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Study of Antimicrobial Activities of *Citrus limetta.*

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Abstract:
The Methanolic extract of *Citrus limetta* screened against various bacterial and fungal pathogens and showed significant antibacterial activity against *Pseudomonas aeruginosa*, *Escherichia coli, Staphylococcus aureus* and zero or no antifungal activity against *Candida albicans, Trychophyton rubrum, Microsporum* and *Aspergillus niger.* It is seen that methanol column has maximum antibacterial activity in *E. coli* JH-2 (17mm) among all solvents and minimum in *S. aureus* (12mm) in case of peel and juice whereas in pomace methanol suspension maximum (14mm) and no antimicrobial activity seen some extracts and in seeds cold water extract had shown partial inhibition. Among all extracts *Citrus limetta* juice cold water has lowest MIC *i.e.*22.85 mg/ml in *S. aureus* and maximum in pomace column extract 42.42 mg/ml in *E. coli* but no MIC seen in seeds.

Keywords: Antimicrobial activity, Disc diffusion method, Phytochemicals, MIC, Citrus peel, juice, pomace and seed extract.

Introduction:

In this world everybody wants to be fit and for that all wants to get rid of diseases through medicines without any side effects. And for that now a day's an interest in natural antimicrobial has been growing to the zenith's height. More recent research into the health benefits of citrus fruits has focused on the medicinal properties of the phytochemicals found in their peels. In addition to pulp, peel, seeds and pomace has many important properties like antimicrobial which include antifungal and antibacterial activities with many phytochemicals and essential oils. The present paper aims to evaluate antihyperglycemic activity of methanol extract of *Citrus limetta* fruit peel (MECL) in streptozotocins-induced (STZ; 65 mg/kg) diabetic rats [1].
Recent studies indicate that peel yields thousands fold more phenolic compound than pulp. Reviews suggest that flavonoids and phenolics were significantly greater in peel than the pulp, seeds and hence their fruit husk extracts shows antiproliferative activity against a panel of human oral, colon and prostate cancer cell lines. Similarly citrus fruits were historically used for their high content of vitamin C. various studies elucidate their radical trapping anti-oxidant potential (TRAP) that the TRAP was significantly higher in peels than in peeled fruits. Citrus fruit contain high concentration of phenols, flavonoids, glycosides, hesperidins, hydrixycinnamates and its flavones analogue, diosmin etc. that all have exhibited anti-carcinogenic activity in various *in vitro* studies [3].
Now as the urbanization is increasing day by day and use of drugs as medicine to cure diseases is establishing deep roots in society (which is having many side effects also), it becomes necessary to introduce naturally made medicines which can assure to have a cure without any side effects.

One of the best example for this is peel, seed, juice and pomace extract of *Citrus limetta* (Mosambi). There are conflicting scientific demonstrations of the efficacy of *Citrus limetta*. Suspicions about the true nature of the active compounds in *C. limetta* arose when synthetic additives were found in commercial products. When preservatives were not present in some of the extracts, laboratory tests found the natural extracts had no natural antimicrobial attributes of their own.

Materials and Methods:

Sample collection:

The peel, juice, seeds and roughage of *Citrus limetta* was collected from fruit juice shop at Vibhuti Khand, near MRD LifeSciences, Gomti Nagar, Lucknow, U.P in the month of July 2011. Then all were dried under sunlight for a week and grinded up to fine powdered form and preserved at dry place as stock throughout the project.
All bacterial cultures namely *P. aeruginosa*, *E. coli, S. aureus* were procured from IMTECH Chandigarh and were maintained in lab by repeated sub-culturing in plates while fungal pathogens *A. niger, C. albicans, Trychophyton, Microsporum* were collected from Gaurang Homeopathic clinic & Research Center, Aliganj, Lucknow, UP and maintained in the laboratory by repeated sub culturing to fresh media.
Single colony from each of the cultured was added to 50ml of steriled liquid media & incubated at 37^0C for bacteria 28^0C for fungal pathogen for overnight. Media was removed by centrifuging the culture at 10,000 rpm for 5 min. and cells were resuspended in DW. Optical density of culture was maintained to 0.1 at 600 nm before use by diluting with steriled water.

Extract preparations:

5.0 gm plant material was weighed and material was mixed with 50.0 ml DW in both cases. Then it was kept in water-bath at 100 ^{0}C for two hrs for hot water and for cold water it was kept in dark for a week. After that it was cooled, filtered with Whattman filter paper No.1 and concentrated by evaporation in Hot air oven at 50-60^{0}C for at least 12 hrs.

