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Study on antimicrobial activity and prebiotic effect of orange (*Citrus reticulata* L.) peel extracts

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Abstract
The main objective of the study is extraction, identification of Prebiotic effect and demonstration of Antibacterial activity (Agar well diffusion method) of Orange (Citrus reticulata L.) peel against certain pathogenic bacteria. As microorganism are becoming more resistant and less effective to present day antibiotics and antimicrobials, this study focuses on antibacterial activity, prebiotic effect and future prophylactic potential of the orange peel. Biologically active compounds and ingredients present in the medicinal plants and its by products have always been of great interest to scientists. The antibacterial activity of orange peel extracts against bacterial strains like Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Salmonella typhi, Enterococcus using agar well diffusion method with different concentrations of Samples (25, 50, 75, 100µl/well) were studied. The disc agar diffusion method was used to determine the antibacterial activity of the extracts. The antibacterial activity was determined by measuring the diameter of the zone of inhibition. Antibacterial activity was more effective against *Pseudomonas aeruginosa* with a zone of inhibition of 20mm and 16mm [at conc 50 & 75 µl], *Salmonella typhi* showed a zone of 24mm diameter [at conc 100 µl]. The Prebiotic activity assay, the lactobacilli cultures were streaked on MRS agar and incubated at 37 °C for 48 hours, *Bifidobacterium animalis* subsp. *lactis* was streaked on Bifidobacterium agar in anaerobic conditions. The dilutions 10^{-5}, 10^{-6} and 10^{-7} were placed on an agar plate, MRS for lactobacilli cultures and Bifido for *Bifidobacterium animalis.* The prebiotic effect of lactobacillus and bifidobacterium at 8% dilution with 10^{-6} concentration were found maximum as $8x10^{7}$ and $7.2x10^{7}$ respectively.

Keywords: orange peel extracts, antibacterial activity, prebiotic effect

Introduction
Orange peels and seeds are considered as wastes, hence are discarded as agricultural fruit wastes (Madhuri *et al.*, 2014) [5]. This could contribute to environmental pollution with adverse public health implications. Citrus species are known for an abundance of bioactive components, nutraceuticals, and functional compounds in the flavedo and albedo of the peels. In Citrus fruits, flavonoids are present as flavanones (neohesperidosides, rutinosides), flavanol glycosides, flavones (polymethoxyflavones, hydroxylated polymethoxyflavones) with predominant bioactive compounds like naringin and hesperidin. Phenolic compounds like flavonoids are known to exhibit antioxidant, antiatherogenic, anti-inflammatory, anti-carcinogenic, antiviral, antimicrobial and antiallergenic activities (Yashaswini *et al.*,2018) [9]. Based on history, plants have provided a good source of anti-infectious agents such as berberine, quinine, emetine which remain effective tools in the war against microbial infections. Plants that contain protoberberines, oligosaccharides, saponin, flavonoid, alkaloid, and phylates are used in the African traditional system and have been found to be active against a wide variety of microorganisms (Uchechi *et al.*, 2010) [8].
Thus, this study examined the Mandarine (Tangerines) variety of sweet orange peels and also the anti-bacterial activity of the (aqueous and ethanol extracts) on some bacterial pathogens (*E. coli*, *S. aureus*, etc.,) to obtain a scientific basis for their possible beneficial use and its prebiotic effect which are generally thought to have health-promoting properties. The percentage yield for the seeds sample (54.49%) was lower (p<0.05) than that of the peels (90.21%). The peel of citrus fruits are rich source of flavanones and many polymethoxylated flavones, which are very rarely seen in other plant varieties. These compounds, not only play an important physiological and ecological role, but are also of commercial interest because of their multitude of applications in the food and pharmaceutical industries which has to be effectively utilized.

Materials and Methods

This study was carried out at the Department of LPT (Dairy Science) Madras Veterinary College, TANUVAS, Chennai, Tamilnadu.

Agar well diffusion method

Different concentrations of extract samples (25, 50, 75, 100µl /well) were used in this study. Nutrient agar plates were inoculated with test organisms. The plates were evenly spread out. Then wells were prepared in the plates with a cork borer. Each well was loaded with 0.1ml of corresponding concentration of extract sample and 10 µg of Tetracycline dissolved in 1 ml of DMSO as a Positive control for assessing the antibacterial activity. The plates were incubated for 24h at 37 °C. The development of inhibition zone around the well was measured and recorded.

