research Center (ARC), Giza, Egypt. These phytopathogenic bacteria were previously isolated by the second author and others from different hosts. These isolates of phytopathogenic bacteria were; *Erwinia amylovora* isolated from pear trees [36], *Pectobacterium aroidearum* isolated from guava root [37], *Pseudomonas aeruginosa* isolated from onion plants (Unpublished data) and *Ralstonia solanacearum* isolated from potato tubers [38]. These isolates were previously identified using different identification methods. *Erwinia amylovora*, and *Ralstonia solanacearum* were identified using polymerase chain reaction (PCR), using the two primers AMSJ14258 and AMSK14892c [39] for *Erwinia amylovora* [36] and the two primers RS-1-F and RS-1-R [40] for *Ralstonia* [41].While *Pectobacterium aroidearum* [37] and *Pseudomonas aeruginosa* (Unpublished data) were identified using16S rRNA analysis.

2.3 Emulsion Preparation

Oil-in-water emulsions were prepared via a high-shear stirring emulsification method. Cinnamaldehyde (10wt %) and surfactant were mixed to form the oil phase. Deionized water, preservative, Arabic gum, and propylene glycol as the aqueous phase. Emulsions were prepared by progressively incorporating the oil phase into the aqueous phase using a high shear dispersion homogenizer Ultra-Turrax homogenizer (T25D IKA, Germany) at 18000 rpm for 5 min at room temperature.

2.4 Characterization of the Emulsion

2.4.1 Stability tests

The formulation stability tests included emulsion characteristics, stability at 0℃, accelerated storage procedure at 54℃, and persistent foaming. All test methods followed the official CIPAC standard method outlined in the CIPAC handbook [42]. In the emulsion characteristics experiment, 5 ml of the emulsion were separately mixed with CIPAC standard water in a 100 ml measuring cylinder to produce 100 ml of diluted emulsion. The stopper was placed on the cylinder, which was subsequently turned upside down 10 times. Subsequently, the amount of free oil or cream that separated at the top or the bottom of the emulsion was observed after the emulsion could stand undisturbed for various intervals (0, 0.5, 1, 2, 24, and 24.5 h).For the stability test at low temperature (0℃), 100 ml of the sample was transferred to a glass tube. For cooling, the tube and its contents were placed in a refrigerator and remained at 0±2℃ for 7 days. At the end of 7 days, the tube was removed from the refrigerator, and allowed to remain undisturbed at room temperature for 3 h. The volume of any separated material at the bottom of the tube was subsequently recorded. Accelerated storage procedure was executed by placing the sample (about 50 ml) in the bottle and placing the capped bottle and contents in an oven of 54 ± 2℃ for 14 days. Persistent foam is a measure of the amount of foam likely to be present in a spray tank or other application equipment following dilution of the product with water. The specified amount of the prepared formulation is added to CIPAC standard waters A and D (95 ml) in the measuring cylinder and made up to the mark. The cylinder is stoppered and inverted 30 times. Stand the cylinder on the bench and left undisturbed for the specified time. The volume of foam was noted.

2.4.2 pH measurement

The pH of a 1% solution of the prepared formulation was measured using a pH meter (Jenway® model pH 3510),it was recalibrated before testing using a standard buffer solution at pH 7.0 and pH 4.0; the measurements were carried out at 25℃ by direct immersion of pH glass electrode into the formulation samples.

2.4.3 Surface tension measurements

The surface tension was measured by the Wilhelmy plate method using a tensiometer “Sigma 700” instrument (Biolin Scientific). The platinum plate was burned under an alcohol flame after being washed by deionized water and alcohol to remove impurities before each measurement. To guarantee the cleanliness of the plate, the surface tension of water was used as a control to calibrate the tensiometer.

2.4.4 Flash point

Measurement of the flash point of the prepared formulation was carried out by the tag open cup method by Koehler instrument company, INC, USA. The flash point was recorded as the temperature at the thermometer when a flash appeared.

