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IN-VITRO ASSESSMENT OF ANTIMICROBIAL ACTIVITY OF INDIAN HERBS AND CITRUS FRUIT JUICES AGAINST ENTEROPATHOGENIC BACTERIA

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ABSTRACT
The worldwide increase of multidrug resistance in both community- and health-care associated bacterial infections has impaired the current antimicrobial therapy, warranting the search for other alternatives. We aimed to find the in vitro assessment of antibacterial activity of Indian herbal plant extracts viz., Aloe vera, *Neem*, Bryophyllum, Lemongrass, T*ulsi*, Oregano, Rosemary and Thyme and four citrus fruit juices viz. Amla, Carrot, Beet and Lemon against a series of MDR bacteria of clinical relevance viz *Staphylococcus aureus, Escherichia coli, Klebsiella* pneumoniae, *Pseudomonas aeruginosa, Proteus mirabilis, Vibrio cholerae* and *Salmonella typhi*. Results showed the inefficiency of all the tested antibiotics against the given test organisms and isolates showed high degree of resistance (70%-100%) against the therapeutic agents. The MDR isolates were treated with extracts and juices. The inhibition was observed with the individual extracts and citrus fruit juices. Plant extracts have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes.

KEYWORDS: Enteropathogenic bacteria, Indian herbs, Citrus fruits.

INTRODUCTION
The emergence and spread of multidrug-resistant (MDR) bacterial pathogens have substantially threatened the current antibacterial therapy. MDR bacterial infections often lead to increased mortality, longer length of stays in hospitals, and higher cost of treatment and care. The most problematic bacteria include, but are not limited to, extended-spectrum *β*-lactamase-producing *Escherichia coli* (ESBL-EC) and *Klebsiella pneumoniae* (ESBL-KP), carbapenem-resistant *Enterobacteriaceae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, hospital-acquired methicillin-resistant *Staphylococcus aureus* (MRSA), and vancomycin resistant *Enterococcus* (VRE). Infectious diseases caused by resistant microorganisms are associated with prolonged hospitalizations, increased cost, and greater risk for morbidity and mortality. Resistance is an especially vexing problem for people with impaired immune systems, such as AIDS, cancer patients and recipients of organ transplants. The promiscuous use of antibiotics accounts for a major part of the community burden of antibiotic use and contributes dramatically to the rising prevalence of resistance among major human pathogens.[1]

The effect is pronounced in third world as the costly replacement drugs for treating the highly resistant infectious diseases are unaffordable.[2] The therapeutic options for these pathogens are extremely limited and physicians are forced to use expensive or previously discarded drugs, such as colistin, that are associated with significant side effect to the patients' health. Therefore, it is necessary to search the other alternatives that can potentially be effective in the treatment of these problematic bacterial infections. The usefulness of plant extracts for antimicrobial therapy and/or other diseases have been observed to be promising remedies since ancient time in Chinese medicine, Ayurveda, Arabic, and Unani medicine.

The most important of these bioactive compounds of plants are alkaloids, flavonoids, tannins, phenolic compounds, steroids, resins, fatty acids and gums which are capable of producing definite physiological action on body. Another driving factor that encouraged scientists to search for new antimicrobial substances from various sources including medicinal plants has been the rapid rate of plant species extinction.

