



Aerobic Microbiota of Neem (*Azadirachta indica*) Leaves: Possible Risks of Chewing Raw Neem Leaves

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Abstract

Raw Neem (*Azadirachta indica*) leaves' chewing is an often-recommended healthy practice in India, but little is understood about the microbial quality of leaves chewed. This study conducted to analyse the presence of potential microbiological hazards associated with Neem leaves. It was assessed through isolation and antimicrobial resistance (A.M.R.) profiling of different aerobically growing bacteria using standard bacteriological methods. A total of 110 samples of Neem leaves collected from four localities (IVRI = 62, CARI = 10, Mahanagar = 18, and Suncity = 20) analysed yielded 357 bacterial isolates belonging to more than 63 species of 24 genera. Isolation of Gram-negative bacteria from samples of IVRI and CARI was significantly more frequent ($p < 0.05$) than those from Mahanagar and Suncity, while the picture was in reverse for Gram-positive bacteria isolates. The most prevalent potentially pathogenic bacteria included *Enterobacter (Pantoea) agglomerans* detected in 37 samples, followed by *Hafnia alvei* (20), *Escherichia coli* (11), *Serratia marcescens* (8), *Bacillus cereus* (7), *Raoultella terrigena* (7), *Serratia odorifera* (7), *Aeromonas bestiarum* (4), *Enterococcus faecium* (2), *Klebsiella oxytoca* (2), *K. pneumoniae* (1), *Pseudomonas aeruginosa* (2), *Acinetobacter ewofflii* (1), *Aeromonas caviae* (1), *Proteus mirabilis* (1), *Stenotrophomonas maltophilia* (1), and *Pseudomonas pseudoalcaligenes* (1). In 26 samples, carbapenem-resistant (CR) and 72 samples, extended-spectrum β -lactamase (ESBL) producing bacteria were detected. Herbal antimicrobial drug resistance was also seen in a large number of bacterial isolates. The study indicated that *A. indica* leaves may be harbouring potentially pathogenic multiple drug-resistant bacteria, which may harm the health of leaves' consumers. Therefore, it may be suggested that a fresh *A. indica* leaves should only be consumed after proper cleansing and decontamination.

Keywords: Aerobic Microbiome; Microbiota of Neem Leaves; *Escherichia coli*; Carbapenem Resistance; ESBL; M.D.R; *Klebsiella*; *Aeromonas*; *Enterococcus faecium*

Introduction

Neem (*Azadirachta indica*) tree is considered a divine tree in many parts of the world due to its multiple medicinal uses [1-3]. All parts of the *A. indica*, including leaf, bark, twigs, and seeds, are shown to possess antibacterial [4], antiviral [5], anti-inflammatory

[6], anti-parasitic activities [7], anti-carcinogenic and curative for many skin diseases [8]. The antimicrobial activity of *A. indica* is supposed to be either due to metabolites of the plant itself or endophytes on the plant. Endophytes of *A. indica* leaves are reported to inhibit the growth of several potential pathogens including *Staphy-*

Staphylococcus aureus, *Streptococcus pyogenes*, *Escherichia coli*, *Salmonella Typhimurium*, *Bacillus cereus*, and *Klebsiella pneumoniae*, due to their antibacterial activity [1,9-13]. The *A. indica* oil, extracted from seeds, is shown to inhibit *S. aureus*, *Mycobacterium tuberculosis*, *Salmonella Typhi* [14,15], *Pseudomonas aeruginosa*, *S. pyogenes*, *E. coli*, *Proteus* group, and *Klebsiella aerogenes* [16]. Even the smoke of *A. indica* leaves is shown to significantly reduce the bacterial load in an environment [17]. However, at the same time *A. indica* may also get affected by some bacteria as *Pseudomonas* species causing shoot-hole and vein blight symptoms [18].

Chewing a handful of *A. indica* fresh leaves is claimed to remove toxins from the body, thus curing skin and hair problems to act as dermocosmetic [19], improving eyesight and liver functions, boosting the immune system, ensuring good oral health [20], anticarcinogenic i.e., protecting from dental caries [21], and diabetes [22]. The frequent recommendations and observations on people chewing fresh *A. indica* leaves made us to study the diversity of cultivable bacteria on tender *A. indica* leaves chewed raw often without any washing or cleansing for claimed health benefits. All the bacterial isolates were tested for their antimicrobial susceptibility against conventional antimicrobials and herbal antimicrobials to assess the potential risks of chewing raw *A. indica* leaves.

Materials and Methods

Collection of *A. indica* leaves

Considering the use of *A. indica* leaves on an empty stomach in the morning, all samples were collected in the morning hours during May and June 2020. Tender fresh five to ten leaves from trees of *A. indica* (on the roadside, on easily approachable plants and often used by the leaf chewers) were picked up with sterile forceps from 3-5 twigs of each tree at the height of 5-7 feet and immediately transferred into a sterile screw-capped tube. A separate sterile pair of forceps and collection tube was used for each of the samples. Samples were collected from four different localities 1) ICAR-Indian Veterinary Research Institute (62), Izatnagar, 2) ICAR-Central Avian Research Institute, Izatnagar (10), 3) Suncity Vistaar, Bareilly (20) and 4) Mahanagar, Bareilly (18), Uttar Pradesh, India (Figure 1).

Processing leaf samples for isolation of bacteria

The leaf samples were brought to the laboratory within an hour of collection and processed. In each sample tube, 10 mL of buffered

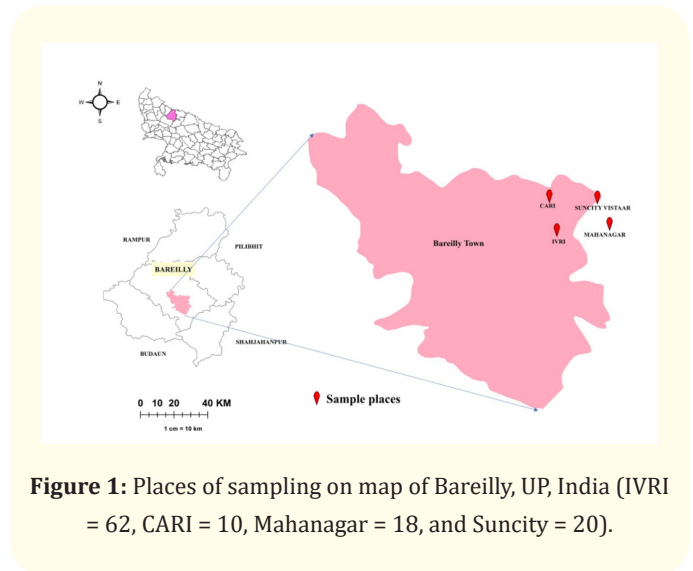


Figure 1: Places of sampling on map of Bareilly, UP, India (IVRI = 62, CARI = 10, Mahanagar = 18, and Suncity = 20).

peptone water (B.P.W., Difco, U.S.A.) containing 0.1% of tween-80 (Sigma, U.S.A.) was added, and tubes were incubated on a shaking platform at 37°C for 6-8 h. After that, growth from the tubes was streaked on to blood agar (containing 5% defibrinated sheep blood) and MacConkey agar (Difco) plates and incubated at 37°C for 18-24 h. Isolated colonies of different type (3-5) were picked and re-streaked on blood agar for checking the purity of the isolates. Pure growth of each isolate was characterised using growth, morphological, staining and biochemical characteristics to identify the bacteria [23-25]. Isolated pure cultures were maintained on nutrient agar slants at 4°C and in 25% glycerol broth [25] at -20°C till tested for antimicrobial susceptibility. All the isolates were reconfirmed for identity using MALDI-ToF MS performed with a MALDI Biotyper Sirius system (Bruker Daltonics) for the identity.

