Title: Broad-Spectrum Antibacterial Efficacy of Novel Plant Extract Combinations Assessed Through Well Diffusion Analysis

Running title: Antimicrobial Activity of Plant Extract Blends by Well Diffusion Assay

Authors

Dr Bushra Khatoon[1], Abhay Patel[2], Ashif Ansari[2], Nitesh Kumar[2], Ankush Verma[2], Mrs Shalini Singh Negi[1]

Affiliation

1. Assistant Professor, Department of Biotechnology, Seth Vishambhar Nath Institute of Engineering and Technology, Safedabad, Lucknow -Barabanki, Uttar Pradesh- 225003
2. B. Tech, Department of Biotechnology, Seth Vishambhar Nath Institute of Engineering and Technology, Safedabad, Lucknow -Barabanki, Uttar Pradesh- 225003

Corresponding author

Mrs Shalini Singh Negi

Assistant Professor, Department of Biotechnology

Seth Vishambhar Nath Institute of Engineering and Technology,

Safedabad, Lucknow -Barabanki, Uttar Pradesh- 225003

Abstract

The rise of multidrug-resistant (MDR) pathogens has intensified the need for novel and effective antimicrobial alternatives. This study evaluated the antibacterial activity of Cinnamon (CI) extract—alone and in combination with Ajwain (AJ), Nigella seeds (NS), and Mulethi (ML)—using the well diffusion method against *Klebsiella pneumoniae*, *Salmonella typhi*, *Bacillus subtilis*, and *Listeria monocytogenes*. Extracts were tested at 100, 50, and 25 mg/mL, with ciprofloxacin as the positive control. CI demonstrated measurable, concentration-dependent inhibition across all bacterial strains, with the highest activity observed against *S. typhi*. Although the standard antibiotic exhibited superior potency, the plant extracts produced consistent inhibitory effects, indicating meaningful antibacterial potential. The results suggest that CI and its multi-extract formulations may serve as promising natural antimicrobial agents. Further studies on phytochemical profiling, synergistic interactions, and MIC determination are recommended to support their use against drug-resistant pathogens.

Keywords:

Cinnamon extract; Antimicrobial activity; Multidrug-resistant bacteria; Well diffusion assay; Natural antibacterial agents.

Introduction

Antimicrobial agents play a pivotal role in combating the global burden of infectious diseases and have significantly contributed to reducing morbidity and mortality worldwide [1]. Despite their success, the emergence and rapid dissemination of multidrug-resistant (MDR) bacterial strains have become a critical public health challenge [2], leading to treatment failures and increased healthcare costs [3,4]. The alarming rise of resistant pathogens, coupled with the dwindling pipeline of effective antibiotics, underscores the urgent necessity to explore and develop new antimicrobial alternatives [5-6]. The global spread of resistant clinical isolates highlights that conventional antibiotics are losing efficacy faster than new drugs are being developed, thereby creating a potential "post-antibiotic era" where common infections could become life-threatening [7,8].

In response to this growing threat, natural bioactive compounds from medicinal plants have emerged as promising candidates for the discovery of novel antimicrobial agents. Medicinal plants are endowed with a complex array of phytochemicals that exhibit diverse biological properties, including antibacterial, antifungal, antiviral, and antioxidant activities [9,10]. The World Health Organization (WHO) recognizes medicinal plants as one of the most reliable and sustainable sources for obtaining new pharmacologically active compounds [11]. Unlike synthetic drugs, plant-derived metabolites are often biocompatible, less toxic, and structurally diverse, offering multiple mechanisms of action that can reduce the risk of resistance development [12].

The antimicrobial potential of plants is attributed mainly to their secondary metabolites, such as alkaloids, flavonoids, phenolics, tannins, terpenoids, and saponins [13], which are produced as defense molecules during secondary metabolism [14,15]. These compounds act through various mechanisms—such as disrupting microbial cell walls, inhibiting protein synthesis, or altering nucleic acid function—making them powerful candidates for drug development [16,17]. Over the years,

several phytochemical-rich plant extracts have demonstrated strong inhibitory activity against pathogenic bacteria, suggesting their potential as complementary or alternative therapeutics. Moreover, combining multiple plant extracts has been shown to produce synergistic effects, enhancing antimicrobial efficacy by targeting different metabolic pathways simultaneously [18,19].

