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Antimicrobial Activity and Phytochemical Analysis of Solvent Extraction of *Citrus reticulata* Peels

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ABSTRACT

Oranges account for a majority of the citrus fruit and it is used widely in the juice manufacturing industry. These industries produce a large amount of waste which majorly includes orange peels. As these peels contain a wide diversity of bioactive compounds, they can be utilized in the synthesis of various value-added products which aid in reducing environmental pollution and also address the economic issues. This paper focuses on finding the antimicrobial activity and phytochemical analysis of solvent extraction of peels of Citrus reticulata. The Citrus reticulata peels were dried, ground, and extracted using various solvents like acetone, ethanol, ethyl acetate, and petroleum ether, and their antimicrobial activity was determined against Gram-positive bacteria like Enterococcus faecalis and Staphylococcus aureus and Gram-negative bacteria like Salmonella typhi. Phytochemical analysis was also carried out to know the presence or absence of various bioactive compounds in solvent extraction. Antimicrobial analysis like agar well diffusion assay, minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) was conducted and the outcomes acquired revealed that ethanol extract of Citrus reticulata peel was more efficient when compared to peel extracts using other solvents. The phytochemical analysis also showed that ethanol extract showed the presence of more secondary metabolites which can be the reason for the increased antimicrobial activity of the ethanol extract.

Keywords: *Orange peels, Antimicrobial assay, Solvent extraction, Minimum inhibitory concentration, Minimum bactericidal concentration*

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INTRODUCTION

Citrus fruits are predominantly employed in the juice processing sector, with oranges accounting for 50-60% of overall production (Satari & Karimi, 2018). Peels, segment membranes, seeds, and pith remnants account for 50-70% of citrus juice production waste (Patsalou *et al*., 2017). Peels account for the majority of this waste, accounting for 50-60% (Jiménez-Castro *et al*., 2020). An abundance of citrus juice manufacturing waste has resulted in major environmental and economic problems. It includes pollution of the environment and a decrease in the efficiency of energy. Transforming citrus juice manufacturing waste into value-added items can significantly alleviate these concerns (Mckay *et al*., 2021). Citrus peels are high in flavonoids, essential oils, organic acids, flavone glycosides, and phenols, which are uncommon in other plants, according to studies (Mitsagga *et al*., 2021). Distinctive polyphenol compounds present in citrus include hesperidin, neohesperidin, naringin, and narirutin (Li *et al*., 2020). These compounds are not only crucial for human health and the environment, but they also have a wider array of economic uses in the food and pharmaceutical sectors (Saramanda & Kaparapu, 2017). Severe side effects and resistance of microorganisms to antibiotics have paved the way for development of new antibacterial agents (Güvensen *et al*., 2019). Plants are a source of antimicrobials that are both renewable and cost-effective, with low toxicity and a wide chemical diversity (Ruddaraju *et al*., 2020). Aldehydes, esters, and alcohols among other active terpenes, add to the plants overall antibacterial activities (Harfouch *et al*., 2019).

The Rutaceae family includes oranges, which are medicinally important plants (Saleem & Saeed, 2020). Lime (*Citrus aurantifulia*), lemon (*Citrus Limonum*) tangerine or mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), and grapefruit (*Citrus Vitis*) are the most significant fruits in the Citrus genus (Sharma *et al*., 2017). Citrus peels are differentiated into flavedo or epicarp (colored peripheral surface) and albedo or mesocarp (white soft center layer) (Rafiq *et al*., 2018). The orange peel has a high concentration of pectin (42.5%), soluble sugar (16.5%), hemicellulose (10.5%), and cellulose (9.5%) (Abd-alla *et al*., 2018). After *Citrus sinensis*, *Citrus reticulata* is the most extensively consumed citrus crop globally (Feng *et al*., 2018).

Mandarin (*Citrus reticulata*) is grown majorly in temperate climate countries (Januário *et al*., 2021). India produces approximately 31.75 × 10^8 kg of oranges per year on an area of 1.96 ×10^9 m^2 (Divyabharathi & Subramanian, 2022). The fruits are flattened, with loose and rough peels and few seeds, making them easy to consume (Colodel *et al*., 2018). Pharmacological analysis has revealed that the peel of *Citrus reticulata* has a variety of biological activities which include anti-inflammatory, antitumor, antiatherosclerosis, antioxidant, and antitumor properties (Duan *et al*., 2017). Many of citrus polyphenol's pharmacological properties are due to its ability to scavenge reactive nitrogen species and reactive oxygen species (Bashandy *et al*., 2020).

