

Isolation Of Plumbagin From *Plumbago Zeylanica* And Its Effect On The MDR Heavy Metal Tolerant Sewage Canal Bacterial Isolates

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Natural plant products have an important contribution in drug development programmes in the pharmaceutical industry. There is an urgent need to identify novel active substances against multiple drugresistant pathogens (MDR). *Plumbago zeylanica* Linn. is one of the well-known natural remedy of the traditional ayurvedic medicine. Plumbagin, a naphthoquinone derived from *Plumbago zeylanica* shows excellent antibacterial activity against a variety of pathogenic bacteria. The inhibitory effects of isolated and purified phytochemicals in *Plumbago zeylanica* against the identified MDR and heavy metal tolerant natural isolates have been performed.

Test bacteria are identified MDR, tolerant against heavy metals like Sr, Cd, Cu, Ni, Pb. 10% W/V methanolic extract of *Plumbago zeylanica* leaves have been prepared and purified to isolate the active compounds. Isolated active compound (plumbagin) from *Plumbago zeylanica* exhibit remarkable inhibitory effect against all selected MDR, heavy metal tolerant bacteria. Plumbagin may play an important role against such antibiotic resistant, metal tolerant, potentially pathogenic bacteria and may open up a new vista in the field of production of natural antibiotics.

Keywords: MDR, Metal tolerant, Natural isolates, Plumbagin, *Plumbago*, Sewage bacteria.

Introduction:

Polluted industrial wastewater is a big threat now a days globally for its altered physicochemical and biological parameters. It is characterised by high BOD (biological oxygen demand), TSS (total suspended solids), oily and greasy substances (Mohan et al.,2009; Alade et al.,2011; Porwal et al.,2015). Wastewater contaminated with organic materials is also responsible for several environmental ailments (Manh,2008). It affects different fauna and flora and making the pure drinking water unsafe for human and animal health as well (Manu et al.,2011; Utsev & Ekwu,2020; Rajan,2020). Microorganisms are

capable to accumulate heavy metals in their body. Industrial effluents with heavy metals have high chance of contamination to local communities and lead major hazards to natural water, animal as well as human health (Sudhin,2020). High concentrations of heavy metals in the water have deleterious effects on the environment (Cheng,2003). Heavy metals like Cr, Hg, Cu, Zn, Sr, Pb and Cd are toxic, mutagenic and carcinogenic (Adriano,2001). Toxic metals resistance is governed by specific genetic factors in many bacteria (Silver and Misgra,1988; Foster,1983). In nature, metals may select these resistant bacteria in a manner similar to the selection of drug resistant strains. Same plasmid possesses both the antibiotic resistance and metal tolerance genes is very frequent in nature (Silver and Bostian,1993). Phytochemicals showing antimicrobial properties against MDR, heavy metal tolerant bacteria already been documented by several workers (Cragg et al.,1997; Hamburger M, Hostettmann,1991). Plumbagin is the main active compound found in the plant *Plumbago zeylanica*. (Richa Tyagi et al.,2014). Leaf extract of *Plumbago zeylanica* has antibacterial activity. (D A Dhale et al.,2011). Naphthaquinone compound found from this plant is responsible for this activity. (Selma Ribeiro de Paiva et al.,2003). Several chromatographic analysis are to do to reveal the types, nature, purification and isolation of active natural drugs (A. H. Dar et al., 2020)

Materials and Methods:

Bacterial samples

Test bacteria were isolated from sewage canal samples and identified as *Weigmannia coagulans*, *Aeromonas caviae*, *Enterococcus ludwigii*, *Bacillus albus* and *Bacillus subtilis*. They were MDR in nature. (Das RC, Mandal S,2022)

Screening of heavy metal tolerance

Five heavy metals (Sr,Cd,Ni,Cu,Pb) were used to see their effect on the test bacteria. 5,10 and 15 mM of each metals have been applied on the inoculated (10⁸ CFU) Muller-Hinton agar plates. ZDIs were recorded after 24 hrs incubation at 37° C. Any growth inhibitory zones in the plate more than 6mm in the plate indicates susceptibility of the test bacteria against heavy metals.

