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Antibacterial potential of indigenous plant extracts against multidrug-resistant bacterial strains isolated from New Delhi region

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Abstract

The extensive use of antibiotics to treat bacterial infections has led to the widespread emergence of multidrug-resistant (MDR) pathogens, becoming increasingly difficult to treat with currently available antibacterial agents. The present study is based on prospecting the ethnomedicinal potential of Indian plant varieties for the treatment of MDR bacteria. Plants produce an array of diverse pharmacological compounds in defence against microbial pathogens which may be employed as a novel intervention strategy to combat MDR human pathogens. In the present study, the antimicrobial activity of extracts of four common Indian plants: *Azadirachta indica* (Neem), *Murraya koenigii* (Kadipatta), *Phyllanthus emblica* (Amla), and *Ocimum sanctum* (Tulsi) prepared in four solvents, water, methanol, ethanol, and chloroform was tested against nine MDR bacterial isolates. Kirby-Bauer well diffusion assays were adopted to assess the antimicrobial activity of plant extracts against the MDR strains. The potency of plant extracts was examined by determining the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). All MDR isolates including *Staphylococcus haemolyticus*, *Bacillus subtilis*, *B. thuringiensis*, *B. cereus*, *Enterobacter xiangfangensis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa* were significantly inhibited by the plant extracts. Test extracts showed promising antibacterial potential against MDR *P. aeruginosa* and *Bacillus* sp. with low MIC values ranging between 0.02–1.56 mg/ml, while most plant extracts exhibited either moderate MBC values or bacteriostatic effects. To the best of our knowledge, this is the first study that demonstrates the potential use of endemic *A. indica*, *M. koenigii*, *P. emblica*, and *O. sanctum* as therapeutic agents against circulating MDR human pathogens in the national capital.

Keywords: Plant extracts; Antimicrobial; Minimum inhibitory concentration; Minimum bactericidal concentration; MDR pathogens; *Pseudomonas aeruginosa*.

1. Introduction

Antibiotics are small antimicrobial drugs administered to cure bacterial infections in patients. They revolutionized medical science in the 20th century and emerged as wonder drugs, especially prolonging life expectancy and decreasing mortality due to microbial infections. However, their rampant and irrational use has led to dissemination in environmental ecosystems escalating the problem of Antimicrobial Resistance (AMR) [1, 2]. The emergence of AMR is a natural evolutionary process used by microorganisms in response to selective pressure against antimicrobial compounds in their surroundings [3]. Sir Alexander Fleming spoke about the emergence of AMR in his Nobel lecture. AMR to Penicillin was first reported in several strains of *Staphylococcus aureus* [4]. With the evolution of “superbugs” such as ‘ESKAPE’ pathogens: *Enterococcus* sp., *S. aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *P. aeruginosa*, and *Enterobacter* sp., AMR has now manifested in the form of a global health crisis. The problem of AMR has become more serious today due to the lack of newer antimicrobial scaffolds [1]. India has been called the “AMR capital of the

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world” due to the rise of MDR pathogens along with indiscriminate consumption of antibiotics [5]. It is estimated that by 2050, the world economy might have to face an additional cumulative burden of US\$100 trillion and 10 million deaths a year because of AMR, which at present is claiming as many as 35,000 lives annually infecting more than 2.8 million people in USA alone [6]. We are entering the post-antibiotic era where common infections and minor injuries can be fatal. Realizing this, the United Nations General Assembly at New York (2016) conducted a high-level meeting to address the AMR issue [7]. In this scenario, there is a global need to look for alternative treatment strategies against the rising burden of AMR. ~~To the rescue,~~ In recent times, medicinal plants and herbal products have regained prominence amongst researchers and the scientific community.

The origin of Ayurveda, Siddha, and Unani, the ancient healthcare systems of Indian medicine can be traced back to the “Vedas” ~~which are now being revived and modernized~~ [8]. The antimicrobial properties of plants have found favor worldwide due to their abundant natural availability, pharmacological properties, and negligible cytotoxicity as compared to antibiotics [9]. Research on traditional medicines has proven to be a cynosure for pharmaceutical industries as plants are rich repository of secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, saponins, and polyphenols which have been shown to resist pathogens, and boost immunity [10]. The anti-malarial drug Artemisinin obtained from *Artemisia annua* has been used in traditional Chinese medicine for over two thousand years [11]. Other popular examples include Morphine, obtained from *Papaver somniferum*, Tiotropium from *Atropa belladonna*, Dronabinol and Cannabidiol from *Cannabis sativa*, Quinine and Quinidine from *Cinchona* sp., etc. [12]. The numbers of bioactive molecules derived from medicinal plants have been estimated to be more than 200,000, which still accounts for only a fraction of all the compounds produced by plant species [13]. Many plants have been found to cure respiratory diseases, urinary tract infections, cutaneous ailments and, even gastrointestinal disorders [14, 15]. Therefore, the problem of AMR may be tackled using plant bioactives as traditional remedies based on Ayurveda.

