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In-vitro Comparative Study on Antimicrobial Activity of five Extract of Few Citrus Fruit: Peel & Pulp vs Gentamicin

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ABSTRACT

Background: The emergence of microbial strains with multi-drug resistance or reduced susceptibility to antibiotics is continuously increasing. This increase has been ascribed to haphazard use of broad-spectrum antibiotics or immunosuppressive agent. In addition to this, particularly in developing nations; synthetic drugs are not only costly and inadequate for management of disease but also are highly adulterated causing enlarged scope of side effects. Therefore, there is need to natural and cost effective alternatives in infection-fighting approach to manage microbial infections. Objective: In the present study the two candidate microbes, *E. faecalis* & *P. putida* were isolated from waste land soils samples from Lucknow region and were identified through various biochemical tests. The PCR amplification and sequencing of 16s rRNA gene was also performed for accurate identification of candidate microbes. The antimicrobial activities of five different extract of peel and pulp of *C. reticulate*, *C. maxima* & *C. sinensis* fruits have also been investigated on the candidate microbes. Results: The PCR amplification of 16s rRNA gene showed up amplicon size of 248bp & 281bp respectively on agarose gel electrophoresis and same was confirmed by sequencing. Kinnow peel and pulp showed maximum antimicrobial activity in methanolic extracts form, against *P. putida*, which was ~73% & ~64% respectively comparatively to gentamicin. The orange peel and pulp show maximum antimicrobial activity in methanolic and ethanolic extracts form respectively, against *P. putida*, corresponding to about 75% to 80% antimicrobial activity of positive control i.e. gentamicin. Likewise, the maximum antimicrobial activity among the chakotra peel and pulp is shown in ethanolic extracts form, against *E. faecalis*, and comparing to 75% (peel) and 66% (pulp) activity of gentamicin. Conclusion: This study highlight that citrus fruits (peel and pulp) are extremely vital as natural antimicrobial agent, nutritional add-on and food product stabilizer.

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INTRODUCTION

Citrus fruits: have been collected and used by man for centuries for medicinal, herbal and agricultural purposes (Dawood *et al*., 1952; Waterman and Grundon, 1983). Citrus fruits, belong to the family of Rutaceae, are one of the main fruit tree crops grown throughout the world (Okwu and Emenike, 2006).

In all the continents, it is one of the most chief viable fruit crops grown (Tao, 2007). Juice processing industries devour citrus fruits in large quantity while the peels are usually wasted. The yield of juice of citrus fruits is less than 50 percent of it weight and result in huge amount of by-product wastes, such as peels, every year (Manthey and Grohmann, 2001). There is always an augmented interest in producing useful products from waste supplies and citrus wastes are no exceptions. Appropriate intervention should be adopted to utilize them for the switch into value-added products (Nand, 1998). In the current era, with an increase in number of antibiotic resistance pathogens, researchers feel an up thrust of a safer alternative from of drug. Both peel and pulp of citrus fruits if demonstrate an effective antibacterial activity; they can be used as valuable source as food preservative in food industry itself. Food fundamentalist, regulatory bodies and food safety researchers have been progressively more alarmed with the increasing number of food-borne illness epidemic caused by some pathogens (Friedman *et al*., 2002; Wilson and Droby, 2000). Nowadays, with an increasing pressure of consumers or legal authorities, the food industry have initiated towards the lesser use of chemical preservatives

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in their products and moved to either completely remove or to implement more natural alternatives to enhance the maintainability and shelf life of there products.

Flavanones and many polymethoxylated flavones are richly found in both peel and pulp of citrus fruits, whereas they are scantly found in other plants (Ahmad, 2006). The citrus oils have great potency as antimicrobial agent and as such shows a penetrating application in food and cosmetic industries (Caccioni, 1998). They also have an anti-diabetic properties (Hamendra and Anand, 2007), hypotensive agent (Kumamoto *et al*., 1986), antioxidant (Kanaze *et al*., 2008; Proteggente *et al*., 2003), antifungal (Stange *et al*., 1993), antimicrobial (Caccioni, 1998), insect repellent, larvicidal, antiviral, antimutagenic and antihepatotoxic (Han, 1998).

Citrus maxima (chakotra or shaddock or pomelo) is indigenous vegetation to tropical parts of Asia. In ancient and medieval literature, the peel and pulp of *C. maxima* is mentioned as cardiac stimulant, appetizer, antitoxic, and stomach tonic. Recently, leaves are found to demonstrate antitumour activity (Sen *et al*., 2011). Alcoholic extracts of the fruit shows evidences of antidiabetic and anti hyperlipidaemic activity (Bhandurge *et al*., 2010). Its essential oil shows in vitro activity against *S. aureus* and *E. coli* (Oyedepo, 2012).

