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ASCERTAINING DRUG RESISTANCE BACTERIA FROM HOSPITAL DRAINAGE WATER AND EVALAUTING ITS SUSCEPTIBILITY WITH LEAF EXTRACT OF *BARLERIA PRIONITIS*

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ABSTRACT

To study the identification of drug resistant bacteria from hospital sewage water especially from drainage canal. The sample was processed for enumerating the total bacterial count and showed a total population of 2.3×10^4. The BOD level of the sample as it is a liquid was evaluated using titration method. The major part of the work denoted by isolating the drug resistant bacteria by cultivating the bacteria in media containing different class of drug. The antibiotic is applied in the drug resistant bacteria were Vancomycin, Amoxicillin, Cephalexin, Amikacin. The identified bacteria S. aureus, Klebsiella species, Pseudomonas species was determined for its multidrug resistant property by antibiogram using different generation antibiotic and zone integration. The antibiogram of the isolated bacteria expression of antibiotic resistant characters by producing beta lactamase (penicillin, cephalexi), 6'-N. acetyl transferases (Amikacin), D-alanine TD-alanine transpeptidase (vancomycin), OXA-1 beta-lactamase (amoxicillin). The enzyme activity was determined by nitrocefin test. The drug resistant bacteria is treat with Barleria prionitis methanolic leaf extract which yield 13.93% of the phytoconstituent by qualitatively. The B.prionitis was resistant to the drug resistant bacteria.

KEYWORDS: Drug resistant bacteria, *Barleria prionitis*, Antibiotics, Enzymes.

INTRODUCTION

In recent years, bacterial resistance to antibiotics has raised to the forefront of world health issues. Overuse of antibiotics has been identified as a leading cause for the development of bacterial antibiotic resistance. A unique feature of enzymes that physically modify antibiotics is that these mechanisms alone actively reduce the concentration of drugs in the local environment; therefore, they present a unique challenge to researchers and clinicians considering new approaches to anti-infective therapy (Gerard D. Wright et al, 2005). Understanding the mechanisms of resistance is important in order to define better ways to keep existing agents useful for a little longer but also to help in the design of better antimicrobial agents that are not affected by the currently known, predicted, or unknown mechanisms of resistance (Denis K. Byarugaba et al, 2009). Aminoglycosides modified at amino groups by AAC enzymes or at hydroxyl groups by ANT or APH enzymes lose their ribosome-binding ability and thus no longer inhibit protein synthesis. Besides aminoglycoside-modifying enzymes, efflux systems and rRNA mutations have been described (Quintiliani and Courvalin, 1995). Multidrug resistance among many organisms has become a big challenge to infectious disease management. It is increasingly being reported in bacteria and is often mediated by genetic mobile elements such as plasmids, transposons, and integrons (Dessen et al., 2001).

Kingdom	:	Plantae.
Subkingdom	:	Tracheobionta.
Division	:	Magnoliophyta.
Class	:	Magnoliopsida.
Subclass	:	Asteridae.
Order	:	Scrophulariales.
Family	:	Acanthaceae.
Genus	:	Barleria.
Species	:	prionitis.
Common Name	:	Bajradantip.
Tamil Name	:	Cemmulli.

Barleria prionitis is used to treat fever, respiratory diseases, toothache and joint pains. A mouthwash made from root tissue is used to relieve toothache and treat bleeding gums. The leaves are used to healing of wounds and to relieve joint pains. Extracts of the plant are

incorporated into herbal cosmetics and hair products to cure skin and scalp problems (Abha Khare et al., 2016, Patel Bharatkumar et al., 2015, Sattya Narayan et al., 2015). In this present investigation we have collected the sewage from hospital drainage, determination of Biological Oxygen Demand of the water sample, isolation of antibiotic resistance bacteria, determination of bacteria for its multidrug resistant property, extraction of Total Phytochemcials from Barleria prionitis leaf, screening for potential phytoconstituents from the *B. prionitis* leaf extract, antibacterial activity using the multi drug resistant bacteria towards plant derivative and enzyme activity measurement assay from the drug resistant bacteria along with the antibiotics and the leaf extract.

