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Evaluation Of Synergistic Antimicrobial Activity Of Selected Medicinal Plant Extracts Against Multidrug-Resistant Bacteria From Hospital And Pharmaceutical Waste Soils In Himachal Pradesh

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Abstract
The increasing prevalence of multidrug-resistant (MDR) bacteria, particularly in environments contaminated with antibiotics, poses a significant public health challenge. The present study aimed to isolate multidrug-resistant (MDR) bacterial strains from soil samples collected at pharmaceutical industry and hospital waste disposal sites in Himachal Pradesh, India, and to evaluate the synergistic antimicrobial potential of selected medicinal plant extracts against these isolates. Ten MDR bacterial strains, including Pseudomonas aeruginosa, Escherichia coli, Providencia rettgeri, Bacillus cereus, and Cellulosimicrobium cellulans, were identified using standard microbiological and molecular techniques. The antimicrobial activity of aqueous and ethanolic leaf extracts of Aegle marmelos, Terminalia chebula, Terminalia bellerica, Eucalyptus camaldulensis, and Jatropha curcas was assessed using the agar well diffusion assay. Synergistic combinations of extracts (S1–S4) showed enhanced antimicrobial effects compared to individual extracts, with S1 (A. marmelos + T. chebula, aqueous) and S3 (T. bellerica + T. chebula, ethanolic) demonstrating the highest activity. MIC and MBC values determined by the broth microdilution method confirmed the increased efficacy of these combinations. The results highlight the potential of synergistic plant extract formulations as alternative therapeutic agents against MDR bacterial infections.
Keywords: *Multidrug-resistant (MDR) bacteria, medicinal plant extracts, synergistic antimicrobial activity, agar well diffusion, MIC, MBC.*

INTRODUCTION

The rapid emergence of antimicrobial resistance (AMR), driven largely by the overuse and improper disposal of antibiotics, presents a growing threat to both environmental and public health. Waste from hospital and pharmaceutical sits is often rich in antibiotics, disinfectants, and heavy metals. This creates selective pressure in nearby environments by promoting the proliferation of multidrug-resistant (MDR) bacteria (Kumari et al., 2024; Kunhikannan et al., 2023). Several studies have confirmed the presence of MDR strains such as *B. cereus*, *P. aeruginosa*, and *K. pneumoniae* in soil adjacent to medical facilities (Soundhararajan and Srinivasan, 2024; Kumari et al., 2024). Traditional medicinal plants have emerged as valuable alternatives in the search for effective antibacterial agents. Compounds found in *T. bellirica*, *T. chebula*, *E. officinalis*, and other plants have demonstrated promising antimicrobial properties against antibiotic-resistant strains (Tiwana et al., 2024; Gupta et al., 2019; Khan et al., 2009). Of particular interest are synergistic combinations of plant extracts and antibiotics, which have demonstrated increased effectiveness and a reduction in microbial resistance in both clinical and environmental isolates (Alam et al., 2022; Haroun and Al-Kayali, 2016; Saquib et al., 2021). Recent innovations, such as polyherbal nanoformulations incorporating plant-derived bioactive compounds and nanoparticles, have shown significant potential in disrupting MDR biofilms and inhibiting bacterial growth (Soundhararajan and Srinivasan, 2024). Such formulations offer novel strategies to manage resistance in contaminated sites before waste is discharged into ecosystems. The present study aims to evaluate the synergistic antimicrobial activity of selected medicinal plant extracts against MDR bacteria isolated from hospital and pharmaceutical waste soils in Himachal Pradesh. By combining ethnomedicinal knowledge with microbiological and phytochemical analysis, this study explores the natural alternatives to conventional

antibiotics and supports sustainable strategies for combating antimicrobial resistance in environmental settings.

