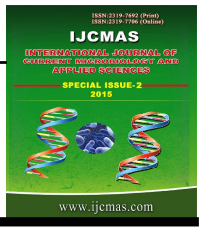
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Special Issue-2 (May-2015) pp. 222-239**
http://www.ijcmas.com



Original Research Article

Anti-Microbial Potential of Plant Extracts against Multi-drug Resistant Uropathogens

S.Ogale Sneha*, Patel Mansi and Tank Jagruti

Department of Microbiology, PES Modern College of Arts, Science and Commerce, Ganeshkhind, Pune, India

**Corresponding author*

Keywords

Uropathogens, Multi-drug resistant, Plasmid, curing, Plant extracts, Garlic (*Allium sativum*), Curry (*Murraya koenigii*)

A B S T R A C T

Urinary tract infection (UTI) is one of the most common infections, second in occurrence only to respiratory tract infections. UTI are difficult to treat due to the drug resistance of the uropathogens. In the case of multi-drug resistant bacteria, antibiotic resistance may be chromosomally encoded or plasmid borne. Plant extracts hold promise as alternatives for inhibiting the growth of uropathogens and as curing agents which would render the pathogens susceptible to antibiotics. Ten multi-drug resistant uropathogens were obtained from Joshi Hospital, Pune. Aqueous and ethanolic extracts of various plants were tested for antimicrobial activity by agar diffusion method. Ethanol extract of Curry *(Murraya koenigii*) leaves showed the ability to inhibit growth of multi-drug resistant Gram positive uropathogens. Garlic extract showed the highest antimicrobial potential being able to inhibit the growth of a broad spectrum of multi-drug resistant uropathogens viz. Gram positive and Gram negative bacteria and also yeast (*Candida sp.*). MIC, SIC and MBC for garlic extract were determined. Effect of exposure to garlic extract was studied. Higher concentrations of garlic extract killed the cultures. The cultures exposed to lower concentrations of garlic extract showed increase in sensitivity to some of the antibiotics. This work highlights the bioactivity of plant extracts such as garlic extract in effectively killing multi-drug resistant uropathogens and in increasing the susceptibility of the surviving pathogens to antibiotics.

Introduction

Urinary tract infections are very common infections and lead to significant amount of morbidity and mortality. Of the causative agents of UTI, *E. coli* is found to occur most frequently (65-80% of the time) whereas the others such as *Klebsiella sp., Proteus sp., Pseudomonas sp., Enterococcus sp., Enterobacter sp., Candida sp.* etc. occur less frequently (2–18% of the time). The occurrence of multi-drug resistant uropathogens is a cause for concern as the infections become difficult to treat (Alon *et al*, 1987; Yuksel *et al.*, 2006). Alternative strategies are being explored such as the potential of plants in providing antimicrobials (Zwetlana *et al.*, 2014). We

have examined the potential of aqueous and ethanolic extracts of various plants: Hirda (*Terminalia chebula*), Behada (*Terminalia bellirica*), orange (*Citrus sinensis*) peel, curry (*Murraya koenigii*) leaves, onion (*Allium cepa*) and garlic (*Allium sativum*) in inhibiting the growth of uropathogens by agar diffusion assay and by broth dilution method. Garlic Extract exhibited the highest potential being able to inhibit the growth of a broad spectrum of uropathogens such as Gram positive and Gram negative bacteria as well as yeast (*Candida sp*.). Hence MIC, SIC and MBC of garlic extract against the uropathogens was determined. Exposure to garlic extract killed many uropathogens and increased the susceptibility of the survivors to antibiotics. Extract of curry leaves was effective against Gram positive bacteria. *Candida sp.* was inhibited by garlic, onion and orange peel extracts. Drug resistance may be chromosomally encoded or plasmid-borne. Hence plasmid profile of the uropathogens was determined. We examined the possibility of sub-inhibitory concentrations of garlic extract curing the uropathogens of their plasmids (Trevors, 1986) leading to loss of multi-drug resistance. We found that though the uropathogens, upon exposure to garlic extract showed increased susceptibility to some of the drugs, curing had not occurred.

Materials and Methods

Cultures and their maintenance

Ten cultures, causative agents of UTI were obtained from MMF's Joshi Hospital, Pune. They were multi-drug resistant and of the following types: Gram negative (*E. coli (4 isolates), Proteus mirabilis, Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), Gram positive *(Enterococcus sp*.- 2 isolates) genera and yeast (*Candida albicans*). *Staphylococcus aureus* (NCIM) was used as a standard antibiotic-sensitive culture. They were cultured on Luria Agar (HiMedia Laboratories Pvt. Ltd.) and MacConkey's Agar (Gram negative cultures) (HiMedia Laboratories Pvt. Ltd.) plates, incubated at 37^0C for a period of 24 hrs and maintained on slants (of the same media) at 4^0C till further use.

Antimicrobial susceptibility testing

The ten isolates were tested for susceptibility against various antimicrobials using CLSI guidelines. Suspensions of freshly grown cultures (18-20 hr old) were made in sterile saline and their turbidity was matched with 0.5 McFarland standard. These culture suspensions were then spread on the surface of sterile Mueller Hinton Agar (HiMedia Laboratories Pvt. Ltd.) plates using sterile swabs. The antibiotic impregnated polydiscs (for use against UTI pathogens provided by Dynamicro Labs. Pvt. Ltd.) were then applied. For *Candida sp.* wells were bored using a surface sterilized cork borer. 30 µl of fluconazole (0.833 mg/ ml and 1.666 mg/ml) was added to the wells to give a final concentration of 25 µg/well and 50 µg/well. The plates were incubated at 37^0C for a period of 24 hrs. The diameters of the zones of inhibition obtained were measured. The mean zone diameter is reported.

Plasmid profile

Plasmids were extracted from the uropathogens by Alkaline Lysis Method (Sambrook and Russel, 2001). The plasmid preparation was electrophoresed in 1% agarose (Merck Genie) gel at 6V/cm, which was then stained by ethidium bromide (0.5 µg/ml) and observed using UV-transilluminator as per the standard protocol (Sambrook and Russel, 2001). Supercoiled Plasmid DNA Ladder (size range: 2, 3, 4, 5,

6, 7, 8 and 10 kb) (Merck Genei) was used as the Molecular Weight Marker during electrophoresis. The Molecular weights of the plasmids of the isolates were determined by plotting a graph of log Molecular weight (in kb) versus the distance migrated by the plasmid bands of the Molecular Weight Marker. From the distance migrated by the plasmids of the uropathogens, their Molecular weights were determined from the graph (Fig. 3: Migration of plasmid bands as a function of Molecular Weight. Determination of Molecular Weight of the plasmids of the isolates).

Preparation of plant extracts (Zwetlana *et al.*, 2014)

Extracts of onion (*Allium cepa*) bulb and garlic (*Allium sativum*) cloves were prepared as follows: Hundred grams of onion bulb and garlic bulb were peeled, surface disinfected and then crushed in a blender or mortar and pestle respectively. The extracts from these were squeezed out and then filtered through sterile muslin cloth followed by filter sterilization (through Millipore filters of pore size 0.25µ). This (original concentration) and dilutions made in sterile distilled water were further used.

