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RESEARCH ARTICLE OPEN ACCESS

Differential Resistance of Soil Borne Microbes to Commonly Used Antibiotics

Ranjana Shee[1]*, Adrija Chakraborty[1], Ankita Chakraborty[1], Krittika Rao[1], Ritu Upadhyay[1], Arup Kumar Mitra[2]
[1]*(Post Graduate Department of Microbiology, St. Xavier's College, Kolkata, affiliated to University of Calcutta, India)*
[2]*(Associate Professor, Department of Microbiology, St. Xavier's College, Kolkata, affiliated to University of Calcutta, India)*

ABSTRACT
Three antibiotic resistance bacteria were isolated from Belur Sramajibi Hospital, Howrah district, on the outskirts of Kolkata. The microscopic, biochemical and pathogenicity tests suggested them to be Pseudomonas sp (isolate 1), Salmonella sp (isolate 2) and Enterococcus sp (isolate 3). The major aim was to check the effect of several aqueous plant extract on these multiple drug resistant strains and also study the change in antibiotic susceptibility pattern of the isolates, after UV irradiation using disc diffusion method. It was found that clove, neem and tulsi were the most effective agents in inhibiting growth of these pathogens. UV exposure (180-200 nm) of 10-20 minutes changed the susceptibility pattern of isolate 3 from resistance to sensitive towards all common and a few advance antibiotics (Ciprofloxacin and Imipenem).
Keywords: antibiotic susceptibility, common antibiotic, disc diffusion, multiple drug resistant, natural extract, UV exposure.

I. INTRODUCTION

Antimicrobial resistance has developed since ages but the number of resistant organisms, their geographic distribution and their spread has been an ever-emerging problem. Overuse of antimicrobial agents has increased the rate of resistance development in different organisms particularly pathogenic bacteria in developing countries. Poor sanitization and use of drugs without prescription have added to this problem [1]. Drug resistant infectious microbes have become an important public health concern warranting organizations in public and private sectors worldwide to work together (Blanch et al., 2003 and NIAID, 2011).Hospital environments specially act as major source of outbreaks of infectious diseases (Boyce *et al*, 2007 and Mario *et al*, 2013).Extensive use of antibiotics in hospitals and their release into the environment exert a selection pressure on bacteria[2]. These selective environments had lead to a high rate of emergence of resistant bacteria [3].The main threat is the transmission of resistant genes from these environmental bacteria to human pathogens. [4]. Sulfonamide-resistant *Streptoccoccus pyogenes* were identified in military hospitals in 1930s [1]. Following the introduction of penicillin, penicillin resistant *Staphylococcus aureus* were found in London civilian hospitals in 1940 [5]. Multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis*, *Enterococcus faecium*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *S. aureus*, *Acinetobacter baumanii* and *Pseudomonas aeruginosa* are some prominent global examples of pathogens encountered in hospitals and community(World Health Organization website) [4].Among other nosocomial pathogens, there are *Bacillus* spp, *Staphylococcus* spp. and *Streptococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans*, *Klebsiella*, *Proteus* and *Enterobacter* species [6]. Even the incidence of MRSA (Methicillin resistant *Staphylococcus aureus*) and NDM-1(New Delhi Metallo-β-lactamase) bacterial infections have now become quite predominant in Kolkata as well as New Delhi [7].

For the increased cause of hospital infections caused by these organisms, two major mechanisms can be held responsible. Resistant pathogen may be endemic within the hospitals and may be acquired by the patient during his stay in the hospitals. Secondly, a small number of endogenous resistant bacteria of the patient might express their resistant property efficiently under the selective pressure exerted by the use of antibiotics during hospitalization and thus emerge as dominant resistant opportunistic flora in the patient's body [8]. This antimicrobial selective pressure has now become a major contributor to the emergence of MDR strains in hospital and anthropogenic settings thus threatening public health [9].

Early and adequate detection of such new mechanisms of resistance is crucial for infection control and prevention. Therefore, continuous epidemiologic monitoring by repeated local revisions of susceptibility patterns to antibiotics is necessary to establish a rational treatment strategy.

As a necessary step, this study was undertaken to isolate and characterize the multidrug resistant pathogens from soil in a hospital premises of Kolkata and characterize them and also investigate their susceptibility patterns against several antibiotics. And also the pathogenicity of isolate(s) on CHROMagar® medium was detected. Due to the increase of resistance to antibiotics, there is a pressing need to develop new and innovative antimicrobial agents. Among the potential sources of new agents, plants and few spices have been investigated here because they contain many bioactive compounds and low toxicity that can be of interest in therapeutic. Along with this, the effect of UV irradiation on their antimicrobial susceptibility was investigated.

