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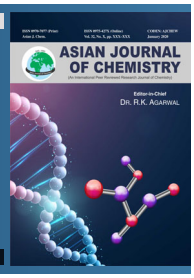

Assessment of *Murraya koenigii* Leaf Extract against New Multiple Drug Resistance Human Enteric Pathogens

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Sewage waters are the primary habitats to harbour antibiotic resistance bacteria (ARB) especially multi-drug resistance (MDR) human enteric pathogens. Microorganisms acquire resistance towards many commercial antibiotics due to their inappropriate use. In this study, human enteric pathogens were isolated, identified and characterized and shows the resistance against five different clinically significant commonly prescribed antibiotics. The bacterial strains were isolated from different sewage treatment plants located in Delhi city, India. Samples were analyzed for the detection of pathogenic human enteric bacteria through morphological, biochemical and molecular analysis. Methanolic leaf extract of *Murraya koenigii* showed the significant antibacterial activity against multi drug resistant human enteric pathogens. Thus, *Murraya koenigii* leaves would be a potential alterantive to antibiotic regimens for the prevention of gastrointestinal infections.

Keywords: Sewage water, Antibiotics, *Murraya koenigii*, Multi drug resistance bacteria.

INTRODUCTION

In today's world, inappropriate use of antibiotics is major problem as it leads to rise in antibiotic resistant pathogenic strains. This multi drug resistance (MDR) pathogen does not respond to these antibiotics anymore, so there is a need to discover new types of antibiotics, which currently remain unchallenged. Now a days scientists are looking for herbal medicine in addition with antibiotics and chemically synthesized drugs because of low toxicity and zero side effects. The phenomenon of antibiotic resistance among bacterial pathogens leads researchers to discover novel sources of antimicrobial drugs; thus, there has been a renewed interest in natural products from plants. These natural products would be able to provide biological functionality, which is indispensable for new drug discovery [1-4].

Murraya koenigii, commonly known as curry leaf or karipatta in local language (Hindi), is under the Family Rutaceae. Leaves of this plant are natural flavouring agent [5,6]. This leaf contains several medicinal properties such as antidiabetic, antioxidant, antimicrobial, antifungal, anti-inflammatory and hepato-protective properties. Medicinally, these leaves found use in gastrointestinal disorders like diarrhoea, dysentery [7]. Gastrointestinal disease caused by human enteric pathogen is the third most common cause of death in the world [8,9].

Sewage water is one of the major sources of human enteric pathogens. Sewage contains human feces and therefore often contains human enteric pathogens. These pathogens can prompt genuine gastrointestinal sickness, which is huge reason for water borne well-being plagues. For fighting against these pathogens, anti-infection agents are commonly utilized which has prompted advancement of multi sedate safe strains of these pathogens. There is an extraordinary need to find novel antitoxins because of wide-spread development of opposition among pathogenic microorganisms against accessible anti-infection agents [10,11]. Standard medication is progressively responsive to the utilization of antimicrobial and different medications got from plants, as conventional anti-infection agents (results of microorganisms or their blended subsidiaries) become insufficient and as new sicknesses stay recalcitrant to this sort of medication. Because of the opposition rising in living beings

against antimicrobial medications, it is a quick need to create substitute route, for example, to search novel antimicrobial medications increasingly dynamic against pathogens with high protection from cook the issue. Therapeutic plants are a wellspring of incredible financial worth everywhere throughout the world. Since prenotable occasions, man has utilized different pieces of various plants against basic sicknesses winning in the general public with changing level of achievement. The information on drugs has created alongside the advancement of logical and social advancement. Medications which are separated from plants are exceptionally successful, effectively accessible and more affordable and they once in a while have symptoms related with them. In the course of the last 40 years, there have been great deals of examinations on normal cures of therapeutic plants as wellspring of new microbicidal movements [12-15].