Extracts of Hirda (*Terminalia chebula*) fruit powder, Behada (*Terminalia bellirica*) fruit powder, orange (*Citrus sinensis*) peel and curry (*Murraya koenigii*) leaves were prepared in 70% ethanol as follows:

Curry leaves and orange peel were rinsed with distilled water and shade dried. They were then powdered.

Commercially available powders (used as Ayurvedic medecines) of fruit of Hirda (*Terminalia chebula*) and Behada (*Terminalia bellirica*) were used.

Twenty-five gram of each powder (curry leaves/ orange peel/ hirda/ behada) was added to 250 ml of 70% ethanol and kept on rotary shaker at 37^0C for a period of 24 hrs. It was then centrifuged at 10,000 rpm for 10 min. The supernatant was filtered through Whatman No. 1 filter paper. The filtrate was then taken in a crucible and the solvent (ethanol) was evaporated from it by keeping it on a boiling water bath. The residue obtained was then weighed and dissolved in DMSO (Dimethyl sulfoxide) to give a concentration of 50 mg/ml.

Anti-microbial activity of plant extracts

Antimicrobial susceptibility testing using plant extracts prepared as above against the drug resistant isolates was carried out by agar well diffusion method following CLSI guidelines. Suspensions of freshly grown cultures (18-20 hr old) were made in sterile saline and their turbidity was matched with 0.5 McFarland standard. These culture suspensions were then spread on the surface of sterile Mueller Hinton Agar plates. Wells (of diameter 5 mm) were bored using surface sterilized cork borer in the Mueller Hinton agar plates; 30 µl of each extract was then added to the respective wells. Sterile distilled water/ DMSO was added to a well as negative control. 30 µl each of Ampicillin (10 mg/ ml) and fluconazole (0.833 mg/ml and 1.667 mg/ml to give 25 µg/ well & 50 µg/well respectively) were added as standard antibiotics against bacterial cultures and against *Candida albicans* respectively.

Determination of MIC range by plate assay

Since garlic extract gave the best results it was used for further study. Suspensions of freshly grown cultures (18-20 hr old) were made in sterile saline and their turbidity was matched with 0.5 McFarland standard. These culture suspensions were then spread on the surface of sterile Mueller Hinton

Agar plates using sterile swabs. For determination of range of MIC, a gradient of concentration of garlic extract on the surface of the plate was prepared in the following manner. Eight sterile Whatman No. 1 filter paper discs were placed on the surface of the plate in alignment with a diameter of the plate and 2 mm apart from each other. Five microliters of each two-fold dilution (16, 32, 64, 128, 256, 512, 1024 and 2048) was then applied to each disc. The plates were incubated at 37^0C for a period of 24 hrs. The concentration gradient produces an ellipse of inhibition. The MIC is determined as the concentration of antimicrobial where the ellipse of inhibition intersects the concentration gradient.

Determination of MIC (Minimum Inhibitory Concentration), MBC (Minimum Bactericidal Concentration for bacterial cultures and Minimum Biocidal Concentration for *Candida sp*.) and SIC (Sub-Inhibitory Concentration) by broth dilution method:

Four milliliters of sterile Luria broth were taken in tubes to which was added sterile garlic extract to give appropriate range of concentrations (less than, equal to and greater than MIC determined by plate assay mentioned above). To this was added 100 µl of culture suspension. For each such tube a negative control was maintained to which culture suspension was not added. For each culture a positive control was maintained where garlic extract was not added. The Optical Density ($O.D_{600}$) of each tube was noted at 'zero' hour i.e. just after completion of all necessary additions. The tubes were incubated at 37^0C for a period of 24 hrs. The OD_{600} was noted after 24 hrs of incubation. For each culture the MIC of garlic extract was defined as the highest fold dilution that gave no increase in turbidity indicating no growth of the culture.

SIC was defined as one fold dilution greater than the MIC. At this concentration of garlic extract some growth was evident.

The MBC of garlic extract was determined in the following manner. Loopful of suspension from each tube showing no increase in turbidity at the end of 24 hrs of incubation was streaked on sterile Luria agar/ MacConkey's Agar (For Gram negative cultures) plates. The plates were incubated at 37^0C for a period of 24 hrs and checked for growth of culture at the end of the incubation period. MBC was defined as the highest fold dilution that gave no growth of culture on the plate.

Determination of effect of SIC of plant extract (garlic) on the uropathogens

Two *E. coli* cultures, *Enterococcus sp.* and *Candida sp.* were considered for further study as representative of Gram negative, Gram positive and fungal (yeast) cultures. From the above mentioned Luria broth tubes containing SIC of garlic extract (after 24 hrs of incubation), 100 µl of culture suspension was spread on Luria agar and MacConkey agar (Gram negative cultures) plates respectively. The plates were incubated at 37^0C for a period of 24 hrs. The isolated colonies that appeared on these plates were then considered as 'garlic exposed cultures'. These cultures were then identified with the added label of 'G' to their Culture Isolate numbers.

Antibiotic sensitivity profile of the cultures exposed to garlic extract versus the unexposed cultures

This was determined following CLSI guidelines. Suspensions of freshly grown (garlic-exposed and corresponding unexposed) cultures (18-20 hr old) were

made in sterile saline and their turbidity was matched with 0.5 McFarland standard. These culture suspensions were then spread on the surface of sterile Mueller Hinton Agar plates using sterile swabs. The antibiotic impregnated polydiscs/ single discs were then applied. The plates were incubated at 37^0C for a period of 24 hrs. The diameters of the zones of inhibition obtained were measured. The mean zone diameter is reported.

To check whether exposure to sub-inhibitory concentrations of garlic extract causes plasmid curing, the plasmid profiles of selected cultures exposed to garlic extract and those not exposed to it were checked

Plasmids were extracted from the selected isolates (*E. coli* 2945 and *E. coli* 2927 cultures 'Exposed' and 'Unexposed' to garlic extract) by Alkaline Lysis Method (Sambrook and Russel, 2001). The plasmid preparation was electrophoresed (Sambrook and Russel, 2001) in 1% agarose (Merck Genie) gel at 6V/cm, which was then stained by ethidium bromide (0.5 µg/ml) and observed using uv-transilluminator. Supercoiled Plasmid DNA Ladder (Merck Genei; size range: 2, 3, 4, 5, 6, 7, 8 and 10 kb) was used as Molecular Weight Marker in the gel. Molecular weights of the plasmids were determined.

Results and Discussion

Antibiotic Resistance pattern of isolates obtained from MMF's Joshi Hospital

Please refer to figure 1 (Antibiotic susceptibility testing)

Candida albicans (2942): was resistant to 25 µg and 50 µg of fluconazole added per well.
Enterococcus sp.(2304): was resistant to gentamicin (no zone) and nitrofurantoin and showed intermediate resistance to ciprofloxacin.

Enterococcus sp. (2289): was resistant to gentamicin (no zone) and ciprofloxacin (no zone) and had intermediate resistance to nitrofurantoin.

The Gram negative uropathogens were found to be highly resistant to most of the drugs. Please refer to table 1 (antibiotic susceptibility of the Gram negative isolates). *Proteus mirabilis* (2912) and *E. coli* (2927) were resistant to all the drugs tested whereas *Klebsiella pneumoniae* (2969) and *E. coli* (2272) were each resistant to all but one drug.

Plasmid profile of the selected isolates: Please refer to Fig. 2 (Agarose gel electrophoresis of the plasmid preparations).

