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STRESS-MODULATED ANTIBACTERIAL INTERACTION OF CINNAMALDEHYDE AND CIPROFLOXACIN AGAINST GRAM-NEGATIVE BACTERIA IN VITRO

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ABSTRACT - *Antibiotic resistance in Gram-negative bacteria necessitates alternative strategies to improve antimicrobial efficacy. This study investigated the antibacterial activity and interaction profiles of cinnamon oil, ciprofloxacin, and proline against* Escherichia coli*. Antimicrobial effects were evaluated using minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and checkerboard assays. Cinnamon oil and ciprofloxacin exhibited strong, dose-dependent antibacterial activity individually. However, combination treatments demonstrated indifferent interactions based on fractional inhibitory concentration index (FICI) analysis. The inclusion of proline reduced bactericidal efficacy and increased tolerance, highlighting stress-mediated modulation of bacterial susceptibility. These findings emphasize the influence of bacterial stress responses on antimicrobial performance and combination therapy outcomes*.

KEY WORDS: **Antibacterial interaction, cinnamaldehyde, ciprofloxacin, Gram-negative bacteria, proline-induced stress, MIC, and checkerboard assay**.

1. INTRODUCTION:

Escherichia coli (E. coli) is a Gram-negative, rod-shaped bacterium belonging to the family Enterobacteriaceae. While most strains are harmless commensals of the human intestinal tract, pathogenic variants are responsible for a wide range of intestinal and extraintestinal infections, including urinary tract infections, pneumonia, diarrhea, bacteremia, and neonatal meningitis. The pathogenicity of E. coli is attributed to multiple virulence mechanisms such as adhesion to host tissues, evasion of immune defenses, toxin production, and the ability to form biofilms. Biofilm formation significantly enhances bacterial survival, persistence, and resistance to antimicrobial agents, making infections difficult to eradicate. Consequently, biofilm-producing E. coli is a frequent cause of hospital-acquired infections. The prolonged and indiscriminate use of antibiotics has further contributed to the emergence of multidrug-resistant E. coli strains, posing a major global public health challenge.

Cinnamomum verum (cinnamon), a member of the Lauraceae family, has been used for centuries in traditional medicine and is well known for its natural antimicrobial properties. Cinnamon and its bioactive compounds exhibit inhibitory effects against both Gram-positive and Gram-negative bacteria, primarily by disrupting bacterial cell structures and interfering with essential cellular processes. In addition to antimicrobial activity, cinnamon demonstrates antioxidant, antifungal, antidiabetic, anti-inflammatory, and cholesterol-lowering properties. Cinnamon oil is widely used as a natural flavoring and food preservative due to its broad-spectrum activity against food-borne pathogens.

Proline is a multifunctional proteinogenic amino acid that plays a critical role in cellular stress adaptation across diverse biological systems. Beyond protein synthesis, proline contributes to stress signaling, redox balance, osmoprotection, and metabolic regulation. Under conditions such as osmotic stress, oxidative stress, heat shock, nutrient limitation, and antibiotic exposure, intracellular proline levels increase significantly.

Quantitative evaluation of antimicrobial activity is essential for assessing antibacterial efficacy. Methods such as minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and checkerboard assays provide comprehensive insights into inhibitory, bactericidal, and synergistic effects, which are particularly important in the context of rising antimicrobial resistance and the exploration of combination therapies.

2 . MATERIALS & METHODS

2.1 Materials:

a) Minimum inhibitory concentration (MIC)

E. coli - 443 was purchased from MTCC, Chandigarh, India. Nutrient broth was purchased from Himedia, India. Sterile 96-well plates were purchased from Tarsan, India. The conical flasks were from Borosil, India. Spirit lamp, double-distilled water, and test samples.

b) Minimum bactericidal concentration (MBC)

E. coli - 443 was purchased from MTCC, Chandigarh, India. Nutrient agar medium, nutrient broth, was purchased from Himedia, India. Test samples, petri plates, test tubes, beakers, and conical flasks were purchased from Borosil, India. A spirit lamp and double-distilled water were used.

C) Checkerboard assay

E. coli – 443 was purchased from MTCC, Chandigarh, India. Nutrient broth and gentamycin solution were purchased from Himedia, India. Test samples, 96-well plates, test tubes, beakers, and conical flasks were from Borosil, India. Spirit lamp, double distilled water.

Luria Bertani Broth (LB Broth)

LB broth was prepared by dissolving tryptone (1 gm), NaCl (1 gm), and yeast extract (0.5 gm) in 100 ml distilled water and boiling to dissolve the medium completely. The medium was dispensed as desired and sterilized by autoclaving at 15 lbs pressure (121ºC) for 15 minutes.

2.2 Test Samples for MIC, MBC & Checkerboard

Sample A – Cinnamon Oil

Sample B – Ciprofloxacin

Sample C – Proline

2.3 Methods/Methodology

a) Minimum inhibitory concentration (MIC)

The broth micro dilution assay method was used to determine the MICs for the susceptibility of E. coli-443 in sterile disposable flat-bottomed 96-well microtiter plates. Briefly, the 0.5 McFarland inoculum suspensions were further diluted 1:100 in nutrient broth before inoculation; 50 µL of the bacterial suspension was seeded into a 96-well plate containing 50 µL of test sample with serial concentrations. The final inoculum of the bacteria was approximately 5×10^5 CFU/mL. The final concentrations of Sample A, Sample B, Sample A+B, and Sample A+B+C were 500, 250, 125, 62.5, 31.25, 15.6, 7.8, 3.9, 1.9, and 0.9 µg/mL. Gentamicin was used as a positive control. The plates were then incubated at 37 °C for 18–24 h. The results were read at 600 nm using a microplate reader. Changes of color were observed and recorded. All the tests were done in triplicate.

b) Minimum bactericidal concentration (MBC)