MATERIALS AND METHODS

Isolation of multi drug resistant (MDR) isolates from soil samples

Soil samples were collected from potentially antibiotic contaminated sites, including pharmaceutical industry and hospital waste disposal areas in Himachal Pradesh, using sterile polybags and labelled adequately for traceability. Samples were processed in the Microbiology Laboratory at Career Point University, Hamirpur, for bacterial isolation using the serial dilution and pour plate method. Distinct bacterial colonies were sub-cultured and screened for multidrug resistance (MDR) traits. Antimicrobial susceptibility was assessed using the Kirby-Bauer disc diffusion method with 15 broad-spectrum antibiotics on Mueller-Hinton agar. Bacterial inoculants were standardised to 0.5 McFarland, and zones of inhibition were measured after 24 hours of incubation at 37°C. Isolates resistant to more than two antibiotics were identified as MDR and preserved for further analysis (Kumari et al., 2024).

Plant extract preparation

Aqueous and ethanolic extracts were prepared using Soxhlet extraction (Mahire and Patel, 2020). Leaf powder (200 g) was placed in the thimble chamber with 500 mL of distilled water or ethanol as solvent. Extraction continued until the thimble contents became colorless. The extracts were concentrated by evaporating excess solvent over a hot water bath and stored in clean containers for further use.

Evaluation of synergistic antimicrobial activity of selected medicinal plants

The antimicrobial activity of aqueous and ethanolic leaf extracts from selected medicinal plants was assessed against MDR bacterial isolates using the agar well diffusion method (Sharma et al., 2015). Bacterial inocula were prepared by suspending 4–5 colonies in 5 ml of normal saline and adjusting the turbidity to 0.5 McFarland standards (~ 1×10^8 CFU/ml). A uniform lawn was spread on Mueller-Hinton Agar (MHA) plates using sterile cotton swabs, and 6 mm wells were created using a sterile cork borer. Each well was loaded with 100 µL of plant extract at a concentration of 100 mg/ml (prepared by diluting 100 µL of a 1000 mg/ml stock in 900 µL of DMSO). Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured in millimeters.

For synergistic evaluation, equal quantities (500 mg each) of two active plant extracts were combined to prepare a 1000 mg/ml stock solution. This was similarly diluted (100 µL stock + 900 µL DMSO) to obtain a final concentration of 100 mg/ml, and 100 µL of the mixture was introduced into the wells. The same incubation and measurement procedures were followed. The combinations tested were: S1 (*A. marmelos* + *T. chebula,* aqueous), S2 (*A. marmelos* + *J. curcas*, aqueous), S3 (*T. bellerica* + *T. chebula*, ethanolic), and S4 (*T. bellerica* + *E. camaldulensis,* ethanolic).

Determination of Minimum inhibitory and minimum bactericidal concentration (MIC and MBC) of plant leaf extracts

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of effective plant leaf extract combination against selected MDR isolates were determined using the broth micro-dilution method (Elshikh et al., 2016; Osungunna, 2020). Inocula were adjusted to 5×10^5 CFU/ml by serially diluting colonies to achieve a 0.5 McFarland standard turbidity in Mueller-Hinton broth (CLSI, 2016). Resazurin dye (0.02%) was prepared, filter-sterilized, and stored at 4°C. The assay was conducted in a sterile 96 well microtiter plate, with wells 1–10 used for serial dilutions of the plant extract (starting from 1000 mg/ml), and wells 11 and 12 serving as positive and negative controls. After adding 50 µl of inoculum (except in the negative control), plates were incubated at 37°C for 24 hours, followed by addition of 30 µl Resazurin and further incubation for 1 hour. MIC was identified as the lowest concentration showing no colour change (blue), while MBC was determined by sub-culturing samples from MIC and higher wells on nutrient agar; the absence of bacterial growth confirmed the MBC.

RESULTS

Isolation of multidrug-resistant (MDR) isolates

Molecular identification of ten multidrug-resistant (MDR) bacterial isolates obtained from ten soil samples revealed a diverse array of species. *P. aeruginosa* (PH6), *P. mendocina* (SS3), *E. coli* (PP4), and *P. rettgeri* (BH2) were among the Gram-negative isolates identified. Gram-positive isolates included *C.*

cellulans (HH3) and *B. cereus* (AB5, HH1, GP1). One isolate (BH5) was identified as *Enterobacter sp.,* while isolate BH6 could not be identified through molecular methods.

