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Antimicrobial activity of different *Citrus species* against different pathogenic bacteria

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Abstract
The rapid growth of the food processing industry and the consumption of processed foods are demanding some natural antimicrobial agents because of their non-toxic nature in long-term uses. The present study was conducted to determine the antimicrobial potential of peel extract of four *Citrus species* collected from four different districts of Uttrakhand, India viz. (*Citrus aurentifolia, Citrus jambhiri, Citrus lemon* and *Citrus sinensis*) having different altitude. The activity was performed by agar well diffusion method against two gram-positive and three gram-negative bacteria. The results concluded that out of four citrus species, the *Citrus lemon and Citrus sinensis* showed a significant antimicrobial potential against all isolated bacterial strains but among these five bacteria the antimicrobial activity against bacillus subtilis showed maximum and hence they can be used as a natural antimicrobial agent in the food processing industry.

Keywords: Antimicrobial, citrus, phytochemical, peel waste

Introduction
Citrus is the one of the most important commercial fruit crops grown in all continents of the World. Citrus fruits are widely consumed around the world because of their specific flavors and nutritional benefits (Yao *et al*., 2004) [13]. Citrus fruits are mainly used by juice processing industries, while the peels are generally wasted. Since the juice yield of citrus is less half of the fruit weight, very large amounts of by-product wastes, such as peels are formed every year (Manthey and Grohmann, 2001) [8]. Peel waste are highly decomposable and are seasonal, is a problem to the processing industries and pollution monitoring agencies. There is always an increased attention in bringing useful products from waste materials and citrus waste are no exceptions. By-product recovery from fruit wastes can improve the processing units of overall economics. Besides this, the problem of environmental pollution also can be reduced considerably. The citrus peels are rich in nutrients and contain many phytochemical polyphenolic compounds can be efficiently used as drugs or as food supplements too (Wilkinson *et al*., 2003) [12]. The structural diversity of plant-derived compounds is immense and, the impact of antimicrobial, action they produce against microorganism depends on their structural configuration. The discovery of natural drugs from natural sources is highly important because many isolated molecules are complex (Dhanik *et al*., 2017) [3]. Peels are used in numerous desserts, jams and marmalades, candied peels, as well as cookies, cakes, and candies. Oil derived from orange peels, as well as flowers, leaves, and twigs is used as an essential oil in perfumes; orange seed oil may also be used in cooking or as a component in plastics. Orange blossoms produce more nectar than any other source in the U.S., and are important for honey production (more than 25% of honey produced in California is from orange groves (Morse and Calderone, 2000) [9]. Since there is an increase in the number of antibiotic resistance pathogens, there is always a search of an alternative drug. One of the possible solutions is the development of new drugs to meet the challenge of antimicrobial resistance (Sharma *et al*., 2005) [10]. Citrus peels if proved to have antibacterial activity; they can be also used in same food industry which generates large peel wastes as food preservatives (Ippolito *et al*., 2000) [5]. In the present study the fruit peel of four important plants such as *Citrus aurentifolia*, *Citrus lemon*, *Citrus jambhiri* and *Citrus sinensis* belonging to the family *Rutaceae* were selected for assessing their antibacterial properties. The antimicrobial agents are the agents that kill or inhibit the growth of other micro organisms. These have potential benefits over synthetic antimicrobials (Tagoe *et al*., 2010) [11].

Natural antimicrobials received popularity from a series of issues related to microorganisms control and as a source of pharmaceutical active compounds (Kummerer, 2009) [7].

Materials and Methods: Sources of test organism: The antibacterial screening of extract of Citrus accession was evaluated against four pathogenic bacterial strains *Escherichia coli, Salmonella typhi, Bacillus subtils, Pseudomonas aerogienosa* and *Microbacterium species*. The bacterial strains used for the antibacterial study was isolated from different meant samples in a department of Veterinary and Public Health, Pantnagar, India. Antibacterial screening of the extract against these bacteria was done by disc diffusion method with slight modification of and was measured by the zone of inhibition.