The medium was prepared by dissolving peptone (0.5 gm), NaCl (0.5 gm), yeast extract (0.2 gm), beef extract (0.1 gm), and agar powder (1.75 gm of the commercially available Nutrient Agar Medium) in 100 ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 15 minutes. The autoclaved medium was mixed well and poured onto 100 mm petri plates (25-30 ml/plate) while still molten. 1 ml cultures were treated with IC50 concentrations of the test sample A–E. *coli* (20.14 µg/ml), sample B– *E. coli* (15.93 µg/ml), sample A+B– *E. coli* (23.03 µg/ml), and sample A+B+C– *E. coli* (25.37 µg/ml), obtained from MIC. The cultures were serially diluted, and the 10^6 dilution was plated on the nutrient agar medium by the spread plate method, and the plate was incubated at 37°C for 24 hrs. After incubation, colonies were counted, and MBC was calculated using the formula.

$$\text{CFU/ml} = \frac{(\text{No. of colonies} \times \text{dilution factor})}{\text{Volume of culture plated in ml}}$$

C) Checkerboard assay

Sample A & B

The stock solution of each drug was prepared so that the concentration is at least double the MIC of sample A (20.14 µg/ml) and sample B (15.93 µg/ml). 100 µl of the LB broth was added to each well of the flat-bottomed 96-well microdilution plate. Row 1 contains the serially diluted concentrations (40, 20, 10, 5, 2.5, 1.25, 0.75 µg/ml) of Sample A, and Column A contains the serially diluted concentrations (32, 16, 8, 4, 2, 1, 0.5 µg/ml) of Sample B. From Column 2 to 8 and Row B to H, the samples were added in combinations. Column 12 was taken as a negative control. 50 µL of 0.5 McFarland inoculum suspensions was inoculated into a 96-well plate. The plates were then incubated at 37 °C for 48 h. The results were read at 600 nm using a microplate reader. Changes of color were observed and recorded.

FORMULA:

The Fractional Inhibitory Concentration Index (FICI) was calculated using the formula:

$$\text{FICI} = \frac{\text{MIC A in combination}}{\text{MIC A}} + \frac{\text{MIC B in combination}}{\text{MIC B}}$$

Sample A+B & C

The stock solution of each drug was prepared so that the concentration is at least double the MIC of sample A+B (23.03 µg/ml) and sample C (10 mM). 100 µl of the LB broth was added to each well of the flat-bottomed 96-well microdilution plate. Row 1 contains the serially diluted concentrations (46, 23, 11.5, 5.75, 2.875, 1.437, 0.718 µg/ml) of Sample A+B, and Column A contains the serially diluted concentrations (1000, 500, 250, 125, 62.5, 31.25, 15.6 µg/ml) of Sample C. From Column 2 to 8 and Row B to H, the samples were added in combinations. Column 12 was taken as a negative control. 50 µL of 0.5 McFarland inoculum suspensions was inoculated into a 96-well plate. The plates were then incubated at 37 °C for 48 h. The results were read at 600 nm using a microplate reader. Changes of color were observed and recorded.

FORMULA:

The Fractional Inhibitory Concentration Index (FICI) was calculated using the formula:

$$\text{FICI} = \frac{\text{MIC A+B in combination}}{\text{MIC A+B}} + \frac{\text{MIC C in combination}}{\text{MIC C}}$$

3) RESULTS

3.1 MIC for Cinnamon Oil (Sample A)

Figure 1: Well broth microdilution MIC plate for cinnamon oil

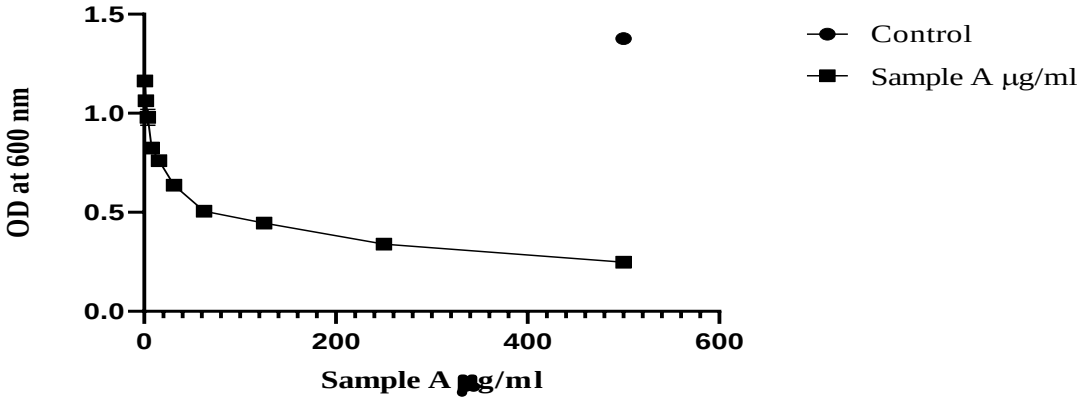

Figure 2: MIC OD_{600} analysis of Sample B

The antimicrobial evaluation demonstrated a potent dose-dependent inhibition of bacterial growth, with the highest concentration (500 µg/ml) reaching an OD of 0.248, closely mirroring the positive control (0.215). The Minimum Inhibitory Concentration (MIC) was identified at 500 µg/ml, representing the threshold for near-complete suppression of microbial proliferation. Significant potency was further evidenced by the IC50 value, which was approximately 31.25 µg/ml, where growth was reduced by roughly 54% compared to the negative control. In contrast, concentrations below 15.6 µg/ml failed to provide substantial inhibition, with optical density values trending toward the untreated growth baseline. Triplicate analysis across all concentrations confirmed high experimental precision and reproducibility of the sample's inhibitory effects. These findings characterize the tested sample as a functional antimicrobial agent with a clear therapeutic window for growth suppression.

