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ISOLATION AND CHARACTERIZATION OF SOME ANTIBIOTIC RESISTANT BACTERIA FROM HOSPITAL DRAINAGE SAMPLE

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ABSTRACT

Hospital waste possesses a significant impact on health and environment. In this present study hospital waste dumped soil sample was collected from private hospital at Tiruchirappalli Dt. and analysis the bacterial population in the soil. Again the soil samples were enriched onto nutrient broth medium incorporated with an antibiotic Streptomycin (200mg/100ml) for the screening of antibiotic resistant bacteria present in the soil. Colonies developed on the plates were identified using standard manual. Based on the morphological and biochemical characters, six bacterial species were isolated and identified namely *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. All the four isolates were again screened for their susceptibility against ten different antibiotics. Among the isolates tested, *Escherichia coli* and *Staphylococcus aureus* showed maximum resistance against most of the antibiotics followed by *Enterobacter aerogenes*. *Andrographis paniculata*, *Leucas aspera* and *Ruellia tuberose* plant leaves were collected and phytochemical compounds were analysed in the plant extract. Among the study maximum compounds were present in acetone and methanol extracts. In this study *Andrographis paniculata* plant leaves maximum antibacterial activity was recorded against isolated all antibiotic resistant bacteria. From this study, it can be said that there is an urgent need to raise awareness and education on medical waste issues. Proper waste management strategy is needed to ensure health and environmental safety.

KEYWORDS: Antibiotic resistance, phytochemical compounds, *Andrographis paniculata*, *Leucas aspera* and *Ruellia tuberose*.

INTRODUCTION

Untreated liquid hospital waste containing unmetabolized antibiotics in low concentration contributes largely to the development of antibiotic resistance in our natural microflora/environmental micro flora. Peoples of developing countries often bear antibiotic-resistant fecal commensal organisms (Calva *et al.,* 1996; Lamikanra.,1997). Visitors to developing countries passively acquire antibiotic-resistant gut *Escherichia coli*, even if they are not taking prophylactic antibiotics, which suggests that they encounter a reservoir of antibiotic-resistant strains during travel (Murray *et al*.,1972). Apparently health people in developing countries carry potentially pathogenic, antibiotic-resistant organisms asymptomatically (Woolfson *et al.,* 1997).

Human activities create waste and the ways that waste is handled, stored, collected and disposed off can pose risks to the environment and public health. Solid waste can be defined as non liquid material that no longer has any value to the person who is responsible for it (Zhu *et al.,* 2008).

Disposal of medical wastes is a growing environmental concern in the developing world. The problem is growing with an ever-increasing number of hospitals, clinics and diagnostic laboratories universally (Hassan.,2008). Medical waste is infectious and hazardous; posing serious threats to environmental health and requires specific treatment and management prior to its final disposal (Manyele, 2004). Therefore the present study was planned isolation and identification of antibiotic resistant bacteria from hospital soil sample in Tiruchirappalli district. To evaluate the antibiotic resistant nature of the selected bacteria against the commonly used antibiotics in an *invitro* condition and studied the antibacterial activity of medicinal plant extracts against isolated antibiotic resistant bacteria.

MATERIALS AND METHODS

Sample Collection

The soil samples were collected from hospital wastes drainage and from soil adjacent to the drainage at Private Hospital, Tiruchirappalli district. The collected soil samples were transported and processed within 24 hours of collection.

Isolation of bacterial colonies

One gram of soil sample was inoculated in to the 100 ml of nutrient broth enrichment with streptomycin (200mg). From the collected and enriched 1g of soil sample was serially diluted

from 10^{-1} to 10^{-8} with 0.85% saline separately. 0.1 ml of sample was taken from 10^{-6} dilution and inoculated on to the respective plates separately. The plates were incubated at 37˚C for 24 hours. After incubation, the total number of bacterial colonies developed on the agar plates were counted using Quebec colony counter.

$$Number\ of\ colines/g = \frac{Number\ of\ colonies \times Dilution\ factor}{Dry\ weig\square t\ of\ t\square e\ sample}$$

Characterization of Bacteria

Bacteria were isolated and characterized using cultural identification, morphological identification using Gram staining reaction and other biochemical tests which include: Indole, Methyl Red, Voges Proskauer (MR-VP), Citrate utilization test, Triple sugar iron agar(TSI), Urease, Oxidase, Catalase, Starch hydrolysis and Carbohydrate fermentation as described by Ogbulie *et al.*, (1998) and Cheesbrough (2003).