Antimicrobial effect

Sterile molten nutrient agar at around 40 ºC was taken and seeded with different microbial cultures and plates were prepared. After solidification 4 mm wells were prepared. In these wells solvent extracts of the peel were added. The plates were incubated overnight at 37 ºC. After incubation the zones of inhibition were measured and recorded. Respective solvent controls were also run simultaneously.

Table 1: Composition of nutrient broth

Components	(%)
Peptone	1.0
Yeast extract	1.0
Sodium chloride	0.5
Agar	2.5

Preparation of orange peel extracts

The orange peel were homogenized in different solvents individually and mixed well. The solvents used were water, ethanol, acetone, and methanol. The extracts were collected separately for further study.

Cultures used for antimicrobial activity

The microorganisms used were Escherichia coli NCIM-2067, *Pseudomonas aeruginosa* NCIM 2036, *Salmonella typhimurium* NCIM 5021, *Staphylococcus aureus* NCIM – 2079 and *Enterococcus faecalis* NCIM 5367. Microbial cultures were procured from NCDC - NDRI Karnal, Haryana.

Culture medium

Nutrient agar medium and a mineral based medium were used in this studies.

Prebiotic Effect

For the Prebiotic activity assay, the *Lactobacilli* cultures were streaked on MRS agar and incubated at 37 °C for 48 hours, *Bifidobacterium animalis* subsp. *lactis* was streaked on Bifidobacterium agar in anaerobic conditions using an anaerobic chamber and incubated at 37 °C for 72 hours. The developed microbial colony from the MRS plates were transferred into 10 ml MRS broth and incubated at 37°C for 16-24 hours. *Bifidobacterium animalis* subsp. *lactis* was transferred 10 ml bifido broth and incubated in the anaerobic chamber.

The sample being tested was prepared by taking different concentrations of powdered orange (at different concentration viz.2, 4, 6, 8, 10%) and dissolved in 10 ml of 1X PBS in test tubes. Approximately 100 µl of the diluted overnight cultures were added to the sample mixture. The samples were then incubated at 37 °C for 24 hours. The overnight inoculated samples were then diluted by taking 1ml of the sample mixture and adding it to 9 ml saline solution. Once the dilutions were made, 20 µl from the dilutions 10^{-5}, 10^{-6} and 10^{-7} were placed on an agar plate, MRS for lactobacilli cultures and Bifido for *Bifidobacterium animalis.* The plates were then incubated at 37 °C for 24 hours and colonies were counted.

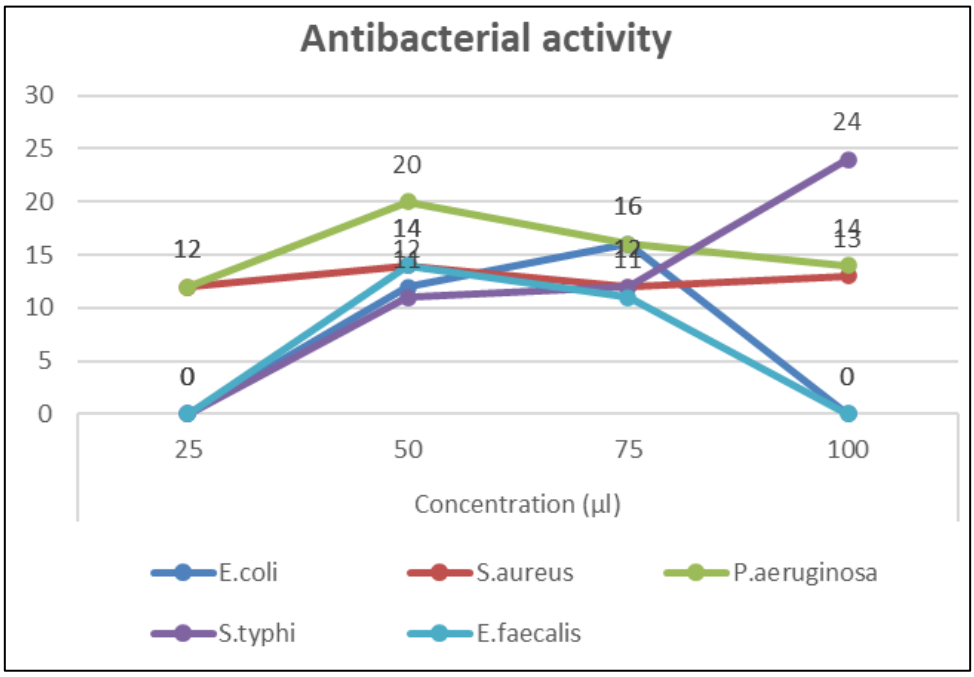
Results and Discussion

The antibacterial activity and prebiotic effect of orange peel extract were assessed in the present study and were tabulated and discussed in table 2 and 3.