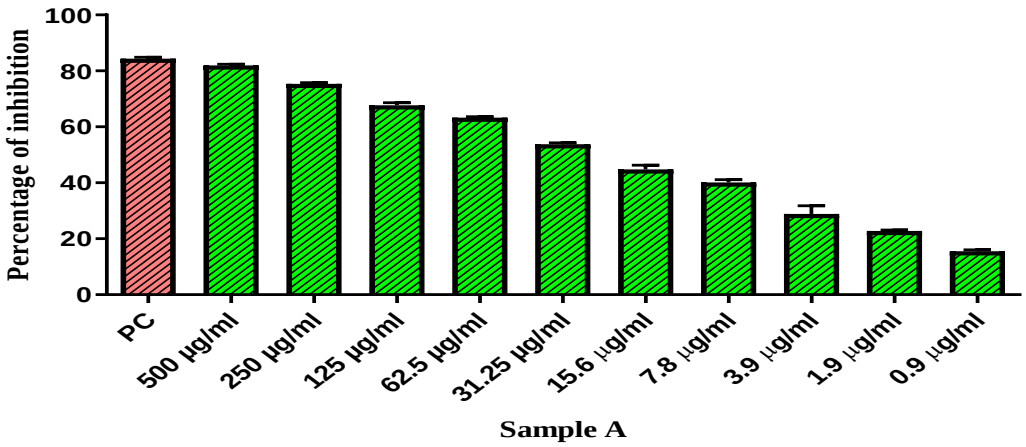

Figure 3: Percentage Inhibition of E. coli by Cinnamon Oil at Different Concentrations

The antibacterial activity of cinnamon oil (Sample A) against Escherichia coli demonstrates a clear dose-dependent relationship between oil concentration and bacterial inhibition. The highest inhibitory effect was observed at a concentration of 500 µg/ml, yielding approximately 80% inhibition, which is statistically comparable to the efficacy of the positive control (PC). As the concentration of Sample A decreased, a gradual reduction in antimicrobial activity was noted, though inhibition levels remained substantial (>60%) down to 62.5 µg/ml. A marked decline in efficacy was recorded at concentrations below 31.25 µg/ml, with the lowest tested concentration of 0.9 µg/ml resulting in less than 20% bacterial inhibition. The narrow error bars observed across all data points indicate high experimental reproducibility and consistent antimicrobial action. Collectively, these results suggest that cinnamon oil possesses significant potential as an antibacterial agent against E. coli, particularly at concentrations exceeding 60 µg/ml.

Table 1:

S.NO	Name of the test sample	Name of the Microorganisms	MIC	MIC- 50
1.	Sample A	*E. coli – 443*	0.9 µg/ml	20.14 µg/ml

Result: Sample A showed an MIC of **0.9 µg/mL** (no visible growth) and an IC_{50} of **20.14 µg/mL** (95% CI 18.04–22.48, R^2 = 0.9848) from the dose–response curve.

Interpretation: Sample A strongly inhibits *E. coli* with complete growth suppression at 0.9 µg/mL, while the dose–response (IC_{50} ≈ 20 µg/mL) indicates a clear, well-fit, concentration-dependent inhibitory effect.

3.2 MIC for Ciprofloxacin (Sample B)

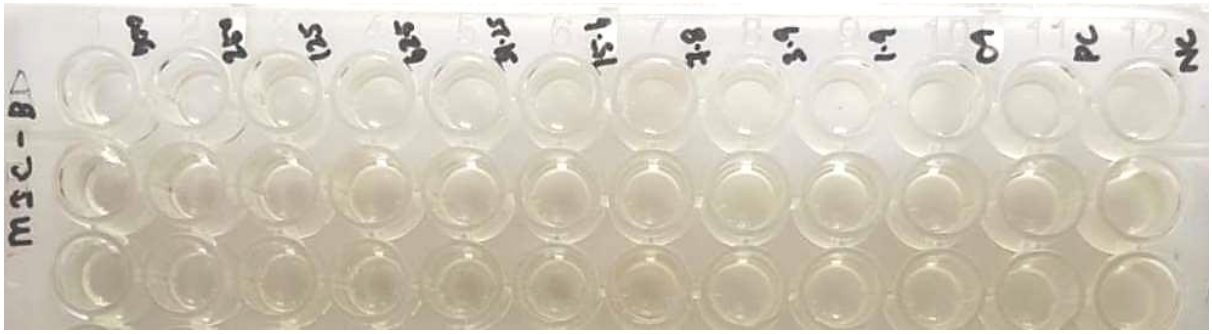

Figure 4: Well broth microdilution MIC plate for Ciprofloxacin

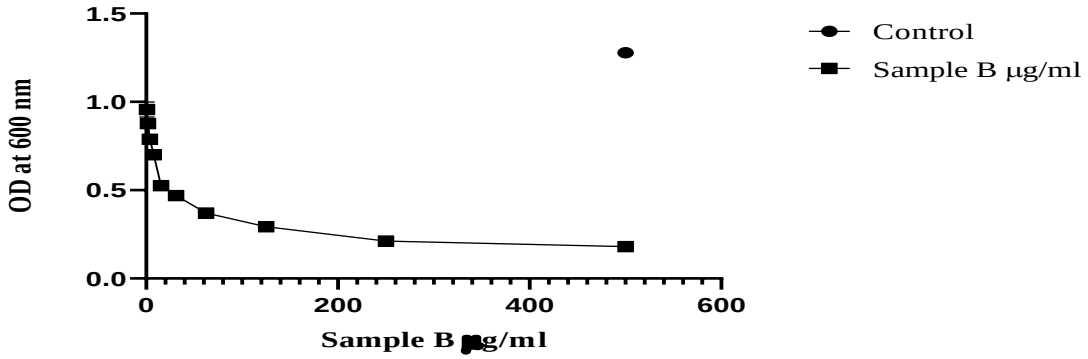

Figure 5: MIC OD_{600} analysis of Sample B

The antimicrobial evaluation revealed a significant, dose-dependent inhibition of bacterial growth, with the highest concentration (500 µg/ml) yielding a mean absorbance of 0.180, closely aligning with the positive control baseline of 0.138. The Minimum Inhibitory Concentration (MIC) was identified at 500 µg/ml, marking the threshold for near-total suppression of microbial proliferation. Significant potency was further evidenced by the IC50 value, located at approximately 15.6 µg/ml, where growth was reduced by roughly 59% compared to the negative control.

A transition from high to moderate inhibition was observed between 125 µg/ml and 31.25 µg/ml, indicating a well-defined bacteriostatic gradient. In contrast, concentrations below 1.9 µg/ml failed to provide substantial suppression, with optical density values trending toward the untreated growth saturation point (OD approx 1.277). The high degree of consistency across triplicates confirms the reproducibility of the sample's inhibitory effects, making it a viable candidate for further antimicrobial characterization.