Table 1 Previously reported properties and traditional uses of Indian plants selected for the study.

Common Name	Scientific name [Herbarium Voucher No.]	Family	Plant part used	Reported properties	Reported Compounds
Kadipatta/ Sweet neem	<i>Murraya koenigii</i> (14666)	Rutaceae	Leaves	Antibacterial and antiviral properties [20]	Murrayastine, murrayaline, carbazole alkaloids [21].
Neem	<i>Azadirachta indica</i> (14668)	Meliaceae	Leaves	Antibacterial, antifungal, antimalarial and antiviral properties [22]	Alkaloids, flavonoids, tannins, saponins, anthraquinones glycosides, triterpenes and reducing sugars [23]. Azadirachtin, Nimbolinin, Nimbin, Nimbidin, Nimbolide, Nimbandiol, Limonoids, beta-sitosterol, Salannin, and Quercetin [24].
Amla/Indian gooseberry	<i>Phyllanthus emblica</i> (14667)	Euphorbiaceae	Fruit	Antipyretic, cardioprotective, gastroprotective, antianemia, and antidiarrheal properties [25]	Emblicanin A & B, Ellagic acid, Gallic acid, Phyllemblicin, Quercetin, Ascorbic acid, Tannins [26]
Tulsi/Holi Basil	<i>Ocimum sanctum</i> (14665)	Lamiaceae	Leaves	Treatment of diabetes mellitus, arthritis, bronchitis [27]	Oleanolic acid, Rosmarinic acid, Ursolic acid, Eugenol, Linalool, Carvacrol, β -elemene, β -caryophyllene, Germacrene [28]

Considering the rich reserves of flora and medicinal plants in developing countries like India, their bioactive phytochemicals can act as game-changers in controlling the national medical burden. Among the thousands of Indian medicinal plants, four were selected for this study against 9 MDR bacterial isolates on the basis of their therapeutic

properties and traditional use as given in Table 1. Previous studies have highlighted the prevalence of various antibiotic-resistant bacteria in numerous water sources such as wastewater [16], hospital wastewater [17], surface and groundwater [18], *etc.* which may eventually find their way into drinking water [19]. Therefore, this study aims to investigate the antimicrobial activities of 4 Indian plant varieties in different solvent formulations against 9 MDR human pathogenic bacterial strains isolated in a previous study from different sources across Delhi, India.

2. Materials and methods

2.1. Bacterial cultures

Nine pre-isolated bacterial strains from our previous study were employed for the study [19]. Their antibiotic resistance profiles have been listed in Table 2. Bacterial cultures were grown till mid-log phase (OD₆₀₀: 0.8-1.0); mixed with 40% glycerol and frozen at -20°C till further use. Cultures were revived in Mueller-Hinton broth (HiMedia Labs, Mumbai, India) and maintained on LB agar, unless indicated otherwise.

Table 2 Gram characteristics and resistance profiles of the bacterial strains used in the study. *Antibiotics: Ampicillin (AMP); Cefuroxime (CXM); Nitrofurantoin (NIT); Co-trimoxazole (COT); Meropenem (MRP).

S. No.	Bacterial Isolate	Gram Character	Identification based on 16S rRNA	Resistant to Antibiotics*
1.	MTP-1(1)	Gram +ve	<i>Bacillus thuringiensis</i>	AMP, CXM, NIT
2.	MTP-1(3)	Gram +ve	<i>Bacillus subtilis</i>	AMP, CXM, NIT
3.	MTP-3(10)	Gram -ve	<i>Enterobacter xiangfangensis</i>	AMP, COT, NIT
4.	JPR-1	Gram -ve	<i>Pseudomonas aeruginosa</i>	CXM, NIT
5.	JPR-9	Gram +ve	<i>Bacillus cereus</i>	CXM, NIT
6.	YRW-2(15)	Gram -ve	<i>Klebsiella pneumoniae</i>	CXM, MRP, NIT
7.	MW 4	Gram +ve	<i>Staphylococcus haemolyticus</i>	AMP, COT, NIT
8.	OW-1	Gram -ve	<i>Pseudomonas aeruginosa</i>	AMP, COT, NIT
9.	GW-16	Gram +ve	<i>Staphylococcus haemolyticus</i>	AMP, COT, CXM, NIT

2.2. Preparation of plant extracts

Plant parts were collected from locations in New Delhi, India and verified at the Department of Botany, University of Delhi with voucher numbers (Table 1). Plant parts were washed with distilled water, air-dried in shade at room temperature (RT) for 7 days and stored at 4°C until further use. Plant extracts were prepared as described previously with minor modifications [29]. 100 g of dried plant material was subjected to Soxhlet extraction for 4 days using the following solvents (400ml): chloroform, ethanol, methanol, and water. The extracts were filtered using Whatman filter paper No.1 and concentrated by drying at RT or using a hot-plate magnetic stirrer. The plant concentrates were stored at 4°C. For further experiments, plant extracts were prepared in 10% DMSO.

