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Evaluation of antimicrobial activity and phytochemical analysis of *Citrus limon*

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Abstract: The present study was carried out to find out the antimicrobial activity of ethanolic, methanolic, ethyl acetate & hot water extract of lemon fruit parts like peels & seeds. Antimicrobial analysis was done by using agar well diffusion method against bacterial and fungal pathogens. Methanolic extract of lemon peels exhibited the maximum zone of inhibition against *Pseudomonas aeruginosa* whereas hot water extract of lemon peels exhibited least zone of inhibition. Ethanolic extract of lemon seeds showed maximum zone of inhibition against *Pseudomonas aeruginosa* whereas hot water extract showed least zone of inhibition. MIC value was determined by using micro broth dilution method. The least concentration was obtained 2.4 mg/ml for ethanolic and hot water extracts of lemon peels against *S. aureus*. The MBC value also determined and phytochemical analysis showed the presence of tannins, glycosides, reducing sugars and flavonoids.

Keywords: Antimicrobial analysis, methanolic, ethanolic & hot water plant extract, MIC, MBC, zone of inhibition, phytochemical analysis.

Introduction:

An antimicrobial is a substance that kills or inhibits the growth of microbes such as bacteria, fungi, protozoa or viruses. Antibiotics are those substances which are produced by microorganism that kills or prevents the growth of another micro-organism. Antibiotics are generally used against bacteria, antiviral are used specifically for treating viral infections. Antifungal are used to treat fungal infections, some of these side effects can be life threatening if the drug is not used properly. Several microorganisms derived antibiotics are currently in use to treat a variety of human disease, therefore the action must be taken to control the use of antibiotics, develop new drugs either synthetic or natural, for a long period of time, plant have a valuable source of natural products for maintaining human health. India has a rich tradition in use of medicinal plants to develop drugs.

According to world health organization (WHO), any plant which contain substances that can be used for therapeutic purpose or which are precursor of chemo-pharmaceuticals semi synthetic new drugs is referred as medicinal plant [1]. Medicinal plant would be the best source to obtain a variety of drugs as the phytochemical are more specific. Phytochemical offer unique platform for structural diversity & biological functionality which is indispensable for drug discovery. *Citrus limon* belongs to *Rutaceae* family, common name is lemon and this originated from South East Asia, probably in India or Southern China. Lemon is a pale yellow, elliptically shaped berry fruit. Citrus fruit in general contain sugar, polysaccharide, organic-acid, lipids, carotenoids, vitamins, minerals, flavonoids, bitter lemonoids and volatile compounds. Lemon is a good source of potassium, calcium & vitamin *C. Limon* or lime juice have been reported to exhibit antimicrobial activity against *Vibrio cholera* [2].

The antimicrobials potential of some Indian herbal oils with a view to exploring their potential to application in food industries as botanical preservation [3]. Peel waste are highly perishable & seasonal is a problem to the processing industries & pollution maintaining agencies. Suitable methods have to be adopted to utilize them for the conversion in to value added products [4]. Citrus peels is employed for a variety of uses, as fodder at fisheries, activated carbon, raw materials for traditional paper [5]. Citrus fruit products are known to potent antimicrobial agents like, bacteria, fungus [6]. The antimicrobial activity of plants had been received attention many years ago as one of the most effective mechanism for the control of microorganisms [7]. Pharmacologically, lemon is primarily important for its vitamin C & potassium content. German studies in the late 1980s related to effect of peel lemon oil also posses antioxidant activity clinical trials are lacking. Lemon juice may increase iron absorption.

The present study is carried out to evaluate the antimicrobial activity of different solvents extracts (ethanolic, methnolic, ethyl acetate & hot water) of lemon fruit parts (peels, seeds, juice) against bacterial (*S. aureus, P. aeruginosa, E. coli*) and fungal (*C. albicans, M. canis, T. rubrum* and *A. niger*) pathogens and their phytochemical analysis which are responsible for antimicrobial activity.