Table 2: Antibacterial activity of orange peel extracts by using Agar well diffusion method

Name of the Pathogen	Antibacterial Activity				
	Concentrations of the Sample (µl) Zone of Inhibition (mm)				STD (Positive control)
	25	50	75	100	
Escherichia coli	-	12	16	-	26
Staphylococcus aureus	12	14	12	13	26
Pseudomonas aeruginosa	12	20	16	14	32
Salmonella typhi	-	11	12	24	34
Enterococcus faecalis	-	14	11	-	30

STD- Standard (Tetracycline 10µg/well)



Graph 1: Antibacterial activity of orange peel extract on different pathogenic bacteria

Despite the ethanol extract exhibited higher inhibitory activities than aqueous extract, the antibacterial activity was low. Results ranged from 11-20 mm in diameter. Consequently, the minimum inhibitory concentration (MIC) determined did not show any zone of inhibition. The MIC result was traceable to the fact that the two fold serial dilution reduced its initial concentration of 100mg/ml to 25mg/ml and then to 0.1mg/ml which had no visible effect on the organisms resulting in no zone of inhibition.

A low level of activity at a low extract concentration might suggest that the concentrations of the active constituent in the extracts were too low for any appreciable antibacterial activity (Ashebir and Ashenati, 1999) [1]. Further, low concentration of diffusible water soluble active constituents or excessive heating which often affect biologically active substances such as flavonoids, essential oils and other heterogeneous phyto-constituents present in the extract (Scalbert, 1991) [7] might also influence their respective activity. Preliminary phytochemical analysis revealed the presence of alkaloids, tannins, saponins, flavonoids, steroids and terpenes. It was also possible that the plant showed low antibacterial potential because all the aforementioned secondary metabolites were present in low concentration and the concentration of plant extract used was also low. In addition, the positive control (Tetracycline) had the widest zones of inhibition on all the organisms where the Dimetyl sulphoxide (DMSO) (negative control) had no effect on all the test organisms.

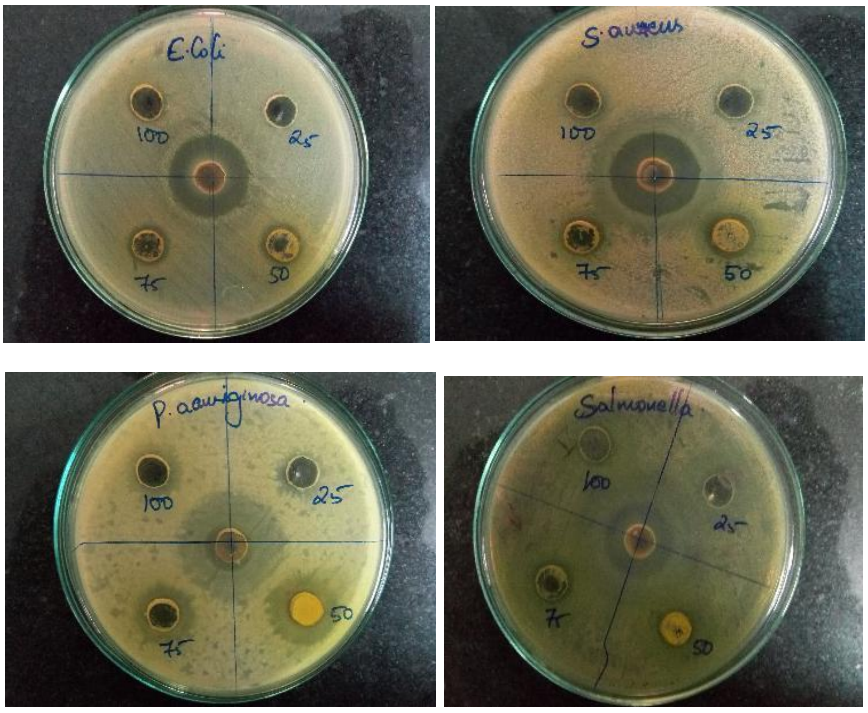


Fig 1: Antibacterial activity of Orange Peel Extract of different pathogenic bacteria