Several plant species have shown promising antimicrobial activities against a wide range of enteric pathogenic bacteria. Aloe vera, turmeric (haldi), *Azadirachta indica* (neem), *Ocimum sanctum* (tulsi), pomegranate (anar) are some of the natural products that is now a day frequently used in the field of ethnopharmacology for its exceptionally wide range of medicinal properties [16-18]. The aim of this study was to evaluate the antimicrobial activity of *Murraya koenigii* leaf extract against human enteric pathogenic bacteria which were isolated from different sewage waters collected from sewage treatment plant of Delhi city, India.

EXPERIMENTAL

Collection of water samples: Gamma irradiated sterilized bottles were used for sampling of water from ten different sewage treatment plants located in different locations of Delhi are shown in Table-1.Precautions were maintained to prevent contamination during sampling. Samples were analyzed within 6 h of collection.

Isolation and identification of human enteric pathogens

***Escherichia* isolates:** Water sample (250 mL) was passed through 0.45 micron filter and the filter paper was inoculated in MacConkey Broth. Confirmatory identification was done by streaking on eosin methylene blue agar and on MacConkey agar plates. Plates were observed after incubation for characteristic colonies. Further confirmation was done by Gram′s staining and HiMedia IMViC biochemical kit for *E. coli*.

***Salmonella* isolates:** Water sample (250 mL) was passed through 0.45 micron filter and the filter paper was inoculated in buffer peptone water and incubated at 37 ºC for 24 h. Above enriched sample (0.1 mL) was inoculated in 10 mL of Rappaport vassiliadis medium and incubated at 42 ºC for 24 h. After incubation streaked on the plates of brilliant green agar and bismuth sulphide agar to observe characteristic colonies. Further confirmation was done by Gram′s staining and HiMedia IMViC biochemical kit for *Salmonella* as per IS: 5887(Part-3) 1999, Reaffirmed 2018 [19].

***Pseudomonas* isolates:** Water sample (250 mL) was passed through 0.45-micron filter and the filter paper was inoculated in cetrimide broth and then incubated at 37 ºC for 28 h. Streaked on the plates of cetrimide agar plates were observed for characteristic green fluorescent colonies and further confirmation was done by Gram′s staining and biochemical test as per reported method [20].

***Vibrio* isolates:** Water sample (250 mL) was passed through 0.45-micron filter and the filter paper was inoculated in alkaline peptone water and then incubated at 37 ºC for 24 h. Streaked on the plate of thiosulfate-citrate-bile salts-sucrose agar and further confirmation was done by Gram′s staining and biochemical test as per IS: 5887 (Part-5) 1976, Reaffirmed 2018 [21].

***Shigella* isolates:** Water sample (250 mL) was passed through 0.45 micron filter and the filter paper was inoculated in nutrient broth. Confirmatory identification was done by streaking on deoxycholate citrate agar. Further confirmation was done by Gram′s staining and HiMedia IMViC biochemical kit for *Shigella* sp. as per IS: 5887 (Part-7)1976, Reaffirmed 2018 [22].

Molecular identification: All the bacteria isolated from sewage water were further identified by 16srRNA sequencing. The reaction was done by using the Sanger dideoxy sequencing kit. All the trimmed nucleotide sequences of bacterial isolates were locally aligned using Basic Local Alignment Search Tool (BLAST) algorithm provided by National Centre for Biotechnology Information (NCBI).

Inoculum preparation: Bacterial cultures (24 h old) were taken for adjustment of 0.5 McFarland density in densitometer by using normal saline (0.85% NaCl) to get bacterial population of 1.0×10^8 cfu/mL.