Antibiotic Resistance studies

The standardized disc diffusion method also known as Kirby Bauer method (Bauer,1966) was used for the *invitro* determination of sensitivity of on the bacterial isolates obtained from hospital waste drainage against the above mentioned antibiotics. Bacterial inoculums were prepared by suspending the freshly grown bacteria and the inoculums suspension was spread on a Muller Hinton agar plate with a sterile swab. After spreading the discs were applied with as Ampicillin (10µg), Chloramphenicol (100µg), Clindamycin (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamicin (100µg), Levofloxacin (5µg), Penicillin-G (10µg), Tetracyclin (30µg) and Vancomycin (30 µg). All the plates were incubated at 37°C for 24 hrs .After incubation the zone of inhibition around antibiotic disc were measured using Zone scale (HiAntibiotic ZoneScale, HiMedia Laboratories Limited, Mumbai) and recorded.

Molecular Characterization

The effective isolate IARB2 was successively transferred to fresh nutrient agar medium plate to obtain the pure culture and used for molecular characterization. Isolation of genomic DNA, PCR amplification and gene Sequencing was analysed. The identification of phylogenetic neighbors and calculation of pair wise 16S rRNA gene sequence similarity were achieved using the NCBI-BLAST search and EzTaxon server (Chun *et al*., 2007). The CLUSTAL-W algorithm of MEGA 4 was used for sequence alignments and MEGA 4 (Tamura *et al.*, 2007) software was used for phylogenetic analysis of the individual sequences. Distances were

calculated by using the Jukes and Cantor correction in a pair-wise deletion manner (Tamura *et al.*, 2007).

Collection of Plant Material

Plants were selected for this study based on their medicinal use. Fresh *Andrographis paniculata, Leucas aspera* and *Ruellia tuberose* leaves were collected from the village area in Tiruchirappalli Dt. of Tamil Nadu, India in Jan, 2016.

Preparation of Plant Powder

The leaves were air-dried. After drying at 37°C for 7 days the plant material was ground in a grinding machine (Thomas Wiley laboratory mill, model # 4, screen size-1mm) made for the laboratory. Exposure to direct sunlight was avoided to prevent the loss of active components. The soxhlet apparatus were used for the extracting antimicrobial active compounds from the plant leaves.

Phytochemical Screening

Chemical tests were carried out on the aqueous, acetone and methanol extracts and on the powdered specimens were using standard procedures to identify the constituents are described by Trease and Evans,1989 and Harborne (1973).

Antibacterial Susceptibility Test

Disc diffusion method (Bauer *et al.*, 1966) was adopted for evaluation of antibacterial activity of *Andrographis paniculata, Leucas aspera* and *Ruellia tuberose* plant leaves.

Statistical Analysis

The results obtained in the present investigation were subject to statistical analysis like Mean $(\bar{x})$ and Standard Deviation (SD) by Zar (1984).

RESULTS AND DISCUSSION

In the present study, the bacteriological parameters monitored included total viable aerobic counts to isolated bacteria in the hospital waste drainage site soil sample. Results in Fig. - 1 shows the total bacterial population from 87×10^8 CFU/ml to 267×10^6 CFU/ml. The highest bacterial population of 267×10^6 CFU/ml was recorded in normal hospital soil sample followed by antibiotic enriched soil (116×10^6 CFU/ml) was recorded. Aluyi *et al.*,(2006)

reported high faecal load with high concentration of *Escherichia coil* in Udu River, Warri, Delta State, Nigeria, which was attributed to human activities.

In Streptomycin (50µg/ml) antibiotics incorporated plate bacterial growth were noted. Bacterial isolates were identified based on their morphological and biochemical characteristic using standard manuals (Table – 1). Tentatively four bacterial isolates were identified namely *Escherichia coli, Enterobacter aerogenes, Pseudomonas aeruginosa* and *Staphylococcus aureus*.

