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Research Article

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-*

lococcus 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], anticariogenic 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 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

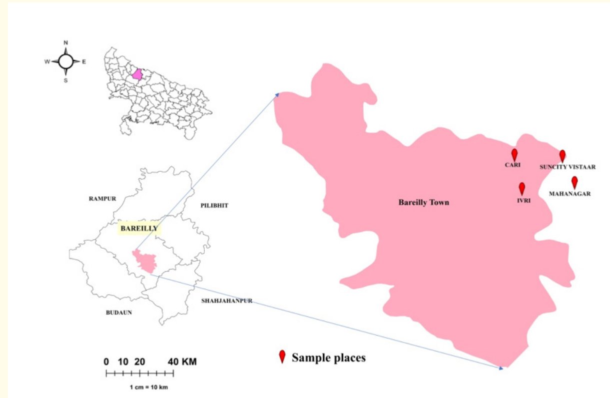

Figure 1: Places of sampling on map of Bareilly, UP, India (IVRI = 62, CARI = 10, Mahanagar = 18, and Suncity = 20).

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 Siriussystem (Bruker Daltonics) for the identity.

Antimicrobial susceptibility assays

All the isolates from Neem leaves were tested for their susceptibility to amoxycillin (30 μg), amoxycillin (30 μg) + clavulanic acid (10 μg), ampicillin (10 μg), ampicillin (10μg) + 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), cefoxitin (10 μg), cefpodoxime (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), fosfomycin (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 (Biomerieux , 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 panthothenticus* (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
Achromobacter xyloxidans	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Acinetobacter calcoaceticus	3	3	0	1	1	0	0	0	0	1	1	0	5	5	0
*Acinetobacter ewofflii**	1	2	0	0	0	0	0	0	0	0	0	0	1	2	0
*Acinetobacter lwoffii**	1	1	0	0	0	0	0	0	0	1	1	0	2	2	0
Aerococcus spp.	1	1	1	1	1	0	0	0	0	9	15	0	11	17	1
*Aeromonas besti-arum**	1	1	0	0	0	0	0	0	0	3	4	1	4	5	1
*Aeromonas caviae**	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1
Alcaligenes denit-rificance	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
*Alcaligenes fae-calis**	2	2	1	0	0	0	0	0	0	0	0	0	2	2	1
Arsenophonus nasoniae	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
Bacillus spp. **	1	2	0	0	0	0	1	1	0	0	0	0	2	3	0
*Bacillus amyloliq-uefaciens***	0	0	0	0	0	0	0	0	0	4	6	0	4	6	0
Bacillus badius	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
*Bacillus cereus**	0	0	0	0	0	0	6	15	0	1	1	0	7	16	0
*Bacillus licheni-formis***	0	0	0	0	0	0	3	9	0	0	0	0	3	9	0
Cedecea lapagii	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
*Enterobacter (Pantoea) agglo-merans**	30	53	10	6	10	1	1	1	0	0	0	0	37	64	11
*Enterobacter amnigenus**	1	3	0	0	0	0	0	0	0	0	0	0	1	3	0
*Enterobacter cloacae**	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
*Enterobacter gregoviae**	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0

