Effect of PRA-5 on Elimination of Antibiotic Resistance in Methicillin Resistance *S Aureus* (Hospital Strain)

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Summary

80% ethanolic extracts of *Curcuma longa* (rhizome), *Ocimum sanctum* (leaves), *Withania somnifera* (leaves), *Tinospora cordifola (*stem*) and Terminalia belerica* (fruit pulp) was prepared by soxhlet method. Dried form of each extract was mixed in equal amount was dissolved in water, and labled as PRA-5. Antimicrobial activity of PRA-5 for *B. Subtilis, Klebsiella Pnemonia, Pseudomonas aeruginosa, S.aureus* (MDR) was studied. PRA-5 was found to have synergistic (potentiating) effect with gentamycin on *S.aureus* as evidenced by reduced MIC. Plasmid curing activity of PRA-5 on methicillin resistant *S. aureus* was also observed.

Keywords; PRA -5, Antimicrobial activity, potentiating activity, methicillin resistant *S. aureus,* plasmid curing activity.

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Introduction

Radiation therapy is considered to be one of the most popular and important therapeutic modalities for the cure of cancer[1]. Despite its benefits, radiation is known to induce oxidative stress through generation of free radicals resulting in imbalance of prooxidants and antioxidants in the cells[2] this imbalance between the prooxidants and anti oxidants culminate to lead to the cell death. We have also shown in previous experiments that exposure of mice to radiation resulted in destruction of intestinal mucosa, bone marrow and leucopenia. It is possible that, the susceptibility to systemic infection from endogenous and exogenous organisms increases after exposure to ionizing radiations[3]. Infection probably plays a major role in radiation death[4].

Germ free rats survive for a longer time than conventional animals under the lethal doses of radiation[5]. Human beings can not live in a germ free environment and exposure to radiations adversely affects their natural defense system making them more prone to infections.

Due to an alarming increase in the incidence of new and reemerging infections diseases and development of resistance to the antibiotics in current clinical use, there is continuous and urgent need to discover new antimicrobial compounds with diverse chemical structures and novel mechanisms[6-7].

Thus, the use of plant extract/s containing various phytochemicals with known antimicrobial properties[8] can be of great significance in therapeutic treatment.

Material & Methods

Preparation of plant extract

The authentic plant material *Curcuma longa* (rhizome) *Ocimum sanctum*(leaves) *withania somnifera*(leaves) *Tinospora cordifola(*stem*) Terminalia belerica* (fruitpulp) was shade dried, Each material was powdered in mechanical grinder and was separately soxhletes in 80% ethanol till the solvent was colourless.The extract was filtered and dried till constant weight was obtained. The extracts were stored at -4◦C. Equal amount of each extract (w/w) was mixed and dissolved in distilled water and filtered. This mixture is called as PRA-5.

Antimicrobial activity

Agar well bioassay[9] was employed for testing antimicrobial activity of PRA 5. Briefly, nutrient broth/ or agar Sabouraud broth/ or agar was used to cultivate microorganisms. Fresh overnight cultures of inoculums (0.1 ml) of each bacterium containing about 10^8 cells were spread on Mueller Hinton agar. Using a sterile cork borer of 6 mm, wells were made on the plates and 30µl of PRA-5 of different concentration were loaded with positive control and negative control. The plates were incubated at 37^0C for 24 hrs and the size of the inhibition zone was measured. The experiments were done in triplicates and average zone diameter was noted. Table no-1.1

Minimum inhibitory concentrations (MIC)

The minimum inhibitory concentrations (MIC) were determined by broth dilution method[9]. Different concentrations of PRA-5 in nutrient broth were serially diluted in triplicate. Control tube was avoided of PRA-5. Later 10^3 cells of *S. aureus* in 30µl were added into each tube and incubated at 37^0C for 24 hour. The lowest concentration of PRA-5 which inhibited the growth was considered as MIC. The MIC for PRA-5 was found to be 50 mg/ml and with gentamycin the MIC in 8 mcg/ml. Table no-1.2

Potentiating effect

The potentiating effect of PRA-5, with gentamycin was carried out on *S. aureus* by broth dilution method at sub inhibitory concentration in triplicate. Table no-1.3

Plasmid curing activity

Plasmid curing activity[10] of PRA-5 on methicillin resistant *S. aureus* was determined by method. The sub MIC concentration was selected.0.1 ml of freshly grown culture was incubated with PRA-5 Negative control were without addition of PRA-5 All tubes were incubated at 37^0 C for 18 hrs.