MATERIALS AND METHODS

COLLECTION OF SAMPLE

The sample was collected from Government General Hospital, Pondicherry. The location defined in sample collection was from inpatient room wash area and the drained water was collected in the day hour's. The seepage water was collected in a aseptic container and immediately transported to the laboratory and stored in the refrigerator (Cruickshankk 1980, Radha 2009). The sample was evaluated for its Biological Oxygen Demand. The procedure is done for the all the BOD bottles before and after the incubation. For the determination of BOD of the given beverage sample the readings are tabulated as.

Table 1: BOD groundwork.

Sl. No.	Day	Volume of sample (ml)	Burette Reading (ml)		Volume of Titrant ($Na_2S_2O_3$ used)	DO (mg/L)
			Initial	Final		
Blank (A)	0	100	0	***	***	***
Sample (A)	0	100	0	***		
INCUBATION FOR 5 DAYS IN B.O.D INCUBATOR						
Blank (B)	5	100	0	***	***	***
Sample (B)	5	100	0	***		

Therefore,

$$\text{Biological Oxygen Demand} = \frac{(D0-D5-BC)X\ Volume\ of\ sample\ titrated}{Volume\ of\ sample\ taken}$$

Determination of total bacterial count

The sample was evaluated for its total bacterial population by serial dilution and spread plate method.

$$\text{CFU/ml} = \frac{\text{Total no.of colonies}}{\text{Volume taken}} \text{ x } 1(\text{dilution factor})$$

Isolation of bacteria from hospital drainage water

The bacteria harboured in the hospital drainage water sample identified by inoculating the sample in selective media by spread plate method and the colony morphology inferred with its bacteria and different biochemical tests performed to identify the bacteria.

Microscopic observation

Hanging drop technique

In this technique, a drop of medium containing cells to be observed is allowed to hang in the cavity of slide. The advantage of this preparation over the wet mount preparation is the increased capacity of the aeration of aeration as the drop is surrounded by an air space. This is the best method available for the routine use to observe the motility of bacteria. This is because, it is relatively easy to make and less time consuming. It is essential to differentiate true motility from the Brownian movement of bacteria. In true motility, the organism changes its position, while in the Brownian movement; the organism oscillates at its place and does not change the position in the field.

Gram staining

A clean grease free slide was taken and a smear of the bacterial culture was made on it with a sterile loop. The smear was air – dried and then heat fixed.

Physiological charcterization

Biochemical characterization

Indole test, methyl red test, voges proskauer test, citrate utilization test, catalase, trilpe sugar iron agar.

Determination of drug resistance from the bacteria isolated from hospital drainage water sample

The drug resistance was determined by adding single and combination of antibiotic into the media and then the resistance bacteria was isolated. The media used Mueller Hinton agar and the antibiotic combination used were.

Table 2: Antibiotics for drug resistance bacterial determination.

ANTIBIOTICS	Concentration (mcg)
Vancomycin	30
Penicillin	30
Amoxicillin	30
Tetracycline	30
Coxacilline	30
Cephalexin	30
Linezoid	30
Amikacin	30
Ciproflacin	30

To this antibiotic combination the bacterial culture inoculated and the antibiotic resistance colonies were selected and further pure cultured. The drug resistance bacteria were isolated by inoculating the sample into selective media and the colony morphology was observed.

Assay of bacterial resistance

The procedure adopted to identify the bacteria isolated from the selective media and characterized by biochemical tests were used in determining the antibiotic resistance ability by diffusion method and the resistance bacteria were selected and applied with the plant leaf extract for susceptibility tests and enzyme assay.

Procedure for performing the disc diffusion test

Inoculum preparation- growth method

Bacterial samples respectively were inoculated in the sterile nutrient broth prepared obtained from Himedia Mumbai LOT no. 0000132645 and incubated for the overnight at 37^{o}C. The bacteria employed for the study include, *Klebsiella species, Staphylococcus aureus* and *Pseudomonas species.*

Phytochemical extraction from leaf of B. prionitis

Collection of plant

The leaf of B.prionitis collected from Thenpasiyar, Villupuram District during the autumn season.