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

Micrococcus spp.	1	1	0	0	0	0	0	0	0	4	5	0	5	6	0
Paenibacillus amylolyticus	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Paenibacillus larvae	0	0	0	0	0	0	0	0	0	1	1	0	1	1	0
*Paenibacillus pantothenticus***	0	0	0	0	0	0	5	10	0	0	0	0	5	10	0
Pantoea dispersa	5	6	0	0	0	0	0	0	0	0	0	0	5	6	0
Peptococcus spp. **	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
Proteus mirabilis	1	1	1	0	0	0	0	0	0	0	0	0	1	1	1
*Pseudomonas aeruginosa**	2	2	0	0	0	0	0	0	0	0	0	0	2	2	0
Pseudomonas pseudoalcaligenes	0	0	0	0	0	0	1	1	0	0	0	0	1	1	0
*Raoultella terrigena**	4	5	0	1	1	0	1	1	0	1	1	0	7	8	0
Serratia ficaria	4	4	0	0	0	0	0	0	0	0	0	0	4	4	0
Serratia fonticola	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Serratia grimesii	2	5	0	0	0	0	0	0	0	0	0	0	2	5	0
Serratia marcescens	6	6	0	0	0	0	2	2	1	0	0	0	8	8	1
Serratia odorifera	7	9	0	0	0	0	0	0	0	0	0	0	7	9	0
Serratia plymuthica	0	0	0	0	0	0	2	2	0	0	0	0	2	2	0
*Serratia rubidaea**	1	1	0	0	0	0	4	5	1	0	0	0	5	6	1
Staphylococcus schleiferi ssp. *schleiferi**	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
Staphylococcus xylosus	0	0	0	1	1	0	0	0	0	0	0	0	1	1	0
Stenotrophomonas *maltofila**	1	1	0	0	0	0	0	0	0	0	0	0	1	1	0
*Xenorhabdus bovienni**	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 (*Azadireahta 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 boviennni* (1).

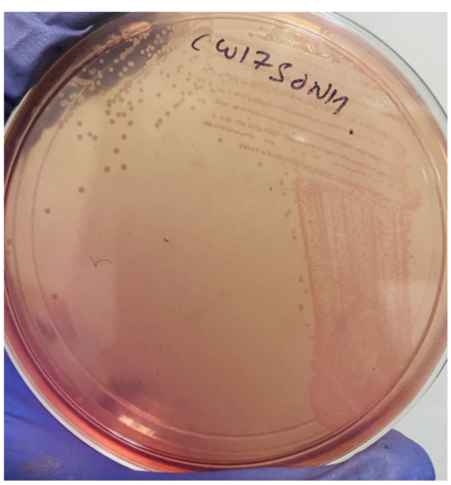

Figure 2: *Acinetobacter lwoffii* growing on McConkey agar, 24 h incubation at 37°C.

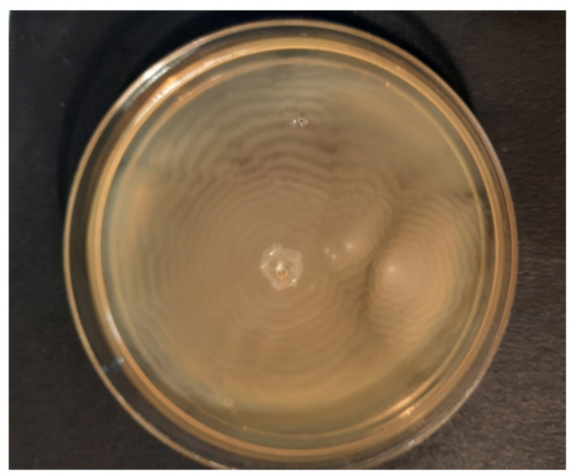

Figure 3: *Proteus mirabilis* growing on nutrient agar, 24 h incubation at 37°C.

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.

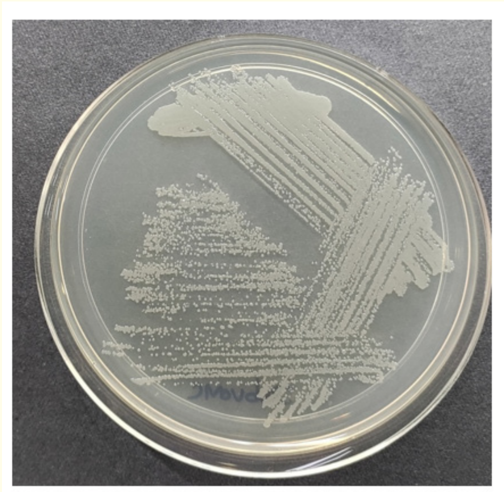

Figure 4: *Stenotrophomonas maltophilia* growing on nutrient agar, 24 h incubation at 37°C.

