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Antimicrobial activity and phytochemical analysis of orange (*Citrus aurantium* L.) and pineapple (*Ananas comosus* (L.) Merr.) peel extract

Biradar Balasaheb Gunwantrao, Sonawane Kaveri Bhausaheb, Barge Sagar Ramrao* and Kharade Sachin Subhash

K.K. Wagh College of Agricultural Biotechnology, Nashik-422 003, Maharashtra, India
**SRM University, Department of Biotechnology, Chennai-603 203, Tamil Nadu, India*

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Abstract

Orange (*Citrus aurantium* L.) and pineapple (*Ananas comosus* (L.) Merr.) are major fruit crops, cultivated in India. Peels represent between 50 to 65 % of total weight of the fruits and remain as the primary byproduct. Orange and pineapple fruits are majorly used for juice extraction in industrial leads to cause huge amounts of residues. If these residues are not processed further, it becomes waste and produce serious environmental pollution. The present study was aimed to extract the metabolites from waste peels, using ethanol and methanol solvent system and checked for the presence of various biomolecules and secondary metabolites like carbohydrates, proteins, steroid, lavonoid, alkaloids, tannins, saponines and tritepinoids. The ethanol solvent was showing most of the positive test as compared to methanol solvent in both the samples. Further, the antimicrobial properties of orange and pineapple peels were checked against pathogenic bacterial strains. The antimicrobial activity was performed, using agar well diffusion method against pathogenic bacterial strains (*Klebsiella pneumonia* K2044, *Pseudomonas aeruginosa* MTCC4676, *Bacillus subtillis* Py79 and *Xanthomonas axonopodis pv. malvacearum* LMG859). From the results, it was clearly observed that both orange and pineapple peel sample dissolved in ethanol, showing maximum zone of inhibition, against all the test pathogens. Methanol extract of both the samples, showing lower zone of inhibition in all the test pathogen as compared to ethanol extract samples. When both the samples, ethanol and methanol extracts were combined in equal amount and test against the pathogenic bacteria, they were showing maximum or equal zone of inhibition as the individual samples zone of inhibition. Finally, we can conclude that orange and pineapple fruits can be used as a antimicrobial agents for the protection from selected plant and animal pathogens, but the use of a standard method for investigation is essential. Similarly, the concentrations or dilutions used, must be appropriate with proper information about its safety.

Key words: Phytochemical, antimicrobial, pineapple, orange

1. Introduction

Orange and pineapple constitutes near about 70 % of total world production. Orange cultivation is probably one of the most important commercial and industrial agricultural activities of the world (Ahmed *et al.*, 2006). Similarly, peels of these two fruits constitute near about 50 to 65 % of total weight of the fruits and remain as the primary byproduct for various purposes. Large production of these fruits in world is mostly used for juice extraction in the industry which leads to produce huge amounts of residues, including peel and other segment membranes. If residues are not processed further, it becomes waste and produces odor, soil pollution, harborage for insects and can be responsible for serious environmental pollution (Mandalari *et al*., 2006). But unfortunately, major quantities of the peel are not further processed for anything, sometime farmers try to use these residues as livestock feed, but these are not giving much nutritional value to the livestock (Bampidis and Robinson, 2006). Therefore, there is a need to look for alternative that will give the beneficial activity from this waste material. So, there was an attempt made to look for substances from these sources which will give the antimicrobial activity. Antimicrobial agents are the substances which either kill or inhibit the growth of microorganisms. As per the reports, citrus fruit products act as antimicrobial agents against bacteria and fungus (Manthley and Grohmann, 1996; Anagnostopoulou *et al.*, 2006). Therefore, citrus product has an important role and nutritional value in food industry as well as commercial value in pharmaceutical industries in entire world (Chanda *et al*., 2010). Consequently, citrus family byproducts found to be very good source of naturally occurring flavonoids and many polymethoxylated flavones, which are very rare in other plants (Horowitz, 1961; Ahmad *et al.*, 2006). The antimicrobial abilities of orange and pineapple oils, are also shown to be a particularly interesting field for applications within the food and cosmetic industries (Caccioni *et al*., 1998). From this brief introduction, the present study was designed to investigate the antimicrobial potential of fruits peel waste (orange and pineapple) extracted in solvents (ethanol, methanol) against pathogenic bacteria and search for more effective antimicrobial agents among materials