Antibiotics and their solutions: Five antibiotics *viz.* amoxicillin, metronidazole, cephalosporin, norfloxacin and nitroimidazole were used to study antibiotic resistance pattern

TABLE-1
MICROBIOLOGICAL PROFILING OF SEWAGE WATER

Sampling location	Sample code	Pathogen identified with accession number
Noida Industrial Effluents	SW01	*Salmonella enteric*, AE006468.2; *Escherichia albertii*, NR_025569
Badarpur Power out	SW02	*Pseudomonas stutzeri*, NR_113652.1
Badarpur Power in	SW03	*Salmonella typhimurium*, AE006468.2
Okhla Head	SW04	*Shigella dysenteriae*, NR_026332.1
DND Highway	SW05	*Pseudomonas aeruginosa*, NR_117678.1
Nizamuddin	SW06	*Escherichia coli* JCM1649, NR_112558.1
IP Powerhouse	SW07	*Escherichia marmotae* HT073016, NR_136472.1
Rajghat Power House	SW08	*Pseudomonas fluorescence*, NR 113647.1
Wazirabad Highway	SW09	*Vibrio cholerae*, NR_119302.1
Nijafarbad Industrial Effluents	SW10	*Pseudomonas baetica*, NR 116899.1

of above bacterial isolates. Working solution having 100 mg/mL concentration of each antibiotic was used for the study.

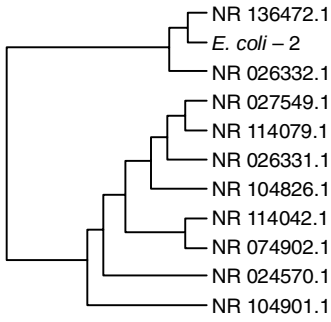
Preparation of *Murraya koenigii* leaf extract: Curry leaf powder (10 g) was added to 100 mL of 70% aqueous methanol solution (w/v) covered with filter paper kept on rotary shaker for 24 h and then kept in a dark area at room temperature for three days. The supernatant was collected and the solvent was evaporated to make the final volume of the curry leaf methanol extract for this experiment. The following six concentration of leaf extract *viz*. 6.25, 12.5, 25, 50, 100 and 200 mg/mL were used for standardization (Table-2). Leaf extract having concentration of 100 mg/mL was used for the study.

Agar-well diffusion assay: Antibiotic assay were evaluated by agar well diffusion method. Plates of sterile Muller-Hinton agar (MHA) with 100 µL of each adjusted cultures was punched to make wells of 6 mm diameter with the help of sterile cork borer at different locations of the plates. Each working solution (100 µL) of antibiotics and standardized leaf extract were added into the well in assay plates. After overnight incubation at 37ºC, petri-plates were observed for the zone of inhibition in mm.

RESULTS AND DISCUSSION

In this study, three different strains of *Escherichia i.e*. *Escherichia albertii*, *Escherichia coli* JCM1649 and *Escherichia marmotae*, four different strains of *Pseudomonas i.e*. *Pseudomonas stutzeri*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescence* and *Pseudomonas baetica*, two different strains of *Salmonella i.e*. *Salmonella enteric* and *Salmonella typhimurium,* one *Shigella dysenteriae* and one *Vibrio cholerae* were identified (Table-1). All the isolated strains were identified in this study showed 96 to 99% sequence similarity to the pathogenic enterobacteriacae sequence available in NCBI database having lowest E-value and maximum query coverage and maximum identity. The multiple alignment file was used to prepare phylogram (Figs. 1-5).

The susceptibility patterns of all isolated human enteric pathogenic strains were checked against five antibiotics as well as *Murraya koenigii* leaf extract by using agar well diffusion assay. The antibiotic resistance patterns in terms of average zones of diameter considering four plates for bacterial isolates



Primer information

PCR Primer name, Primer sequences	Sequencing Primer name, Primer sequences
27F 5' (AGAGTTTGATCMTGGCTCAS) 3'	785F 5' (GGATTAGATACCCTGGTAS) 3'
1492R 5' (TACGGYTACCTTGTTACGACTT) 3'	907R 5' (CCGTCAATTCMTTTRAGTTT) 3'

