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Study of the antimicrobial activity of consortiums of plant extracts and actinomycetes against multi-drug resistant bacteria

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Abstract
In order to ease the burn wound healing the bacterial accumulation has to be alleviated. It successively upsurges the necessity to improve substitute approach to counter the growing apprehension against the multi-drug resistant infection in burn wounds. In the present study, two combinations of herbal alcoholic extracts have been assessed for antimicrobial activity against the isolates collected from human burn wound sample and biopsies. Total of 42 strains have been isolated and screened for its antibiotic resistance.
It was found that a significant amount of bacteria adapted resistance to traditional antibiotics such as Gentamycin and Imipenem. Out of the 42 isolates, 28 isolates were found to be multi-drug resistant strains. The study demonstrated the inhibition of resistant strain of Vancomycin-resistant *Staphylococcus aureus* (VRSA) by extract of *Terminalia chebula* fruit, *Terminalia bellirica* fruit, *Syzygium aromaticum* fruit, *Syzygium cumini* fruit (HBLJ) while multi-drug resistant *Escherichia* was inhibited by consortium of *Ocimum tenuiflorum* Leaves, *Ficus religiosa* Bark and *Piper betel* Leaves (TPB) extract. Chloramphenicol, Kanamycin and Tetracycline effectively inhibited All 7 gram positive bacteria. Significantly, actinomycetes showed greater antimicrobial activity, which has inhibited the great number of multidrug resistant bacteria.
Kill time study indicated that HBLJ extract inhibited *Pseudomonas spp* inhibited within 1 hour of contact time. But TPB extract showed relatively high contact time for efficient bioactivity. Moreover, both of extract showed no anticancer activity in 10µg/ml to 80µg/ml in case of SK-MEL-2 cell line, indicates extract selectively inhibited bacteria.

Keywords: *Actinomycetes,* plant extract, Antibiotic resistance, Kill time study, Antimicrobial activity. Cytotoxicity

Introduction
A major concern in the health care industry is antibiotic resistance. Genuses belonging to *Enterobacter spp* [1], *Escherichia coli* [2], *Pseudomonas spp* [3], and *Klebsiella spp* [4] reported with acquired resistance.
Burn injuries are a troublesome problem in health care field. The prime attention has been given to mollify the pain and mainly reducing the infections [5]. Infections causing pathogenic flora at burn wounds can initiate distinct immune response followed by sepsis thus hinder the wound healing process [6]. Therefore Burn wound healing process significantly depends upon the management of the infections.
Numerous antibiotics are functional to the target against moderately intricate bacterial accumulation [7]. As a result, cell to cell communication and vertical gene transmission affects the bacterial attribute and serves to be the reason to develop the antibiotic resistance [8]; therefore it employs pressure on health care specialists to hunt for the substitute antimicrobial approach to regulate infections.
Seven million burn injuries per year have estimated in India. Out of them, 10 % require hospital admission. Lead to excess use of antibiotics and on consequence the bacteria are getting resistance to broad spectrum of antibiotics [9] .This increasing antibiotic resistance makes us thrive for alternative source of antimicrobial agents.
In the science of the Ayurveda, plants with plentiful of clinical benefits emerged out to be the promising counter solution [10]. Plants are rich with different phenolic compounds alkaloids, saponins, steriods, terpenols, tannins, Glycosides, resins, flavonoids, and Volatile oils etc. Many of them possess significant bactericidal activity [11].

In the present study, two combinations of hot alcoholic plant extracts were studied for their antimicrobial activity. Isolates from the burn wounds were screened for the antibiotic resistant strain and its control was studied by two combinations of alcoholic plants extract at different concentration and different contact time. Extracts are also studied for its anticancer activity against human melanoma cell line (SK-MEL-2) cell line.

Material and method
Selection of plants for extract preparation
The plant parts were obtained from the local market and authenticated by a botanist. The plant parts were selected and used as combinations. Two combinations were selected for preparation Combination TPB contains hot alcoholic extract of Ocimum tenuiflorum (Tulsi) Leaves, Ficus religiosa (Pipal) Bark and Piper betel (Betel) Leaves.
The second combination HBLJ contains hot alcoholic extract of *Terminalia chebula* (Hirada) fruit, *Terminalia bellirica* (Behda) fruit, *Syzygium aromaticum* (Lavang) fruit, and *Syzygium cumini* (Jambhul) fruit.