Materials and method

Sample collection:

The Sample *Citrus limon* was collected from Vibhav Khand (Gomti Nagar) Lucknow. Used parts of sample were juice, peels and seeds.

Test microorganisms: Bacterial and fungal cultures were obtained from IMTECH, Chandigarh. Subcultures

were maintained by MRD LifeSciences, Lucknow. Bacterial cultures used were- *Staphylococcus aureus, Pseudomonas aeruginosa* and *E. coli* and Fungal cultures used were- *A. niger, C. albicans, M. canis* and *T. rubrum*.

Solvents: Four solvents were used in this experiment- 80% methanol**,** 70% ethanol**,** 100% ethyl acetate and hot water.

Preparation of plant extract:

Sample should be washed with distilled water air dried in oven and grind properly to get powder form. Weigh 5gm of sample for each solvent. All sample kept in dark region for 2-4 days (to dissolve secondary metabolites properly). Air dried the filtrate and dissolve in double amount of Di methyl sulfoxide (DMSO). Plant extracts were ready for further work, except lemon juice; all parts were used for preparation of solvent mixture.

Antibiogram analysis:

Antibiogram analysis method was performed to evaluate the antimicrobial properties of plant extract with the help of agar well diffusion method [8]**.** Nutrient agar plates were prepared for all extracts, 50 µl inoculum of each selected bacterium was uniformly spreaded on agar plates with the help of glass spreader, after five minutes three wells approximately 5mm diameter was bored with the help of borer. The equal volume (50µl) of antibiotic (tetracycline), distilled water and plant extract were poured into the wells. The plates were incubated at 37°C for 24 hrs and observed zone of inhibition and for fungus cultures, PDA plates were prepared for all extracts, 50µl inoculum of each selected fungus was uniformly spreaded on PDA plates with the help of glass spreader, after five minutes three wells approximately 5mm diameter was bored with the help of borer. The equal volume (50µl) of antibiotic (tetracycline), distilled water and plant extract were poured into the wells. The plates were incubated at room temperature for 24-48 hrs and observed zone of inhibition.

MIC (Minimum Inhibitory Concentration):

Minimum inhibitory concentration is the lowest concentration of an antimicrobial that inhibits or kills the visible growth of microorganisms. MIC is generally regarded as the most basic laboratory measurement of the activity of an antimicrobial agent against an organism [9]**.** Prepare nutrient broth then add antibiotics in first test tube, mixed properly and transferred 1 ml in second tube and so on. In last test tube discard same amount of solution. After this step, add 20µl of the bacterial pathogen and kept at shaker incubator for overnight at 37^{0}C and observe result in the form of turbidity and take OD at 600 nm and for fungus cultures, prepare potato dextrose broth then add antibiotics in first test tube, mixed properly and transferred 1ml in second tube and so on. In last test tube discard same amount of solution. After this step, add 20 µl of the fungal pathogen. Incubate at room temperature for 24-48 hrs. Observe result in the form of turbidity and take OD at 600 nm.

MBC and MFC (Minimum Bacteriocidal/ Fungicidal Concentration): The bacteriostatic or bacteriocidal concentration in the form of growth of bacterial culture which can be seen in the form of colonies on petriplates. Prepare NA plates. Spread 20µl of MIC tube cultures according to their arrangement. Incubate at 37^{0}C for overnight. Observe results in the form of bacterial colonies. Prepare PDA plates. Spread 20µl of MIC tube cultures according to their arrangement. Incubate at room temperature for 24-48 hrs. Observed result in the form of fungal colonies.

Phytochemical analysis:

Phytochemical are the main constituents of any plant sample, which are responsible for secondary metabolites also. The other works of these phytochemical are flavouring, colors etc [10].

