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PHYTOCHEMICAL SCREENING AND ESTIMATION OF ANTIMICROBIAL ACTIVITY OF CITRUS PEEL

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A B S T R A C T

The present research was conducted in Lab. to evaluate the efficacy of aqueous and methanolic extract of *Citrus reticulate* fruit. The antimicrobial activity of *C. reticulate* fruit peel extracts were tested against ten bacterial and five fungal strains. The stock solution of extract was prepared and 1%, 2%, 3%, 4% and 5% concentrations of aqueous and crude extracts were used for antimicrobial assays by agar well diffusion technique. Afterwards results of zone of inhibition (mm) of different bacterial and fungal agents at various concentrations of *C. reticulate* are expressed as mean ± standard deviation. Extracts obtained from the organic solvent and water showed different antibacterial activity with the same bacterial strains. The antibacterial efficacy of methanolic peel extract against pathogenic bacteria showed varied level of inhibition. Moreover, in case of methanolic peel extract the growth of *Syntrophospora* sp., *A. xylinum*, *M. visconsensis*, *X. nematophilis* and *Oscillospira* sp. were exhibited maximum inhibition zone ranging from 36.0-47.0mm. However no antifungal activity was observed against aqueous and solvent extracts. The results of the phytochemical screening indicated the presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroids. The success of present investigation could lead to the development of cheap, easily available and relatively safe bactericides from citrus plant.

Keywords: Citrus, fruit peel, antimicrobial activity, methanolic extract, phytochemical tests

INTRODUCTION

Citrus belongs to family Rutaceae, common name is orange and this descends from South Eastern Japan, probably in Indian or Southern Chinese suppliers (Ekwenye and Edeha, 2010). Citrus in general contain sugar, polysaccharide, organic-acid, fats, carotenoids, vitamins, minerals, flavonoid and it's an excellent resource of potassium, calcium mineral and vitamin C (Cowan, 1999). Major flavanones and poly-methoxy flavones of citrus farming is possibly one of the most essential professional and commercial agricultural activities of the world (Ortuno *et al.,* 2006). Citrus juice has been revealed to demonstrate anti-microbial activity against *Vibrio cholera* (Hiroyuki *et al.,* 2006).

The peel of citrus or lime fruits and vegetables is a rich resource of flavanones and many polymethoxylated flavones, which are very rare in other plants. These substances, not only perform an essential physical and ecological role, but are also of commercial attention because of their wide range of applications in the food and pharmaceutical sectors. An anti-microbial is a substance that destroys or stops the development of bacteria, fungus, or protozoa. Medications are generally used against bacteria and anti-fungal are used to cure fungus attacks, some of these adverse reactions can be debilitating if the drug is not used properly. Several microbial derived antibiotics are currently in use to cure a variety of individual disease, therefore the action must be taken to control the use of antibiotics, develop new drugs either artificial or organic, for a long period, plant have a valuable source of organic products for maintaining individual health (Pandey *et al.,* 2011). The purpose of present research was to estimate the antimicrobial activity of peel extract (methanolic and aqueous) of *C. reticulata* against bacterial and fungal

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strains in vitro and their pytochemical analysis which are responsible for antimicrobial activity.

MATERIALS AND METHODS

Test microorganisms: The tested bacterial strains [*Xanthomonas axonopodis* (FCBP: 007), *Pseudomonas syringae* (FCBP: 010), *Xanthomonas nematophilis* (FCBP: 112), *Xenorhabdus luminescens* (FCBP: 119), *Kurthia gibsonii* (FCBP: 225), *Syntrophospora sp.* (FCBP: 226), *Acidovorax temperans* (FCBP: 227), *Oscillospira sp.* (FCBP: 231), *Acetobacter xylinum* (FCBP: 239) and *Moellerella visconsensis* (FCBP: 253)] and fungal cultures [(*Alternaria alternata* (FCBP: 1238), *Fusarium oxysporum* (FCBP: 1211), *Curvularia clavata* (FCBP: 1251), *Aspergillus niger* (FCBP: 1214) and *Aspergillus flavus* (FCBP: 1142)] were procured from First Fungal Culture Bank of Pakistan (FCBP), Institute of Agricultural Sciences (IAGS), Lahore Pakistan.