After incubation both was detected using sterile normal saline and then spread over the surface of sterile LB agar plates & the plates were incubated at 37^0 C for 18 hrs. Isolated colonies were selected and replica plated on to LB agar plates containing antibiotics to which the test bacterium was resistant. Table no-1.4

Plasmid isolation was performed to confirm the plasmid curing in potentially cured derivatives obtained after replica plating technique by alkaline lysis method[11]. Briefly, the MRSA strain was grown overnight in 1.5 ml vial, pellet was obtained by centrifugation at 12,000 rpm for 5 min which was suspended in 100 µl of ice cold solution of glucose- tris EDTA. 200 µl of freshly prepared lysis solution was mixed and vials were kept on ice for 5 min. To this 150 µl of ice cold solution of 3M potassium acetate, LBM (Luria Bertani medium), ampicillin (1µg/ml) Tris EDTA buffer pH 8, containing RNase enzyme was added, mixed and kept on ice for 4 minutes. Then the vials were centrifuged at 10,000 rpm for 10 min at 4^0C. Supernatant was transferred to fresh vial. 300 µl of absolute ethanol was added, mixed by vortexing and allowed to stand for 3 min at room temperature. The vials were then centrifuge at 12, 000 rpm for 5 min at 4^0c and the supernatant was discarded. The pellet was rinsed with 1 ml of 70% ethanol and centrifuged at 5000 rpm for 2 min. The supernatant was discarded and the pellet was allowed to air dry & then suspended in normal saline. Physical loss of the plasmid in the cured derivative was confirmed by agarose gel electrophoresis of the plasmid DNA preparation of respective culture. The bands were observed in Kodak Image viewer.

Results

Table: 1.1 Antimicrobial activity of PRA -5 and zone of inhibition in mm

Conc. of PRA-5	***B. Subtilis***	***Klebsiella Pnemonia***	***Pseudomonas aeruginosa***	***S.aureus* (MDR)**
100 mg/ml	10	12	16	10
200 mg/ml	12	14	18	13
300 mg/ml	16	18	20	18
Tetracyclin (20mcg/ml)	18	20	22	22

Antibiogram of methicillin resistance *S. aureus* (hospital strain) was determined by disc diffusion method on Muller Hinton agar plates by the method.

Table: 1.2 Antibiogram of MRSA (methicillin resistance *S aureus***)**

Antibiotic	**Conc(mcg/disc)**	**Sensitive (S)/ Resistance (R)**
Amoxycilin	10	R
Cotrimaxazole	25	R
Ampicllin	10	R
Cloxacillin	5	R
Erythromycin	15	R
Tetracyclin	10	S
Penicillin	15	S

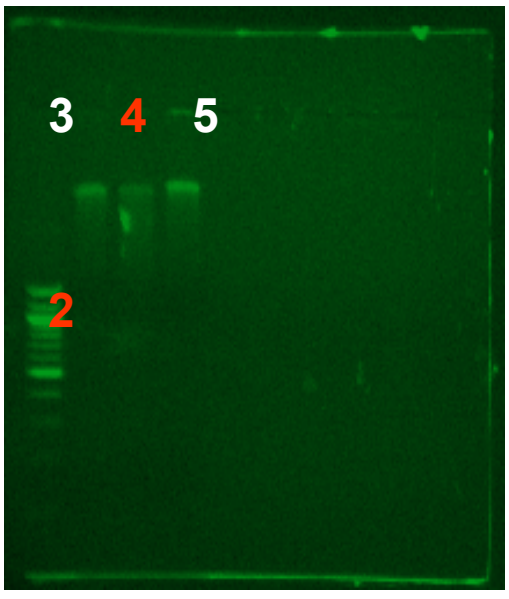
Table1.3: Antibiotic potentiation activity of PRA 5

Drug	MIC
Gentamycin	8 mcg/ml
PRA-5	50 mg/ml
Gentamycin + PRA 5 20 mg/ml	2 mcg/ml

Table 1.4: Plasmid curing activity of PRA-5 on MRSA

Antibiotic	Concentration mcg/disc	Before Plasmid curing	After Plasmid curing
Amoxicillin	10	R	S
Cotrimaxazole	25	R	S
Cloxacillin	5	R	S
Erythromycin	15	R	S
Ampicillin	10	R	R

Band intensity uncured (lane 2) was 21.17, for cured (lane3) was 8.02 and uncured (lane-4) was 25.19. (Fig 1.5)



Lane No. 2: 100bp ladder
Lane No. 3: Sa (Resistant/uncured)
Lane No. 4: C1 (Cured/Treated with PRA-5)
Lane No. 5: C2 (Resistant/uncured)