Preparation of bacterial inoculation

For the preparation of bacterial inoculums Luria Bartani a Broth for *Escherichia coli*, buffered peptone water (Hi-media) for *Salmonella typhi*, nutrient broth for *Bacillus subtilis*, *Salmonella typhi, Microbacterium species* and *Pseudomonas aeruginosa* were weight and poured in distilled water as per manufactures instruction. The test tubes containing culture media was sterilized in an autoclave at 120 ºC and 15-20 lbs for 0.5 hours. Bacterial colonies were inoculated in the test tube in above-prepared broths. The test tube containing bacterial colonies showed a marked turbidity in the tubes and were used to conduct the further experiment.

Preparation of agar plates

(Alper *et al*., 1958) [1] Difco Nutrient Agar (1.5%) was used for the preparation of plate's media. The media was prepared in distilled water, autoclaved and gently cooled. Thereafter, the prepared media was poured in Petri plates (dia. 9cm) in laminar flow and kept undisturbed as such till it got solidified. After solidification, these plates were incubated at 37 ºC overnight for sterile testing.

Antibacterial screening of extract by disc diffusion method

Antibacterial screening of methanolic extract was performed by disc diffusion method, which is the most common method to evaluate the antimicrobial activity (Bauer *et al*., 1966)[2]. Bacterial inoculums 100 µl was added to the agar plates and uniformly spread over the surface using the spreader. Sterilized disc of 5 mm diameter soaked in the different methanolic concentration of extracts (250,500,750 &1000 µl) was placed on the inoculated plate. These plates were incubated at 37 ºC overnight to observe the zone of inhibition formed by the standard antibiotic gentamicin (10 µg/disc). The sterile paper discs impregnated with methanol served as negative control. After incubation relative susceptibility of each organism was determined by the clear zone of inhibition of growth around the disc impregnated with the extracts as well as the antibiotic. Zone of inhibition (mm) was measured with the help of scale.

Peel extract of citrus fruits

Preparation of extract: The peel of Citrus species was homogenized in methanol solvents individually and mixed well. The extract obtained from peel of *Citrus jambhiri* (Rough Lemon), *Citrus aurantifolia* (key lime), *Citrus lemon* (galgal) and *Citrus sinensis* (sweet orange) were individually tested against pathogenic and non-pathogenic microorganism including three gram-negative (*Salmonella typhi, Escherichia Coli, Pseudomonas aeruginosa*) and two gram-positive (*Bacillus sebtilis* and *Microbacterium species*). These were collected from Department of Microbiology, college of basic science and humanities Pantnagar.

Statistics Analysis

All the experiments were carried out in triplicates. The data represent the mean of triplicate values. An analysis of variance (ANOVA) was done using STPR programming to compare the mean values when two way ANOVA showed significant differences. P-value of less than 0.05 was considered statistically significant.

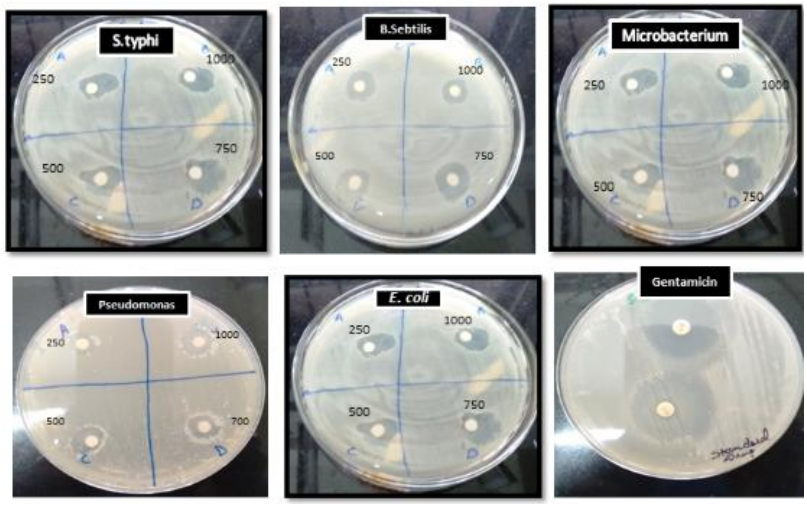

Photo slide: Antimicrobial Activity of peel extracts showed zone of inhibition in (mm) against different bacteria

