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Antibacterial activity of some plant extracts alone and in combination with antibiotics against multidrug resistant potential pathogenic bacteria from sewage water in Kolkata, India

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ABSTRACT

Reports are available on multidrug resistant (MDR) potential pathogenic bacteria from industrial sewage from different parts of the globe. This study explores the antibacterial activity of plant extracts as well as phytochemicals against bacteria isolated from sewage-water from Kolkata (West Bengal), India. Methanolic extract of *Plumbago zeylanica (Plumbaginaceae), Costus specious (Costaceae)* and *Clerodendrum indicum (Lamiaceae)*, using different concentrations, were tested against five sewage bacteria from Kolkata, India: DC-1, DC-4, DC-8, DC-9 and DB-11, (selected on the basis of their highest antibiotic resistance out of total 30 isolates) by agar-well diffusion method. Three plant extracts viz. *Plumbago zeylanica (leaves) and Costus specious* (rhizome*) and Cerodendrum indicum* (leaves) were tested in combination following checker board agar dilution method. The antibiogram for the test bacteria were determined by disc diffusion method using five antibiotics. All the test bacteria were resistant to chloramphenicol (30μg/ml) and ampicillin (10 μg/ml). Among the three plants *Plumbago zeylanica* showed remarkable antibacterial efficiency even at 250μg/ml against the test bacteria. In combination the plant extracts had better activity against DC-4 and DC-8 strains. Phytochemicals individually and in combinations showed antibacterial activity against sewage bacteria that are potential to cause human infection. Current findings help understand in developing non-antibiotic treatment protocol. However, this require further studies (purification of active phytochemicals, detection of combinational products and their mode of action) to be used as therapeutics.

Keywords: Plant extracts, sewage bacteria, MDR, phytochemicals, *Plumbago zeylanica*.

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1. INTRODUCTION

Industrial waste water shows higher BOD (biological oxygen demand), TSS (total suspended solids), oily substances than non-polluted water (Porwal et al., 2015; Alade et al., 2011; Mohan et al., 2009). This type of water contaminated with organic materials is responsible for several ecological problems (Manh, 2008). It affects both flora and fauna, making drinking water contaminated and unfit for health (Manu et al., 2011; Gaikar et al. 2010; Dhanan, 2009; Utsev & Ekwu, 2020; Rajan, 2020; Afangideh & Udokpoh, 2022). Microorganisms, which are exposed in the aquatic environment with heavy metal contamination, accumulate different kinds of heavy metals inside the cell. Because of the presence of heavy-metals in the effluents from industries, major hazards to natural water, animal and human health are known to be caused. Also, high concentration of such heavy metal accumulation exerts hazardous effects on the environment (Cheng, 2003). Several heavy metals, such as Pb, Hg, Zn, Cu, Cr, and Cd are toxic, mutagenic and carcinogenic to many living organisms, including human (Adriano, 2001). Microorganism found in this water may have the potentiality to survive against wide array of different physical and chemical conditions. They may have ability to tolerate wide range of pH, salt, metal concentrations. Wastewater could play a role in the selection of antibiotic-resistant bacteria in sewage (Mindlin et al., 2001). Resistance to toxic metals is governed by genetic factors in many bacteria (Silver and Misra, 1988; Foster, 1983). Due to the selection pressure of heavy metals, the resistant microorganisms are emerged with a similar manner as the antibiotic-resistant bacteria are evolved. The association of metal and drug resistance is common since both resistance genes are frequently located on the same plasmid (Silver and Bostian, 1993). Antibacterial effects of phytochemicals have already been documented by Silver and Bostian (Cragg et al., 1997). Therefore, it is urgent and imperative to discover novel substances having effects on drug resistant pathogens. In this regard, many researchers have screened various plant extracts in order to detect the secondary metabolites displaying antibacterial activities. Several methods have been developed for screening of phytochemicals (Hamburget M, Hostettmann, 1991). Phytotherapeutics have many advantages over the synthetic drugs. Combination between phytocompounds and antibiotics may have synergistic effects on such MDR strains (Shariff). The inhibitory effects of isolated and purified phytochemicals and their combinations against the MDR clinical isolates also an important aspect of this research. Kolkata is a crowded city; its industrial waste load is very high. Heavy metals resistant and MDR bacteria from industrial sewage is very natural as they continuously facing a wide array of unusual chemicals. They have great impacts on the surrounding local communities in respect of health-related issues. There is a chance of infection of MDR bacteria of such polluted wastewater to the peoples of local area (Sudhin, 2022). There are several works on the canal water bacteria of Kolkata but my focus of work is on detailed study and to find out some phytoremedies that may take an important role for the improvement of public health.

