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Effect of Herbal Extract on Antimicrobial Susceptibility Profile of Drug Resistant Burn Wound Isolates

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Abstract

Major problems of wound management and therapy are bacterial infection and wound sepsis, which in worst case may lead to mortality. The status of infection determines the class of wound. Invasive infection is injurious to host cells. Wound healing is becoming challenging due to the emergence of antibiotic resistant microbes. The present study evaluates the use of neem, turmeric, kuppaimeni, aleovera along with penicillin, ciprofloxacin, metrogel, cefadroxil, , gentamycin, and neomycin against pathogens isolated from burn wound. Among the isolates the predominant pathogens were *Pseudomonas aeruginosa*, *E.coli*, and *Staphylococcus aureus.* Sensitivity of the isolates to antibiotics and herbal formulations were performed by agar well diffusion and disk diffusion method. All *P. aeruginosa* isolates were resistant to penicillin, metrogel, cefadroxil,, gentamycin,and neomycin but sensitive to ciprofloxacin and bacitracin. *E.coli* isolates were resistant to vancomycin, penicillin, metrogel and cefadroxil. *S. saureus* was resistant to gentamycin, neomycin, ciprofloxacin, penicillin and silvergel. But use of herbal extracts increased the sensitivity of organisms to antibiotics. *P. aeruginosa* showed an inhibition zone of 20mm to ciprofloxacin but addition of turmeric, neem, kuppaimeni and aloevera shifts the inhibition zone from resistant to intermediate sensitivity with an inhibition zone of 26mm, 29mm and 33mm respectively. Similarly addition of turmeric increased the zone of inhibition from 10mm which is intermediate to 15mm which is sensitive zone for *P.aerugenosa* to cefadroxil. Thus the study reveals the prevalence of multi drug resistant pathogens in burn wound. Use of herbs is an easy and efficient way of reducing the opportunities for microbes to develop resistance to drugs by combination with antibiotics. Chitosan(0.5%) was used as an antimicrobial agent and it showed 25 mm, 27mm and 20 mm against *P.aeruginosa*, *S.aureus and E.coli.* But when chitosan was used with 5mg of ethanolic extract of neem, the respective inhibition zones increased to 29mm,32mm and 25mm. 5mg of ethanol extract of aloevera gel showed an increase in zone to 28mm,30mm and 24mm respectively.

Keywords: Burn wound, Multi Drug Resistance , *Pseudomonas*, neem, turmeric,kuppaimeni and aleovera.

Introduction

Skin is a mechanical barrier between internal organs and the external environment and a protective barrier against disease-causing organisms. Wound damages the skin integrity. Burn injuries are more common in the elderly population and young children. Burns are injuries to skin tissue caused by heat, electricity, radiation or chemicals. According to the National Institute of Health, more than 2 million people in United States require treatment for burns each year, and between 3,000 and 4,000 die of severe burns. Sivakasi is an active town and a municipality in Virudhunagar District in the Indian state of Tamil Nadu. It is the capital of India's firecracker industry with about 8,000 factories. It produces 90 percent of the total fireworks output. Looking at the activeness of the people, Pandit Jawaharlal Nehru, the first Prime Minister of India nicknamed Sivakasi

as “Kutti Japan” which means “Little Japan”. It contribute 80% of India’s total safety matches production, 90% of India’s total fireworks production, and 60% of India’s total offset printing solutions. It is one of the highest Sales/ Excise/Customs Duty paying towns in India. Fire accidents is becoming common occupational hazard in sivakasi.Huge explosion in a fire cracker unit happened in September 2012 which costs a life of many individuals. Recovery from fire accidents is very hard as the internal system is completely exposed to heat. Moreover infection of the tissues delays wound healing.

Exposure of sterile subcutaneous tissue to external environment provides a favourable route to the entry of microbes. Progress in different stages of wound healing is hampered by infection. Non healing wounds contribute to extended stay in health care centres and associated health care expenses. Microorganisms can get access into a wound either by direct contact of air borne dispersal or by contamination (Rahman and Anson 2004).An ideal dressing should inhibit the colonization and proliferation of pathogens. Hydrogels provide a moist wound healing microenvironment with good fluid absorbance and are transparent to allow the monitoring of healing (Biji Balakrishnan *et.al*., 2012).