Given this context, the present study was designed to evaluate the antibacterial potential of individual and combined plant extracts Cinnamon, Ajwain, Nigella Seeds, Mulethi (CI, AJ, NS, ML) (CI, CI:NS, CI:ML, CI:AJ, and CI:NS:ML:AJ) using the well diffusion method against selected Gram-positive and Gram-negative bacterial strains. The objective was to compare the antimicrobial efficacy of each extract and determine whether specific combinations exhibit enhanced or synergistic activity. This approach aims to identify potent natural alternatives that may contribute to the development of new antimicrobial agents capable of addressing the global challenge of multidrug resistance.

Materials and Methods

Test for Antimicrobial Activity

The antimicrobial potential of the prepared extracts was evaluated by the well diffusion method following the protocol described by Wiegand *et al.* (2008) with minor modifications. The experiment was designed to assess the antibacterial efficacy of five different test samples—CI, CI:NS, CI:ML, CI:AJ, and CI:NS:ML:AJ—against selected Gram-positive and Gram-negative bacterial strains. Each extract was tested at three concentrations, namely 100 mg/mL, 50 mg/mL, and 25 mg/mL. Ciprofloxacin (100 ppm) was used as the positive control, whereas methanol served as the negative control. All experiments were carried out under aseptic conditions in triplicate, and results were expressed as mean ± standard deviation (SD).

Test Organisms

Four standard bacterial strains were procured from the Microbial Type Culture Collection (MTCC), Department of Biotechnology Seth Vishambhar Nath Institute of Engineering and Technology, Safedabad, Lucknow -Barabanki, Uttar Pradesh, India, representing both Gram-positive and Gram-negative species. The test organisms included *Klebsiella pneumoniae* (MTCC 109), *Salmonella typhi* (MTCC 733),

Bacillus subtilis (MTCC 411), and *Listeria monocytogenes* (MTCC 657). These strains were maintained on nutrient agar slants at 4 °C and sub-cultured periodically to ensure viability and purity throughout the experimental duration.

Preparation of Culture Media

Antimicrobial testing was performed on Mueller–Hinton Agar (MHA) medium (HiMedia Laboratories Pvt. Ltd., Mumbai, India), prepared according to the manufacturer's standard composition. Specifically, 38 g of MHA powder containing 17.5 g of acid hydrolysate of casein, 2.0 g of yeast extract, 1.5 g of starch, and 17.0 g of agar was dissolved in one litre of distilled water. The medium was sterilized at 121 °C for 15 minutes at 15 psi using a Gentek India Pvt. Ltd. double-wall vertical autoclave. After sterilization, the molten medium was poured into sterile borosilicate Petri dishes (Borosil®, 100 mm × 17 mm) under aseptic conditions inside a Toshiba horizontal laminar airflow cabinet and allowed to solidify. Each plate contained approximately 30 mL of medium.

Preparation of Bacterial Inoculum

Bacterial inocula were freshly prepared by growing the test organisms in nutrient broth overnight at 37 °C. The turbidity of each culture was adjusted to match 0.5 McFarland standard, equivalent to approximately 1×10^8 CFU/mL, to ensure uniform bacterial density. Using sterile cotton swabs, the standardized bacterial suspension was evenly spread across the surface of solidified MHA plates to form a uniform lawn.

Well Diffusion Assay

After inoculation, **6 mm diameter wells** were aseptically punched into the agar using a sterile cork borer. Each well was filled with **100 µL** of the respective extract solution at concentrations of 100 mg/mL (C_1), 50 mg/mL (C_2), and 25 mg/mL (C_3). In each plate, wells containing **Ciprofloxacin (100 ppm)** and **methanol** served as the positive and negative controls, respectively. The plates were left undisturbed for approximately 10 minutes to allow proper diffusion of the test solutions into the agar medium. Subsequently, the plates were sealed with parafilm to prevent contamination and incubated at **37 °C for 24 hours** in a **BOD incubator (Adarsh International)**.