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), cefotaxime (83.3) and tigecycline (83). Other antibiotics could inhibit the growth of only less than 80% isolates and many common antibiotics, 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-

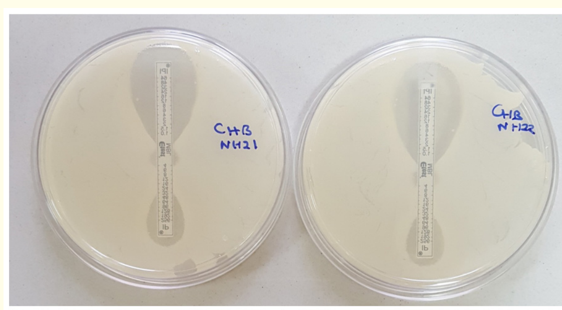

Figure 5: E-test (Biomerieux, France) for determining metallo-β-lactamase production by *Alcaligenes faecalis* (tested in duplicate).

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)
Amoxycillin	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
Amoxycillin 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
Chlorampheni-col	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
Minocyc;line	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. badius*, *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.

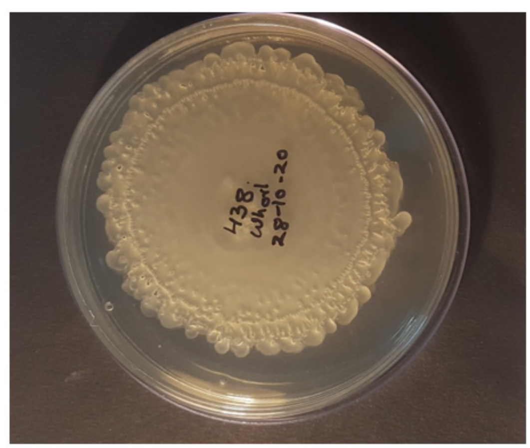

Figure 6: *Bacillus licheniformis* growing on nutrient agar, 48 h incubation at 37°C.

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%).

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
Acinetobacter spp. (9)	22.2	37.5	0	0	55.6	12.5	44.4	0	50	66.7	50	66.7	50	0.3
Aerococcus 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
Bacillus spp. (36)	75	91.2	58.3	8.8	77.8	52.9	72.2	30.6	NT	NT	NT	NT	NT	0.59
Enterobacter 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
Enterococcus 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
Erwinia 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
Escherichia 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
Hafnia alvei (38)	7.9	57.1	10.5	0	89.5	0	57.9	10.5	NT	87.5	NT	91.7	NT	0.35
Paenibacillus spp. (12)	75	83.3	25	25	83.3	41.7	58.3	16.7	100	100	100	100	100	0.52
Serratia 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, Ajovan 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 xylosus* (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 isolates 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, cefoxitin, lincomycin and fosfomycin 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 2nd group to	First group was less often (statistically significant, p <0.05) susceptible than 2nd 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, cefoxitin, lincomycin, fosfomycin	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,
Acinetobacter/Enterobacter	LGO, penicillin, ciprofloxacin, erthromycin, cefoperazone	Cotrimoxazole
Acinetobacter/Erwinia	Penicillin, azithromycin, erythromycin,	Cotrimoxazole, ceftriaxone, cefepime
Acinetobacter/Escherichia	LGO, BLO, penicillin, streptomycin, erythromycin,	Cotrimoxazole, ceftriaxone
Acinetobacter/Hafnia	LGO, ampicillin, amoxycillin+clavulanic acid, azithromycin, erythromycin	TO, cotrimoxazole, ceftriaxone
Acinetobacter/Serratia	SWO, ampicillin+sulbactam, azithromycin, erythromycin, colistin	Cotrimoxazole, ceftriaxone