Collection of plant material and extract preparation

Plumbago zeylanica L. (plumbaginaceae) leaves have been collected from the Gurudas College medicinal plants garden, Kolkata-700054, India. 10% w/v methanolic extract of dried, crude powdered drugs were done by using Soxhlet apparatus (Borosil, India) (Das

RC, Mandal S,2022). Solvents have been condensed by rotary evaporator at 60°C for 4 hrs at reduced pressure (-20 hg).

Thin Layer Chromatography (TLC) of the *P. zeylanica* crude leaf extract.

Crude methanolic extract have been spotted on a TLC sheet (stationary phase) with the help of capillary tube. n-Hexane: Ethyl acetate (8:2) were used as mobile phase. Spots were marked chronologically and acropetally and viewed in visual light and under UV light at 254 nm. (fig-1a&b)

Characterization of the TLC spots

Rf values of the distinct visual spots have been measured(table-1) by using the following formula:

$$R_f = \frac{\text{Distance travelled by the visual spot}}{\text{Distance travelled by the mobile phase(solvent)}}$$

Column Chromatography of the *P. zeylanica* crude leaf extract

About 50 cm of the Borosilicate column(diameter-30cm) was filled with the 60grade silica gel powder (stationary phase). Column was filled with the mobile phase solvent (n-Hexane: Ethyl acetate 8:2), then some mobile solvent was passed through the silica gel stationary phase to pack the column. 1g of crude extract was mixed thoroughly with the silica gel and then applied at the top of the column. Column have been runed slowly by opening the lower tap so that the mobile solvent was passing drop wise. Eluted mobile solvent have been collected in the clean and autoclaved glass tubes, maintaining the same volume (10 ml) in each tube. 17 column fractions have been collected (fig-2b). Column Fractions were blended to make 5 pools on the basis of their TLC behavior and spectroscopic properties. (table-3)

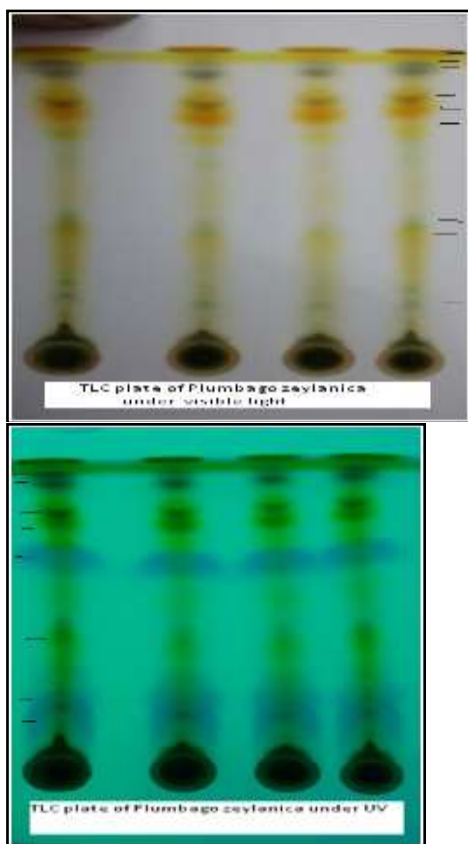
Bacterial sensitivity against isolated plumbagin and its MIC detection:

Pooled fractions were evaporated to make it dry and calculate yield. 500 µg/ml of different pools have been applied on the five MDR isolates (108 cfu) to detect their antimicrobial activity by agar well diffusion method using Mueller-Hinton agar (Hi-Media, India) plates. After incubation for 24 hours at 37°C, the assessment of antibacterial activity was done on the basis of measurement of the zone diameter of the inhibition (ZDI) formed (table-4). That determines the pool actually responsible for growth inhibition of said bacteria.