Antimicrobial susceptibility assays

All the isolates from Neem leaves were tested for their susceptibility to amoxicillin (30 µg), amoxicillin (30 µg) + clavulanic acid (10 µg), ampicillin (10 µg), ampicillin (10 µg) + sulbactam (10 µg), azithromycin (15 µg), aztreonam (30 µg), bacitracin (10IU), cefepime (30 µg), cefexime (5 µg), cefoperazone (75 µg), cefoperazone (75 µg) + sulbactam (10 µg), cefotaxime (10 µg), ceftazidime (30 µg), ceftazidime (30 µg) + clavulanic acid (10 µg), ceftriaxone (10 µg), chloramphenicol (25 µg), ciprofloxacin (10 µg), cloxacillin (10 µg), colistin (10 µg), cotrimoxazole (25 µg), doxycycline (30 µg), erythromycin (15 µg), fosfo-

mycin (50 µg), gentamicin (30 µg), imipenem (10 µg), kanamycin (5 µg), lincomycin (10 µg), linezolid (10 µg), meropenem (10 µg), minocycline (30 µg), moxalactam (30 µg), nalidixic acid (30 µg), nitrofurantoin (300 µg), novobiocin (30 µg), oxacillin (5 µg), penicillin G (10 IU), piperacillin (100 µg), streptomycin (10 µg), tetracycline (30 µg), tigecycline (15 µg) and vancomycin (30 µg) discs (Difco, USA) as per CLSI guidelines [26,27] on Mueller Hinton agar (MHA, Difco) plates. Incubated at 37°C overnight and diameter of zone of growth inhibition around the antibiotic discs was measured in mm and susceptibility of isolates was determined using appropriate tables in CLSI guidelines [26,27]. An *E. coli* strain (E-382) susceptible to most of the antimicrobials was used as a reference strain. Bacteria resistant to therapeutically used antimicrobials of three or more classes were classified as multi-drug-resistant (MDR). Extended spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) activity of bacterial isolates was determined using ESBL E-test and MBL strips (Biomérieux, France), respectively and results were interpreted as per guidelines of the manufacturer.

Besides, all the isolates were also tested for their susceptibility to ajowan (*Trachyspermum ammi*) oil (AO), betel (*Piper betel*) leaf oil (BLO), carvacrol (Sigma, U.S.A.), cinnamaldehyde (CNH) (Sigma, U.S.A.), Cinnamon (*Cinnamomum verum*) oil (CO), citral (Sigma, U.S.A.), guggul (*Commiphora mukul*) oil (GO), holy basil (*Ocimum sanctum*) oil (HBO), lemongrass (*Cymbopogon citratus*) oil (LGO), marjoram (*Origanum majorana*) essential oil (MEO), rosewood (*Dalbergia latifolia*) oil (RWO), sandalwood (*Santalum album*) oil (SWO), and thyme (*Thymus vulgaris*) oil (TO). Except for guggul oil, all herbal oils were procured with >99.5% purity from Shubh Flavours and Fragrance Ltd, New Delhi. The pure guggul oil was received as a kind gift from Dr MZ Siddiqui, Processing and Product Development Division, ICAR - Indian Institute of Natural Resins and Gums, Namkum, Ranchi, India. The discs loaded with 1µL of herbal compound/oil were prepared as described earlier and stored in sealed vials at 4°C till used for disc diffusion assays [28]. The zone of growth inhibition around herbal disc was measured in mm, and the isolate with measurable inhibition zone was classified as susceptible to the herbal test preparation. No measurable zone of growth inhibition (Z.I.) was taken as an indicator of resistance in the bacterial isolate. The multiple herbal antimicrobial resistance indices (HAMRI) of bacterial isolates were calculated using a formula, HAMRI= number of herbal drugs resisted ÷ number of herbal drugs tested.

Statistical analysis

Data of identification and susceptibility of isolates and their source entered in Excel sheet were analysed for Chi-square and Odds Ratio to understand various relations among different factors.

Results

Altogether 110 samples of Neem leaves collected from four localities (IVRI 62, CARI 10, Mahanagar 18 and Suncity 20) and processed for isolation of bacteria, all were found carrying one or more types of bacteria, and a total of 357 bacterial isolates were identified (Table 1). From 62 samples of IVRI 204, from 10 samples of CARI 39, from 18 samples of Mahanagar 55, and 20 samples of Suncity 59 bacterial isolates were identified and tested for the 110 samples of Neem leaves collected from four localities (IVRI 62, CARI 10, Mahanagar 18 and Suncity 20) processed for detection of bacteria, all were found carrying one or more types of bacteria, and a total of 357 bacterial isolates were identified (Table 1) and tested for their antimicrobial susceptibility (Table 1).

The 357 bacterial isolates belonged to more than 63 species of 24 genera (Table 1). A total of 269 isolates of Gram-negative bacteria of 17 genera belonging to 44 species were detected on 88 samples (61, 9, 8 and 10 samples from IVRI, CARI, Mahanagar and Suncity, respectively), while 88 Gram-positive bacteria isolates belonging to 7 genera and more than 19 species were detected on 42 samples (9, 3, 13 and 17 samples from IVRI, CARI, Mahanagar and Suncity, respectively). Isolation of Gram-negative bacteria from IVRI and CARI samples was significantly more frequent ($p, <0.05$) than those from Mahanagar and Suncity. The picture was in reverse for Gram-positive bacteria isolates.

The most prevalent bacteria on Neem leaves (Table 1) was *Enterobacter (Pantoea) agglomerans* detected in 37 samples followed by *Hafnia alvei* (20), *Aerococcus* species (11), *Escherichia coli* (11), *Erwinia amylovora* (10), *Serratia marcescens* (8), *Bacillus cereus* (7), *Raoultella terrigena* (7), *Serratia odorifera* (7), *Erwinia chrysanthemi* (6), *E. mallotivora* (6), *Acinetobacter calcoaceticus* (5), *Enterococcus faecalis* (5), *Micrococcus* species (5), *Paenibacillus panthothenicus* (5), *Pantoea dispersa* (5), and *Serratia rubidaea* (5). Other bacteria were isolated from four or less number of samples. Though isolated from a few samples some public health concern bacteria detected include *Aeromonas bestiarum* (4), *En-*

Bacteria	Indian Veterinary Research Institute (n = 62)			Central Avian Research Institute (n = 10)			Mahanagar, Bareilly (n = 18)			Suncity Vistaar, Bareilly (n = 20)			All (n = 110)		
	Samples positive	Isolates	CR	Samples positive	Isolates	CR	Samples positive	Isolates	CR	Samples positive	Isolates	CR	Samples positive	Isolates	CR
<i>Achromobacter xyloxidans</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Acinetobacter calcoaceticus</i>	3	3	0	1	1	0	0	0	0	1	1	0	5	5	0
<i>Acinetobacter ewofflii</i> *	1	2	0	0	0	0	0	0	0	0	0	0	1	2	0
<i>Acinetobacter lwoffii</i> *	1	1	0	0	0	0	0	0	0	1	1	0	2	2	0
<i>Aerococcus</i> spp.	1	1	1	1	1	0	0	0	0	9	15	0	11	17	1
<i>Aeromonas bestiarum</i> *	1	1	0	0	0	0	0	0	0	3	4	1	4	5	1
<i>Aeromonas caviae</i> *	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1
<i>Alcaligenes denitrificans</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Alcaligenes faecalis</i> *	2	2	1	0	0	0	0	0	0	0	0	0	2	2	1
<i>Arsenophonus nasoniae</i>	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
<i>Bacillus</i> spp. **	1	2	0	0	0	0	1	1	0	0	0	0	2	3	0
<i>Bacillus amyloliquefaciens</i> **	0	0	0	0	0	0	0	0	0	4	6	0	4	6	0
<i>Bacillus badius</i>	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
<i>Bacillus cereus</i> *	0	0	0	0	0	0	6	15	0	1	1	0	7	16	0
<i>Bacillus licheniformis</i> **	0	0	0	0	0	0	3	9	0	0	0	0	3	9	0
<i>Cedecea lapagii</i>	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
<i>Enterobacter (Pantoea) agglomerans</i> *	30	53	10	6	10	1	1	1	0	0	0	0	37	64	11
<i>Enterobacter amnigenus</i> *	1	3	0	0	0	0	0	0	0	0	0	0	1	3	0
<i>Enterobacter cloacae</i> *	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Enterobacter gregoviae</i> *	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0