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

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

Hafnia/Enterococcus	AO, CNH, carvacrol, TO, ceftazidime+clavulanic acid, aztreonam	Ampicillin, ampicillin+sulbactam, nitrofurantoin, amoxycillin, amoxycillin+clavulanic acid, erythromycin, vancomycin, novobiocin, cloxacillin, linezolid
Hafnia/Paenibacillus	AO, carvacrol, TO, ceftriaxone, cefepime, aztreonam, cefoperazone	Penicillin, ampicillin, amoxycillin, amoxycillin+clavulanic acid, erythromycin, vancomycin, novobiocin, cloxacillin, linezolid
Hafnia/Bacillus and allied	AO, HBO, CNH, TO, CO, ceftazidime+clavulanic acid, colistin, cefpodoxime, aztreonam	Penicillin, ampicillin, amoxycillin, amoxycillin+clavulanic acid, erythromycin, cloxacillin, oxacillin, cefoxitin, linezolid
Serratia/Aerococcus	AO, HBO, CNH, carvacrol, TO	Penicillin, ampicillin, ampicillin+sulbactam, gentamicin, amoxycillin, amoxycillin+clavulanic acid, erythromycin, cloxacillin, oxacillin, colistin, cefepime, lincomycin, linezolid, minocycline, cefoperazone
Serratia/Enterococcus	AO, HBO, CNH, CO, ceftazidime+clavulanic acid	Ampicillin, ampicillin+sulbactam, amoxycillin, amoxycillin+clavulanic acid, erythromycin,novobiocin, linezolid, fosfomycin
Serratia/Paenibacillus	AO, HBO, carvacrol, TO, nitrofurantoin, moxalactam	Penicillin, amoxycillin, erythromycin,novobiocin, cloxacillin, linezolid, minocycline
Serratia/Bacillus and allied	AO, HBO, CNH, TO, CO, cefoperazone	Penicillin, ceftazidime, amoxycillin, amoxycillin+clavulanic acid, erythromycin, oxacillin, fosfomycin
Aerococcus/Enterococcus	Cefoperazone	Penicillin, azithromycin, fosfomycin
Aerococcus/Paenibacillus	Ampicillin+sulbactam, nitrofurantoin, cefepime, cefoperazone	ND
Aerococcus/Bacillus and allied	Amoxycillin, ceftriaxone, lincomycin, bacitracin, cefoperazone	ND
Enterococcus/Paenibacillus	Ampicillin+sulbactam, nitrofurantoin	Ciprofloxacin
Enterococcus/Bacillus and allied	Nitrofurantoin, vancomycin, novobiocin, ceftriaxone, fosfomycin,	Ciprofloxacin
Paenibacillus/Bacillus and allied	CNH, vancomycin, novobiocin,	ND
Erwinia amylovora/E. chrysanthemiae	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, Ajovan 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 *Panibacillus* 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 pneumonia, Acinetobacter baumanii, 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 Erwiniaceae 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 leave 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*, *Pseudomonas 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.

Bibliography

1. Singh AK., *et al*. "Isolation, morphological identification and *in vitro* antibacterial activity of endophytic bacteria isolated from *Azadirachta indica* (Neem) leaves". *Veterinary World* 10 (2017): 510-516.
2. Strobel G and Daisy B. "Bioprospecting for microbial endophytes and their natural products". *Microbiology and Molecular Biology Reviews* 67 (2003): 491-502.
3. Upma A., *et al*. "The nature's gift to mankind: Neem". *International Research Journal of Pharmacy* 2 (2011): 13-15.
4. Elavarasu S., *et al*. "Evaluation of anti-plaque microbial activity of *Azadirachta indica* (Neem oil) *in vitro*: A pilot study". *Journal of Pharmacy and Bioallied Sciences* 4 (2012): S394.
5. Xu J., *et al*. "Antiviral activity and mode of action of extracts from Neem seed kernel against duck plague virus *in vitro*". *Poultry Science* 91 (2012): 2802-2807.