Plasmid bands were visible in all lanes other than Lane No.9 (Plasmid preparation of *E. coli* 2296). The following cultures were seen to have plasmids: *E. coli* 2286, *E. coli* 2927, *K. pneumoniae* 2969, *E. coli* 2945, *Proteus mirabilis 2912*, *E. coli* 2272, and *Pseudomonas aeruginosa* 2972.

From the graph (Please refer to fig. 3: Migration of plasmid bands as a function of Molecular Weight. Determination of Molecular Weight of the plasmids of the isolates) the plasmids for the various isolates range in size from 2 to 3 kb (Isolate No 2286, 2927 & 2969) and 5-6 kb (Isolate No.s 2286, 2927, 2969, 2945, 2912, 2272 & 2972).

Preparation of plant extracts:

Extracts of Hirda (*Terminalia chebula*) fruit powder, Behada (*Terminalia bellirica*) fruit powder, orange (*Citrus sinensis*) peel and curry (*Murraya koenigii*) leaves were prepared in 70% ethanol and aqueous

extracts of onion (*Allium cepa*) bulb and garlic (*Allium sativum*) cloves were prepared. (Please refer to Fig. 4: Plant Extracts prepared).

Antimicrobial activity of plant extracts: Please refer to table 2 (Antimicrobial activity of plant extracts).

Garlic Extract gave the best result:

It showed **antimicrobial activity against** Gram negative and Gram positive bacterial cultures as well as against yeast (*Candida sp*.)

The **inhibition zone** obtained by garlic extract was greater than that obtained by any of the other extracts and it was comparable with or greater than that obtained by standard antibiotic.

Gram positive uropathogens such as *Enterococcus spp* were inhibited only by garlic extract, onion extract, **curry leaves extract** and ampicillin.

Curry leaves extract exhibited antimicrobial activity only against Gram positive cultures.

The **MIC range for garlic extract** was determined for the selected isolates by **plate assay** (Please refer to Fig. 6: MIC range determination by Plate Assay). This range was then used in the broth dilution assay for determination of MIC, SIC and MBC. (Please refer to Tables 3 through 7: Broth dilution Method for determination of MIC, SIC and MBC for garlic extract; Table 8: MIC, SIC and MBC in terms of fold dilutions of Garlic Extract for various isolates) and Figures 7 (Growth of *Candida albicans* in absence and presence of Garlic Extract) and 8 (Determination of MBC of Garlic Extract for *Pseudomonas aeruginosa* 2972).

Garlic Extract is highly effective in inhibiting the growth of all the isolates tested.

The negative $O.D_{600}$ values in the given tables (Tables 3, 4, 6 and 7) are indicative of the probable lysis of the cultures in the presence of higher concentrations of garlic extract.

Please refer to Table 8 (MIC, SIC and MBC in terms of fold dilutions of Garlic Extract for various isolates) for the MIC, SIC and MBC values determined for the various isolates.

MIC values range from: 16 to 512 fold dilutions of Garlic Extract. SIC values range from: 32 to 1024 fold dilutions of Garlic Extract.

MBC values range from: 4 to 512 fold dilutions of Garlic Extract for all isolates other than *Klebsiella pneumoniae* (2969).

The MBC value for *Klebsiella pneumoniae* (2969) was in the range of original to 4 fold dilution of Garlic Extract.

Determination of effect of SIC of plant extract (garlic) on the uropathogens:

There was some change in the colony morphologies of the 'Garlic Exposed' cultures

E. coli (2945G) (3 types of colonies obtained viz. Large, medium- sized and small) and *E. coli* (2927G): was turning yellow on MacConkey's agar plates within 16-18 hrs as compared to the unexposed culture which took 24 hrs to turn yellow indicating a probable faster metabolism of lactose. The medium sized colony of *E. coli* 2945G was further tested for antibiotic resistance and plasmid curing. There was no

observable difference in colony morphologies of 'garlic exposed' and 'unexposed' *Enterococcus sp*. and *Candida sp*. Please refer to Fig. 9 to observe the colony morphologies/ growth pattern of the 'Garlic Exposed' cultures.

Antibiotic sensitivity profile of the cultures exposed to garlic extract versus the unexposed cultures

Please refer to Table 9 (Antibiotic resistance profile of Garlic Exposed and unexposed *E. coli* cultures) and Fig. 10 (Antibiotic susceptibility of garlic exposed and unexposed cultures).

For the two *E. coli* cultures (2945 and 2927): exposure to garlic resulted in an increase in sensitivity to various antibiotics: *E. coli* 2945G became susceptible to ofloxacin, gentamicin and netilmicin whereas the 'unexposed' culture was having resistance or intermediate resistance to them. For *E. coli* 2945G: Even if the status of susceptibility did not change for some of the antibiotics the zone of inhibition increased for them indicating an increase in sensitivity.

E. coli 2927G: became susceptible to amikacin for which the 'unexposed' culture was resistant and had intermediate resistance to nitrofurantoin for which the 'unexposed culture' was resistant. For *E. coli* 2927G: Although the status of resistance did not change for netilmicin and ciprofloxacin, the zone size increased for the former and decreased to no zone for the latter.

Table.1 Antibiotic susceptibility of the Gram negative isolates Inhibition zone (IZ) diameter (mm) and Interpretation

Cultures		*E. coli* 2945		*E. coli* 2927		*E. coli* 2272		*Klebsiella pneumoniae* 2969		*Pseudomonas aeruginosa* 2972		*Proteus mirabilis* 2912	
Antibiotic	Disc content (μg)	IZ	Interpretation	IZ	Interpretation	IZ	Interpretation	IZ	Interpretation	IZ	Interpretation	IZ	Interpretation
Amikasin AN	30	20	**S**	14	**R**	19	**S**	14	**R**	15	**I**	NZ	**R**
Ofloxacin OX	5	15	**I**	NZ	**R**	6	**R**	NZ	**R**	25	**S**	NZ	**R**
Gentamicin G	10	10	**R**	NZ	**R**	6	**R**	17	**S**	NZ	**R**	NZ	**R**
Norfloxacin NR	10	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	20	**S**	NZ	**R**
Cefaclor CFC	30	10	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**
Ciprofloxacin CIP	5	15	**R**	9	**R**	NZ	**R**	NZ	**R**	29	**S**	NZ	**R**
Nitrofurantoin NF	300	24	**S**	12	**R**	9	**R**	NZ	**R**	NZ	**R**	NZ	**R**
Cefoperazone CFP	75 Units	22	**S**	NZ	**R**	NZ	**R**	NZ	**R**	12	**R**	NZ	**R**
Ceftriaxone CTX	30	22	**S**	NZ	**R**	NZ	**R**	NZ	**R**	24	**S**	NZ	**R**
Cefuroxime CR	30	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**
Cefadroxil CD	30	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**	NZ	**R**
Netilmicin NET	30	14	**I**	NZ	**R**	6	**R**	NZ	**R**	NZ	**R**	NZ	**R**

Key: IZ: Inhibition zone diameter (mm), NZ: No zone of Inhibition indicating Resistance to the antibiotic. R: Resistant to the antibiotic; I: Intermediate; S: Susceptible; NG: No Growth indicating sensitivity to the antibiotic