2. MATERIALS AND METHODS

2.1. Bacteriological analysis of sewage waste water

Bacterial samples of canal waste-water were collected from the Bagjola canal of East Kolkata, West Bengal, India. The samples were labelled properly and transported to the laboratory for analysis. The samples collected were stored in the refrigerator at 4 °C to avoid any physical-chemical changes in the wastewater. Serially diluted samples were cultured on media to get different types of colonies. Each of the water sample, taking 100 µl from each, was inoculated into 2 ml of sterile nutrient broth medium (purchased from Hi-Media, India), and incubated at 37°C for 24h; thereafter, using an inoculating loop the broth cultures (from each sample) thus obtained were utilized for streak- dilution culture on different agar plates: nutrient agar, blood agar, cetrimide agar, and MacConkey agar (Hi-Media, India). After an incubation for 24 h at 37°C, the a single and morphologically distinct colonies seen on the agar plates were selected and stored in stabs prepared with cystine-tryptone agar (Hi-Media, India). Gram nature, biochemical test results and 16s rRNA sequencing data (neighbor joining method) are given in the result part in Table 1, and Figure 1 to Figure 5, respectively. On the basis of antibiotic resistance properties 5 isolates have been selected among the collected samples.

2.2 Collection of plant materials and extract preparation

On the basis of ethnobotanical sources *Plumbago zeylanica, Clerodendrum indicum* and *Costus speciosus were* collected and washed thoroughly in distilled water and dried under shade for overnight, then placed in hot air oven at 50 ℃ till proper drying according to WHO Guidelines (2003). Dried plant materials then crushed to make fine powder by using electrical grinder. 10% w/v methanolic extracts of crude powdered drugs were done by using Soxhlet apparatus. Extracted solvents then filtered by using Whatmann number 1 filter paper after cooling. Solvents were then condensed by evaporating in Rotary evaporator under reduced pressure. Weighed semisolid extracts were preserved at 4 ℃ for further analysis.

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2.3 Assessment of plant extracts for antibacterial activity

The effect of plant extracts (*Plumbago zeylanica, Clerodendrum indicum and Costus speciosus)* on the selected bacterial strains have been done by agar-well diffusion method (CLSI, 2011), in order to measure the zone diameter of the inhibition (ZDI), agar dilution method (Adriano, 2001), was also used to assess the MIC (minimum inhibitory concentration) values, as represented in Figure 3, the details of which are described elsewhere.

2.4 Antibiotic susceptibility test

The antibiotic-susceptibility test was done for the bacterial isolates following disk diffusion method (Kirby et al., 1966), using Mueller-Hinton agar (Hi-Media, India) medium. The results, in terms of zone diameter of inhibition (ZDI) obtained around each of the antibiotic discs for the isolates, were interpreted following the criteria of the Clinical Laboratory Standards Institute (CLSI, 2011). Results were summarized in Table 2. Five samples viz. DC1, DC4, DC8, DC9 and DB11 were selected, based on their highest antibiotic resistant properties.

2.5 Combined antibacterial activity of plant extract and antibiotics

Tetracycline (30mcg/ml) Streptomycin (10mcg/ml) Ampicillin (10mcg/ml) Norfloxacin (10mcg/ml) Chloramphenicol (30mcg/ml) were combined with the *Plumbago zeylanica* leaf extracts to determine their combined effects on the selected MDR isolates. *Plumbago zeylanica* leaf extracts were combined with other two plant extracts as well.

The GII (growth inhibitory index) values calculated were interpreted (Table 4) following the criteria published elsewhere, in order to express the nature of the interaction (synergistic, additive, or antagonistic) between antibiotics and plant extract. Combined drug effects have been summarized in the result section.

3. RESULTS AND DISCUSSION

Gram nature, morphology and biochemical tests of the five natural sewage canal isolates have showed that there are four Gram positive bacteria and one Gram negative bacteria. *Enterococcus cloacae,* Gram positive coccus is a potential human pathogen and others are rod shaped. Biochemical test responses of the said bacteria have been summarized in Table 1.

