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ANTIBACTERIAL AND ANTIBIOTIC-POTENTIATION ACTIVITIES OF LEMON AGAINST DRUG RESISTANT PHENOTYPES

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ABSTRACT: Antibiotic resistance continues to pose a significant problem in the management of bacterial infections despite advances in antimicrobial chemotherapy and supportive care. Therefore, it is necessary to search the other alternatives that can be effective in the treatment of these infections. The present work was aimed to evaluate the antibacterial and antibiotic-potentiation activities of lemon against drug resistant phenotypes. The sensitivity of *S. aureus* and *P. aeruginosa* was determined by performing antibiotic sensitivity test (AST). They were found to be resistant to tested antibiotics and since *S. aureus* was resistant to oxacillin it was classified as methicillin resistant *S. aureus* (MRSA). The phytochemical analysis of lemon juice and lemon peel extract showed presence of flavonoids, phenols, steroids, reducing sugar and alkaloid. Antioxidant activity which was determined by Ferric Reducing Antioxidant Power (FRAP) assay method showed increasing trend in reducing power with the increase in concentration for both samples. The antibacterial activity of lemon juice, lemon peel extract and citric acid was screened against resistant isolates by using agar well diffusion method. All three samples were found to show good antibacterial effect against MRSA and *P. aeruginosa*. The MIC was performed and minimum concentration of sample inhibiting test organism was found to be 1:4. The antibiotic-potentiation activity of lemon juice, lemon peel extract and citric acid was carried out by agar dilution method. It was found that all above three samples at certain dilution in combination with antibiotic modulated the activity of antibiotic which resulted in the inhibition of drug resistant bacteria. Liquid sanitizer was prepared using lemon juice showed reduction in bacterial growth after treatment which proves its efficacy as a sanitizer.

INTRODUCTION: Infectious diseases of bacterial origin are a recurrent problem in public health and have a substantial impact on morbidity and mortality in populations in general. Therefore, in recent years, the pharmaceutical industry has been prompted to develop new antibiotic drugs, in particular because of the emergence of microorganisms resistant to conventional drugs.

This occurs because bacteria have the genetic capacity to acquire and transmit resistance to the antibacterial agents currently available¹. Therefore there is dire need to search for new classes of antibacterial substances to which there is lesser resistance.

Plants as a source of medicinal compounds have continued to play a dominant role in the maintenance of human health since ancient times². These are known to be significant source of natural products having antioxidant, antitumor, antimutagenic and antimicrobial properties and confer substantial health benefits by providing protections against many diseases. It is considered that antimicrobial activities of medicinal plants are

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due to the presence of phytochemicals, mostly secondary metabolites. Fruits are an important part of human diet that provides essential nutrients as well as many bioactive compounds like vitamins and secondary metabolites for promotion of health and prevention of diseases³.

The genus citrus is one of the most effective herbs in traditional medicine that belongs to the family of Rutaceae. The members of this genus are characterized by many biologically active secondary metabolites such as flavonoids, limonoids, coumarins and furanocoumarins, sterols, volatile oils, organic acids, and alkaloids.

Many citrus species are recognized for their medicinal, physiological, and pharmacological activities including antimicrobial, antioxidant, anticancer, anti-inflammatory, and hypoglycaemic activities. Citrus by-products, if utilized fully, could be major sources of phenolic compounds. The peels, in particular, are an abundant source of natural flavonoids, and contain higher amount of phenolics compared to the edible portions⁴.

Lemon is very rich in important natural compounds, including citric acid, ascorbic acid, minerals, flavonoids, and essential oils⁵. Citric acid is a weak tricarboxylic acid that is naturally concentrated in citrus fruits. It increases the acidity of the bacterial environment making it difficult for it and microbes to survive and most importantly to reproduce⁶.

Many plants have been evaluated not only for direct antimicrobial activity, but also as a resistance modifying agent. The resistance modifying agents are compounds which potentiate the activity of an antibiotic against resistant strain. These compounds enhance the activity of a specific antibiotic by reversing the natural resistance of bacteria, promoting the elimination of plasmids from bacteria and inhibiting transport functions of the plasma membrane with regards to a given antibiotic. The inhibition of plasma membrane-based efflux pumps has also been observed.

