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Evaluation of Antimicrobial Activity of Peel and Pulp Extracts of *C. Paradise, C. Medica* & *C. Limon* Against *B. Cereus* & *M. Luteus*

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ABSTRACT

Background: Antibiotic resistance in microbes has become a global apprehension. The clinical effectiveness of many antibiotics in existence is being diminished by the emergence of multi drug-resistant in microbes. Because of immeasurable availability of diversities of chemical, the natural herbal products either as their extracts or in pure compounds has provided limitless prospect for new drug leads. This results to an endless approach and pressing need to discover new antimicrobial agents for new as well as re-emerging infectious diseases. Objective: In the present study, biochemical and molecular identification of two microbes *Bacillus cereus* & *Micrococcus luteus* from soils samples was done through various biochemical tests and 16s RNA gene PCR amplification & sequencing. Five extracts each of peel & pulp of *Citrus paradise* (Grapefruit), *Citrus medica* (Citron) & *Citrus limon* (Lemon) was evaluated for antimicrobial activity against targeted microbes. Synthetic antibiotic, Gentamicin was used for correlation studies against natural antimicrobial agents. Results: The PCR amplification and sequencing of 16s rRNA gene was carried out for precise identification of candidate microbes and showed up amplicons size of 261bp & 287bp respectively on agarose gel electrophoresis and sequencing. Grapefruit peel and pulp showed maximum antimicrobial activity against *B. cereus* in isopropanol and ethanol extracts form respectively, it was ~56% & ~59% comparatively to gentamicin, respectively. While *M. luteus* was highest inhibited by Grapefruit peel & pulp in ethanol and isopropanol extract form and it was ~73% & ~63% comparatively to gentamicin, respectively. Citron peel and pulp showed maximum antimicrobial activity against *B. cereus* in chloroform and ethanol extracts form respectively; it was ~63% & ~67% comparatively to gentamicin, respectively. While *M. luteus* was highest inhibited by Citron peel & pulp in chloroform and aqueous extract form respectively and it was ~71% & ~60% comparatively to gentamicin, respectively. Similarly, Lemon peel and pulp showed maximum antimicrobial activity against *B. cereus* in methanol and ethanol extracts form respectively; it was ~67% & ~64% comparatively to gentamicin, respectively. While *M. luteus* was highest inhibited by Lemon peel & pulp in isopropanol extract form respectively and it was ~63% & ~69% comparatively to gentamicin, respectively. Conclusion: This study underline that extracts of peel & pulp of grapefruit, citron & lemon are highly potent natural antimicrobial agent, which poses their enormous potential in cosmetic, natural antimicrobial drug formulation and food industry.

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INTRODUCTION

Citrus originated from south-eastern Asia, India and China around 2000 BC (Swingle, 1943; Gmitter and Hu, 1990; Webber *et al*., 1967). The fruit has been introduced to western hemisphere in 1493 (Samson, 1980) and South Africa in 1654 (Oberholzer, 1969) and previously to other parts of the world via the trade course of Africa to eastern Mediterranean basin around 1000 AD (Scora, 1975). Currently, citrus is cultivated throughout world in over 137 countries on six continents and generate about 105 billion dollar per year in the world fruit

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market (Ismail and Zhang, 2004; Okwu and Emenike, 2006) and it is one of the most chief viable fruit crops grown (Tao, 2007).

The citrus fruits group plants are of immense medicinal importance and continual to play a central role in the maintenance of human health since ancient times (Dawood *et al*., 1952; Waterman and Grundon, 1983). Limonoids in citrus as anticancerous property (Jacob, *et al*., 2000), Maliheh, *et al*., (2009), reported antimutagenicity and anticancer effects of *citrus medica* fruit juice. The extracts of different plants parts of citrus fruits have reported to demonstrate antimicrobial properties and as such shows a penetrating application in food, drug formulation and cosmetic industries (Caccioni, 1998, Kanaze *et al*., 2008; Proteggente *et al*., 2003, Stange *et al*., 1993). The peel extracts of *C. sinensis* and *C. limon* was studied to be as equally effective as the synthetic antibiotics, penicillin and metacillin (Kumar *et al*., 2011).