The growth inhibition of antibiotic resistant of *E. coli* and *Staphylococcus aureus* was achieved when the strains were inoculated into an antibiotic (streptomycin, rifampicin) mixed medium and showed a delayed growth due to the resistance. However, the growth was completely prevented when the bacteria were grown in medium with combination of antibiotic and Plumbagin. (Durga *et al.*, 1991). All the test bacteria showed resistance against chloramphenicol 30mcg/ml and ampicillin 10 µg/ml. DC 9 and DB11 showed their resistance against all antibiotics applied. Summary are given in Table 2.

Table 1: Characterization of isolated sewage waste water bacteria

Bacterial isolates	Tests											
	Indole	VP	MR	Citrate	TSI	UTI	Catalase	Protease(mm)	Lipase	Amylase	Gram Nature	Morphology
Weizmannia coagulans (DC1)	-ve	-ve	-ve	+ve	Yellow slant	Blue small	+ve	7	P	+ve	+ve	Rod shaped
Aeromonas caviae (DC4)	+ve	-ve		+ve	Yellow slant	Blue small	+ve	14	...	...	-ve	Rod shaped
Enterococcus cloacae (DC8)	-ve	+ve		-ve	Red slant yellow bud		-ve	13	P	N	+ve	Cocci
Bacillus albus (DC9)	-ve	-ve		P	-ve	No growth	+ve	13	N	P	+ve	Rod shaped
Bacillus subtilis (DB11)	-ve	+ve	-ve	+ve	Red slant yellow bud	No growth	+ve	17	N	P	+ve	Rod shaped

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Table 2: Antibiotic susceptibility of test bacteria

Bacteria	Zone diameter of inhibition				
	Tetracycline 30 µg/disc	Streptomycin 10 µg/disc	Ampicillin 10 µg/disc	Norfloxacin 10 µg/disc	Chloramphenicol 30 µg/disc
Weizmannia coagulans (DC1)	7	7	6	**18**	7
Aeromonas caviae (DC4)	22	8	6	6	8
Enterococcus cloacae (DC8)	6	23	7	19	6
Bacillus albus (DC9)	6	6	7	6	6
Bacillus subtilis (DB11)	7	6	7	7	6

Table 3: Bacterial sensitivity and combinational study

Concentrations → Bacteria ↓	PL	CL	CS	A+ PL	C+ PL	S+ PL	T+ PL	CL+ PL	CS+ PL	CL+ CS
	Zone diameter of inhibition (mm)									
Weizmannia coagulans (DC1)	25	06	15	25	24	25	25	23	25	13
Aeromonas caviae (DC4)	24	13	06	26	28	26	25	20	26	11
Enterococcus cloacae (DC8)	23	12	06	25	24	23	31	22	24	11
Bacillus albus (DC9)	27	12	13	27	26	31	26	26	26	14
Bacillus subtilis (DB11)	28	15	12	27	28	27	30	26	27	14

PL: *Plumbago zeylanica* leaf crude extract (500mcg/ml), CL:*Clerodendrum indicum* leaf extract(500mcg/ml), CS- *Costus speciosus* rhizomqqqe extract 500mcg/ml, A: Ampicillin 10 mcg/ml, C: Chloramphenicol 30mcg/ml; S: Streptomycin 10mcg/ml, T: Tertacyclin 30 mcg/ml

Table 4: The nature of interactions and Growth Inhibitory Index (GII) values from combined antibacterial activity of antibiotics (A, C, S, T) and *Plumbago zeylanica (PL)* leaf extract.

Bacterial strains	Combined agents	GII	Interaction*
DC1	A+PL	25/(25+6)=0.80	Synergistic
	C+PL	24/(25+6)=0.77	Synergistic
	S+PL	25/(25+6)=0.80	Synergistic
	T+PL	25/(25+6)=0.80	Synergistic
DC4	A+PL	26/(24+6)=0.86	Synergistic
	C+PL	28/(24+6)=0.93	Synergistic
	S+PL	26/(24+6)=0.86	Synergistic
	T+PL	25/(24+22)=0.54	Synergistic

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DC8	A+PL	25/(23+6)=0.86	Synergistic
	C+PL	24/(23+6)=0.82	Synergistic
	S+PL	23/(23+23)=0.50	Synergistic
	T+PL	31/(23+6)=1.06	Synergistic
DC9	A+PL	27/(27+6)=0.81	Synergistic
	C+PL	26/(27+6)=0.78	Synergistic
	S+PL	31/(27+6)=0.93	Synergistic
	T+PL	26/(27+6)=0.78	Synergistic
DB11	A+PL	27/(28+6)=0.79	Synergistic
	C+PL	28/(28+6)=0.82	Synergistic
	S+PL	27/(28+6)=0.79	Synergistic
	T+PL	30/(28+6)=0.88	Synergistic

GII (growth inhibitory index); *values ranges from 0.5 to 1.06 and all are synergistic in nature.