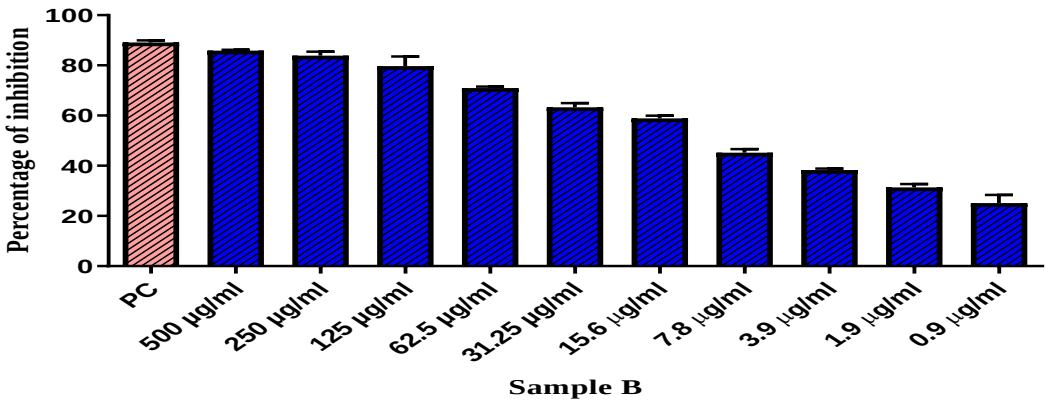

Figure 6: Percentage Inhibition of E. coli by Ciprofloxacin at Different Concentrations

The antimicrobial evaluation revealed a significant, dose-dependent inhibitory profile, with efficacy increasing from 25.10% at the lowest dose to 85.86% at 500 µg/ml. The maximum concentration of 500 µg/ml exhibited near-total growth suppression (85.86 ± 0.37%), closely approximating the performance of the positive control (89.15 ± 0.79%). Based on the inhibition gradient, the half-maximal inhibitory concentration (IC_{50}) was determined to be approximately 10.0 µg/ml, highlighting the high intrinsic potency of the tested sample. Potent bioactivity was sustained across the mid-range concentrations, with 31.25 µg/ml maintaining a substantial 63.27 ± 1.66% inhibition. Even at the extreme dilution of 0.9 µg/ml, the sample retained a notable 25.10% inhibitory capacity, suggesting a persistent antimicrobial effect. The minimal variance observed across triplicate measurements indicates high experimental precision and confirms the reliability of the dose-response relationship.

Table 2:

S.NO	Name of the test sample	Name of the Microorganisms	MIC	MIC- 50
1.	Sample B	*E. coli – 443*	0.9 µg/ml	15.93 µg/ml

Result: MIC = **0.9 µg/mL** (no visible growth); **IC_{50} = 15.93 µg/mL** (95% CI 14.39–17.65), dose–response fit **R^2 = 0.9866**.

Interpretation: Sample B strongly inhibits *E. coli* (complete growth suppression at 0.9 µg/mL); the IC_{50} ≈16 µg/mL indicates the concentration for half-maximal inhibition, and the high R^2 shows the dose–response data are reliable.

3.3 MIC for Cinnamon Oil & Ciprofloxacin (Sample A+B)

Figure 7: Well broth microdilution MIC plate for Cinnamon Oil & Ciprofloxacin

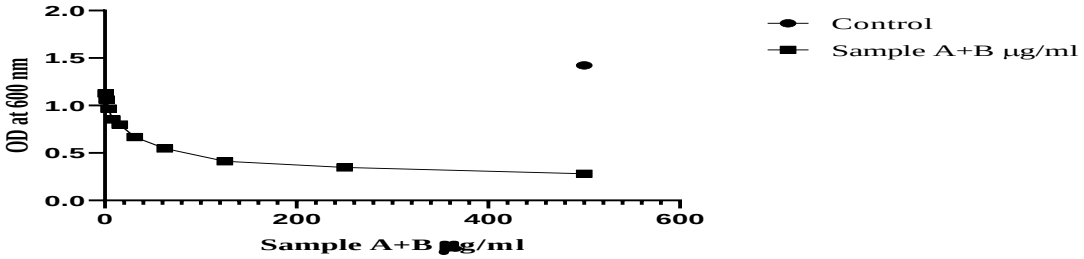

Figure 8: MIC OD_{600} analysis of Sample A+B

The antimicrobial evaluation revealed a significant, dose-dependent reduction in bacterial turbidity across the tested range (0.9–500 µg/ml), with the negative control (NC) maintaining a high mean absorbance of 1.423 ± 0.010. The tested sample demonstrated potent inhibitory effects at higher concentrations, with 500 µg/ml achieving an 80.30% reduction in growth (OD = 0.280 ± 0.006) relative to the NC. The Minimum Inhibitory Concentration (MIC) is identified at 500 µg/ml, as it represents the lowest concentration where the absorbance most closely approached the baseline established by the positive control (0.187 ± 0.011). The half-maximal inhibitory concentration (IC_{50}) was determined to be approximately 24.78 µg/ml, marking the point of significant metabolic suppression. Moderate inhibitory activity was observed between 31.25 and 125 µg/ml, whereas concentrations below 7.8 µg/ml showed diminished efficacy with OD values exceeding 0.85. The high degree of consistency across triplicates, reflected in low standard deviation values, confirms the precision and reproducibility of the sample's antimicrobial properties.

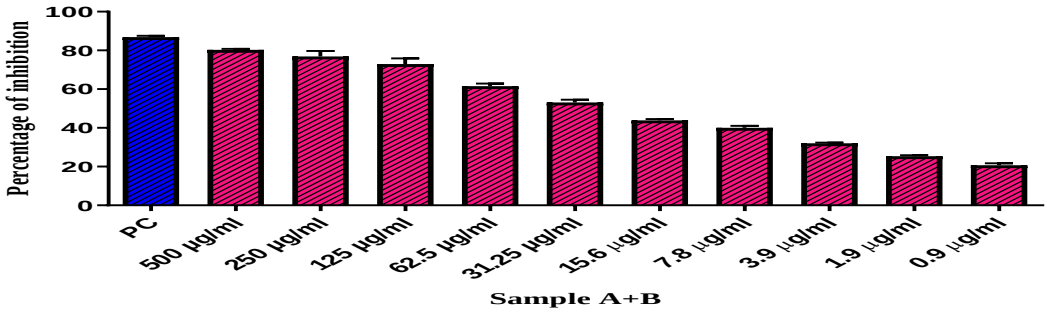

Figure 9: Percentage Inhibition of E. coli by Cinnamon Oil and Ciprofloxacin at Different Concentrations