Results and Discussion

The *in-vitro* antimicrobial activity of extracts of four different *Citrus species* against five bacterial strains, estimated by the zone of inhibition varied according to samples and bacterial strains with different altitude, on the increasing of altitude the antimicrobial activity of *citrus* peel was increases against most of the bacteria. Samples of citrus species collected from four different districts of uttarakhand. In (Table-1 or Fig-1), extract of *citrus aurentifolia* results revealed that the inhibition of bacterial growth was dose-dependent which showed strongest antimicrobial activity towards *bacillus subtilis* and *E. coli* in comparison to other bacteria, on increasing concentration towards to 1000 ppm. In (Table-2 or fig-2), extract of *citrus lemon* the inhibition of bacterial

growth was dose-dependent and showed strongest antimicrobial activity towards all bacteria. In (Table-3or fig-3), extract of *Citrus jambhiri* showed strongest antimicrobial activity towards *Bacillus subtilis* and *Salmonella typhi, E. coli* and *Pseudomonas aeroginosa* in comparison to micro bacterium and In (Table-4 or fig-4), extract of *Citrus sinensis* showed strongest antimicrobial activity towards *Bacillus subtilis* in comparison to other. Major or trace compounds in the extract give rise to the antimicrobial activity exhibited. Results found are in accordance with (Hayes and Markovic, 2002) [4] *citrus* oil and extracts was shown to possess significant antimicrobial activity against the organisms *Salmonella typhi, Escherichia coli, Pseudomonas aeruginosa, Candida albicans, Methicillin-resistant S. aureus, Aspergillus niger, Klebsiella pneumoniae and Propionibacterium acnes* comparable to its major component-citral (Iturriaga *et al*.,2012)[6] which confirmed that Citrus species show potential inhibition of against *E.coli* and *S.typhi*.

Table 1: showing diameter of zone of inhibition (in mm) of Citrus Aurantifolia (CA) extract collected from four different district of Uttarakhand.

Altitude	Conc. *ppm*	*S.typhi*	*Bacilus sebtilis*	*E.coli*	*P.aeruginosa*	*Microbacterium.*
Pithoragarh CA1 1514 m	250	13.33±1.53de	11.33±1.15def	13.67±1.15de	13.33±1.53c	11.33±0.58fg
	500	14.00±2.65de	12.00±1.00de	15.00±1.00cd	14.00±2.65cd	12.00±0.00ef
	750	15.00±1.00cd	13.00±0.00de	15.33±0.58cd	15.67±0.58bc	13.00±0.00ef
	1000	16.00±1.00cd	12.67±2.52de	16.00±1.00cd	16.00±1.00bc	13.00±2.00ef
Almora CA2 1642 m	250	10.67±0.58ef	13.00±2.00de	11.33±0.58e	10.67±0.58de	13.33±0.58e
	500	11.67±1.15ef	14.33±2.08d	12.00±1.00e	12.33±0.58d	14.00±1.00de
	750	12.67±1.15d	16.67±1.53cd	13.00±1.00de	12.67±1.15cd	15.00±1.00d
	1000	15.00±0.00cd	17.67±1.53bc	15.33±0.58cd	15.00±0.00bc	15.33±0.58c
Nainital CA3 2084 m	250	10.33±0.58f	12.33±0.58de	12.33±0.58de	10.33±0.58e	12.00±1.00ef
	500	12.33±1.15ce	12.33±3.06de	12.33±1.15de	12.33±1.15d	12.67±3.21ef
	750	15.67±1.15cd	13.00±2.65de	15.67±1.15cd	15.67±1.15bc	13.00±2.65e
	1000	16.33±0.58bcd	14.33±0.58d	16.33±0.58bc	16.33±0.58b	14.00±1.00de
Rudrprayag CA4 895 m	250	13.33±2.08cde	11.00±1.00ef	13.33±2.08de	11.33±0.58d	11.33±0.58fg
	500	13.67±3.06cde	12.00±0.00ef	13.67±3.06de	13.67±3.06cd	12.00±0.00ef
	750	16.67±1.53bc	13.00±0.00de	16.67±1.53bc	16.67±1.53ab	13.00±0.00ef
	1000	17.00±1.00b	13.00±2.00de	17.00±1.00bc	17.00±1.00ab	13.00±2.00ef
Gentamicin		22.33±0.58[a] | 22.33±0.58[a] | 24.33±0.58[a] | 24.00±1.00[a] | 14.81±0.58[bc] | 20.67±0.58[a]	24.33±0.58[a]	24.00±1.00[a]	24.81±0.58[a]	20.33±0.58[a]
Cd at 5%		2.380	1.784	2.555	1.432	2.669