Citrus paradise (grapefruit) an important member of the genus *Rutaceae*. It is indigenous to the island of Barbados. Grapefruit are also grown commercially in Morocco, Spain, Mexico, Israel, South Africa, Jordan, Brazil, Asia and Jamaica (Bhattacharya *et al*., 2007). Mainly in Florida and Texas, USA, further varieties of grapefruit were developed (Ortuno *et al*., 2006). At present grapefruit is the second most important citrus fruit, globally. Pharmacologically the grape fruit has very important activity, like, anti HIV (Kupferschmidt *et al*., 1998; Chinsembu and Hedimbi *et al*., 2010), anti-inflammatory effect (Ojewole, 2004), anti-atherogenic (Samman *et al*., 1999; Wilcox *et al*., 1999), anti-bacterial (Anthonia and Olumide, 2010; Roussenova, 2011), apoptotic activity (Hata *et al*., 2003) and anxiolytic and antidepressant (Gupta *et al*., 2010).

C. medica (citron) was introduced between 250 and 200 BC. In Europe, citron was the first among citrus fruits emigrant. In India, citron is found under wild conditions particularly in Nilgiris, Assam and lower Himalayas (Dugo and Di 2002). It is used in folk medicine as antispasmodic, tonic, expectorant, inhaler and antiemetic.(Simoons, 1991)**,** owing to its major constituent coumarin compounds, *p*-coumaric acids, steroids, triterpenoids, limonin, nomilin, *etc*. (Feng *et al*., 2004; Yin and Lou 2004).

Citrus Limon (lemon) is also a vital medicinal plant of family *Rutaceae*. It is cultivated chiefly for its alkaloids contents, which are having anticancer activities**.** Its peel show strong antimicrobial activity **(**Dhanavade, *et al*., 2011; Guimaraes *et al*., 2010).

Many studies have reported antimicrobial and antioxidant effect of edible part as well as non-edible part of citrus fruits. This study was aimed to evaluate antimicrobial activity of pulp as well as peel of grape fruit; citron and lemon to put forward deep insight as antimicrobial agent and minimize waste of fruit juice processing industries.

MATERIALS AND METHODS

Collection of fruit materials:

The candidate citrus fruits were purchased from local fruits market. After collection, the peels and pulp were dried in shade at room temperature (30 - 35ºC) for 5 days. 150 g of each plant dried peel and pulp were coarsely milled using a mortar and pestle and finally reduced to fine powder via an electric blender. The powder was stored in air tight containers at 4º temperature until further use.

Preparation of extracts:

To study their antimicrobial activity various extracts were prepared namely methanolic, ethanolic, chloroform, aqueous and isopropanol extracts individually for peel & pulp of all candidate citrus fruits under study. 20g of each material was soaked in different solvents for 72 h with constant stirring at an interval of 24 h. Finally, the extracts were filtered through Whatmann filter (paper no. 1) and the filtrate was collected and incubated in water bath to obtain the crude extract (Alade and Irobi, 1993).

Identification Of Candidate Microbes:

Isolation of Bacterial Strains:

Two soil samples were randomly collected from different areas of waste land area in Lucknow, U.P. (India) region. Such samples were placed in separate sterile bottles/polythene bags and stored in a refrigerator at 4 °C till use.

The Luria Bertani Agar (LBA) and Nutrient Agar media (NAM) were prepared following the manufacturer's instructions for isolations. Using appropriate isolation technique (Ali and Naseem, 2011; Beishir, 1991; Hampton, 1990), inoculation of bacteria was carried out under aseptic conditions. Inoculated petriplates were incubated for 2-3 at days 37°C. Different single colonies from two different plates were sub-cultured for purification. After 24 hours of incubation at 37°C, single colonies were streaked on two fresh media plates.

Biochemical Tests:

The two strain isolated from different soils samples were identified by various conventional biochemical tests as per Bergey's Manual of Systematic Bacteriology (Buchanan and Gibbons, 1974; Holding and Colle,

1971; Sneath, 1986; Taiwo and Oso, 2004). They are gram reaction, motility test, Methyl red test, Indole production test, Voges proskauer tests, mannitol test, Starch Hydrolysis, Citrate utilization test, 6.5 % NaCl, Catalase Activity, urease activity test and glucose fermentation test.

Genomic DNA Isolation:

Genomic DNA was extracted from two different cultures according to the following method. In two flask, 50 mL LB broth was prepared and inoculated with different single bacterial colony and grown until an OD_{600} of 0.5–1.5. Bacterial cells were collected by centrifugation for 10 min at 5000 rpm, at 4°C. Bacterial Genomic DNA isolation kit (Qiagen, USA) was used to isolate genomic DNA from each bacterial sample. The quantity and quality of isolated genomic DNA is estimated using Nanodrop spectrophotometer and finally stored at -20°C for further use.