Author for correspondence: Dr. Biradar Balasaheb Gunwantrao
Assistant Professor, K.K. Wagh College of Agricultural Biotechnology, Nashik-422 003, Maharashtra, India
E-mail: bbiradar04@gmail.com
Tel.: +91-8722103647; 8856862144

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of plant origin, with the aim of discovering potentially useful active ingredients that can serve as source for the synthesis of new antimicrobial drugs.

2. Materials and Methods

2.1 Collection and preparation of sample

Orange and pineapple waste peels were collected from Nashik local fruit shops, thoroughly washed and shade dried at room temperature (35-37°C) until get completely dried. The dried peels were blended in an electric blender and powder was stored in close containers until further use.

2.2 Preparation of plant extracts

2.2.1 *Hot water extraction*

5 g of dried plant material powder was taken in a beaker and 200 ml of distilled water was added. The mixture was heated on a hot plate with intermitted stirring at 30-40ºC for 20 min. Then extract was filtered through Whatman filter paper No.1 and the filtrate was used for the phytochemical analysis. The filtrate was kept in refrigerator for further use.

2.2.2 *Solvent extraction*

Crude plant extract was prepared by using Soxhlet extraction method. About 25 g of powdered plant material was uniformly packed into a filter paper and extracted with 250 ml of different solvents separately. Solvents used were methanol and the ethanol. The process of extraction continues for 24 h. or till the solvent from the extractor become colorless. Further, extract was collected in a beaker and filtered through Whatman filter paper No.1 and concentrated till all the solvent get evaporated, using rotary evaporator. Dried extract weights were taken and percent yield of plant material was calculated. Each of the dried extract of the fruit peel was stored in closed vial at 4ºC. Working stocks were prepared by dissolving each fruit peel extract in appropriate amount of respective solvent.

2.3 Antimicrobial activity

Antimicrobial activity was performed against pathogenic bacterial strains (*Klebsiella pneumonia* K2044, *Pseudomonas aeruginosa* MTCC4676, *Bacillus subtillis* Py79 and *Xanthomonas axonopodis pv. malvacearum* LMG859) which were obtained from the culture collection centre, Department of Applied Botany and Biotechnology, University of Mysore, India, used for the present investigation. The antimicrobial activity was analyzed, using agar well diffusion method (Javed *et al*., 2011). For each bacterial test strain, 100 µl of 24 h. old bacterial suspension was thoroughly spread on nutrient agar medium with L-shaped spreader. In each plate, four wells of 6 mm diameter were prepared, using a sterile cork borer. From these four wells, the two wells were loaded with 100 µl of orange and pineapple fruit peel extract stock, respectively. In remaining two wells, one well was loaded with 50 µl mixture of each orange and pineapple peel extract. In last, wells were loaded with the respective solvents (ethanol, methanol) with no plant extract was served as the negative controls. Then the plates were incubated overnight at 37ºC to observe the antimicrobial activity of the extract against the test bacteria. It was indicated by free "zone of inhibition" near the respective well, the diameter of the zones of inhibition around each well was taken and measured the antibacterial activity. Each experiment was carried out in triplicate and average mean diameter of the inhibition zone was recorded (Sen and Batra, 2012).

2.4 Phytochemical screening assay

After metabolite extraction, phytochemical screening assay was performed for preliminary detection of specific compounds, present in the given plant extract.

2.4.1 *Ferric chloride test*

2 ml of peel extract was taken and three drops of $FeCl_3$ diluted solution was added, production of a blue or greenish-black color clearly indicates the presence of tannins (Evans, 2002). This is the simple, quick, and inexpensive procedure for the detection of tannin from given sample.