II. METHODOLOGIES

2.1. Collection of sample

The soil sample was taken from hospital wastages dumping site. Soil sample was chosen because of higher probability of finding bacterial strains of localized zone mainly obtained from dump hospital wastages which may include medicines, edibles, patient's dressings etc, so there might be probability of finding large amount of pathogenic bacteria. The soil sample was taken from a hospital in Kolkata, West Bengal.

2.2. Isolation of Organism

A stock culture was prepared according to this ratio, soil sample: water = 1 gm: 9 ml. After mixing, it was kept for 30 minutes so that the heavy soil particles get precipitated. After 30 minutes, 200 µl of stock solution was gently taken from the upper liquid portion and spread on two Nutrient agar plates, one having Ampicillin (50µg/ml) & another with Streptomycin (50µg/ml) and incubated over night at 37^0C. On the next day observation was taken and gram staining was performed.

2.3. Pure Culture Maintenance

Pure cultures were also made for each of the isolates on nutrient agar plates and in nutrient broth tubes (both were supplemented with respective antibiotics of 50µg/ml) for further use. The stock solution for both the antibiotics was of 1mg/ml. Three strains were incubated and maintained at 37°C.

2.4. Characterization of the isolates

2.4.1. Detection of pathogenic bacteria on CHROMagar® medium

CHROMagar® plates were taken (each containing 30ml medium), divided into three sectors and labelled for three different isolates obtained. Isolates were inoculated in their respective sectors by streak plate method. Incubation was done at 37ºC for24-48 hours to determine if the isolated organisms were pathogenic.

2.4.2. Characterization by biochemical analysis

All the isolated strains were further characterized by all these biochemical tests catalase test (using hydrogen peroxide), phenylalanine deaminase test (using $FeCl_3$), nitrate reduction test (using Reagent A(α- naphthylamine, acetic acid) and Reagent B (Sulfanilic acid, Acetic acid), urease test (using Christensen's Urea broth), starch utilization test(using iodine solution) and citrate test (using Simmon's citrate agar).

2.5. Antimicrobial susceptibility profiling of isolates using common and advanced antibiotics

To check their growth potential all the isolates were selected for antibiotic sensitivity test. Antibiotic sensitivity test was performed by disc diffusion method by taking different antibiotics. Routine antibiotics used were Ampicillin, Tetracycline, Streptomycin and Chloramphenicol. Advance antibiotics used were Ciprofloxacin, Vancomycin, Linezolid and Imipenem. Nutrient Agar plates were taken (each containing 30ml medium) and labelled for three different isolates obtained. Isolates were inoculated in their respective nutrient agar plate by spread plate method. Respective antibiotic discs were placed in all the nutrient agar spread plates. Then the plates were incubated at 37°C for 24 hours. After 24 hours, plates were observed for zone of inhibition and zone diameters were measured.

2.6. Well diffusion assay for the isolates by the aqueous extract of natural substances

To perform this assay at first nutrient agar was made, sterilized in autoclave in 121°C at 15lbs for 15 minutes, then 30ml media was poured in each sterile petri dish and allowed to solidify. We have chosen Neem, Tulsi, Turmeric, Clove and Cinnamon. Next the aqueous extract was prepared. To make it, 2gm of washed and dried leaves of Neem and Tulsi, 2gm of Turmeric and 2gm of powdered form of Clove and Cinnamon was weighed separately and mixed in 10 ml of distilled water (the stock conc. become 200mg/ml) and centrifuged at 5000rpm for 15mins. The different concentrations (50 mg/ml, 100 mg/ml and 150 mg/ml) of each substance were prepared by diluting the supernatant of the stock.
After the solidification of the plates, all the three isolates were spread and four wells were made in each plate. Then 200µl of each concentration of each samples were added in each wells and the plates were incubated for 24 hours at 37°C.

2.7. Antimicrobial resistance, susceptibility profiling of organisms after UV irradiation and comparative study

Nutrient agar (NA) plates were prepared for this purpose. Among the three isolates, the organism found resistant (Isolate 3) to the action of most antibiotics (to be discussed in the result portion) was subjected to mid UV radiation of 100-280 nm for three time intervals of 5 minutes, 10 minutes and 20minutes in a UV chamber (source was germicidal UV lamp mostly used in the laminar air flow hood). 200 µl of UV treated culture was spread on NA plates. Antibiotics discs (both advance and routine) were placed carefully on the plates and left for diffusion for some time. After that the plates were incubated at 37^0C and later observations were noted.