5.0 gm plant material was weighed and mixed with 50.0 ml of 80% methanol or different solvents and kept in dark for a week for suspension. Plant materials and silica gel were mixed (1:1) thoroughly for Column and for Soxhlet and was filled in extraction chamber after small piece of cotton, 200ml 80% methanol in boiling flask and the apparatus was assembled properly and run at 80 ^{0}C for three days. Compound thus obtained was then concentrated by evaporation in Hot air oven at 50-60^{0}C for 12 hrs. Finally solid material was collected and used throughout the project.

Screening of bioactive compounds against various pathogens:

The antimicrobial activity of *C. limetta* peel, juice, seeds and pomace extract was determined by agar well diffusion method against *S. aureus, P. aeruginosa* and *E. coli* [1]. About 20µl of each test bacterium was inoculated by pour plate method in sterile agar plate. After solidification of media wells were prepared by sterile borer and wells were filled by 30µl test sample, positive (Tetracycline if 50µg/ml) and negative control (autoclaved distilled water). Plates were incubated aerobically at 37 ^{0}C for 18 hours. The diameters of zones of inhibition were measured and thus the antibacterial activity of each extract was expressed in terms of the mean of diameter of zone of inhibition (in mm) produced by each extract at the end of the incubation period.

Determination of Minimum Inhibitory Concentration (MIC) of extract:

This is carried out by double agar plate gradient method. Active extracts obtained by agar well diffusion assay were further subjected to determine the Minimum Inhibitory Concentration (MIC) required for the bacteriostatic effects by standard micro-dilution agar double layer methodology. Sterilized Nutrient agar media (5.0 ml) was poured into sterilized Petri dishes. Leave the plate in slanting position so that gradient plates prepared and wait for its solidification up to half an hour. Mark the low and high concentration on plates. Take another 5.0ml media and mixed sample of concentration 50mg/ml and pour in previous gradient plate and label the high and low concentration on the bottom of the plate. Now spread the prepared inoculums culture of 1/100 dilution of actual concentration. Incubate the plate in inverted position at 37^{0}C for 24hrs. After incubation, the MIC was calculated as the lowest concentration of the extract inhibiting the visible growth of bacterial stain.

Qualitative analysis of phytochemicals:

Plant sample may contain some significant phytochemicals can be detected by using best solvent and extraction methods; chemical test are conducted on the aqueous extracts of each plant material and also the powdered form of plant as well as powdered extract of sample.

Test for reducing sugar: Take 1 ml and 1gm of sample in a test tube and 10ml deionized water then add few drop of Fehling solution and heat at 40^{0}C in water bath. Brick red precipitate indicates positive result.

Test for tannins: 2gm of aqueous extract in test tube add 2 drops of 5% ferric chloride if gives green color then test will be positive.

Test for phlobatannins: Take 10ml of aqueous extract and boil with few drop of 1% HCL. Deposition of red precipitation gives positive result.

Test for saponins: Take of aqueous extract in test tube and add 5ml de-ionized distilled water and shake it vigorously allow it for few minutes if froth last for 15 minutes means presence of saponins. Olive oil can also be added for checking oil emulsion.

Test for terpenoids: Take 5ml of aqueous extract add 2ml $CHCl_3$ followed by addition of 3ml conc. H_2SO_4 observe the reddish brown interface for presence of terpenoids.

Results:

Yields of different parts of C. limetta extracts

Table 1: Yield (in g) of *C. limetta* peel extracts

S. No.	Extracts	Yield (in g/100g of sample)			
		Peel	Juice	Pomace	Seed
1	Methanol suspension	0.516	60.48	19.44	15.30
2	Methanol column	0.416	64.58	16.60	17.12
3	Methanol Soxhlet	2.33	72.92	10.62	11.16
4	Hot water extract	0.436	74.84	17.00	7.96
5	Cold water extract	0.400	40.70	18.88	15.73

Antimicrobial activity of Citrus limetta peel extract:

Table 2: Antimicrobial of activity of aqueous and organic extracts of *C. limetta* peel against *P. aeruginosa, E. coli* and *S. aureus*

	Extracts (400mg/ml)	Zone of inhibition in mm		
		P. aeruginosa	*S. aureus*	*E. coli*
	Methanol column	16	12	**17**
	Methanol suspension	11	11	11
	Methanol Soxhlet	12	00	11
	Ethanol column	12	10	10
	Ethanol suspension			
	70%Ethanol Soxhlet	00	00	00