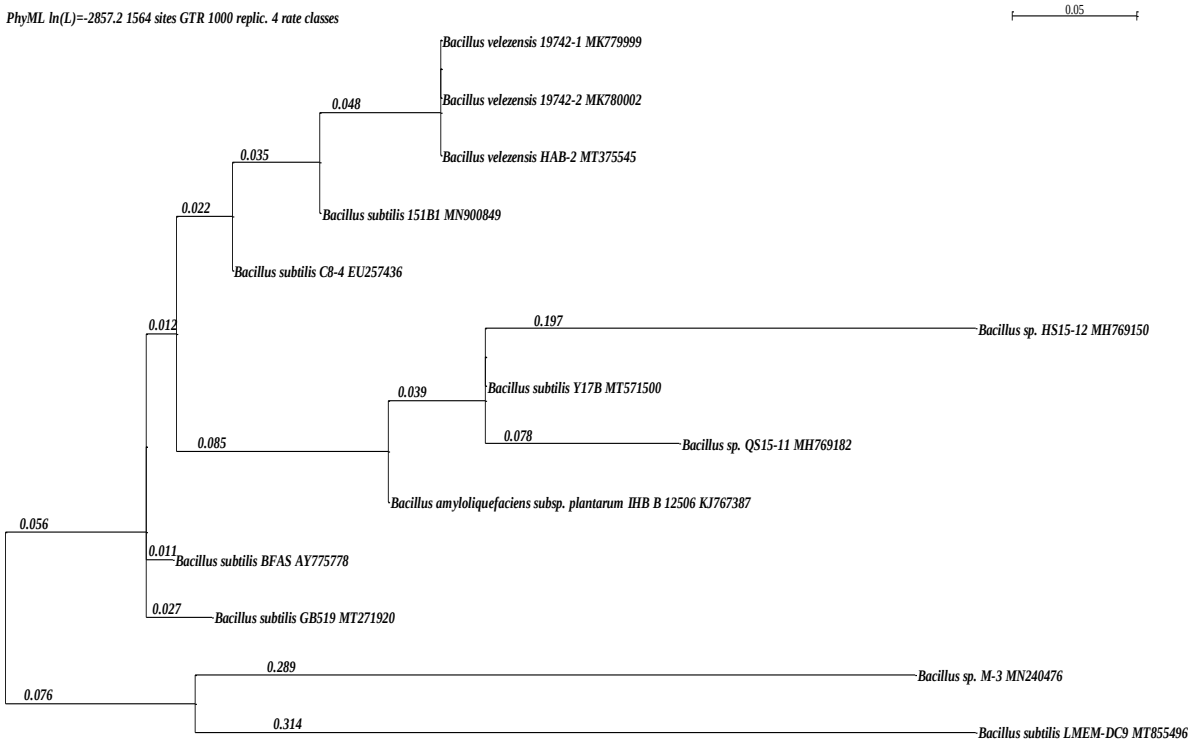

Figure 1: Phylogenetic tree of LMEM_DC9-UGB-bs, NCBI ACCESSION NUMBER MT855496 on the basis of 16s rRNA gene sequencing data that was compared with the sequences of closely related reference bacterial strains retrieved from the NCBI Gene Bank database.