Amplification of the 16s rRNA Gene of candidate microbes :

The PCR amplification (Mullis, 1990) of the DNA coding for 16s rRNA is the most potent tool to identify the unknown bacteria. The PCR was set up in 25 µl reaction volume. Based on initial trial, the reaction mixture was optimized as follows, 2.5 µl of 10 X Assay buffer (100 mM Tris- HC1, pH 9.0, 15 mM $MgC1_2$, 500mM KC1 and 0.1% gelatin), 0.20 mM of dNTP mix, 10 pm of forward and reverse primer, 1U Taq DNA polymerase, 100ng of purified DNA and autoclaved milliQ water to make up the volume. The primers used were designed by DNAStar software using sequences from NCBI database, Table 1.

The cycling conditions were, initial denaturation at 94 °C for 5 min followed by 35 cycles of 50 sec denaturation at 94 °C, 50 sec annealing at 62 °C and 55 sec elongation at 72°C and final elongation for 5 min, for *B. cereus*, whereas annealing condition for microbe *M. luteus*, was 55 sec annealing at 58 °C. The products of PCR amplification were electrophoretically determined on a 1.6% agarose gel containing ethidium bromide (0.5 μg/mL) in 1XTAE buffer (Tris–Acetate–EDTA buffer) and visualized by UV transillumination. The PCR product was gel purified and subjected for sequencing (outsourced). The nucleotide sequence so obtained was compared with available sequences from the NCBI database and the candidate bacterial sequence was finally confirmed.

Evaluation of Antimicrobial activity of candidate citrus fruit's peel and pulp:

In nutrient agar medium/broth the candidate bacteria were grown. Antimicrobial activity of different extracts was measured using well diffusion method (Sawhney and Singh, 2000). To prepare a fresh culture, 25 ml of nutrient broth was taken in two sterilized test-tubes and inoculated with different candidate bacterium and incubator for 18-20 h. Gentamicin at the rate of 1mg/ml of bacterial cultures was used as positive controls and milli Q water as negative control. 1ml of freshly prepared inoculums of different bacterial cultures was introduced in freshly prepared different sterilized molten medium plates. After hardening, the plates were perforated with a sterilized borer. The various solvent extracts of both peel and pulp of citrus fruits were added separately in different wells. Finally, the bacterial culture plates were incubated for 24 h at 37°C to determine the zonal inhibition.

RESULTS AND DISCUSSION

In the present investigation, various results reveal that the different extracts of peel and pulp of candidate citrus fruits has great potential antimicrobial activity against candidate microbes.

Preparation of extracts:

The candidate citrus fruits were purchased from local fruits market, and the peels and pulp were shade dried at room temperature (32 - 35ºC) and coarsely powdered and stored in closed containers & in 4ºC temperature. The five extracts were prepared in order to study the antimicrobial activity, namely aqueous, ethanolic, methanolic, chloroform and isopropanol extract of each peels and pulps of the candidate citrus fruits and stored for further use.

Identification Of Candidate Microbes:

Biochemical Tests:

The two bacteria isolated from different soils samples were identified by various conventional biochemical tests. The result of these biochemical tests revealed the probability of following two microbes, *B. cereus & M. luteus.* The results of biochemical test are summarized in Table 2.

Identification of the Isolated Bacteria by PCR Amplification & Sequencing of 16S rRNA Gene:

The genetic based identification of microbes through PCR based amplification and sequencing of 16s rRNA gene is most accurate, sensitive and potent tool for identification of microbes. The genomic DNA from two

different pure cultures was isolated and visualized on 0.8 % agarose gel electrophoresis (Figure 1a). The good quality DNA was further used in PCR amplification reaction.

Fig. 1a: Genomic DNA extracted from two pure cultures of candidate microbes.

The 16S rRNA gene specific for two candidate microbes was amplified using specific primers Table-1. In order to ensure the amplification of specific fragment with higher yield, the PCR protocol was optimized with respect to reaction conditions as well as cycle parameters as mentioned in the materials and methods. Following PCR, the amplicons were checked using agarose gel electrophoresis. As expected a single and specific band were amplified from the DNA of respective candidate microbe. The amplicon sizes were 261bp & 287bp for *B. cereus & M. luteus* respectively (Figure 1b).

The two PCR products were gel purified and were sequenced (outsourced). The 5'-3' sequence of 16S rRNA gene of the respective microbes is shown in Figure 2. The sequences were BLASTED on NCBI website for sequence confirmation. The Sequencing confirmed the size of the amplified fragments to be 261bp, & 287bp for *B. cereus & M. luteus*, respectively.