Synergistic effect of selected medicinal plants

Ethanolic extracts of *T. bellerica* (7E), *E. camaldulensis* (5E), and *T. chebula* (8E), along with aqueous extracts of *A. marmelos* (1A), *T. chebula* (8A), and *J. curcas* (6A), exhibited significant antimicrobial activity against ten MDR bacterial isolates. Based on these results, the synergistic antimicrobial potential of selected extract combinations was evaluated (Table 1). The combination of ethanolic extracts of *T. bellerica* and *T. chebula* (S3) demonstrated an additive antimicrobial effect compared to the individual extracts. Among aqueous extracts, the combination of *A. marmelos* and *T. chebula* (S1) exhibited enhanced activity compared to individual treatments (Table 2). Overall, all four tested combinations (S1–S4) exhibited synergistic effects against MDR isolates, with combinations S1 and S3 showing the most pronounced activity (Table 2(a, b); Fig. 1–4).

Table 1. Combination of plant extracts prepared for determination of antimicrobial effect.

Combination of plant extracts*	Combinations
A.marmelos(1A)+*T.chebula*(8A)	S1
A.marmelos(1A)+*J.curcas*(6A)	S2
T.bellerica(7E)+*T.chebula*(8E)	S3
T.bellerica (7E)+*E.Camaldulensis*(5E)	S4
*__A:__*Aqueousextract**;E:**Ethanolic extract	

Table 2(a). Zone of inhibition (mm) observed for different plant extracts (individual and in combinations) against MDR isolates (AB5, BH2, BH5, BH6 and GP1)

Plant extracts*	**Zone of inhibition (mm) against MDR isolates (Mean±S.D.)**				
	AB5	**BH2**	**BH5**	**BH6**	**GP1**
1A	27.66 ±0.26	29.16 ±0.224	26.83 ±0.49	25.5 ±0.46	25.16 ±0.22
6A	24.83±0.53	19.16 ±0.267	23.33 ±0.49	21.83±0.70	24±0.00
8A	21.66±0.26	25±0.00	25.16 ±0.70	26.83 ±0.22	26.83±0.27
5E	16.83±0.26	21.83±0.35	20±0.00	22.33±0.27	20.5 ±0.46
7E	28±0.46	25.83±0.70	24.16±0.27	21 ±0.0	25.16±0.27
8E	27.83±0.26	27±0.00	21.83 ±0.70	15.33 ±0.53	27.83±0.27
S1 (1A+8A)	**33.66±0.26**	**35.33±0.26**	**31±0.00**	**29.67 ±0.53**	**33.16 ±0.27**
S2 (1A+6A)	28±0.46	25.33±0.53	34.66 ±0.53	27 ±0.46	30.5 ±0.46
S3 (7E+8E)	**34.5±0.46**	**31.83±0.26**	**30.5 ±0.46**	**31.83±0.35**	**34.16 ±0.27**
S4 (7E+5E)	37.16±0.26	30.5 ±0.46	27.83±0.35	31±0.00	32±0.00
* **1:** *A. marmelos*;**5:** *E. Camaldulensis*; **6:** *J. curcas;* **7:** *T. bellerica*; **8:** *T. chebula* **A:** Aqueous extract**; E:** Ethanolic extract					

Table 2(b). Zone of inhibition (mm) observed for different plant extracts (individual and in combinations) against MDR isolates (HH1, HH3, PH6, PP4 and SS3)

Plant extracts*	**Zone of inhibition (mm) against MDR isolates (Mean±S.D.)**				
	HH1	**HH3**	**PH6**	**PP4**	**SS3**
1A	27.5 ±0.46	27±0.00	25.83±0.27	26±0.00	25.83±0.59
6A	21.83±0.70	20.83±0.70	22±0.00	16.83±0.27	18.5 ±0.46
8A	28.33±0.53	28.83±0.70	21±0.47	24.5 ±0.46	30.67 ±0.53
5E	20.83±0.35	23 ±0.46	24±0.47	22 ±0.46	25 ±0.46
7E	24.5 ±0.46	26.83±0.27	19.83±0.27	28.83±0.70	24±0.00
8E	29±0.00	22.33 ±0.45	27.66±0.27	28±0.00	26.16 ±0.27
S1 (1A+8A)	**35.33 ±0.53**	**31±0.00**	**33±0.46**	**34 ±0.46**	**30.83±0.70**
S2 (1A+6A)	30±0.00	27±0.00	28.83±0.27	27.83±0.27	27±0.00
S3 (7E+8E)	**32.83 ±0.71**	**34.16 ±0.27**	**40.16±0.27**	**36.33±0.53**	**34.83±0.35**
S4 (7E+5E)	32.83 ±0.267	31.33 ±0.35	34.5±0.46	34±0.00	30.33±0.45