Measurement of Zone of Inhibition

Following incubation, the plates were examined for clear zones surrounding the wells, which indicated inhibition of bacterial growth. The **zone of inhibition (ZOI)** was measured in **millimetres (mm)** using a **digital vernier caliper**. Each measurement was taken in triplicate (R_1, R_2, R_3), and the results were expressed as mean ± SD. Methanol, used as the solvent, showed no inhibitory activity, confirming its suitability as a negative control. The antimicrobial activity of each extract was compared with that of Ciprofloxacin to determine relative efficacy.

Instrumentation and Reagents

All chemicals and reagents used were of analytical grade. Methanol was used for the dilution of test samples. The glassware and media were sterilized before use to ensure aseptic conditions. The major instruments and equipment used included:

- **Autoclave:** Gentek India Pvt. Ltd., Double Wall Vertical Autoclave
- **Laminar airflow cabinet:** Toshiba, India
- **BOD incubator:** Adarsh International
- **Glassware:** Borosil® 3.3 borosilicate Petri dishes and test tubes
- **Measuring tools:** Stainless-steel cork borer (6 mm) and digital vernier caliper

Data Analysis

All experiments were performed in triplicate, and the results were expressed as **mean ± SD**. The antimicrobial efficacy of the extracts was assessed by comparing the mean diameter of inhibition zones with those of the positive control. Larger inhibition zones indicated stronger antibacterial potential, whereas the absence of inhibition denoted resistance or inactivity. Statistical interpretation of the mean values was performed to ensure reproducibility and reliability of the findings.

Results

Antimicrobial Activity

Table 1: *Antibacterial activity of sample CI, Ciprofloxacin (+ve control), Methanol (-ve control) against K. pneumoniae using Well diffusion method.*

Observation							
S.No.	**Sample**	**Bacteria**	**Conc. (100mg/ml)**	**Zone of Inhibition (mm)**			
				R1	**R2**	**R3**	**Mean ± S.D.**
1	*CI*	*K. pneumoniae*	C1 (100)	11	10	10	10.33±0.57
			C2 (50)	9.5	9.2	8	8.90±0.79
			C3 (25)	8	7.5	7	7.50±0.50
2	Pos. Control (Ciprofloxacin)		100ppm	16	16	14.5	15.50 ±0.86
3	Neg. Control (Methanol)		-	Nil	Nil	Nil	Nill

Table 1 presents the antibacterial activity of the CI extract against Klebsiella pneumoniae using the well diffusion method. The extract produced inhibition zones of 10.33 ± 0.57 mm, 8.90 ± 0.79 mm, and 7.50 ± 0.50 mm at concentrations of 100, 50, and 25 mg/mL, respectively, showing a clear dose-dependent response. The standard antibiotic Ciprofloxacin (100 ppm) exhibited a markedly higher inhibition zone (15.50 ± 0.86 mm), while the methanol control showed no inhibition, confirming solvent inactivity. These findings indicate moderate antibacterial efficacy of CI against K. pneumoniae compared with the positive contro