Citrus reticulata (kinnow) is a beautiful golden-orange colour fruit and has special economic value and export demand because of its high juice content, rich source of vitamin C, extraordinary flavor and refreshing taste. Chutia (2009) reported antifungal activity and chemical composition of essential oil of *C. reticulata* against many phytopathogens. Kangralkar *et al*. (2010) reported that essential oils of *C. reticulata* have protective effect on isoniazid stimulated hepatotoxicity in wistar rats.

Citrus sinensis, commonly known as sweet orange, belongs to *Rutaceae* family (Bakshi *et al*., 1999) and is the most commonly grown tree fruit in the world (Maimi and Morton, 1987). *C. sinensis* fruit has intrinsic properties of strengthening, laxative, cardiotonic, anthelmintic and anti-fatigue (Kirtikar and Basu, 1984). It also possesses very effective anti-inflammatory, antibacterial and antioxidant properties (Ramachandran *et al*., 2002). Its fruit is said to lower cholesterol and support digestion of fatty foods (Cesar, 2010). The orange peel and the white layer beneath its peel are concentrated source of vitamin C. Its peel contains citral, a vital aldehyde that can antagonize the action of vitamin A (Audrey, 1983). Flavonoids and limonoids present in the peel impart distinguishing peculiar fragrance. It is also reported to have antifungal property (Sawalha *et al*., 2009; Hegazy and Ibrahium, 2012; Velázquez-Nuñez *et al*., 2013).

Clinical microbiologists have several reasons to be intensely inclined towards exploration of antimicrobial potential of plant extracts. One is very likely that, the phytochemicals present in them, will occupy the central place in the repository of antimicrobial drugs. Researchers realize that the antibiotics have limited effectual life span; as such new sources especially from plant parts are needed to be investigated. Second the public is now aware enough of the harms with the over prescription and misuse of conventional antibiotics. A huge number of plants compounds, though of unreliable purity, are easily available with herbal suppliers and the self medication with these natural forms is a common practice to certain extent.

MATERIALS AND METHODS

Collection of fruit materials:

The citrus fruits, [*C. reticulata* (kinnow), *C. maxima (*Chakotra*), C. sinenis* (orange)], were purchased from local fruits market. After collection, the peels and pulp were dried in shade at room temperature (30 - 35ºC) for 5 days. Using a mortar and pestle, 150 g of each plant parts were coarsely powdered 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 aqueous, methanolic, ethanolic, chloroform and isopropanol extracts individually for peel & pulp of all 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 at 37°C for 2-3 days. 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 Methyl red test, Voges proskauer tests, mannitol test, Indole production test, Oxidase Production, Starch Hydrolysis, 6.5 % NaCl Citrate utilization test, Catalase Activity, urease activity test, and glucose fermentation test.

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

The PCR amplification of the DNA coding for 16s rRNA is the most potent tool to identify the unknown bacteria. The 16s rRNA gene is amplified using the Polymerase Chain Reaction (Mullis, 1990), and the purified amplified product was outsourced for sequencing and the final sequence obtained was compared with the sequence obtained from the NCBI database.

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 untill 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 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, 50ng 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 45 sec denaturation at 94 °C, 50 sec annealing at 60 °C and 1 min elongation at 72°C and final elongation for 5 min, for *E. faecalis,* whereas annealing condition for microbe *P. putida*, was 55 sec annealing at 62 °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:

The candidate bacteria were grown in nutrient agar medium/broth. 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.

Collection of fruit material:

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.

Preparation of extracts:

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, *Enterococcus faecalis & Pseudomonas putida.* 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 248bp & 281bp for *E. faecalis & P. putida* respectively (Figure 1b).

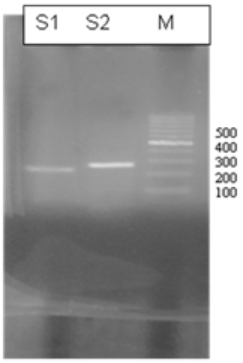

Fig. 1b: PCR amplified product 248bp and 281bp of 16s rRNA gene of *E. faecalis* (S1) *& P. putida* (S2) respectively, M = 100bp ladder.

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 248bp, & 281bp for *E. faecalis & P. putida*, respectively.