This study examines the antimicrobial properties of *Citrus reticulata* peel extract against Gram-positive bacteria like *Enterococcus faecalis* and *Staphylococcus aureus* and Gram-negative bacteria like *Salmonella typhi*. This study also aims at finding out the components of orange peel and determining the relation between the components and the antimicrobial nature of the orange peel.

Scope of the work

The increased production of solid waste from various sources like industries, households, and commercial sites has led to the rise of pollution of the environment thereby seriously affecting human health as well as animals. Orange peel being the major waste produced can be utilized for the production of various value-added products leading to a decrease in the harm produced to the environment. It also adds more benefits to the health of human beings since it is paraben and harmful chemicals free. Various studies have been carried out to study the antimicrobial activity and phytochemical analysis of orange peel extract using various solvents and various microorganisms as test material. The current paper addresses the compatibility of the different solvent's efficiency according to their antimicrobial activity and also the most feasible method of preparation of the disinfectant. The ultimate goal of this paper is to identify the most efficient and cost-effective method of preparation of organic disinfectant from the waste material of the fruit. Because nowadays people are more conscious of organic products in order to avoid the harmful effects of the chemical added disinfectants.

MATERIALS AND METHODS

Materials

Acetone, Ethanol, Ethyl acetate, Petroleum ether

Nutrient agar: Peptone - 5.00g/L; Sodium chloride – 5.00g/L; Meat extract – 1.50g/L; Yeast extract – 1.50g/L; Agar – 15.00g/L; pH (at 25^{0}C) – 7.4±0.2

Nutrient broth: Peptone - 5.00g/L; Sodium chloride – 5.00g/L; Meat extract – 1.50g/L; Yeast extract – 1.50g/L; pH (at 25^{0}C) – 7.4±0.2

Microorganism selection

Extractions of *Enterococcus faecalis; Staphylococcus aureus* and *Salmonella typhi* were used for antibacterial testing as Gram-positive and Gram-negative bacteria, respectively.

The pure cultures of *Enterococcus faecalis; Staphylococcus aureus* and *Salmonella typhi* are taken from the master plate and then subcultures into nutrient broth by inoculation technique. Overnight cultures of bacteria were prepared on nutrient broth.

Microbiological media and agar plate preparation

28 g of nutrient agar was liquefied in one litter of distilled water. A small amount of agar-agar was added to it. The mixture was stirred to dissolve all the components. The mixture was autoclaved for 15 mins at 15 lbs pressure. The nutrient agar was cooled after autoclaving. Once the media is cooled, it is poured on the Petri plates and allowed to solidify

Collection of plant extracts

The peel of the plant used in this study is *Citrus reticulata* (common name: Mandarin orange). The peels were obtained from local juice shops. The peels were washed using distilled water to remove dust particles. They were then shade dried at a temperature of 32-35^{0}C for 5 days. The dried peels were ground and made to powder. The powder was stored in a closed container.

Preparation of extracts

25g of dried and powdered orange peel was extracted using 100 ml of each solvent (acetone, ethanol, ethyl acetate, and petroleum ether). In a conical flask, the peel powder and solvents were placed and thoroughly mixed using a shaking machine for 48 hrs at 150 rpm (except petroleum ether- 24 hrs). The extracts were then filtered using Whatman filter paper. The filtrate obtained was then dried using the open-dish evaporation method and the dried extract obtained was placed in small vials and stored in the refrigerator at 4 ^{0}C (El-Desoukey *et al*., 2018).

Estimation of percentage yield

The percentage yield of the extract obtained using various solvents was calculated using the following formula (Giwa *et al*., 2018):

$$\text{Percentage yield (\%)} = \frac{\text{Weight of extract recovered}}{\text{Weight of dry powder}} \times 100 \quad (1)$$

Antimicrobial activity/assay

Agar well diffusion assay

The extracts agar well diffusion assay was conducted to determine the antimicrobial assay. Two Gram-positive bacteria (*Enterococcus faecalis* and *Staphylococcus aureus*) and one Gram-negative bacteria (*Salmonella typhi*) were used to test for antimicrobial activity. Petri plates used for this assay were autoclaved and all the steps were carried inside the laminar air flow chamber. Nutrient agar was used as a nutrient medium for assay. Bacteria was poured and spread on the solidified nutrient agar and wells of 6 mm diameter were punched on the agar. 100 µL of 100 mg/ml of test samples were transferred to the labeled wells. The test samples were allowed to diffuse through the media and the plates were incubated in the upright position for 24 hrs at 37 ^{0}C. 1 mg/ml of ciprofloxacin was used as positive control and 25% Dimethyl sulfoxide (DMSO) was used as a negative control. The diameter of the zone of inhibition around the wells was measured to evaluate the antimicrobial assay.