* **1:** *A. marmelos*;**5:** *E. Camaldulensis*; **6:** *J. curcas*; **7:** *T. bellerica*; **8:** *T. chebula* **A:** Aqueous extract**; E:** Ethanolic extract

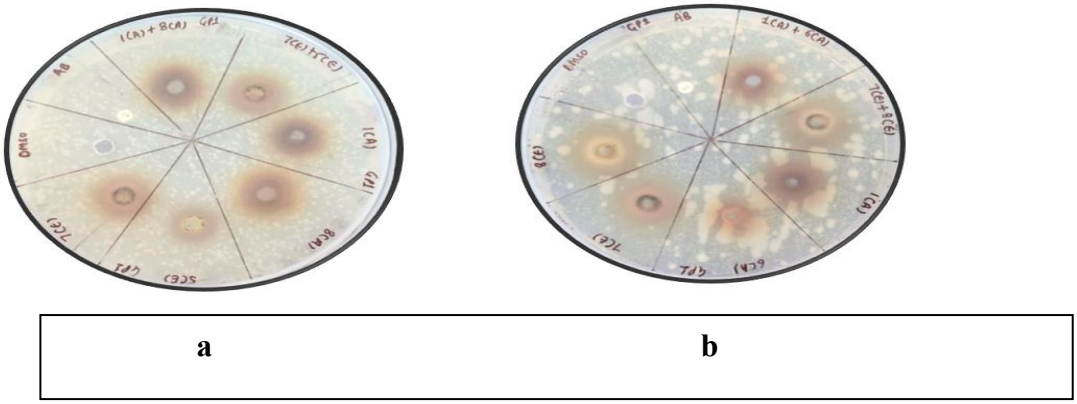


Fig.1 Antimicrobial activities of individual and in combination of selected plant extracts against MDR isolate GP1 **a.** S1 (1A+8A), S4 (7E+5E), 1A, 8A, 7E, 5E **b.** S2 (1A+6A), S3 (7E+8E), 1A, 6A, 7E, 8E. Control of antibiotic (AB: VA30) and DMSO was also included in each plate.