Antimicrobial activity:

It was found that the orange peel possessed maximum antimicrobial activity against *E. faecalis* & *P. putida* in the ethanolic & methanolic extract from respectively. This maximum activity against *P. putida* was almost equivalent to positive control i.e. gentamicin, hence suggest a very good antimicrobial alternative in natural

form. Correspondingly for orange pulp maximum antimicrobial activity was by isopropanolic & ethanolic extract from respectively. The ethanolic extract form also exert a high thrust as natural antimicrobial agent against *P. putida* being equivalently comparable to gentamicin, Figure 3 (a, b & c) & Table 3.

E. faecalis 248bp
GAGGAGTGGCGGACGGGTGAGTAACACGTGGGTAACCTACCCATCAGAGGGGGATAACACTT
GGAAACAGGTGCTAATACCGCATAACAGTTTATGCCGCATGGCATAAGAGTGAAAGGCGCTTT
CGGGTGTCGCTGATGGATGGACCCGCGGTGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAA
GGCCACGATGCATAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCC
P. putida 281bp,
GCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGGTG
CTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCC
TTGTCCTTAGTTACCAGCACGTAATGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGA
GGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACACACGTGCTACAA
TGGTCGGTACAGAGGGTTGCCAAGCCGC

Fig. 2: Nucleotide sequence of 16s rRNA gene of S1 = *E. faecalis* 248bp and S2 = *P. putida* 281bp.

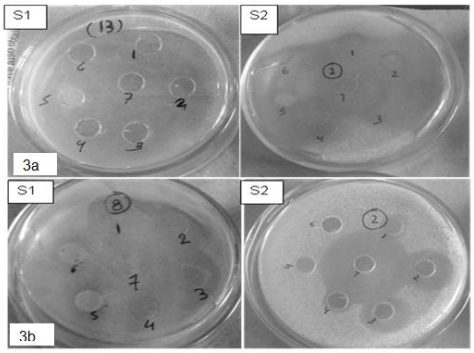

Fig. 3 a & b: Zone of inhibition of orange (a) peel and (b) pulp extracts against micro-organism S1 & S2..

Similarly, orange peel possessed minimum antimicrobial activity against *E. faecalis* & *P. putida* in the chloroform & aqueous extract from respectively. While correspondingly for orange pulp minimum antimicrobial activity against said microbes was in aqueous extract from respectively. This shows that aqueous form of extract is least effective in comparison to gentamicin, Figure 3 & Table 3.

It was found that the Kinnow peel possessed maximum antimicrobial activity against *E. faecalis & P. putida* in the isopropanolic & methanolic extract from respectively which is almost good 60% & 73% respectively in comparison to gentamicin activity and correspondingly minimum in ethanolic & aqueous extracts form respectively**.** Similarly, kinnow pulp showed maximum antimicrobial activity against *E. faecalis & P. putida* in the isopropanolic & methanolic extract from respectively and this was also more than half the activity of gentamicin. Correspondingly minimum antimicrobial activity against *E. faecalis* was both in ethanolic and chloroform extract form and against *P. putida* in aqueous extract from, Figure 4 (a, b & c) & Table 4.

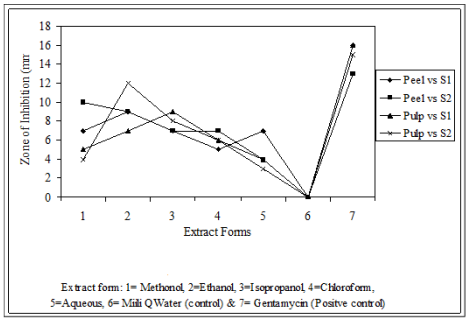

Fig. 3c: Antimicrobial activity of orange peel & pulp extract against micro-organism of S1 & S2.

It was found that, in case of Chakotra peel, both the microbes were maximum inhibited in the ethanolic extract from and this is almost 75% of gentamicin activity hence demonstrate its great potential for natural antimicrobial agent. The Chakotra peel showed minimum activity in aqueous extract form. Similarly, the Chakotra pulp possessed maximum antimicrobial activity against both the microbes in ethanolic extract form which was also about 60% activity of gentamicin. The chakotra pulp reveals minimum activity in aqueous extract form, Figure 5 (a, b & c) and Table 5.

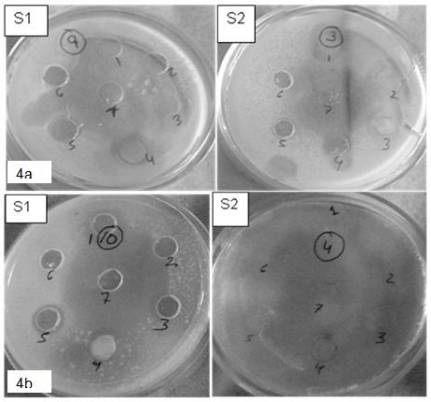

Fig. 4 a & b: Zone of inhibition of kinnow (a) peel and (b) pulp extracts against micro-organism S1 & S2.