Material collected: Plant used in this study was *Citrus reticulate L.* (Bitter Orange or lemon orange). The peels were collected from the local fruit juice shops. After collection, the peel was shade dried at room temperature (30–35 ^{o}C). 20g of peel of oranges and lemon was coarsely powdered using a mortar and pestle and were further reduced to powder using an electric blender for 15 min. The powder was transferred into closed containers (150 ml) for further use.

Extract Preparation: Aqueous extract: 20 g of powdered samples of citrus peel was weighed and suspended in 200 ml of water for half an hour followed by filtering through Whattman filter paper. These extracts were evaporated to get paste which was transferred and stored in sterile bottles under refrigerator at 4 ^{o}C for further use. *Methanolic extract:* 20 g of powdered samples were added to 200 ml of methanol and extraction was done for 72 hours, followed by filtering through Whattman filter paper. The extracts were later evaporated to get paste of samples which were stored and refrigerated at 4 ^{o}C for supplementary tests.

Concentration of extract: The stock solution of the methanoloc extract was prepared by dissolving 10 mg of the fruit peel extract in 10 ml dimethyl sulfoxide (DMSO). The following concentrations were prepared: 5%, 4%, 3%, 2% and 1% of aqueous and crude extract. For antimicrobial sensitivity testing, concentrations taken were from 1 to 20 µg/ml. Standard antibacterial agents, Tetracycline, and antifungal agent, Fluconazole (Belco Pharmaceuticals, Bahadurgarh, India) served as positive control. DMSO (HPLC grade) was used as negative control.

Antimicrobial assay: The fungal and bacterial spore suspensions were prepared in normal saline by transferring the organism from fresh cultures (1×10^8 cells/ml). Antimicrobial assay was performed with the help of agar well diffusion method (Tanaka *et al.,* 2002). Nutrient agar plates were prepared for bacterial species while Malt extract agar for fungal isolates. 50µl inoculum of each species was uniformly spread on agar plates with the help of spreader, after 5 minutes one well 8 mm diameter was with the help of sterilized borer. The equal volume (50 µl) of each concentration of aqueous and methanolic extract was poured into the wells on separate plates. The plate was incubated overnight at 37 ^{o}C for bacterial species and at 25±2 ^{o}C for fungal isolates. After incubation the zones of inhibition were measured and recorded. These studies were performed in triplicate. Data was statistically analyzed.

Phytochemical screening of extract: The citrus peel extract was analyzed for presence of various bioactive compounds.

1. Ninhydrin test: This was performed by following the method described by Ali *et al.* (2014). Crude extract was boiled at 45 ^{o}C with 2 ml of 0.2% solution of ninhydrin, appearance of violet color indicated the presence of amino acids and proteins.

2. Iodine test: Iodine test was carried out according to the method used by Bansode and Chavan (2012). Crude juice was mixed with 2 ml of iodine for 10 min in beaker. Appearance of dark purple or blue coloration indicated the existence of carbohydrates.

3. Phenols and Tannins test: Phenols and Tannins indication test was carried out according to the process described by Lawal *et al.* (2013). Crude juice was mixed with 5 ml of distilled water and heated at 45 ^{o}C for 10 minutes, then added 5-6 drops of 1% ferric chloride. Appearance of dark green or blue black color shows the existence of phenols and tannins.

4. Alkaline reagent test: According to Lawal *et al.* (2013) test was done by accumulation of 2 ml of NaOH in crude juice then mixture appeared into yellow. After wards yellow color turned into colorless by adding 2 ml conc. H_2SO_4 which indicated the presence of flavonoides.

5. Saponins test: This test was done by mixing of 0.2 ml of crude juice with 4.8 ml of distilled water in a test tube and it was shaken vigorously with the help of vortex machine. The presence of saponins was

indicated by the presence of stable foam in the test tube (Lawal *et al.,* 2013).