<i>Enterobacter sakazaki</i> *	3	5	0	0	0	0	0	0	0	0	0	0	3	5	0
<i>Enterococcus casseliflavus</i>	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
<i>Enterococcus durans</i>	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
<i>Enterococcus faecalis</i> *	3	3	0	1	1	0	0	0	0	1	1	0	5	5	0
<i>Enterococcus faecium</i> *	1	1	0	0	0	0	0	0	0	1	1	0	2	2	0
<i>Erwinia amylovora</i> #	10	13	3	0	0	0	0	0	0	0	0	0	10	13	3
<i>Erwinia ananas</i> #	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Erwinia aphidicola</i> #	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Erwinia carotovora</i> #	3	3	0	1	3	0	0	0	0	0	0	0	4	6	0
<i>Erwinia chrysanthemi</i> #	6	9	7	0	0	0	0	0	0	0	0	0	6	9	7
<i>Erwinia mallovora</i> #	3	4	0	1	1	0	2	2	0	0	0	0	6	7	0
<i>Erwinia nimipressuralis</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Erwinia rhapontici</i> #	4	8	0	0	0	0	0	0	0	0	0	0	4	8	0
<i>Erwinia tasmaniensis</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Escherichia blattae</i>	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
<i>Escherichia coli</i> *	7	14	2	2	7	2	0	0	0	2	5	0	11	26	4
<i>Escherichia vulneris</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Geobacillus steaerothermophilus</i>	0	0	0	0	0	0	0	0	0	2	4	0	2	4	0
<i>Hafnia alvei</i> *	8	12	0	3	8	1	4	6	0	5	12	2	20	38	3
<i>Kleb. pneumoniae ssp. pneumoniae</i> *	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
<i>Klebsiella oxytoca</i> *	2	2	0	0	0	0	0	0	0	0	0	0	3	2	0
<i>Lysinibacillus sphaericus</i>	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0

<i>Micrococcus</i> spp.	1	1	0	0	0	0	0	0	0	4	5	0	5	6	0
<i>Paenibacillus amylolyticus</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Paenibacillus larvae</i>	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
<i>Paenibacillus pantothenicus</i> **	0	0	0	0	0	0	5	10	0	0	0	0	5	10	0
<i>Pantoea dispersa</i>	5	6	0	0	0	0	0	0	0	0	0	0	5	6	0
<i>Peptococcus</i> spp.**	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
<i>Proteus mirabilis</i>	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1
<i>Pseudomonas aeruginosa</i> *	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
<i>Pseudomonas pseudoalcaligenes</i>	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0
<i>Raoultella terrigena</i> *	4	5	0	1	1	0	1	1	0	1	1	0	7	8	0
<i>Serratia ficaria</i>	4	4	0	0	0	0	0	0	0	0	0	0	4	4	0
<i>Serratia fonticola</i>	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Serratia grimesii</i>	2	5	0	0	0	0	0	0	0	0	0	0	2	5	0
<i>Serratia marcescens</i>	6	6	0	0	0	0	2	2	1	0	0	0	8	8	1
<i>Serratia odorifera</i>	7	9	0	0	0	0	0	0	0	0	0	0	7	9	0
<i>Serratia plymuthica</i>	0	0	0	0	0	0	2	2	0	0	0	0	2	2	0
<i>Serratia rubidaea</i> *	1	1	0	0	0	0	4	5	1	0	0	0	5	6	1
<i>Staphylococcus schleiferi</i> ssp. <i>schleiferi</i> *	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Staphylococcus xylosum</i>	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
<i>Stenotrophomonas maltophilia</i> *	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
<i>Xenorhabdus bovienni</i> *	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Total	62	204	26	10	39	4	18	55	2	20	59	3	110	357	35

Table 1: Types of bacteria isolated and their carbapenem resistance (CR) from Neem (*Azadirachta indica*) leaves from trees in different localities in Bareilly, Uttar Pradesh.

*Bacteria reported to be associated with zoonotic infections or infections in humans and animals

**Bacteria with known probiotic potential

#Bacteria known to cause infection in plants.

terobacter sakazaki (3), *Acinetobacter lwoffii* (2, Figure 2), *Alcaligenes faecalis* (2), *Enterococcus faecium* (2), *Klebsiella oxytoca* (2), *K. pneumoniae ssp. pneumoniae* (1), *Pseudomonas aeruginosa* (2), *Acinetobacter ewofflii* (1), *Aeromonas caviae* (1), *Proteus mirabilis* (1, Figure 3), *Stenotrophomonas maltophilia* (1, Figure 4), *Pseudomonas pseudoalcaligenes* (1) and *Xanthorhabdus bovienni* (1).

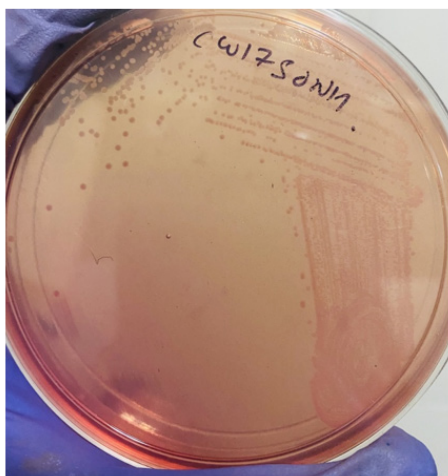


Figure 2: *Acinetobacter lwoffii* growing on McConkey agar, 24 h incubation at 37°C.

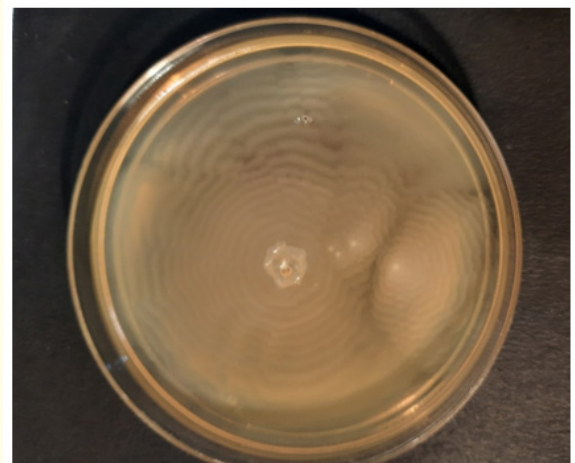


Figure 3: *Proteus mirabilis* growing on nutrient agar, 24 h incubation at 37°C.

Bacillus and *Paenibacillus* species often considered GRAS bacteria of potential probiotic isolates in 23 samples (2, 1, 13 and 7 samples from IVRI, CARI, Mahanagar and Suncity, respectively). Their isolation was more common (OR >16; p <0.05) on Neem leaves collected from Mahanagar and Suncity than those from both institutions.

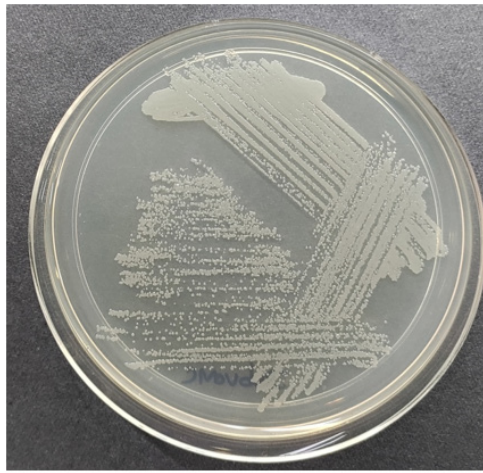


Figure 4: *Stenotrophomonas maltophilia* growing on nutrient agar, 24 h incubation at 37°C.

A total of 26 samples (17, 4, 2 and 3 samples from IVRI, CARI, Mahanagar and Suncity, respectively) were resistant to carbapenem drugs. There was no statistically significant ($p > 0.05$) difference in the rate of detection of carbapenem-resistant (CR) bacteria from samples of different localities (Table 1).

A total of 35 bacteria isolated from 26 Neem leaves samples had resistance to one or more carbapenem antibiotics. The Most common CR bacteria detected on Neem leaves was *Enterobacter (Pantoea) agglomerans* (11) followed by *Erwinia chrysanthemi* (7), *E. coli* (4), *Hafnia alvei* (3), *Erwinia amylovora* (3), *Aerococcus* spp. (1), *Serratia marcescens* (1), *Serratia rubidaea* (1), *Aeromonas bestiarum* (1), *Alcaligenes faecalis* (1), *Aeromonas caviae* (1) and *Proteus mirabilis* (1, Figure 3). Though 26 isolates had carbapenem-resistance (CR) but only one isolate of *Alcaligenes faecalis* produced metallo- β -lactamase (NDM-5, Figure 5). Though CR strains were isolated from about one-fourth of the samples, on comparing the susceptibility of all bacterial isolates (Table 2) imipenem, (94.6%) was the most effective antibiotic followed by doxycycline (94.4%), meropenem (93.2%), minocycline (88.7%), ceftriaxone (85.7), chloramphenicol (85.4), gentamicin (85.3), tetracycline (84.1), ceftaxime (83.3) and tigecycline (83). Other antibiotics could inhibit the growth of only less than 80% isolates and many common an-

tibiotics, including colistin, ampicillin, erythromycin, ceftazidime, and oxacillin, amoxicillin, penicillin and lincomycin failed to inhibit ≥ 70 of the bacterial isolates from Neem leaves.