The minimum inhibitory concentration (MIC) values of the plumbagin against the MDR isolates were determined by agar dilution method using different concentrations of isolated plumbagin ranging from 25 to 500 µg/ml in Muller-Hinton Agar plates. Plates were inoculated with approximately 10⁴ cfu /spot and then incubated at 37°C for 24h. The MICs were considered as the minimum concentration of plumbagin at which no visible growth was found. Hazy growth and one or two colonies on the spot were ignored.

Results:

Fig.1: TLC plates of *Plumbago zeylanica* leaf extracts: a) spots visualized under visible light. b) spots underUV.



a

b

Ten spots under visible light and nine spots under UV. Spots have been marked chronologically and acropetally with Rf values ranging from 0.1 to 0.96 (fig-1)

Table-1: Different spots under visual light and UV light with Rf values and color.

Visibility of the TLC plate					
Under Visible light			Under UV (254 nm)		
Spots	Rf values	Color	Spots	Rf values	Color
1	0.17	Green	1	0.17	Blue
2	0.21	Green	2	0.21	Blue
3	0.43	Yellow	3	0.48	Green
4	0.48	Green	4	0.71	Blue
5	0.75	Yellow	5	0.79	Blackish
6	0.79	Orange	6	0.82	Blackish
7	0.82	Blackish	7	0.90	Blackish
8	0.90	Blackish	8	0.92	Blackish
9	0.92	Blackish	9	0.96	Blackish
10	0.96	Orange			

Table-2: Effect of collected scratched spots (under visual light) on the isolated MDR bacteria represented in respect of their ZDI.

TLC Spots	Zone diameter of inhibition (mm $\pm$ SD)				
	DC1	DC4	DB6	DC9	DB11
1	6	8	6	6	6
2	7	6	6	6	7
3	8	9	8	7	7
4	12	8	9	9	10

5	21	20	18	22	21
6	8	6	12	10	11
7	8	7	6	7	8
8	6	8	6	7	7
9	6	6	6	6	6
10	6	6	7	7	6

Phytochemicals present in different spots showed more or less antibacterial activity against isolated bacteria. Out of the ten spots, spot number 5 have shown distinct effects against all test bacteria. Other spots have shown no or negligible antibacterial activity. Rf value and Color of spot 5 was 0.75 and bright yellow respectively in case of visual light spot but 0.79 and blackish respectively under the UV light.

Figure 2: Column chromatography of *Plumbago zeylanica* leaf extract. a) column showing separated colored bands. b) column fractions were collected in the test tubes.