2.3. Evaluating the antimicrobial activity of plant extracts

Well-diffusion assays were performed by Kirby-Bauer method for primary screening of plant extracts against the 9 MDR strains [30]. Bacterial cultures with turbidity comparable to 0.5 McFarland Standard were swabbed onto MH agar plates and 6 wells of diameter 7 mm each were punched, 4 of which were loaded with 50µl of appropriate concentrations (25 mg/ml, 50 mg/ml, 75 mg/ml, 100 mg/ml) of plant extract. One well was loaded with 50µl of 50% solvent (Control 1) and the final one with 50µl of 10% DMSO (Control 2). Following incubation at 37°C for 18 h; zones of inhibition were measured and normalized to those of corresponding controls. All extracts were tested in triplicates to obtain statistically significant results.

2.4. Determining the MIC and MBC of plant extracts

The MICs of plant extracts were determined using 96-well microtiter plate-based cell viability assay for 3 selected MDR bacterial strains using previously established protocols [31]. These strains were selected based on their resistance profiles and prevalence rates in human infections. 100µl of double-strength MH broth was added to all the wells and 100µl of 2X plant extract (50 mg/ml) was dispensed in the first well. 1:1 serial dilutions of plant extracts were prepared by transferring 100µl suspension from the previous well and to the next well. This was repeated serially until desired lowest dilutions of plant extracts were achieved. 10µl of standardized bacterial inoculum was added to each well, barring the negative controls. Wells with only MH broth and MH broth-containing plant extracts served as negative controls, while those with inoculated MH broth lacking plant extracts served as positive growth controls. The plates were incubated for 18 h at 37°C. 20µl of p-INT solution (0.2 mg/ml stock) was added to each well and incubated at 37°C for 30 min. MIC was regarded as the lowest sample concentration which failed to produce a visible color change from colorless to pink after the addition of p-INT indicating complete inhibition of microbial growth. For the evaluation of MBCs, cell suspensions containing plant extracts that failed to show a color change in the MIC assays were dispensed in fresh MH broth and appropriate dilutions were plated onto LB agar and incubated at 37°C for 24 h. MBC was defined as the minimum concentration of the plant extract that completely abolishes-inhibited colony formation in the candidate bacterial strains.

2.5. Statistical analysis

All assays were performed in triplicates, and the mean values \pm standard deviations were calculated. Experiments were conducted in three independent events and a representative set of data has been reported.

3. Results and discussion

3.1. Plant extracts inhibit the growth of MDR strains.

Previous reports have affirmed the antimicrobial efficacy of medicinal plant extracts against human MDR pathogens [32-34]. In view of the rapid global spread of MDR bacteria, there is a pressing requirement to discover new antimicrobial agents. Identifying the antimicrobial action of indigenous plant extracts and plant secondary metabolites is important for exploring their potential as natural antimicrobial agents [30] and alternative therapeutic agents [35, 36] which can be used alone or in combination with antibiotics [37, 38]. To extend these findings, we assessed the antimicrobial properties of plant extracts obtained from endemic Neem, Amla, Tulsi, and Kadipatta leaves using various solvents against 9 MDR bacterial strains. The resistance profile of MDR strains procured was confirmed and found to show resistance against 2nd generation antibiotics including Cefuroxime, Co-trimoxazole, Nitrofurantoin, and Ampicillin. Zone of inhibition diameters for plant extracts varied from no inhibition (0 mm) to 15.67 mm. From the well diffusion assays, it was evident that aqueous plant extracts significantly inhibited the growth across all MDR genera. Tulsi, Amla, and Kadipatta extracts proved to be more promising in terms of antimicrobial activity as compared to Neem extracts. Considering the notorious nature of *P. aeruginosa* and its implications in nosocomial infections, the tested plant extracts can prove to be worthwhile in devising treatment strategies. A representative set of images has been shown in Figure 1. Data obtained in the form of inhibition zones (maximum 15.67 mm) has been summarized in the form of heat maps in Figure 2. We found that all plant extracts exhibited promising antimicrobial activity against both Gram-positive and Gram-negative MDR pathogens.