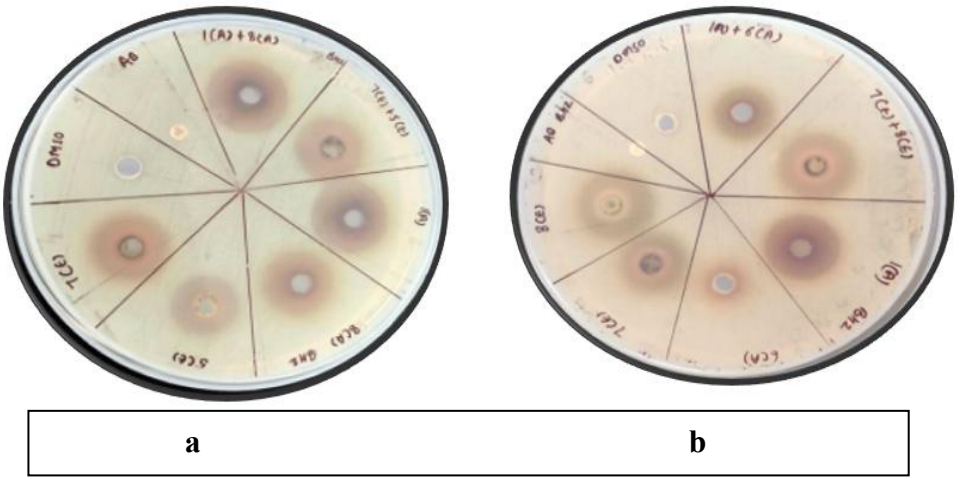
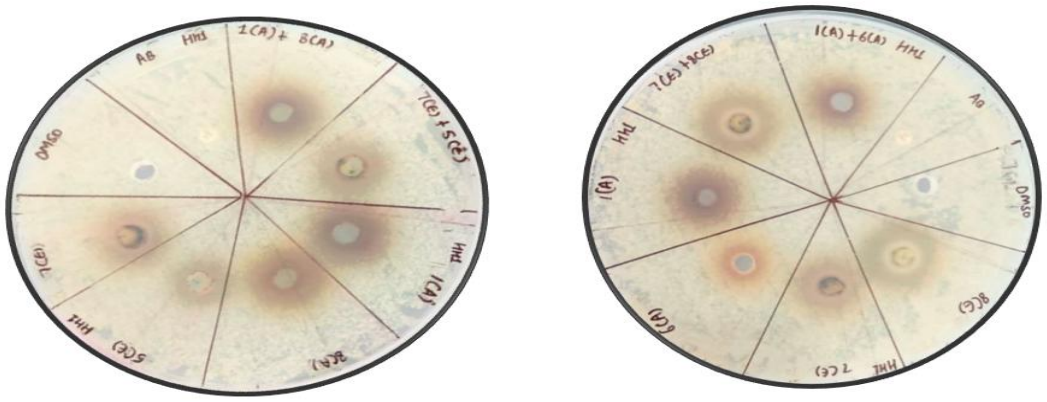
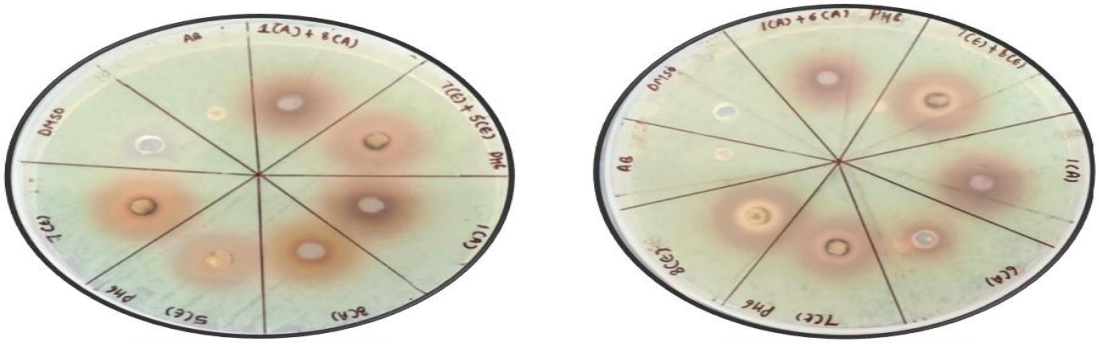


Fig.2 Antimicrobial activities of individual and in combination of selected plant extracts against MDR isolate BH2 **a.** S1 (1A+8A), S4 (7E+5E), 1A, 8A, 7E, 5E **b.** S2 (1A+6A), S3 (7E+8E), 1A, 6A, 7E, 8E. Control of antibiotic (AB: VA30) and DMSO was also included in each plate.



a **b**

Fig.3 Antimicrobial activities of individual and in combination of selected plant extracts against MDR isolate HH1 **a.** S1 (1A+8A), S4 (7E+5E), 1A, 8A, 7E, 5E **b.** S2 (1A+6A), S3 (7E+8E), 1A, 6A, 7E, 8E. Control of antibiotic (AB: CPM30) and DMSO was also included in each plate.



a **b**

Fig.4 Antimicrobial activities of individual and in combination of selected plant extracts against MDR isolate PH6 **a.** S1 (1A+8A), S4 (7E+5E), 1A, 8A, 7E, 5E **b.** S2 (1A+6A), S3 (7E+8E), 1A, 6A, 7E, 8E. Control of antibiotic (AB: AMC30) and DMSO was also included in each plate.