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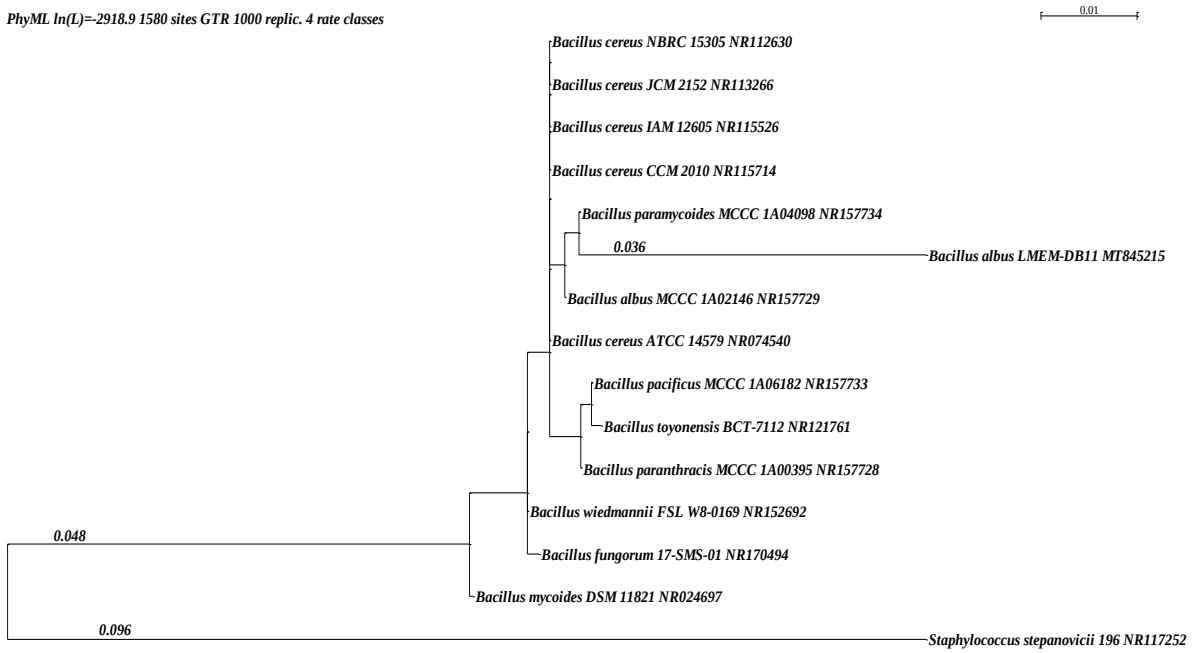

Figure 2: Phylogenetic tree of LMEM DB11-UGB-ba, MCBI ACCESSION NO- MT845215 on the basis of 16s rRNA gene sequencing data that was compared with the sequences of closely related reference bacterial strains retrieved from the NCBI Gene Bank database.

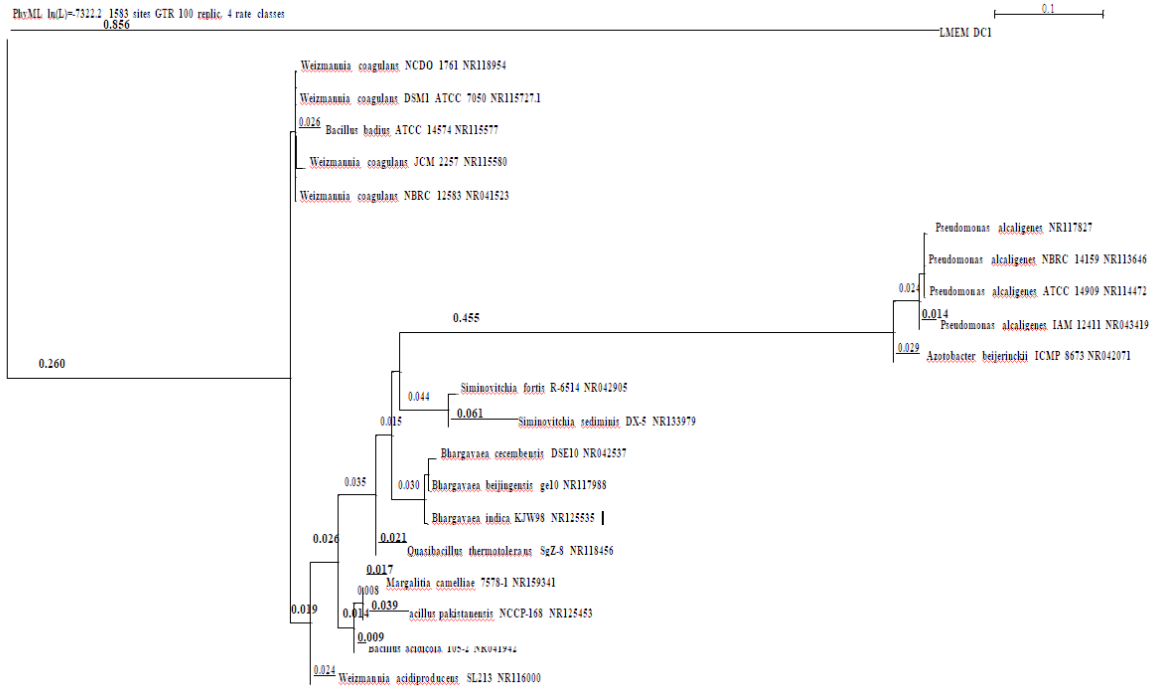

Figure 3: Phylogenetic tree of LMEM DC-1 on the basis of 16s rRNA gene sequencing data that was compared with the sequences of closely related reference bacterial strains retrieved from the NCBI Gene Bank database.