In combination with an antibiotic that is a substrate for these mechanisms, an inhibitor will increase the cellular concentration of the antibiotic therefore restoring its efficacy. It has also been shown that the use of such resistance modifying agents can

also reduce the emergence of antibiotic resistant variants⁷⁻⁸.

The efficacy of citric acid have been reported and commended by many researchers. Citric acid has been found highly effective against bacterial pathogens like *S. aureus* and *P. aeruginosa* and also there are findings that an effective concentration of citric acid can potentiate the activity of antibiotics against resistant strains of these bacteria (mentioned in discussion). Since lemon is a rich source of citric acid, an attempt was made to check antibacterial and antibiotic-potential activity of lemon against drug resistant phenotypes. Simultaneously antibacterial and antibiotic-potential activity of citric acid was also carried out.

MATERIALS AND METHODS:

Clinical Isolates Used: The clinical isolates used in this study were *S. aureus* (obtained from Suburban Diagnostic Centre, Andheri) and *P. aeruginosa* (obtained from Jaslok hospital Peddar road, Mumbai).

Collection of Lemon Crude Juice: Fresh ripen samples of lemon were collected and washed in running tap water. They were then surface sterilized with 70% alcohol and then rinsed with sterile distilled water and cut open with a sterile knife. The juice pressed out into a sterile universal container separately and then filtered into another sterile container to remove the seeds and other tissues and used freshly as crude without refrigeration.

Preparation of Lemon Peel Extract: Lemon peels were dried in an oven at 50 °C for 48 h followed by grinding into a fine powder. 10 g of peel powder was weighed separately and soaked in 100 ml of ethanol in separate 250 ml flasks and covered with aluminium foil to avoid evaporation and kept at rotary shaker at R.T. for 48 h. This was filtered and filtrate collected was kept at 37 °C to evaporate solvents and residue was recollected by adding DMSO.

Preliminary Phytochemical Investigation: The Lemon juice and lemon peel extract obtained were subjected to phytochemical screening to identify the chemical constituents viz., carbohydrates, reducing sugar, glycosides, amino acids, phenols,

flavonoids, saponins, steroids, alkaloids and terpenoids.⁹

Estimation of Antioxidant Activity:

Ferric Reducing Antioxidant Power (FRAP)

Assay: The reducing capacity of lemon juice and lemon peel extract were determined by FRAP assay method. Fruit extracts of varying concentration in deionized water was mixed in 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml 1% potassium ferricyanide. The reaction mixture was incubated at 50 °C for 20 min. Then 2.5 ml 10% TCA was added to the mixture and centrifuged for 10 min at 3000 rpm. 2.5 ml of the centrifuged clear solution was mixed with 2.5 ml distilled water and 0.5 ml of freshly prepared 1% FeCl₂. Absorbance of the final solution was measured at 700 nm. Increased absorbance of reaction mixture indicated the increased reducing power¹⁰.

Preparation of Citric Acid: The commercially available pure citric acid was diluted in the concentration of 0.05 mg/ml by appropriate mixing with sterile distilled water.

Preparation of Sample Dilution: Four different concentration of three different samples *viz.* lemon juice, lemon peel extract and citric acid were prepared by serially diluting the sample with diluent. The dilutions prepared were 1:1, 1:2, 1:4 and 1:8. The diluent used for lemon juice and citric acid were sterile distilled water and for Lemon peel extract DMSO was used.

Inoculum Standardization: The test organisms were subcultured on nutrient agar slants and incubated for 24 h at 37°C. The slants were maintained at 4 °C until required. Inoculum was prepared by touching a loopfull of bacterial colony with sterile loop and transferred it into 4 to 5 ml nutrient broth. It was then incubated at 37 °C until it achieves or exceeds the turbidity of the 0.5 McFarland standards (usually two to six hours).