Table.2 Antimicrobial activity of plant extracts

	Isolate No.	Inhibition Zone Diameter in mm **Extract used**										
		Orange peel-DMSO	Behada-DMSO	Hirda-DMSO	Onion	**Garlic**	Antibiotic*	Orange peel	Behada	Hirda	DMSO	Curry leaves
E. coli	2927	NZ	8	6	NZ	**30**	NZ	NZ	14	12	6	NZ
E. coli	2945	7	NZ	11	NZ	**30**	32	7	NZ	11	NZ	NZ
E. coli	2286	NZ	2	4	NZ	**38**	18	NZ	10	12	8	NZ
E. coli	2272	9	NZ	NZ	NZ	**28**	NZ	9	NZ	NZ	NZ	NZ
Klebsiella pneumoniae	2969	4	13	2	8	**20**	NZ	13	22	11	9	NZ
Proteus mirabilis	2912	NZ	10	16	8	**30**	15	9	19	25	9	NZ
Pseudomonas aeruginosa	2972	NZ	9	7	10	**22**	20	NZ	17	15	8	NZ
Enterococcus sp.	2304	NZ	NZ	NZ	15	**21**	50	NZ	NZ	NZ	NZ	11
Enterococcus sp.	2289	NZ	NZ	NZ	16	**26**	21	NZ	NZ	NZ	NZ	10
S. aureus	NCIM	11	25	26	17	**44**	50	11	25	26	NZ	9
Candida albicans	2942	10	NZ	NZ	8	**18**	NZ	10	NZ	NZ	NZ	NZ

Key:1) NZ: No Zone of Inhibition; 2)* Antibiotic: Ampicillin 10 mg/ml for bacterial cultures and fluconazole (0.833 mg/ml to give 25 µg/well and 1.666 mg/ml to give 50 µg/well) for *Candida sp.*
3) Hirda, Behada and Orange peel were used at a concentration of 50 mg/ml
4) Onion and Garlic Extracts were used without dilution i.e. in their original concentrations
5) 30 µl of extract/ antibiotic were added per well

Table.3 Broth dilution Method for determination of MIC, SIC and MBC for garlic extract

Culture	Culture No.	Fold dilution Of Garlic Extract	$O.D_{600}$24 hrs-OD_{600}0 hrs	
			Positive control	Test
E. coli	2945	8	0.36	-0.06
		16		-0.11
MIC		**32**		**-0.04**
SIC		**64**		**0.22**
		128		0.24

Table.4 Broth dilution Method for determination of MIC, SIC and MBC for garlic extract

Culture	Culture No.	Fold dilution	$O.D_{600}$24 hrs-OD_{600}0 hrs	
		Of Garlic Extract	Positive control	Test
Proteus mirabilis	2912	64	0.44	-0.04
MIC		**128**		**-0.02**
SIC		**256**		**0.22**
		512		0.14
		1024		0.2

Table.5 Broth dilution Method for determination of MIC, SIC and MBC for garlic extract

Culture		Fold dilution	$O.D_{600}$24 hrs-OD_{600}0 hrs	
	Culture No.	Of Garlic Extract	Positive control	Test
Klebsiella pneumoniae	2969	4	0.35	Out of Range
		8		0
MIC		**16**		**0**
SIC		**32**		**0.24**
		64		0.3

Table.6 Broth dilution Method for determination of MIC, SIC and MBC for garlic extract

Culture	Culture No.	Fold dilution Of Garlic Extract	$O.D_{600}$24 hrs-OD_{600}0 hrs	
			Positive control	Test
Pseudomonas aeruginosa	2972	4	0.36	Out of range
		8		0
		16		-0.18
		32		-0.09
		64		-0.06

Table.7 Broth dilution Method for determination of MIC, SIC and MBC for garlic extract

Culture	Culture No.	Fold dilution of Garlic Extract	$O.D_{600}$24 hrs-OD_{600}0 hrs	
			Positive control	Test
Candida albicans	2942	4	0.33	-0.13
		8		-0.1
		16		-0.08
MIC		**32**		**-0.01**
SIC		**64**		**0.32**

Table.8 MIC, SIC and MBC in terms of fold dilutions of garlic extract for various isolates

Culture	No.	MIC	SIC	MBC
E. coli	2945	32	64	16
Proteus mirabilis.	2912	128	256	128
Candida albicans	2942	32	64	16
Enterococcus sp.	2304	16	32	16
E. coli	2927	512	1024	512
Klebsiella pneumoniae	2969	16	32	>4
Pseudomonas aeruginosa	2972	>64	>= 128	4

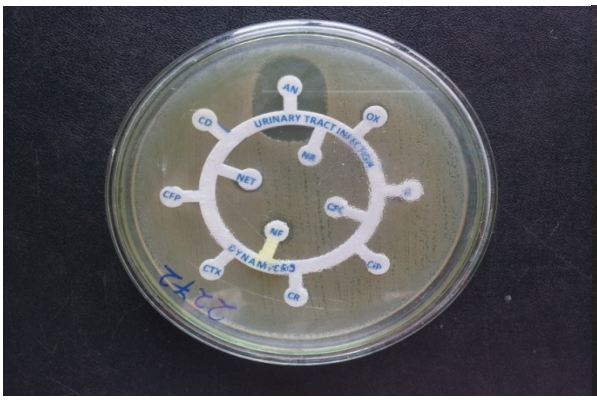
Table.9 Antibiotic resistance profile of Garlic Exposed and unexposed *E. coli* cultures

		Inhibition Zone Diameter in mm				Inhibition Zone Diameter in mm			
Cultures		*E. coli* 2945				*E. coli* 2927			
Antibiotic	Disc content (µg)	Exposed	Intrprtn	Unexposed	Intrprtn	Exposed	Intr prtn	Unexposed	Intr prtn
Amikasin AN	30	23	S	20	**S**	**20**	**S**	**14**	**R**
Ofloxacin OX	5	**20**	**S**	**15**	**I**	**10**	**R**	**NZ**	**R**
Gentamicin G	10	**15**	**S**	**10**	**R**	NZ	R	NZ	R
Norfloxacin NR	10	NZ	R	NZ	R	NZ	R	NZ	R
Cefaclor CFC	30	**16**	**I**	**10**	**R**	NZ	R	NZ	R
Ciprofloxacin CIP	5	**17**	**I**	**15**	**R**	**NZ**	**R**	**9**	**R**
Nitrofurantoin NF	300	26	S	24	S	**15**	**I**	**12**	**R**
Cefoperazone CFP	75 Units	**30**	**S**	**22**	**S**	NZ	R	NZ	R
Ceftriaxone CTX	30	**30**	**S**	**22**	**S**	NZ	R	NZ	R
Cefuroxime CR	30	**9**	**R**	**NZ**	R	NZ	R	NZ	R
Cefadroxil CD	30	NZ	R	NZ	R	NZ	R	NZ	R
Netilmicin NET	30	**18**	**S**	**14**	**I**	**11**	**R**	**NZ**	**R**