Tannins*:* Take 5ml of lemon peel filtrate and add 5ml distilled water then heat at 80-100 ^{0}C for 10 min in water bath, then filter it after that add 1% Ferric chloride (5-6 drops). Dark green color indicates the presence of tannins.

Glycosides: Extract was hydrolyzed with HCl solution and neutralized with NaOH solution. Few drops of fehling's solution A & B were added, red precipitate showed the presence of glycosides.

Reducing sugar: Extract was shaken with distilled water and filtered. Filtrate was boiled with fehling's solution A&B for 10 min. Orange & red precipitate indicates the presence of reducing sugar.

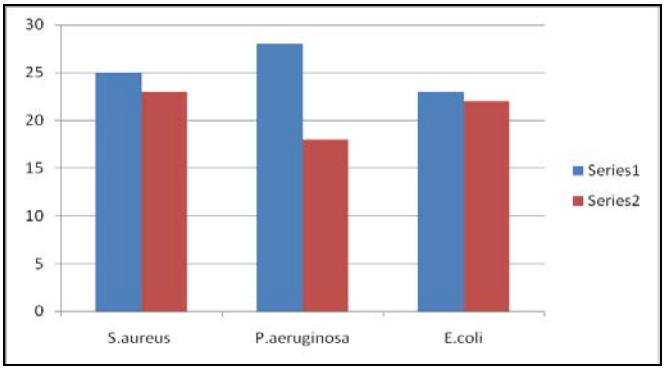
Saponins: Take 0.2ml sample, add 4.8 ml distilled water and then heated to boil frothing showed the presence of saponins.

Flavonoids: 0.2 ml of sample was added in 0.2ml of NaOH. And then add 1-2 drops of HCl, yellow to colorless showed the positive result.

Phlobatannins: Take 10 ml of sample and add 0.2ml HCl, then boiled in water bath for 10 min. Red precipitate indicates positive result.

Table 1: **Antibacterial activity of lemon juice**

Pathogens	Plant extract (ZOI in mm)	Tetracycline (ZOI in mm)
S. aureus	25	23
P. aeruginosa	28	18
E. coli	23	22

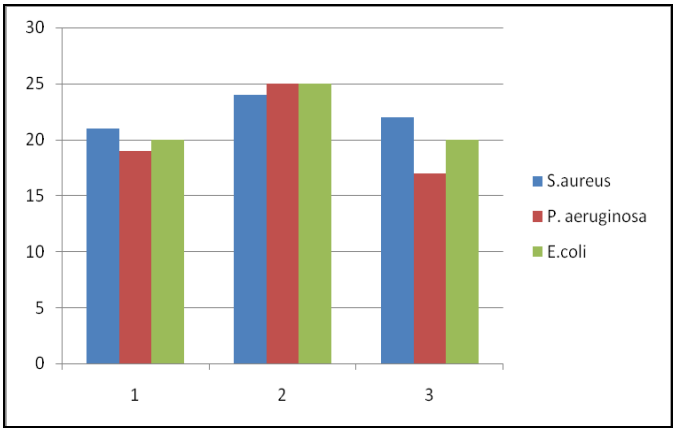


Graph 1: **Antibiogram analysis of lemon juice**

Table 1 and Graph 1 showed that the antibacterial property of lemon juice was found to be maximum against *P. aeruginosa*.

Table 2: **Antibacterial activity of ethanolic extract of lemon peels and seeds**

Pathogens	Ethanolic extract of peels, ZOI (mm)	Ethanolic extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
S. aureus	21	24	22
P. aeruginosa	19	25	17
E. coli	20	25	20