III. RESULTS

3.1. Isolation of Organism

Three types of colonies from two antibiotic contained plates were chosen. The detailed descriptions are listed below:

Table-1-Table showing comparative colony morphology and Gram characters of the bacterial isolates from collected sample

Strains	NA Plates	Gram character and morphology	Colony Morphology	Pigmentation
Isolate 1	Streptomycin supplemented	Gram Negative, short rods	Flattened, round colonies with slimy appearance	Light orange
Isolate 2	Streptomycin supplemented	Gram Negative, very short rods	Flattened, round shaped colonies with slimy appearance	Yellow
Isolate 3	Ampicillin supplemented	Gram Positive, very small cocci	Small, round shaped colonies	White

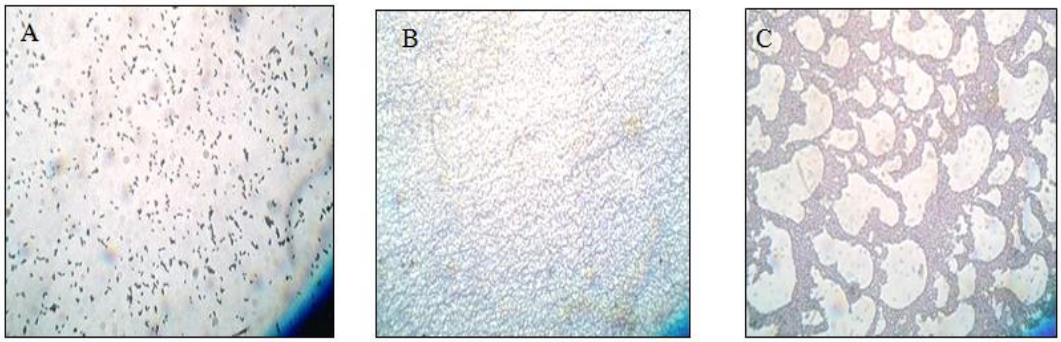


Figure 1: Gram characteristics of the bacterial isolates from collected sample; A: Isolate 1, Gram Negative, short rods; B: Isolate 2, Gram Negative, very short rods; C: Isolate 3, Gram Positive, very small cocci.

3.2. Pure Culture Maintenance

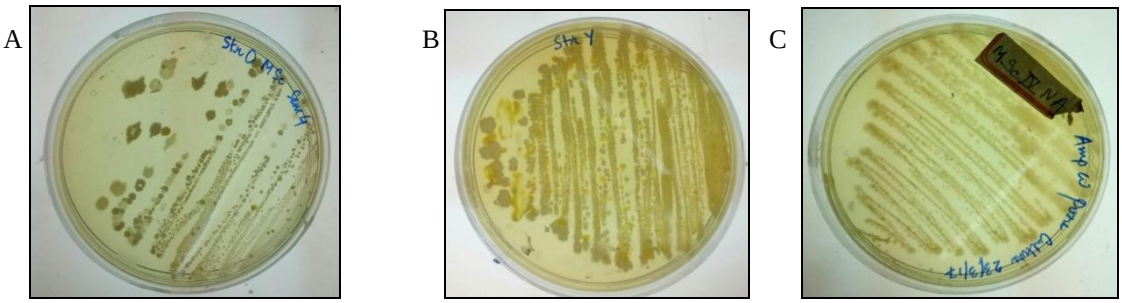


Figure 2: pure culture on nutrient agar plates of the bacterial isolates from collected sample, a: isolate 1 (slight orange colony), b: isolate 2 (yellowish colony), c: isolate 3 (whitish colony)

3.3. Characterization of isolates

3.3.1. Detection of pathogenic bacteria on CHROMagar® medium

Table 2: Showing results of CHROMagar® detection for pathogenic bacteria

Organisms	Colony Description
Isolate 1	Yellowish white
Isolate 2	Off white colonies with black grape pulp coloured /purple pigmentation, colonies small in size
Isolate 3	Medium sized bluish green colonies with slimy colour

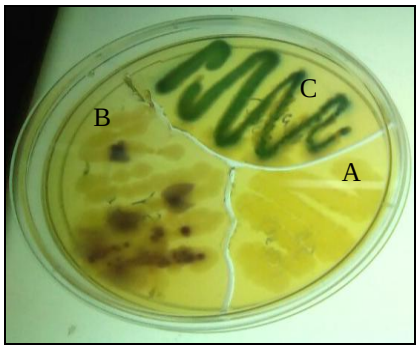
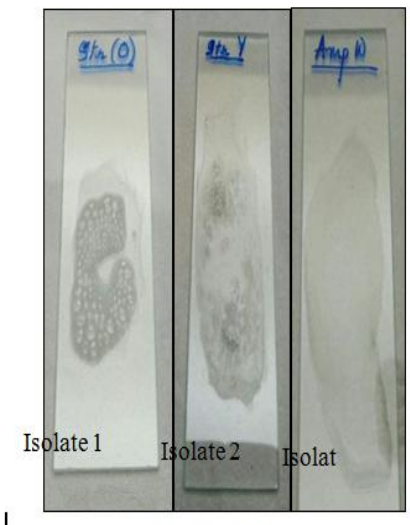


Figure 3: Bacterial colonies as observed on CHROMagar® plate, A: isolate 1 (yellowish white) B: isolate 2 (off white colonies with black grape pulp coloured /purple pigmentation); C: isolate 3 (bluish green colonies with slimy colour).