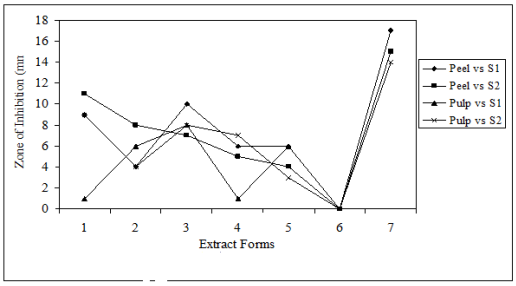

Fig. 4c: Antimicrobial activity of kinnow peel & pulp extract against micro-organism of S1 & S2.

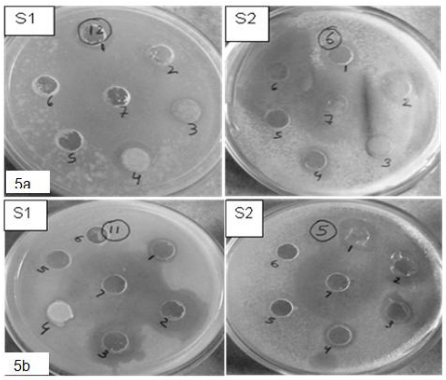

Fig. 5 a & b: Zone of inhibition of chakotra (a) peel and (b) pulp extracts against micro-organism S1 & S2.

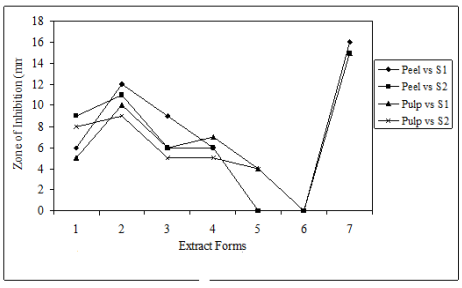

Fig. 5c: Antimicrobial activity of chakotra peel & pulp extract against micro-organism of S1 & S2.

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

Organism	Forward primer 5' – 3'	Reserve primer5' – 3'	NCBI Accession No.
Enterococcus faecalis	Gaggagtggcggacgggtgag	Ggccgtgtctcagtcccagtgtg	FJ378704
Pseudomonas putida	Gccttgacatgcagagaactttc	Acggcttggcaaccctctgt	D37923

Table 2: Results of biochemical tests.

Test	Sample S1	Sample S2
Gram reaction	+	-
Motility	-	-
VP test	+	+
Methyl red	+	-
Indole test	-	-
Catalase	+	+
Oxidase test	+	+
Urease activity	+	+
Mannitol	-	-
Citrate	-	+
Starch	-	-
6.5 % NaCl	-	+
Glucose fermentation	-	+
Probable identify	*Enterococcus faecalis*	*Pseudomonas putida*

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

Organism	Orange: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	E
S1	7	9	7	5	7	0	16
S2	10	9	7	7	4	0	13

Organism	Orange: Diameter of zone of inhibition (mm)						
	Pulp Extract						
	A	B	C	D	E	F	G
S1	5	7	9	6	4	0	16
S2	4	12	8	6	3	0	15

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

Organism	Kinnow: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	G
S1	9	4	10	6	6	0	17
S2	11	8	7	5	4	0	15

Organism	Kinnow: Diameter of zone of inhibition (mm)						
	Pulp Extract						
	A	B	C	D	E	F	G
S1	1	6	8	1	6	0	15
S2	9	4	8	7	3	0	14

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

Organism	Chakotra: Diameter of zone of inhibition (mm)						
	Peel Extract						
	A	B	C	D	E	F	G
S1	6	12	9	6	0	0	16
S2	9	11	6	6	0	0	15

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

Conclusion:

From this study, it can be concluded that the peel and pulp of candidate citrus fruits encourage a very high potential to be used as natural antimicrobial agents against studied microbes in comparison to gentamicin. Further research may also be done to study the antimicrobial properties of these citrus fruits against other microbes as well as in comparison to other synthetic antibiotics also. It can also be concluded that the peel and pulp of candidate citrus fruits are valuable for human consumption and health. Further, this study put forward a new insight towards formulation of new & effective antimicrobial drug and can be used as a powerful natural antimicrobial stabilizer for food products and as nutritional supplement.

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