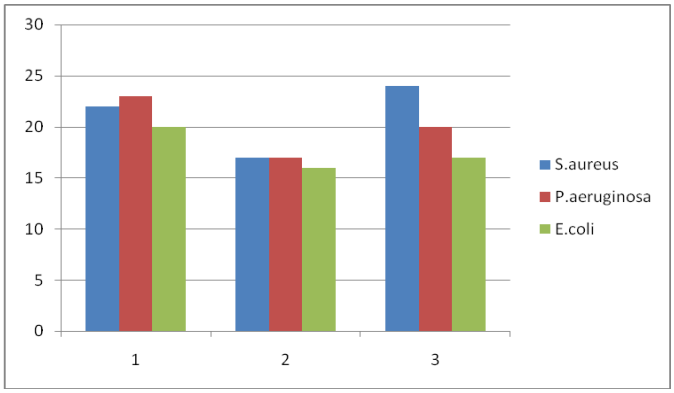
1= Peels, 2= Seeds 3= Tetracycline

Graph 2: **Antibiogram analysis of ethanolic extract of lemon peels and seeds**

Table 2 and Graph 2 showed that the antibacterial property of ethanolic extract of lemon seed was found to be maximum against *P. aeruginosa*.

Table 3: **Antibacterial activity of methanolic extract of lemon peels and seeds**

Pathogens	Methanolic extract of peels, ZOI (mm)	Methanolic extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
S. aureus	22	17	24
P. aeruginosa	23	17	20
E. coli	20	16	17



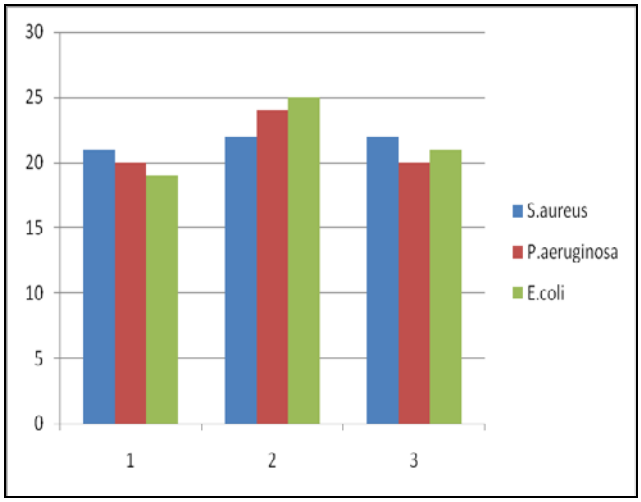
1= Peels, 2= Seeds 3= Tetracycline

Graph 3: **Antibiogram analysis of methanolic extract of lemon peels and seeds**

Table 3 and Graph 3 showed that the antibacterial property of methanolic extract of lemon peels was found to be maximum against *P. aeruginosa*.

Table 4: **Antibacterial activity of ethyl acetate extract of lemon peels and seeds**

Pathogens	Ethyl acetate extract of peels, ZOI (mm)	Ethyl acetate extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
S. aureus	21	22	22
P. aeruginosa	20	24	20
E. coli	19	25	21



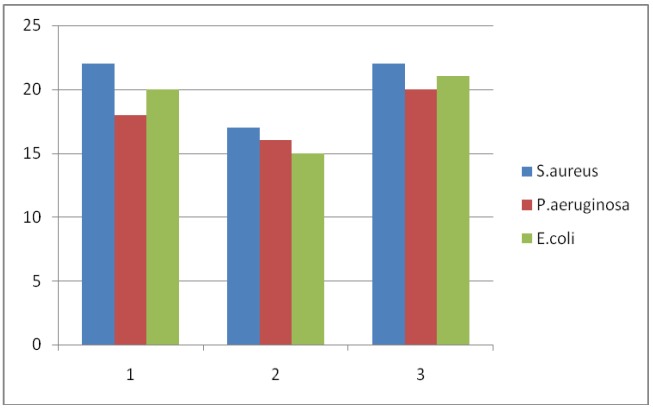
1= Peels, 2= Seeds 3= Tetracycline

Graph 4: Antibiogram analysis of ethyl acetate extract of lemon peels and seeds

Table 4 and Graph 4 showed that the antibacterial property of ethyl acetate extract of lemon seeds was found to be maximum against *E. coli*.