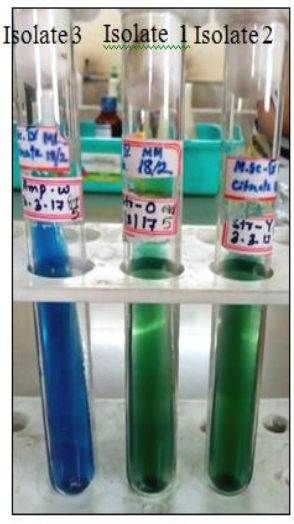
3.3.2. Characterization by biochemical analysis

Table 3: Showing results of different biochemical test on isolates 1, 2 and 3

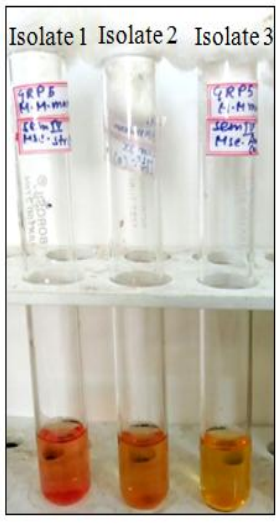
Serial no.	Tests	Test organisms		
		Isolate 1	Isolate 2	Isolate 3
1.	Catalase	+	+	-
2.	PA Deaminase	+	-	-
3.	Citrate Utilization	-	-	+
4.	Urease	+	+	-
5.	Nitrate Reduction	+	+	+
6.	Starch Utilization	+	+	+



A. Catalase Test



B. Citrate utilization test



C. Urease test

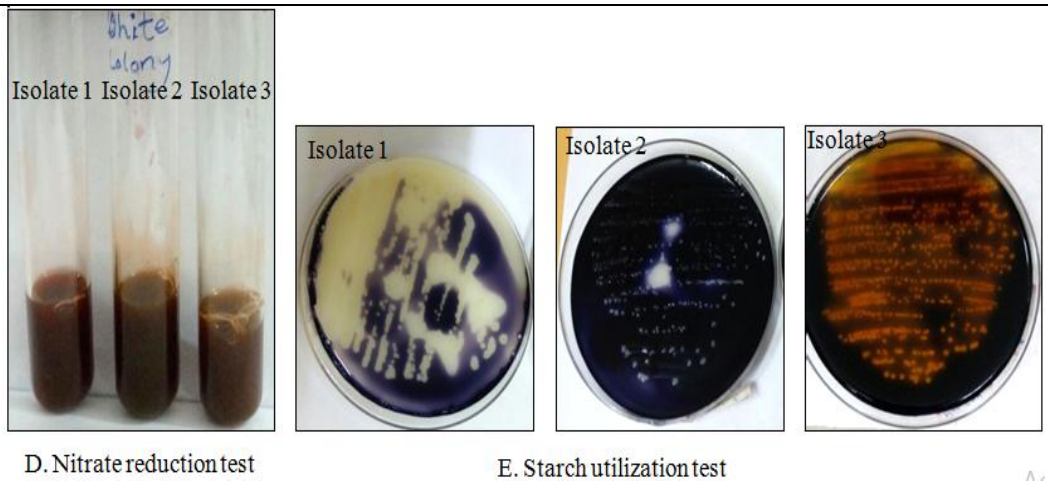


Figure 4: Biochemical characterization of the isolated bacterial colonies; A: Isolate 1 and 2 are showing positive catalase test (formation of effervescence) and isolate 3 is not showing; B: Isolate 1 are showing positive result (blue colouration) in citrate utilization test but isolate 2 and isolate 3 is showing the negative result; C. Isolate 1 and 2 is showing positive result (pink colouration) and isolate 3 is showing negative urease test (yellowish orange colouration); D. Isolate 1, 2 and 3 are showing to reduce nitrate (red colouration); E. Three isolates are showing to utilize starch (formation of bluish black colour)

3.4. Antibiotics sensitivity test by disc diffusion method:

Table 4: Table showing zones of inhibition for all the three isolates against different routine antibiotics