3.2. Evaluating MIC of plant extracts against candidate MDR strains

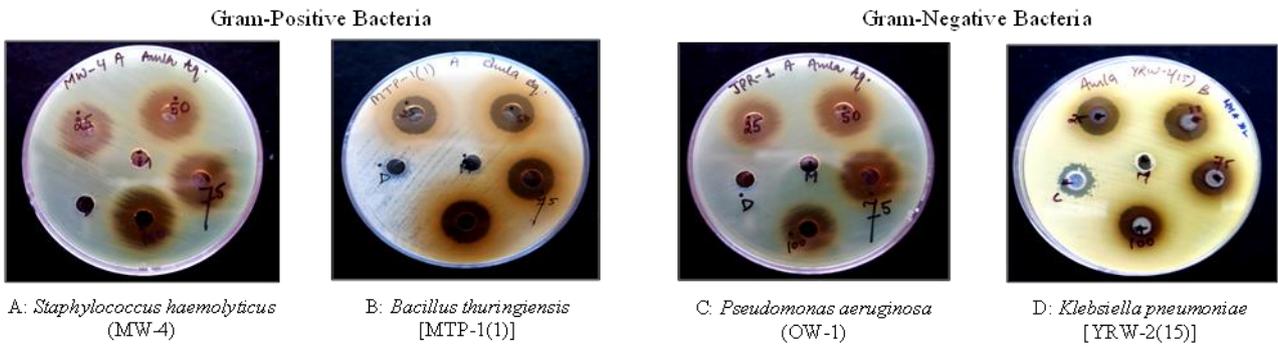
The MIC of a compound serves as a pharmacological parameter for the evaluation of its antimicrobial potency. Several investigations have reported plant extracts to exhibit MIC values on a higher scale, raising concerns over their dosage and efficacy [39, 40]. Therefore, in an attempt to resolve this, MIC of the plant extracts was determined using a rapid microdilution assay by examining the reduction of p-INT in a colorimetric assay. As per the results, our plant extracts indicated high efficacy with comparatively lower MIC values for three selected MDR isolates, *B. thuringiensis*, *B. cereus*, and *P. aeruginosa* than reported in previous studies [41]. The MIC values ranged from 0.02 mg/ml to 6.25 mg/ml in the current investigation for the mentioned extracts. Amla and Kadipatta extracts showed promisingly lower MIC values (0.02 mg/ml), indicating their prolific antimicrobial activities. The Amla fruits have been reported to exhibit antimicrobial activities owing to tannins: Emblicanin A and B [42]. The results of the present investigation are in consonance with previous studies which also reported *E. officinalis* to have a broad-spectrum antibacterial potential against *B. subtilis*, *K. pneumoniae* and *P. aeruginosa* [43, 44]. Aqueous, ethanolic, and acetic extracts of Amla and Neem have previously been shown to possess antimicrobial properties against *Enterococcus faecalis*, *Candida albicans* and *Streptococcus mutans* [45]. *E. officinalis* has also been reported to be more effective against Gram-positive than Gram-negative bacteria, owing to differences in structure and composition of the cell wall [24]. The leaves of *M. koenigii*

contain numerous bioactives including murrayastine, murrayaline, pyrayafoline carbazole alkaloids, and glycosides [21]. These constituents have been demonstrated to exhibit antibacterial properties, even better than Amikacin and Gentamycin against *Staphylococcus*, *Streptococcus*, *Escherichia coli*, *Pseudomonas*, *Klebsiella* and *Proteus* sp. [46]. Previously, Handral *et al.* reported much higher MIC values of 25 mg/ml of Kadipatta extracts against *Pseudomonas* sp. [47]. In our study, we report Kadipatta aqueous extract to be most effective against *P. aeruginosa* with the lowest MIC of 0.09 mg/ml. Kadipatta methanolic extract was most potent against *B. cereus* with MIC of 0.02 mg/ml than Amla aqueous extract. Ethanolic extracts of Amla and Kadipatta and aqueous extract of Amla proved equally effective for *B. thuringiensis*.