All the four bacterial isolates were subjected to antibiotic sensitivity method against 10 different antibiotics (Table – 2). The observed results were interpreted with CLSI zone size interpretative chart. Among the organisms tested *Enterobacter aerogenes* showed maximum resistant (9) against most of the antibiotics tested followed by *Escherichia coli* and *Staphylococcus aureus. Escherichia coli* show resistant once against 7 antibiotics, only Ciprofloxacin, Erythromycin and Tetracyline show intermediate effect.

In the present study based on partial 16S rRNA sequencing where the sequences obtained from the effective isolate IARB2 when subjected to BLAST. The IARB2 alignment sequencing was presented in showed sequence similarities 99% with *Enterobacter aerogenes.* The sequence has been deposited at GenBank Bethesda, MD, USA. After the alignment, the tree building option can be activated using Bioedit Software. The tree viewing software nj plot is used to generate a cladogram and grouped the bacterial isolates as shown in Fig - 2.

The use of medicinal plants plays a vital role in covering the basic health needs in developing countries and these plants may offer a new source of antibacterial, antifungal and antiviral agents with significant activity against infective microorganisms (Bhatta et al., 2010). The *Andrographis paniculata Leucas aspera* phytochemical and *Rueilla tuberose* compounds were screened (Table – 3). While steroids, also present in *Leucas aspera* and *Ruellia tuberose* leaves acetone extract is recognized to have anticancer, antiviral and antihemorrhagic properties (DeRoos, 1974).

The effect of different extracts of *Andrographis paniculata* test plant on bacteria was shown in Fig - 3. The results clearly showed that plant extracts were specific in action against the growth of bacteria. Aqueous, acetone and methanol extract was most effective followed by other aqueous extract. *Escherichia coli* were more sensitive for methanol extract of leaves of

the *Andrographis paniculata* plant (21±0.55 mm). The *Escherichia coli* (17±1.00; 16±0.27; 21±0.55 mm in diameter) exhibit relatively higher zone of inhibition followed by aqueous, acetone and methanol extracts and then compared then other test organisms. Tannins possess antibacterial, antiviral, moluscicidal and antitumoral properties (Chitnis 2000).

In this study *Leucas aspera* maximum antibacterial activity was recorded against *Enterobacter aerogenes* (17±0.14 mm in diameter). Moderate antibacterial activity was noted against *Staphylococcus aureus*. Among the three plant extract, aqueous extract has low inhibitory effect against all bacterial isolates (Fig – 3).

The results clearly showed that plant extracts were specific in action against the growth of bacteria. Methanol extract was most effective followed by other acetone and aqueous extract. *Pseudomonas aeruginosa* was more sensitive for acetone and methanol extract of leaves of the tested plants. Aqueous extracts was low inhibition against the tested organism compared to other test plant extracts (Fig – 3). In this study Supported by Bhatta and Kapadnis, 2010 have been analyzed as *Solanum surratense*, *Andrographis paniculata* showed concentration dependent activity against all the tested micro-organisms with the zone of inhibition ranges from 12-24mm at various concentrations.

Finally concluded, it was observed that hospital wastes have negative influence on the microbiological and physiological parameters on the environment. The microbial load as well as the high densities of the physiochemical parameters suggests that activities of hospital wastes in the environment is a major health and environmental threat, which therefore call for a proper regulatory system on disposal of hospital waste in the world, especially in the developing countries Like India.