Preparation of herbal extracts: Hot alcoholic extract preparation was carried out by continuous hot extraction by the soxhlet apparatus. 30 g of crude dried powder of equal proportion of respective herbals was made into a thimble using filter paper and was introduced into the siphon tube of

the extractor. 300 ml of ethanol was poured into the siphon tube and allowed to settle down in the round bottom flask. The apparatus was set up with the condenser above the central tube. The apparatus was placed into the heating mantle and the temperature was adjusted to 70°C. The extraction was carried out for 36 hours until the ethanol in the siphon tube turns colorless, indicating complete extraction. The extract was dried at 50°C into powder form and stored at room temperature [12].

Extraction of Secondary metabolite from gamma tolerant *Actinomycetes*: The organisms isolated from gamma irradiated soil were inoculated in a respective medium such as bacteria in Nutrient broth, fungi in Potato dextrose broth and *Actinomycetes* in *Actinomycetes* broth. They were incubated for three weeks and cell free supernatant was separated by centrifuging it at 15000 rpm. The organic solvent like ethyl acetate was used to extract secondary metabolite in two phase solvent extraction system. The separated secondary metabolite was dried at RT for evaporation of ethyl acetate. Dimethyl sulphoxide was used as solvent to dissolve the dried secondary metabolite and stored in refrigerator [13]

Sample collection and isolation: Samples of patients with burn wounds and biopsies were collected. The collection was done at Masina Hospital, Byculla and J.J. Hospital, Mumbai. The surface samples of the wound were collected by sterile swabs and transported in sterile Ringer's solution. The samples were processed immediately in the laboratory for isolation and identification of isolates. Inoculated ringer's solutions were streaked on Sterile Nutrient agar plates and sterile Mac Conkey agar plates. The plates were incubated at 37°C for 24 hours and Gram nature of all isolates was identified.

Antibiotic susceptibility test by disc diffusion method: Inoculums was prepared from the isolates isolated and identified from various burn wounds as well as Soft and skin tissue**.** The density of the culture was adjusted to McFarland Standard No. 1 and the culture was spread on the plate using sterile swabs. Antibiotic discs were placed on the surface with at least 24mm of the distance between centers of each disc. The antibiotics of different groups were used for the study including Amikacin, Chloramphenicol, Colistin, Ciprofloxacin, Imipenem Gentamicin, Kanamycin, Penicillin G, Polymyxin B, Tetracycline, Tobramycin, and Vancomycin. The plates were incubated at 37°C for 24 hours. After incubation, the zone diameter was measured in millimeters and interpreted using Kirby Bauer chart. Colonies grown on agar were further selected for identification.

Identification of multidrug-resistant strains: Strains which showed resistance to more than two antibiotics was selected for identification. On the basis of colony characters, Gram's nature and further biochemical tests, the isolates were identified using Bergey's Manual of Determinative Bacteriology 9th edition [14].

Agar ditch method: The antibacterial activity of the combination of plant extracts and secondary metabolites of actinomycetes was studied against broad-spectrum antibiotic resistant isolates by Agar ditch method. A ditch of 8.0cm X 1.5cm was made in the sterile nutrient agar plate. The extract was weighed according to the concentration required, then dissolved in Dimethyl sulfoxide (DMSO). This was then poured into the ditch prepared. The plate was cooled and then dried. The cultures were streaked perpendicular to the ditch and parallel to each other. The plates were incubated at 37°C for 24 hours. Inhibition of the growth of organisms was observed after incubation. Solvent control plates were also maintained [15].

Minimum inhibitory concentration: Minimum inhibitory concentration MIC was determined using a concentration gradient of herbal extract in Mueller and Hinton broth. The dissolved extracts were mixed in sterile molten agar butts in concentrations of 10µg/ml to 20µg/ml of extract. Extract and molten agar butts were poured on the sterile plates. The test cultures were spot inoculated on the extract plates and plates were incubated at 37°C for 24 hrs. After 24 hrs. The inhibition of growth of the isolates was observed [16].