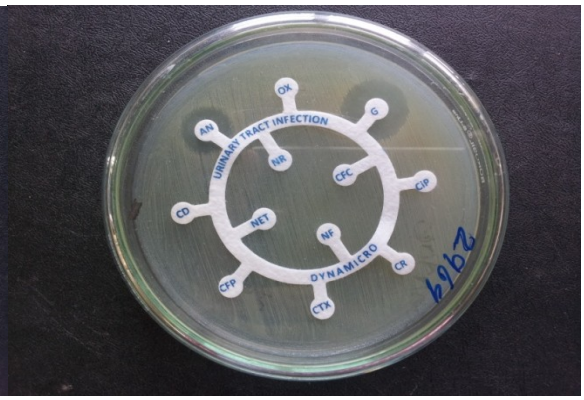
Key: NZ: No zone of inhibition ie it is highly resistant to the drug.
Intrprtn: Interpretation
R: Resistant; I: Intermediate resistance; S: Susceptible

Fig.1 Antibiotic susceptibility testing

1) E. coli (2272)

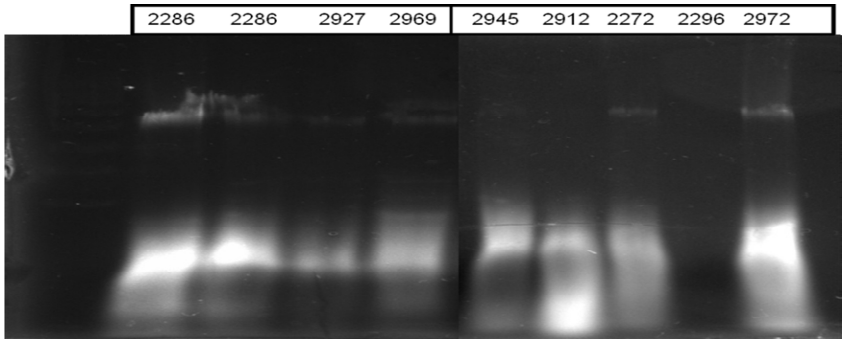


2) *Klebsiella sp*. (2969)



3) *Pseudomonas aeruginosa* (2972): 4) *E. coli* (2927)

Fig.2 Agarose gel electrophoresis of the plasmid preparations



Lane No. 1: Molecular Weight Marker (Supercoiled Plasmid DNA Ladder) 2: Plasmid preparation of *E. coli* 2286;
3: Plasmid preparation of *E. coli* 2286; 4: Plasmid preparation of *E. coli* 2927;
5: Plasmid preparation of *K. pneumoniae* 2969 6: Plasmid preparation of *E. coli* 2945;
7: Plasmid preparation of *Proteus mirabilis* 2912 8: Plasmid preparation of *E. coli* 2272
9: Plasmid preparation of *E. coli* 2296 10: Plasmid preparation of *Pseudomonas aeruginosa* 2972

Fig.3 Migration of plasmid bands as a function of Molecular Weight. Determination of Molecular Weight of the plasmids of the isolates

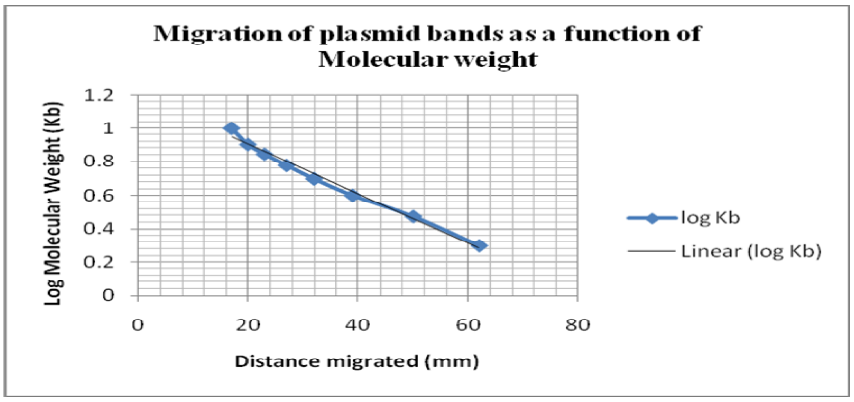
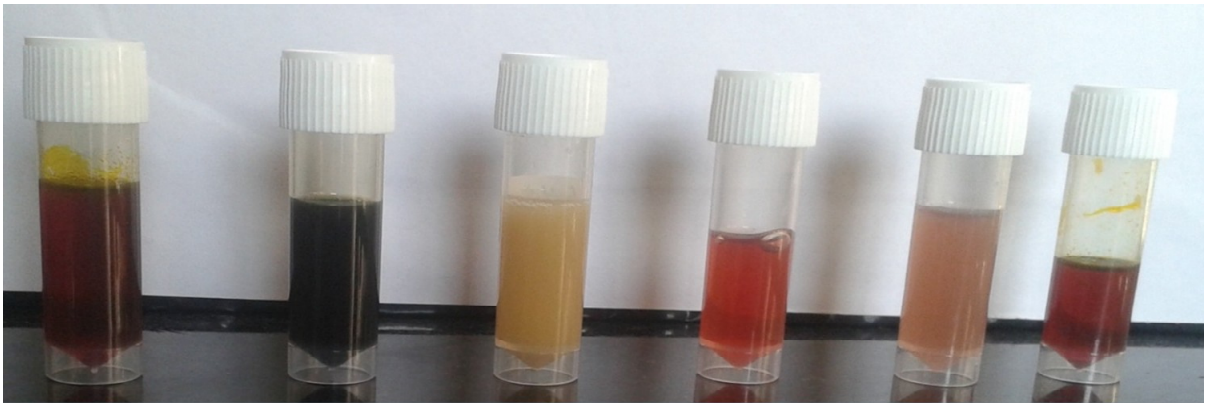