The knowledge of the infectious agents of wound is helpful in the treatment option to control infection.The widespread use of antibiotics together with the duration of exposure has led to the emergence of resistant pathogens contributing to morbidity and mortality. The control of wound infections has become more challenging due to the emergence of multi drug resistant pathogens. Significance of antibiotic sensitivity profile in guiding the treatment strategy was reported (Wright *et.al*., 1998; Shittu *et.al*., 2002). Since the presence of drug resistant bacteria in the environment is a threat for public health up-to-date information on local pathogens and drug sensitivity pattern is very crucial to treat patients. Wound colonization with multidrug resistant organisms limit the choice of antibiotic for treatment and complicate the healing process. Steroids used for wound healing has side effects like Adverse Drug Reactions (ADR). But relatively lower incidence of adverse reactions to herbal preparations has been reported (Nair *et al*., 2005). Natural products can be used in conjunction with synthetic drugs to reduce their concentration and hence subsequent side effects.

Medicinal plants are a source of great economic value all over the world. Nature has bestowed on us a very rich botanical wealth and a large number of diverse types of plants grow in different parts of India. Plants are the richest resource of drugs of traditional systems of medicine, modern medicine, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Hammer *et al*., 1999). Herbal medicine is still the mainstay of about 75 - 80% of the whole population, and the major part of traditional therapy involves the use of plant extract and their active constituents (Akerele, 1993).

Hence the present study is aimed to assess the use of herbal extracts in conjunction with antibiotics against drug resistant pathogens from burn wound.

Materials and Methods

Isolation and Identification of Pathogens from Burn Wound.

A major fire accident explored on September 5th 2012, at Sivakasi, Virudhunagar district, Tamilnadu.Many were injured severely and admitted in Government Hospital, at Aruppukkottai, Virudhunagar District, Tamilnadu. From the patients with third degree burn wound fifteen samples were collected in the Presence of duty in charge Physician Dr.Priya. Samples were taken by cotton swabbing at the wound site. It was then suspended well in phosphate buffered saline. Pathogens were isolated by serial dilution in blood agar medium by spread plate technique. Identification of the isolates were executed as given in Bergeys Manual of Systemic Bacteriology.

Herbal Extracts Antimicrobial Susceptibility Test

Dried neem leaves, kuppaimeni leaves, turmeric powder , aloevera gel and chitosan were used to evaluate their antimicrobial activity against burn wound isolates. 10g of plant materials were extracted with 50 ml of ethanol. The extract was centrifuged and the supernatant was dried till the solvent evaporates leaving dried residue. The residue was resuspended in ethanol at concentration of 500mg/ ml. 500mg of chitosan was dissolved in 100ml of 1.5% acetic acid. 100µl of inoculum with 1x 10^8cfu/ml was swabbed with cotton on Mueller and Hinton Agar medium. Well of 8mm diameter was punctured at the centre of the petriplate.100µl of the experimental antimicrobial agent was added to the well and incubated at 37°C for 24 hours **(**Agarry *et al.,*2005). Diameter of Zone of inhibition was recorded in mm. Interpretation of zone of inhibition as R (resistant), I (intermediate) and S (susceptible) were done as per CLSI (2007) guidelines.

Minimum Inhibitory Concentration (MIC)

The extracts of aleovera, turmeric, neem, kuppaimeni and chitosan which showed antibacterial activity in agar well assay were subjected to MIC assay (Jones and Barry, 1987).In order to determine MIC, two fold serial dilution of the extracts and chitosan were prepared with concentration ranging from 320 mg to 5mg /ml and 40mg to 0.625mg/ml for herbal extracts and chitosan respectively. The MIC values were interpreted as the highest dilution (lowest concentration) of the sample, which was transparent. All tests were performed in triplicates.

Statistical Analysis

Experiments were conducted in triplicates and the values represented are a mean of all three replicates and the standard deviation is also represented.

Results and Discussion

Burn injuries are more common in the elderly population and young children. Burns are injuries to skin tissue caused by heat, electricity, radiation, or chemical. Burns cause aesthetic problems as well as acute physical problems and if not taken proper care of can cause serious complication in the form of secondary bacterial infection, various degrees of contractures which restrict the daily activities, septicaemia, etc. The results of Biochemical tests performed to identify the isolates are represented in Table 1. From the fifteen wound samples five different organisms were isolated. Two different Gram negative organism and one Gram positive organism was predominant. They were identified as *P.aeruginosa* , *E.coli* and *S.aureus* respectively. Only the predominant organisms were taken for the study. Prevalence of these organisms was in accordance with the reports of Church, *et al*., 2006.