Antibiotics	Tetracycline					Chloramphenicol				
	D1	D2	D3	Avg. Diamete	Interpret ation	D1	D2	D3	Avg. Diameter (Cm)	Interpretati on
D. Nitrate reduction test						E. Starch utilization test				
Isolate 1	1.8	1.5	1.6	1.6	Sensitiv e	1.6	1.5	1.4	1.5	Sensitive
Isolate 2	2.6	3	2.7 (Zone Contai ning 79 Coloni es)	2.7	Partially Resistan t	3.2	2.9	2.8(Zone Containi ng 80 Colonies)	3	Partially Resistant
Isolate 3	0	0	0	0	Resistan t	0	0	0	0	Resistant

Antibiotic s	Ampicillin					Streptomycin				
	D1	D2	D3	Avg. Diameter (Cm)	Interpretatio n	D1	D2	D3	Avg. Diameter (Cm)	Interpretat ion
Isolate 1	0	0	0	0	Resistant	0	0	0	0	Resistant
Isolate 2	3.3	3.2	2.7 Zone Contai ning 124 Coloni es)	3	Partially Resistant	0	0	0	0	Resistant
Isolate 3	0	0	0	0	Resistant	0	0	0	0	Resistant

*D= diameter of zone of inhibition - diameter of the antibiotic disc (6 mm)

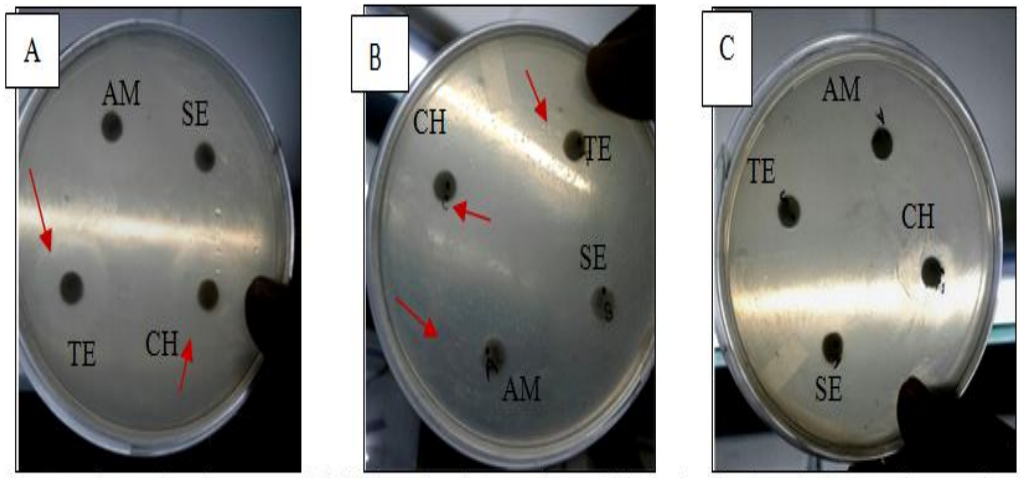


Figure 5: Figure showing zone of inhibition of various routine antibiotics for the three isolates, red arrow shows the zone if inhibition, A: Isolate 1, showing zone of inhibition for Chloramphenicol and Tetracycline drug; B: Isolate 2, showing zone of inhibition for Chloramphenicol and Tetracycline and Ampicillin drug; C: Isolate 3, showing no zone of inhibition for those used drugs, indicates its resistance to those.

Table 5: Table showing zones of inhibition for all the three isolates against different advance antibiotics

Antibiotics	Vancomycin					Linezolid				
	D1	D2	D3	Avg. Diameter (Cm)	Susceptibility	D1	D2	D3	Avg. Diameter (Cm)	Interpretation
Isolate 1	1.3	1.1	1.1	1.2	Sensitive	1.6	1.7	1.6	1.6	Sensitive
Isolate 2	1.7	1.4	1.5	1.5	Sensitive	2	2.1	1.9	2	Sensitive
Isolate 3	0	0	0	0	Resistant	0	0	0	0	Resistant

Antibiotics	Imipenem					Ciprofloxacin				
	D1	D2	D3	Avg. Diameter (Cm)	Interpretation	D1	D2	D3	Avg. Diameter (Cm)	Interpretation
Isolate 1	1.2	1.1	1.4	1.2	Sensitive	1.7	1.7	1.7	1.7	Sensitive
Isolate 2	2.1	1.6	2.2(Zone Containing 40 Colonies	1.9	Partially Resistant	1.7	1.9	1.6	1.7	Sensitive
Isolate 3	0	0	0	0	Resistant	1.7	1.5	1.9	1.7	Sensitive