Most of the studies are directed to see the activity of plant extracts against a variety of test bacteria including both pathogenic and nonpathogenic strains. Several workers have made targeted screening against MDR bacteria such as MRSA, VRE, *M. tuberculosis*, enteric bacteria and others.[3-6] It was documented that acetone and ethanol extracts obtained from fifteen plants used in folk medicine by tribals of Mandla region exhibited significant activity against urinary tract infection (UTI) causing pathogens[7] Aqil *et al*[8] reported significant inhibitory effect of ethanol extracts of various Indian medicinal plants on both clinical isolates of β-lactamase producing MRSA and methicillin-sensitive *S. aureus* (MSSA). In another study, oregano oil exhibited antibacterial activity against methicillin-sensitive and methicillin-resistant bacteria.[9] Ayachi *et al*.[10] detected the antibacterial activity of methanol, dichloromethane and ether extracts of *Thymus vulgaris* against MDR *Salmonella typhimurium*, Despite abundant literature on the antimicrobial properties of plant extracts, none of the plant derived chemicals have successfully been exploited for clinical use as antibiotics. A significant part of the chemical diversity produced by plants is thought to protect plants against microbial pathogen. Hence, they have been proven to have antimicrobial importance both *in vivo* and *in vitro*.[11] This research was designed to study the antimicrobial potentiality of eight medicinal plants viz. *Aloe barbadensis* (aloe vera), *Azadirachta indica* (*neem*), *Bryophyllum pinnatum* (bryophyllum), *Cymbopogon citratus* (lemongrass), *Ocimum sanctum* (*tulsi*), *Origanum vulgare* (oregano), *Rosmarinus officinalis* (rosemary) and *Thymus vulgaris* (thyme) against a series of MDR bacteria of clinical relevance viz *Staphylococcus aureus, Escherichia coli, Klebsiella* spp, *Pseudomonas* spp, *Proteus* spp, *Vibrio cholerae* and *Salmonella* spp. In addition to medicinal plants, antimicrobial activity of some Citrus fruits *Phyllanthus emblica* (Amla), *Beta vulgaris* (Beet), *Daucus carota (*Carrot) and lemon, were also tested against the said pathogen.

MATERIALS AND METHODS

Bacterial strains

A total of 70 prevalent bacterial isolates of *Staphylococcus aureus* (n=10), *Escherichia coli* (n=10), *Klebsiella Pneumoniae* (n=10), *Pseudomonas aeruginosa* (n=10), *Proteus mirabilis* (n=10,) *Vibrio cholerae* (n=10) and *Salmonella typhi* (n=10) were screened and identified on the basis of morphological, biochemical and cultural characteristics. The further confirmation was carried out by using VITEK 2 Compact identification system. In the procedure, isolated colonies were selected from the primary plate. Theses colonies were transferred asceptically into 3.0ml of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5-7.0) into clear plastic (polustyrene) test tubes (12mm× 75mm). This homogenous suspension was then checked for MacFarland No. 0.05 to 0.63 using calibrated VITECK® 2 DENSICHEK (BioMerieux, MarcyI' Etoile, France). These suspension tubes and ID cards were placed into the VITECK® 2 Cassette. The cassette is finally loaded into the VITECK® 2 systems attached to the workstation. Inoculated cards are passed by a mechanism, which cuts off the transfer tube and seals the card prior to loading into the carousel incubator.

Antibiotic Susceptibility testing

All the 70 confirmed bacterial isolates were further tested for antibiotic susceptibility testing by VITEK 2 Compact AST system. All the isolates were diluted to a standardized concentration in 0.45% saline before being used to rehydrate the antimicrobial medium within the card. The card was then filled, sealed, and placed in VITECK® 2 incubator. VITECK® 2 instrument monitors the growth of each well contained in this card over a defined period of time. At the completion of incubation cycle, MIC values were determined for each antimicrobial contained in the card. MIC results must be linked to an organism identification to determine category interpretation. The category interpretation will be reported along with the MIC, according to the interpretation defined by CLSI guidelines. The GN AST card of VITECK® 2 contain ampicillin, amoxicillin + clavulanic acid, cephalothin, cephazolin, cefexime, cefaleten, cefixitim, cefuroxime, ceftriaxone, cefotaxime, nalidixic acid, trimethoprime sulfamethaxazole (TMP-SMZ), chloramphenicol, norfolxacin, ceprofloxacin, and cotrimoxazole.