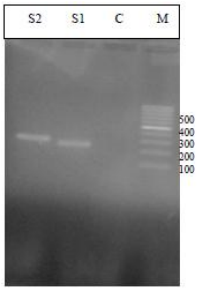

Fig. 1b: PCR amplified product 261bp and 287 bp of 16s rRNA gene of *B. cereus* (S1) *& M. luteus* (S2) respectively, C = control, M = 100bp ladder.

Antimicrobial activity:

Grapefruit peel and pulp showed maximum antimicrobial activity against *B. cereus* in isopropanol and ethanol extracts form respectively, it was ~56% & ~59% comparatively to gentamicin, respectively while least in ethanol and aqueous extract from of peel and pulp respectively. While *M. luteus* was highest inhibited by Grapefruit peel & pulp in ethanol and isopropanol extract form and it was ~73% & ~63% comparatively to gentamicin, respectively, while least in aqueous and chloroform, respectively. This activity is reasonably good comparative to synthetic antibiotic, Figure 3 (a, b & c) & Table 3.

B. cereus 261bp
AAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATAAGACTGGGATAACTCCG
GGAAACCGGGGCTAATACCGGGTAACATTTTGAACCGCATGGTTCGAAATTGAAAGGCGGCTTC
GGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGG
CAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGAC
TCCTACG
M. luteus 287 bp,
TTTTGGATGGACTCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGG
GTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGG
AGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGA
AGAAGCACCGGCTAACTACGTGCCAGCAGCCGC

Fig. 2: Nucleotide sequence of 16s rRNA gene of S1 = *B. cereus* 261bp and S2 = *M. luteus* 287bp.

It was found that the citron peel showed maximum antimicrobial activity *B. cereus* (~63.% vs gentamicin) and *M. luteus* (~71.% vs gentamicin) in chloroform extract form and least in isopropanol and aqueous extract from respectively. This is substantially good comparable to Gentamicin. While citron pulp showed maximum

antimicrobial activity against *B. cereus* (~67.% vs gentamicin) and *M. luteus* (~60.% vs gentamicin) in ethanol and aqueous extract form, respectively, and correspondingly least in aqueous and ethanol extract form, Figure 4 (a, b & c) & Table 4.

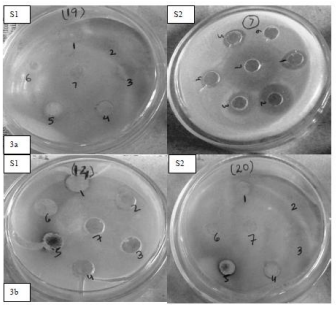

Fig. 3a & b: Zone of inhibition of Grapefruit (a) peel and (b) pulp extracts against micro-organism S1 & S2.

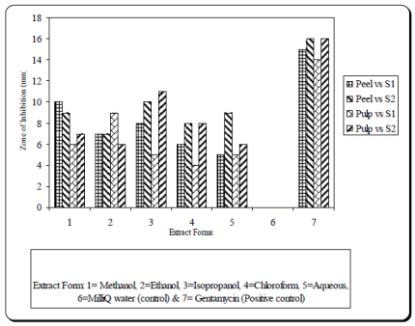

Fig. 3c: Antimicrobial activity of grapefruit peel & pulp extract against micro-organism of S1 & S2.

In case of lemon peel, it was found that, maximum antimicrobial activity against *B. cereus* (~67.% vs gentamicin) and *M. luteus* (~63.% vs gentamicin) in methanol extract and isopropanol extract form, respectively and least in aqueous and ethanol extract from respectively. While lemon pulp showed maximum antimicrobial activity against *B. cereus* (~64% vs gentamicin) and *M. luteus* (~69.% vs gentamicin) in ethanol and isopropanol extract form, respectively, and correspondingly least in chloroform and aqueous extract form, Figure 5 (a, b & c) & Table 5.

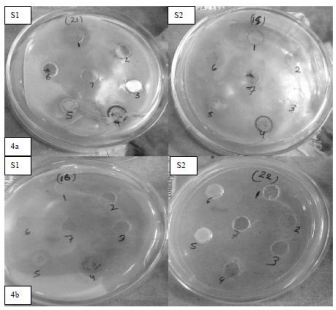

Fig. 4 a & b: Zone of inhibition of Citron (a) peel and (b) pulp extracts against micro-organism S1 & S2.