Antibiotic Sensitivity Testing: The AST was performed following the disc diffusion technique. Within 15 min after adjusting the turbidity of inoculum suspension, a sterile cotton swab was dipped into it and spread over the entire surface of Muller Hinton agar plate. After 15 min, sterile antibiotic disc were placed on the surface of agar plate seeded with bacterial inoculum. Antibiotic

discs used were oxacillin (1 mcg), norfloxacin (10 mcg), cotrimoxazole (25 mcg), cefoxitin (30 mcg) and vancomycin (30 mcg) for *S. aureus* and piperacillin (100 mcg), norfloxacin (10 mcg), ciprofloxacin (5 mcg) and amikacin (30 mcg) for *P. aeruginosa*. The plates were then incubated at 37°C for 24 h. After incubation, each inhibition zone diameter (IZD) was measured and analyzed according to CLSI guidelines¹¹ and the antibiotic sensitivity was expressed in millimetres (mm).

Antibacterial Activity of Lemon juice, Lemon Peel Extract and Citric Acid:

The antimicrobial activity was evaluated using agar well diffusion method. The bacterial inoculum was prepared and was spread with a sterile cotton swap into petri dishes (90 mm of diameter) containing 20 mL of MH agar. 6 mm wells were made with sterile cork borer into Muller Hinton agar plates containing the bacterial inoculum. 100 µl volumes of three different samples was poured into a well of inoculated plates. The plates was allowed to stay for 15 min for pre-diffusion to take place followed by incubation at 37 °C for 24 h and then examined for appearance of inhibition zone. The diameter of inhibition zone (DIZ) was measured and expressed in millimetres (mm).

Determination of Minimum Inhibitory Concentration (MIC):

Minimum inhibitory concentration of lemon juice, lemon peel extract and citric acid were determined by agar well diffusion method. The bacterial inoculum was prepared and spread into petri dishes containing 20 mL of MH agar with a sterile cotton swap. 6 mm wells were made with sterile cork borer into MH agar plates containing the bacterial inoculum. 100µl volume of each dilution was poured into a well. Diluent without sample were taken as a control. The plates were then incubated for 24 h at 37 °C. The MIC was the highest dilution of sample showing clear zone of inhibition.

Antibiotic-potential activity of Lemon juice, Lemon peel extract and Citric acid:

This procedure is a modification from the Kirby-Bauer disc diffusion method. Different dilution of the sample was prepared ranging from 1:10, 1:20 and 1:30 with diluent. 1 ml of each dilution was added to 9 ml of MH agar (after autoclaved and cooled) yielding the final concentration desired and the

medium was poured into petri dishes. MH agar plate without sample was taken as a control. Within 15 min after adjusting turbidity (0.5 McFarland standards) of inoculum suspension, a sterile cotton swab was dipped into it and was swabbed over the entire surface of MH agar medium containing different dilution of the sample. The antibiotic discs were placed on the agar plate seeded with the respective bacteria. Antibiotic disc used were oxacillin (1mcg), norfloxacin (10 mcg) and co-trimoxazole (25 mcg) for MRSA and piperacillin (100 mcg) and norfloxacin (10 mcg) for resistant *P. aeruginosa*. The plates were incubated at 37°C for 24 h. After incubation, each inhibition zone diameter (IZD) was measured and analysed according to CLSI guidelines¹¹ and was expressed in millimeters (mm).

Preparation of Lemon Liquid Sanitizer: Liquid hand sanitizer was prepared by adding 7 ml of filtered crude lemon juice to 6ml ethanol (60%), 0.4 ml sterile glycerol and 0.6 ml rose water.

Determination of Efficacy of Lemon Liquid Sanitizer: The efficacy of lemon liquid sanitizer was determined to check its effectiveness in reducing bacterial growth. The efficacy is tested against MRSA and resistant *P. aeruginosa* and also

on the baseline counts of resident flora on the hands. Hand swab sample was taken using a sterile cotton swab dipped into sterile saline and moistened swab was then rubbed and rolled firmly on the hand palm area. Swab was then dipped and mixed into the saline suspension. Inoculum of other two cultures was prepared in sterile saline suspension and turbidity adjusted to 0.5 McFarland standards. To check the efficiency of lemon liquid sanitizer, 0.1 ml of inoculum was added to three different sterile coverslips. Then one coverslip was treated with control sample and other with lemon liquid sanitizer by adding 0.5ml each of these samples on the coverslip. One coverslip was used without any treatment. The coverslips were treated for 20 sec.