Preparation of Plant Extract

Thoroughly washed leaves of five plants of *Aloe barbadensis* (aloe vera), *Azadirachta indica* (*neem*), *Cymbopogon citratus* (lemongrass), *Ocimum sanctum* (*tulsi*), *Origanum vulgare* (oregano, Maruga in Marathi), *Rosmarinus officinalis* (rosemary) and *Thymus vulgaris* (thyme) were taken. These plant materials were dried in shade for five days and then powdered with the help of waring blender. 25 g of shade dried powder was filled in the thimble and extracted with methanol solvent. The extracts were concentrated under reduced pressure and preserved at 5˚C in airtight bottle until further use. In addition to the plant extracts, citrus fruit juices were also taken to study antimicrobial activity against the above said clinical isolates.

Antimicrobial activity of plant extract and fruit juices

The above prepared extracts and fruit juices were used to check their antimicrobial activity against the well confirmed clinical bacterial isolates by agar well diffusion method and results were incorporated on the basis of zone of clearance according to CLSI guidelines.

Data Analysis

Microsoft Excel® was used to enter and capture data. Various graphs and tables were extracted from this data. The data was tabulated and analyzed through descriptive statistics- mean and standard deviation. The Antimicrobial Resistance pattern for each microorganism was analyzed using one way analysis of variance (ANOVA). P value < 0.05 was considered as significant.

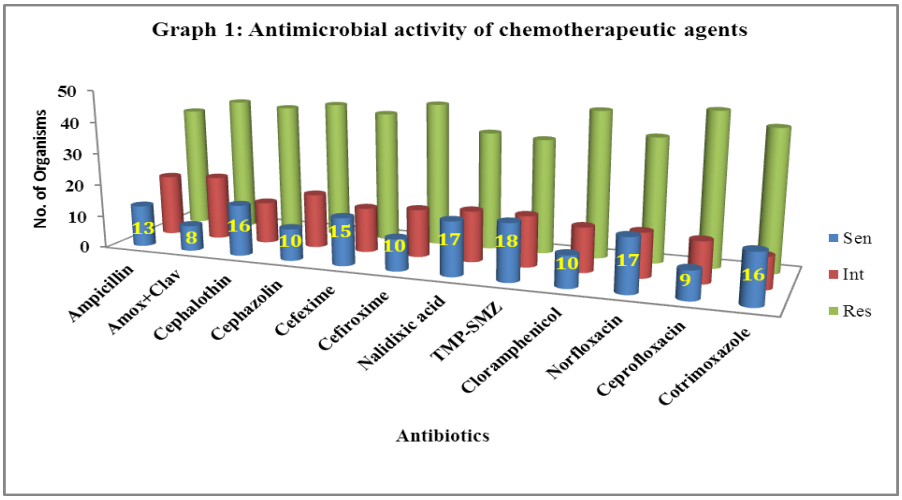
Student t-test-two-sample assuming equal variances was employed for statistical analysis between antibiotics and plant extracts and juices.

RESULTS

Results obtained in the present investigation revealed that the given isolates of enteropathogenic and pyogenic bacteria (n=70) showed greater degree of resistance against the battery of tested antibiotics. *S. aureus* showed 80% resistance towards cefexime and ciprofloxacin. Similarly *E. coli* and *Klebsiella* pneumonia showed 80 to 100 % resistance against chloramphenicol and ciprofloxacin respectively. Isolates of opportunistic pathogen viz., *Pseudomonas aeruginosa* showed greater degree of resistance against cephazolin (80%). Other enteropathogenic bacteria viz., P mirabilis, V. Cholerae and S. typhi showed highest resistance against cotrimoxazole, chlorampgenicaol and cephazolin respectively (Graph 1).