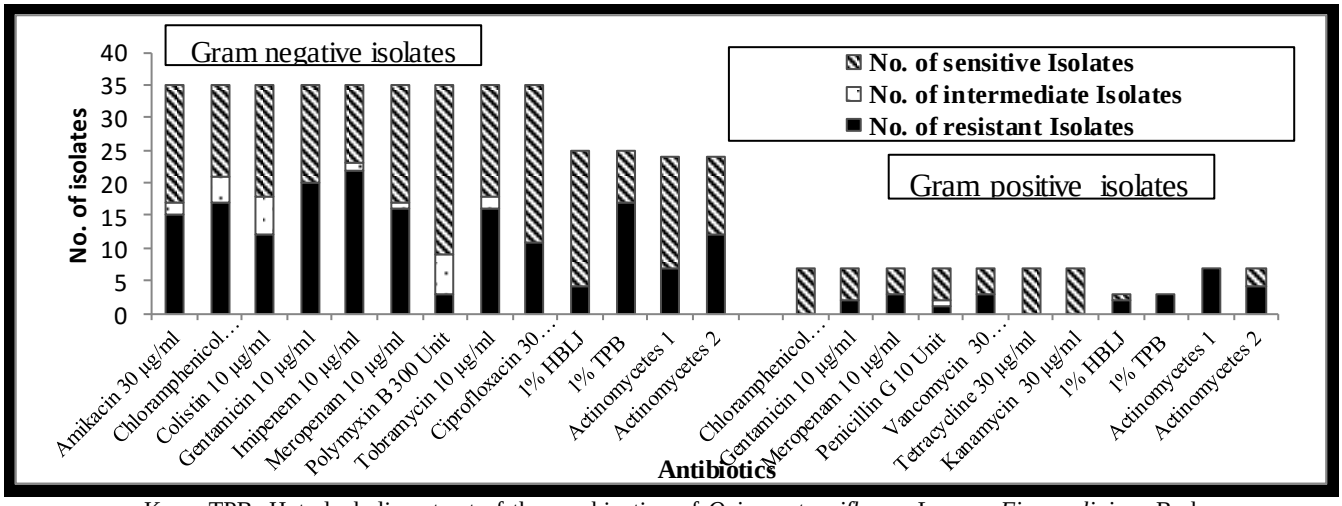
Kill time studies of herbal extracts: Combination of plant extracts were subjected to bactericidal time efficacy (contact time period) test. This method assesses the antibacterial effect of the herbal extracts in relation to time under study. The organisms are maintained in contact with the plant extract and its bactericidal effect is monitored at regular time intervals ranging from 0 to 8 hours at an interval of 1 hour. The herbal ointment was formulated using petroleum jelly and incorporated with 50mg of herbal extract, which makes the final concentration 1% of the combination extract. 2ml of inoculums was mixed with 5gm of petroleum jelly based ointment in a sterile plate. This was then spot inoculated on a sterile nutrient agar plate at a time interval of 1 hour for 8 hours. The plates were incubated for 24 hours at 37°C. After incubation, the plates were seen for the growth. The hour at which the plate showed no growth indicates the time taken for the extract to exert its bactericidal action [15].

Sulforhodamine-B (SRB) assay*:* In vitro cytotoxicity of the combination extract against Human melanoma cell line (SK-MEL-2) was determined by using Sulforhodamine-B (SRB) assay. The cell line SK-MEL-2 was harvested from an exponential growth phase, grown on RPMI 1640 medium containing 10% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated in 96 wells microtiter plate. After cell inoculation, the microtiter plates were incubated at 37°C, in 5% CO_2, 95% air and 100 % relative humidity for 24 h prior to the addition of experimental drugs. After 24 hours, 50% Tri chloro-acetic acid (50µl/well) was added at 40C for 1 hour, allowing cells to be fixed by Protein Precipitation. Aliquots of 10µl of 100, 200, 400 and 800µg/ml dilutions were added to the appropriate microtiter wells already containing 90 µl of cell suspension, resulting in the required final drug concentrations of 10, 20, 40 and 80 µg/ml. fixed cells were stained for 15 min with 0.4% SRB dissolved in 1% Acetic acid (50µl/well). Acetic acid was used to remove the unbound stain. Then plates were air-dried. Bound protein stain was solubilized with 150µl 10Mmol/1 un-buffered Tris base and Optical Density was measured on the Elisa Plate reader at wavelength 540nm [17].