2.4.2 *Test for protein*

Take 1 ml of extract and add 1 ml of 40 % sodium hydroxide solution and 2 drops of 1 % $CuSO_4$ solution, keep at room temperature for 5 min., formation of pinkish or purple violet color indicates the presence of proteins **(**Arun Kumar and Muthuselvam, 2009).

2.4.3 *Carbohydrates*

Moliseh's test: Take 2 ml of the peel extract and add l ml of α-napthol solution and mix the mixture thoroughly, then add l ml of concentrated sulphuric acid through the side of the test tube. Purple or reddish violet colour in between two liquids indicates the presence of carbohydrates (Arunkumar and Muthuselvam, 2009).

2.4.4 *Saponins*

The presence of saponins was determined by Frothing test. The crude dry powder of each peel extract was vigorously shaken with distilled water and was kept to stand for 10 min. No froth in solution indicates absence of saponins and stable froth in sample indicated the presence of saponins (Parekh and Chanda, 2007).

2.4.5 *Alkaline reagent test*

2 ml of crude peels extract was mixed with 2 ml of 2 % solution of NaOH. An intense yellow colour was formed which further turned colorless on addition of few drops of diluted acid which indicated the presence of flavonoids.

2.4.6 *Test for terpenoids*

Salkowski test: 5 ml of peels solvent extract was mixed in 2 ml of chloroform, followed by addition of 3 ml concentrated (H_2SO_4). A layer of the reddish brown color was formed at the top of solvent, thus indicating a positive result for the presence of terpenoids (Okwu, 2004).

2.4.7 *Benedict's test for carbohydrate*

Take 1 ml test solution was mixed with few drops of Benedict's reagent (alkaline solution containing cupric citrate complex) and boiled in water bath for 2 min, observed for the formation of reddish brown precipitate to show a positive result for the presence of carbohydrate.

2.4.8 *Test for steroids*

Take 0.5 ml of peel extract and add 2 ml of acetic anhydride mixed thoroughly, followed by 2 ml of sulphuric acid. The color changed from violet to blue or green indicated the presence of steroids.

2.4.9 *Hager's test for alkaloid*

Take 1ml of test solution was treated with few drops of Hager's reagent (saturated picric acid solution). Formation of yellow precipitate would show a positive result for the presence of alkaloids (Satheesh *et al*., 2012).

2.5 Statistical analysis

A simple statistical analysis was carried out to calculate the mean and the standard deviation. Each experiment was carried out in triplicate and average mean diameter of the inhibition zone was recorded (Sen and Batra, 2012)

3. Results and Discussion

In the present investigation, orange and pineapple peels were collected and after drying, the fine powder was prepared. Extraction of metabolites was carried out, using ethanol and methanol as solvent by using Soxhlet apparatus and dry recovery of compounds was measured in grams. The highest recovery was observed from orange peel in ethanol extract (Table 1). Further, these crude extracts were dissolved in pure ethanol and methanol solvents (50 mg/ml) and used for phytochemical analysis and antimicrobial activity.

Table 1: Extract recovery from orange and pineapple peels

Samples	Solvent	Peels powder wt. taken for extraction (g)	Solvent taken extraction (ml)	Dry extract wt. (g)	Recovery %
Orange peel	Ethanol	25	250	2.79	11.16
	Methanol	25	250	2.02	8.08
Pineapple peel	Ethanol	25	250	1.99	7.96
	Methanol	25	250	2.10	8.40

3.1 Phytochemical analysis

Extracts of both orange and pineapple peels were screened for presence of various biomolecules and secondary metabolites like proteins, carbohydrates, alkaloids, tannins, flavanoids, steroid, saponins and terpinoids (Table 2). This table showed the presence and absence of metabolites in the extract.