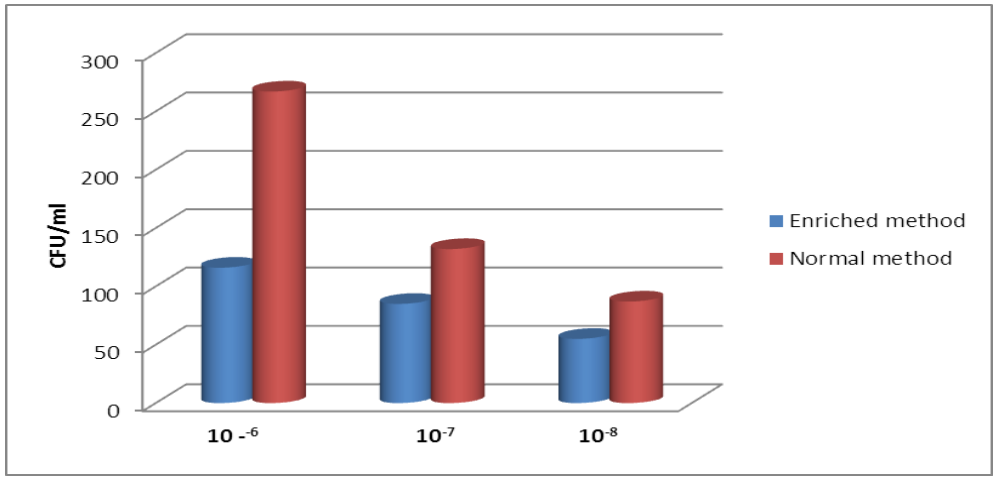

Fig. – 1 Isolation of antibiotic resistant bacteria from hospital contaminated soil.

* Enriched by streptomycin (200mg\100ml).

Table - 1 Biochemical tests for isolated organisms.

Isolated organisms	Gram staining	Shape	Indole	MR	VP	Citrate	Urease	TSI	Catalase	Oxidase	Carbohydrate fermentation		
											Lactose	Dextrose	Sucrose
Escherichia coli	Negative	Rod	+	+	-	-	-	A/A	+	-	+	A	+/-
Enterobacter aerogenes	Negative	Cocci	-	-	-	+	+	-	-	-	-	-	-
Pseudomonas aeruginosa	Negative	Rod	+	-	-	+	±	+	+	+	-	A	-
Staphylococcus aureus	Positive	cocci	-	+	±	-	-	A/A	+	-	A	-	A

+ - positive

- - Negative

± - variable

A- Acid

A/A – Acid slant and acid butt

Table – 2 Antibiotic sensitivity.

S. No.	Antibiotics	Zone of inhibition (mm in diameter) (Mean ± Standard Deviation)			
		IARB1	IARB2	IARB3	IARB4
1.	Ampicillin	-	-	-	08±0.14
2.	Chloramphenicol	16±1.22	-	23±0.27	12±0.53
3.	Clindamycin	13±0.50	-	16±0.18	10±0.46
4.	Ciprofloxacin	26±0.74	14±0.61	24±2.10	11±0.38
5.	Erythromycin	15±0.28	-	-	10±0.22
6.	Gentamicin	19±0.47	-	15±0.23	14±0.61
7.	Levofloxacin	26±0.64	-	22±1.29	16±1.25
8.	Penicillin – G	-	10±0.13	-	-
9.	Tetracyline	21±0.24	12±0.52	17±0.49	12±0.84
10.	Vancomycin	14±0.36	-	13±0.91	14±0.14