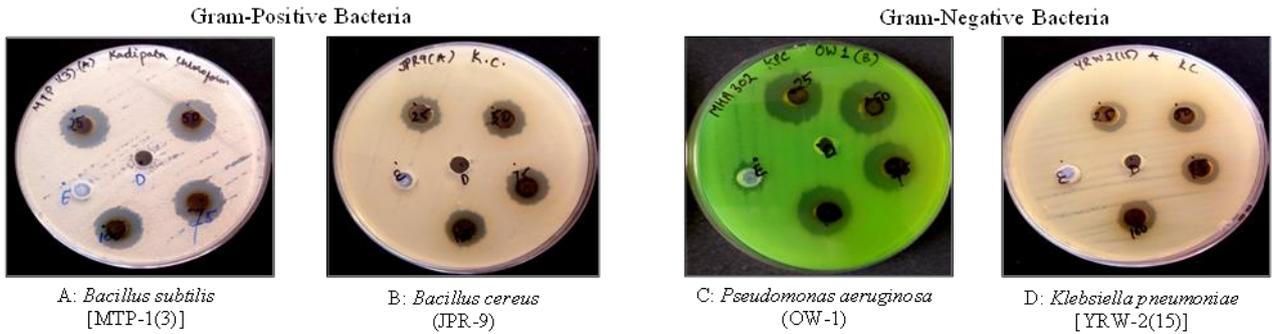
Neem is widely regarded for its therapeutic potential as it contains numerous bioactives including azadirachtin, nimbolinin, nimbin, nimbidin, nimbidol, salannin, and quercetin [48]. Alzohairy *et al.* reported antimicrobial activity of neem due to inhibition of microbial growth and cell wall development [24]. Our Neem aqueous formulations showed a lower MIC value of 190 µg/ml [0.09 mg/ml] against MDR *P. aeruginosa* as compared to an MIC value of 500 µg/ml reported by a previous study [49]. However, MIC values for its ethanolic extract were on the higher end (3.125-6.25 g/ml). Indian Tulsi varieties are known to possess antimicrobial phytochemicals like eugenol, cyclooctene, methyl eugenol, camphor, and β-caryophyllene [50]. Previous studies have shown Tulsi extracts to possess extremely high MIC values of 200 mg/ml against *E. coli* and *S. aureus* [51]. On the contrary, our Tulsi extracts inhibited the growth of notorious human pathogens at extremely low MIC values ranging between 0.09 mg/ml and 0.78 mg/ml. As is evident from Figure 3, the order of potency was Kadipatta, Tulsi, Amla, and Neem. Kadipatta extracts were most potent at low concentrations, exhibiting low MIC values (mean 0.045 mg/ml) against the 3 test strains. While Amla and Tulsi extracts showed MIC values in the moderate range averaging 1.56 and 0.39 mg/ml. This accounts for the prolific antimicrobial activities of these endemic plant extracts against the commonly circulating MDR pathogens in the Delhi region.

3.3. Plant extracts exhibit both bacteriostatic and bactericidal properties

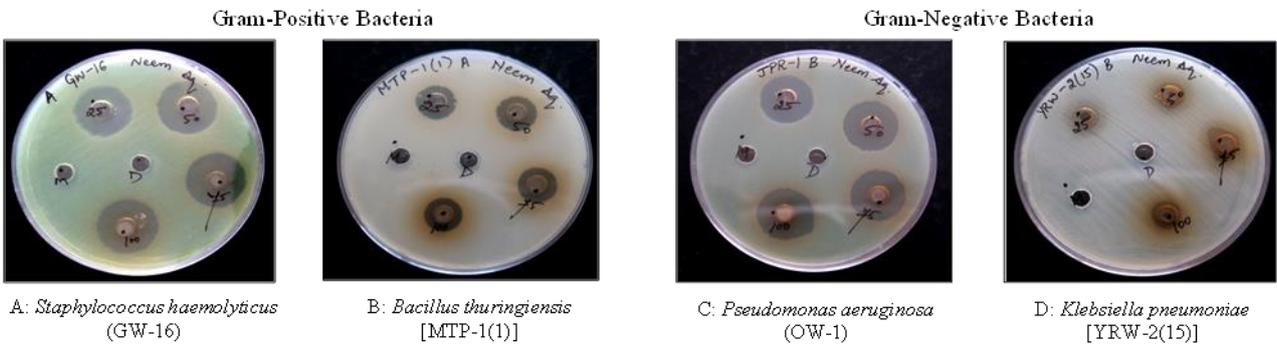
The Minimum Bactericidal concentration (MBC) of the extracts was determined by appropriately diluting the cell suspensions from plant extract containing-wells which did not show any growth after incubation during MIC assays in fresh broth. The test MBC values ranged from 0.045 to 25 mg/ml, while extracts for which MBCs could not be estimated in the tested range appeared to be bacteriostatic in nature. Kadipatta extracts exhibited strong bactericidal activity against all the test strains at extremely low concentrations ranging between 0.09 mg/ml and 1.56 mg/ml. While Amla and Tulsi extracts did not exert killing effects but rather exhibited bacteriostatic properties. Neem extracts on the other hand showed both bacteriostatic as well as bactericidal effects with varying solvents. Chloroformic and ethanolic extracts of Neem showed bactericidal properties against *B. cereus* and *B. thuringiensis* within the range of 0.78-6.25 mg/ml. We observed a significant variation in the antibacterial potential of plant extracts prepared in water, chloroform, methanol, and ethanol. The differences in the bactericidal properties of plant extracts can be accounted for the use of different solvents. Solvents with different polarities including water, ethanol, methanol, cyclohexane, and chloroform have been reported to exert varying antibacterial effects [52]. Salem *et al.* also reported a difference in antibacterial activities of plant extracts with the solvents used for extraction [53]. Our results indicate that aqueous, chloroformic, and methanolic plant extracts possess strong antimicrobial activity corresponding to lower MIC and MBC values against the MDR strains tested (Table 3)]. However, ethanolic extracts exhibited moderate or higher MIC and MBC values as compared to other solvents. Generally, organic solvents have been shown to exhibit greater antimicrobial potential due to greater solubility of phytoconstituents. In a recent study by Samman *et al.*, aqueous and methanolic extracts of Amla fruits have been shown to exert varying antibacterial activity against pathogenic bacteria [54]. Raghu and Ravindra reported that the MICs exhibited by methanolic extract of Amla against the tested organisms ranged between 0.261-0.342 mg/ml and were more potent than chloroform and diethyl ether extracts [55]. These justify the varying bactericidal and bacteriostatic potential of the plant extracts tested. Our results are also in agreement with Fatima *et al.* showing prolific antibacterial potential of Neem against UTI pathogens like *S. aureus*, *E. coli*, and *Pseudomonas* sp. with ethanolic extract [23]. At a glance, our findings reinforce the potential of natural plant extracts as effective therapeutic formulations against MDR bacteria.