Determination of moisture content

For drying the leaf(g) taken separately and dried at 45°C oven for 24 hours continuously. The dried leaf were weighed and the losses on drying were noted. The dried leaf was grinded

using a mechanical blender and made to a fine powder. The powdered sample's were used for extraction.

$$Moisture\ \% = \frac{\text{Wet weight} - \text{dry weight}}{\text{Wet weight}} \times 100$$

Extraction of total phytoconstituents

The powdered leaf of *B.prionitis* added with the solvent methanol along with ethanol ie in the ration 8: 2 (80% Methanolic Ethanol v/v) was used for the extraction of leaf. 20g each of leaf powder was separately taken in a beaker and soaked it in 100 ml methanol and the beaker was covered using aluminum foil to avoid evaporation and kept undisturbed for 24 hours at room temperature. After the incubation period the extract was then filtered using filter paper and was followed by filtration using filter paper. The filtrate was collected and then the 80% methanolic ethanol kept for evaporation in a water bath at 65°C until complete saturation occurs. The crude extract obtained evaluated for its total content gravimetrically.

$$\%\ of\ yield = \frac{\text{FINAL WEIGHT} - \text{INITIAL WEIGHT}}{\text{SAMPLE WEIGHT}} \times 100$$

Preparation of phytochemical concentration

The concentrated extract of each was diluted using 25 ml of 80% methanolic ethanol and the diluted extract was used for further studies. The concentration of the extract was noted in table: The extract was screened for its total phyto-nutrient by qualitatively.

Phytochemical screening

1. **Test for Alkaloids (Test):** Treat the jujube seed extract with few drops of s reagents. Formation of reddish brown precipitate indicates presence of alkaloids.
2. **Test for phenols/Tannins (ferric chloride Test):** Mix the crude extract with 2ml of 2% solution of Fecl3. Blue-green or black coloration indicates presence of phenols & tannins
3. **Test for Terpenoids (salkowski Test):** To the seed extract few drops of con.H2SO4 and 2ml chloroform and shaken then allow standing appearance of golden yellow colour indicates the presence of triterpenoids.
4. **Test for flavonoids (Ferric Chloride):** To the extract a pinch of magnesium ribbon added and followed by concentrated Hydrochloric acid which gives a magenta color with effervescence.

5. **Test for fixed oils (paper Test):** Few drop of the extract applied over the surface of Whattman filter and allowed to air dry for 10 minutes. If the filter paper retains the oil stain it confirms the presence of Fixed oil.

Antibacterial activity using the multi drug resistant bacteria towards plant derivative

Testing the antibacterial activity by well diffusion method

Muller Hinton agar was use to check antimicrobial activity by well diffusion method. Autoclaved medium was poured in to petriplates in the laminar air flow hood. On cooling the medium within petriplates the microorganism from 24hrs old broth were spread then wells were made on the petriplates with the help of stainless steel borer of diameter 6- 8 mm. Three wells were made on entire surface of medium; one is for control to the test organism and remaining for different concentration of the sample. Now increasing volume (µl) of phytochemicals preparations was poured in the first well and subsequently for the other well. These plates were incubated for 24-48hrs and the diameter of zone of inhibition was measured with the help of scale.

The freshly prepared inoculum was swabbed all over the surface of the MHA plate using sterile cotton swab. Five wells of 6mm diameter were bored in the medium with the help of sterile cork-borer having 6mm diameter and were labeled properly and fifty micro-liters of the working suspension/solution of different medicinal plant extract and same volume of extraction solvent for control was filled in the wells with the help of micropipette. Plates were left for some time till the extract diffuse in the medium with the lid closed and incubated at 37°C for 24 hour and measured using scale and mean were recorded after incubation, plates were observed for zone of inhibition.

Preparation of culture broth

Bacterial samples respectively were inoculated in the sterile nutrient broth prepared obtained from Himedia Mumbai, India and incubated for the overnight at 37^{o}C.

Preparation of the agar lawns

The sterile pre incubated bored plates were taken for the analysis and the lawn was made by using the sterile cotton swabs and lawn was made.

Preparation of the test solution

Test solution was prepared by the equivalent of 200 mg/ml concentration and used for analysis. The secondary metabolites were added into the well at a concentration of 50µl and kept for incubation. And the results were recorded by measuring the zone using calibrated reader scale.

The plate incubated were observed for the zone of inhibition and the zone measurement was made using standard measuring scale- HiAntibiotic Zone Scale (HIMEDIA Laboratories)and the readings were noted in mm. Note: The plant material used were leaf of *B.prionitis* extract obtained from 80% Methanolic Ethanol (v/v).