2.4.5 Viscosity measurements

The viscosity of the prepared formulation was measured at different shear rates, without dilution, using ″Brookfield DV II + PRO″ Digital Viscometer. (Brookfield, USA). UL rotational adaptor. The temperature was kept at 25℃ during the measurement by water bath (Model: TC-502 USA). Preliminary experiments showed that the appreciated value of torque to obtain reliable results was between 10 and 100% of the measuring range. The flow curve of the prepared formulation was obtained by directly reading the viscosity (mPas) and shear rate(s^{-1}) from the viscometer**.**

2.4.6 Size distribution of the emulsions

The oil-droplet size of oil-in-water emulsions was measured using a Mastersizer 3000 (Malvern Instruments, Malvern, UK) after preparation at ambient temperature (~25 ℃). Distilled water (25℃) was used as the dispersion medium; the refractive index values input was 1.333. The emulsion was added into the water dropwise. When the laser intensity reached 10% to 20%, the measurement was started. The average droplet size was expressed in terms of volume mean diameter D [43].

2.5 Antibacterial Activity of Cinnamaldehyde Formulation

2.5.1 Preparation of bacterial suspensions

Each isolate of the tested phytopathogenic bacteria was separately inoculated in Petri plates containing King's agar B medium (proteose peptone (20.0 g), dipotassium phosphate (1.5g), magnesium sulfate heptahydrate (1.5g), glycerol 15.0 ml, Agar (20.0 g), distilled water 1000.0 ml and final pH 7.2±0.2). Inoculated plates were incubated at 28℃ for 48 hrs. Bacterial growth was harvested using sterilized 10 mM phosphate buffer (2.7 g $Na_2HPO_4.12H_2O$;0.4 g $NaH_2PO_4.2H_2O$; distilled water to 1.0 liter and final pH 7.2) and the cell suspension was adjusted to 10^8CFU/ ml using UV/Visible spectrophotometer (MODEL:2000 UV-UNICO INSTRUMENTS CO., LTD,USA). The optical density (at λ=600 nm) is between 0.07 and 0.1 and this is roughly equivalent to 1 × 10^8 CFU/ml [44].

2.5.2 Antibacterial activity

Antibacterial activity of the prepared cinnamaldehyde-water emulsion (without dilution) was tested against phytopathogenic bacteria using a well diffusion method [45]. YPG agar medium (5 g yeast extract; 5 g peptone; 10 g glucose; 15g agar; distilled water to 1000.0 ml) was prepared. The medium was sterilized in an autoclave for 15 minutes at 121℃. The sterile medium temperature was lowered to 50℃ in a water bath. Then, the medium flask containing 250 ml was inoculated with 1.00 ml suspension (10^8CFU/ml) of a tested isolate of phytopathogenic bacteria to obtain a final concentration of about 4 x10^5 CFU/ml of bacteria in the medium. Then, 25 ml of inoculated medium were poured into each sterilized Petri plate (9 cm).Once the agar was solidified, a hole with a diameter of 5 mm has punched aseptically with a sterile cork borer, and then wells were filled with 50 µl of the prepared formulation. Wells filled with 50 µl of sterilized water used as a control. The experiment was performed in three replicates. The Plates were incubated at 28°C for 48 h. Clear zones that formed around the wells were measured to the nearest millimeter.