A total of 245 isolates (68.6%) were resistant to β -lactam antibiotics (Table 2). 72 samples were carrying ESBL producing bacteria (50, 9, 3 and 10 samples from IVRI, CARI, Mahanagar and Suncity, respectively). A total of 188 ESBL producing G-ve bacteria isolated from 72 samples of Neem leaves belonged to 36 different species. The most common ESBL producers in the study were *Enterobacter* species (53), followed by *Erwinia* species (36), *Serratia* species (22), *E. coli* (15), *Acinetobacter* species (7), *Aeromonas bestiarum* (4), *Raoultella terrigena* (4), *klebsiella* (3) and a few isolates of other species (Table 2). The occurrence of ESBL positive bacteria was significantly higher in Neem leaves samples from ICAR-IVRI, campus than those in samples from Mahanagar (OR, 20.8; CI99, 3.34-129.93), and Suncity (OR, 5; CI99, 1.16-21.52) but had no significant difference from CARI samples ($p > 0.05$). Similarly, isolates from CARI Neem leaves samples had significantly higher odds of being ESBL producers than those from Mahanagar (OR, 45; CI99, 1.89-1072.97) and Suncity (OR, 9; CI90, 1.37-59.19) samples. Further analysis proved that bacteria isolated from Mahanagar Neem leaves were significantly less often ESBL types than those from Neem leaves sampled from Suncity (OR, 5; CI95, 1.10-22.82).

A total of 57 Gram-positive bacteria isolated from 34 Neem leaves samples (5, 3, 11 and 15 samples from IVRI, CARI, Mahanagar and Suncity, respectively) were β -lactam antibiotics resistant (Ta-



Figure 5: E-test (Biomérieux, France) for determining metallo- β -lactamase production by *Alcaligenes faecalis* (tested in duplicate).

Antimicrobials	G+ve (88)	G-ve (269)	O+ve (57)	O-ve (357)	O+ve G+ve (42)	O+ve G-ve (14)	O-ve G+ve (46)	O-ve G-ve (255)	Acn (9)	Enb (75)	Erw (47)	Esc (29)	Ha (38)	Ser (35)	Arc (17)	Enc (9)	Pnb (12)	Bc (36)	All (357)
Amoxicillin	51.2	83.8	68.8	76.0	65.9	85.7	37.2	83.7	75.0	80.7	84.1	80.0	92.1	90.3	29.4	37.5	36.4	65.7	74.8
Amoxicillin clavulanic acid	26.2	50.8	41.1	45.5	31.7	64.3	20.9	50.0	37.5	35.2	52.2	28.6	76.3	65.7	17.6	0.0	36.4	37.1	44.8
Ampicillin	40.7	79.8	57.9	72.5	47.6	85.7	34.1	79.4	66.7	79.5	82.6	71.4	94.4	76.5	26.7	22.2	50.0	55.6	70.1
Ampicillin sul-bactam	37.8	60.7	65.5	56.8	46.7	84.6	31.8	59.4	25.0	53.4	51.2	53.6	71.0	76.9	20.0	12.5	100	50.0	57.7
Azithromycin	21.7	42.5	36.4	40.6	27.3	50.0	16.7	42.0	0.0	21.7	52.9	35.0	52.4	58.1	100	0.0	0.0	30.0	40.2
Aztreonam	78.8	45.2	74.5	50.2	80.5	53.8	77.3	44.7	62.5	43.8	45.9	40.7	35.3	53.1	70.6	87.5	83.3	85.7	54.6
Bacitracin	90.8	NT	97.3	82.1	97.3	NT	82.1	NT	NT	NT	NT	NT	NT	NT	71.4	100	90.9	100	90.8
Cefepime	33.8	36.4	39.5	31.0	35.9	75.0	31.0	31.0	100	100	0.0	50.0	15.4	42.9	6.7	50.0	54.5	41.2	34.7
Cefexime	60.0	64.9	100	63.4	100	NT	50.0	64.9	100	60.0	50.0	100	100	75.0	NT	50.0	NT	NT	64.3
Cefoperazone	23.3	64.2	42.4	47.6	29.2	75.0	15.8	62.2	50.0	100	80.0	80.0	10.0	60.0	0.0	50.0	100	36.8	45.8
Cefoperazone+ Subactam	25.0	34.9	55.6	32.1	NT	50.0	25.0	33.3	0.0	32.0	21.1	44.4	NT	50.0	0.0	NT	100	NT	34.5
Cefotaxime	18.3	16.1	23.6	15.3	17.1	46.2	19.5	14.5	16.7	13.7	4.3	21.4	22.2	26.7	12.5	25.0	16.7	20.0	16.7
Cefoxitin	30.6	60.0	29.2	48.1	30.4	0.0	30.8	64.3	NT	50.0	100	NT	100	50.0	NT	100	30.0	31.8	39.2
Cefpodoxime	46.7	25.0	54.5	25.0	52.4	100	33.3	18.2	NT	0.0	0.0	NT	0.0	40.0	NT	NT	33.3	52.4	40.5
Ceftazidime	66.1	75.3	60.6	74.9	57.1	80.0	73.5	75.2	71.4	77.1	76.0	68.4	71.9	87.5	75.0	66.7	75.0	54.5	72.8
Ceftazidime Clavulanic acid	33.8	15.5	28.3	19.1	27.5	33.3	41.9	14.9	0.0	17.0	11.1	21.1	8.3	17.4	16.7	55.6	30.0	25.7	20.8
Ceftriaxone	16.0	13.7	23.6	12.5	17.5	42.9	14.6	12.0	33.3	11.4	6.8	17.9	8.3	17.1	5.9	16.7	18.2	20.6	14.3
Chloramphenicol	9.2	16.2	20.0	13.6	10.0	42.9	8.3	14.7	12.5	12.7	15.2	10.7	8.1	17.6	0.0	0.0	16.7	8.3	14.6
Ciprofloxacin	12.6	25.4	16.1	23.4	9.8	35.7	15.2	24.8	0.0	33.8	21.7	25.0	18.4	21.2	11.8	44.4	0.0	11.4	22.2
Cloxacillin	57.6	94.7	64.7	81.3	61.3	100	54.3	94.4	80.0	96.2	88.9	100	100	88.9	50.0	60.0	45.5	72.0	77.3
Colistin	83.3	65.8	80.4	68.0	95.1	42.9	72.1	67.1	50.0	63.9	78.3	67.9	60.5	85.7	58.8	87.5	83.3	94.3	70.0
Cotrimoxazole	29.5	28.8	42.1	26.4	38.1	50.0	21.7	27.6	66.7	23.0	26.1	11.1	31.6	25.7	23.5	11.1	16.7	41.7	29.0
Doxycycline	1.4	8.8	2.7	6.5	0.0	25.0	2.7	8.0	0.0	7.1	0.0	25.0	11.8	7.7	0.0	0.0	9.1	0.0	5.6
Erythromycin	31.9	82.9	34.0	78.9	23.7	57.1	41.2	84.4	14.3	83.3	77.8	92.9	91.2	90.3	37.5	50.0	25.0	27.3	71.5
Fosfomycin	71.7	55.6	68.2	58.6	76.5	40.0	69.0	56.3	66.7	75.0	52.0	26.3	50.0	68.8	87.5	25.0	100	72.7	59.8
Gentamicin	5.1	18.4	10.9	15.6	7.5	21.4	2.6	18.1	16.7	11.1	24.4	22.7	11.5	20.6	0.0	20.0	0.0	8.8	14.7
Imipenem	1.2	6.8	3.6	5.8	0.0	14.3	2.3	6.4	0.0	9.6	4.3	7.1	7.9	5.7	5.9	0.0	0.0	0.0	5.4
Kanamycin	0.0	22.1	22.2	21.7	NT	25.0	0.0	21.7	NT	19.2	11.1	33.3	NT	0.0	NT	NT	0.0	NT	21.7
Lincomycin	78.4	100	88.4	90.8	87.2	100	68.6	100	100	100	100	100	100	100	50.0	80.0	81.8	93.9	90.1
Linezolid	21.9	86.2	18.6	70.9	15.4	50.0	29.4	88.0	80.0	88.9	80.0	87.5	100	78.6	25.0	20.0	27.3	18.2	56.9