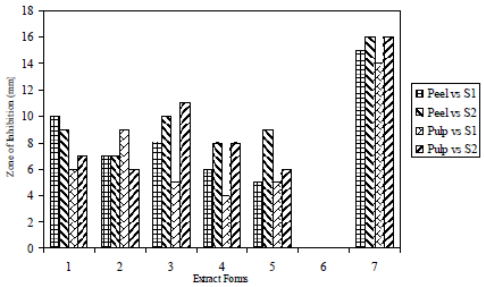

Fig. 4c: Antimicrobial activity of Citron peel & pulp extract against micro-organism of S1 & S2.

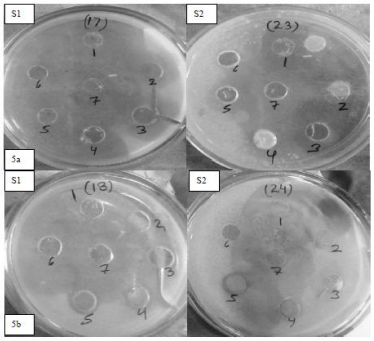

Fig. 5a & b: Zone of inhibition of Lemon (a) peel and (b) pulp extracts against micro-organism S1 & S2.

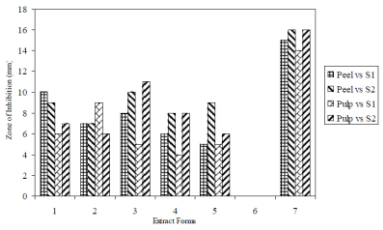

Fig. 5c: Antimicrobial activity of Lemon peel & pulp extract against micro-organism of S1 & S2.

Table 1: List of 16s rRNA gene primers used were designed using sequences from NCBI database.

Organism	Forward primer 5' – 3'	Reserve primer 5' – 3'	NCBI Accession No.
Bacillus cereus	aagttagcggcggacgggtgag	cgtaggagtctgggccgtgtc	Z84576
Micrococcus luteus	ttttggatggactcgcggcctat	gcggctgctggcacgtagtta	AJ536198

Table 2: Results of biochemical tests.

Test	Sample S1	Sample S2
Gram reaction	-	+
Motility	-	-
VP test	+	+
Methyl red	+	-
Indole test	-	-
Catalase	+	+
Urease activity	+	+
Mannitol	-	+
Citrate	+	-
Starch	-	-
6.5 % NaCl	+	+ (slow)
Glucose fermentation	-	+
Probable identify	*Bacillus cereus*	*Micrococcus luteus*

Table 3 (A & B): Antimicrobial activity of grapefruit peel & pulp extract against micro-organism of S1& S2. Extract form: A= Methonol, B=Ethanol, C=Isopropanol, D=Chloroform, E=Aqueous, F= Water (control) & G= Gentamicin (Positive control).

(A)

Organism	Grapefruit: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	E
S1	8	3	9	7	8	0	16
S2	9	11	7	8	5	0	15

(B)

Organism	Grapefruit: Diameter of zone of inhibition (mm)						
	Pulp Extract						
	A	B	C	D	E	F	G
S1	8	10	6	8	6	0	17
S2	7	7	10	4	5	0	16

Conclusion:

This study suggests that the peel and pulp of candidate citrus fruits have high potential as natural antimicrobial agents against *B. cereus* and *M. luteus* in comparison to gentamicin. Future study may also be

done to assess the antimicrobial properties of peel and pulp extracts of these citrus fruits against other microbes as well as in comparison to other antibiotics also. Further, this study also indicates new formulation approach for new & effectual antimicrobial preparation that can be used as a powerful natural antimicrobial stabilizer in cosmetic, food and drug industry.

Table 4(A & B): Antimicrobial activity of citron peel & pulp extract against micro-organism of S1 & S2.

(A)

Organism	Citron: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	G
S1	6	4	3	10	6	0	16
S2	9	7	9	10	5	0	14

(B)

Organism	Citron: Diameter of zone of inhibition (mm)						
	Pulp Extract						
	A	B	C	D	E	F	G
S1	9	10	8	6	4	0	15
S2	4	8	5	7	9	0	15

Table 5(A & B): Antimicrobial activity of lemon peel & pulp extract against micro-organism of S1 & S2.

(A)

Organism	Lemon: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	G
S1	10	7	8	6	5	0	15
S2	9	7	10	8	9	0	16

(B)

Organism	Lemon: Diameter of zone of inhibition (mm)						
	Pulp Extract						
	A	B	C	D	E	F	G
S1	6	9	5	4	5	0	14
S2	7	6	11	8	6	0	16

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