MIC and MBC values of plant extracts

Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values varied among the combinations and isolates tested. Overall, S3 exhibited relatively more potent antimicrobial activity, with lower MIC (0.8-6.3 mg/mL) and MBC (1.6-12.5 mg/mL) values, compared to S1 and S2 (Table 3(a,b), Fig.5,6). Notably, S3 was particularly effective against isolate PH6, while S4 showed the broadest activity across all tested strains. These findings suggest a synergistic effect of ethanolic plant extracts in enhancing antimicrobial potency against MDR bacteria.

Table 3(a): The overall range of MIC for plant extracts S1, S2, S3 and S4.

Plant extracts	**Overall range for minimum inhibitory concentration (MIC)**
S1	6.3 – 12.5 mg/ml.

S2	6.3-12.5 mg/ml
S3	0.8- 6.3 mg/ml
S4	1.6-6.3 mg/ml

Table 3(b): The overall range of MBC for plant extracts S1, S2, S3 and S4.

Plant extracts	Overall range for minimum bactericidal concentration (MBC)
S1	12.5 - 25 mg/ml
S2	12.5-25 mg/ml
S3	1.6 – 12.5 mg/ml
S4	3.2- 12.5 mg/ml

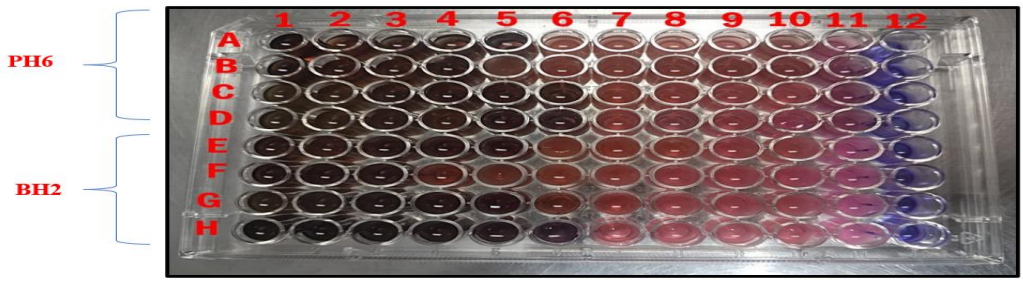


Fig. 5 Determination of MIC against PH6 and BH2

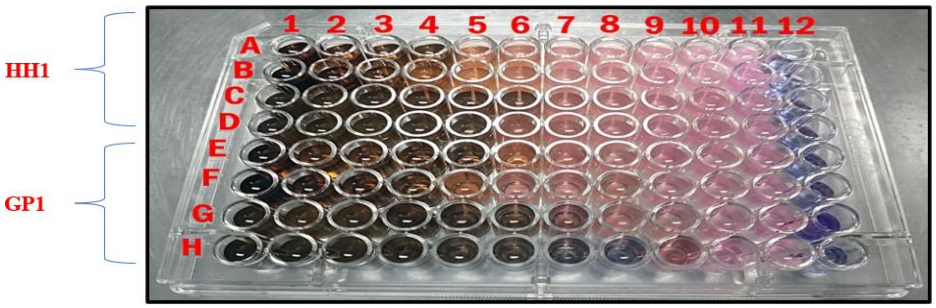


Fig. 6 Determination of MIC against HH1 and GP1

DISCUSSION

The escalating prevalence of multidrug-resistant (MDR) bacteria in environmental reservoirs, particularly near hospital and pharmaceutical waste sites, represents a critical public health and ecological challenge (Chandan *et al.*, 2013). Our findings are consistent with both global and regional trends, highlighting the presence of robust multidrug-resistant (MDR) strains such as *P. aeruginosa* (PH6), *E. coli* (PP4), *P. rettgeri* (BH2), *C. cellulans* (HH3), and multiple *B. cereus* isolates in contaminated soils of Himachal Pradesh. These results were previously reported by our group (Kumari et al., 2024) and are further substantiated in the present study, underscoring the persistence of these MDR species across diverse contaminated environments. `Supporting this, Singh and co-workers (2023), isolated and characterized 42 bacterial strains from pharmaceutical contaminated soil, identifying 17 genera with 78.57% preliminarily classified as extended-spectrum beta-lactamase (ESBL) producers. Confirmatory double dick synergy tests (DDST) verified ESBL activity in 51.51% of isolates, while 55% exhibited high multiple antibiotic resistance