2.5.3 Minimum Inhibitory Concentration (MIC)

The minimum inhibitory concentration (MIC) of cinnamaldehyde-in-water emulsion formulation against *Erwinia amylovora*, *Pectobacterium aroidearum*, *Pseudomonas aeruginosa*, and *Ralstonia solanacearum* was determined using well diffusion method [46]. Seven serial twofold dilutions ranged from 500.0 to 7.81 µl/ml of the formulation in sterile distilled water were prepared. Eight agar wells (5 mm diameter) were prepared around the center of Petri plates (15 cm) containing the YPG agar medium (60

ml/plate) previously inoculated with the tested isolate of phytopathogenic bacteria, as previously explained. Each well was filled with 50 µl of one of the tested concentrations. Wells filled with 50 µl of sterilized water was used as a control. The experiment was performed in three replicates. The plates were incubated at 28°C for 48 h. Clear zones that formed around the wells were measured to the nearest millimeter. The lowest concentration formed clear zones around the wells are considered the minimum inhibitory concentration to the tested isolate of phytopathogenic bacteria.

3. RESULTS AND DISCUSSION

3.1 Emulsion Stability

Creaming is best defined as the tendency of droplets for moving upward, which occurs if the droplets have a lower density than the liquid phase [47]. The oil-in-water emulsions stability can be described and monitored during a lifetime. An emulsion system is stable if no phase separation occurs and a single phase is formed over the whole period. The emulsion stability with different levels of water hardness is an important factor for a product of agricultural use due to the type of water hardness present in different agricultural regions. Some agricultural regions use groundwater that often has significant levels of hardness, but some surface water supplies also have the same issue. Calcium concentrations up to and exceeding 100 mg. L^{-1} is common with groundwater. In contrast, magnesium usually occurs at lower concentrations compared to calcium in groundwater (about 50 mg. L^{-1} and rarely about 100 mg. L^{-1}), and calcium-based hardness usually predominates [48]. Considering its importance, the water of different hardness was used, called waters CIPAC A and D [49]. The data presented in Table 1 showed the emulsion stability and re-emulsification of the prepared cinnamaldehyde-in-water emulsion after storage at 54℃ for 14 days when diluted with the different hardness of water (CIPAC A and D) presented appropriated bloom, formed milky bluish emulsion, and did not show creamy layer until 24 h. Such signs are a preliminary indication of physical stability. In the experiment of low temperature stability, the formulation was stored in a freezer (0±2℃) for 7 days, no precipitation or a separate material was observed in the formulation which indicated that the prepared formulation was stable without phase separation; these results demonstrated that the emulsion would have a satisfactory shelf life either in the tropics or in temperature zones and maintain the quality of the emulsion. Finally, the volume of foam from the samples in CIPAC standard waters A and D is low and passed through the recommended rat of foam.

3.2 Physical Properties

The physical properties of the prepared formulation are important to expand the applicability. pH plays an important role in the stability of the emulsion system [50]; pH results revealed that no obvious chemical degradation occurred in the prepared formulation. The pH values of the prepared formulation were in the range of (4.12-4.16), indicating that the prepared formulation in the different storage conditions having acidic character implying that it will have good biological activity.

The prepared formulation had a value of surface tension range (33.65-34.87 mN/m); the lower surface tension of a solution indicates a higher wetting property and further promotes the deposition and retention of pesticide droplets on target surfaces [51,52]. Flash point is an important parameter to measure product safety for the storage and transportation, the prepared formulation in the storage conditions having the high value of flash point more than 65℃, which showed good security for storage and transportation, thus demonstrating that the cinnamaldehyde-in-water emulsion formulation had higher flash points suitable for spraying.

Rheological properties are some of the most vital factors for characterizing emulsions, because they are directly related to formation, creaming stability, the sensory attributes, and the shelf life [53]. For instance, the creaming of oil particles in O/W emulsions was effectively dependent on the viscosity of the water phase. Fig. 1 displays the emulsion exhibited the typical non-Newtonian behavior of shear-thinning in the shear rate (reduced in apparent viscosity with enhancing shear rate).The rheological behavior of the emulsions was attributed mainly due to the presence of polysaccharides in aqueous solutions. When the shear rate was increased, the entanglements between the polysaccharide-chains were disrupted, and the molecular chains were more randomly orientated. Finally, the interactions between the adjacent chains were reduced, and the viscosity was decreased [54-56]. Based on the obtained results the particle size distribution varied from 1.19 µm to 1.24 µm.