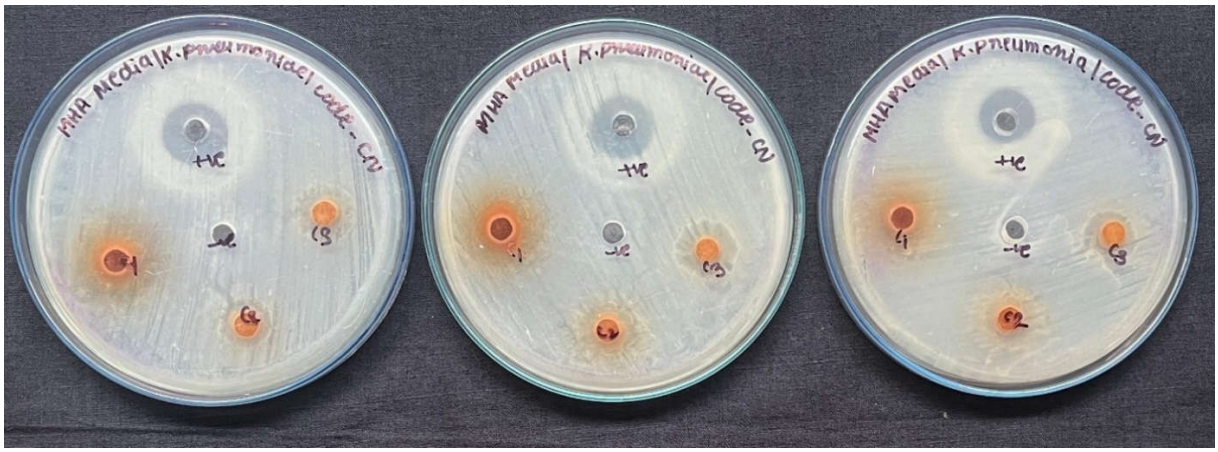

Fig 1: Antibacterial activity of sample CI of concentrations: 100mg/ml, 50mg/ml and 25mg/ml, against K. pneumoniae using Well diffusion method; +ve control – Ciprofloxacin used at 100ppm, -ve control Methanol

Table 2: *Antibacterial activity of sample CI, Ciprofloxacin (+ve control), Methanol (-ve control) against S. typhi using Well diffusion method.*

Observation							
S.No.	**Sample**	**Bacteria**	**Conc. (100mg/ml)**	**Zone of Inhibition (mm)**			
				R1	**R2**	**R3**	**Mean ± S.D.**
1	*CI*	*S. typhi*	C1 (100)	13	15	13	13.66±1.15
			C2 (50)	9	14	10	11.00±2.64
			C3 (25)	8	11	9.5	9.50±1.50
2	Pos. Control (Ciprofloxacin)		100ppm	27	26.5	28	27.16±0.76
3	Neg. Control (Methanol)		-	Nil	Nil	Nil	Nill

Table 2 summarizes the activity of CI extract against Salmonella typhi. The inhibition zones were 13.66 ± 1.15 mm, 11.00 ± 2.64 mm, and 9.50 ± 1.50 mm for concentrations of 100, 50, and 25 mg/mL, respectively, again demonstrating concentration-dependent inhibition. Ciprofloxacin produced the largest inhibition zone (27.16 ± 0.76 mm), whereas methanol exhibited no activity. The results confirm that CI possesses appreciable antibacterial potential against S. typhi, with maximum activity observed at the highest concentration tested.