PEEL	Methanol + 70% Ethanol	00	00	00
	70% Ethanol	00	00	00
	80% Methanol +20% Ethyl acetate	00	00	00
	50%Methanol + 50% Ethyl acetate	00	00	00
	Hot water	11	13	13
	Cold water	15	13	16
	Tetyracycline	20	31	25
POMACE	Methanol column	00	12	00
	Methanol suspension	00	13	14
	Methanol Soxhlet	00	11	00
	Hot water	00	00	00
	Cold water	00	00	00
	Tetracycline	20	31	25
JUICE	Methanol column	16	15	15
	Methanol suspension	17	16	15
	Methanol Soxhlet	15	15	17
	Hot water	1.9	1.5	1.6
	Cold water	1.9	1.9	1.6
SEED	Tetracycline	20	31	25
	Methanol column	00	00	00
	Methanol suspension	00	00	00
	Methanol Soxhlet	00	00	00
	Hot water	00	00	00

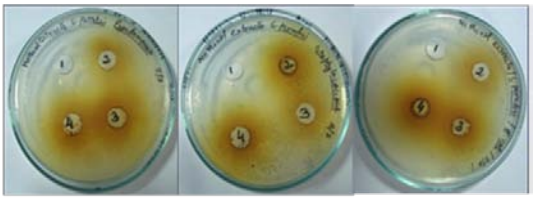

Fig.1: Sensitivity of Methanol extracts of *Citrus limetta* performed against different pathogens (a) *P. aeruginosa* (b) *S. aureus* (c) *E. coli* (1-DW, 2-Column, 3-Suspension, 4-Soxhlet)

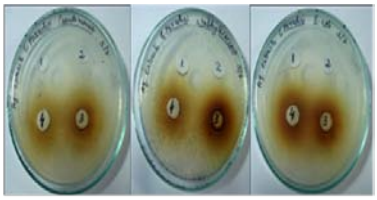

Fig.2: Sensitivity of Aqueous extracts of *Citrus limetta* performed against different pathogens (a) *P. aeruginosa* (b) *S. aureus* (c) *E. coli* (1-DW, 2-Column, 3-Suspension, 4-Soxhlet)

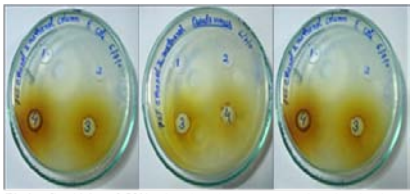

Fig.3: Sensitivity of 70% ethanol column extract and methanol column column extract of *Citrus limetta* performed against different pathogens (a) *P. aeruginosa* (b) *S. aureus* (c) *E. coli* (1-DW, 2-Column, 3-Suspension, 4-Soxhlet)

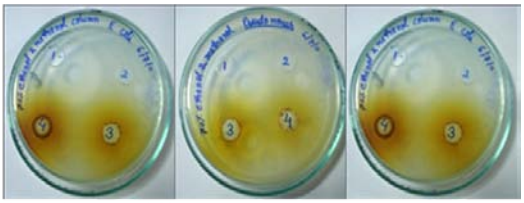

Fig.3: Sensitivity of 70% ethanol column extract and methanol column column extract of *Citrus limetta* performed against different pathogens (a) *P. aeruginosa* (b) *S. aureus* (c) *E. coli* (1-DW, 2-Column, 3-Suspension, 4-Soxhlet)

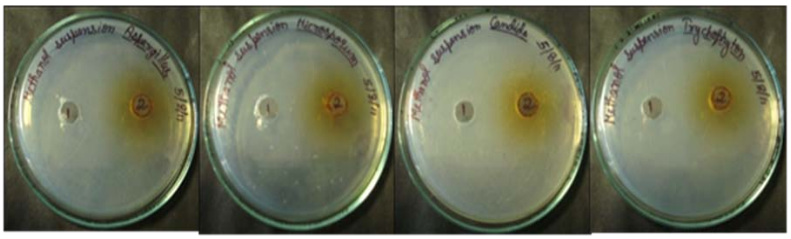

Fig.4: Negative senstivity of methanol suspension extract of *Citrus limetta* peel against fungi (a) *Aspegillus niger* (b) *Microsporum* (c) *Candida albicans* (d) *Trychophyton* (1-DW,2- Sample)