Values are expressed in Mean ± Standard Deviation; n=3

IARB – Isolated Antibiotic Resistant Bacteria

IARB1 – *Escherichia coli*

IARB2 – *Enterobacter aerogenes*

IARB3 – *Pseudomonas aeruginosa*

IARB4 – *Staphylococcus aureus*

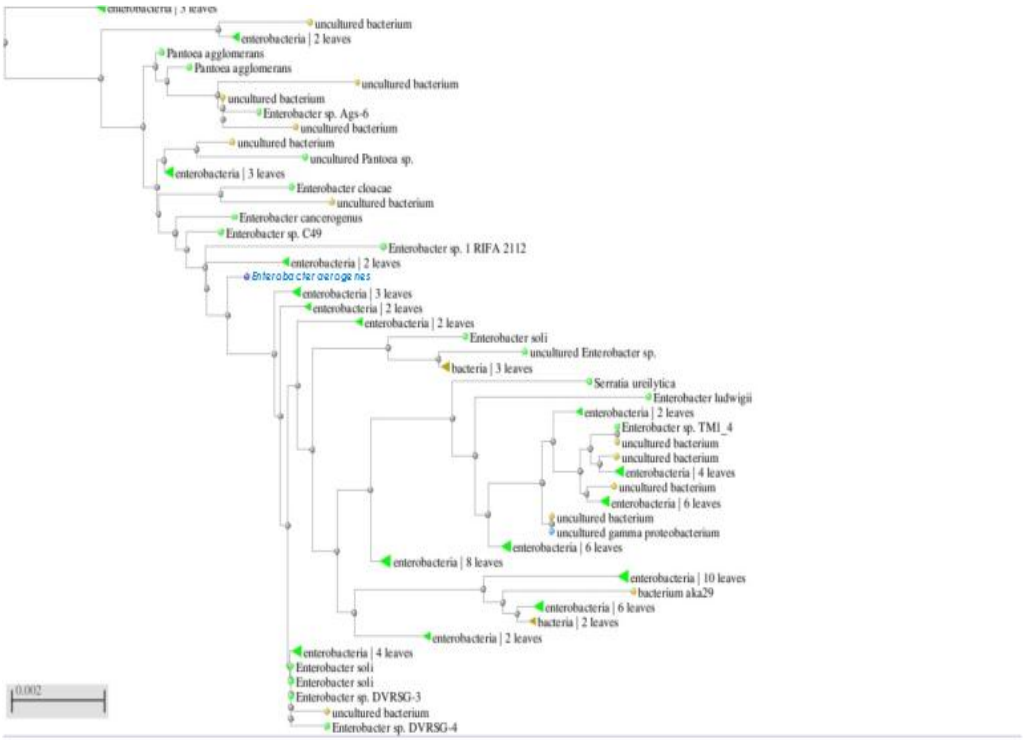

Fig – 2 Tree viewing software Neighbor Joining plot.

Table – 3 Screening of phytochemical component.

S. No.	Phytochemical test	*Andrographis paniculata*			*Leucas aspera*			*Rueilla tuberosa*		
		Aqueous	Acetone	Methanol	Aqueous	Acetone	Methanol	Aqueous	Acetone	Methanol
1.	Tannis	-	+	+	-	+	+	-	-	-
2.	Saponins	-	+	+	-	+	+	-	+	+
3.	Flavonoids	-	+	-	-	+	-	-	+	-
4.	Anthrocyanin	-	+	+	-	+	-	-	-	-
5.	Betacyanin	-	_	-	-	_	+	-	+	+
6.	Glycoids	-	+	-	-	+	-	-	+	-
7.	Steroids	-	+	-	-	-	+	-	+	-
8.	Phytoserol	-	_	+	-	+	-	-	-	+
9.	Phenol	-	-	+	-	-	+	-	+	+
10.	proteins	-	+	+	-	+	_	-	+	-

+ indicates present of compound; - indicates absent of compound.

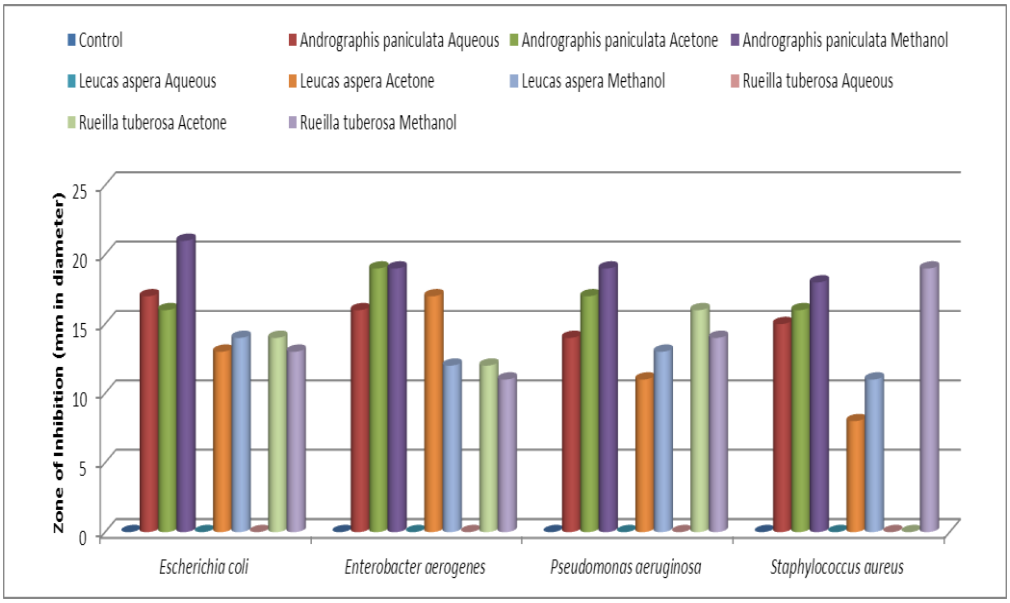

Fig. – 3 ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata.*

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