They were then placed on the surface of sterile LB agar touching the treated side to the agar surface. Coverslips were then removed and discarded. Control sample used was liquid sanitizer without lemon juice.

RESULTS:

Antibiotic Sensitivity Testing (AST): Antibiotic sensitivity test was performed using disc diffusion technique and is depicted in **Table 1** for *S. aureus* and **Table 2** for *P. aeruginosa*.

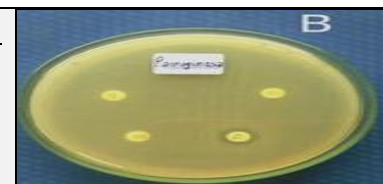
TABLE 1: ANTIBIOTIC SENSITIVITY TESTING OF S. AUREUS

Antibiotic discs (Conc.)	Zone of Inhibition (mm)	Resistance / Sensitive
Oxacillin (1 mcg)	0 mm	R
Norfloxacin (10 mcg)	9 mm	R
Co-trimoxazole (25 mcg)	0 mm	R
Cefoxitin (30 mcg)	14 mm	R
Vancomycin (30 mcg)	14 mm	S



TABLE 2: ANTIBIOTIC SENSITIVITY TESTING OF P. AERUGINOSA

Antibiotic discs (Conc.)	Zone of Inhibition (mm)	Resistance / Sensitive
Piperacillin (100 mcg)	10 mm	R
Norfloxacin (10 mcg)	0 mm	R
Ciprofloxacin (5 mcg)	0 mm	R
Amikacin (30 mcg)	0 mm	R



Phytochemical Analysis of Lemon Juice and Lemon Peel Extract: The lemon juice and lemon peel extract was analysed for its phytochemical constitution and is listed in **Table 3**.

Ferric Reducing Antioxidant Power Assay: The reducing capacity of lemon juice and lemon peel extract was determined to find out its antioxidant

activity. Absorbance at 700 nm is represented graphically in **Graph 1**.

Antibacterial activity of Lemon juice, Lemon Peel Extract and Citric Acid: Antibacterial activity of lemon juice, lemon peel extract and citric acid were determined using agar well diffusion method were shown in **Fig. 1**.

TABLE 3: PHYTOCHEMICAL ANALYSIS OF LEMON JUICE AND LEMON PEEL EXTRACT

Test	Lemon juice	Lemon peel extract
Carbohydrate	+	+
Amino acids	+	-
Phenols	+	+
Flavonoids	+	+
Saponins	-	-
Steroids	+	+
Reducing sugar	+	+
Glycosides	-	-
Alkaloids	+	+
Terpenoids	-	+