Meropenem	1.1	8.6	3.5	7.4	0.0	14.3	2.2	8.3	0.0	5.4	19.6	10.7	5.3	5.7	5.9	0.0	0.0	0.0	6.8
Minocycline	5.5	16.1	9.5	11.9	7.9	25.0	2.9	15.7	0.0	12.0	27.3	0.0	18.8	28.6	0.0	0.0	0.0	6.3	11.3
Moxalactam	100	23.9	88.9	15.3	NT	87.5	100	15.3	NT	15.4	0.0	11.1	NT	0.0	NT	NT	100	NT	25.0
Nalidixic acid	25.7	25.0	24.0	26.7	25.0	0.0	27.3	26.3	NT	0.0	50.0	NT	16.7	33.3	NT	NT	30.0	25.0	25.5
Nitrofurantoin	63.2	64.1	74.4	62.2	70.0	83.3	55.6	63.1	71.4	64.9	50.0	54.2	80.8	57.1	0.0	16.7	90.9	72.4	63.9
Novobiocin	78.4	97.6	90.5	88.0	89.5	100	66.7	97.5	100	96.2	100	100	100	92.9	81.3	40.0	54.5	93.8	88.7
Oxacillin	41.5	94.9	40.0	81.3	29.4	100	50.0	94.6	100	92.3	100	85.7	90.9	100	37.5	50.0	100	45.5	73.0
Penicillin	45.1	94.0	64.7	82.5	52.6	100	36.4	93.5	60.0	95.8	96.4	100	90.9	89.5	20.0	75.0	41.7	59.4	78.4
Piperacillin	45.2	53.6	54.3	50.8	54.2	60.0	33.3	53.0	33.3	50.0	45.5	63.6	36.4	57.1	100	0.0	27.3	54.2	51.5
Streptomycin	12.8	37.0	10.5	27.7	5.9	50.0	18.2	36.0	0.0	33.3	50.0	80.0	27.3	NT	18.8	25.0	0.0	8.3	22.7
Tetracycline	17.0	15.5	19.3	15.3	11.9	42.9	21.7	14.0	0.0	12.2	8.7	22.2	13.5	11.4	11.8	22.2	16.7	11.1	15.9
Tigecycline	10.6	19.1	19.6	16.5	14.6	28.6	6.8	18.5	12.5	12.5	15.2	21.4	26.3	23.5	11.8	0.0	8.3	17.1	17.0
Vancomycin	86.2	94.7	100	87.9	100	100	74.3	94.4	80.0	100	100	71.4	100	88.9	81.3	60.0	63.6	100	90.7
ESBL	64.8	69.9	59.6	70.3	69.0	35.7	60.9	71.8	77.8	70.7	76.6	62.1	86.8	62.9	52.9	66.7	66.7	69.4	68.6
CR	1.1	12.8	5.3	10.8	0.0	21.4	2.2	12.3	0.0	14.9	21.7	14.3	7.9	5.7	5.9	0.0	0.0	0.0	9.9

Table 2: Antimicrobial resistance among different types of bacteria including major genera isolated from *Azadirachta indica* leaves in Bareilly.

G+ve, Gram Positive; G-ve, Gram Negative; O+ve, Oxidase Producer; O-ve, Non-Oxidase Producer; Acn, *Acinetobacter* Species; Enb, *Enterobacter* Species; Erw, *Erwinia* Species; Esc, *Escherichia* Species; Ha, *Hafnia Alvei*; Ser, *Serratia* Species; Arc, *Aerococcus* Species; Enc, *Enterococcus* Species; Pnb, *Paenibacillus* Species; Bc, *Bacillus* and Allied Species; ESBL, Extended Spectrum B-lactamase Producing; CR, Carbapenem (meropenem/imipnem) Resistant; NT, Not Tested.

ble 2). The most common Gram-positive β-lactam resistant bacteria on Neem leaves belonged to *Bacillus* and allied species (*B. cereus* 13, *B. amyloliquefaciens* 6, *Geobacillus stearothermophilus* 3, *B. licheniformis* 2 (Figure 6), and one each of *B.adius*, *Lysinibacillus sphaericus*, and an unidentified species) detected on 16 samples, followed by *Aerococcus* strains (9) on eight samples, *Enterococcus* species (*E. faecalis* 3, *E. faecium* 2, *E. durans* 1) strains on five samples, *Paenibacillus* species (*P. pantothenticus* 7, *P. larvae* 1) on four samples and *Staphylococcus schleiferi* and *Peptococcus* species strains on one sample each. The occurrence of β-lactam antibiotic-resistant Gram-positive bacteria was significantly higher ($p < 0.05$) in samples from Suncity and Mahanagar than on samples collected at IVRI and CARI.

Herbal drug susceptibility pattern of the bacteria from Neem leaves (Table 3) revealed that carvacrol was the best inhibiting 89.29% of the isolates followed by cinnamon oil (85.27%), thyme (79.17%), cinnamaldehyde (76.16%) and ajowan oil (71.47%).

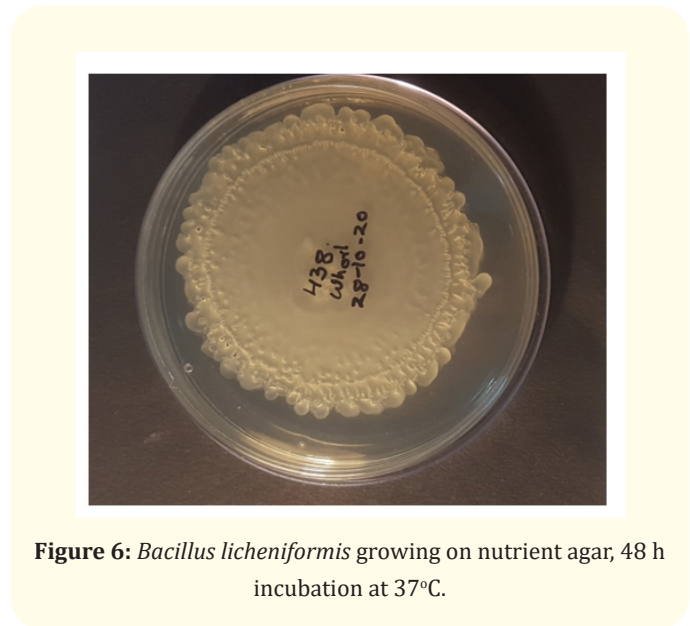


Figure 6: *Bacillus licheniformis* growing on nutrient agar, 48 h incubation at 37°C.

Other herbs including HBO (44.81%), citral (41.81%), BLO (39.77%), GO (30.52%), LGO (21.65%), RWO (20.25%), MEO (16.75%) and SWO (14.77%) failed to inhibit even 50% of the isolates. Analysis of multiple herbal drug resistance indices (HAMRI) revealed that Gram-negative bacteria (HAMRI, 0.55) were more

resistant to herbs than Gram-negative bacteria (HAMRI, 0.39). Similarly, oxidase-positive bacteria (HAMRI, 0.57) were more often multiple herbal drug-resistant than oxidase negative bacteria (HAMRI, 0.4).