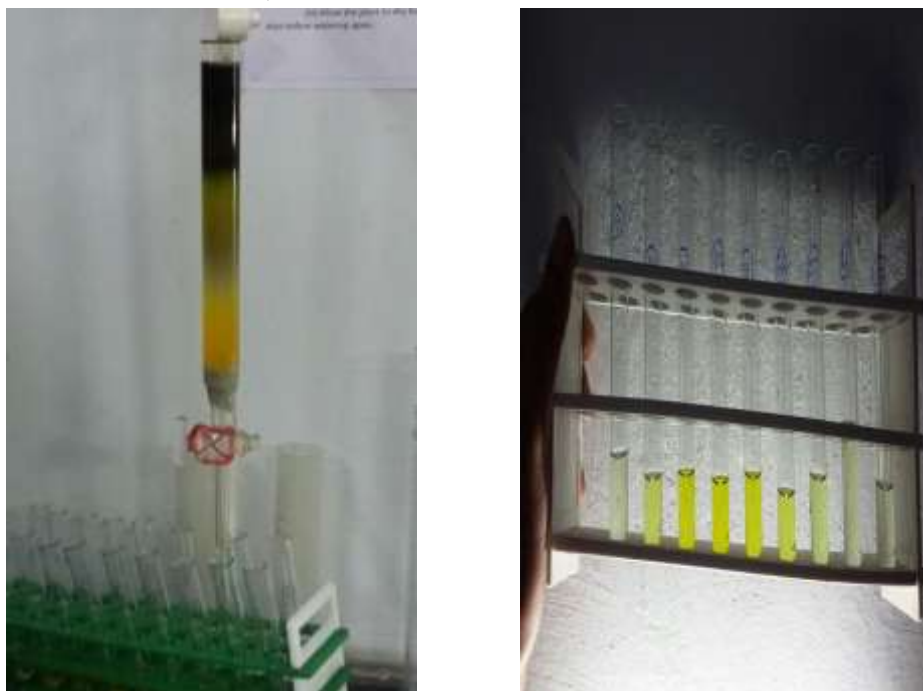
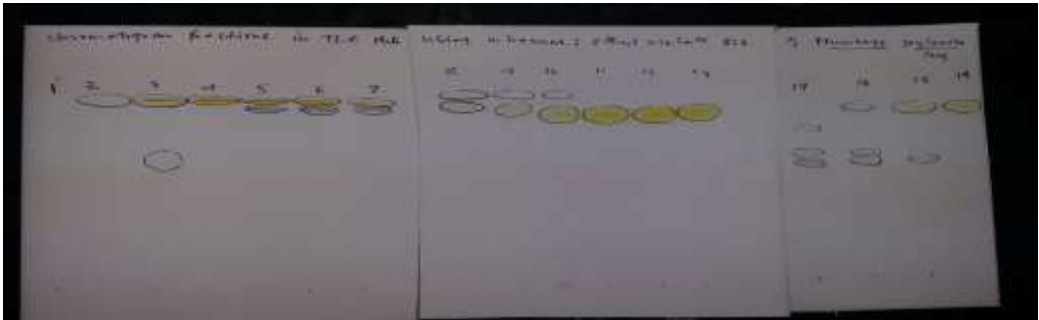


Figure 3: TLC behavior of the column fractions.



Seventeen column fractions have been blended to get five pools on the basis of their spectrophotometric behaviour and TLC spots homology.

Table 3: Column fractions with their spectroscopic and TLC behavior

Column fraction s	Peaks in (nm)	λ - max with absorptio n	No of spots in TLC	Rf values of the spots	Pool
1			----	----	
2			1	0.95	1
3			2	0.96, 0.68	
4			1	0.96	
5	240,340,450,476	450(2.86)	2	0.96, 0.93	2
6	423,450	450(1.00)	2	0.96, 0.93	
7	289,412,448,476	289(0.46)	2	0.95, 0.93	
8	2.89,411,445,472	289(0.38)	2	0.91, 0.84	3
9	262,411	262(0.43)	2	0.91, 0.83	
10	408	408(0.95)	2	0.91, 0.79	
11	262,270,403.5	262(4.10)	1	0.78	4
12	261,274,403,412	261(4.20)	1	0.78	
13	410	410(3.16)	1	0.78	
14	260,408	260(4)	1	0.77	5
15	262,409	262(1.68)	2	0.77, 0.61	
16	262,305,321,409	409(0.56)	3	0.77, 0.64, 0.61	

17	266,320,409.5	409.5(.86)	3	0.74, 0.64, 0.61	
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Column fractions 11,12,13 and 14 have been grouped in pool 4, they showed distinct bright yellow color with Rf values 0.78,0.78,0.78 and 0.78 respectively. They have shown their absorption homology in spectroscopic analysis.

Table 4: Antibacterial activity of pooled column fractions.

Pools (100µg/ml)	Zone diameter of inhibition (mm)				
	W. coagulans	A. caviae	E. ludwigii	B. subtilis	B. albus
1	6	6	6	7	6
2	7	6	7	6	8
3	12	12	9	8	7
4	21	22	32	28	28
5	9	12	6	10	10

Pool 4 contains the potent phytochemicals that is responsible for the antimicrobial activity. Other pools have shown negligible activity against isolated bacteria. Pool 4 has been evaporated in water bath to collect the dry sample.