The tested compound demonstrated a clear dose-dependent inhibitory effect across a concentration range of 0.9 µg/mL to 500 µg/mL. At the highest concentration of 500 µg/mL, the sample achieved a mean percentage of inhibition of 80.30%, closely approaching the positive control (PC) efficacy of 86.84%. Notably, the IC_{50} value—the concentration required to inhibit 50% of growth—falls between 15.6 µg/mL and 31.25 µg/mL, indicating potent biological activity. Even at a minimal concentration of 0.9 µg/mL, the sample maintained a basal inhibitory effect of 20.75%. The narrow variance observed across the triplicates confirms the high reproducibility and precision of the experimental assay. These findings suggest that the sample possesses significant antimicrobial or enzymatic inhibitory properties comparable to standard benchmarks.

Table 3:

S.NO	Name of the test sample	Name of the Microorganisms	MIC	MIC- 50
1.	Sample A+B	*E. coli – 443*	0.9 µg/ml	23.03 µg/ml

Result: MIC = **0.9 µg/mL** (complete growth suppression); **IC_{50} = 23.03 µg/mL** (95% CI 20.90–25.37), dose–response fit **R^2 = 0.9881**.

Interpretation: Sample A+B strongly inhibits *E. coli* (complete inhibition at 0.9 µg/mL); the IC_{50} ≈ 23 µg/mL indicates the concentration for half-maximal inhibition, and the high R^2 shows the dose–response data are highly reliable.

3.4 MIC for Cinnamon Oil, Ciprofloxacin & Proline (Sample A+B+C)

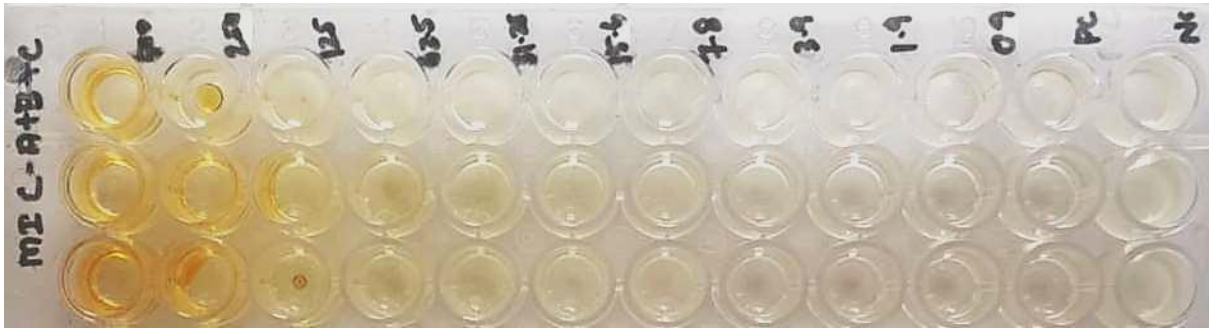

Figure 10: Well broth microdilution MIC plate for Cinnamon Oil & Ciprofloxacin & Proline

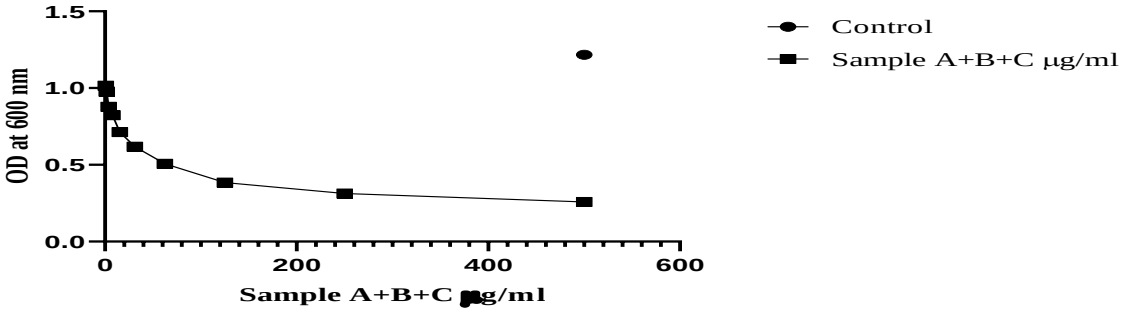

Figure 10: MIC OD_{600} analysis of Sample A+B+C

The tested sample exhibited a significant, dose-dependent inhibition of microbial growth, with OD values decreasing from 1.016 at 0.9 µg/mL to 0.258 at 500 µg/mL. . At the highest concentration (500 µg/mL), the sample achieved 78.8% growth suppression compared to the negative control, approaching the efficacy of the positive control (84.5%). The IC_{50} (half-maximal inhibitory concentration) was identified near the 31.25 µg/mL threshold, where growth was reduced by approximately 49.1%. The low standard deviation across triplicates (SD < 0.023) confirms high experimental precision and the reproducibility of the antimicrobial effect. These results validate the potent bacteriostatic activity of the sample, providing a quantitative basis for its potential application as a therapeutic agent.

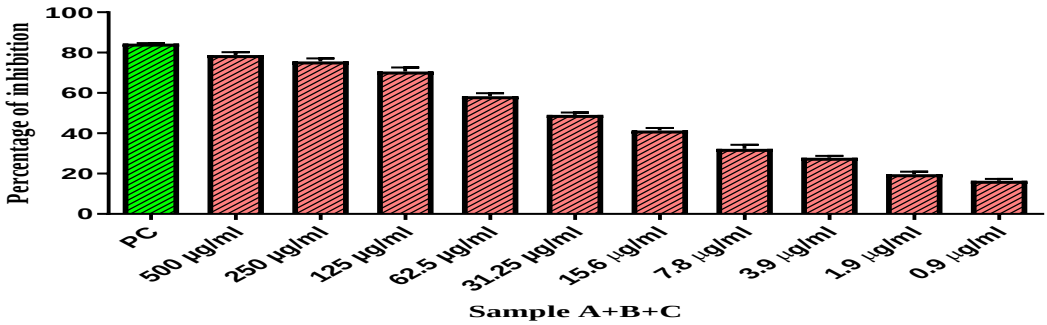

Figure 11: Percentage Inhibition of E. coli by Cinnamon Oil, Ciprofloxacin and Proline at Different Concentrations