Enzyme assay

The bacteria showed susceptible and resistant in both diffusion assays were evaluated for its total enzyme activity spectrophotometrically.

Bacterial cultivation

The isolated bacterial culture was inoculated in sterile nutrient broth and kept for incubation at 37^0C for 24 hours. The broth culture as sonicated for 5 minute as top release the resistance enzyme present in the periplasmic membrane of the cell. After sonication the suspension was centrifuged at 10000 rpm for 10 minute and the supernatant was collected.

Total enzyme precipitation

The supernatant was added with 500 µl of 100% Acetone (ice cold) to precipitate the enzyme. The mixture was kept for incubation in refrigerator for 15 minute. The suspension was centrifuged to collect the precipitate. The collected precipitate was washed with 0.01M phosphate buffer and finally the pellet was suspended in phosphate buffer.

Determination of enzyme activity

The enzyme activity was determined by applying antibiotics as a substrate. The following table shows the combination of preparations,

Table 3: Antibiotic with enzyme extract for Activity assay.

	BLANK	CONTROL	T1	T2	T3	T4	T5
Dis.H_2O	3ml	3ml	3ml	3ml	3ml	3ml	3ml
ENZYME	20µl	20µl	20µl	20µl	20µl	20µl	20µl
ANTIBIOTICS	0	0	k.linezoid	k.cephalexin	P.Amikacin	P.Cloxacillin	S.Vancomycin

RESULTS AND DISCUSSION

The aim of the study describe the identification of drug resistance bacteria from seepage of hospital sewage system. The drainage water sample collected from the hospital during the day hours without any prior bleaching and transported to the laboratory. The sample was processed for enumerating the total bacterial count which showed a total population of 2.366 x $10^{4.}$.The BOD level of the sample as it is a liquid was evaluated using titremtric method and found to be 6 mg/l for day 0 and 12 mg /l for day 5 of the hospital drainage water which falls in the range prescribed by BSI, as the sewage sample was by nature its flowing water. The major part of the work denoted by isolating the drug resistance bacteria by cultivating the bacteria in media containing different class of drug. The anitibiogram study put forth the resistant bacteria by formation of no zone formation. The antibiotic applied in detecting the drug resistant bacteria were vancomycin, penicillin, amoxicillin, coxacillin, cephalexin, Linezoid, amikacin by the bacteria obtained from the hospital drainage water.

Figure 1: Hospital Drainage water.

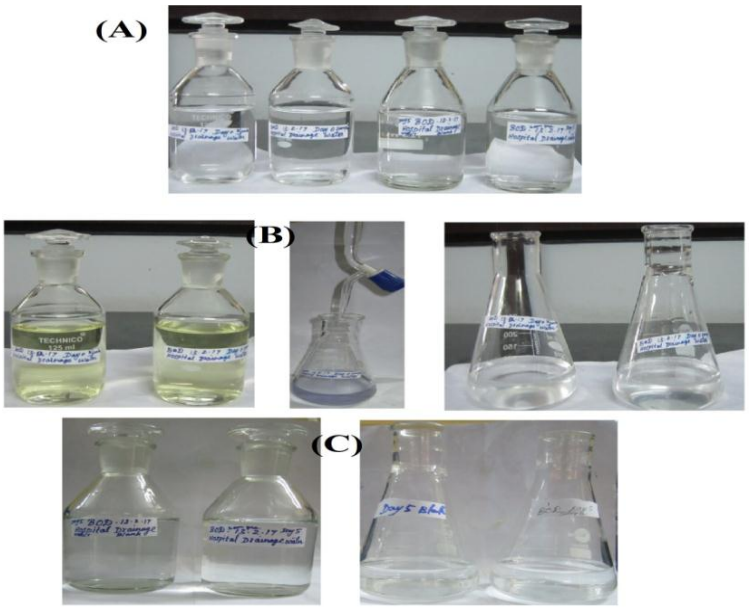

Figure 2: BOD (A) preparation – Day 0, (B) Titration – Day 0, (C) Titration – Day 5.

Table 4: BOD Limit.