Microbes	AO	HBO	CNH	Carvacrol	LGO	TO	Citral	CO	BLO	GO	RWO	MEO	SWO	HAMRI
Gram Positive (88)	71.6	82.9	47.7	14.6	69.3	47.6	64.8	22.7	50.0	80.0	75.0	72.7	100	0.55
Gram Negative (269)	14.3	46.3	15.6	9.4	81.4	12.2	56.0	12.1	60.7	68.8	80.0	83.9	84.5	0.39
Oxidase Positive (57)	68.4	87.3	49.1	16.4	75.4	49.1	73.7	24.6	66.7	90.9	77.8	81.8	77.8	0.57
Oxidase Negative (357)	20.9	48.9	18.8	9.6	78.9	15.3	55.2	12.8	59.5	67.8	80.0	83.3	86.1	0.40
Oxidase +ve G+ve (42)	76.2	90.0	52.4	10.0	71.4	52.5	71.4	26.2	NT	100	NT	100	NT	0.57
Oxidase +ve G-ve (14)	42.9	78.6	42.9	28.6	85.7	35.7	78.6	21.4	62.5	88.9	75.0	77.8	75.0	0.56
Oxidase -ve G+ve (46)	67.4	76.2	43.5	19.0	67.4	42.9	58.7	19.6	50.0	77.8	75.0	70.0	100	0.53
Oxidase -ve G-Ve (255)	12.7	44.4	14.0	8.3	81.1	10.8	54.8	11.6	60.5	67.4	80.6	84.2	85.5	0.38
<i>Acinetobacter</i> spp. (9)	22.2	37.5	0	0	55.6	12.5	44.4	0	50	66.7	50	66.7	50	0.3
<i>Aerococcus</i> spp. (17)	64.7	76.5	58.8	23.5	58.8	58.8	58.8	11.8	0	0	100	0	100	0.52
<i>Bacillus</i> spp. (36)	75	91.2	58.3	8.8	77.8	52.9	72.2	30.6	NT	NT	NT	NT	NT	0.59
<i>Enterobacter</i> spp. (75)	17.6	35.7	20.3	7.2	86.3	5.8	43.2	8.1	57.7	70	80.8	84.1	88.5	0.37
<i>Enterococcus</i> spp. (9)	88.9	85.7	44.4	14.3	66.7	28.6	66.7	33.3	NT	100	NT	66.7	NT	0.59
<i>Erwinia</i> spp. (47)	6.5	42.2	13.9	22.2	68.2	25	54.3	28.3	50	46.2	66.7	80.6	83.3	0.4
<i>Escherichia</i> spp. (29)	14.3	66.7	3.6	3.7	92.9	7.4	60.7	11.1	100	94.1	88.9	82.4	77.8	0.41
<i>Hafnia alvei</i> (38)	7.9	57.1	10.5	0	89.5	0	57.9	10.5	NT	87.5	NT	91.7	NT	0.35
<i>Paenibacillus</i> spp. (12)	75	83.3	25	25	83.3	41.7	58.3	16.7	100	100	100	100	100	0.52
<i>Serratia</i> spp. (35)	11.4	42.9	11.4	2.9	74.3	14.3	71.4	5.7	60	75	80	88.5	100	0.35
All (357)	28.5	55.2	23.8	10.7	78.3	20.8	58.2	14.7	60.2	69.5	79.7	83.2	85.2	0.43

Table 3: Herbal antimicrobial resistance among different types of bacteria including major genera isolated from *Azadirachta indica* leaves in Bareilly.

AO, Ajowan Oil; HBO, Holy Basil Oil; CNH, Cinnamaldehyde; LGO, Lemongrass Oil; TO, Thyme Oil; CO, Cinnamon Oil; BLO, Betel Leaf Oil; GO, Guggul Oil; RWO, Rosewood Oil; MEO, Marjoram Essential Oil; SWO, Sandalwood Oil; HAMRI, Herbal Antimicrobial Multiple Drug Resistant Index.

Six isolates including one each of *Erwinia chrysanthemi* (IVRI), *Lysinibacillus sphaericus* (CARI), *Staphylococcus xylosum* (Suncity), *Aerococcus* spp. (Suncity) and *Bacillus licheniformis* (Mahanagar) had a total herbal drug-resistant (HAMRI, 1.0). Though *E. chrysanthemi* strains had the highest HAMRI (0.61), other erwineae strains had lower resistance indices than isola-

tes belonging to *Bacillus* (0.59), *Enterococcus* (0.59), *Aerococcus* (0.52), *Paenibacillus* (0.52), and *Escherichia* (0.41) species.

Both herbal antimicrobial and antibiotic resistance varied significantly among different species/genera (Table 4). Gram-positive bacteria were more often resistant to AO, HBO, CNH, TO, CO

and ceftazidime+clavulanic acid than Gram-negative bacteria. In contrast, oxidase-positive bacteria were less often resistant to penicillin, ampicillin, erythromycin, oxacillin, cloxacillin, ceftriaxone, cefpodoxime, ceftaxime, lincomycin and fosfomicin than oxidase

negative bacteria. The common bacteria of plants like *Erwinia* isolates were less often resistant to lincomycin and nitrofurantoin than *Bacillus* and *Paenibacillus* strains.

Group of microbes compared	First group was more often (statistically significant, p <0.05) susceptible than 2 nd group to	First group was less often (statistically significant, p <0.05) susceptible than 2 nd group to
Gram positive/Gram negative	LGO, penicillin, ampicillin, doxycycline, gentamicin, ciprofloxacin, carbapenems, Amoxycillin, erythromycin, novobiocin, oxacillin, cloxacillin	AO, HBO, CNH, TO, CO, ceftazidime+clavulanic acid
Oxidase positive/Oxidase negative	Penicillin, ampicillin, erythromycin, oxacillin, cloxacillin, ceftriaxone, cefpodoxime, ceftaxime, lincomycin, fosfomicin	AO, HBO, CNH, TO, Citral, CO, ceftazidime+clavulanic acid, cotrimoxazole, linezolid
Oxidase positive and Gram positive/Oxidase positive and Gram negative	Penicillin, ampicillin, tetracycline, doxycycline, streptomycin, ciprofloxacin, chloramphenicol, carbapenems, amoxycillin+clavulanic acid, erythromycin, oxacillin, cefotaxime, ceftriaxone, cefoperazone	AO, colistin
Oxidase positive and Gram positive/Oxidase negative and Gram positive	NA	Amoxycillin, colistin, ceftriaxone, lincomycin, bacitracin, novobiocin, vancomycin
Oxidase positive and Gram positive/Oxidase negative and Gram negative	Penicillin, ampicillin, ciprofloxacin, ceftazidime, carbapenems, amoxycillin, erythromycin, cloxacillin, oxacillin, lincomycin, linezolid,	AO, HBO, CNH, TO, citral, CO, colistin, aztreonam, ceftraxone
Oxidase positive and Gram negative/Oxidase negative and Gram negative	Erythromycin, linezolid	AO, HBO, CNH, Carvacrol, TO, tetracycline, chloramphenicol, cefotaxime, ceftriaxone, moxalactam
Oxidase negative and Gram positive/Oxidase negative and Gram negative	Penicillin, ampicillin, gentamicin, amoxycillin+clavulanic acid, erythromycin, vancomycin, novobiocin, erythromycin, cloxacillin, oxacillin, tigecycline, lincomycin, linezolid, minocycline, cefoperazone	AO, HBO, CNH, Carvacrol, LGO, TO, ceftazidime+clavulanic acid, moxalactam, aztreonam,
<i>Acinetobacter/Enterobacter</i>	LGO, penicillin, ciprofloxacin, erythromycin, cefoperazone	Cotrimoxazole
<i>Acinetobacter/Erwinia</i>	Penicillin, azithromycin, erythromycin,	Cotrimoxazole, ceftriaxone, cefepime
<i>Acinetobacter/Escherichia</i>	LGO, BLO, penicillin, streptomycin, erythromycin,	Cotrimoxazole, ceftriaxone
<i>Acinetobacter/Hafnia</i>	LGO, ampicillin, amoxycillin+clavulanic acid, azithromycin, erythromycin	TO, cotrimoxazole, ceftriaxone
<i>Acinetobacter/Serratia</i>	SWO, ampicillin+sulbactam, azithromycin, erythromycin, colistin	Cotrimoxazole, ceftriaxone