The analysis of the percentage inhibition data reveals a strong, dose-dependent antimicrobial profile, with the tested sample demonstrating maximum inhibitory activity at 500 µg/mL (78.82% ± 1.37%). This peak efficacy closely parallels the performance of the positive control (PC), which yielded a mean inhibition of 84.49% ± 0.21%. A clear linear-log relationship was observed between concentration and microbial suppression, with the IC_{50} value identified at

approximately 31.25 µg/mL (mean: 49.14%). Significant bacteriostatic effects (>50%) were maintained across the higher concentration gradient from 62.5 µg/mL to 500 µg/mL, whereas activity diminished to 16.50% at the lowest concentration of 0.9 µg/mL. The tight standard deviations across triplicates underscore the robustness of the assay and the consistency of the sample's biological activity. Overall, these results categorize the sample as a potent antimicrobial agent with high therapeutic potential, warranting further investigation into its mechanism of action.

Table 4:

S.NO	Name of the test sample	Name of the Microorganisms	MIC	MIC- 50
1.	Sample A+B+C	*E. coli – 443*	0.9 µg/ml	25.37 µg/ml

Result: The sample showed dose-dependent antibacterial activity against *E. coli* with an IC_{50} of **25.37 µg/mL** and a strong curve fit (R^2 = **0.989**). The MIC was reported as **0.9 µg/mL**.

Interpretation: The data indicate moderate antibacterial potency; however, the low % inhibition at 0.9 µg/mL suggests the reported MIC may need re-verification based on standard MIC criteria.

3.4 MBC Assay: Cinnamon Oil, Ciprofloxacin & Proline

Images for Minimum bactericidal concentration of microorganism from the test sample

Figure 12: Bactericidal of E. coli by by Cinnamon Oil (Sample A)

Figure 13: Bactericidal of E. coli Ciprofloxacin (Sample B)

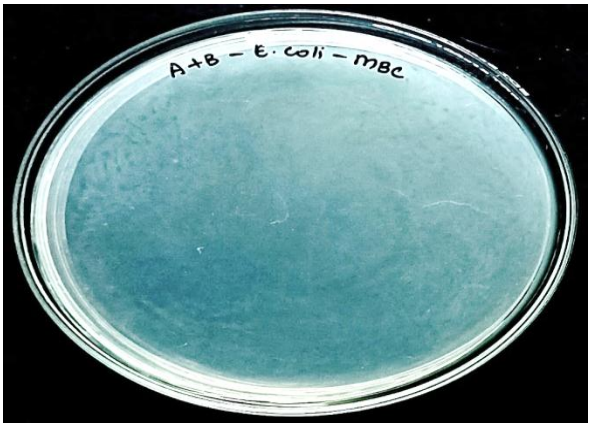

Figure 14: Bactericidal of E. coli by Cinnamon Oil + Ciprofloxacin (Sample A+B)

Figure 15: Bactericidal of E. coli by Cinnamon Oil + Ciprofloxacin + Proline (Sample A+B+C)

Table 5:

S. No	Name of the test samples	No. of bacterial colonies- 24 hrs	Total volume of test sample	CFU/ml/gm-24 hrs
1.	A	0	100 µl	0
2.	B	0	100 µl	0
3.	A+B	1	100 µl	1×10^7
4.	A+B+C	2	100 µl	2×10^7

The Minimum Bactericidal Concentration (MBC) assay was conducted to evaluate the antibacterial efficacy of the test samples against the target organism over a 24-hour incubation period. The results demonstrated that individual test samples A and B possessed strong bactericidal activity, as evidenced by the complete absence of bacterial growth (0 colonies) and a final count of 0 CFU/ml. In contrast, the combined formulations failed to achieve complete eradication of the bacteria. The combination of "A+B" resulted in the survival of a bacterial population at 1 x 10^7 CFU/ml (1 colony), while the "A+B+C" combination exhibited slightly higher growth with 2 x 10^7 CFU/ml (2 colonies). These findings indicate that while samples A and B are effective as standalone agents, their combined application in this specific assay did not result in total bacterial clearance.

3.5 Checkerboard Assay for Cinnamon Oil & Ciprofloxacin (Samples A & B)

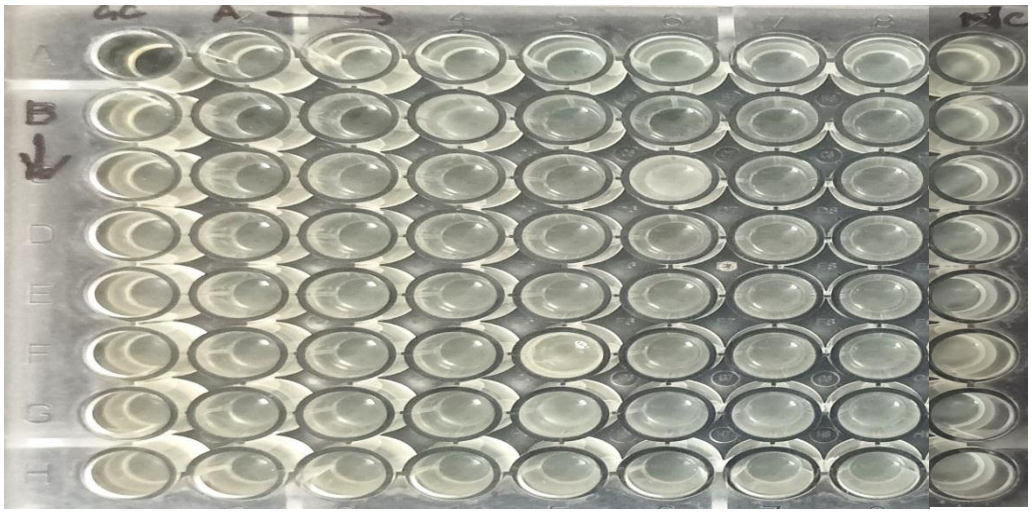

Figure 16: The FICI of the samples in combination is 2.06, which infers that the samples act indifferent to each other.