Sl. No	BOD		Titre value - I	Titre value- II	BOD (mg/l)
1	Day 0	Blank	0.1 ml	0.1 ml	4
2	Day 0	Sample	0.2 ml	0.1 ml	6
3	Day 5	Blank	0.4 ml	0	0
4	Day 5	Sample	0.7 ml	0	12

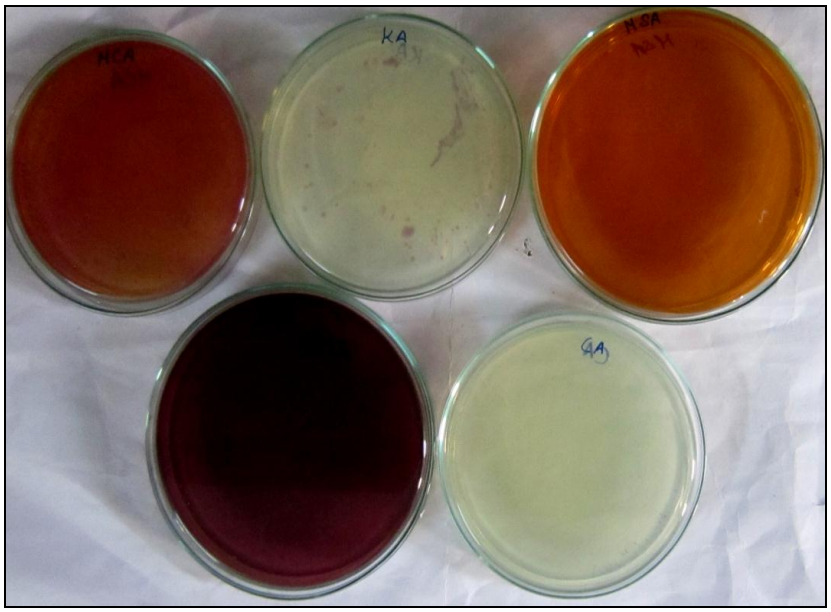

Figure 3: Selective media inoculated with hospital drainage water for screening of drug resistant bacteria.

The isolated drug resistant bacteria were pure cultured by identifying those using selective media and different biochemical characterization was performed to identify the bacteria class as mentioned in the Table-8.

Table 5: Total bacterial count in Hospital drainage water.

Sl. No	Dilution	No. of colonies
1	10^{-1}	1575
2	10^{-2}	704
3	10^{-3}	81
4	10^{-4}	5
5	10^{-5}	1
	Total bacterial population	23660
	CFU	**2.3×10^{4}**

Table 6: Colony Morphology on Selective media.

Selective medium	Bacteria	Morphology	Color	Colony appearance
Mac Conkey Agar	*E.coli*	Concave	surrounding yellow & centre in pale pink	Large & small colonies form
Eosin Methylene Blue Agar	*Bacilli*	Concave	Pale pink	small colonies
Mannitol Salt Agar	*Staphlococci*	Concave	Yellow	small colonies
Hichrome Klebsiella Agar	*Klebsiella*	Raised	Violet & white	luxurious growth
Cetrimizde Agar	*Pseudomonas*	Raised	White (Resistant growth in 18 hours) & growth in 45 hours	Small size colonies

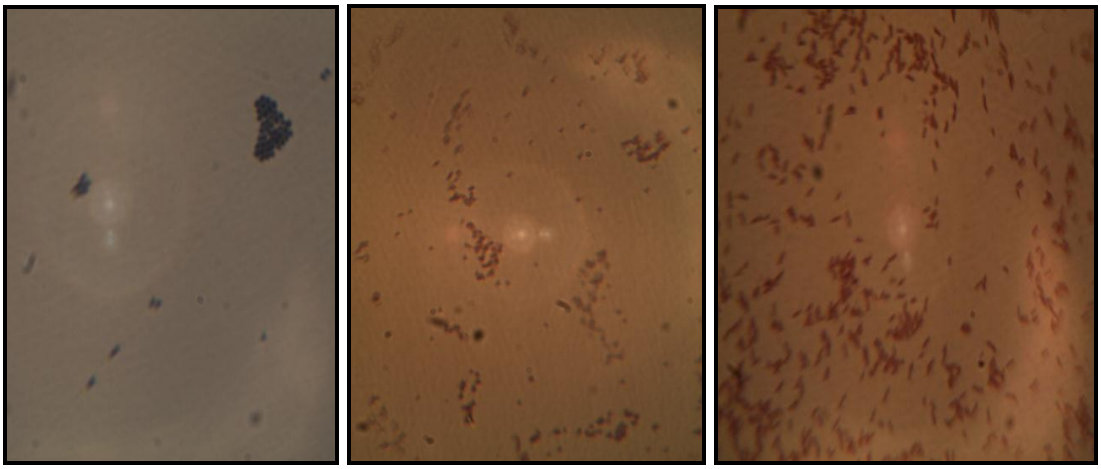

Figure 4: Gram Staining - S. aureus, Klebsiella and Pseudomonas species.