6. Salkowski's test: Lawal *et al.* (2013) illustrated this test by merging of 2 ml of chloroform and 2 ml of concentrated H_2SO_4 in crude extract with gentle shaking. The steroidal reddish ring around the test tube showed the glycone portion of the glycoside.

7. Steroid test: Bansode and Chavan (2012) described this test by sidewise adding of 2 ml chloroform, 2 ml conc. H_2SO_4 and 2 ml acetic acid into crude extract. After that mixture turned into greenish coloration indicated the presence of steroids.

8. Cardiac glycosides test: This test was exhibited followed by the method described by Ali *et al.* (2014). Crude extract (2 ml) was mixed with 1ml of glacial acetic acid containing 1-2 drops of $FeCl_3$ solution; this was under layered with 1ml of conc. H_2SO_4. Brown coloration shows the characteristics of cardiac glycoside.

9. Phlobotannins test: This test was carried out according to the method used by Pandey *et al.* (2011) method. For phlobotannins test citrus extract was boiled with 1% HCl at 45 ^oC and then red precipitates were observed.

10. Terpenoids test: Five milliliter of sample was added to 2 ml of chloroform and 3 ml of conc. H_2SO_4. Reddish brown color formed shows the presence of terpenoids (Ali *et al.,* 2014).

11. Carbonyl test: Two milliliter of crud extract was mixed with few drops of 2, 4-dinitrophenyl hydrazine. Appearance of yellow crystals shows the presence of carbonyl (Kumar *et al.,* 2011).

RESULTS AND DISCUSSION

In the present study the antimicrobial activities of the peel of citrus fruits has been investigated. The antimicrobial effect of *C. reticulate* fruit peel study involves a comparison of the inhibition zones of its aqueous and methanolic extracts (Table 1-3) with those of commercially developed antibiotics.

Methanolic extract citrus peel showed a significant antibacterial activity against all the test organisms. The activity of *C. reticulate* fruit peel extract against both gram-positive and gram-negative bacteria is an indication of its broad spectrum activity, and thus can be used as source antibiotic substances for drug development that can be used for control of these bacterial infections. Results of zone of inhibition (mm) of different bacterial and fungal agents at various concentrations of *C. reticulate* are expressed as mean ± standard deviation (Table 1&2), respectively. As per the antimicrobial investigations, the methanolic extract exhibited antibacterial activity against *Oscillospira sp.* with 47.0 mm, and appreciable activity with *X. nematophilis* (41.3 mm) (Table 2). Consequently no antifungal activity was observed against aqueous and methanolic extracts of citrus fruit peel (Table 3). The phytochemical screening results indicated the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides, gums, and mucilages), flavonoid glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils as the main constituents (Figure 1). Finally phytochemical analysis exposed the existence of secondary metabolites like steroids, alkaloids; phenols and flavonoids etc. were present in trace amounts in the peel (Table 4).

Table 1. Antibacterial Activity of aqueous extract of citrus peel against different bacterial species

FCBP Acc no	Name of Bacterial pathogens	Control	Inhibition zone in at various concentrations of Aqueous Extract				
			1%	2%	3%	4%	5%
007	*Xanthomonas axonopodis*	28.9±0.09d	11.8±0.12a	12.4±0.14a	13.0±0.09a	13.4±0.09b	14.0±0.12b
010	*Pseudomonas syringae*	35.8±0.05c	--- ND	--- ND	--- ND	--- ND	--- ND
119	*Xenorhabdus luminescens*	35.8±0.04c	--- ND	10.0±0.21cd	11.3±0.15c	12.0±0.09c	12.0±0.14c
225	*Kurthia gibsonii*	35.8±0.09c	--- ND	--- ND	--- ND	10.0±0.09e	10.3±0.15e
226	*Syntrophospora sp.*	40.9±0.09b	--- ND	--- ND	10.3±0.07e	11.0±0.04d	11.0±0.09d
227	*Acidovorax temperans*	28.9±0.09d	--- ND	--- ND	--- ND	--- ND	--- ND
239	*Acetobacter xylinum*	40.9±0.06b	11.0±0.01ab	12.0±0.15ab	12.0±0.09b	13.2±0.07bc	14.0±0.04b
253	*Moellerella visconsensis*	40.9±0.04b	10.0±0.08b	10.3±0.09c	11.0±0.08cd	12.0±0.09c	12.00.07c
112	*Xanthomonas nematophilis*	40.9±0.09b	--- ND	--- ND	--- ND	--- ND	--- ND
231	*Oscillospira sp.*	45.8±0.03a	10.0±0.09b	11.3±0.13b	12.5±0.24ab	14.8±0.09a	16.00.04a