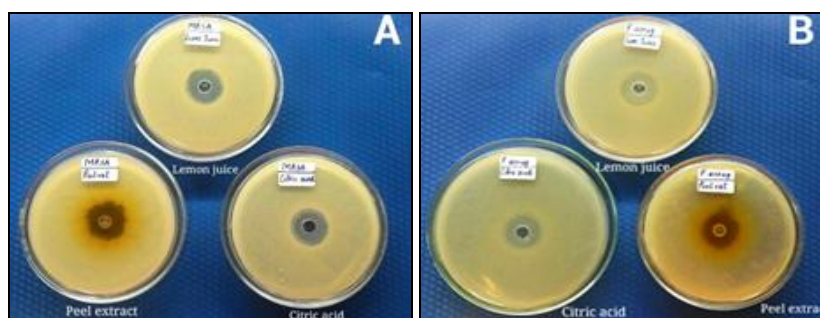


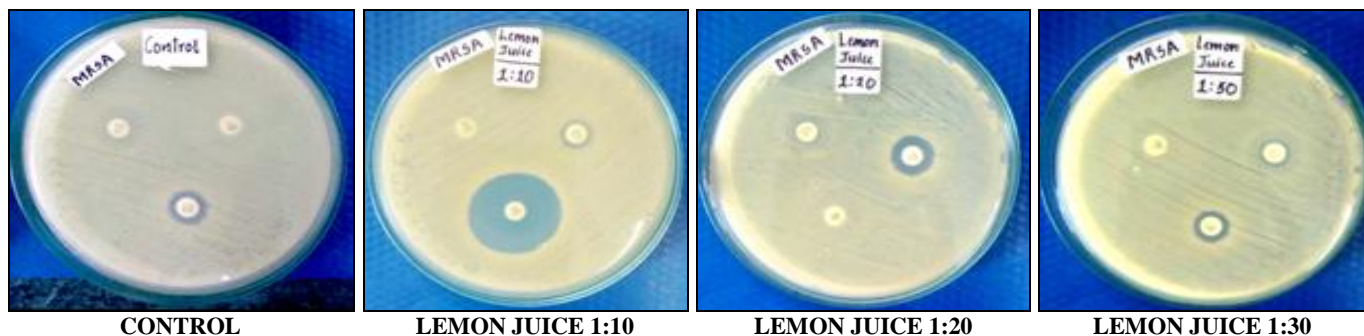
FIG. 1: ANTIBACTERIAL ACTIVITY OF LEMON JUICE, LEMON PEEL EXTRACT AND CITRIC ACID AGAINST A) MRSA AND B) P. AERUGINOSA

MIC of Lemon Juice, Lemon Peel Extract and Citric Acid: MIC of lemon juice, lemon peel extract and citric acid was found out using agar well diffusion method. MIC was found to be 1:4 for all three samples against both MRSA and *P. aeruginosa*.

Antibiotic-Potential Activity of Lemon Juice, Lemon Peel Extract and Citric Acid: Antibiotic - potentiation activity of lemon juice, lemon peel extract and citric acid was determined by modified Kirby-Bauer disc diffusion method and it is presented in **Table 4** and **Table 5**.

TABLE 4: ANTIBIOTIC-POTENTIATION ACTIVITY OF LEMON JUICE, LEMON PEEL EXTRACT AND CITRIC ACID AGAINST MRSA

Dilutions	Zone of Inhibition (mm)								
	Oxacillin (1 mcg)			Norfloxacin (10 mcg)			Co-trimoxazole (25 mcg)		
	Lemon Juice	Lemon peel extract	Citric acid	Lemon Juice	Lemon peel extract	Citric acid	Lemon Juice	Lemon peel extract	Citric acid
Control	0mm	0mm	0mm	10mm	10mm	10mm	0mm	0mm	0mm
1:10	19mm	0mm	14mm	10mm	12mm	12mm	0mm	0mm	0mm
1:20	0mm	0mm	0mm	13mm	12mm	12mm	7mm	0mm	7mm
1:30	0mm	0mm	0mm	11mm	11mm	11mm	8mm	8mm	8mm