Table 7: Inference for microscopy and biochemical tests.

Microscopic /Biochemical tests	Bacteria isolated from selective media				
	HKA	EMB	MSA	CA	MCA
Gram staining	Gram negative rod	Gram negative short rod	Gram positive cocci in cluster	Gram negative slender rod	***
Motility	Negative	Negative	Negative	Positive	Positive
Indole	Negative	Negative	Negative	Negative	Negative
Methyl red	-	-	+	+	-
V.P	-	-	-		+
Citrate	+	-	No change	+	+
TSI	Alk/A/G	-	No change	Alk/Nogas	Alk/Acid/Gas
Urease	(+)	**	Pink color	Alk/No gas	Alk/Acid/Gas
Catalase	**	**	+	**	**

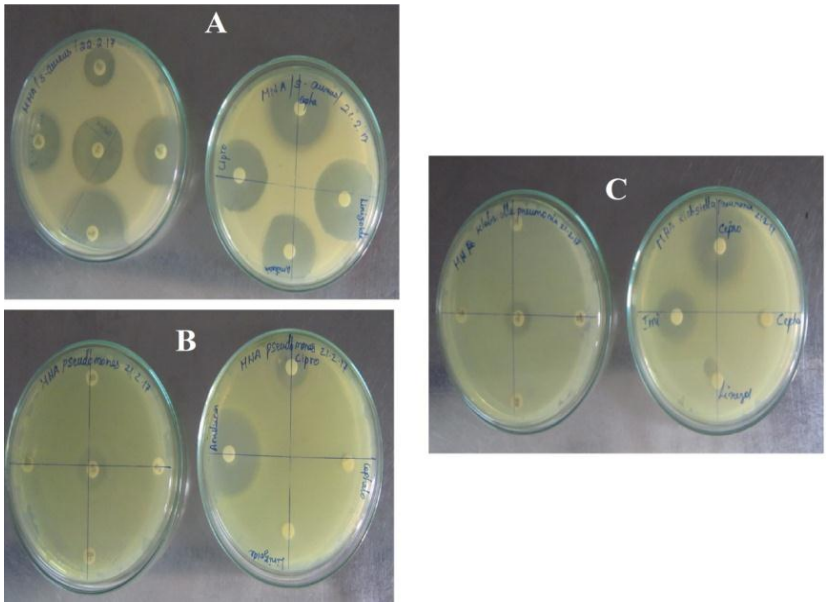

Figure 5: Antibiotic Resistant assay A. *Pseudomonas* B. *S. aureus* C. *Klebsiella.*

The identified bacteria S.aureus, Klebsiella species, Pseudomonas species was determined for its multidrug resistant property by antibiogram assay using different generation antibiotic and the zone interpretation was noted in table 10.The antibiogram of the isolated bacteria defines its expression of antibiotic resistance character by producing beta lactamase (Penicillin, Coxacillin, Cephalexin), 6'-Nacetyltransferases (Amikacin), aminoglycoside phosphoryltransferase (Amikacin), D-alanine-D-alanine transpeptidase (Vancomycin), OXA-1 beta-lactamase (Amoxycillin) exhibiting extended spectrum of beta lactamase and beta metallo lactamase activity. The drug resistance bacteria showed susceptible character towards the antibiotic used represented in table-4.

Table 8: Antibiotic resistant bacteria detection.

ANTIBIOTICS	S. aureus	Pseudomonas	Klebseilla
Vancomycin	17mm	Resistant	Resistant
Penicillin	30mm	Resistant	Resistant
Amoxicillin	26mm	Resistant	Resistant
Tetracycline	28mm	15mm	13mm
Coxacilline	29mm	Resistant	Resistant
Cephalexin	32mm	Resistant	Resistant
Linezoid	33mm	Resistant	22mm
Amikacin	30mm	30mm	Resistant
Ciproflacin	31mm	17mm	27mm

Figure 6: Fresh and dried leaf of *Barleria prionitis* and extract.