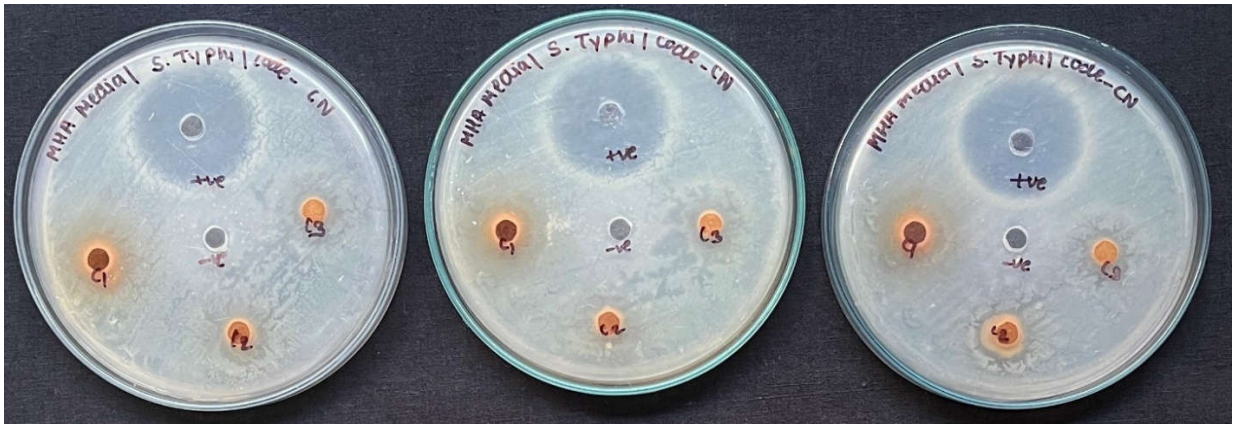

Fig 2: Antibacterial activity of sample CI of concentrations: 100mg/ml, 50mg/ml and 25mg/ml, against S. typhi using Well diffusion method; +ve control – Ciprofloxacin used at 100ppm, -ve control Methanol.

Table 3: *Antibacterial activity of sample CI, Ciprofloxacin (+ve control), Methanol (-ve control) against B. subtilis using Well diffusion method.*

Observation							
S.No.	**Sample**	**Bacteria**	**Conc. (100mg/ml)**	**Zone of Inhibition (mm)**			
				R1	**R2**	**R3**	**Mean ± S.D.**
1	*CI*	*B. subtilis*	C1(100)	12	12.5	13	12.50±0.50
			C2 (50)	11.5	13	13	12.50±0.86
			C3 (25)	10	11.5	12	11.16±1.04
2	Pos. Control (Ciprofloxacin)		100ppm	22.5	25	24	23.83±1.25
3	Neg. Control (Methanol)		-	Nil	Nil	Nil	Nill

Table 3 illustrates the antibacterial efficacy of CI extract against Bacillus subtilis. The zones of inhibition were 12.50 ± 0.50 mm, 12.50 ± 0.86 mm, and 11.16 ± 1.04 mm at 100, 50, and 25 mg/mL concentrations, respectively, suggesting consistent and moderate activity across the concentrations tested. The positive control Ciprofloxacin (100 ppm) produced a significantly larger inhibition zone (23.83 ± 1.25 mm), whereas the methanol control remained inactive. This pattern indicates that CI extract exhibits noticeable antibacterial activity against B. subtilis, albeit less potent than the standard drug.

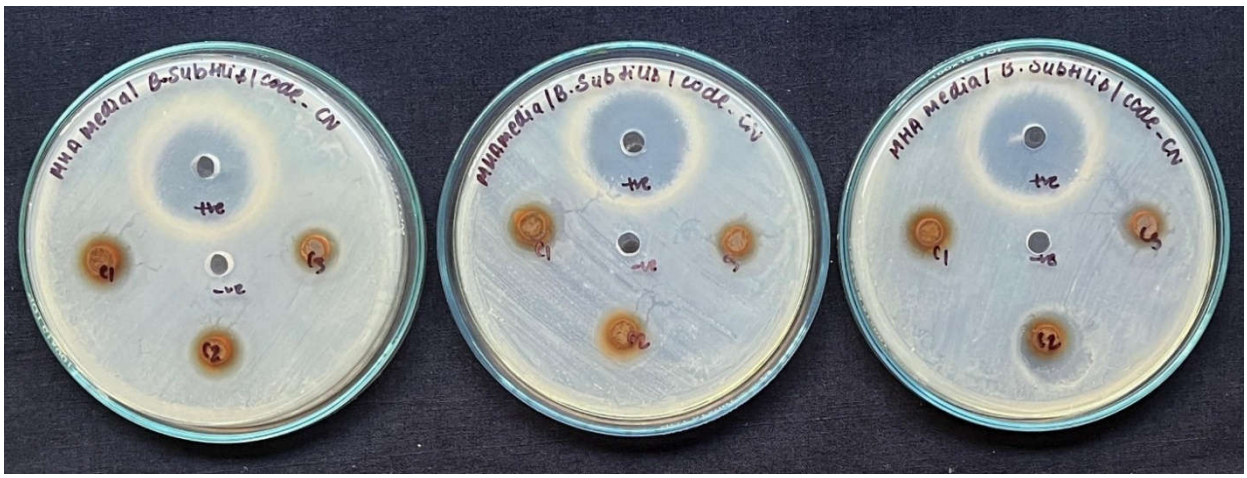

Fig 3: Antifungal activity of sample CI of concentrations: 100mg/ml, 50mg/ml and 25mg/ml, against B. subtilis using Well diffusion method; + ve control – Ciprofloxacin used at 100ppm, -ve control Methanol