The interpretation of antibiotic sensitivity of burn isolates was based on the CLSI, 2007 guidelines. The susceptibility of the isolates to antibiotics is given in Table 2. From the table it is clearly evident that *P.aeruginosa* was resistant to all antibiotics. *E.coli* was resistant to all except ciprofloxacin and cefadroxil. To these two antibiotics *E.coli* was intermediately sensitive. *S.aureus* is also resistant to all antibiotics except Cefadroxil. These pictures well the present scenario of wide distribution of Multi drug resistant organisms. Genes that confer resistance can be transferred between bacteria in a horizontal fashion. Antibiotic resistance evolves via natural selection. Continuous exposure to antibiotics selects for the antibiotic resistance as a process of natural selection. Many antibiotic resistance genes reside on plasmids, facilitating their transfer. So the isolates can be considered as multidrug resistant (MDR) or a superbug. β lactamase activity is present in *Staphylococcus* and *Pseudomonas.* Resistance to antibiotics are also developed by alternative efflux pump.

Exposure of bacteria to suboptimal doses of antibiotics due to inappropriate dose or failure to take the prescribed dose for stipulated time contributes to the development of resistance among microbes. (Robicsek *et.al.* 2006). Some types of efflux pumps can act to decrease intracellular quinolone concentration. Resistance to quinolones in Gram-

Table 1. Identification of the Burn Isolates

S.No.	Burn Isolate	Grams Staining	Pigmentation	Indole	Methyl Red	VP	Citrate	Catalase	Oxidase
1.	*P.aeruginosa*	-	+	-	-	-	+	+	+
2.	*S.aureus*	+	-	-	+	+	-	+	-
3.	*E.coli*	-	-	+	+	-	-	-	+

Table 2. Antibiotic Sensitivity of the Burn Isolates to Antibiotics (diameter of inhibition zone in mm)

S.No.	Burn Isolate	Metrogel 5µg	Ciprofloxacin 5µg	Cefadroxil30µg	Penicillin 10Units	Gentamycin 10µg	Neomycin 30µg	Silverge l30µg
1.	*P.aeruginosa*	0 (R)	20±3 (R)	0 (R)	0 (R)	7 ±1(R)	9±2 (R)	2±1(R)
2.	*S.aureus*	0 (R)	13±2 (R)	12±1 (I)	0 (R)	6±1(R)	10 ±1 (R)	3±1 (R)
3.	*E.coli*	0(R)	17±1 (I)	14±1 (I)	0(R)	9 ±1(R)	11±1(R)	2±1 (R)

negative bacteria is mediated by plasmid coded proteins which binds to DNA gyrase. Mutations at key sites in DNA gyrase or topoisomerase IV can also decrease their binding affinity to quinolones and decrease the effectiveness of drug. Low antibiotic susceptibility of *P. aeruginosa* is attributable to a concerted action of multidrug efflux pumps. They have mexAB-*oprM, mexXY* etc responsible for antibiotic resistance encoded in chromosomes. Hyper mutation favours the selection of mutation-driven antibiotic resistance in *P. aeruginosa* strains producing chronic infection. Clustering of several different antibiotic resistance genes in integrons favour the concerted acquisition of antibiotic resistance. (McCollister, 2011).

Peptidoglycan layer of bacterial cell wall continuously undergoes remodeling by transpeptidase. Penicillins inhibit transpeptidase and prevent the reformation of the peptide bonds. This leads to the loss of cell wall integrity. Death of bacteria is caused by the leakage of cellular contents (Pirt, and Righelato, 1987). Resistance to penicillin arises due to mutations in the active site of the transpeptidase enzyme(Yocum *et al*.,1980).