*****Each data represent the mean value of five samples. Means with the same letters are not significantly differed at $P \leq 0.05$

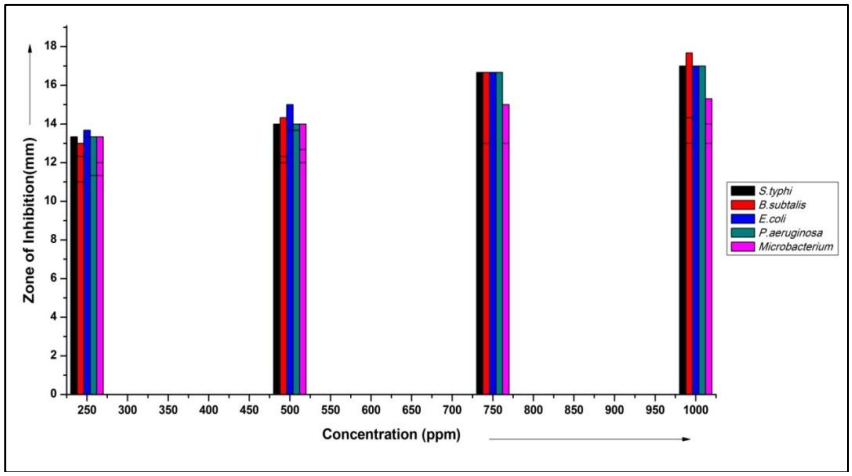

Fig 1: Zone of inhibition in different bacterial strains by *Citrus aurentifolia*.

Table 2: showing diameter of zone of inhibition (in mm) of Citrus Lemon (CL) extract collected from four different district of uttarakhand.