6. Alam A., *et al*. "Novel anti-inflammatory activity of epoxyazadiradione against macrophage migration inhibitory factor: inhibition of tautomerase and proinflammatory activities of macrophage migration inhibitory factor". *The Journal of Biological Chemistry* 287 (2012): 24844-24861.

7. Abdel-Ghaffar F., *et al*. "Efficacy of a single treatment of head lice with a Neem seed extract: an *in vivo* and *in vitro* study on nits and motile stages". *Parasitology Research* 110 (2012: 277-280.

8. Aravindan S., *et al*. "Molecular basis of 'hypoxic' breast cancer cell radio-sensitization: phytochemicals converge on radiation induced Rel signaling". *Radiation Oncology* 8 (2013): 1-12.

9. Ebrahimi A., *et al*. "Antimicrobial activities of isolated endophytes from some Iranian native medicinal plants". *Iranian Journal of Pharmaceutical Science* 6 (2010): 217-222.

10. Jalgaonwala RE., *et al*. "Evaluation of endophytes for their antimicrobial activity from indigenous medicinal plants belonging to North Maharashtra region India". *International Journal of Pharmacy and Biomedical Research* 1 (2010): 136-141.

11. Pal A., *et al*. "Diversity and antimicrobial spectrum of endophytic bacteria isolated from *Paederia foetida* L". *International Journal of Current Pharmaceutical Research* 4 (2012): 123-127.

12. Roy S and Banerjee D. "Isolation of antimicrobial compound by endophytic bacteria from *Vinca rosea*". *International Journal of Curreent Research* 5 (2010): 47-51.

13. Verma VC., *et al*. "Endophytic actinomycetes from *Azadirachta indica* A. Juss.: isolation, diversity, and anti-microbial activity". *Microbial Ecology* 57 (2009): 749-756.

14. Chaurasia SC and Jain PC. "Antibacterial activity of essential oils of four medicinal plants". *Indian Journal of Hospital Pharmacy* 15 (1978): 166-168.

15. Rao DVK. "*In vitro* antibacterial activity of Neem oil". *Indian Journal of Medical Research* 84 (1986): 314-316.

16. Jahan T., *et al*. "Effect of Neem oil on some pathogenic bacteria". *Bangladesh Journal of Pharmacology* 2 (2007): 71-72.

17. Khan SA and Aslam J. "Study on the effect of Neem (*Azadirachta indica*) leaves smoke in controlling airborne bacteria in residential premises". *Current Research in Bacteriology* 1 (2010): 64-66.

18. Srivastava SK. "Symptoms of a bacterial disease of Neem". *PANS Pest Articles and News Summaries* 16 (1970): 518-521.

19. Baby AR., *et al*. "*Azadirachta indica* (Neem) as a potential natural active for dermocosmetic and topical products: A narrative review". *Cosmetics* 9 (2022): 58.

20. Sengupta S. "Amazing benefits of chewing Neem regularly". *NDTV Food* (2021).

21. Murthy GS., *et al*. "Effect of chewing *Azadirachta indica* (Neem) and *Ocimum sanctum* (tulsi) leaves on salivary acidogenicity: A comparative study". *Journal of Oral Maxillofacial Pathology* 24 (2020): 479-483.

22. Purohit H. "Amazing benefits of eating neem leaves on empty stomach". Sehat (2019).

23. Carter GR. "Diagnostic Procedures in Veterinary Microbiology". 2nd edn, Charles C Thomas Publishers: Springfield (1975).

24. Bergey D., *et al*. "Bergey's manual of determinative bacteriology". William and Wilkins, Baltimore, MD. Bergey's manual of determinative bacteriology. 9th ed. Williams and Wilkins, Baltimore, MA. (1994).