The descriptive statistical analysis showed mean and standard deviation of all the enteropathogenic bacteria which are represented in table 1. Further, according to statistical analysis results of one way ANOVA, for all the sensitive isolates, the P-value was 0.510, F-calculated was 0.884 and F-critical was 2.21 (Table2), for all the intermediate isolates, the P-value was 0.57, F-calculated was 0.79 and F-critical was 2.21 (Table 3) and for resistant isolates, the P-value was found to be 0.77, F-calculated was 0.5390 and F-critical was 2.20 (Table 4), Since P-values were greater than 0.05 (P >0.05). Hence, we fail to reject the null hypothesis and conclude that there is no significant variation in sensitivity, intermediate and resistance pattern of all the stains of enteropathogenic bacteria with respect to given tested antibiotics at 95% confidence Interval.

Further in the current investigation, for in vitro assessment of antibacterial activity, all the above tested MDR isolates were treated with previously said Indian herbal plant extracts and citrus fruit juices by well diffusion method and results were recorded according to CLSI guidelines (Table 5, Graph 2). Surprisingly, the susceptibility pattern was profoundly increased for all the enteric pathogen and the resistance percentage was found to be almost nil. Statistical analysis by student t test for two samples assuming equal variance, showed P=0.000, and results were significantly proved that the herbal extracts and juices have significant antibacterial activity against the given MDR isolates.