Altitude	Conc. *ppm*	*S.typhi*	*Bacilus sebtilis*	*E.coli*	*P.aeruginosa*	*Microbacterium.*
Pithoragarh CL1, 514 m	250	14.67±0.58cd	13.67±0.58de	14.67±0.58cd	14.67±0.58bc	13.33±0.58e
	500	15.33±0.58bc	14.67±1.53cd	15.33±0.58cd	15.33±0.58bc	14.00±0.00de
	750	16.33±1.53bcd	15.67±0.58cd	16.33±1.53bc	16.33±1.53b	15.00±0.00d
	1000	17.33±1.53bc	16.67±1.15bc	17.33±1.53b	17.33±1.53ab	15.67±0.58cd
Almora CL2 1642 m	250	16.33±0.58bc	13.67±3.06de	16.33±0.58bc	16.33±0.58b	15.33±1.53cd
	500	17.00±1.00bc	15.33±1.15c	17.00±1.00bc	17.00±1.00ab	17.00±1.00bc
	750	18.00±0.00bc	16.67±2.52c	18.00±0.00b	18.00±0.00ab	18.00±1.00bc
	1000	18.00±1.73bc	17.00±2.65bc	18.00±1.73b	18.00±1.73ab	18.00±0.00bc
Nainital CL3 2084 m	250	16.00±1.00bc	12.33±1.53de	16.00±1.00cd	16.00±1.00bc	15.67±0.58cd
	500	17.00±1.00bc	13.00±1.00de	17.00±1.00bc	17.00±1.00ab	16.33±1.15cd
	750	17.33±1.53bc	14.67±0.58c	17.33±1.53b	17.33±1.53ab	16.67±1.53c
	1000	18.00±1.00b	15.67±0.58c	18.00±1.00b	18.00±1.00ab	18.33±0.58b
Rudrprayag CL4 895 m	250	15.67±0.58c	13.00±1.00de	15.67±0.58cd	15.67±0.58bc	15.33±1.53cd
	500	16.33±1.15c	14.00±1.00d	16.33±1.15bc	16.33±1.15b	17.00±1.00bc
	750	16.67±1.53bc	15.00±1.00cd	16.67±1.53bc	16.67±1.53ab	18.00±1.00bc
	1000	18.33±0.58b	15.33±0.58cd	18.33±0.58b	18.33±0.58a	18.33±0.58b
Gentamicin		22.33±0.58[a] | 22.33±0.58[a] | 24.33±0.58[a] | 24.00±1.00[a] | 14.81±0.58[bc] | 20.67±0.58[a]	24.33±0.58[a]	24.00±1.00[a]	24.81±0.58[a]	20.33±0.58[a]
Cd at 5%		1.805	1.02	1.927	2.856	1.519

*****Each data represent the mean value of five samples. Means with the same letters are not significantly differed at $P \leq 0.05$

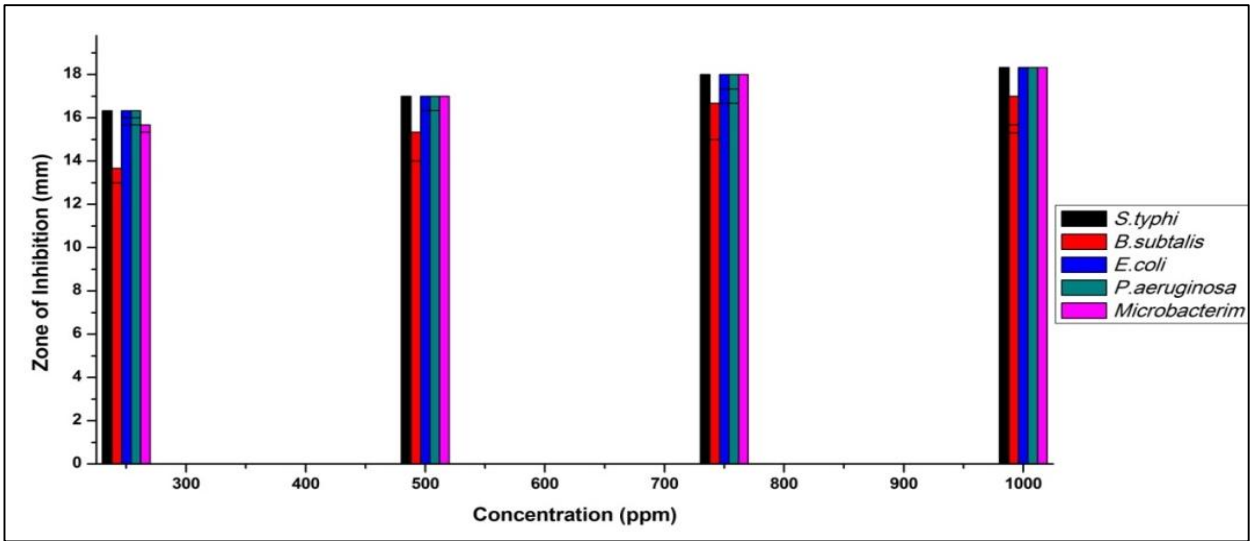

Fig 2: Zone of inhibition in different bacterial strains by *Citrus lemon*.