ND: Activity not detected

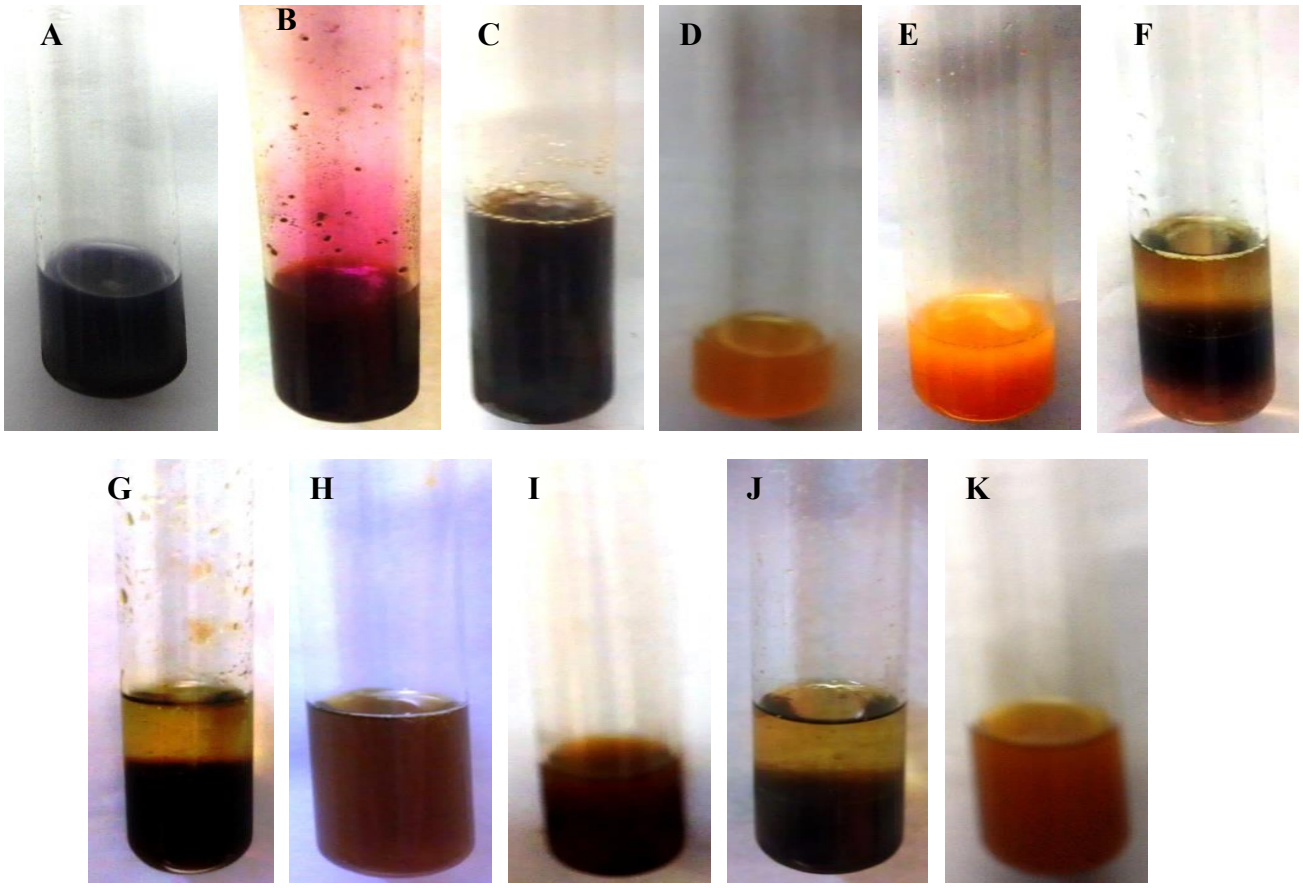

Figure 1. Phytochemical analysis of citrus peel extract. (A) Ninhydrine test (B) Iodine test (C) Tannin test (D) Flavenoides test (E) Saponins test (F) Salkowski test (G) Steroid test (H) Cardiac glycoside test (I) Phlobabatannis test (J) Terpenoids test (K) Carbonyl test.

Table 2. Antibacterial Activity of methanolic extract of citrus peel against different bacterial species.

FCBP Acc no	Name of Bacterial pathogens	Control	Inhibition zone at various concentrations of Methanolic Extract				
			1%	2%	3%	4%	5%
007	*Xanthomonas axonopodis*	28.9±0.09d	13.0±0.12e	14.3±0.14f	18.0±0.09g	19.3±0.09h	26.3±0.04g
010	*Pseudomonas syringae*	35.8±0.05c	--- ND	12.3±0.06g	18.3±0.08g	24.0±0.09e	31.0±0.14f
119	*Xenorhabdus luminescens*	35.8±0.04c	--- ND	15.0±0.21e	22.3±0.15e	29.0±0.09c	34.0±0.09e
225	*Kurthia gibsonii*	35.8±0.09c	--- ND	11.0±0.09h	15.0±0.06h	20.0±0.09g	34.0±0.21e
226	*Syntrophospora sp.*	40.9±0.09b	15.0±0.07d	17.0±0.09de	19.3±0.07f	26.0±0.04ef	37.0±0.09c