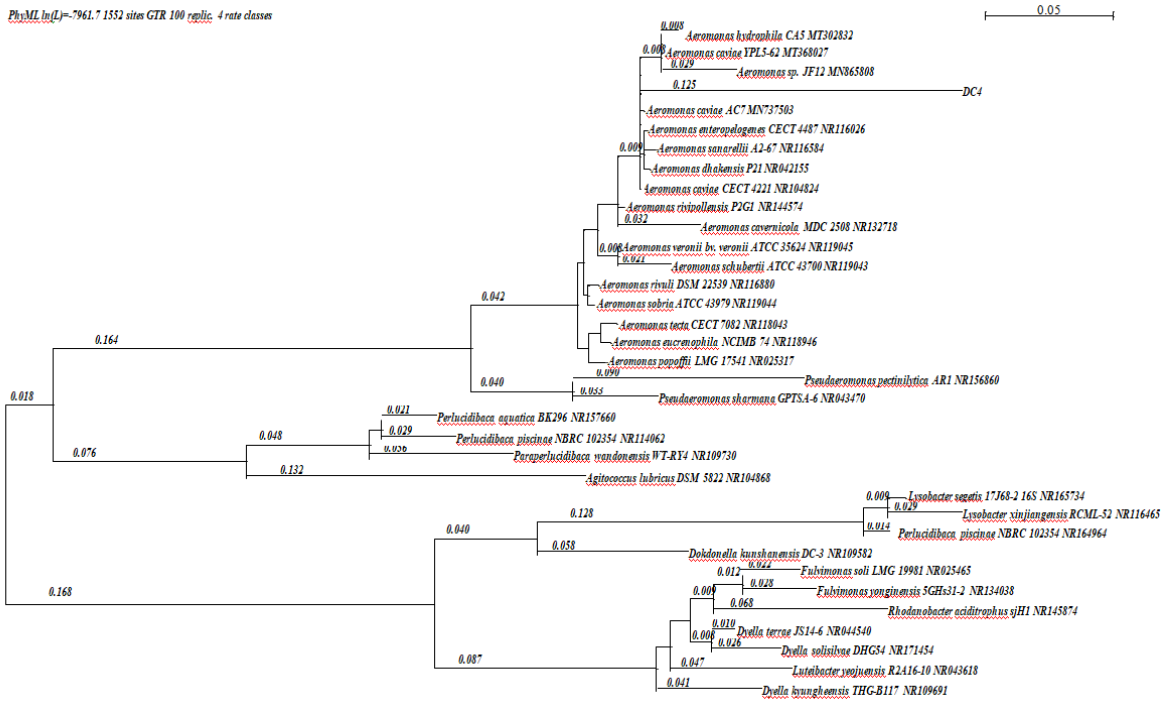

Figure 4: Phylogenetic tree of LMEM DC-4 on the basis of16s rRNA gene sequencing data that was compared with the sequences of closely related reference bacterial strains retrieved from the NCBI Gene Bank database.

Figure 5: Phylogenetic tree of LMEM DC-8 on the basis of16s rRNA gene sequencing data that was compared with the sequences of closely related reference bacterial strains retrieved from the NCBI Gene Bank database.