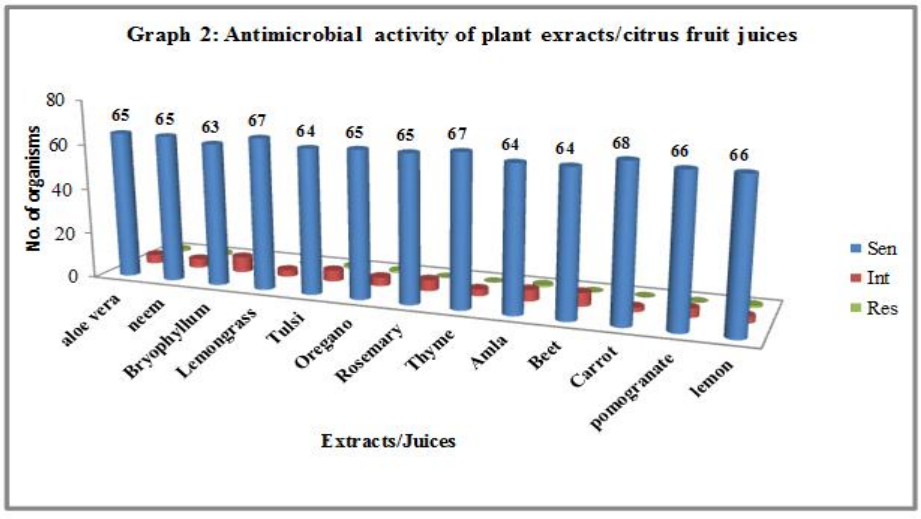


Table 1: Antimicrobial activity of chemotherapeutic agents

Antibiotics	*S. aureus (n=10)*			*E. coli (n=10)*			*K. pneumoniae (n=10)*			*P. aeruginosa (n=10)*			*P. mirabilis (n=10)*			*V. cholerae (n=10)*			*S. typhi (n=10)*		
	Sen	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**
Ampicillin	1	3	6	1	4	5	1	3	6	5	1	4	0	3	7	2	3	5	3	2	5
Amox+Clav	1	4	5	0	3	7	2	4	4	3	1	6	1	2	7	1	4	5	0	2	8
Cephalothin	2	2	6	1	3	6	3	0	7	3	1	6	2	3	5	1	3	6	4	1	5
Cephazolin	0	3	7	2	3	5	4	1	5	0	2	8	1	4	5	3	1	6	0	3	7
Cefexime	0	2	8	1	4	5	2	1	7	1	3	6	5	1	4	3	2	5	3	1	6
Cefiroxime	2	3	5	1	3	6	1	1	8	2	3	5	3	1	7	1	1	8	1	3	6
Nalidixic acid	0	4	6	4	0	6	2	3	5	2	5	3	3	0	7	4	1	5	5	3	1
TMP-SMZ	5	1	4	3	2	5	2	4	4	1	3	6	2	2	6	2	1	7	3	3	0
Cloramphenicol	3	1	6	0	2	8	2	2	6	3	2	5	1	3	6	0	1	9	1	3	6
Norfloxacin	3	0	7	4	1	5	2	3	5	1	4	5	1	4	5	3	0	7	3	2	5
Ceprofloxacin	0	2	8	0	3	7	0	0	10	2	3	5	2	0	8	4	1	5	1	4	5
Cotrimoxazole	1	2	7	3	1	6	4	0	6	2	2	6	1	0	9	3	2	5	2	3	5
Mean	**1.50**	**2.25**	**6.25**	**1.67**	**2.42**	**5.92**	**2.08**	**1.83**	**6.08**	**2.08**	**2.50**	**5.42**	**1.75**	**1.92**	**6.33**	**2.25**	**1.67**	**6.08**	**1.92**	**2.50**	**5.58**
Standard Deviation	**1.57**	**1.22**	**1.22**	**1.50**	**1.24**	**1.00**	**1.16**	**1.53**	**1.73**	**1.31**	**1.24**	**1.24**	**1.29**	**1.51**	**1.44**	**1.29**	**1.15**	**1.38**	**1.31**	**0.90**	**1.08**

Table 2: P and F values for all the sensitive isolates enteropathogenic bacteria

Antibiotics	*S. aureus (n=10)*	*E. coli (n=10)*	*K. pneumoniae (n=10)*	*P. aeruginosa (n=10)*	*P. mirabilis (n=10)*	*V. cholerae (n=10)*	*S. typhi (n=10)*
	Sen	**Sen**	**Sen**	**Sen**	**Sen**	**Sen**	**Sen**
Ampicillin	1	1	1	5	0	2	3
Amox+Clav	1	0	2	3	1	1	0
Cephalothin	2	1	3	3	2	1	4
Cephazolin	0	2	4	0	1	3	0
Cefexime	0	1	2	1	5	3	3
Cefiroxime	2	1	1	2	2	1	1
Nalidixic acid	0	4	2	2	3	4	2
TMP-SMZ	5	3	2	1	2	2	3
Cloramphenicol	3	0	2	3	1	0	1
Norfloxacin	3	4	2	1	1	3	3
Ceprofloxacin	0	0	0	2	2	4	1
Cotrimoxazole	1	3	4	2	1	3	2
Anova: Single Factor							
ANOVA							

Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*	
Between Groups	8.571428571	6	1.428571429	0.884125921	**0.510751402**	2.218816738	
Within Groups	124.4166667	77	1.615800866				
Total	132.9880952	83					

Table 3: P and F values for all the intermediate isolates of enteropathogenic bacteria

Antibiotics	***S. aureus (n=10)***	***E. coli (n=10)***	***K. pneumoniae (n=10)***	***P. aeruginosa (n=10)***	***P. mirabilis (n=10)***	***V. cholerae (n=10)***	***S. typhi (n=10)***
	Intermediate	**Intermediate**	**Intermediate**	**Intermediate**	**Intermediate**	**Intermediate**	**Intermediate**
	1	**2**	**3**	**4**	**5**	**6**	**7**
Ampicillin	3	4	3	1	3	3	2
Amox+Clav	4	3	4	1	2	4	2
Cephalothin	2	3	0	1	3	3	1
Cephazolin	3	3	1	2	4	1	3
Cefexime	2	4	1	3	1	2	1
Cefiroxime	3	3	1	3	1	1	3
Nalidixic acid	4	0	3	5	0	1	3
TMP-SMZ	1	2	4	3	2	1	3
Cloramphenicol	1	2	2	2	3	1	3
Norfloxacin	0	1	3	4	4	0	2
Ceprofloxacin	2	3	0	3	0	1	4
Cotrimoxazole	2	1	0	2	0	2	3
ANOVA: Single Factor							
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*	
Between Groups	8.30952381	6	1.384920635	0.798793175	**0.573808949**	2.218816738	
Within Groups	133.5	77	1.733766234				
Total	141.8095238	83					