*****D= diameter of zone of inhibition - diameter of the antibiotic disc (0.6 cm)

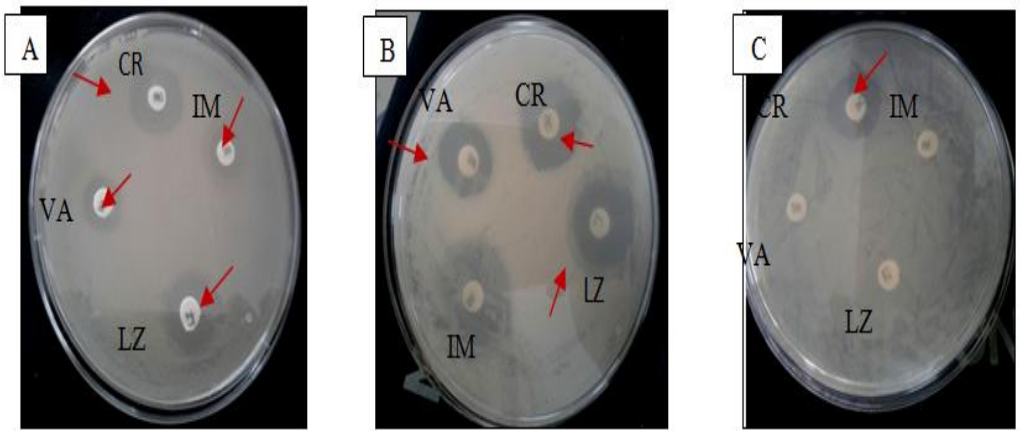


Figure 6: Figure showing zone of inhibition of various advance antibiotics for the three isolates, red arrow shows the zone if inhibition; A: Isolate 1, showing zone of inhibition for Vancomycin, Ciprofloxacin, Linezolid and Imipenem drugs; B: Isolate 2, showing zone of inhibition for Vancomycin, Ciprofloxacin and Linezolid drugs; C: Isolate 3, showing zone of inhibition for only Ciprofloxacin.

3.5. Results of well diffusion assay for the isolates by the aqueous extract of natural substances (Neem, Tulsi, Turmeric, Clove and Cinnamon):

Table 6A: Result of well diffusion assay of isolate 1 after using antimicrobial natural substances

Isolate 1	Used natural substances									
	Neem		Tulsi		Turmeric		Clove		Cinnamon	
Used conc.	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)
50mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	1.5cm	Resistant	No zone
100mg/ml	Resistant	No zone	Resistant	No zone	Sensitive	1.4cm	Sensitive	1.8cm	Resistant	No zone
150mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	2.6cm	Resistant	No zone
200mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	2.9cm	Resistant	No zone

Table 6B: Result of well diffusion assay of isolate 2 after using antimicrobial natural substances

Isolate 2	Used natural substances									
	Neem		Tulsi		Turmeric		Clove		Cinnamon	
Used conc.	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)
50mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	0.9cm	Resistant	No zone
100mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	1cm	Resistant	No zone
150mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	1.1cm	Resistant	No zone
200mg/ml	Resistant	No zone	Resistant	No zone	Resistant	No zone	Sensitive	1.4cm	Resistant	No zone

Table 6C: Result of well diffusion assay of isolate 3 after using antimicrobial natural substances

Isolate 3	Used natural substances									
	Neem		Tulsi		Turmeric		Clove		Cinnamon	
Used conc.	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)	Susceptibility	Avg. Diameter (cm)
50mg/ml	Resistant	No zone	Sensitive	2.06cm	Resistant	No zone	Resistant	No zone	Resistant	No zone
100mg/ml	Resistant	No zone	Sensitive	2.9cm	Resistant	No zone	Resistant	No zone	Resistant	No zone
150mg/ml	Sensitive	2.6cm	Sensitive	2.76cm	Resistant	No zone	Resistant	No zone	Resistant	No zone
200mg/ml	Sensitive	3.3cm	Sensitive	1.43cm	Resistant	No zone	Resistant	No zone	Resistant	No zone