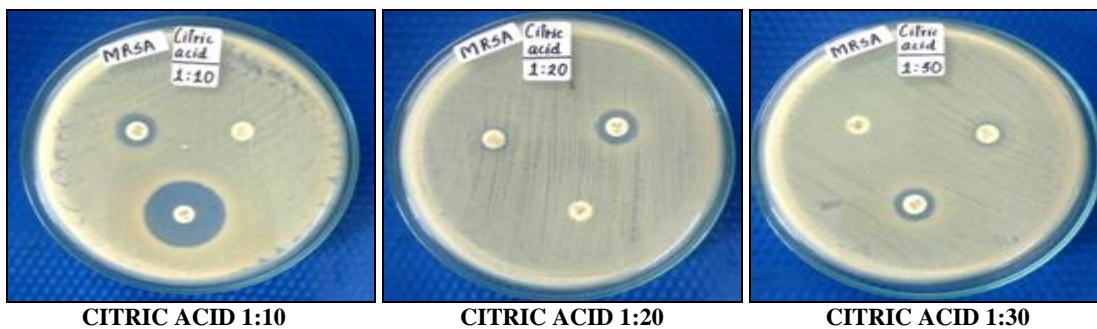


FIG. 2: ANTIBIOTIC-POTENTIATION ACTIVITY OF LEMON JUICE, PEEL EXTRACT AND CITRIC ACID AGAINST MRSA

TABLE 5: ANTIBIOTIC-POTENTIATION ACTIVITY OF LEMON JUICE, LEMON PEEL EXTRACT AND CITRIC ACID AGAINST P. AERUGINOSA

Dilutions	Zone of Inhibition (mm)					
	Piperacillin (100 mcg)			Norfloxacin (10 mcg)		
	Lemon Juice	Lemon peel extract	Citric acid	Lemon Juice	Lemon peel extract	Citric acid
Control	8mm	8mm	8mm	0mm	0mm	0mm
1:10	16mm	13mm	15mm	0mm	0mm	0mm
1:20	11mm	12mm	9mm	0mm	0mm	0mm
1:30	10mm	8mm	8mm	0mm	0mm	0mm

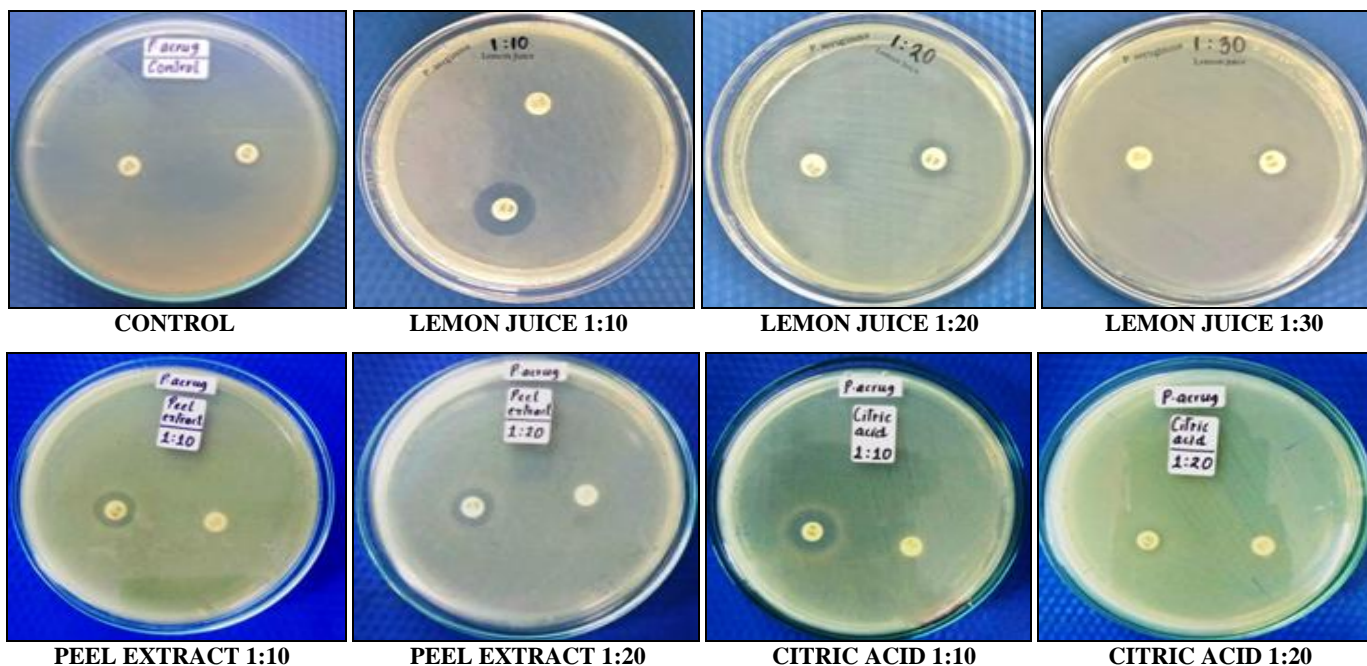
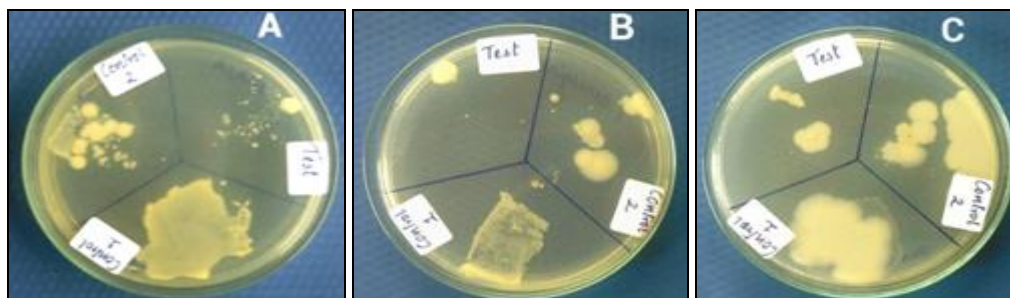