Table 4: P and F values for all the resistant isolates of enteropathogenic bacteria

Antibiotics	***S. aureus (n=10)***	***E. coli (n=10)***	***K. pneumoniae (n=10)***	***P. aeruginosa (n=10)***	***P. mirabilis (n=10)***	***V. cholerae (n=10)***	***S. typhi (n=10)***
	Res	**Res**	**Res**	**Res**	**Res**	**Res**	**Res**
	1	**2**	**3**	**4**	**5**	**6**	**7**
Ampicillin	6	5	6	4	7	5	5
Amox+Clav	5	7	4	6	7	5	8

Cephalothin	6	6	7	6	5	6	5
Cephazolin	7	5	5	8	5	6	7
Cefexime	8	5	7	6	4	5	6
Cefiroxime	5	6	8	5	7	8	6
Nalidixic acid	6	6	5	3	7	5	5
TMP-SMZ	4	5	4	6	6	7	4
Cloramphenicol	6	8	6	5	6	9	6
Norfloxacin	7	5	5	5	5	7	5
Ceprofloxacin	8	7	10	5	8	5	5
Cotrimoxazole	7	6	6	6	9	5	5
ANOVA: Single Factor							
ANOVA							
Source of Variation	*SS*	*df*	*MS*	*F*	***P-value***	*F crit*	
Between Groups	7.208791209	6	1.201465201	0.539030403	**0.777110825**	2.208553806	
Within Groups	187.2307692	84	2.228937729				
Total	194.4395604	90					

Table 5: Antimicrobial activity of Plant extracts and citrus fruit juices

Plant extracts/Fruit juices	***S. aureus (n=10)***			***E. coli (n=10)***			***K. pneumoniae (n=10)***			***P. aeruginosa (n=10)***			***P. mirabilis (n=10)***			***V. cholerae (n=10)***			***S. typhi (n=10)***		
	Sen	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**	**Sen**	**Int**	**Res**
aloe vera	9	1	0	10	0	0	9	1	0	10	0	0	8	1	1	9	1	0	10	0	0
neem	10	0	0	8	1	1	10	0	0	9	1	0	9	1	0	10	0	0	9	1	0
Bryophyllum	8	2	0	9	1	0	9	1	0	8	2	0	10	0	0	9	1	0	10	0	0
Lemongrass	9	1	0	10	0	0	10	0	0	9	1	0	10	0	0	10	0	0	9	1	0
Tulsi	10	0	0	10	0	0	9	1	0	10	0	0	8	1	1	9	1	0	8	2	0
Oregano	10	0	0	10	0	0	8	1	1	10	0	0	10	0	0	8	2	0	9	1	0
Rosemary	10	0	0	9	1	0	9	1	0	8	2	0	10	0	0	9	1	0	10	0	0
Thyme	9	1	0	8	2	0	10	0	0	10	0	0	10	0	0	10	0	0	10	0	0
Amla	8	1	1	9	1	0	10	0	0	10	0	0	9	1	0	10	0	0	8	2	0
Beet	9	1	0	10	0	0	8	2	0	10	0	0	9	1	0	8	2	0	10	0	0
Carrot	10	0	0	9	1	0	10	0	0	9	1	0	10	0	0	10	0	0	10	0	0
lemon	10	0	0	8	1	1	10	0	0	10	0	0	8	2	0	10	0	0	10	0	0
Mean	**9.333**	**0.583**	**0.08**	**9.17**	**0.7**	**0.17**	**9.33**	**0.58**	**0.083**	**9.42**	**0.6**	**0**	**9.3**	**0.583**	**0.17**	**9.33**	**0.67**	**0**	**9.42**	**0.58**	**0**
Standard Deviation	**0.778**	**0.669**	**0.29**	**0.83**	**0.7**	**0.39**	**0.78**	**0.67**	**0.289**	**0.79**	**0.8**	**0**	**0.9**	**0.669**	**0.39**	**0.78**	**0.78**	**0**	**0.79**	**0.79**	**0**