227	*Acidovorax temperans*	28.9±0.09d	12.0±0.09f	14.0±0.08f	15.0±0.09h	20.0±0.05g	26.3±0.09g
239	*Acetobacter xylinum*	40.9±0.06b	16.0±0.01c	20.0±0.15c	24.0±0.09d	27.0±0.07e	36.3±0.09d
253	*Moellerella visconsensis*	40.9±0.04b	15.3±0.08d	18.0±0.09d	25.0±0.08c	28.0±0.09d	37.0±0.04c
112	*Xanthomonas nematophilis*	40.9±0.09b	17.0±0.05b	22.0±0.09b	29.0±0.09b	36.0±0.08b	41.3±0.09b
231	*Oscillospira sp.*	45.8±0.03a	20.0±0.09a	24.3±0.13a	30.0±0.24a	37.0±0.09a	47.0±0.09a

ND: Activity not detected

Table 3. Antifungal Activity of aqueous and methanolic extract of citrus peel against different fungal species.

FCBP Acc no	Name of fungal pathogens	Inhibition zone in mm		
		Control	Aqueous extract	Methanolic extract
1238	*Alternariaalternata*	29.5±0.12	---- ND	---- ND
1211	*Fusarium oxysporum*	36.9±0.06	---- ND	---- ND
1251	*Curvulariaclavata*	41.7±0.09	---- ND	---- ND
1214	*Aspergillus nige*r	27.8±0.06	---- ND	---- ND
1142	*Aspergillus flavus*	37.8±0.09	---- ND	---- ND

ND: Activity not detected

Table 4. Phytochemical analysis of peel extract of citrus fruit.