Table 5: **Antibacterial activity of hot water extract of lemon peels and seeds**

Pathogens	Hot water extract of peels, ZOI (mm)	Hot water extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
S. aureus	22	17	22
P. aeruginosa	18	16	20
E. coli	20	15	21



1= Peels, 2= Seeds 3= Tetracycline

Graph 5: **Antibiogram analysis of hot water extract of lemon peels and seeds**

Table 5 and Graph 5 showed that the antibacterial property of hot water extract of lemon peels was found to be maximum against *S. aureus.*

Table 6: **Antifungal activity of ethanolic extract of lemon peels and seeds**

Pathogens	Ethanolic extract of peels, ZOI (mm)	Ethanolic extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
T. rubrum	15	0	0
M. canis	0	0	0
C. albicans	0	0	0
A. niger	0	0	0

Table 6 showed that the antifungal activity was observed only in ethanolic extract of lemon peels against *T. rubrum*.

Table 7: **Antifungal activity of methanolic extract of lemon peels and seeds**

Pathogens	Methanolic extract of peels, ZOI (mm)	Methanolic extract of seeds, ZOI (mm)	Tetracycline ZOI (mm)
T. rubrum	18	0	0
M. canis	0	0	0
C. albicans	0	0	0
A. niger	0	0	0

Table 7 showed that the antifungal activity was observed only in methanolic extract of lemon peels against *T. rubrum*.

Table 8: **MIC of methanolic, ethanolic, ethyl acetate & hot water extract of lemon peels against bacterial pathogens**

Test tubes	Conc. of extracts (mg/ml)	Ethanolic extract against *S. aureus* (OD at 600 nm)	Methanolic extract against *P. aeruginosa* (OD at 600 nm	Ethyl acetate extract against *S. aureus* (OD at 600 nm)	Hot water extract against *S. aureus* (OD at 600 nm)
1	83.33	0.77	0.95	0	0.65
2	**14**	0.38	0.28	**0.07**	0.53
3	**2.4**	**0.06**	**0.05**	0.20	**0.08**
4	0.4	0.20	0.19	0.70	0.20
5	0.066	0.25	0.27	0.25	0.25
6	0.011	0.28	0.33	0.66	0.38

Table 8 showed that ethanolic extract of lemon peels was subjected to get the MIC against *S. aureus* and it was found to be 2.4mg/ml, methanolic extract was subjected to get the MIC against *P. aeruginosa* and it was found to be also 2.4 mg/ml, for ethyl acetate in *S. aureus,* MIC observed at 14mg/ml & for hot water extract against *S. aureus,* MIC was observed at 2.4mg/ml.

Table 9: **MIC of methanolic, ethanolic, ethyl acetate & hot water extract of lemon seeds**

Test tubes	Conc. of extracts (mg/ml)	Ethanolic extract against *P. aeruginosa* (OD at 600 nm)	Methanolic extract against *S. aureus* (OD at 600 nm	Ethyl acetate extract against *E. coli* (OD at 600 nm)	Hot water extract against *S. aureus* (OD at 600 nm)
1	83.33	0.09	0.55	0.64	0.21
2	**14**	0.57	**0.15**	**0.13**	**0.18**
3	2.4	0.49	0.35	0.30	0.37
4	**0.4**	**0.42**	0.38	0.35	0.45
5	0.066	0.46	0.36	0.18	0.38
6	0.011	0.50	0.57	0.39	0.40

Table 9 showed that ethanolic extract of lemon peels was subjected to get the MIC against *P. aeruginosa* and it was found to be 0.4mg/ml, methanolic extract was subjected to get the MIC against *S. aureus* and it was found to be also 14 mg/ml, for ethyl acetate in *E. coli*, MIC observed at 14mg/ml & for hot water extract in *S. aureus,* MIC was observed at 2.4mg/ml.