Table 3: showing diameter of zone of inhibition (in mm) of Citrus Jambhiri (CJ) extract collected from four different district of uttarakahnd

Altitude	Conc. *ppm*	*S.typhi*	*Bacilus sebtilis*	*E. coli*	*P.aeruginosa*	*Microbacterium.*
Pithoragarh CJ1, 1514 m	250	11.00±1.00de	11.33±0.58ef	11.00±1.00e	11.00±1.00de	11.33±1.15fg
	500	11.33±0.58de	13.00±2.00de	11.33±0.58e	11.33±0.58de	12.00±1.00ef
	750	12.67±0.58de	14.33±0.58d	12.67±0.58de	12.67±0.58cd	13.00±0.00ef
	1000	12.33±3.06de	16.00±1.00cd	12.33±3.06de	12.33±3.06d	13.67±2.52ef
Almora CJ2 1642 m	250	13.33±1.53cde	10.67±0.58ef	13.33±1.53de	13.33±1.53cd	11.67±0.58f
	500	15.00±1.00cd	11.33±0.58de	15.00±1.00cd	15.00±1.00bc	12.67±0.58ef
	750	17.00±1.00bc	12.67±1.15de	17.00±1.00bc	17.00±1.00ab	13.33±0.58e
	1000	18.00±1.00b	14.33±1.15de	18.00±1.00b	18.00±1.00ab	14.33±0.58de
Nainital CJ3 2084 m	250	12.00±1.00e	10.00±0.00f	12.00±1.00e	12.00±1.00de	11.67±0.58f
	500	12.67±3.21d	12.33±1.15de	12.67±3.21de	12.67±3.21cd	12.67±0.58ef
	750	13.00±2.65cde	15.33±0.58cd	13.00±2.65de	13.00±2.65cd	13.33±0.58e
	1000	14.00±1.00cde	16.00±0.00cd	14.00±1.00d	14.00±1.00cd	14.33±0.58de
Rudrprayag CJ4 895 m	250	11.33±0.58e	14.67±0.58cd	11.33±0.58e	11.33±0.58de	9.00±1.00g
	500	12.00±0.00def	14.00±3.61d	12.00±0.00e	12.00±0.00de	9.33±0.58g
	750	13.00±0.00cde	16.33±1.15cd	13.00±0.00de	13.00±0.00cd	11.00±0.00fg
	1000	13.00±2.00cde	16.67±0.58c	13.00±2.00de	13.00±2.00cd	12.33±0.58ef
Gentamicin		22.33±0.58[a] 22.33±0.58[a] 24.33±0.58[a] 24.00±1.00[a] 14.81±0.58[bc] 20.67±0.58[a]	24.33±0.58[a]	24.00±1.00[a]	24.81±0.58[a]	20.33±0.58[a]
Cd at 5%		2.523	2.380	1.161	1.861	2.240

*****Each data represent the mean value of five samples. Means with the same letters are not significantly differed at $P \leq 0.05$