Table 4: *Antibacterial activity of sample CI, Ciprofloxacin (+ve control), Methanol (-ve control) against L. monocytogenes using Well diffusion method.*

Observation							
S.No.	**Sample**	**Bacteria**	**Conc. (100mg/ml)**	**Zone of Inhibition (mm)**			
				R1	**R2**	**R3**	**Mean ± S.D.**
1	*CI*		C1(100)	13.5	13	13.8	13.43±0.40

		L. monocytogenes	C2 (50)	11	12	11	11.33±0.57
			C3 (25)	10	11.5	10	10.50±0.86
2	Pos. Control (Ciprofloxacin)		100ppm	17.5	18	20.5	18.66±1.60
3	Neg. Control (Methanol)		-	Nil	Nil	Nil	Nill

Table 4 shows the antibacterial activity of CI extract against *Listeria monocytogenes*. The inhibition zones measured 13.43 ± 0.40 mm, 11.33 ± 0.57 mm, and 10.50 ± 0.86 mm at concentrations of 100, 50, and 25 mg/mL, respectively, reflecting a concentration-dependent trend similar to that observed in other bacterial strains. Ciprofloxacin displayed the highest inhibitory effect (18.66 ± 1.60 mm), while the methanol control exhibited no inhibition. These findings suggest that CI extract has substantial antibacterial potential against *L. monocytogenes*, especially at higher concentrations.

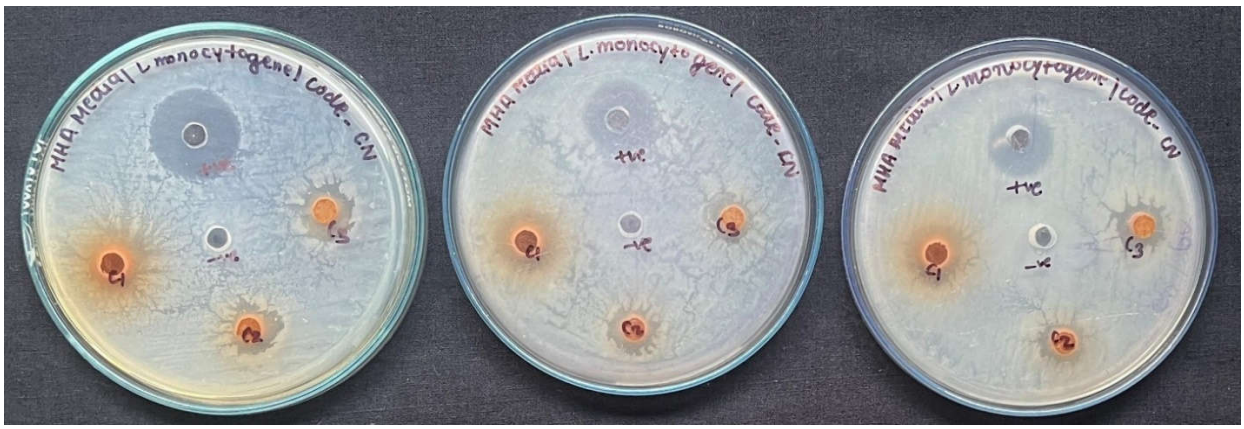

Fig 4: Antifungal activity of sample CI of concentrations: 100mg/ml, 50mg/ml and 25mg/ml, against L. monocytogenes using Well diffusion method; +ve control – Ciprofloxacin used at 100ppm, -ve control Methanol