Table 1. Emulsion characteristics of the cinnamaldehyde-in-water emulsion formulation

Test code	Temperature(25±1℃)		Accelerated storage (54±2℃, 14 days)	
Water hardness	A	D	A	D
Emulsion stability				
0h	0/0	0/0	0/0	0/0
0.5h	0/0	0/0	0/0	0/0
1h	0/0	0/0	0/0	0/0
2h	0/0	0/0	0/0	0/0
24h	0/0	0/0	0/0	0/0
24.5 h (REE)	0/0	0/0	0/0	0/0

0/0: no creamy or oily layer was observed
REE: 24.5 h : 30 minutes after reemulsification

Table 2. Physicochemical properties of the cinnamaldehyde-in-water emulsion formulation

Test Code	Temperature (25±1℃)	Accelerated storage (54±2℃, 14 days)
pH value (1%)	4.12	4.16
Surface tension (mN/m)	33.65	34.87
Flash point (℃)	Over 60	Over 60
particle size distribution (µm)	1.24	1.19

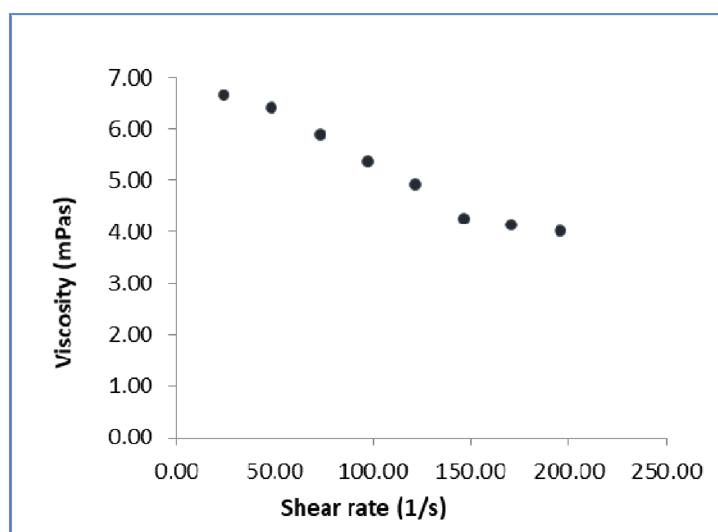

Fig. 1. Rheological properties of the cinnamaldehyde -in-water emulsion formulation

3.3 Antibacterial Activity of Cinnamaldehyde Formulation

In the present study, *in vitro* antibacterial activity of cinnamaldehyde formulation (without dilution) was determined as the diameter of the inhibition zone against phytopathogenic bacteria using well diffusion method Table 3 and Fig.2. The diameter of the inhibition zone mostly indicates the degree of antibacterial effect that exists in the product or sample; in this case, a higher zone means that the antimicrobial is more effective. The antimicrobial activity based on these inhibition diameters was determined in the tested treatment. Among the plant pathogenic bacteria, *Ralstonia solanacearum* was the most sensitive to the prepared formulation with 52.0 mm inhibition zone diameter. The prepared formulation showed inhibition zones of 29.7and 25.7mm against *Erwinia amylovora* and *Pectobacterium aroidearum*, respectively. However, *Pseudomonas aeruginosa* displayed less sensitivity to the oil-in-water emulsion formulation with a 10.3 mm zone diameter. The antibacterial mechanism of cinnamaldehyde was studied by [57-59]. The target of cinnamaldehyde is mainly the bacterial membrane; the contact of cinnamaldehyde with the bacterial membrane might cause the loss of membrane functionality or cause the loss of channel proteins in the

membrane, resulting in the death of bacterial cells. Also [60,61] suggested an interaction with the cell membrane induces rapid inhibition of energy metabolism. The disruption of the proton motive forces results in leakage of small ions without the leakage of large components such as ATP accompanied by the inhibition of ATP generation and inhibition of membrane-bound adenosine triphosphatase (ATPase) activity. Other mechanisms of cinnamaldehyde action include perturbing the cell membrane and altering the lipid profile of the membrane [62,63].