Fig.5: Negative sensitivity of methanol soxhlet extract of *Citrus limetta* peel against fungi (a) *Aspergillus niger* (b) *Microsporum* (c) *Candida albicans* (d) *Trycophyton* (1- DW,2-Sample)

Fig.6: Sensitivity of Methanol + 70% Ethanol extract of *Citrus limetta* peel against different bacterial pathogens. (1) *P. aeruginosa* (2) *S. aureus* (3) *E. coli* (1-DW, 2-Tetracycline, 3-Sample)

Fig.7: Sensitivity of 70% Ethanol Soxhlet extract of *Citrus limetta* peel extract against different bacterial pathogens. (1) *P. aeruginosa* (2) *S. aureus* (3) *E. coli* (1-DW, 2-Tetracycline, 3-Sample)

Fig.8: Sensitivity of solvent extract of *Citrus limetta* pulp extract against different bacterial pathogens. (1) *P. aeruginosa* (2) *S. aureus* (3) *E. coli* (1-DW, 2-column, 3-Suspension, 4- Soxhlet)

Minimum Inhibitory Concentration of Citrus limetta against P. aeruginosa, S. aureus and E. coli

Table3: Minimum inhibitory concentration of aqueous and organic extracts of *C. limetta* against *P. aeruginosa, E. coli* and *S. aureus*

Part	*Extracts*	*MIC in mg/ml*		
		P. aeruginosa	*S. aureus*	*E. coli*
PEEL	Soxhlet	34.28	45.72	45.72
	Column	30.00	24.28	28.57
	Suspension	22.86	27.14	24.28
	Hot	25.00	26.43	30.00
POMACE	Soxhlet	00.00	26.42	37.14
	Column	00.00	00.00	46.42
	Suspension	00.00	00.00	45.00
	Hot	00.00	00.00	00.00
	Cold	00.00	00.00	00.00
JUICE	Methanol column	31.42	30.00	30.00
	Methanol suspension	28.57	27.14	29.28
	Methanol Soxhlet	27.85	25.71	26.42
	Hot water	25.00	27.14	30.71

Phytochemical quantification was performed from Citrus limetta extracts and results are as follows:

Table4: Phytochemical quantification of *C. limetta* extracts.

Phytochemical constituents	Results
Reducing sugar	+++
Tannins	++++
Phlobatannins	+
Saponins	+++
Terpenoids	++

Discussions:

Plant metabolites play crucial role in plant defense system and thus are always in spotlight for their *in vitro* applications and uses. To study the antimicrobialproperties of such plant metabolites, solvent extraction procedure was adopted using various solvents and extraction conditions. Among all methods adopted, for several plant parts, yield was maximum for dried juice followed by pomace, seed and peel ***(Table 1).*** Plant extracts responded differently when screened against various pathogens, seeds and pomace extracts were found less active against pathogens in comparison to peel and juice ***(Table 2)***. Best results of screening were found in *P. aeruginosa* 13mm in comparison *S. aureus* and *E. coli*. Antifungal activity of extracts against *Trychophyton rubrum, Microsporum, Candida albicans, Aspergillus niger* was found to be negative. Among all the parts selected and screened in the study, pomace and seeds were found less effective for almost all of the microorganisms. Among different phytochemicals it was observed that Tannins had highest percentage ratio *i.e.* 30.07% and Phlobatannins had lowest percentage ratio *i.e.* 7.60%.

References:

1. Bonev B, Hooper J, Parisot J. 2008 Principles of assessing bacterial susceptibility to antibiotics using the agar diffusion method. J. Antimicro. Chemotherapy. 65(3): 01-07.
2. Sriparna, K. S.; Pallab, K. H.; Gupta, M.; Mazumder, K.; Saha, B.; Bhattacharya, B.; and Kar, B. 2011. Evaluation of antihyperglycemic activity of *Citrus limetta* fruit peel in streptozotocin - induced diabetic rats. Endocrinology.134: 22-36.
3. Li, R. 2006; Citrus peel extract shown to reduce insulin resistance; may help diabetics. Natural News; 79: 365-73.
4. Manthey, J.; Grohmann. K. Phytochemicals (flavanone) of *Citrus limetta* peel; U.S. Citrus and Subtropical Products Laboratory, Agricultural Research Service, South Atlantic Area, U.S. Department of Agriculture ,American Chemical Society. 2001; 3268–73.

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