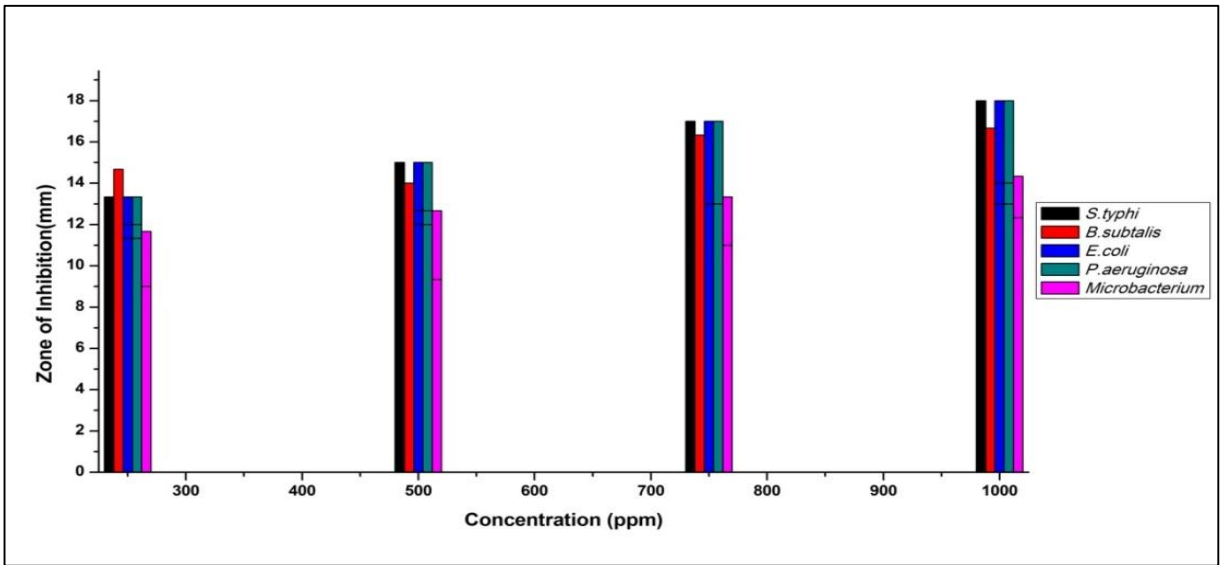

Fig 3: Zone of inhibition in different bacterial strains by *Citrus jambhiri*.

Table 4: Showing diameter of zone of inhibition (in mm) of Citrus sinensis (CS) extract collected from four different district of uttarakhand.

Altitude	Conc, *ppm*	*S. typhi*	*Bacilus sebtilis*	*E.coli*	*P.aeruginosa*	*Microbacterium.*
Pithoragarh CS1 1514 m	250	14.00±1.00cde	17.00±1.00bc	14.00±1.00d	14.00±1.00cd	8.67±1.53g
	500	14.33±1.53cde	17.67±1.53bc	14.33±1.53cd	14.33±1.53c	9.33±0.58g
	750	15.33±0.58cde	18.00±1.00bc	15.33±0.58cd	15.33±0.58bc	10.67±0.58fg
	1000	17.00±1.00bc	19.00±1.00b	17.00±1.00bc	17.00±1.00ab	12.33±0.58ef
Almora CS2 1642 m	250	15.33±1.53cd	16.33±0.58c	15.33±1.53cd	15.33±1.53bc	9.33±0.58g
	500	17.00±1.00bc	17.00±1.00bc	17.00±1.00bc	17.00±1.00ab	9.67±0.58g
	750	18.00±1.00b	18.00±0.00bc	18.00±1.00b	18.00±1.00ab	10.67±0.58fg
	1000	18.00±0.00b	18.00±1.73bc	18.00±0.00b	18.00±0.00ab	12.33±0.58ef
Nainital CS3 2084 m	250	13.67±0.58de	16.67±0.58c	13.67±0.58de	13.67±0.58cd	9.33±0.58g
	500	14.00±0.00de	17.67±0.58bc	14.00±0.00d	14.00±0.00cd	9.67±0.58g
	750	15.00±0.00cd	17.67±0.58bc	15.00±0.00cd	15.00±0.00bc	10.67±0.58fg
	1000	15.67±0.58cd	18.00±1.00bc	15.67±0.58cd	15.67±0.58bc	12.67±0.58ef
Rudrprayag CS4 895 m	250	13.33±0.58de	15.67±0.58cd	13.33±0.58de	13.33±0.58cd	10.00±1.00g
	500	14.00±1.00cde	16.33±1.15c	14.00±1.00cd	14.00±1.00cd	10.33±0.58fg
	750	15.00±1.00cde	16.67±1.53c	15.00±1.00cd	15.00±1.00bc	10.67±0.58fg
	1000	15.33±0.58cde	18.33±0.58bc	15.33±0.58cd	15.33±0.58bc	12.33±0.58ef
Gentamicin		22.33±0.58^{a} | 22.33±0.58^{a} | 24.33±0.58^{a} | 24.00±1.00^{a} | 14.81±0.58^{bc} | 20.67±0.58^{a}	24.33±0.58^{a}	24.00±1.00^{a}	24.81±0.58^{a}	20.33±0.58^{a}
Cd at 5%		1.523	1.469	1.927	1.161	2.240