Protein and alkaloid tests were observed positive in both orange and pineapple peel extract in both the solvents. The test for carbohydrates was positive only in orange extract in both the solvents and negative in pineapple (Table 2). Tannin and saponin tests were positive in only ethanol solvents of both orange and pineapple and negative in methanol solvents. Tannin and Saponin tests were positive in only ethanol solvents of both pineapple and orange and negative in methanol solvents. But these results are not matching with similar work reported by Huda *et al*., 2016, because presence or absence of secondary metabolites may be depends on type of solvents, nature of extraction or climatic condition of the zone from where plant samples were collected. The growth of many yeast, fungus, bacteria and viruses were inhibited by tannin (Chung *et al*., 1998). Traditionally, saponins have been extensively used as detergents and pesticides, in addition to their industrial applications as foaming and surface active agents and also have beneficial health effects (Shi *et al*., 2004). Flavanoids and terpenoids tests were positive only in methanol extract in case of both the samples (Table 2). Terpenoid and tannins were also found in orange and pineapple sample which can be attributed for analgesic and anti-inflammatory activities. Apart from these, they are known for astringency, *i.e*., faster the healing of wound and inflamed mucous membrane (Okwu and Josiah, 2006). The tests for steroids are positive in orange sample ethanol extract and negative in methanol extract but interestingly same test was positive in case of pineaple extract in both the solvents (Table 2). It should be noted that steroidal compounds are having high importance in pharmaceuticals due to their relationship with sex hormones (Santhi *et al.*, 2011). Tannin is present in both the peel extract, *i.e*., orange and pineapple. Carbohydrate is present in orange peel extract but absent in pineapple peel extract of both solvents.

Table 2: Phytochemical analysis of orange and pineapple peel extract

Tests	Orange peel		Pineapple peel	
	Ethanol	Methanol	Ethanol	Methanol
Protein	+	+	+	+
Carbohydrate	+	+	–	–
Alkaloids	+	+	+	+
Tannins	+	–	+	–
Flavanoids	–	+	–	+
Steroids	+	–	+	+
Saponin	+	–	+	–
Terpenoids	–	+	–	+

Note: **+** : sign indicates the positive test for that compound and **–** : sign indicates the negative test for the compound

3.2 Antimicrobial activity

The antimicrobial activity of orange and pineapple peel extract, were checked against the selected plant and animal pathogens. Antimicrobial activity was calculated as per the zone of inhibition, formed around the wells, the zone of inhibition was recorded in millimetre (mm) (Table 3). The extracts of both orange and pineapple peels were tested against the pathogenic bacteria, namely; *Xanthomonus, Bacillus subtilis, Azotobacter, Pseudomonas, Klebsiella* (Figures 1 and 2).

From the results, it is directly observed that pineapple sample dissolved in ethanol extract showing highest zone of inhibition (27 mm) as against *Klebsiella* and lowest was observed in pineapple peel extract dissolved in ethanol against *Bacillus subtilis* (17 mm) (Table 3). Similarly, from the results mentioned in Table 3, it is clearly observed that both orange and pineapple peel sample dissolved in ethanol showing maximum zone of inhibition against all the test pathogens (Figure 3). Methanol extract of both the samples showing lower zone of inhibition in all the test pathogen as compared to ethanol extract samples (Figure 4). When both the samples ethanol and methanol extracts, were combined separately and test against the pathogenic bacteria, they are showing maximum or equal zone of inhibition as the individual samples zone of inhibition.