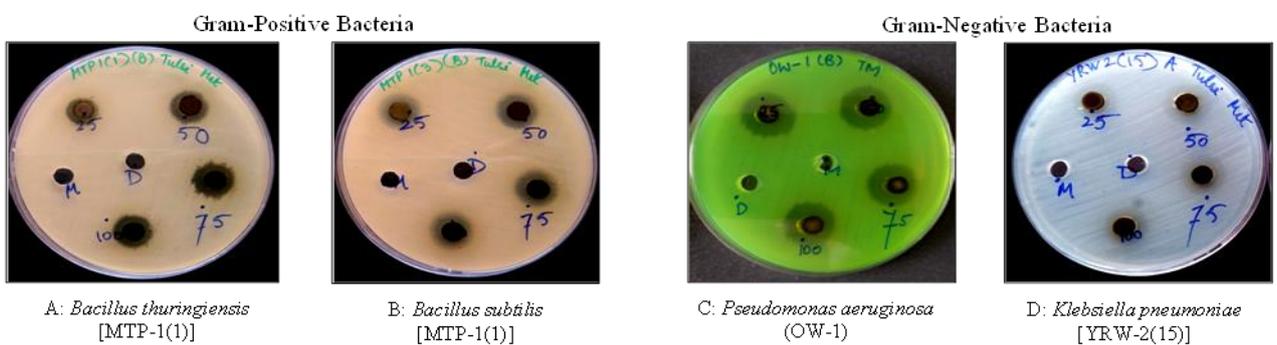
1.1. Inhibition of growth by *Phyllanthus emblica* (Amla) aqueous extracts.



1.2. Inhibition of growth by *Murraya koenigii* (Kadipatta) chloroformic extracts.



1.3. Inhibition of growth by *Azadirachta indica* (Neem) aqueous extracts.



1.4. Inhibition of growth by *Ocimum sanctum* (Tulsi) methanolic extracts.

Figure 1 Representative images for growth inhibition of MDR isolates by Amla Aqueous Extract (1.1); Kadipatta chloroformic extract (1.2); Neem aqueous extract (1.3); Tulsi methanolic extract (1.4).