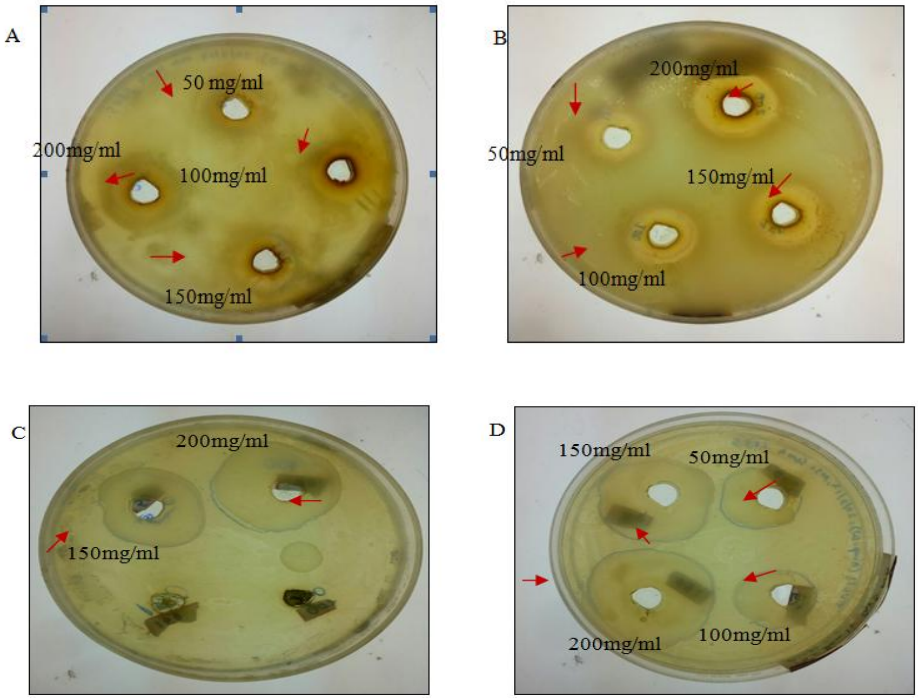


Figure7. Result of well diffusion assay of three isolates by using antimicrobial natural substances; A: Isolate 1 is inhibited by four concentrations of clove (50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml); B: Isolate 2 is inhibited by four concentrations of clove (50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml); C and D: Isolate 3 is showing the zone of inhibition by the action of neem (150mg/ml and 200mg/ml) and tulsi (50mg/ml, 100mg/ml, 150mg/ml, 200mg/ml)

3.6. Antimicrobial resistance, susceptibility profiling of organisms after UV irradiation and comparative study:

Table 7: Zone of inhibition given by the microorganism against different antibiotics after UV treatment

Time of exposure (in minutes)	Common Antibiotics(cm)							Advanced Antibiotics(cm)					
	SM		CH		TE		AM	VM	CR		IM		LZ
5	1.9	S	1.6	S	R		R	R	0.9	S	1.1	S	R
10	1.8	S	2.1	S	1	S	R	R	2.1	S	1.5	S	R
15	1.7	S	1.4	S	1	S	R	R	1.9	S	1.1	S	R

***** Mean diameters have been considered, approximate diameter of each disc was 0.6 cm.
S= Sensitive, R= Resistance, I= Intermediate; Ampicillin-AM, Tetracycline-TE, Chloramphenicol-CH, Streptomycin-SE; Vancomycin-VM, Ciprofloxacin-CR, Linezolid-LZ, Imipenem- IM

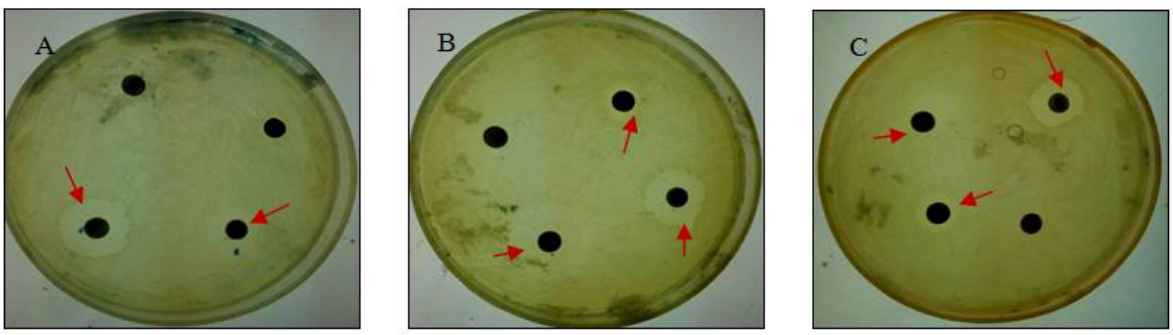


Figure 8: Result of susceptibility test of chosen isolate (isolate 3) against common antibiotic (ampicillin, tetracycline, streptomycin and chloramphenicol) after treatment of UV with different time period, A: 5 minutes exposed culture; B: 10 minutes exposed culture; C: 20 minutes exposed culture, red arrow shows the zone of inhibition