Result and Discussion

Isolation and Antibiotic susceptibility testing of the

bacteria: Human burn wound samples from Masina hospital, Byculla and J.J. hospital, Mumbai were collected in ringer's solutions, and headed for isolation by using selective media. Gram staining of the isolates was carried out. Total 42 isolates were isolated, which contains 35 isolates, of which 28 isolates were Gram negative and 7 were Gram positive. The antibiotic susceptibility testing of the isolates was carried out by disk diffusion method. Several antibiotics were selected for the testing. 28 isolates among 42 isolates revealed resistance to more than two antibiotics. Isolates were found to be unaffected by Amikacin, Chloramphenicol, Meropenem, Gentamicin, Imipenem and Tobramycin antibiotics. In the case of Gram positive strains, Polymyxin B showed the great cidal effect on Gram negative strains correspondingly Penicillin, Tetracycline and kanamycin were effective against Gram positive strains. Significantly, a great quantity of strains is found to be resistant against Amikacin, Chloramphenicol, Meropenem, Gentamicin, Imipenem and Tobramycin antibiotics. It was observed that more than 50% of bacteria exhibited resistance to Gentamycin and Imipenem. That suggests the threatening implications of the overuse of antibiotics in hospital facilities.



Keys: TPB- Hot alcoholic extract of the combination of *Ocimum tenuiflorum* Leaves, *Ficus religiosa* Bark and *Piper betel* Leaves, HBLJ- Hot alcoholic extract of *Terminalia chebula* (Hirada) fruit, *Terminalia bellirica* (Behda) fruit, *Syzygium aromaticum* (Lavang) fruit, *Syzygium cumini* (Jambhul) fruit.

Fig 1: Study of the antibiotic susceptibility pattern of the isolates Number of resistant intermediate and sensitive isolates to various tropical antibiotics and plant extracts

Identification of the multi-drug resistant strains: These resistant strains were further subjected to several biochemical tests and microscopic techniques. It was observed that the *Klebsiella spp*. was the predominant organism found in the burn wounds lesions which represent 25% of all of the isolates followed by *Escherichia coli (18%), Pseudomonas spp (14%), Alcaligenes spp.(14%), Enterobacter spp. (14%), Staphylococcus aureus (11%),* and *Citrobacter spp. (4%)* were identified using Bergey's Manual of Determinative Bacteriology 9th edition. The results are shown in table no 1 Colistin has seen to be effective against Gram negative bacteria such as *Pseudomonas aeruginosa, Klebsiella pneumonia* and *Acinetobacter* [18]. It attacks bacterial cell membrane by displacing magnesium and calcium through the membrane [16], as well as Tobramycin is known to be effective against *Pseudomonas spp*. It binds to ribosome which in result inactivates mRNA [19]. Most of the bacteria found to be resistant with Imipenem and Gentamicin. Whereas, Imipenem inhibits bacterial cell wall synthesis and gentamicin also affects protein synthesis. In order to inhibit Gram positive strains, Chloramphenicol, Tetracycline and Kanamycin effectively showed inhibition of all Gram positive strains. Moreover, all three antibiotics disrupt protein synthesis.

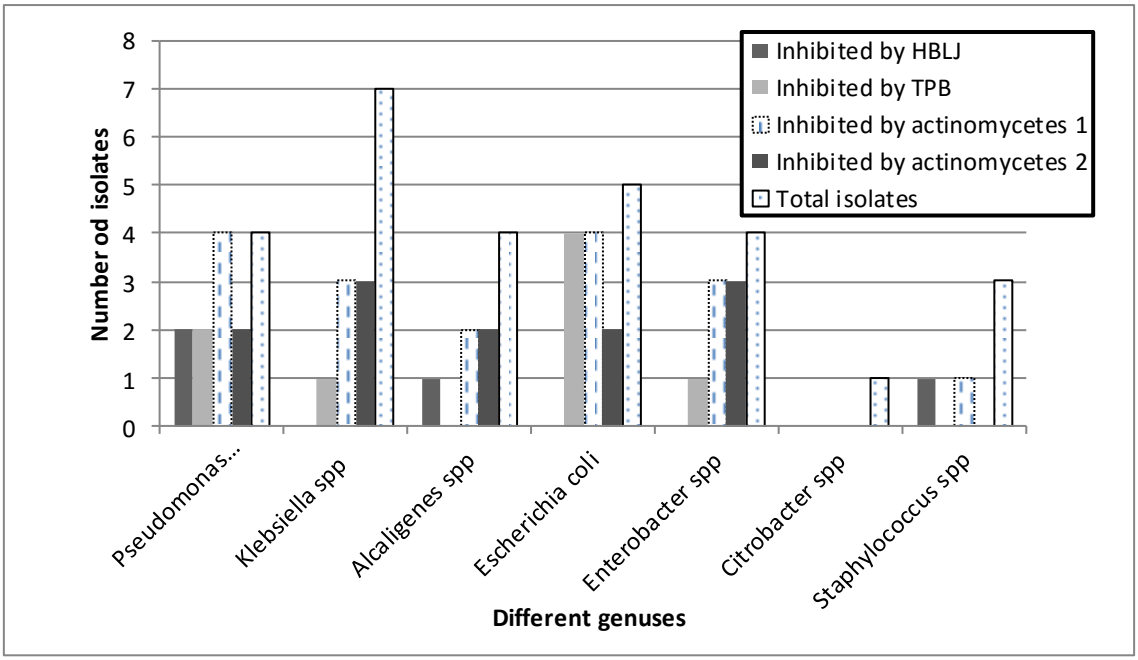
Table 1: Identification of multi-drug resistant isolates from clinical samples