<i>Acinetobacter/Aerococcus</i>	AO, HBO, TO, azithromycin	Ampicillin, cotrimoxazole, amoxycillin, oxacillin, ceftriaxone, cefepime, lincomycin, cefoperazone
<i>Acinetobacter/Enterococcus</i>	AO, CNH, ciprofloxacin, ceftazidime+clavulanic acid	Nitrofurantoin, cotrimoxazole, ceftriaxone
<i>Acinetobacter/Paeni Bacillus</i>	AO, HBO, TO, azithromycin, ampicillin + sulbactam,	Cotrimoxazole, ceftriaxone, linezolid
<i>Acinetobacter/Bacillus</i> and allied	AO, HBO, vancomycin	Ceftriaxone, linezolid
<i>Enterobacter/Erwinia</i>	Carvacrol, TO, CO, meropenem, azithromycin	LGO, ceftriaxone
<i>Enterobacter/Escherichia</i>	HBO, BLO, GO, ceftriaxone,	CNH, fosfomycin
<i>Enterobacter/Hafnia</i>	HBO, ampicillin, amoxycillin+clavulanic acid, azithromycin,	Ceftriaxone, cefepime, cefoperazone
<i>Enterobacter/Serratia</i>	Citral, ampicillin+sulbactam, amoxycillin+clavulanic acid, azithromycin, colistin, ceftriaxone,	ND
<i>Enterobacter/Aerococcus</i>	AO, HBO, CNH, Carvacrol, TO	LGO, penicillin, ampicillin, amoxycillin, erythromycin, vancomycin, cloxacillin, oxacillin, lincomycin, linezolid, cefoperazone
<i>Enterobacter/Enterococcus</i>	AO, HBO, TO, CO, ceftazidime+clavulanic acid, ceftriaxone, aztreonam, lincomycin,	Ampicillin, ampicillin+sulbactam, nitrofurantoin, amoxycillin, amoxycillin+clavulanic acid, erythromycin, novobiocin, vancomycin, cloxacillin, oxacillin, lincomycin, linezolid, fosfomycin, cefoperazone
<i>Enterobacter/Paenibacillus</i>	AO, HBO, TO, ceftriaxone, aztreonam, moxalactam	Penicillin, ampicillin, ciprofloxacin, amoxycillin, erythromycin, novobiocin, vancomycin, cloxacillin, lincomycin, linezolid
<i>Enterobacter/Bacillus</i> and allied	AO, HBO, CNH, TO, Citral, CO, cotrimoxazole, colistin, ceftriaxone, aztreonam	Penicillin, ampicillin, ciprofloxacin, erythromycin, cloxacillin, oxacillin, linezolid, cefoperazone
<i>Erwinia/Escherichia</i>	HBO, carvacrol, cefotaxime	Amoxycillin+clavulanic acid
<i>Erwinia/Hafnia</i>	Penicillin, ampicillin, nitrofurantoin, vancomycin, novobiocin, cefoxitin, lincomycin,	AO, CNH, carvacrol, TO, cotrimoxazole, ciprofloxacin, gentamicin, chloramphenicol, ceftazidime+clavulanic acid, carbapenems, cefotaxime, ceftriaxone, cefepime, aztreonam, fosfomycin, minocycline, cefoperazone
<i>Erwinia/Serratia</i>	Penicillin, erythromycin, colistin, lincomycin,	AO, CNH, carvacrol, TO, Co, tetracycline, doxycycline, gentamicin, kanamycin, cotrimoxazole, ciprofloxacin, chloramphenicol, ceftazidime+clavulanic acid, carbapenems, tigecycline, cefotaxime, ceftriaxone, minocycline

<i>Erwinia/Aerococcus</i>	NA	Carvacrol, CO, ampicillin, ampicillin+sulbactam, tetracycline, doxycycline, gentamicin, cotrimoxazole, ciprofloxacin, chloramphenicol, ceftazidime+clavulanic acid, carbapenems, amoxicillin, amoxicillin+clavulanic acid, oxacillin, tigecycline, cefotaxime, ceftriaxone, cefepime, linezolid, minocycline, cefoperazone
<i>Erwinia/Enterococcus</i>	NA	CNH, carvacrol, ampicillin, ampicillin+sulbactam, tetracycline, doxycycline, cotrimoxazole, chloramphenicol, carbapenems, amoxicillin, amoxicillin+clavulanic acid, azithromycin, ceftriaxone, linezolid, fosfomycin, minocycline
<i>Erwinia/Paenibacillus</i>	Nitrofurantoin	CNH, carvacrol, Co, tetracycline, doxycycline, gentamicin, kanamycin, cotrimoxazole, ciprofloxacin, chloramphenicol, ceftazidime+clavulanic acid, carbapenems, amoxicillin, erythromycin, tigecycline, cefotaxime, ceftriaxone, linezolid, minocycline
<i>Erwinia/Bacillus</i> and allied	Lincomycin	AO, HBO, CNH, LGO, TO, citral, CO, penicillin, ampicillin, gentamicin, cotrimoxazole, ciprofloxacin, ceftazidime, carbapenems, amoxicillin+clavulanic acid, erythromycin, vancomycin, coxacillin, colistin, tigecycline, cefotaxime, ceftriaxone, linezolid, minocycline, cefoperazone
<i>Escherichia/Hafnia</i>	Ampicillin, nitrofurantoin, cotrimoxazole, amoxicillin+clavulanic acid	Streptomycin, ceftriaxone, cefoperazone
<i>Escherichia/Serratia</i>	Amoxicillin+clavulanic acid, fosfomycin	BLO, ceftriaxone
<i>Escherichia/Aerococcus</i>	AO, CNH, carvacrol, TO, aztreonam, fosfomycin	LGO, BLO, GO, penicillin, ampicillin, doxycycline, streptomycin, gentamicin, erythromycin, amoxicillin, cloxacillin, oxacillin, ceftriaxone, lincomycin, linezolid, cefoperazone
<i>Escherichia/Enterococcus</i>	AO, CNH, aztreonam	LGO, penicillin, ampicillin, ampicillin+sulbactam, amoxicillin, erythromycin, novobiocin, linezolid
<i>Escherichia/Paenibacillus</i>	AO, CNH, carvacrol, TO, nitrofurantoin, moxalactam, aztreonam	Penicillin, amoxicillin, erythromycin, novobiocin, cloxacillin, linezolid
<i>Escherichia/Bacillus</i> and allied	AO, HBO, CNH, TO, cotrimoxazole, ceftriaxone, colistin, aztreonam, fosfomycin	Penicillin, doxycycline, streptomycin, meropenem, erythromycin, lincomycin, linezolid
<i>Hafnia/Serratia</i>	TO, colistin, cefoperazone	Ampicillin, nitrofurantoin
<i>Hafnia/Aerococcus</i>	AO, CNH, carvacrol, TO, aztreonam, fosfomycin	LGO, GO, penicillin, ampicillin, ampicillin+sulbactam, nitrofurantoin, amoxicillin, amoxicillin+clavulanic acid, erythromycin, cloxacillin, oxacillin, linezolid

<i>Hafnia/Enterococcus</i>	AO, CNH, carvacrol, TO, ceftazidime+clavulanic acid, aztreonam	Ampicillin, ampicillin+sulbactam, nitrofurantoin, amoxicillin, amoxicillin+clavulanic acid, erythromycin, vancomycin, novobiocin, cloxacillin, linezolid
<i>Hafnia/Paenibacillus</i>	AO, carvacrol, TO, ceftriaxone, cefepime, aztreonam, cefoperazone	Penicillin, ampicillin, amoxicillin, amoxicillin+clavulanic acid, erythromycin, vancomycin, novobiocin, cloxacillin, linezolid
<i>Hafnia/Bacillus</i> and allied	AO, HBO, CNH, TO, CO, ceftazidime+clavulanic acid, colistin, cefpodoxime, aztreonam	Penicillin, ampicillin, amoxicillin, amoxicillin+clavulanic acid, erythromycin, cloxacillin, oxacillin, cefoxitin, linezolid
<i>Serratia/Aerococcus</i>	AO, HBO, CNH, carvacrol, TO	Penicillin, ampicillin, ampicillin+sulbactam, gentamicin, amoxicillin, amoxicillin+clavulanic acid, erythromycin, cloxacillin, oxacillin, colistin, cefepime, lincomycin, linezolid, minocycline, cefoperazone
<i>Serratia/Enterococcus</i>	AO, HBO, CNH, CO, ceftazidime+clavulanic acid	Ampicillin, ampicillin+sulbactam, amoxicillin, amoxicillin+clavulanic acid, erythromycin, novobiocin, linezolid, fosfomycin
<i>Serratia/Paenibacillus</i>	AO, HBO, carvacrol, TO, nitrofurantoin, moxalactam	Penicillin, amoxicillin, erythromycin, novobiocin, cloxacillin, linezolid, minocycline
<i>Serratia/Bacillus</i> and allied	AO, HBO, CNH, TO, CO, cefoperazone	Penicillin, ceftazidime, amoxicillin, amoxicillin+clavulanic acid, erythromycin, oxacillin, fosfomycin
<i>Aerococcus/Enterococcus</i>	Cefoperazone	Penicillin, azithromycin, fosfomycin
<i>Aerococcus/Paenibacillus</i>	Ampicillin+sulbactam, nitrofurantoin, cefepime, cefoperazone	ND
<i>Aerococcus/Bacillus</i> and allied	Amoxicillin, ceftriaxone, lincomycin, bacitracin, cefoperazone	ND
<i>Enterococcus/Paenibacillus</i>	Ampicillin+sulbactam, nitrofurantoin	Ciprofloxacin
<i>Enterococcus/Bacillus</i> and allied	Nitrofurantoin, vancomycin, novobiocin, ceftriaxone, fosfomycin,	Ciprofloxacin
<i>Paenibacillus/Bacillus</i> and allied	CNH, vancomycin, novobiocin,	ND
<i>Erwinia amylovora/E. chrysanthemiae</i>	TO, CO, meropenem, ceftriaxone	Ceftazidime

Table 4: Comparison of susceptibility of different groups of bacteria isolated from *Azadirachta indica* leaves in Bareilly to various herbal and conventional antimicrobials.