Tests	Extract of citrus peel	Inference
Test for Proteins: Ninhydrin test	+	Proteins present
Test for Carbohydrates: Iodine test	+	Carbohydrates present
Test for Phenols and Tannins	+	Phenols and tannins present
Test for Flavonoids: Alkaline reagent test	+	Flavonids present
Test for Saponins	+	Saponins present
Test for Glycosides: Salkowski's test	+	Glycosides present
Test for Steroid	+	Steroids present
Test for Cardiac glycosides	+	Cardiac glycosides present
Test for Phlobotannins	+	Phlobotannins present
Test for Terpenoids	+	Terpenoids present
Test for Carbonyl	+	Carbonyl present

The peel of citrus or lime fruits are a prosperous source of flavanones and many polymethoxylated flavones, which are very unusual in other vegetation. These substances, not only play an important physical and environmental role, but are also of commercial interest because of their variety of applications in the food and farming industry. In present investigation the fruit extracts were prepared from dried samples as has been revealed previously by study (Ekwenye and Edeha, 2010). In order to get anti-microbial activity of various plant ingredients against test micro-organism, Agar well diffusion method was used as has been conducted previously by Ramachandra *et al.* (2013).

The anti-bacterial activity of the ingredients suggests their possible use for the treatment of attacks caused by the analyzed bacteria. Prasad (2012) and Maruti *et al.* (2011) revealed that the solvent extract of citrus peel showed activity against different microbial strains while evaluating their results with our findings shows that our peel extract has a high degree of anti-bacterial activity. However, this distinction may be because of the difference in the phytochemical composition in various parts of the plant or may be also due to the extraction method used or ecological factors or difference in the genotypes of the citrus peel used (Osarumwense *et al.,* 2011). The citrus peel extracts revealed higher anti-bacterial activity as that of the standard medications used in the research. All tested bacteria in this research were multiple drug resistant bacteria. The chemotherapeutic potential of the citrus peel could be due to the presence of flavonoids, alkaloids, saponins, tannins, triterpenoids, phytosterols and steroid chemicals. As new drug-resistant microbial stresses appear, herbal drugs are being looked as very important source for finding of new agents for the treatment various conditions related to attacks. Plants from the genus citrus or lime are well-known herbs used in traditional medicine for their efficiency against a variety of illnesses, such as skin disease, due to the advantage of the variety of additional metabolites accountable for their anti-bacterial activity. There has been increasing interest in the development of new types of effective and non-toxic anti-microbial compounds. . Conversely, no antifungal activity revealed against aqueous and methanolic extract of citrus peel and these results are similar to study of Okwu *et al.* (2007) and Osarumwense *et al.* (2011). The phytochemistry of *C. reticulata* methanolic extract revealed the presence of carbohydrates (reducing sugars, hexose sugars, non-reducing polysaccharides, gums, and mucilages),

flavonoid glycosides, coumarin glycosides, volatile oils, organic acids, fats and fixed oils (Kumar *et al.,* 2011). Most of the organic chemical constituents reported are known to possess aromatic phenolic compounds, which are known for their wide spectra of antimicrobial activity (Kabra *et al.,* 2012).

In case of microbial attack a group of flavonoids (hydroxylated phenolic ingredients) produced by plants (Cowan, 1999). The flavonoids present in the citrus peel methanolic extract may be responsible for the anti-microbial action. Therefore, the existence of organic chemicals along with flavonoids might have provided high anti-microbial activity of methanolic extract of *C. reticulata* peel. In present study, tested bacterial strains were controlled with citrus extract with different levels of resistance. This is recommended that the phenolic ingredients might significantly promote the anti-bacterial action of the test extract. Further, medicinal and scientific tests are required to understand the procedure and the actual effectiveness of this herbal extract in treatment of various plant infections and diseases.

CONCLUSION

The outcomes showed that the methanolic extract of *C. reticulata* peel extract was able to inhibit tested bacteria used in this research with different degrees of inhibition. On the other hand, some examined bacterial strains were not scored self-consciousness against *C. reticulata* peel extract. In short, *C. reticulata* peel methanolic extract has a potential source of natural antimicrobials. Primary focus of the research is to minimize the growth of microbial species by using different ingredients of *C. reticulata* peel and the outcomes shows that these ingredients are capable to cure some of the microbial diseases in plants.

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