Table-5: Heavy metal tolerance screening of test bacteria

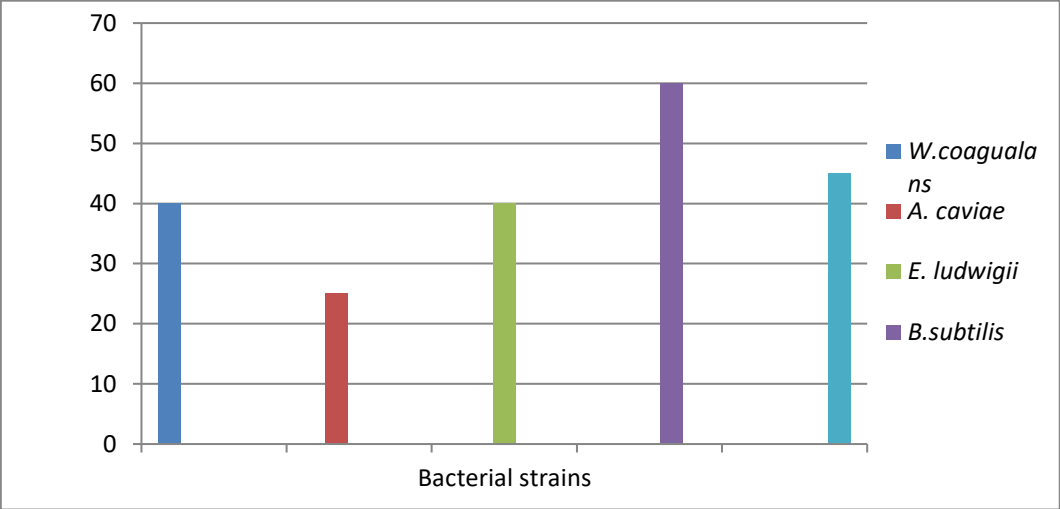
Heavy metals	Sr (mM)			Cd (mM)			Cu (mM)			Ni (mM)			Pb (mM)		
Bacteria	5	10	15	5	10	15	5	10	15	5	10	15	5	10	15
W. coagulans	R	S	S	S	S	S	R	S	R	R	S	S	R	S	S
A. caviae	R	S	S	R	S	S	R	S	R	R	R	S	R	S	S
E. ludwigii	R	S	S	S	S	S	R	S	S	R	R	S	R	R	S
B. subtilis	R	S	S	S	S	S	R	R	R	R	R	S	R	R	S

B. albus	R	R	S	S	S	S	R	R	S	R	S	S	R	R	S
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(Sr-strontium, Cd-cadmium, Cu-copper, Pb-lead, Ni-nickel, mM- 5,10,15 mili Molar, R-resistant, S- succceptible)

All the test bacteria (05) were found resistant against Sr,Cu,Ni,Pb at 0.5 mM concentration except A.caviae that showed resistance against Cd also. Almost 50% bacterial strains showed tolerance against all five heavy metals tested even at 10 mM concentration. Three out of ten tested bacteria showed resistant against 15 mM Copper. Zevenhuizen et al., isolated different strains of Pseudomonads that were tolerant to 10-30 M Cu2+. Tornabene et al.,1972 described Micrococcus luteus and Azotobacter sp. Capable of immobilize 49 and 310 mg of lead/g dry cell weight.

Figure 4: MIC values of plumbagin:



Plumbagin showed remarkable MIC values against all tested MDR bacterial strains. MIC values ranging from 25 μg/ml against A. caviae - 60 μg/ml against B.subtilis.

Identification of the phytochemical(s) present in the Pool-4

Physical characteristics –Bright yellow color Compared by TLC study with the pure synthetic Plumbagin.