FIG. 3: ANTIBIOTIC-POTENTIATION ACTIVITY OF LEMON JUICE, PEEL EXTRACT AND CITRIC ACID AGAINST P. AERUGINOSA



Key: Control 1 - No treatment; Control 2- Treatment with liquid sanitizer without lemon juice; Test - Treatment with lemon liquid sanitizer

FIG. 4: EFFICACY OF LEMON LIQUID SANITIZER ON A- MRSA, B- P. AERUGINOSA, C- RESIDENT FLORA OF HAND

Preparation and Determination of Efficacy of Lemon Liquid Sanitizer: Lemon liquid sanitizer was prepared as mentioned in materials and methods and its efficacy was determined by its ability to reduce the bacterial growth and it is depicted in **Fig. 4**.

DISCUSSION: The number of multidrug resistant microbial strains and the appearance of strains with reduced susceptibility to antibiotics are continuously increasing. This increase has been attributed to indiscriminate use of broad spectrum antibiotics, immunosuppressive agent, intravenous catheters, and organ transplantation. The emergence of drug resistance with patient's poor compliance, drugs adverse effects and higher cost of therapy combinations indicates a strong need for therapy regimes with similar or higher antibiotic beneficial properties but with better adverse effect profiles¹². The usefulness of plants and their extracts have been observed to be promising remedies since ancient time. The natural products with potential antibacterial activity overcome the resistance phenotype of multiple drug resistant bacteria and render them susceptible to the available antibiotics. So in this study antimicrobial and antibiotic-potential activity of lemon against drug resistant strains of *S. aureus* and *P. aeruginosa* was carried out.

The two clinical isolates used in this study were *S. aureus* and *P. aeruginosa*. Their susceptibility was checked by performing antibiotic susceptibility test. *S. aureus* was tested with five different antibiotics viz. oxacillin, vancomycin, norfloxacin, cotrimoxazole and cefoxitin and *P. aeruginosa* with piperacillin, norfloxacin, ciprofloxacin and amikacin. The results showed that *S. aureus* is resistant to all antibiotics tested except vancomycin and this strain was classified as methicillin resistant *S. aureus* since it was found to be resistant to oxacillin. *P. aeruginosa* was found to be resistant to all antibiotics tested.

The medicinal value of plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body. The phytochemical investigation revealed the presence of various constituents of lemon juice and ethanolic extract of lemon peels. The phytochemical analysis showed presence of phenols, flavonoids, steroids,

reducing sugar and alkaloids in both lemon juice and lemon peel extract. Lemon juice also showed presence of amino acid. Lemon peel extract gave positive results for terpenoids and both lemon juice and lemon peel extract gave negative results for saponins and glycosides. These results agree in part with the findings of Kumar A *et al.*, 2011¹³ who reported presence of steroids, terpenoids in ethanolic extract of lemon peel but absence of amino acids, reducing sugars and flavonoids. Also Rauf A *et al.*, in 2014¹⁴ reported presence of reducing sugars, phenols, flavonoids and terpenoids in lemon juice. However they did not detect the presence of alkaloids, saponins, glycosides and steroids in lemon juice. These differences may be due to different extraction methods and conditions used and may be due to differences in species and geographical location.