Fig. 1. Molecular identification of *Escherichia marmotae*

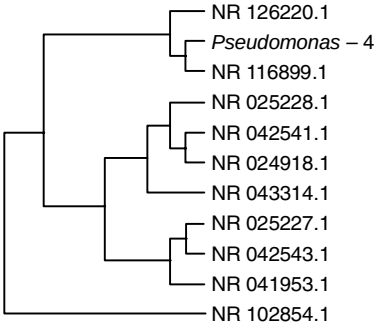


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Fig. 2. Molecular identification of *Pseudomonas baetica*

against five antibiotics and leaf extract were calculated (Table-3), while the percentage of susceptibility against antibiotics are shown in Table-4. In this study, it has shown that minimum concentration 100 mg/mL of *Murraya koenigii* leaf extract have potent antimicrobial activity against all isolated Gram-negative multi-drug resistant human enteric pathogenic bacteria. All three *Escherichia* isolates showed 100% susceptible against amoxycillin and *Murraya koenigii* leaf extract. Intermediate susceptibility was shown by *Escherichia i.e*. 66% towards

TABLE-2
STANDARDIZATION OF *Murraya koenigii* LEAF EXTRACT

Name of pathogens	Concentrations of *Murraya koeniggi* extract (Zone of inhibition in mm)						
	6.25 mg/mL	12.5 mg/mL	25 mg/mL	50 mg/mL	100 mg/mL	200 mg/mL	Methanol as control
Escherichia marmotae	NZI	NZI	9.01	13.33	16.21	16.20	NZI
Escherichia albertii	NZI	NZI	NZI	14.01	16.29	16.29	NZI
Escherichia coli JCM1649	NZI	NZI	8.69	14.79	16.25	16.26	NZI
Pseudomonas aeruginosa	NZI	NZI	NZI	12.21	17.25	17.21	NZI
Pseudomonas fluorescence	NZI	NZI	11.24	12.38	17.89	17.75	NZI
Pseudomonas stutzeri	NZI	NZI	10.09	11.14	17.96	17.94	NZI
Pseudomonas baetica	NZI	NZI	NZI	10.06	18.01	17.99	NZI
Salmonella enteric	NZI	NZI	NZI	14.26	16.65	16.63	NZI
Salmonella typhimurium	NZI	NZI	9.13	13.27	16.47	16.44	NZI
Shigella dysenteriae	NZI	NZI	11.25	15.26	17.71	17.59	NZI
Vibrio cholerae	NZI	NZI	10.36	11.76	16.14	16.11	NZI

Diameter including well diameter of 6.0 mm; NZI = No zone inhibition.

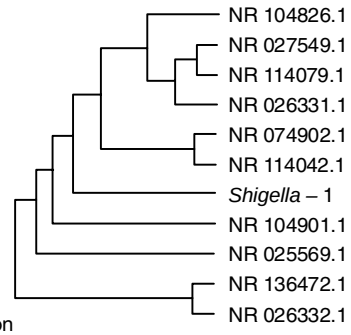

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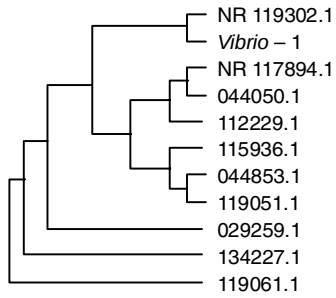
Fig. 3. Molecular identification of *Shigella desynteriae*



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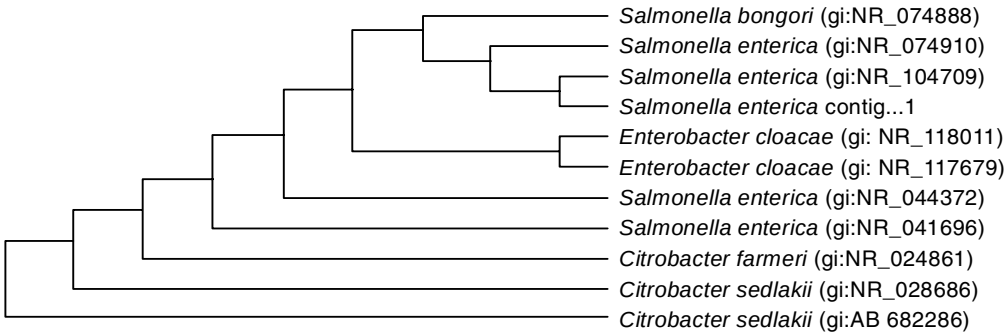
1492R 5' (TACGGYTACCTTGTTACGACTT) 3'	907R 5' (CCGTCAATTCMTTTRAGTTT) 3'