Table 10: **MIC of lemon juice against *P. aeruginosa***

Test tubes	Conc. of lemon juice	OD against *P. aeruginosa*
1	83.33	0.88
2	14	0.62
3	2.4	0.51
4	**0.4**	**0.10**
5	0.066	0.25
6	0.011	0.31

Lemon juice was subjected to get the MIC against *P. aeruginosa* and it was found to be 0.4mg/ml**.**

Table 11: **Phytochemical results:**

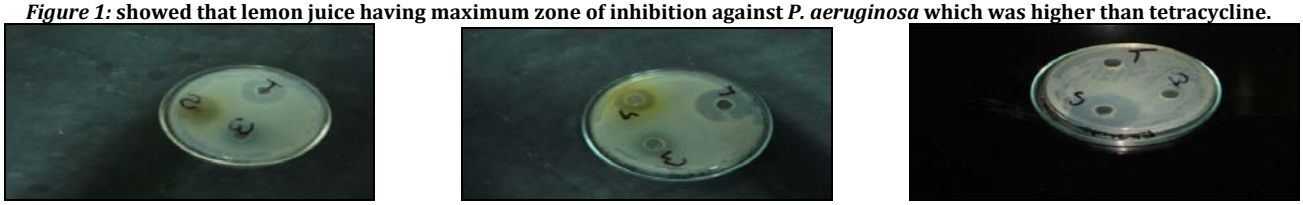
Test	Seeds	Peels
Tannins	(+)	(+)
Glycosides	(+)	(-)
Reducing sugar	(+)	(+)
Saponins	(-)	(-)
Flavonoids	(+)	(+)
Phlobatannins	(-)	(-)

In seeds; tannins, glycosides, reducing sugar & flavonoids were showed positive results, whereas saponins & phlobatannins were absent. In peels; tannins, reducing sugar & flavonoids were showed positive results, whereas glycosides, saponins & phlobatannins were absent in peels.

S. aureus *P. aeruginosa* *E. coli*

Figure 1: **Antibiogram analysis of lemon juice**

Figure 1: **showed that lemon juice having maximum zone of inhibition against *P. aeruginosa* which was higher than tetracycline.**



S. aureus *P. aeruginosa* *E. coli*

Figure 2: **Antibiogram analysis of ethanolic extract of peels**

Figure 2**: showed that maximum antibacterial activity of ethanolic extract of lemon peels was obtained against *Staphylococcus aureus & E. coli* whereas least in *P. aeruginosa.***

S. aureus *P. aeruginosa* *E. coli*

Figure 3: Antibiogram analysis of methanolic extract of peels

Figure 3: **showed that maximum antibacterial activity of methanolic extract of lemon peels was obtained against *P. aeruginosa* & *S. aureus* whereas least in *E. coli.***

S. aureus *P. aeruginosa* *E. coli*

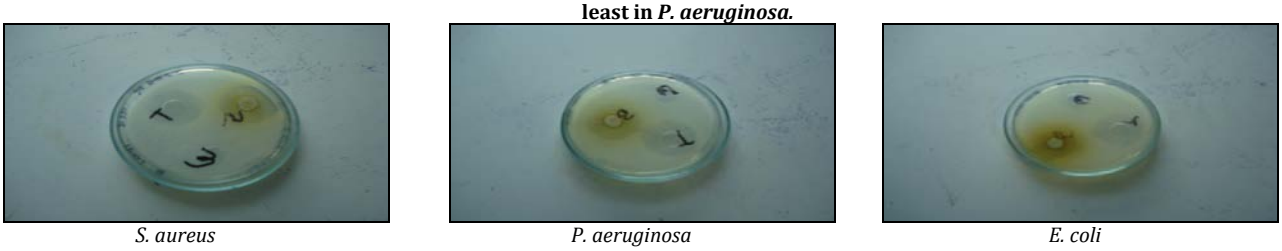
Figure 4: **Antibiogram analysis of ethyl acetate extract of lemon peels**