Microbes have developed resistance to gentamycine via decreased cell permeability, alterations at the ribosomal binding sites or by the production of amino glycoside modifying enzymes. This mechanism is chromosomally mediated and results in cross-reactivity to all amino glycosides. Mutations at the site of amino glycoside attachment may interfere with ribosomal binding. Enzymatic modification is the most common type of amino glycoside resistance. Genes encoding for amino glycoside modifying enzymes are usually found on plasmids and transposons (Mingeot-Leclercq *et al*., 1999). The three types of amino glycoside modifying enzymes are N-Acetyltransferases which catalyzes acetyl CoA-dependent acetylation of amino group of antibiotic, O-Adenyltransferases (ANT) which catalyzes ATP-dependent adenylation of hydroxyl group of antibiotic and O-Phosphotransferases (APH) which catalyzes ATP-dependent phosphorylation of hydroxyl group of antibiotic.Gentamycine resistance in *S.aureus* is also reported by Townsend *et al*. (1983). Bacterial neomycin phosphotransferase gene confers resistance to neomycin and related amino glycoside antibiotics. Ciprofloxacin belongs to fluoroquinolone class. Ciprofloxacin inhibits bacterial DNA gyrase. Cefadroxil is a first-generation cephalosporin antibiotic. It interferes with bacterial cell wall synthesis. The isolates in the present study showed only intermediate sensitivity to cefadroxil and are in the border of developing resistance. So there is an urgent need to develop alternative drugs.

The influence of different herbal extracts on the susceptibility of pathogens is given in Table 3. From the table it is inferred that chitosan is more potent than herbs in controlling the growth of pathogens. The inhibition zone for *P.aeruginosa* was 25mm, 27 mm for *S.aureus* and 20 mm for *E.coli*. Chitosan is a natural polysaccharide derived by *N*-deactivation of chitin, from the shells of crab, shrimp, and crawfish (Orgaz, *et al*., 2011). Our results corroborate well with the reports of Li *et al*., (2010a; 2010b). It is active against both *P.aeruginosa* and *S.aureus* indicating that it has higher and broad spectrum of antimicrobial activity. Positively charged chitosan can interact with the negatively charged bacterial membrane and cause the leaching of low molecular weight materials, nucleic acid and proteins. (Rafat *et al*.,2008). It alters bacterial surface morphology and membrane permeability, (Didenko, *et al*.,2005 ; Eaton *et al*., 2008 ; Tang *et al*.,2010). The inhibition zone showed by neem, turmeric kuppaimeni and alovera against *P.aeruginosa* was 10mm, 7mm ,8mm and 6 mm respectively.The above extracts exhibited 12mm, 12mm, 8mm and 9mm were the zone of inhibition against *S.aureus. P.aeruginosa and E.coli* were comparatively susceptible to neem. *S.aureus* was sensitive to turmeric as they have a highest inhibition zone of 16mm. Antimicrobial activity of herbs is due to variety of secondary metabolites. This is supported by the observations of Parekh *et al*., 2005. Presence of phytochemicals like phenols, unsaturated sterols, triterpenes and saponine phenolic diterpenoids (Fujiwara *et al*., 1984;Joshi *et al*.,20011).

Table 3. Antibiotic Sensitivity of Burn Isolates to Herbal extract and Chitosan (diameter of inhibition zone in mm)

S.No.	Burn isolates	Neem (100mg/ml)	Turmeric (100mg/ml)	Kuppaimeni (100mg/ml)	Aloevera (100mg/ml)	Chitosan(5mg/ml)
1	*P.aeruginosa*	10 ±1	8±1	7±1	6±1	25±3
2	*S.aureus*	12±1	12±1	8±1	9±1	27±2
3	*E.coli*	16±1	14±1	10±1	10±1	20±1

The influence of the combination of herbal extract and antibiotics on antimicrobial activity is shown in Table 4. As evident from the table, combination of herbal extract and antibiotics were found to be significantly effective against the burn wound isolates. Herbal extracts increased the sensitivity of organisms to antibiotics. Ciprofloxacin showed an inhibition zone of 20 mm against *P. aeruginosa.* But addition of turmeric, neem , kuppaimeni and aleovera to ciproflaxacin shifts the inhibition zone from resistant to intermediate sensitivity with an inhibition zone of 26 mm, 29 mm and 33 mm and 33mm respectively. Intensive use of antibiotics often resulted in the development of resistant strains (Cunha, 2001) Medicinal plants have many advantages including less side effects, better patient tolerance, relatively less expensive, and renewable in nature (Vermani and Garg, 2002).