Fig. 5. Molecular identification of *Vibrio cholera*



Primer information

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785F 5' (GGATTAGATACCCTGGTA) 3'	27F 5' (AGAGTTTGATCMTGGCTCAG) 3'
907R 5' (CCGTCAATTCMTTTRAGTTT) 3'	1492R 5' (TACGGYTACCTTGTTACGACTT) 3'

Fig. 4. Molecular identification of *Salmonella enteric*

TABLE-3
ANTIBIOTIC RESISTANCE PATTERNS OF DIFFERENT PATHOGENS

Antibiotics used	Zone of inhibition (mm)										
	EA	EM	EC	PS	PA	PF	PB	SE	ST	SD	VC
Amoxycillin	15.23	15.27	14.69	11.23	0	14.88	0	12.25	15.44	0	0
Cephalosporin	14.32	0	14.25	0	14.12	13.65	15.24	0	11.25	12.39	0
Metronidazole	0	0	15.24	0	0	13.27	0	13.78	15.47	0	0
Nitroimidazole	14.36	13.78	0	15.47	0	0	15.23	12.47	14.29	13.28	15.74
Norfloxacin	0	0	15.17	0	15.24	17.07	0	14.25	14.19	0	12.14
Murraya koenigii	16.29	16.21	16.25	17.96	17.25	17.89	18.01	16.65	16.47	17.71	16.14
Methanol	0	0	0	0	0	0	0	0	0	0	0

EA = *Escherichia albertii*; EM = *Escherichia marmotae*; EC = *Escherichia coli* JCM1649; PS = *Pseudomonas stutzeri*; PA = *Pseudomonas aeruginosa*; PF = *Pseudomonas fluorescence*; PB = *Pseudomonas baetica*; SE = *Salmonella enteric*; ST = *Salmonella typhimurium*; SD = *Shigella dysenteriae*; VC = *Vibrio cholerae*
Diameter including well diameter of 6.0 mm

TABLE-4
PERCENTAGE OF PATHOGENS SUSCEPTIBLE TO ANTIBIOTICS

Antibiotic used	% Susceptible pathogens				
	Escherichia sp	*Pseudomonas sp*	*Salmonella sp*	*Shigella sp*	*Vibrio sp*
Amoxycillin	100	50	100	0	0
Cephalosporin	66	75	50	100	0
Metronidazole	33	25	100	0	0
Nitroimidazole	66	50	100	100	100
Norfloxacin	33	50	100	0	100
Murraya koenigii	100	100	100	100	100