Fig.4 Plant Extracts prepared

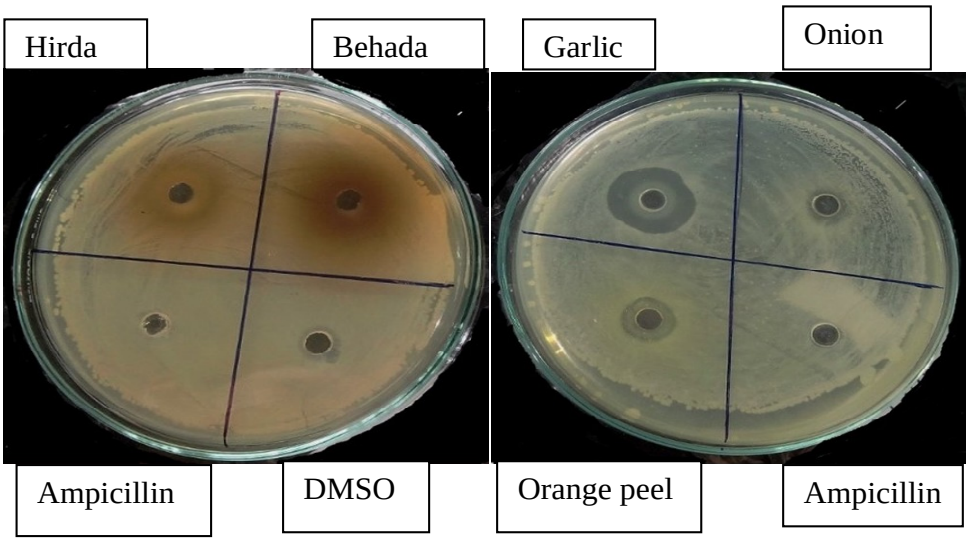


Hirda Curry leaves Garlic Orange peel Onion Behada

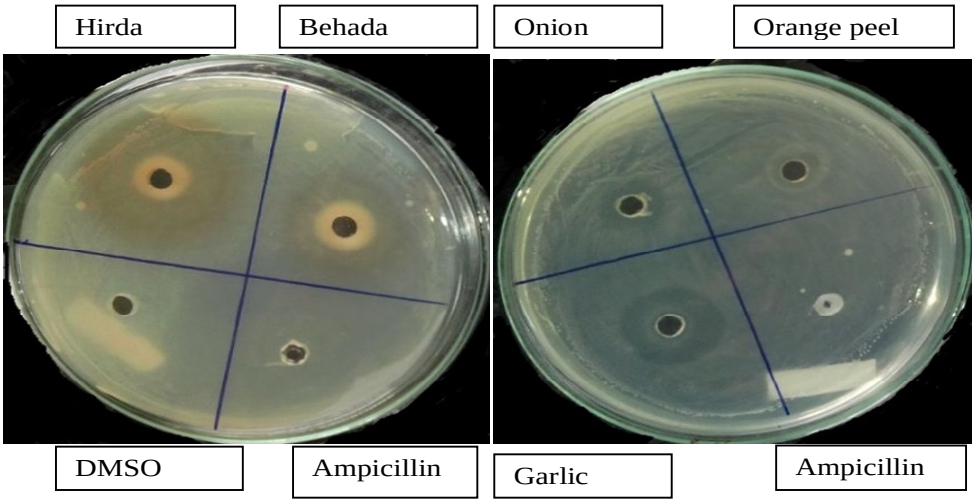
Fig.5 Antimicrobial activity of plant extracts

- Antibiotic: Ampicillin 10 mg/ml for bacterial cultures and fluconazole (0.833 mg/ml to give 25 µg/well and 1.666 mg/ml to give 50 µg/well) for *Candida sp*.
- Hirda, Behada and Orange peel were used at a concentration of 50 mg/ml
- Onion and Garlic Extracts were used without dilution i.e. in their original Concentrations
- 30 µl of extract/ antibiotic were added per well

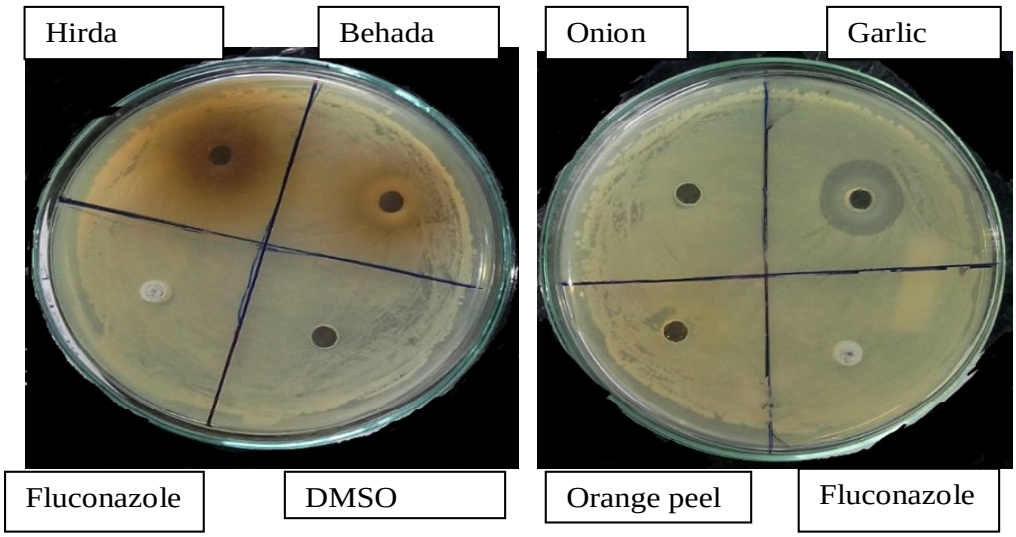
1) Effect of Plant Extracts on *Klebsiella pneumoniae (*2969)*:*



2) Effect of Plant Extracts on *Enterococcus sp.* (2304):



3) Effect of Plant Extracts on *Candida . (2942)*



4) Effect of curry leaves extract on *Enterococcus*

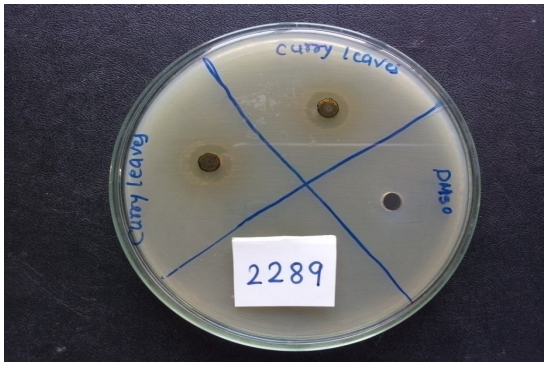


Fig.6 MIC range determination by *Enterococcus sp*. (2289): Plate Assay:

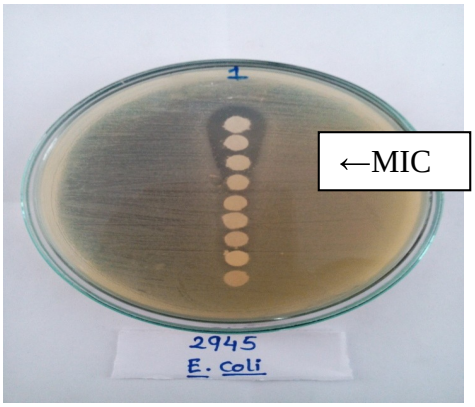


Fig.7 Growth of *Candida albicans* in absence and presence of garlic extract

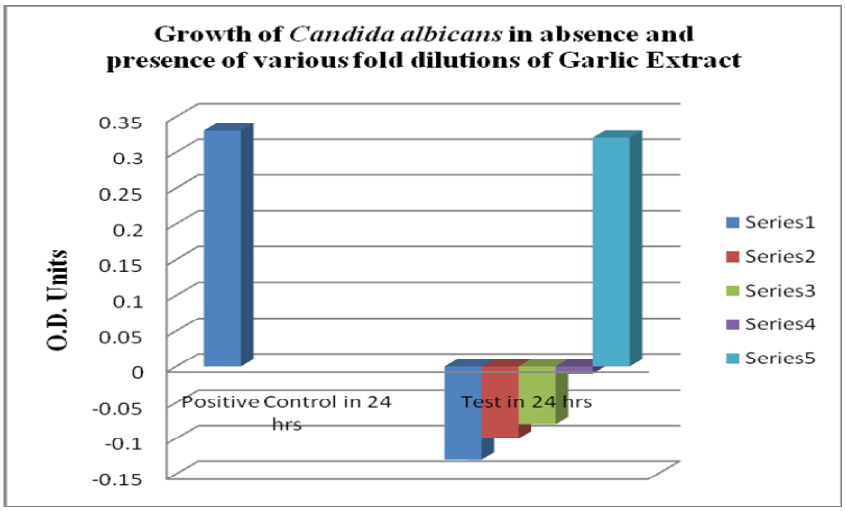


Fig.8 Determination of MBC of garlic extract for *Pseudomonas aeruginosa* 2972

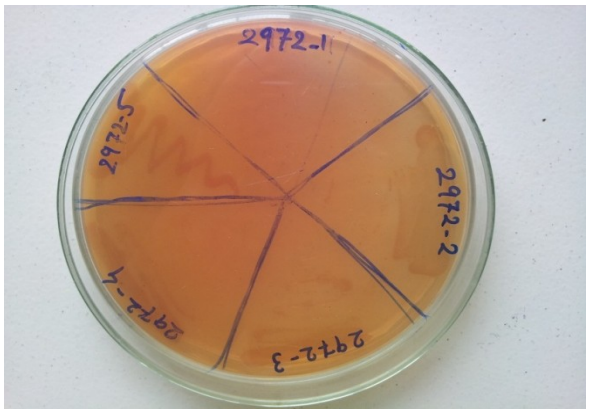
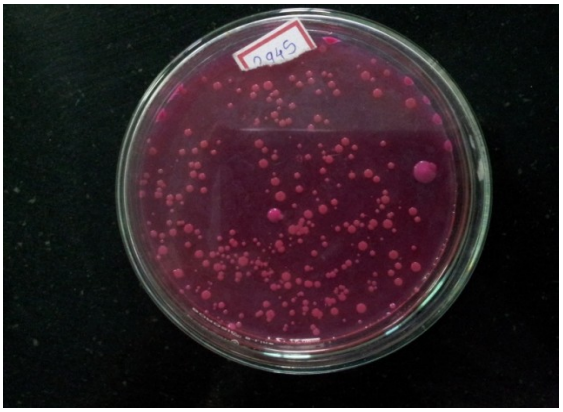
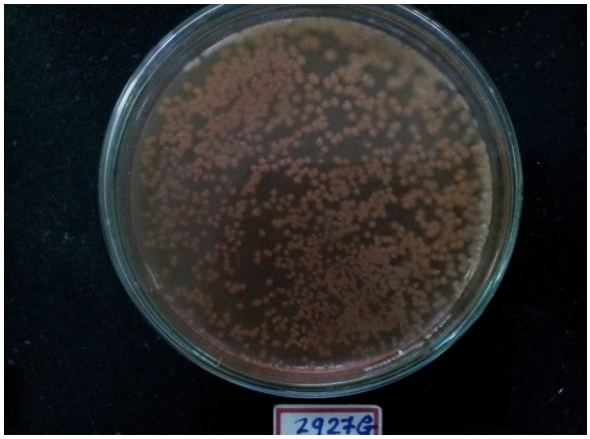
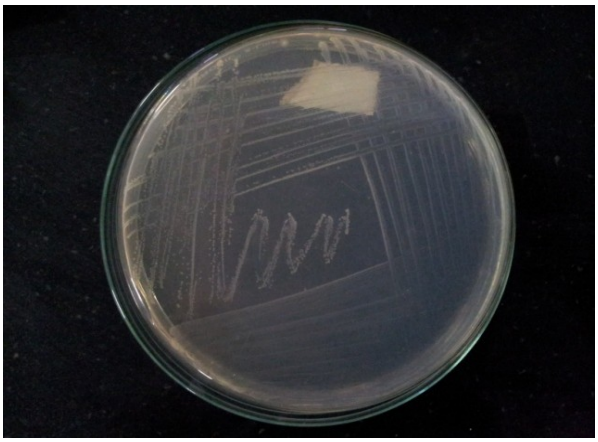


Fig.9 Garlic exposed cultures: 1) *E. coli* (2945G)

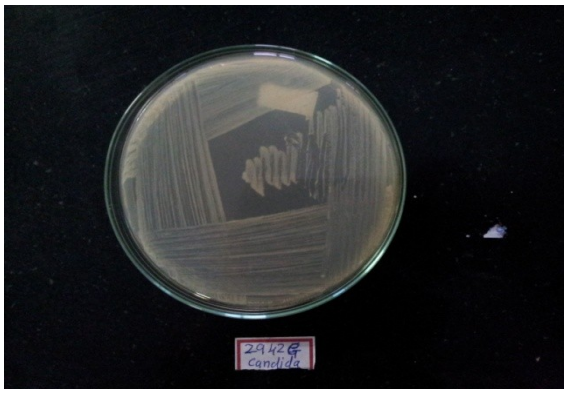


2) *E. coli* (2927G) 3) *Enterococcus sp.*(2304G)





4) *Candida albicans* (2942G) 5) *Klebsiella pneumoniae* (2969G)



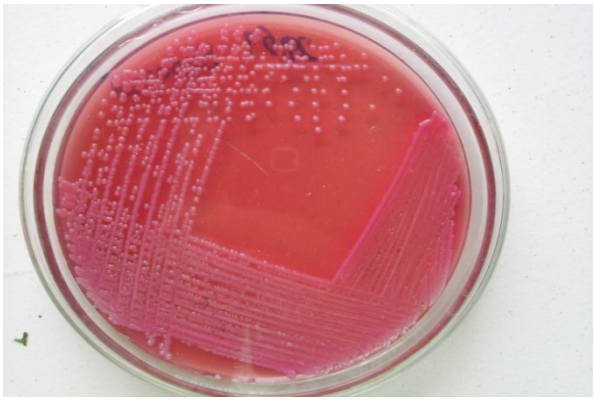
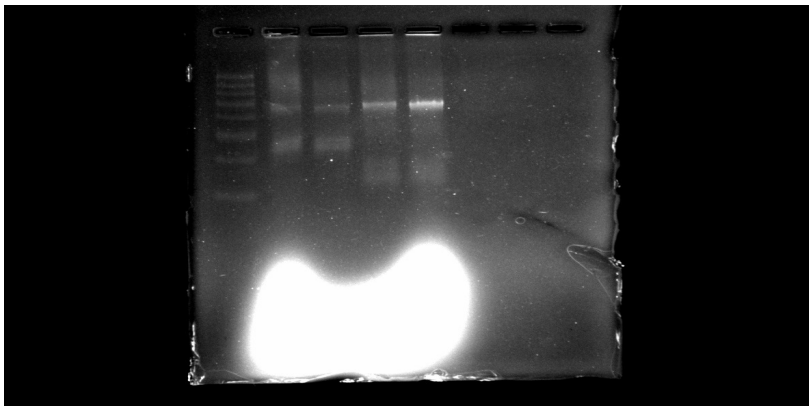


Fig.10 Antibiotic susceptibility of garlic exposed and unexposed cultures:
1) *E. coli* (2927) 'unexposed' and 2) *E. coli* (2927) 'exposed' to garlic extract

Fig.11 Agarose gel electrophoresis of plasmid preparation



Lane No. 1: Molecular Weight Marker (Supercoiled Plasmid DNA Ladder)
2: Plasmid preparation of E. coli 2945
3: Plasmid preparation of E. coli 2945G
4: Plasmid preparation of E. coli 2927
5: Plasmid preparation of E. coli 2927G

Both the garlic 'Exposed' and 'Unexposed' cultures of *Candida albicans* 2942 were resistant to 25 μg and 50 μg of Fluconazole and produced no zones of inhibition.
For *Enterococcus sp.* (2304G), exposure to garlic resulted in it becoming sensitive to Nitrofurantoin (whereas the 'unexposed' culture was resistant), having intermediate resistance to ciprofloxacin (same as 'unexposed' culture) and it remained resistant to gentamicin (although the zone size increased from no zone for 'unexposed' culture to 12 mm).