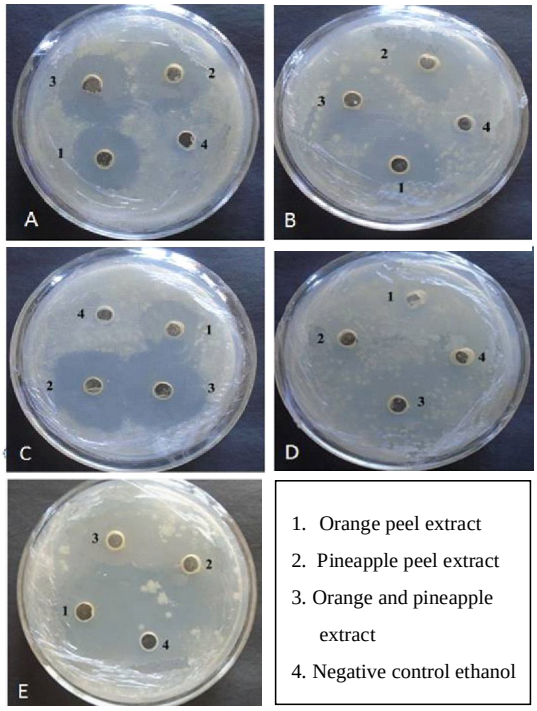

Figure 1: Antimicrobial activity of orange, pineapple and combination of orange and pineapple peel extracts dissolved in ethanol against (A). *Pseudomonas*, (B). *Azotobacter*, (C). *Klebsiella* (D) *Bacillus subtilis* and (E). *Xanthomonas*

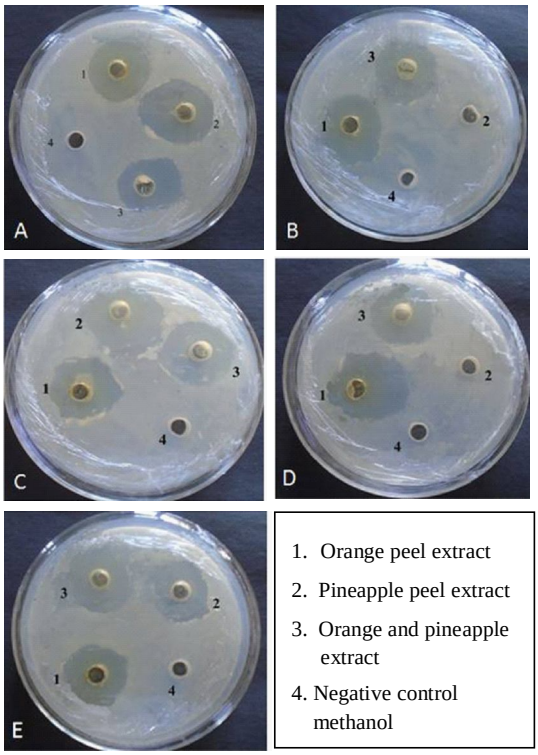

Figure 2: Antimicrobial activity of orange, pineapple and combination of orange and pineapple peel extracts dissolved in methanol against (A). *Pseudomonas*, (B). *Azotobacter*, (C). *Klebsiella* (D) *Bacillus subtilis* and (E). *Xanthomonas*

Table 3: Antimicrobial activity of orange and pineapple peel extract measured in (mm) against selected human and plant pathogens

Organism	Orange		Pineapple		Pineapple + Orange	
	Methanol	Ethanol	Methanol	Ethanol	Methanol	Ethanol
Pseudomonas	19 ± 0.41	22 ± 0.45	20 ± 0.47	19 ± 0.43	24 ± 0 56	24 ± 0.42
Azotobacter	21 ± 0.53	24 ± 0.56	19 ± 0.56	22 ± 0.56	24 ± 0..65	26 ± 0.56
Klebsiella	22 ± 0.45	22 ± 0.43	22 ± 0.46	27 ± 0.65	26 ± 0.54	29 ± 0.49
Bacillus subtilis	23 ± 0.38	22 ± 0.46	23 ± 0.57	17 ± 0.48	25 ± 0.43	26 ± 0.55
Xanthomonas	00	00	22 ± 0.37	22 ±0.45	24 ± 0.44	27 ± 0.48

Note: Zone of inhibition was recorded in millimetre (mm)
Values are mean of triplicate readings (Mean ± S.D)

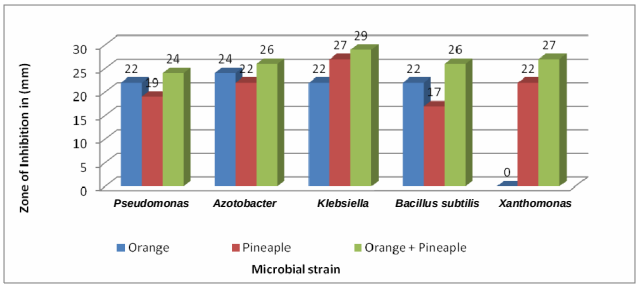

Figure 3: Graphical representation for zone of inhibition of ethanol extract of orange and pineaple peel against different microorganism.