Isolate name	Percentage of isolates
Pseudomonas spp.	14
Escherichia coli	18
Klebsiella spp	25
Alcaligenes spp.	14
Enterobacter spp.	14
Staphylococcus aureus	11
Citrobacter spp.	4

Antimicrobial activity of the combination of extracts: The qualitative activity of 1% of extracts was evaluated against multi-drug resistant strains by agar ditch method. 1% of TPB extract showed antimicrobial activity against four strains of *Escherichia coli*, two strains *Pseudomonas spp* and one strain of each *Klebsiella spp* and *Enterobacter spp*, but none of the *Alcaligenes spp, Citrobacter spp* and *Staphylococcus aureus strains* was inhibited by TPB extract. On the other hand, 1% of the HBLJ extract efficiently inhibited two strains of *P. aeruginosa*, one strains

of *Alcaligenes spp*, one strains of *Staphylococcus aureus*, Though both extracts showed inhibited very few MDR isolates whereas, in case of actinomycetes, secondary metabolites actinomycetes showed greater inhibition activity, which inhibited four P. aeruginosa, three *Klebsiella spp*, two Alcaligenes *spp* and three *Enterobacter Spp*. both of the *actinomycetes* didn't show cidal effect against *Citrobacter spp*.

The quantitation was carried out by Minimum inhibitory concentration by plate dilution technique. HBLJ extract showed significant antimicrobial activity, extract further examined for its minimum inhibitory concentration against MDR isolates. HBLJ extract showed minimum inhibitory concentration as 1% against one *Pseudomonas* spp. isolates, two *Alcaligenes* spp. isolates, isolates, one *Enterobacter* spp. isolate, three *Escherichia coli* isolates and one *Citrobacter spp* isolate, except three of the isolates of *Pseudomonas spp* inhibited by 2%. Results are presented in figure 2.



Keys: TPB- Hot alcoholic extract of the combination of *Ocimum tenuiflorum* Leaves, *Ficus religiosa* Bark and *Piper betel* Leaves, hot alcoholic extract of *Terminalia chebula* (Hirada) fruit, *Terminalia bellirica* (Behda) fruit, *Syzygium aromaticum* (Lavang) fruit, *Syzygium cumini* (Jambhul) fruit.

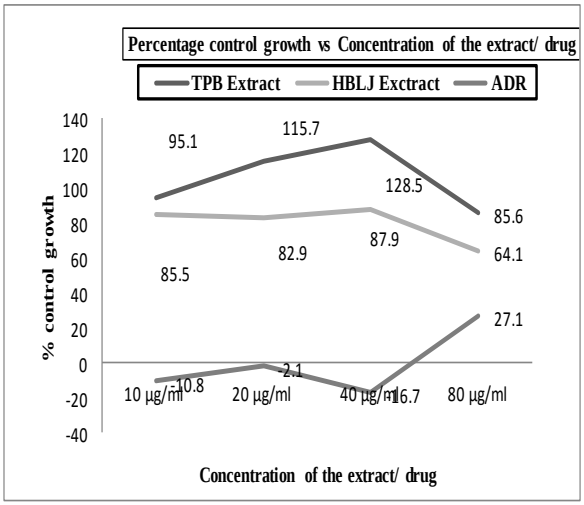
Fig 2: Study of the Antibacterial activity of the combination of the extracts against the isolates by Plate dilution method

Kill time study: Strains inhibited by both of the extracts are selected for this study. The TPB extract inhibited *Enterobacter spp, Pseudomonas spp* within 4 hours of contact time but extract showed inhibition of Klebsiella spp after about 6 hours of contact time. However HBLJ extract results appeared significant. It inhibited *Staphylococcus aureus* within 4 hours of contact time and inhibited *Klebsiella spp* within 2 hours and inhibited *P. aeruginosa spp* within 1 hour.