Appearance of Red color by spraying with 10 % w/v ethanolic solution of KOH followed

by heating at 100°C indicates the presence of Plumbagin in the TLC spots. (Sankaram et al. 1986)



Figure 4: LCMS spectra of purified column fractions (Pool-4)

In LCMS study, molecular analysis revealed that the major peak corresponds 100 % to plumbagin (MW 189.12 g/mol). However, there were appearing other minor peaks of different molecular masses which might be due to similar polarity and homology between the molecules, making them separate at the same retention time, resultantly could not be analyzed.

Discussion:

P. zeylanica leaf extract (200mg/ml) showed zone diameter inhibition against *Bacillus subtilis*, *Klebsiella pneumoniae*, *Escherichia coli* and *Staphylococcus aureus* measured 16mm, 15mm, 15mm and 13.5mm respectively. ZDI by a variety of antibiotic drugs R-CPX-S-SXT-AM-Z-AP- CN-E-PEF against these bacteria were measured as 13-21-15-15-14-20-R-22-14-15mm respectively (Adegbite and Ayoade, 2014). Antibacterial activity of the methanolic leaf extract of *Plumbago zeylanica* detected by Dr. Sao Shweta & Swati Dubey, 2015 against *Bacillus* sp, *Staphylococcus* sp, *E. coli*. ZDI values by using well diffusion method were measured as 7.2, 8, 8 mm respectively. In this study ZDIs recorded between 21- 32 mm even at the lesser concentration i.e. 100 mg/ml) against the test MDR bacteria. MIC value of isolated plumbagin to methicillin resistant *Staphylococcus aureus* (MRSA) was recorded as 4-8 µg/ml by H. Periasamy et. al. (2019). Laxmi VV et al. (1987) noticed a range of MIC values (50- 400 µg/ml) of plumbagin against MDR *Escherichia*

coli strains. In this study MIC values of isolated plumbagin found 25 -65 µg/ml against said MDR isolates. *Plumbago zeylanica* ethanolic root extract showed 3 green colored spots in TLC study with Rf values 0.49, 0.62, and 0.82 under UV (254 nm) and 5 bluish grey spots with Rf values 0.26, 0.28, 0.49, 0.51, 0.64, and 0.72 and 1 pale brown spot with Rf value 0.49 have been noticed under visible light. Toluene: ethyl acetate: formic acid 5:1.5:.05 v/v used as mobile phase (Brijendra Singh et al. 2010). In this study, Plumbagin resolved at Rf 0.75 under UV and 0.79 in visible light. color of the spot was yellow and black respectively.

Spectroscopic analysis of plumbagin showed the maximum absorption at 253 and 406 nm while using methanol as solvent. In our study maximum absorption shows in 260 and 450 nm. LCMS data of plumbagin showed major peak at 189 mol. wt. (Nayana S.K et al. 2005) Plumbagin in our plant sample has been detected Chromato-spectrometrically (LCMS) by its peak at molar mass 189.2 region. Carvalho VR, (1986) showed the TLC of the chloroform fraction of *Plumbago zeylanica* leaf extract on silica gel thin layer plates using chloroform: methanol (8:2) solvent system yields 4 bands. The Rf values of bands calculated were- band I (0.91), band II (0.76), band III (0.58) and band IV (0.42). In this study methanolic fraction of plumbago leaf extract showed very prominent and well separated spots (10 in visual light 9 under UV light) with different colours. Rf value calculated of the plumbagin spot was 0.75 and 0.79 under the visual and UV light respectively. Color of plumbagin was bright yellow in naked eye but turned blackish under UV light. Number of spots and their characteristics are subject to the proper polarity based combination of solvents in mobile phase used.

Conclusion:

Methanolic leaf extract of *Plumbago zeylanica* showed excellent antimicrobial activity against MDR, metal tolerant test bacteria even at 25 µg/ml MIC. It contains plumbagin as a major active constituent along with some other negligible antimicrobial compounds. It is a bright yellow substance well visual in visual light and under UV light and easily separable by chromatographic techniques. Plumbagin can act as novel antimicrobial natural drug against a variety of pathogenic bacteria and can have a great impact in the improvement of human health.

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