Figure 4 : **Showed that maximum antimicrobial activity of ethyl acetate extract of lemon fruit peels was obtained against *S. aureus*, *P. aeruginosa* whereas least in *E. coli* .**

S. aureus *P. aeruginosa* *E. coli*

Figure 5: **Antibiogram analysis of hot water extract of peels**

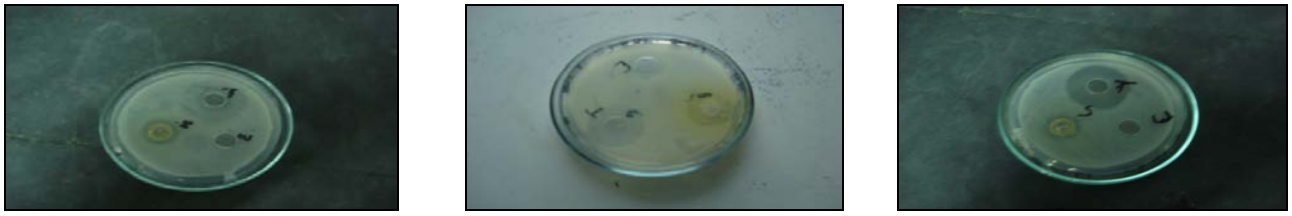
Figure 5: **showed that maximum antibacterial activity of hot water extract of lemon fruit peels was obtained against *S. aureus* & *E. coli* whereas least in *P. aeruginosa.***



S. aureus *P. aeruginosa* *E. coli*

Figure 6: **Antibacterial activity of ethanolic extract of seeds**

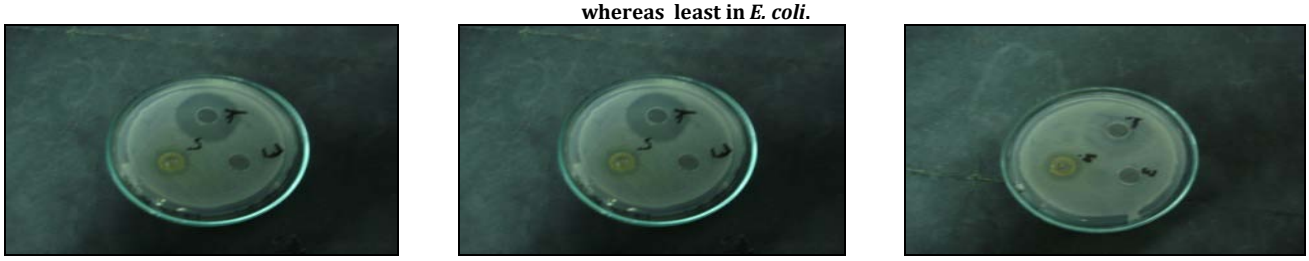
Figure 6: **showed that maximum antimicrobial activity of ethanolic extract of lemon fruit peels was obtained against *P. aeruginosa* & *E. coli* whereas least in *S. aureus.***



S. aureus *P. aeruginosa* *E. coli*

Figure 7: Antibiogram analysis of methanolic extract of seeds

Figure 7 **showed that maximum antimicrobial activity of methanolic extract of lemon fruit peels was obtained against *S. aureus* & *P. aeruginosa* whereas least in *E. coli*.**



S. aureus *P. aeruginosa* *E. coli*

Figure 8: **Antibiogram analysis of ethyl acetate extract of lemon seed**

Figure 8 showed that maximum antibacterial activity of ethyl acetate extract of lemon peels was obtained against *E. coli* & *P. aeruginosa* whereas least in *S. aureus.*

S. aureus *P. aeruginosa* *E. coli*

Figure 9: **Antibiogram analysis of hot water extract of seeds**

Figure 9: **showed that maximum antimicrobial activity of hot water extract of lemon peels was obtained against *Staphylococcus aureus* & *Pseudomonas aeruginosa* whereas least in *E. coli.***

(a) *T. rubrum* (b) *T. rubrum*

Figure 10: **Antifungal activity of ethanolic (a) and methnanolic (b) extract of peels**

Antifungal activity of ethanolic and methanolic extract of lemon peels was obtained only in *Trycophyton*, whereas no activity in *Aspergillus, Candida, Microsporum.* No antifungal activity was obtained in tetracycline.