Similarly addition of turmeric to cefadroxil increased the zone of inhibition from 10mm which is intermediately sensitive to 15mm which is sensitive zone for *P.aerugenosa*. Wound care using herbal remedies can be traced back to early civilization (Mantle *et al.*, 2001). Approximately one third of all traditional medicines are used for treating wounds and skin disorders. These plant based products are could influence specific phases of wound healing such as inflammation, collagenation, epithelialisation and contraction (Khalil *et al*., 2006).

Chitosan showed 25 mm, 27mm and 20 mm against *P.aeruginosa, S.aureus and E.coli.* But when chitosan was used with neem extract, the respective inhibition zones were increased to 29 mm,32 mm and 25 mm. Addition of aloevera gel extract increased the inhibition zone to 28

Table 4. Antibiotic Sensitivity of the Burn Isolates to combination of plant extract and antibiotics (diameter of inhibition zone in mm). The potency of antibiotics and herbal extract is 50 µl of herbal extract + 50 µl of antibiotic.
Stock herbal extract : 5mg/50µl ; Metrogel -2.5 µg/50µl ; Ciproflaxacin- 2.5 µg/50µl;
Cefadroxil-15 µg/50µl; penicillin- 5Units; Gentamycin - 5 µg/50µl; Neomycin -5 µg/50µl

S.No.	Plant Extract+Antibiotics	*P.aeruginosa*	*S.aureus*	*E.coli*
1	Neem+pencillin	15±1	19±1	45±3
2	Neem+metrogel	13±1	12±1	30±3
3	Neem+ciprofloxacin	29±2	22±1	35±2
4	Neem+cefadroxil	24±1	25±2	35±3
5	Neem+gentamycin	21±1	26±2	21±2
6	Neem+neomycin	25±2	23±1	28±3
7	Neem+chitosan	33±2	35±4	44±4
8	Turmeric+pencillin	19±3	21±1	42±4
9	Turmeric+metrogel	11±1	10±1	25±1
10	Turmeric+ciprofloxacin	26±2	20±1	39±2
11	Turmeric+cefadroxil	27±1	20±1	35±3
12	Turmeric+gentamycin	21±1	17±1	32±3
13	Turmeric+neomycin	22±2	20±1	25±1
14	Turmeric+chitosan	28±2	30±2	35±3
15	Kuppaimeni+pencillin	14±1	10±1	22±1
16	Kuppaimeni+metrogel	10±1	15±1	21±1
17	Kuppaimeni+ciprofloxacin	25±3	30±1	36±3
18	Kuppaimeni+cefadroxil	22±1	25±1	30±3
19	Kuppaimeni+gentamycin	18±1	13±1	22±1
20	Kuppaimeni+neomycin	11±1	13±2	25±2
21	Kuppaimeni+chitosan	28±1	33±2	37±2
22	Aloevera+pencillin	20±2	13±1	30±1
23	Aloevera+metrogel	21±1	19±1	23±1
24	Aloevera+ciprofloxacin	29±2	27±1	37±1
25	Aloevera+cefadroxil	30±1	24±1	35±2
26	Aloevera+gentamycin	20±1	26±2	27±2
27	Aloevera+neomycin	21±1	22±2	30±1
28	Aloevera+chitosan	35±1	38±3	45±4

mm,30 mm and 24 mm to *P.aeruginosa, S.aureus and E.coli* respectively. Addition of herbal extract to antibiotics increased the susceptibility of the organism to antibiotics. *Aloe* gel is used to relieve thermal burn, sunburn and promote wound healing. Mucilage of it consists of 99.5% water (Eshun and He,2004). The remaining 0.5–1% solid material consists of a range of compounds including water-soluble and fat-soluble vitamins, minerals, enzymes, polysaccharides, phenolic compounds and organic acids (Boudreau and Beland, 2006). Heterogenous composition of the *Aloe* pulp may contribute to the diverse pharmacological and therapeutic activities (Talmadge et al., 2004).MIC of all plant extracts were 80mg/ml for all organisms. Chitosan showed minimum inhibitory concentration at 10mg/ml. Remarkable increase in antimicrobial activity observed when herbs and antibiotic were used together might be due to the synergistic activity between them. Combination of herbal extract with antibiotics could have increased the permeability of bacterial cells to antibiotics (Sikkema *et al.*, 1994).

Conclusion

Thus the present study clearly demonstrates that the sensitivity of multi drug resistant organisms to antibiotics can be improved by their combination with herbal extracts. This paves a way for limiting the microbes to develop resistance against antibiotics.

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