3.4 Minimum Inhibitory Concentration (MIC)

Results of the inhibitory effect of the prepared formulation against *Erwinia amylovora*, *Pectobacterium aroidearum*, *Pseudomonas aeruginosa*, and *Ralstonia solanacearum*are given in Table 4 and Fig. 3. The MIC value is the lowest concentration of the prepared formulation when a clear zone is formed around the well. MICs of the prepared formulation were15.63, 31.25, 62.5, and 15.63 µl/ml against *Erwinia amylovora*, *Pectobacterium aroidearum*, *Pseudomonas aeruginosa*, and *Ralstonia solanacearum*, respectively. *Ralstonia solanacearum* was the most affected bacterium by the prepared formulation followed by *Erwinia amylovora*, *Pectobacterium aroidearum* and, *Pseudomonas aeruginosa*, respectively. None of the four tested species was affected by the concentration of 7.81 µl/ml of cinnamaldehyde cinnamaldehyde formulation inhibits the growth of *Erwinia amylovora* and *Ralstonia*

Table 3. Inhibition zone (mm) of cinnamaldehyde-in-water emulsion against some phytopathogenic bacteria

Phytopathogenic bacteria	Inhibition zone diameter (mm)
Erwinia amylovora	29.7±2.08
Pectobacterium aroidearum	25.7±1.52
Pseudomonas aeruginosa	10.3±0.58
Ralstonia solanacearum	52.0±2.00
Control (sterile distilled water)	0.0±0.00

Means of three replicates; values expressed as mean ± Standard Deviation (SD)

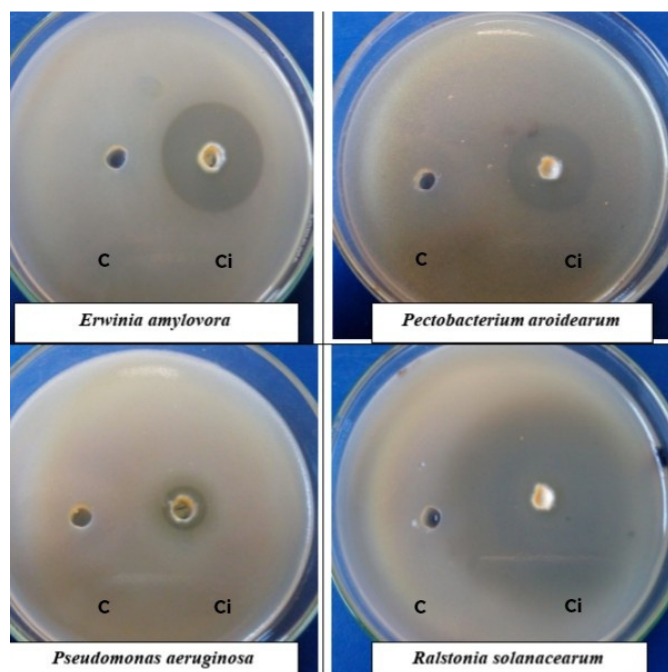

Fig. 2. Antibacterial activity of cinnamaldehyde -in water emulsion against some phytopathogenic bacteria

C = Control i.e. Sterile distilled water,Ci = cinnamaldehyde formulation

Table 4. Minimum Inhibitory Concentration (MIC) of cinnamaldehyde-in-water emulsion against some phytopathogenic bacteria using well diffusion method