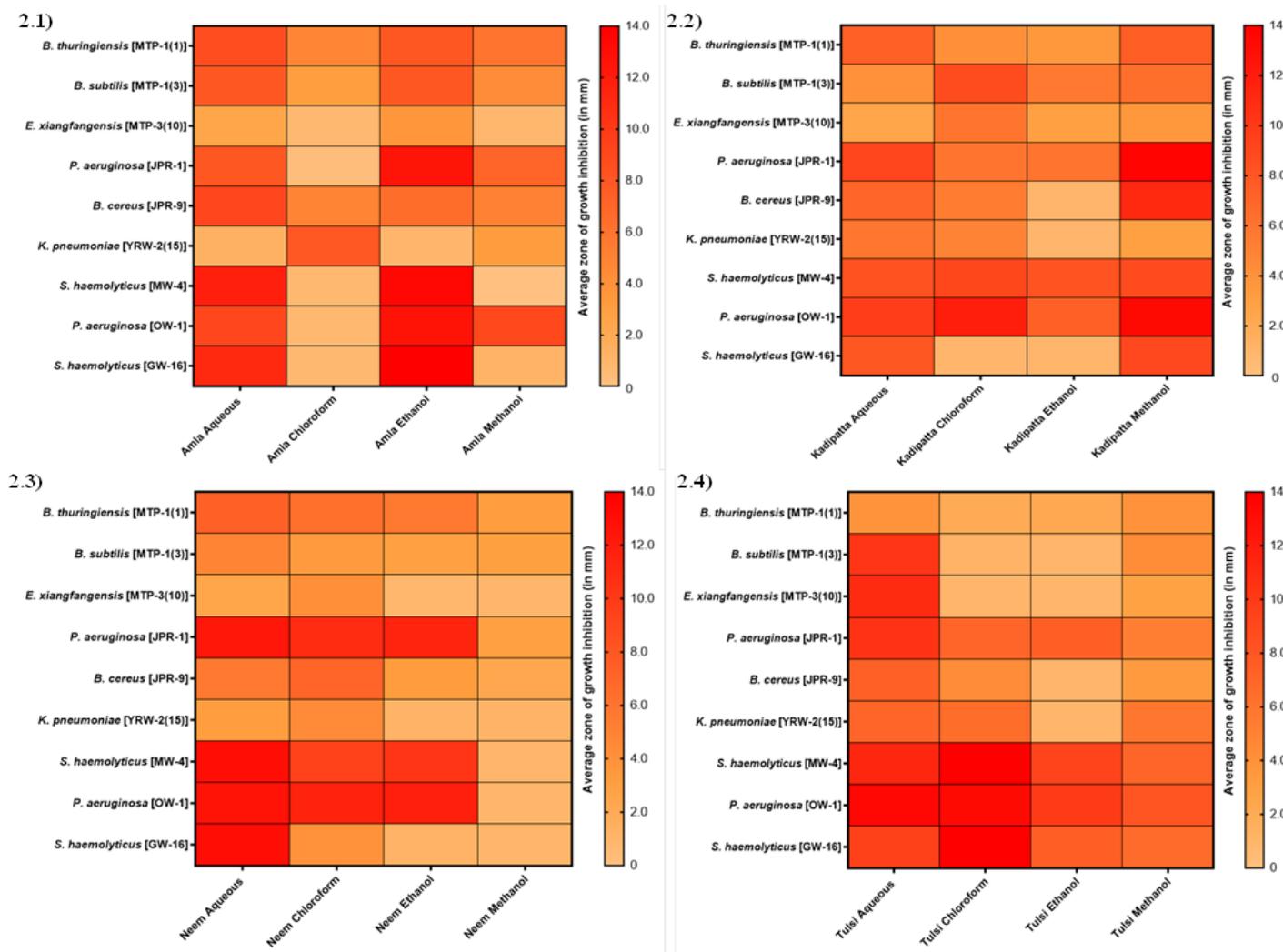


Figure 2 Heat maps representing the inhibitory zone diameters (in mm) of MDR strains obtained against various plant extracts and formulations. (2.1) Amla extracts (2.2) Kadipatta extracts (2.3) Neem extracts (2.4) Tulsi extracts.