Discussion:

Herbal medicines are a valuable and readily available resources for primary health care and complementary health care system, undoubtedly the plant kingdom still holds many species of plants containing substances of medicinal value that have yet to be discovered, though large number of plants are constantly being screened for their antimicrobial effect, these plant may prove to be a rich source of compounds with possible antimicrobial activities, but more pharmacological investigations are necessary.

Plant extract were prepared from dried samples in this research work as has been reported earlier by earlier study [11]. Agar well diffusion method was used here in order to get antimicrobial activity of various plant extracts against test micro-organism as has been performed earlier by earlier study [12,13]. MIC was also done to know the minimum inhibitory concentration of the extract by broth dilution method as experimented earlier by other scientists[14]. The antibacterial property of lemon juice and ethanolic extract of lemon seed were found to be maximum against *P. aeruginosa*. The antibacterial property of methanolic extract of lemon peels was found to be maximum against *P. aeruginosa* and the antibacterial property of ethyl acetat extract of lemon seeds were found to be maximum against *E. coli*.

The antibacterial property of hot water extract of lemon peels was found to be maximum against *S. aureus.* The antifungal activity was observed only in ethanolic and methanolic extract of lemon peels against *T. rubrum*. Ethanolic extract of lemon peels was subjected to get the MIC against *S. aureus* and it was found to be 2.4mg/ml, methanolic extract was subjected to get the MIC against *P. aeruginosa* and it was found to be also 2.4 mg/ml, for ethyl acetate in *S. aureus,* MIC observed at 14mg/ml & for hot water extract against *S. aureus,* MIC was observed at 2.4mg/ml. Ethanolic extract of lemon peels was subjected to get the MIC against *P. aeruginosa* and it was found to be 0.4mg/ml, methanolic extract was subjected to get the MIC against *S. aureus* and it was found to be also 14 mg/ml, for ethyl acetate in *E. coli*, MIC observed at 14mg/ml & for hot water extract in *S. aureus,* MIC was observed at 2.4mg/ml. Lemon juice was subjected to get the MIC against *P. aeruginosa* and it was found to be 0.4 mg/ml. In seeds, tannins, glycosides, reducing sugar & flavonoids showed positive results, whereas saponins & phlobatannins were absent. In peels, tannins, reducing sugar & flavonoids showed positive results, whereas glycosides, saponins and phlobatannins were absent.

Conclusion:

The result of antimicrobial susceptibility assay showed promising evidence for the antimicrobial effects of lemon fruit peels, seeds and juice against bacterial (*S. aureus , P. aeruginosa ,E. coli*) & fungal (*C. albicans, M. canis ,T. rubrum* & *A. niger*) pathogens. Methanolic extract of lemon peels showed maximum inhibition 23 mm against *P. aeruginosa* than other peels extracts. Ethanolic extract of lemon seeds showed maximum inhibition 25mm against *P. aeruginosa* than other lemon seeds extracts. Lemon juice showed maximum inhibition 28mm against *P. aeruginosa* than peels & seeds, by this finding it may be suggested that lemon juice have highest antimicrobial properties. Thus MICs, MBCs assay are capable of verifying that the compound has antimicrobial activity and that it gives reliable indication of the concentration of drug required to inhibit the growth of microorganisms. Phytochemical analysis are responsible for the identification of components which are responsible for antimicrobial activity of plant, thus these traditional species can be used as a potential source of drugs against various diseases.

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