ND, No Statistically Significant Difference; AO, Ajoan Oil; HBO , Holy Basil Oil; CNH, Cinnamaldehyde; LGO, Lemongrass Oil; TO, Thyme Oil; CO , Cinnamon Oil; BLO, Betel Leaf Oil; GO, Guggul Oil; RWO, Rosewood Oil; MEO, Marjoram Essential Oil; SWO, Sandalwood Oil.

Discussion

There are loads of studies on beneficial endophytic bacteria of Neem leaves [1,2,9-13] to reveal benefits of chewing raw Neem leaves empty stomach in the morning [20-22] but only a few on pathogenic bacteria causing the disease to Neem [18] or the Neem leaves consumers. The present study attempted to identify different types of aerobically growing bacteria present on *A. indica* leaves. The study indicated that only a few samples (20 out of 110) of Neem leaves in the study were positive for bacteria with probiotic potential, i.e., *Bacillus* and *Paenibacillus* species [1,29,30] but the majority of samples had many potentially pathogenic bacteria.

Isolation of potentially pathogenic and CR strains of bacteria more often from *A. indica* leaves' samples from the two research Institute campuses is alarming because the two campuses are often considered to have cleaner and greener environment than any of the two colonies from where samples were collected. It may be a reflection of management of wash and wastewater coming out of clinic and laboratories while handling clinical cases and samples from animals, birds and environment and also handling potential pathogens of high-level drug resistance in different laboratories of the Institute, respectively. Beside wash and wastewater management, sanitary and hygienic practices followed in both of the institutes might be an important reason of occurrence of antimicrobial-resistant strains [31] more frequently on *A. indica* leaves from the two institutes. Inadvertent spilling of potential and known pathogens from clinically sick or dead animals and birds or from laboratories and some flaw in treatment of laboratory and clinical waste coming out regularly might be possible reasons of more resistant pathogens on Neem leaves in the two Institutes [31].

Most of the *Bacillus* and *Paenibacillus* strains are recognised as endophytic to *A. indica* leaves with potential antimicrobial activity against several potential pathogens including *S. aureus*, *Streptococcus pyogenes*, *E. coli*, *Salmonella* Typhimurium, and *K. pneumoniae* [1]. The presence of *Bacillus* or *Paenibacillus* strains on *A. indica* leaves was associated with an absence of potentially pathogenic bacteria except in one sample containing *Bacillus* (*B. cereus*) and *E. coli* both and another having *Paenibacillus amylolyticus* along with *E. coli*. It might be due to non-endophytic or non-probiotic potential of *B. cereus* and *Paenibacillus amylolyticus*, not tested in the present study. *Bacillus cereus*, a potentially toxigenic

species, is associated with food poisoning in humans [32]. *Bacillus cereus* was detected on seven samples of Neem leaves indicating the Neem leaves' potential in food poisoning. *Paenibacillus amylolyticus* is also reported as potentially pathogenic without any probiotic value [33]. Though the load of different bacteria on Neem leaves, an essential estimate of microbial safety of any food item, was not determined, presence of some (four out of six) potentially nasty pathogens classified as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species) bacteria causing hardly curable infections revealed the significance of Neem leaves as the potential pathogen carriers. ESKAPE pathogens cause infection in animals and in animals and birds [34].

Though there is scanty information on *A. indica* leaves microbiota [1], a similar spectrum of many bacterial types has been reported on tree leaves [35]. On *A. indica* leaves bacteria of Erwinaceae family, a common symbiont of plants, were detected on 27 samples (24.5%) has been reported on only 2% tea leaves [35] which might be due to several reasons to be understood. The 41 isolates of *Erwinia* genus in the study belonged to nine species. Many of those are reported pathogenic to plants [36], but their significance on Neem diseases is little understood and needs further studies. Pseudomonads were detected on four (3.6%) samples of *A. indica* leaves. It is quite close to the occurrence of pseudomonads on tea leaves reported in 4% samples in Assam [35]. The airborne nature of pseudomonads [37] may be one reason for deposition of these bacteria on Neem leaves.

Detection of enteric bacteria (*E. coli*, *Hafnia alvei*, *Klebsiella* species, *Proteus* species, *Peptococcus* species, *Serratia* species, enterococci, aeromonads and many more) on *A. indica* leaves indicated the poor hygiene and dirtiness in vicinity of Neem trees. Though the presence of enteric bacteria on different foods is not uncommon in the study region and often causes widespread illness in food consumers [38], detection of carbapenem (one of the last resort antibiotic group) resistance among potentially pathogenic enteric bacteria is alarming. The carbapenem resistance in 10 *Erwinia* species isolates from six Neem leaf samples collected in the vicinity of Modular Laboratory building of ICAR-Indian Veterinary Research Institute, Izatnagar is alarming, not due to pathogenic potential of *Erwinia* strains but due to their emergence in a secluded

area and needs further studies on the emergence of carbapenem resistance. Of the 26 samples positive for CR strains of bacteria 21 were from trees around laboratories, many of which handle potentially pathogenic strains of bacteria, in the two Institutes (CARI and IVRI). Though it was not investigated and proved, it may be a probability that there may be a problem with proper laboratory waste disposal management.

Resistance to extended-spectrum- β -lactam (ESBL) antibiotics in a sizeable number of bacteria (68.6%) isolated from *A. indica* leaves indicated the widespread occurrence of ESBL resistant bacteria in the environment of *A. indica* trees. Similar high prevalence of ESBL resistant strains has been reported earlier in animals and environment in Bareilly region [39,40].

Herbal antimicrobial drug resistance detected among bacteria isolated from *A. indica* leaves in the study is in corroboration with the earlier observation in Bareilly region among bacteria associated with clinical infections and environmental samples [41]. However, the proportion of resistant strains to most of the herbal antimicrobials (cinnamon oil, thyme oil, ajowan oil, cinnamaldehyde and holy basil oil) detected on *A. indica* leaves was much more than reported earlier [41] in clinical samples. Either it is due to better survival of herbal drug-resistant bacteria on *A. indica* leaves or antimicrobial activity of *A. indica* might have induced cross-resistance to other herbs in bacteria harboured on its leaves is not clear with the study and needs further elaborate research.

The wide and significant difference among the susceptibilities of bacteria of different genera and different species of the same genus observed in the study is in concurrence to earlier observation reported frequently [30,41]. The difference among Gram-positive and Gram-negative bacteria's antimicrobial susceptibility is often attributed due to cell wall structure difference between the two and to acquisition potential difference of the two types of bacteria for mobile antibiotic resistance traits [42].

Conclusion

The study concluded that *A. indica* leaves may be harbouring potentially pathogenic multiple drug-resistant bacteria including *Hafnia alvei*, *Escherichia coli*, *Serratia marcescens*, *Bacillus cereus*, *Raoultella terrigena*, *Serratia odorifera*, *Aeromonas bestiarum*, *Enterococcus faecium*, *Klebsiella oxytoca*, *K. pneumoniae*, *Pseudomo-*

nas aeruginosa, *Acinetobacter ewofflii*, *Aeromonas caviae*, *Proteus mirabilis*, *Stenotrophomonas maltophilia*, and *Pseudomonas pseudoalcaligenes*. Twenty six samples carried carbapenem-resistant (CR) and 72 were positive for extended-spectrum β -lactamase (ESBL) bacteria, and many of those may seriously harm the health of leaves' consumers. Therefore, either the Neem leaves chewed raw cause any illness or infection or not in leaf chewers may be an exciting area of future research of public health concern. The study suggested that fresh leaves of *A. indica* should only be consumed after proper cleansing and decontamination for real health benefits.

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Conflict of Interest

None to declare.

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