Discussion

The data pertaining to the antimicrobial potential of PRA-5 is presented in table1.1 PRA-5 has shown antimicrobial activity against the tested microorganisms including the MDR *S.aureus*. The antimicrobial activity of constituents of PRA-5 has been documented in literature[12]. Antimicrobial activity of the extract of *T.cordifolia* (bark) and *W. somnifera* (leaves) against *B. subtilis*, *E. coli, P.fluorescens, S.aureus* and antifungal activity against *A. flavus*, *D .tarcia* and *F.verticillionides*[12]. The antibacterial and synergistic action of *W. somnifera* against *S. typhimurium* and *E.coli* [13-14]. Antimicrobial activity of *W. somnifera* against enteric bacteria with multi drugs resistance *V. cholerae*, and *B. subtilis*[15]. Antimicrobial activity of *W. somnifera* against *E.coli*, *P. aerunginosa*, *S.aureus*, *K. Pnuemoniae* and *C.albicans*[16].

Antimicrobial activity of *O.santum* against Gram positive microorganisms *S. aureus* and *B.subtilis* and Gram negative organisms like *K.peumonia*, *E.coli* while no activity was reported for Pseudomonas and *Shigella dysenteries*[17].

Antibacterial activity of *T Belerica* extract was reported[18-20]. Antimicrobial activity of *C.longa* against different strains of bacteria[21-23]. Antibacterial activity of *C.longa* against methicillin resistant *S.aureus*[24]. Since PRA-5 is a mixture of *T.cordifolia, T.belerica O.sanctum W. somnifera and C.longa,* antibacterial activity is expected and proved against the tested microorganisms. PRA-5 is reported to have tannins and polyphenols in previous experiments. The antimicrobial mechanisms of tannins may be due to its astringent property induce complexiting with enzymes or substrates[25]. The inhibition of many microbial enzymes in raw culture filtrate or in purified forms when mixed with tannins[26]. Tannins toxicity may be related to their action on membranes of the microorganisms which may be due to complexation of metal ions[27]. According to Cowan (1991) the phenolic toxicity to micro organisms may be due to enzyme inhibition possibly through reaction with sulfahydryl groups or through more non specific interactions with proteins. Polyphenols are shown to inhibit growth of many bacterial species[28]. The bactericidal effects may be due to membrane perturbation[29]. Phenolic compounds are responsible for antimicrobial activity of Olive (*Olea europaca*)[30].

PRA-5 was found to have synergistic (potentiating) effect with gentamycin on *S.aureus* as evidenced by reduced MIC (Table 15.3). Thus addition of PRA5 with gentacycin reduced the MIC significantly. Synergistic effect for *P.aeruginosa* by various phytochemicals like thyme, jambolan, pomegranate and clove while no synergistic effect was observed when different concentrations of extracts from clove and eugenol were combined with ampicillin to inhibit the growth of *E.aerogenes*[31-32]. Antimicrobial activity of *Elephantopus scaber* extract on MRSA and synergistic interaction between antibiotics like ampicilin, tetracycline and chloramphenicol with crude plant extract[33]. These authors are of the state that phytochemicals such as terpenoids, phenols, glycosides, saponins, steroids may be responsible for antimicrobial effects.

The synergistic effect in reduction of MIC of ethanol extract of *Turnera ulmifolia* with gentamycin and kanamycin in the MIC[34]. According to these authors the extract must be affecting efflux pump inhibition.

It has been well established that most genes which are responsible for antibiotic resistance are borne on plasmid DNA[35]. Elimination of plasmid DNA mediated antibiotic resistance in pathogenic bacteria is of great practical importance in chemotherapy of bacteria and in microbial genetics. Elimination of plasmid DNA can be performed using different chemical materials such as acridin dyes[36] and ethidium bromide [37] by medicinal plant extract[38].

Table 1.2 represent the antibiotic resistance / sensitive profile of plasmid harboring strain and plasmid curing strain. It is observed that after curing the strain become sensitive to antibiotics except ampicillin which may be due to physical loss of the plasmid as revealed by electrophoretic pattern of the isolated cured plasmid. (Fig 1.5) as revealed by band intensity of uncured 21.17(Lane-2), cured 8.02 (lane 3) and uncured 25.19 (lane 4). Thus, PRA-5 has direct antimicrobial activity enhancing the activity of specific antibiotic, reversing the natural resistance of specific bacteria to given antibiotic, promoting the elimination of plasmid from bacteria and inhibiting the plasma membrane based efflux pumps[39] and can also act as resistant modifying agent[40].

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