The Fractional Inhibitory Concentration Index (FICI) value of 2.06 indicates that the two samples in the combination exert an indifferent (or non-synergistic) effect on the target organism. In pharmacological terms, an interaction is typically classified as synergistic if the FICI is ≤ 0.5 and antagonistic if it is > 4.0. Because this result falls within the range of 0.5 to 4.0, it signifies that the combined antimicrobial activity is essentially no more effective than the sum of the individual parts; the drugs do not significantly assist or hinder one another's performance. Consequently, while the combination may still be effective at inhibiting growth, there is no clinical or biochemical advantage to using these specific concentrations together over using the more potent agent alone.

Table 6:

S.NO	1	2 (40 µg/ml)	3 (20 µg/ml)	4 (10 µg/ml)	5 (5 µg/ml)	6 (2.5 µg/ml)	7 (1.25 µg/ml)	8 (0.75 µg/ml)	9 (NC)
A	0.175	1.28	1.468	1.386	1.348	1.475	1.323	1.437	1.95
B (32 µg/ml)	1.087	0.81	0.675	1.218	1.14	0.902	0.95	1.062	1.71
C16 µg/ml)	1.184	1.018	1.211	1.088	1.108	1.714	1.052	1.159	1.711
D (8 µg/ml)	1.189	1.093	1.11	1.22	1.223	1.189	1.231	1.226	1.75
E (4 µg/ml)	1.255	1.303	1.309	1.318	1.297	1.254	1.3	1.246	1.776
F (2 µg/ml)	1.353	1.406	1.24	1.297	1.356	1.318	1.367	1.348	1.829
G (1 µg/ml)	1.362	1.337	1.321	1.393	1.577	1.285	1.407	1.403	1.644
H (0.5 µg/ml)	1.498	1.3	1.453	1.393	1.306	1.245	1.336	1.377	1.646

Based on the optical density (OD) values provided in this checkerboard assay, the combination of the two agents shows a clear dose-dependent inhibitory effect compared to the negative control (NC) values, which consistently hover between 1.64 and 1.95. The most significant synergy or enhanced inhibition is observed at the highest concentrations of both agents, specifically in the intersection of row B (32 µg/ml) and column 3 (20 µg/ml), which yielded the lowest OD of 0.675. As the concentrations of either agent decrease (moving toward row H or column 8), the OD values steadily climb back toward the 1.3–1.5 range, indicating a loss of inhibitory pressure and a return toward baseline microbial growth. Interestingly, row A (40 µg/ml) shows an unusually low OD in column 1 (0.175), which may suggest a potent individual effect or a potential outlier, but the overall trend confirms that the agents work most effectively in tandem at their upper-middle concentration ranges.

MIC of Sample A alone = 20 µg/ml

MIC of sample B alone = 16 µg/ml

MIC of Sample A in combination with Sample B = 1.25 µg/ml

MIC of Sample B in combination with Sample A =32 µg/ml

Table 7:

S. No	Name of the samples	MIC Alone	MIC Combination	FIC	FICI	Interpretation
1	A	20 µg/ml	1.25 µg/ml	0.06	2.06	Indifferent
2	B	16 µg/ml	32 µg/ml	2		

The results of the checkerboard assay reveal a significant disparity in how the two samples behave when combined, ultimately resulting in an indifferent classification with an FICI of 2.06. While Sample A shows a dramatic increase in potency when combined—reducing its MIC from 20 µg/ml to 1.25 µg/ml (an FIC of 0.06)—this benefit is offset by Sample B, which actually performs worse in the presence of Sample A. Specifically, Sample B's MIC doubles from 16 µg/ml to 32 µg/ml (an FIC of 2.0), suggesting that Sample A may interfere with Sample B's mechanism of action or that the organism's resistance to Sample B increases in this environment. Because the sum of these two fractional concentrations is greater than 0.5 but less than 4.0, the relationship is formally defined as indifferent, meaning the combination offers no statistical advantage over using the individual components.

3.6 Checker Board assay for Cinnamon Oil, Ciprofloxacin & Proline (Sample A+B&C)

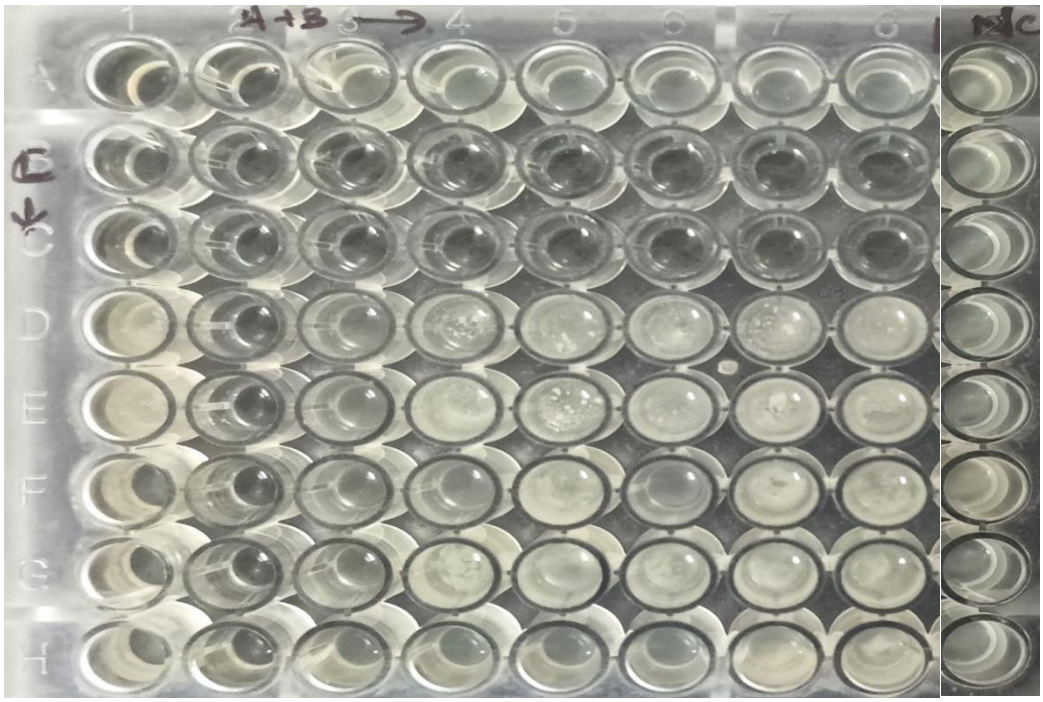

Figure 17: The FICI of the samples in combination is 1.03, which infers that the samples act indifferent to each other.