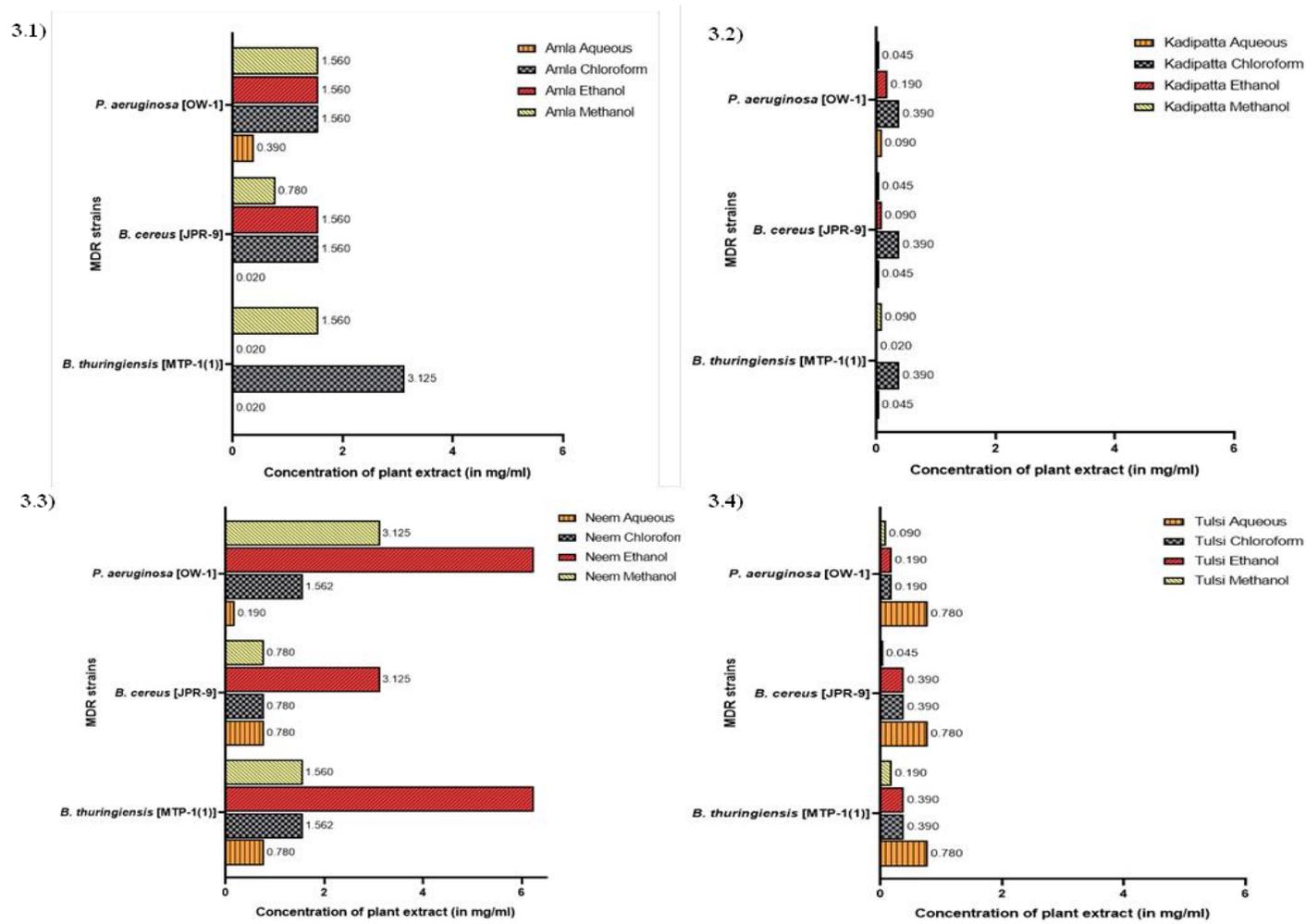


Figure 3 Comparison of MIC values of various plant extracts against selected MDR strains. (3.1) Amla extracts (3.2) Kadipatta extracts (3.3) Neem extracts (3.4) Tulsi extracts.

Table 3 The MBC values for various plant extracts tested against the candidate MDR isolates.

MBC value of plant extract tested (in mg/ml)		Strain Name		
		<i>B. cereus</i>	<i>B. thuringiensis</i>	<i>P. aeruginosa</i>
Amla	Aq.	6.25 [‡]	>25*	>25*
	CHL	>25*	>25*	6.25 [‡]
	EtOH	>25*	>25*	25
	MeOH	6.25 [‡]	3.125 [‡]	>25*
Kadipatta	Aq.	0.09 [‡]	0.09 [‡]	0.19 [‡]
	CHL	1.56 [‡]	1.56 [‡]	1.56 [‡]
	EtOH	0.78 [‡]	0.045 [‡]	0.39 [‡]
	MeOH	0.045 [‡]	0.09 [‡]	0.09 [‡]
Neem	Aq.	>25*	>25*	12.5 [‡]
	CHL	0.78 [‡]	1.56 [‡]	1.56 [‡]
	EtOH	3.125	6.25	6.25 [‡]
	MeOH	>25*	>25*	25 [‡]
Tulsi	Aq.	>25*	>25*	>25*
	CHL	>25*	>25*	>25*
	EtOH	1.56	>25*	0.78 [‡]
	MeOH	>25*	>25*	0.09 [‡]

([‡]) Indicates the bactericidal property of the plant extract. (*) Indicates the bacteriostatic potential of the plant formulation. Plant Extracts with MBC values >25mg/ml were designated to be bacteriostatic in nature.

4. Conclusion

In summary, the ever-evolving threat of AMR is at an unprecedented juncture today. The presently available drugs have become ineffective and compromised our fight against MDR pathogens. ~~To the rescue, p~~ Plant extracts and their bioactive phytochemicals ~~have shined~~ offer a ray of hope as emerging antimicrobials to combat the deadly bacterial manifestations. This study affirmatively establishes the ~~affirmative role~~ antimicrobial potential of Indian medicinal plants: Amla, Neem, Tulsi, and Kadipatta ~~harboring extensive antimicrobial activity~~. Amla, Tulsi, and Kadipatta extracts demonstrated prolific antibacterial activities even at low doses, indicative of their high potency and therapeutic efficacy. Neem extracts also exhibited significant antimicrobial responses, but at comparably high doses. Kadipatta extracts exhibited remarkable bactericidal properties, while Amla, Neem, and Tulsi extracts were bacteriostatic in nature. Considering the common usage of these plants among the Indian population, their phytoconstituents can be repurposed and exploited to serve as “life-saving elixirs”. A better understanding in this direction through systematic modifications and medicinal chemistry can be undertaken for lead identification to develop novel intervention strategies against emerging microbial pathogens and superbugs. As a future medicine and alternative to antibiotics, this can help in reducing the incidence of AMR with improved quality of patient life. Overall, this can reduce the global medical burden and provide substantial societal and economic returns, essential to improve life today and for future generations.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declare no conflict of interest.

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