Plasmid profile of the cultures (*E. coli* 2945 and *E. coli* 2927) exposed to garlic extract versus the unexposed cultures: Please refer to Fig. 11 (Agarose gel electrophoresis of plasmid preparation)

Both the 'Exposed' and 'Unexposed' cultures of: a) *E. coli* 2945 showed the presence of 3 plasmid bands (sizes: 3.5, 3.9 and 5.6 kb) whereas those of b) *E. coli* 2927 showed the presence of a single plasmid band (size: 6 kb).

This indicates that exposure to garlic extract has not cured the cultures of their plasmids.

Patients having UTI face tremendous difficulty with regards to treatment, due to the prevalence of multi-drug resistance amongst the causative agents. The Gram positive and Gram negative bacterial cultures as well as *Candida spp*. obtained from Joshi Hospital were also multi-drug resistant.

The drug resistance may be plasmid borne or chromosomally encoded (Bastarrachea, 1998). Our isolates too showed the presence of plasmids (ranging in size from 2 to 3 kb (Isolate No.s 2286, 2927 & 2969) and 5-6 kb (Isolate No.s 2286, 2927, 2969, 2945, 2912, 2272 & 2972) some of which may encode drug resistance.

Plant Extracts are reported to contain antimicrobial components: alkaloids, flavonoids, saponins, phenolic compounds, steroids, carboxylic acid and tannins have been reported in Hirda (*Terminalia chebula*) fruit powder (Baliah and Astalakshmi, 2014); phenolic compounds including several flavonoid compounds in orange (*Citrus sinensis*) peel (Madhuri *et al.,* 2014); carbazole alkaloids and essential oils in curry (*Murraya koenigii*) leaves (Ganesan *et*

al., 2013); alkyl thiosulfinates, peptides, phenols, flavonoids, alkaloids in onion (*Allium cepa*) bulb and S-containing compounds such as allicin in the case of garlic (*Allium sativum*) cloves (Marinkova *et al.,* 2015).

Extracts of Hirda (*Terminalia chebula*) fruit powder, Behada (*Terminalia bellirica*) fruit powder, orange (*Citrus sinensis*) peel and curry (*Murraya koenigii*) leaves prepared in 70% ethanol and aqueous extracts of onion (*Allium cepa*) bulb and garlic (*Allium sativum*) cloves showed appreciable antimicrobial potential. They could inhibit the growth of highly drug resistant isolates such as *E. coli* (2927), *Proteus mirabilis.* (2912), *Klebsiella pneumoniae.* (2969), *Enterococcus sp*. (2304), *Pseudomonas aeruginosa* (2972) and *Candida albicans* (2942).

Garlic Extract gave the best result:

It showed antimicrobial activity against Gram negative and Gram positive bacterial cultures as well as against yeast (*Candida albicans*). The inhibition zone obtained by garlic extract was greater than that obtained by any of the other extracts and it was comparable with or greater than that obtained by standard antibiotic.

Gram positive uropathogens such as *Enterococcus spp* were inhibited only by garlic extract, onion extract, curry leaves extract and ampicillin.

Curry leaves extract exhibited antimicrobial activity only against Gram positive cultures.

The MIC, SIC and MBC values were determined for garlic extract against the selected drug resistant isolates. They were all susceptible to garlic and the negative $O.D_{600}$ values obtained during performance of MIC indicate possible lysis of culture with increasing concentration of the extract.

The culture tubes containing SIC of the extract were used to study the effect of garlic extract on the isolates: a) Whether there is any increase in the antibiotic sensitivity of the isolates; b) Whether plasmid curing occurs (Trevors, 1986).

The present study found that exposure to garlic extract does increase the antibiotic sensitivity of the isolates; but we did not find any loss of plasmids amongst the 'garlic exposed' cultures probably indicating loss of drug resistance as a result of chromosomal deletions or point mutations (Freifelder, 1987). Plasmid curing is nowadays regarded as a possible approach towards improving the susceptibility of the pathogens. We report here an increase in susceptibility to drugs in the absence of plasmid curing thus increasing the scope of use of plant extracts as sources of antimicrobial agents.

Acknowledgement

We would like to thank MMF's Joshi Hospital, Pune for providing us the necessary UTI isolates for our research work. We are indebted to the Management and Principal of Modern College of Arts, Science and Commerce, Ganeshkhind, Pune for providing us the infrastructural facilities to carry out the work and for their constant encouragement and support.

References

Alon, U., Davidai, G., Berant, M., Merzbach, D. 1987. Five-year survey of changing patterns of Susceptibility of bacterial uropathogens to Trimethoprim-Sulfamethoxazole and other antimicrobial agents. *Antimicrob. Agents Chemother.*, 31(1): 126–128.

Baliah, N.T., Astalakshmi, A. 2014. Phytochemical analysis and antibacterial activity of extracts from *Terminalia chebula* Retz'. *Int. J. Curr. Microbiol. Appl. Sci.*, 3(3): 992–999.

Bastarrachea, F. 1998. On the origin of plasmid-borne, extended-spectrum, antibiotic resistance mutations in bacteria. *J. Theor. Biol.*, 190(4): 379–387.

Freifelder, D. 1987. Microbial genetics. Jones and Bartlett Publishers, Inc., U.S.A.

Ganesan, P., Phaiphan, A., Murugan, Y., Baharin, B.S. 2013. Comparative study of bioactive compounds in curry and coriander leaves: An update. *J. Chem. Pharm. Res.*, 5(11): 590–594.

Madhuri, S., Hegde, A.U., Srilakshmi, N.S., Prashith, K.T.R. 2014. Antimicrobial activity of *Citrus sinensis* and *Citrus aurantium* peel extracts. *J. Pharm. Sci. Innovat.*, 3(4): 366–368.

Marinkova, D., Danalev, D., Genchev, G. 2015. Antimicrobial activity of Bulgarian products and their extracts containing bioactive components. *J. Chem. Technol. Metallurgy*, 50(1): 13–19.

National Committee for Clinical Laboratory Standards (Now Clinical and Laboratory Standards Institute, CLSI). 1997. Methods for dilution antimicrobial susceptibility testing for bacteria that grow aerobically. Approved Standards M7-A4. NCCLS, Villanova, PA.

National Committee for Clinical Laboratory Standards (Now Clinical and Laboratory Standards Institute, CLSI). 2002. Performance Standards for antimicrobial susceptibility testing; 12th information supplement (M100-S1). NCCLS, Villanova, PA.

Sambrook, J., Russel, D.W. 2001. Molecular cloning: a laboratory manual, 3rd edn. Cold Spring Harbour Laboratory Press, Cold Spring harbour, NY.

Trevors, J.T. 1986. Plasmid curing in bacteria. *FEMS Microbiol. Lett.*, 32(3-4): 149–157.

Yuksel, S., Ozturk, B., Kavaz, A., Ozcakar, Z. B., Acar, B., Guriz, H., Avsev, D., Ekim, M., Yalcinkava, F. 2006. Antibiotic resistance of urinary tract pathogens and evaluation of empirical treatment in Turkish children with urinary tract infections. *Int. J. Antimicrob. Agents*, 28(5): 413–416.

Zwetlana, A., Nandini, M., Dorcas, K. 2014. Antimicrobial activity of medicinal plant extracts on Gram negative bacteria. *J. Med. Plants Studies*, 2(5): 51–54.