25. Singh BR. "Labtop for Microbiology Laboratory". Lamber t Academic Publishing, AG and Co. KG, Berlin, Germany (2009).

26. Clinical and Laboratory Standards Institute. "Performance standards for antimicrobial disk susceptibility tests". Clinical and Laboratory Standards Institute, Wayne, USA (2014).

27. Clinical and Laboratory Standards Institute. "Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria". M45, 3rd edn. Clinical and Laboratory Standards Institute, Wayne, USA (2015).

28. Singh BR., *et al*. "Antimicrobial activity of lemongrass (*Cymbopogon citratus*) oil against microbes of environmental, clinical and food origin". *International Research Journal of Pharmacy and Pharmacology* 1 (2011): 228-236.

29. Govindasamy V., *et al*. "*Bacillus* and *Paenibacillus* spp.: potential PGPR for sustainable agriculture, plant growth and health

promoting bacteria". *Microbiology Monographs*. Springer (2010): 333-364.

30. Zulkhairi A., *et al*. "Probiotic properties of *Bacillus* strains isolated from stingless bee (*Heterotrigona itama*) honey collected across Malaysia". *International Journal of Environmental Research (Public Health)* 17 (2020): 278.
31. WHO. "Technical brief on water, sanitation, hygiene (WASH) and wastewater management to prevent infections and reduce the spread of antimicrobial resistance (AMR)". In: World Health Organization, F.A.O., World Organization for Animal Health (Ed.), Global Coordination and Partnership, Surveillance, Prevention and Control, Water, Sanitation, Hygiene and Health. World Health Organization (2020).
32. Singh BR., *et al*. "A bakery product associated *Bacillus cereus* food poisoning outbreak". *Indian Journal of Comparative Microbiology Immunology and Infectious Disease* 16 (1996): 151-152.
33. Wenzler E., *et al*. "Severe sepsis secondary to persistent *Lysinibacillus sphaericus*, *Lysinibacillus fusiformis* and *Paenibacillus amylolyticus* bacteremia". *International Journal of Infectious Diseases* 35 (2015): 93-95.
34. Singh BR. "ESKAPE pathogens in animals and their antimicrobial drug resistance pattern". *Journal Dairy, Veterinary and Animals* 7 (2018): 1-10.
35. Rungsirivanich P., *et al*. "Culturable bacterial community on leaves of assam tea (*Camellia sinensis* var. assamica) in Thailand and human probiotic potential of isolated *Bacillus* spp". *Microorganisms* 8 (2020): 1585.
36. Starr MP and Chatterjee AK. "The genus *Erwinia*: Enterobacteria pathogenic to plants and animals". *Annual Review of Microbiology* 26 (1972): 389-426.
37. Clifton IJ and Peckham DG. "Defining routes of airborne transmission of *Pseudomonas aeruginosa* in people with cystic fibrosis". *Expert Review of Respiratory Medicine* 4 (2010): 519-529.
38. Singh BR., *et al*. "An orange juice-borne diarrhoeal outbreak due to enterotoxigenic *Escherichia coli*". *Journal of Food Science and Technology* 32 (1995): 504-506.
39. Nirupama KR., *et al*. "Molecular characterisation of blaOXA-48 carbapenemase-, extended-spectrum beta-lactamase- and Shiga toxin-producing *Escherichia coli* isolated from farm piglets in India". *Journal of Global Antimicrobial Resistance* 13 (2018): 201-205.
40. Singh BR and Singh SV. "Metallo-β-lactamase and extended-spectrum-β-lactamase production by *Serratia* strains". *Infection and Drug Resistance* 13 (2020): 1295-1297.
41. Singh BR., *et al*. "Potential of herbal antibacterials as an alternative to antibiotics for multiple drug resistant bacteria: An analysis". *Research Journal of Veterinary Science* 13 (2020): 1-8.
42. Kumar S and Singh BR. "An overview of mechanisms and emergence of antimicrobials drug resistance". *Advances in Animal and Veterinary Sciences* 1.2S (2013): 7-14.