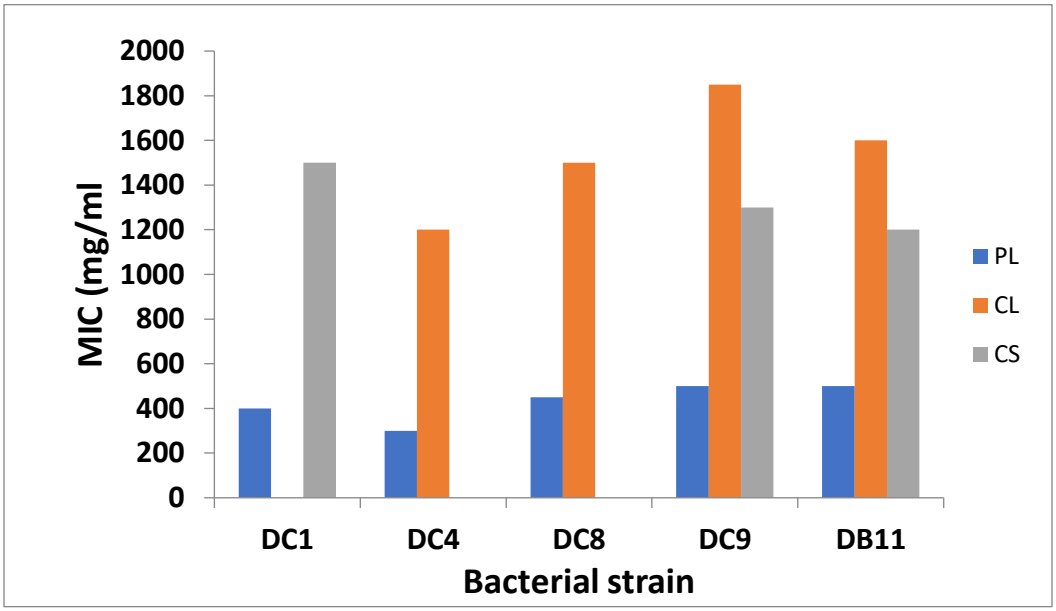

Figure 6: Minimum inhibitory concentration (MIC) values of plant extracts against test bacteria.

The leaf extract of *Plumbago zeylanica* has antibacterial activity (Dhale et al., 2011), and the napthaquinone compound isolated from this plant is responsible for this activity (Paiva *et al.,* 2003). The water and different organic solvents (ethanol, ethyl acetate and acetone) extracts of the pant, *Plumbago zeylanica* were used in order to determine the anti-bacterial activity. The ethyl acetate extract had displayed the MICs from 0.32 to 1.28 mg/ml, against five *Helicobacter pylori* isolates tested, in ascending order of acetone, ethanol and water (Wang and Huang, 2005). The water extract of the plant as reported by the said other, and its partition with petroleum ether, methanol, dichloromethane, and the aqueous constituents were effective against gram-negative (*Salmonella gallinarum, Pseudomonas aeruginosa, Escherichia coli, Proteus vulgaris, Salmonella typhimurium,*), as well as gram-positive (*Staphylococcus aureus)* bacteria. Our MIC test results for the isolated bacteria are displayed in Figure 6.

The *Plumbago zeylanica* root alcoholic extract had been tested against multi-drug resistant of clinical bacteria, both gram-positive and gram-negative: *Salmonella paratyphi, Escherichia coli, Staphylococcus aureus,*and *Shigella dysenteriae.* Methanolic extract of *Plumbago zeylanica* showed remarkable antibacterial activity against all tested isolates. Extracts of *Clerodendrum indicum* and Costus *speciosus* also having inhibitory effects against said bacteria. Summary of antimicrobial effect of selected plant extracts against identified bacterial strains have been summarized in Table 3.

Plumbago zeylanica was tested against the antibiotic resistant *Mycobacterium tuberculosis* (H37, RV), for which the inhibitory activity of *Plumbagin* was < 12.5 mg/ml (Mossa *et al.,* 2004). The antibacterial activity of isonicotinic acid hydrazide against *M. smegmatis*, *Mycobacterium intracellurum*, *M. xenopei* and *M. chelonei*, combined with plumbagin was lowered from a MIC value of 1.25-2.5 mg/ml to 0.15-0.3 mg/ml (Mossa *et al.,* 2004). *Plumbago zeylanica* have lesser value of MIC in comparison to other two plant extracts. It ranges from 250 µg to 450 µg for the *Plumbago zeylanica* methanolic leaf extract. MIC results of our study are graphically represented in Figure 3.

4. CONCLUSION

All the test bacteria were resistant to chloramphenicol (30µg/ml) and ampicillin (10µg/ml). Among the three plants *Plumbago zeylanica* crude leaf extract showed remarkable antibacterial efficiency even at 250µg/ml against the test bacteria. In combination the plant extracts had better activity against DC-4 and DC-8 strains. Phytochemicals individually and in combinations showed synergistic antibacterial activity against sewage bacteria that are potential to cause human infection. Current findings help to understand in developing non-antibiotic treatment protocol. However, this ± further Studies (purification of active phytochemicals, detection of combinational products and their mode of action) to be used as therapeutics. Moreover, the plant extracts, alone or combined with the conventionally used antibiotics, might be effective as biotherapeutics to combat antibiotic resistant bacteria causing serious infection to humans.