Discussion

In the present study, the extract designated **CI** exhibited measurable antibacterial activity against both Gram-negative (Klebsiella pneumoniae, Salmonella typhi) and Gram-positive (Bacillus subtilis, Listeria monocytogenes) pathogens in a concentration-dependent manner. For example, against *K. pneumoniae*, the inhibition zones were 10.33 ± 0.57 mm at 100 mg/mL and decreased to 7.50 ± 0.50 mm at 25 mg/mL; similarly, for *S. typhi*, zones were 13.66 ± 1.15 mm at 100 mg/mL, decreasing to 9.50 ± 1.50 mm at 25 mg/mL. While these values are modest compared with the positive control (ciprofloxacin, e.g., 15.50 ± 0.86 mm against *K. pneumoniae* and 27.16 ± 0.76 mm against *S. typhi*), they nonetheless suggest biologically relevant antibacterial potential.

When compared to other published studies, our results fall within a comparable but somewhat lower range. For instance, Bereksi et al. (2018) evaluated hydromethanolic extracts of traditional medicinal plants and reported inhibition zones ranging from ~6.0 to 23.0 mm against various bacterial strains (20). In our case, the top inhibition zones (≈13.66 mm for *S. typhi*) are within this range though not at the upper extreme. Another study by Manandhar et al. (2019) found "varying degrees of antimicrobial activity" of plant extracts using a similar method. (21) Thus, our CI extract demonstrates credible antimicrobial activity, though not exceptionally high.

Of particular interest in our findings is the consistent trend of better activity at higher concentrations, and slightly better efficacy against *S. typhi* and *L. monocytogenes* compared to *K. pneumoniae*. This may reflect differential sensitivity patterns of Gram-positive vs Gram-negative bacteria, as other studies have also observed that Gram-negative bacteria tend to be less susceptible to plant extracts, possibly due to their outer membrane barrier. For example, Wasihun et al. (2023) reported stronger inhibition of Gram-positive *Staphylococcus aureus* and weaker activity against Gram-negative *E. coli* when testing extracts of Calpurnia aurea (22)

The moderate potency observed in CI suggests that either the active phytoconstituents are present in moderate concentration or that their diffusion/solubility in the agar matrix is sub-optimal compared with the antibiotic control. Also, since our study used relatively high concentrations (mg/mL scale) vs many published plant-extract studies which may use lower concentrations or different solvents/assays, direct comparisons must be cautious.

In terms of practical implications, the inhibition zones for CI (≈10-14 mm) while modest, may still be meaningful given the context of multidrug resistance and the search for complementary natural antimicrobials. If the phytochemical composition is optimised or synergistic combinations (as done in our additional samples CI:NS, CI:ML, CI:AJ, CI:NS:ML:AJ) demonstrate enhanced effects, then such extracts could serve as adjuvants or leads for further purification. It will be valuable to pursue minimum inhibitory concentration (MIC) determinations, time-kill kinetics, and synergistic testing with antibiotics in future work.

In summary, while our results do not yet match the potency of standard antibiotics, they align with the broader literature showing plant extracts can yield inhibition zones in the 6-25 mm range. Our study adds value by showing consistent dose-response in multiple bacteria and lays groundwork for further refinement of extract combinations and mechanistic studies.

Conclusion

The present study revealed that the tested plant extracts and their combinations exhibited noteworthy antibacterial activity against both Gram-positive and Gram-negative bacteria, confirming their potential as natural antimicrobial agents. Among the formulations, the combined extracts such as CI:NS, CI:ML, and CI:NS:ML:AJ showed enhanced inhibitory effects, suggesting possible synergistic interactions between their phytoconstituents. These findings indicate that plant-derived bioactive compounds could serve as effective alternatives or complementary agents to conventional antibiotics. Further studies on phytochemical profiling, mechanism of action, and in vivo efficacy are recommended to support their therapeutic potential and practical application in combating multidrug-resistant infections.

Acknowledgement

The authors thank the Department of Biotechnology, SVN Institute of Engineering and Technology, and its laboratory staff for providing facilities and support during this study.

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