Phytopathogenic bacteria	Diameter of inhibition zone (mm) at different concentrations (µl/ml)								MIC (µl/m)
	500	250	125	62.5	31.25	15.63	7.81	0.0**	
Erwinia amylovora	26.3±1.53	22.3±0.58	20.0±0.00	16.3±1.15	14.0±1.00	9.3±0.58	0.0±0.00	0.0±0.00	1.563
Pectobacterium aroidearum	22.7±1.15	19.3±0.58	16.7±1.53	11.7±1.53	9.7±0.58	0.0±0.00	0.0±0.00	0.0±0.00	3.125
Pseudomonas aeruginosa	14.3±0.58	11.7±1.53	10.0±0.00	8.0±1.00	0.0±0.00	0.0±0.00	0.0±0.00	0.0±0.00	6.25
Ralstonia solanacearum	51.3±3.21	46.3±2.30	35.0±0.00	22.7±2.08	14.7±1.15	11.3±1.53	0.0±0.00	0.0±0.00	1.563

*Means of three replicates; values expressed as mean ± Standard Deviation (SD); *Control (sterile distilled water)*

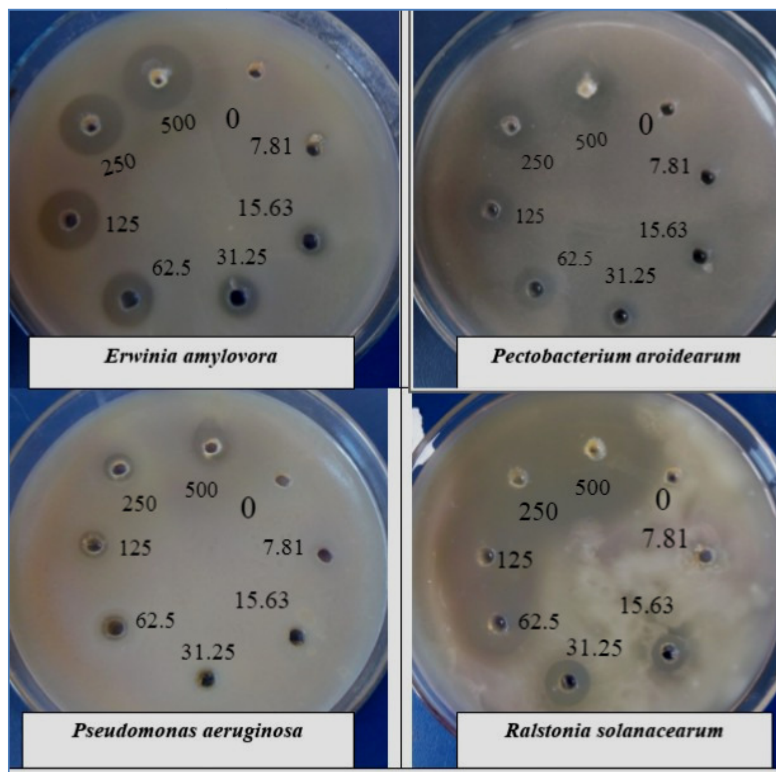

Fig. 3. Minimum Inhibitory Concentration (MIC) of cinnamaldehyde – in -water emulsion formulation against some phytopathogenic bacteria. Different concentrations of formulation (500, 250, 125, 62.5, 31.25, 15.63 and 7.81µl/ml) and Control (0 µl/ml. i.e. Sterile distilled water)

solanacearum, and inhibition zones were 9.3 and 11.3 mm, respectively. While this concentration of 15.63 µl/ml was not inhibited the growth of *Pectobacterium aroidearum* and *Pseudomonas aeruginosa*.

4. CONCLUSION

It can be concluded that cinnamaldehyde could be successfully formulated in the form of an oil-in-water emulsion. The formulation can be used as a natural antimicrobial agent; it had potent antibacterial activity against various species of bacterial pathogens, could be a new alternative to chemical bactericide. Also, it is safe to be used. However, more studies should be done to check the storage stability of the emulsion and further work will focus on the evaluation of the toxicity of the prepared formulation and comparing the activity with the traditional and commercial formulation currently used.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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