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Conflicts of interests

The authors declare that there are no conflicts of interests.

Data and materials availability

All data associated with this study are present in the paper.

REFERENCES AND NOTES

1. Adriano D.C. Trace elements in terrestrial environments: biogeochemistry, bioavailability, and risks of metals: (2001); Springer
2. Afangideh CB, Udokpoh UU. Environmental impact assessment of groundwater pollution within cemetery surroundings. Indian Journal of Engineering, 2022, 19(51), 100-115
3. Alade AO, Jameel AT, Muyibi S.A. Application of semi fluidized bed bioreactor as novel bioreactor system for the treatment of palm oil mill effluent (POME). Afr. J. Biotechnol., 2011; 10:18642–18648.
4. Cheng, S. Heavy metal pollution in China: origin, pattern and control. Environmental Science and Pollution Research, (2003); 10(3), 192- 198.
5. Clinical and Laboratory Standards Institute (CLSI): Performance standards for antimicrobial susceptibility testing (2011), 21st informational supplement M100S21. CLSI, Wayne, Pa.
6. Cragg, G M., Newmann, D. J. and Snadder, K. M. Natural products in drug discovery and development. Journal of Natural Products 1997; 60: 52-60.
7. Dhanan S. Effect of dairy effluent on seed germination, seedling growth and biochemical parameters in paddy. Bot. Res. Int., 2 (2009), 61–63.
8. Foster T.J. Plasmid-determined resistance to antimicrobial drugs and toxic metal ions in bacteria. Microbiol. Rev. 1983; 47(3):361–409.
9. Gaikar RB, Uphade BK, Gadhave AG, Kuchekar SR. Effect of dairy effluent on seed germination and early seedling growth of soybeans. Res J. Chem., 3 (2010), 137–139.
10. Guardabassi L, Petersen A, Olsen J, Dalsgaard A. Antibiotic resistance in Acinetobacter spp. isolated from sewers receiving waste effluent from a hospital and a pharmaceutical plant. Appl Environ Microbiol. 1998; 61:3499–350.
11. Hamburget M, Hostettmann K, Bioactivity in Plants: the link between phytochemistry and medicine 1991; 30(12), 3864-3874.
12. Kirby W, Bauer W, Sherris J, and Turck M, (1966) "Antibiotic susceptibility testing by a standardized single disk method," The American Journal of Clinical Pathology, 45, 4: 493–496.
13. Manh D. Bioremediation of vegetable oil and grease from polluted wastewater in dairy factory. J. Sci. Nat. Sci. Technol., 24 (2008), 56–62.
14. Manu KJ, Mohana VS, Ganeshaiah KN. Effluent generation by the dairy units: characterization and amelioration for irrigation. Int. J. Res. Chem. Environ., 1 (2011), 173–182.
15. Mindlin S., Kholodii G., Gorlenko Z., Minakhina S., Minakhin L., Kalyaeva E., Kopteva A., Petrova M., Yurieva O., Nikiforov V. Mercury resistance transposons of Gram-negative environmental bacteria and their classification. Res. Microbiol. 2001; 152(9):811–822.
16. Mohan S, Mohanakrishna VG, Goud RK, Sarma PN. Acidogenic fermentation of vegetable-based market waste to harness biohydrogen with simultaneous stabilization. Bioresourse Technol., (2009); 100:3061–3068.
17. Porwal J, Manea AV, Velhal SG. Biodegradation of dairy effluent by using microbial isolates obtained from activated sludge. Water Res 2015; 9: 1–15.
18. Rajan DS. An exploratory study and elucidation of the nitrogen content of an Estuary. Discovery, 2020, 56(299), 791-804
19. Silver LL, Bostian KA, Discovery and development of new antibiotics: the problem of antibiotic resistance. Antimicrobial Agents and Chemotherapy1993; 37: 377–383.
20. Silver S., Misra T.K. Plasmid-mediated heavy metal resistances. Annu. Rev. Microbiol. 1988; 42:717–743.
21. Sudhin AS. Characteristic Study of sul3 gene expression, protein profiling and multi resistance in Salmonella typhi. Drug Discovery, 2022, 16(37), 1-8.
22. Utsev JT, Ekwule OR. Effect of pollution due to human activities at Naka dam Benue State, Nigeria. Discovery, 2020, 56(289), 59-65