Results of a current study indicate a superiority of the antibacterial activity of the lemon juice and lemon peel extract. Antibacterial activity of lemon juice, lemon peel extract and citric acid was performed using agar well diffusion method against drug resistant *P. aeruginosa* and MRSA. The zone of inhibition was seen against both isolates, confirming the antibacterial activity of lemon juice, lemon peel extract and citric acid. All three samples, lemon juice, lemon peel extract and citric acid showed maximum zone of inhibition against MRSA. *P. aeruginosa* was equally inhibited by both lemon juice and lemon peel extract. A result of this study was consistent with previous findings where the citrus juice of lemon showed good antibacterial activity against MRSA¹⁵.

The reducing capacity of a compound may serve as a significant indicator for its potential antioxidant activity. From the results of FRAP, it was found that the reducing power of the samples increased with increase in concentration. Both Lemon juice and Lemon peel extract showed increasing trend in reducing power with the increase in concentration. The lemon peel extract displayed highest reducing power than lemon juice. This shows that the lemon juice and lemon peel extract constituent's possesses reducing power capabilities and can act as a potent antioxidant. These results were comparable with Suja D *et al.*, 2017¹⁶ who also found increased reducing power with an increase in concentration of the lemon peel extract.

Since lemon juice, lemon peel extract and citric acid showed good antibacterial activity; their individual MIC was performed and was found to be 1:4 for which inhibition zone was observed for both MRSA and resistant *P. aeruginosa*. Since lemon juice, lemon peel extract and citric acid showed good inhibitory effect on the organisms, these samples may hold promise in the management of MRSA and *P. aeruginosa* infection.

The development of antimicrobial resistance is a natural process which cannot be stopped but can be minimized or reduced by some or the other way. So an attempt was made to potentiate the activity of antibiotics by lemon juice, lemon peel extract and citric acid against MRSA and resistant *P. aeruginosa*. In present investigation above all three samples at 1:10, 1:20 and 1:30 dilutions were tested to determine potentiation activity. 1:10 dilution of lemon juice and citric acid was found to be most effective in potentiating the inhibitory action of oxacillin and all three samples at 1:20 for norfloxacin and 1:30 for cotrimoxazole was found to be most effective against MRSA. Similarly for *P. aeruginosa*, 1:10 dilution of all three samples was found to be most effective in increasing inhibitory action of piperacillin but all three samples failed to change the inhibitory action of norfloxacin. The result of citric acid was consistent with previous researches which found that citric acid can potentiate the activity of antibiotics. In 2013, Chandak N *et al.*,¹⁷ found that citric acid at 0.1% concentration, in combination with β -lactam antibiotic (Oxacillin) modulated the activity of antibiotic against MRSA. In Dharmik P *et al.*, 2012¹⁸, citric acid at 0.05% and 0.1% was found to be effective in increasing the potency of antibiotics against multiple drug resistant *P. aeruginosa*.

Formulation of lemon liquid sanitizer was carried out and its efficacy was tested to determine reduction of bacterial growth after its application. From observation it was found that lemon liquid sanitizer yielded significant reduction in the growth of baseline counts of resident flora on the hands after treatment. It was also found to reduce the growth of drug resistant bacteria like MRSA and *P. aeruginosa*.

CONCLUSION: On the basis of results obtained it can be concluded that lemon juice and lemon peels

possess different phytoconstituents and have potent antioxidant activities. The results obtained from antibacterial study clearly demonstrated broad spectrum antibacterial activity of lemon juice and lemon peel extract against drug resistant bacteria like MRSA and *P. aeruginosa*. So, it can be concluded that lemon juice and lemon peel extract have great potential as an antibacterial compound for clinical use against MRSA and resistant *P. aeruginosa*. Also certain amount of lemon juice, lemon peel extract and citric acid when used in combination with conventional drugs can make big impact on their sensitivity.

Therefore, lemon juice, lemon peel extract and citric acid can be used as potentiating agents against these resistant bacteria. Lemon liquid sanitizer which was formulated showed significant reduction of baseline bacterial counts of resident flora on the hands as well as reduction of drug resistant pathogens like MRSA and *P. aeruginosa*. Formulation of sanitizers with lemon as active constituent can give promising result in reducing the viable number of microorganisms and if their use is included in hospitals, it can play a very important role in reducing healthcare-associated infection.

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CONFLICT OF INTEREST: There are no conflicts of interest.

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