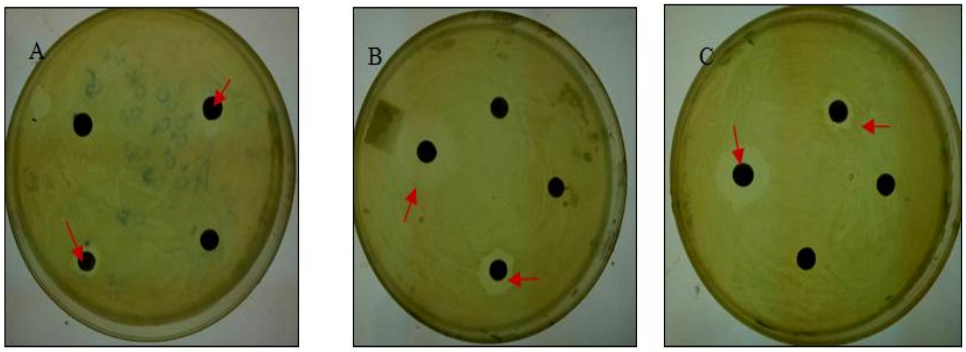


Figure 9: Result of susceptibility test of chosen isolate (isolate 3) against advanced antibiotic (vancomycin, ciprofloxacin, linezolid, imipenem) after treatment of UV with different time period, A: 5 minutes exposed culture; B: 10 minutes exposed culture; C: 20 minutes exposed culture, red arrow shows the zone of inhibition

3.6.1. Comparative Study

A difference between the diameters of the zone of inhibition between UV treated samples and UV untreated ones was observed (for isolate 3).

Table 8: Summary of the diameters of zone of inhibition for Comparative analysis of UV action

	SM	AM	TE	CH	VA	CR	LZ	IM
ISOLATE 3	R	R	R	R	R	S	R	R
ISOLATE 3(UV)	S	R	S	S	R	S	R	S

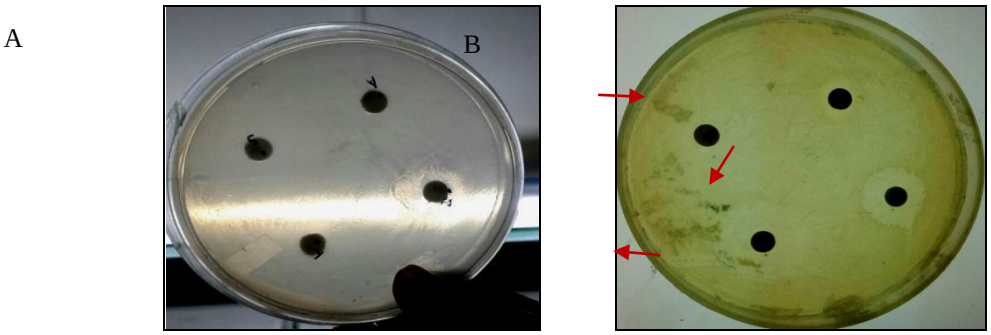


Figure 10 A and B: Presence of no zone of inhibition for all tested common antibiotics (isolate 3 non UV treated sample) Presence of zone of inhibition for all tested common antibiotics except ampicillin (isolate 3 UV treated sample

IV. CONCLUSION

Hospital environment is generally contaminated with several pathogen. In recent years, these pathogens have turned into multi-drug resistant (MDR) organisms, which is a major threat to our society. Use of common antibiotics is not a very effective option and so alternatives are being looked for. Roberts Marilyn C. (1989) has shown that garlic is as an effective alternative due to its pronounced DNase activity owing to its selenium content. Even, neem has been assessed for antimicrobial activity, as it contains a natural compound azadirachtin (Kunjal S.et.al.2014).

Thus concluding the whole set of experiments, three MDR isolates were obtained (Fig. 2) (Table 1). On the basis of microscopic studies, gram characterization (Fig.1) (Table 1), CHROMagar® pathogenicity test (Fig. 3)(Table 2) and biochemical tests (Fig.4) (Table 3) presence of *Pseudomonas* spp. (isolate 1), *Salmonella* spp.(isolate 2), *Enterococcus spp*.(isolate 3) were assumed. All these isolates were resistant to most of the common and advanced antibiotics used in this study (Fig.5 and Fig. 6) (Table 4 and Table 5).Clove were found to be a very potent inhibitory agent against isolate 1 and isolate 2 (Fig.7) (Table 6A, 6B and 6C). But only higher concentrations (150mg/ml and 200mg/ml) of neem and tulsi could suppress the growth of MDR isolate 3 (Fig.7)(Table 6A,6B and 6C). From this view point it can be concluded that clove, neem and tulsi in the form of different formulations can be used to can be used to combat growth of MDR bacteria.

On UV exposure, the MDR isolate 3 turned sensitive against most of the antibiotics used in this study (except ampicillin, linezolid, vancomycin) (Fig. 8, Fig.9 and Fig. 10 A, B) (Table 7 and 8). Previously it has been reported that UV exposure

causes mutation in plasmid turning the MDR strain into sensitive one [10]. However, here it can concluded that the wavelength (100-280nm) of the UV radiation or exposure time was not enough to cause any mutation in the ampicillin resistant gene of isolate 3.Thus UV irradiation can be used as an effective tool to manipulate the degree of antibiotic susceptibility of MDR strains like isolate 3.

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