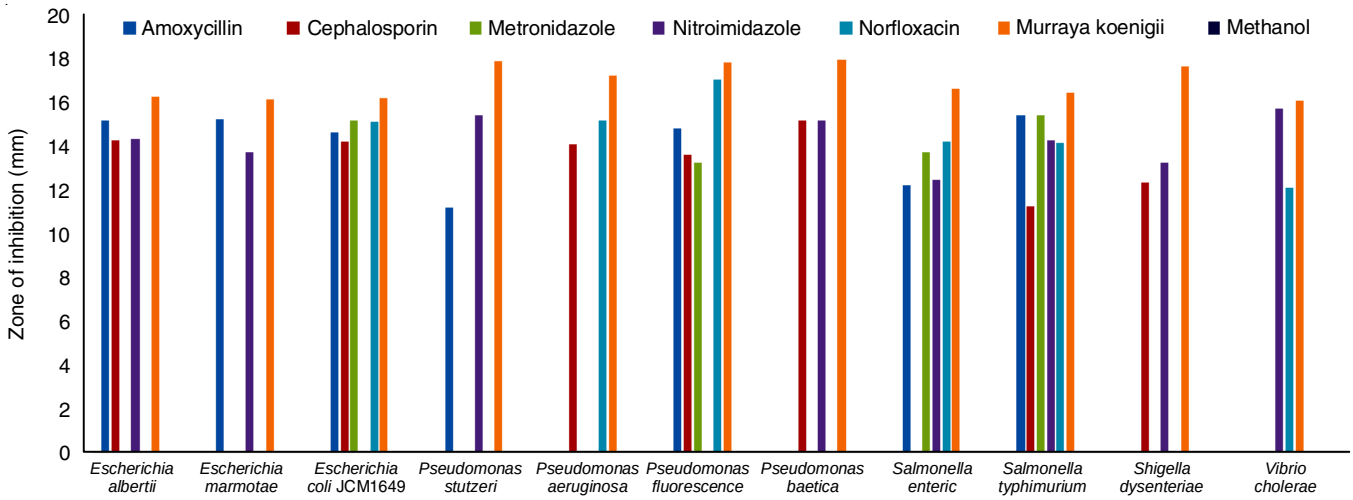

Fig. 6. Susceptibility patterns of bacterial isolates against antibiotics and *Murraya koeniggi* leaf extract

cephalosporin and nitroimidazole. In case of four multi-drug resistant *Pseudomonas* isolates 100% susceptible against only *Murraya koenigii* leaf extract. However, two *Salmonella* isolates have shown more susceptible against five antibiotics as well as *Murraya koenigii* leaf extract. Both *Salmonella enteric* and *Salmonella typhimurium* have shown 50% susceptibility against cephalosporin. In case of *Shigella dysenteriae* and *Vibrio cholera* both have shown 100% resistance against amoxycillin and metronidazole, which are commonly prescribed antibiotics for gastrointestinal disorder. Comparing the inhibition zones of all eleven different human pathogenic enterobacteriacae strain on Muller-Hinton agar, *Murraya koenigii* (methanolic) leaf extract was more effective having minimum 16.14 mm to maximum 18.01 mm inhibition zone (Fig. 6). Multiple antibiotic resistances have shown by 11 human enteric pathogens isolated. *Murraya koenigii* was found to be most promising herbal medicinal plant. The cumulative antimicrobial effectiveness obtained in this study is *Murraya koenigii* > nitroimidazole > norfloxacin > amoxycillin > cephalosporin > metronidazole.

In this study, curry leaves have shown more potential as that natural alternative, especially having antimicrobial property. Curry leaf extracts have demonstrated the strongest inhibition zone against *Proteus mirabilis* (18 mm), *Staphylococcus aureus*, *Corynebacterium pseudotuberculosis* (15 mm), *Klebsiella pneumoniae* (15 mm) *Pseudomonas aeruginosa* (14 mm), *Enterobacter aerogenes* (13 mm) and a moderate level zone of inhibition was observed with *Salmonella enterica* (11 mm) and *Streptococcus pyrogens* (10 mm), respectively. Present study has shown comparable results with the inhibition zone for eleven different strains of human enteric pathogens ranging from 16.14 mm to 18.01 mm. The antibacterial effects of *Murraya koenigii* leaf extract on human enteric pathogenic bacteria were found to be an effective alternative to therapeutic antibiotics in present study.

Conclusion

The emergence of antibiotic resistance among pathogens increases the demand for new treatment strategies. The present investigation showed that *Murraya koenigii* would be the major source in finding metabolites with greater efficacy against multi drug resistant human enteric pathogenic bacteria. The methanolic extracts of *Murraya koenigii* leaf were found to be effective in comparison with five clinically commonly prescribed antibiotics used against all tested human enteric pathogenic bacterial strains. It may be the best natural alternative to treatment against pathogenic microbes.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interests regarding the publication of this article.

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