An FICI of **1.03** confirms that the combination of these two samples results in an **indifferent** (or additive-tending) interaction. In antimicrobial susceptibility testing, an interaction is only considered synergistic if the FICI is ≤ 0.5. Since this value sits slightly above the threshold of 1.0, it indicates that the combined effect of the two agents is essentially equal to the sum of their individual activities; they do not significantly enhance nor interfere with one another. While the samples remain effective at the tested concentrations, there is no "bonus" efficacy gained by mixing them, as the organism responds to each agent independently.

Table 8:

S.NO	1	2 (46 µg/ml)	3 (23 µg/ml)	4 (11.5 µg/ml)	5 (5.75 µg/ml)	6 (2.875 µg/ml)	7 (1.437 µg/ml)	8 (0.718 µg/ml)	9 (NC)
A	0.211	0.401	1.227	1.318	1.300	1.279	1.328	1.380	1.559
B (1000 µg/ml)	0.241	0.264	0.277	0.253	0.251	0.250	0.259	0.298	1.858
C (500 µg/ml)	0.251	0.232	0.251	0.286	0.271	0.274	0.306	0.299	1.505
D (250 µg/ml)	2.289	0.341	0.995	1.603	1.603	1.826	2.178	2.019	1.717
E (125 µg/ml)	2.326	0.351	1.115	1.843	1.894	1.797	2.177	2.171	1.570
F (62.5 µg/ml)	0.568	0.358	1.053	1.254	1.843	1.239	2.262	2.018	1.555
G (31.25 µg/ml)	0.595	0.313	1.056	2.108	1.909	1.911	1.987	2.135	1.633
H (15.6 µg/ml)	0.803	0.316	1.075	1.184	1.219	1.376	1.893	2.273	1.605

Based on the optical density (OD) values provided, this checkerboard assay demonstrates a potent inhibitory effect primarily driven by high concentrations of Sample B, regardless of Sample A's presence. The Negative Control (NC) values average approximately 1.62, while rows B and C (1000 µg/ml and 500 µg/ml of Sample B) show significantly low OD values ranging from 0.232 to 0.306 across all columns, indicating near-complete growth inhibition. A clear "cutoff" or transition zone is visible starting at Row D (250 µg/ml), where OD values jump sharply toward or even exceed the control values (e.g., 2.289), suggesting that the inhibitory effect is lost below the 500 µg/ml threshold for Sample B. Column 2 (46 µg/ml of Sample A) appears to be a unique point of interest, as it maintains low OD values (~0.3) even when Sample B concentrations drop to their lowest (Row H), hinting at a potential individual potency of Sample A at that specific concentration or a possible technical variation in that column.

MIC of Sample A+B alone = 23 µg/ml

MIC of sample C alone = 500 µg/ml

MIC of Sample A+B in combination with Sample C = 0.718 µg/ml

MIC of Sample C in combination with Sample A+B = 500 µg/ml

Table 9:

S. No.	Name of the sample	MIC Alone	MIC Combination	FIC	FICI	Interpretation
1	A+B	23 µg/ml	0.718 µg/ml	0.03		
2	C	500 µg/ml	500 µg/ml	1	1.03	Indifferent

The analysis of the checkerboard data reveals an indifferent interaction between the combined Sample A+B and Sample C, with a calculated FICI of 1.03. While the concentration of Sample A+B required for inhibition dropped drastically from 23 µg/ml to 0.718 µg/ml (a Fractional Inhibitory Concentration of 0.03) when used alongside Sample C, Sample C itself showed no change in potency, maintaining its MIC at 500 µg/ml (an FIC of 1.0). This suggests that while Sample A+B becomes more effective in the presence of Sample C, the interaction does not meet the strict mathematical threshold for synergy (≤ 0.5). Instead, the total effect is essentially dominated by the individual activity of Sample C, and the two agents do not provide a significant mutual enhancement that would justify a synergistic classification.

4) DISCUSSION

The present study evaluated the antibacterial activity and interaction profiles of cinnamon oil, ciprofloxacin, and proline against *Escherichia coli*, with emphasis on stress-modulated effects and combination outcomes. Cinnamon oil showed dose-dependent antibacterial activity with an IC_{50} of ~20 µg/mL, consistent with membrane-disruptive effects attributed to cinnamaldehyde. Ciprofloxacin exhibited stronger activity (IC_{50} ~16 µg/mL) through inhibition of DNA gyrase and topoisomerase IV, although partial survival at low concentrations suggested the presence of tolerant subpopulations. The combination of cinnamon oil and ciprofloxacin enhanced growth inhibition at some concentrations; however, checkerboard analysis indicated an indifferent interaction (FICI = 2.06), suggesting interference rather than synergy. The addition of proline increased IC_{50} values and reduced bactericidal efficacy, reflecting stress-mediated tolerance and protection against antibiotic-induced damage. The A+B+C combination also showed an indifferent interaction (FICI = 1.03). Overall, the findings highlight the significant role of bacterial stress responses in modulating antimicrobial efficacy and caution against the uncritical inclusion of stress-adaptive agents in combination therapies

5) CONCLUSION

The present study demonstrates that cinnamon oil and ciprofloxacin exhibit significant antibacterial activity against Escherichia coli when used individually, with clear dose-dependent inhibitory effects. However, combination treatments did not result in synergistic interactions, as indicated by indifferent FICI values in checkerboard assays. The inclusion of proline reduced bactericidal efficacy and increased tolerance, highlighting the role of stress-modulated bacterial responses in diminishing antimicrobial effectiveness. Overall, these findings emphasize that bacterial stress adaptation can compromise combination therapy outcomes and should be carefully considered when developing strategies to combat Gram-negative antibiotic resistance.

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