Table 9: Moisture content in B. prionitis.

Fresh leaf (g)	Dried leaf (g)	Moisture %
108.7	38.9	69.8

Table 10: Extract yield from leaf of *B. prionitis.*

Final weight (g)	Initial weight (g)	Extract quantity (g)	Yield %
118.3509	114.3378	4.0131	13.93

Table 11: Result for Phytochemical screening.

S. No.	Test	Inference
1	Alkaloid	+
2	Flavanoid	-
3	Tannin	-
4	Phenol	+
5	Turpenoid	-

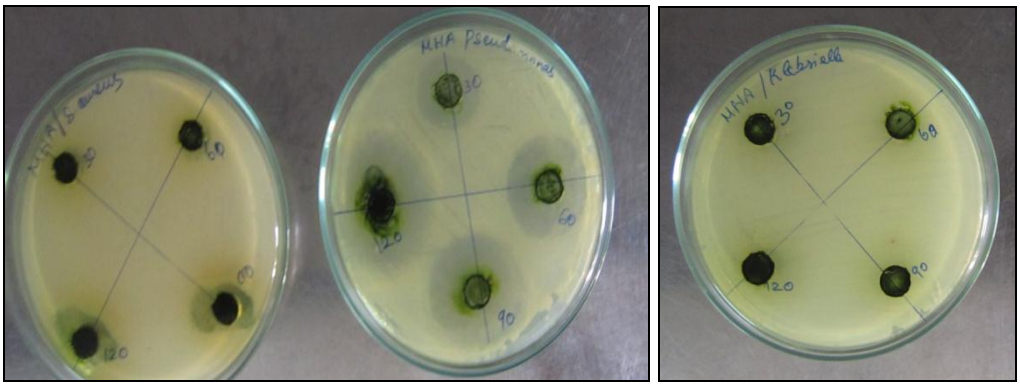

Figure 7: Antibacterial activity of *B. prionitis* leaf extract with drug resistant bacteria (isolated).

The drug resistance was aimed to treat with the herbal extract by preparing the leaf extract of *B. prionitis* and the total quantity of the phytochemical and its constituents were evaluated as recorded in table 11,12 and 13.The drug resistance bacteria was screened for susceptible

character with that of plant secondary materials rather using antibiotic which presently show sensitive but they obtain resistance in the due course. As a experimental trial the bacteria applied for antibacterial assay using plant materials from leaf extract of *B. prionitis*. As with no surprise the bacteria was susceptible with the plant materials .As the study can be further extended with what bioactive molecule has the tendency to kill the bacteria as natural products show a wide property as an antibacterial agent. The effect of the plant extract exhibit a potent bactericidal agent as denoted in table 14.

Table 12: Susceptibility of Drug resistant bacteria with *B. prionitis* leaf extract.

Drug resistant bacteria	Extract Concentration (mg)				MIC (mg)
	6.02	12.03	18.05	27.07	
	Zone Inhibition (mm)				
Pseudomonas species	21	27	30	36	9.87
S.aureus	0	0	12	13	9.11
Klebsiella species	0	0	0	16	10.09

The enzyme activity was qualitatively determined by performing Nitrocefin test which indicated by the presence of red color and similarly Acidimetric test by tube method which was identified by the formation of yellow color (table-15).

Table 13: Enzyme activities by Antibiotic resistance bacteria.

Control	Absorbance	Sample	Absorbance	% Activity
c-Linezoid(klebsiella)	0.378	T1-Linezoid(klebsiella)	1.009	63.1
c-Cephalexin(klebsiella)	0.476	T2-Cephalexin(klebsiella)	1.009	53.3
c-Amikacin(pseudo)	0.01	T3-Amikacin(pseudo)	0.033	2.3
c-Coxacillin(pseudo)	0.008	T4-Coxacillin(pseudo)	0.028	2
c-Vancomycin(s.aureus)	0.018	T5-Vancomycin(s.aureus)	1.71	169.2
c-Plant extract	0.727	ESA	1.71	98.3