*****Each data represent the mean value of five samples. Means with the same letters are not significantly differed at P ≤ 0.05

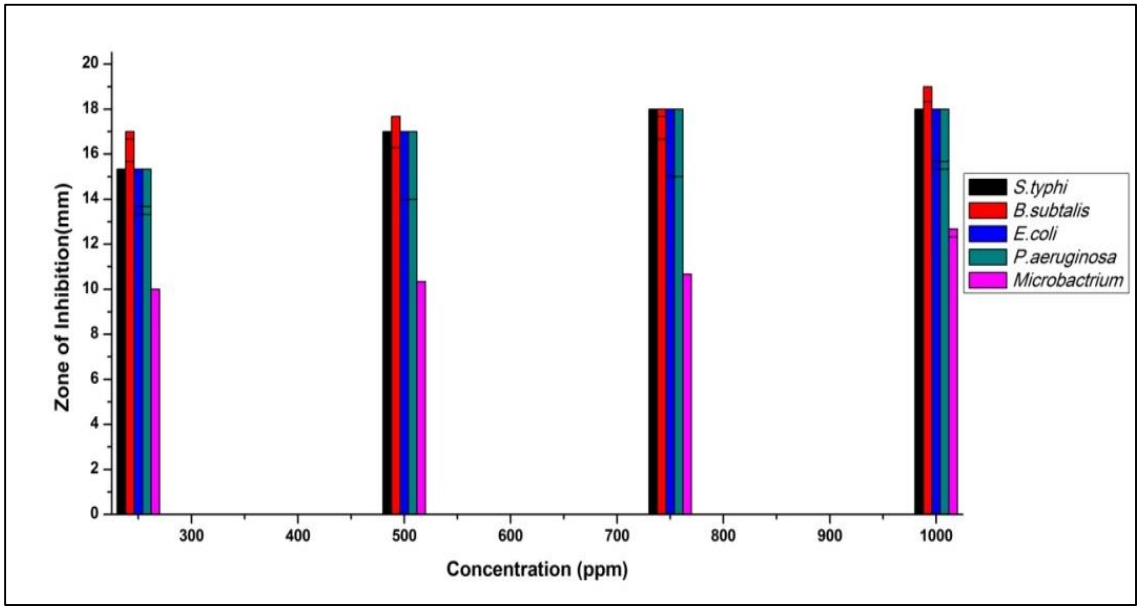

Fig 4: Zone of inhibition in different bacterial strains by *Citrus sinensis*.

Conclusion

Plants are the rich source of drugs used in primary health care for treating human ailments. The advantage of using plants as drugs are safe, low cost and more reliable than the synthetic products. Hence plants can be used as effective pharmacological agents. Since there is a growing demand for food that is free of synthetic chemicals as preservatives, it is necessary to examine and identify alternatives and safe approaches for controlling food born pathogen. Even though many natural products are currently being used for the

preservation and extension of the self-life of foods, there are still many unexplored sources. The use of natural compounds from plants could open up the possibility of using them as novel antimicrobials in food system remains limited mainly due to the side effects of undesirable flavor or aroma. The results of the present study support the recycling of fruit waste. Thereby, yielding new products and meeting the requirements of essential products required in human, animal and plant nutrition as well as in the pharmaceutical industry.

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