TPB extract showed no antimicrobial activity against *Staphylococcus aureus.* Although HBLJ inhibited *Staphylococcus aureus* strain. Kill time study showed that it's possible to inhibit a wide range of bacteria which may have unaffected by 1% of extract if contact time has increased.

Anticancer activity: Both the combinations were examined for anticancer activity against a cancerous cell line i.e. human melanoma cell line (SK-MEL-2) by SRB (Sulforhodamine – B) assay. Both the combinations of the extracts in the range from 10µg/ml to 80µg/ml have shown no significant anticancer activity.

Figure 3 represents % control growth with respective concentration and the percentage control growth was calculated as the ratio of average absorbance of the test well



Keys: TPB- Hot alcoholic extract of the combination of *Ocimum tenuiflorum* Leaves, *Ficus religiosa* Bark and *Piper betel* Leaves, HBLJ- Hot alcoholic extract of the combination of *Terminalia chebula* (Hirada) fruit, *Terminalia bellirica* (Behda) fruit Syzygium aromaticum (Lavang) fruit, *Syzygium cumini* (Jambhul) fruit, and ADR- Adriamycin

Fig 3: Anticancer activity of extracts against SK-MEL-2 cell line

to the average absorbance of the control wells X 100.TPB Extract at concentration 80 µg/ml exhibited 85.6% control growth of Human melanoma (SK-MEL-2) cell lines. Whereas, HBJL extract at concentration 80µg/ml exhibited 64.1% control growth of Human melanoma (SK-MEL-2) cell lines. As compared to standard drug ADR (Adriamycin), known to be an anticancer agent [20] , both of the combination extract appeared with no anticancer activity again

LC_{50} (Lethal concentration, 50%), TGI (Total growth inhibition), and GI_{50} (Growth inhibition, 50%) values found to be greater than 80 µg/ml. indicated that both of the extracts under the concentration range 10 µg/ml to 80 µg/ml possess no anticancer activity against the human melanoma cell line are presented in Table no 2.

Table 2: Determination of LC_{50}, TGI and GI_{50} values of the combination of extracts

Drug concentrations (µg/ml) calculated from the graph				
Parameters		**LC_{50}**	**TGI**	**GI_{50}**
SK-MEL-2	TPB	>80	>80	>80
	HBLJ	>80	>80	>80
	ADR	NE	<10	<10

Keys: TPB- Hot alcoholic extract of the combination of *Ocimum tenuiflorum* Leaves, *Ficus religiosa* bark and *Piper betel* Leaves. HBLJ- Hot alcoholic extract of the combination of *Terminalia chebula* (Hirada) fruit, *Terminalia bellirica* (Behda) fruit *Syzygium aromaticum* (Lavang) fruit, *Syzygium cumini* (Jambhul) fruit, ADR- Adriamycin, LC_{50}- Concentration of drug resulting in a 50% reduction in the measured protein at the end. TGI- Total Growth Inhibition, GI_{50}- Growth inhibition of 50 %, NE-Non Evaluable Data.

Conclusion

Accumulation of bacteria, such as *Klebsiella spp., Pseudomonas spp., Escherichia coli, Alcaligenes spp., Enterobacter spp., Citrobacter spp.* and *Staphylococcus spp* plays vital delaying burn wound healing. However, *Klebsiella spp* is found to be major super bug present in the burn wound. Our study has recommended two combinations of herbal extracts that possess significant antimicrobial potential. Since the various pathogenic bacteria found in burn wound are resistant to more than one antibiotic. A great number of bacteria found with the resistance to Gentamicin and Imipenem antibiotics. This reinforces the necessity for the control in abuse of antibiotics and the hunt for substitute antimicrobial agent. Subsequently *actinomycetes* isolated from soil found with noteworthy antimicrobial activity against MDR isolates. On the summation, Secondary metabolite present in actinomycetes and synergistic activity of medicinal plants has great ability speed up the burn wound healing process by inhibiting the MDR bacteria. However combination extracts of the plant and secondary metabolites of actinomycetes are found to be inactive against gram positive bacteria. Moreover both of the extracts showed no anticancer activity indicates extracts selectively inhibits bacteria. This strengthens the applicability of our recommended combination extracts.

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