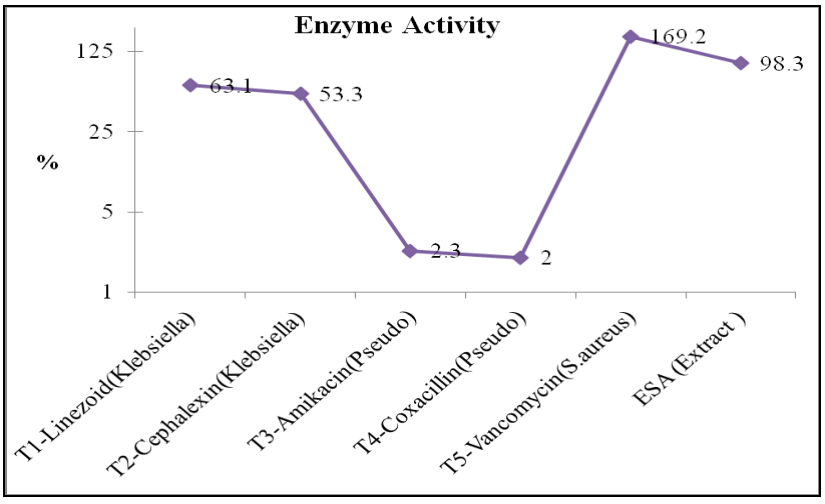

Figure 8: Graphical representation of Enzyme Activity.

CONCLUSION

Antibiotic resistance is a serious threat and challenge to human treatment procedures adopting with different microbial ailments that continues to challenge the countries with poor sanitation. In particular, MDR is common with prominent pathogens such as *Staphylococcus aureus* and *Mycobacterium tuberculosis*, as well as emerging pathogens such as *Acinetobacter baumannii*, *Escherichia coli*, *and Pseudomonas aeruginosa*. Sewage from hospitals contains high number of resistant bacterial strains and high antibiotic residues which contaminate the environment and thus spreads the contagious resistant diseases was mainly contributed by enzyme. Several studies indicate that sewage from hospital provide an excellent environment for the development of antibiotic resistant's and contains high number of pathogens. Thus the present study was investigated to isolate and characterize the antibiotic resistant bacteria from hospital waste especially from drainage canal. Though there is expanding diversity in antibiotic resistance, there is always a continuous need of effort in discovering new therapeutic strategies to address this challenge. This piece of report provides an insight about the prominent resistant bacteria exhibiting enzyme activity.

REFERENCES

1. Gerard D. Wright, Bacterial resistance to antibiotics: Enzymatic degradation and Modification, Advanced Drug Delivery Reviews, 2005; 57: 1451–1470.
2. Denis K. Byarugaba, A. de J. Sosa Mechanisms of Antimicrobial Resistance, Antimicrobial Resistance in Developing Countries, DOI 10.1007/978-0-387-89370-9_2, _ Springer Science Business Media, LLC, 2009.
3. Quintiliani, R. and Courvalin, P. Mechanisms of resistance to antimicrobial agents, In Manual of Clinical Microbiology, ed, 1995.
4. Dessen, A., Di Guilmi, A. M., Vernet, T., and Dideberg, O. Molecular mechanisms of antibiotic resistance in gram-positive pathogens. Curr. Drug Targets Infect. Dis., 2001; 1: 63–77.
5. Abha Khare, Phytochemical and antimicrobial activity of Barleria prionitis leaves, International Journal of Applied Research, 2016; 2(10): 95-97.
6. Patel Bharatkumar K., Chandel B. S., Chauhan H. C., Patel Kirit B., Parth Falguni M., Patel Manoj V., Patel Sanjiv I., Pandya R. P. and Shah Jignesh D. Evaluation of antibacterial activities of Barleria Prionitis Linn, 29 July, 2015; 9(30): 1840-1848. DOI: 10.5897/AJMR2015.7545.

7. Sattya Narayan Talukdar, Md. Bokhtiar Rahman and Sudip Paul, A Review on Barleria prionitis: Its Pharmacognosy, Phytochemicals and Traditional Use Journal of Advances in Medical and Pharmaceutical Sciences, 2015; 4(4): 1-13. Article no.JAMPS.20551 ISSN: 2394-1111.
8. R. Cruickshankk, "Medical Microbiology" 12th eds. (revised reprint) Edinburg: Churchill Livingstone, 1980; 170–189.
9. Radha KV. A Case Study of Biomedical Waste Management in Hospitals, *Global J Health Sci.*, 2009; 1(1): 82-88.