DISCUSSION

Plants are important source of potentially useful structures for the development of new chemotherapeutic agents. The first step towards this goal is the *in vitro* antibacterial activity assay.[12] Many reports are available on the antiviral, antibacterial, antifungal, anthelmintic, antimolluscal and anti-inflammatory properties of plants.[13,14,15,16,17,18,19] The number of multi-drug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. This increase has been attributed to indiscriminate use of broad-spectrum antibiotics, immunosuppressive agent, intravenous catheters, organ transplantation and ongoing epidemics of HIV infection. In addition, in developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also often with adulterations and side effects.

The therapeutic options for these pathogens are extremely limited and physicians are forced to use expensive or previously discarded drugs, such as colistin, that are associated with significant side effect to the patients' health[20] Therefore, it is necessary to search the other alternatives that can potentially be effective in the treatment of these problematic bacterial infections. The usefulness of plant extracts for antimicrobial therapy and/or other diseases have been observed to be promising remedies since ancient time in Chinese medicine, Ayurveda, Arabic, and Unani medicine.[21] The inclusion of traditionally used medicines including phytomedicine, if they prove safe and effective, into national health care system is suggested by World Health Organization.[21]

Hence in the present study, 70 multiple dryg resistant isolates of enteric pathogens were treated with extracts of *Aloe barbadensis* (aloe vera), *Azadirachta indica* (*neem*), *Bryophyllum pinnatum* (bryophyllum), *Cymbopogon citratus* (lemongrass), *Ocimum sanctum* (*tulsi*), In addition to medicinal plants, antimicrobial activity of some Citrus fruits *Phyllanthus emblica* (Amla), *Beta vulgaris* (Beet), *Daucus carota (*Carrot) and lemon, were also tested against the said pathogen. 90 to 100 % sensitivity was observed against the extracts of aloe vera, 100% sensitivity was shown by S. aureus and Klebsiella pneumonia towards neem extract. The susceptibility of this bacterium to different plant extracts has been documented in literature.[22, 23] Previous reports also revealed the antibacterial efficacy of the investigated plant extracts and essential oils against *S. aureus* MRSA.[8,24–26] S. aureus, P. aeruginosa nad Klebsiella pneumonia showed 80 to 90% susceptibility towards bryophylla and *S. typhi* and Proteus mirabilis showed100 % sensitivity to it. This is in fair correlation with Aibinu *et al*. [27] who reported good antibacterial activity in bryophyllum against some Gram-positive and Gram-negative bacteria using methanol, local gin and aqueous extracts.

Findings in this study supported the observations of some other researchers about lemongrass which exhibited antibacterial activity against *E. coli* and *K. pneumonia*.[28, 29] Our results contradicts the results shown by Dahiya et al[1] where lemongrass did not show any activity on Proteus mirabilis whwere as in current studt 100% sensitivity was given by Proteus isolates for lemongrass, Bryophyllum, Rosemary, organo and thyme.Among the citrus fruits, amla, carrot and lemon showed promising results. Citrus fruits belong to the family Rutaceae. These are rich source of vitamin C (Ascorbic acid). The peel and pulp of the citrus fruits contain various active constituents and essential oils. The study carried out by Anwesha Chakraborty et.al,[30] showed that orange juice and other citrus fruit juices have profound antimicrobial activity. Hence, current investigation concludes that the plant extracts and citrus fruit juices have great potential as antimicrobial compounds against microorganisms. Thus, they can be used in the treatment of infectious diseases caused by resistant microbes. The synergistic effect from the association of antibiotic with these agents against resistant bacteria leads to new choices for the treatment of infectious diseases.

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