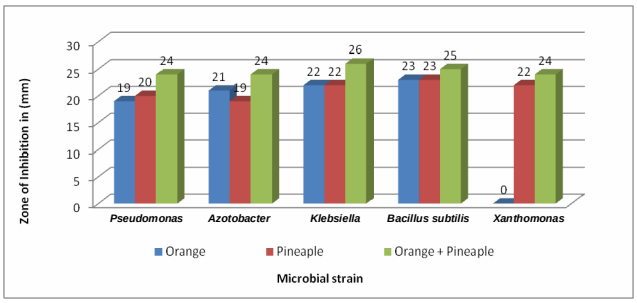

Figure 4: Graphical representation for zone of inhibition of methanol extract of orange and pineaple peel against different microorganism

The antimicrobial property of the orange and pineapple peel extract is due to the presence of many active phytochemicals including vitamins, terpenoids, carotenoids, cumarins, flavonoids, lignin, saponin and plant sterol (Li *et al*., 2006). Citrus fruits and juices are very good source of bioactive compounds and antioxidants such as ascorbic acid, flavonoids, phenolic compounds and pectins that are important for human nutrition (Fernandez *et al*., 2005). Moreover, essential oils from orange and pineapple peel extracts were also exhibited antimicrobial activity against many pathogenic microbes (Samy, 2005; Wannissorn *et al*., 2005; Melendez and Capriles, 2006). Phenolic compounds present in sample have been reported as the main bioactive components for the antimicrobial activity. Most plant phenolic compounds are not toxic for human consumption; therefore, they could be used to prevent growth of many food-borne and food spoilage microorganisms in foods. Citrus essential oils are also been reported to improve the shelf life and the safety of minimally processed fruits (Lanciotti *et al*., 2004). The results from this study are quite promising. The use of orange peel and pineapple peel as a multipurpose antimicrobial medicinal agent, may function in all organisms with several limitations as reported in the current literature. Finally, it is concluded that the *Citrus sinensis* and *Ananas comosus* based on their characterization,

could be a useful source for antibiotic production and has the potential of medicinal and industrial application. Antioxidants and antimicrobial properties of various extracts from many plants have recently been of great interest in both research and the food industry, because their possible use as natural additives emerged from a growing tendency to replace synthetic antioxidants and antimicrobials with natural ones (Deba, *et al*., 2008). Phytochemical analysis tests are preliminary qualitative test, useful in the detection of bioactive compounds and subsequently may useful to drug discovery and development (Mallikharjuna *et al*., 2007).

4. Conclusion

The present study was aimed to investigate the antimicrobial properties and phytochemical analysis of orange and pineapple fruits peel waste extracted in solvents (ethanol, methanol) against human and plant pathogenic bacteria. Both orange and pineapple peels extracts were screened for the presence of biomolecules like carbohydrates and proteins as well as for the secondary metabolites like steroid, alkaloids, tannins, saponines and terpenoids. Most of the tests were found positive for them. The extracts of both orange and pineapple peels were showing the antimicrobial activity, tested against the selected human and plant pathogenic bacteria, namely; *Klebsiella pneumonia* K2044, *Pseudomonas aeruginosa* MTCC4676, *Bacillus subtillis* Py79 and *Xanthomonas axonopodis pv. malvacearum* LMG859. From the study, we can conclude that pineapple and orange fruits can be used as an antimicrobial agents for the protection from selected plants and animal pathogens, but the use of a standard method for investigation is essential. Likewise, the concentrations or dilutions used must be appropriate with proper information about its safety.

Conflict of interest

We declare that we have no conflict of interest.

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