Table 3: Prebiotic effect of Lactobacillus and Bifidobacterium in orange peel extract

Dilutions	***Lactobacillus*** Colony (cfu/100µl)	***Bifidobacterium*** Colony (cfu/100µl)
2%		
10^{-5}	$5x10^6$	$2.6x10^7$
10^{-6}	$2.5x10^7$	$2.5x10^7$
10^{-7}	$2.5x10^8$	$1.8x10^7$
4%		
10^{-5}	$1x10^6$	$3.2x10^7$
10^{-6}	$2x10^8$	$1.2x10^8$
10^{-7}	$4.5x10^8$	$4.3x10^8$
6%		
10^{-5}	$1x10^6$	$1.23x10^8$
10^{-6}	$1.5x10^7$	$5.4x10^7$
10^{-7}	$2x10^8$	$1.2x10^8$
8%		
10^{-5}	$1x10^6$	$5.4x10^6$
10^{-6}	$8x10^7$	$7.2x10^7$
10^{-7}	$1.5x10^8$	$3.2x10^8$
10%		
10^{-5}	$1x10^6$	$1x10^7$
10^{-6}	$1.5x10^7$	$2.7x10^7$
10^{-7}	$3x10^9$	$1.3x10^8$

Now-a-days, there is considerable interest in using the diet to manipulate then gut microflora (Gibson and Roberfroid, 1995; Flint *et al.*, 2007) [4, 3]. Beneficial changes within the gut microbiota have been attributed to increases in *Bifidobacterium* and *Lactobacillus*, which were generally thought to have health promoting properties (Gibson and Roberfroid, 1995) [4]. The use of carbohydrates, known as prebiotics, that resist digestion and can be metabolized by certain gut bacteria has attracted most of this attention (Rastall, 2010) [6]. The prebiotic effect of *Lactobacillus* and *Bifidobacterium* at 8% dilution with 10^{-6} concentration were found to be the maximum as $8x10^7$ and $7.2x10^7$ respectively.

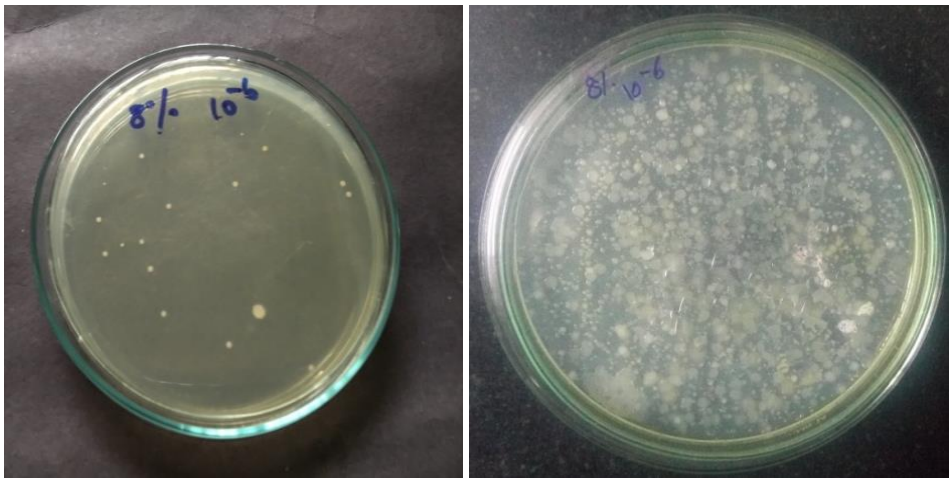


Fig 2: Prebiotic activity of *Lactobacillis* and *Bifidobacterium* cultures at 8% conc. (10^{-6} dilution)

Conclusion

From the present investigation, it was concluded that aqueous extract of orange peel had potent antibacterial activity against gram positive and gram negative bacteria. Plant based antimicrobials have enormous therapeutic potentials as they can serve the purpose with lesser side effects that are often associated with synthetic antimicrobials. There is need for further exploration of plant-derived antimicrobials. Prebiotics have been suggested to have several beneficial effects, including promotion of beneficial bacterial growth, stimulation of intestinal peristalsis, production of short chain fatty acids, and a shortened orofecal transit time (Cummings *et al.*, 2001) [2]. Also further studies will be needed to purify the bioactive compounds of the ethanol extracts and characterize the aqueous fraction of the plants and their phytochemical mode of action along with the prebiotic effect should be further investigated.

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