(MAR) index values (0.8–1.0). A statistically significant correlation ($p < 0.05$) between ESBL production and higher MAR scores further underscores pharmaceutical waste as a major reservoir of MDR bacteria, calling for rigorous waste management and continuous surveillance to prevent environmental resistance spread. Hospital adjacent environments increasingly serve as breeding grounds for resistance due to unchecked antibiotic disposal and anthropogenic pressures (Kunhikannan et al., 2023). Concurrently, Himachal Pradesh's varied altitude and climate foster rich plant biodiversity, particularly in the Shimla hills, where a survey from 2011 to 2013 documented medicinal plants including Aloe, Holy Basil, Indian Gooseberry, Rhododendron, Himalayan Yew, and Thyme with known therapeutic properties (Singh and Thakur, 2014). In the search for alternative antimicrobial agents, plant-derived compounds have attracted renewed attention owing to their diverse phytochemical profiles and reduced likelihood of inducing resistance compared to conventional antibiotics (Cowan, 1999). Addressing the urgent need for alternative antimicrobial strategies, our study evaluated the antibacterial efficacy of selected medicinal plant extracts against MDR strains. Individually, extracts from *T. chebula*, *T. bellirica*, *E. camaldulensis*, and *A. marmelos* demonstrated significant inhibition zones of 20–30 mm. Notably, the ethanolic extract of *T. chebula* (8E) showed consistent potent activity, validating earlier findings by Doye et al. (2023);Tiwana et al. (2024; Aneja and Radhika, 2009. The rich phenolic and tannin content of *T. chebula* underpins its antibacterial mechanisms, including membrane disruption and efflux pump inhibition, as reported by Atta et al. (2023) and Gupta et al. (2019). Medicinal plants provide promising natural therapies against MDR pathogens (Rajan and Banu, 2020, Ghosh et al., 2008). Mehta et al. (2021), employed molecular docking and dynamics simulations to evaluate phytocompounds from 35 North Western Himalayan plants against the RamR protein (PDB ID 6IE9) of *S. typhimurium*, a key regulator of antibiotic and bile resistance. Beta-sitosterol emerged as the top candidate due to strong binding affinity, drug-likeness, and non-toxic profiles, suggesting its potential to inhibit efflux pump mechanisms. Synergistic combinations of extracts exhibited enhanced antibacterial effects. Combinations S1 (*A. marmelos* + *T. chebula*) and S3 (*T. bellirica* + *T. chebula*) yielded inhibition zones of 33–40 mm, significantly surpassing individual extracts. The Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) values varied across the different plant extract combinations and MDR isolates tested. Among the combinations, S3 and S4 demonstrated comparatively stronger antimicrobial activity, as evidenced by their lower MIC and MBC values in relation to S1 and S2. Notably, S3 showed marked effectiveness against the PH6 isolate, while S4 exhibited broad-spectrum activity against all tested MDR strains. These results indicate a potential synergistic effect of the ethanolic plant extracts, particularly in combinations S3 and S4, in enhancing antimicrobial efficacy against resistant bacterial pathogens. Comparable observations were reported by Zouine and coworkers (2024), who analyzed the antibacterial potential of plant extracts over a decade (2014–2024). In their study, extracts with MIC values ≤625 µg/mL were considered highly active. Among 81 plants tested, *Quercus coccifera*, *Ocimum gratissimum*, and *Curcuma longa* demonstrated significant inhibitory effects against key MDR pathogens, including *P. aeruginosa*, *S. aureus,* and *E. coli*. These parallel findings reinforce the potential of certain plant-based combinations in targeting resistant bacteria and support the growing interest in phytotherapeutic alternatives to conventional antibiotics. These findings align with Haroun and Al-Kayali (2016), who documented improved antibiotic efficacy when combined with plant extracts, and Saquib and co-workers (2021), who reported augmented antimicrobial effects from *Punica granatum* and *Commiphora molmol* in co-treatment. Such synergism is attributed to complementary phytochemicals flavonoids, tannins, alkaloids, and terpenoids targeting multiple bacterial pathways (Alam et al., 2022).The potent synergy in the *T. bellirica* and *T. chebula* combination reflects the antimicrobial activity observed in traditional Ayurvedic formulations, such as Triphala, which is composed of *T. bellirica*, *T. chebula*, and *E. officinalis*. Triphala exhibits strong antibacterial and antibiofilm effects against drug-resistant bacteria, and its incorporation into nanoformulations can enhance stability, targeted delivery, and bioavailability, facilitating clinical and environmental applications (Soundhararajan and Srinivasan, 2024). The aqueous combination S1 (*A. marmelos* + *T. chebula*) is especially notable due to its ecological and economic advantages, corroborating Shahin et al. (2025), who demonstrated that essential oils and water-soluble plant constituents boost antibiotic efficacy against pathogens like *P. aeruginosa* and *S. aureus*.Ethnomedicinal plants such as *Cinnamomum zeylanicum*, *Syzygium aromaticum*, and *Acacia nilotica* have also been documented as effective

against hospital acquired MDR infections (Khan et al., 2009; Reda et al., 2017; Masoumian and Zandi, 2017). The integration of these extracts into nanobased delivery systems could amplify pharmacological efficacy while minimizing toxicity a critical aspect underscored by Sarangi and Padhi (2018), and safety concerns addressed by Ekor (2014) and Zhang et al. (2012).Further advancing this approach, Chauhan et al. (2023) demonstrated the synergistic effect of methanolic extracts from Withania somnifera and Catharanthus roseus combined with synthetic antibiotics (ceftazidime, chloramphenicol, trimethoprim) and non-antibiotic drugs (ibuprofen, paracetamol) against 17 MDR *Salmonella Typhi* isolates. The synergy observed particularly with ceftazidime and trimethoprim indicates the potential to enhance treatment efficacy and immune responses through combined phytochemical and synthetic drug therapies. However, we did not evaluate the efficacy of the plant extract in combination with an antibiotic in our study. Importantly, our study provides a novel comparative analysis of ethanolic and aqueous extract combinations, confirming that extract synergy is a reproducible and scalable phenomenon, rather than an incidental one. While other combinations (S2 and S4) also exhibited antimicrobial effects, the superior performance of S1 and S3 highlights the importance of selecting phytochemically complementary extracts for synergistic formulations. In conclusion, our results confirm the presence of highly resistant bacteria in hospital and pharmaceutical waste soils of Himachal Pradesh and identify promising natural solutions for their control. The demonstrated synergism among *T. chebula*, *T. bellirica*, and *A. marmelos* supports continued exploration of phytochemical combinations as viable alternatives or adjuncts to synthetic antibiotics. Given their safety, availability, and affordability, integrating these extracts into nanocarriers or biodegradable delivery systems could represent a sustainable frontier in antimicrobial therapy. Future research should focus on isolating active compounds, elucidating molecular mechanisms, and evaluating in vivo efficacy and safety of these formulations.

CONCLUSION

This study highlights the alarming presence of multidrug-resistant (MDR) bacteria in soil samples collected from hospital and pharmaceutical waste disposal sites in Himachal Pradesh, including strains such as Pseudomonas aeruginosa, Escherichia coli, Providencia rettgeri, Bacillus cereus, and Cellulosimicrobium cellulans. These findings emphasize the role of contaminated environmental sites as significant reservoirs and transmission routes for antimicrobial resistance. Selected medicinal plant extracts, particularly Terminalia chebula, Terminalia bellirica, and Aegle marmelos, showed strong antibacterial activity, with combinations S1 and S3 exhibiting notable synergistic effects against MDR strains. These findings support the potential of plant-based formulations as natural, effective alternatives for managing antimicrobial resistance. Further research is needed to optimize these combinations for safe and practical applications in healthcare and environmental sanitation.

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Conflict of interest

The authors declare no conflicts of interest.

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