Determination of minimum inhibitory concentration (MIC)

The final extract concentration with no apparent microbial growth is known as minimum inhibitory concentration (MIC) (Guo *et al*., 2018). A sterile 96 well microtiter plate was labeled. To all the wells, a nutrient broth of 100 µL was added. Serial dilution of 6.25 mg/ml, 12.5 mg/ml, 25 mg/ml and 50 mg/ml of test samples were prepared with micropipette. 100 µL of the test sample was added to each well in ascending order and tips were discarded after each use. Finally, 25 µL of each bacterial suspension was added to every well. The plate was wrapped loosely with parafilm to avoid contamination and dehydration of bacteria. Added positive control with vegetative broth and test organisms and negative control with the nutrient broth was added. The plate was then kept in the incubator for 24 hrs at 37 ^{0}C. After 24 hrs the microtiter plate was read using a microtiter plate reader and the MIC of the extract is recorded.

Determination of minimum bactericidal concentration (MBC)

MBC was determined by taking a loopful of broth from the wells with no evident signs of bacterial growth. The broth was streaked onto the sterile nutrient agar plates by streaking. The plates were then incubated for 24 hrs at 37 ^{0}C. The concentration in which no visible growth of bacteria was seen was taken as the minimum bactericidal concentration.

Phytochemical analysis (Qualitative analysis)

Test for alkaloids

2 ml of filtrate was mixed with 2 ml of distilled water and continuously shaken. The presence of alkaloids was indicated by the formation of a 1 cm layer of foam.

Test for amino acids

Few drops of Ninhydrin reagent were added to 2 ml of filtrate. The amino acid's presence was indicated through the purple color appearance.

Test for flavonoids

2 ml of 2N sodium hydroxide was mixed with 2 ml of filtrate. The flavonoid's presence was designated by the yellow color appearance.

Test for steroids

The 2 ml of filtrate was diluted using an equal chloroform volume and a few drops of conc. Sulfuric acid. The steroid's presence is shown through the brown ring formation.

Test for tannins

2 ml of filtrate was mixed with a few drops of 0.1% ferric chloride. The tannin manifestation was formed by specified blue-black or brownish-green tint.

RESULTS AND DISCUSSION

In each of the experiments undertaken in this research, the solvent extract of the orange peel employing various solvents generated varied results. There was a significant difference in the percentage yield of the recovered extract using different solvents **(Figure 1)**. Ethanol extract showed the highest percentage yield of 6.5% followed by acetone extract (2.5%) and ethyl acetate extract (1.2%). Petroleum ether extract showed the least percentage yield of 0.7%. The solubility of various plant compounds in various solvents is explained by the difference in yield between various solvents. Studies conducted by Shakya *et al*. (2019) showed that the percentage yield of ethanol extract of peels of *Citrus reticulata* was higher when compared to the yield of other citrus fruits like *Citrus limetta*, *Citrus aurantifolia*, *Citrus limon*, *Citrus maxima* (Shakya *et al*., 2019).

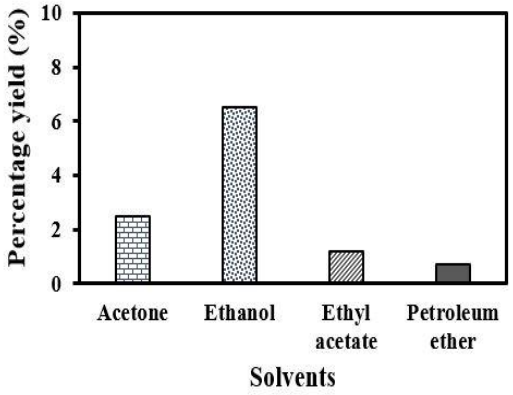

Figure 1. The Percentage yield of *Citrus reticulata* peel extract

The color of the extract was brownish orange. Citrus peel extracts show considerable antibacterial activity against all of the species tested **(Table 1)**. Ethanol is an excellent solvent for extracting antibacterial agents since it has a greater yield as well as antibacterial activity. A greater antibacterial agent is associated with a larger concentration of a variety of phytochemicals which is due to increased yield. Ethanol extract of *Citrus reticulata* showed the highest zone of inhibition against *E. faecalis* and *S. Typhi* (12 mm) followed by *S. aureus* (11.5 mm). Both petroleum ether and ethyl acetate have similar antibacterial actions. When compared to other solvents, petroleum ether had relatively less antibacterial action. This demonstrates that the solvents are capable of extracting antibacterial compounds that may be extremely hazardous to the bacterial strains. As a result, various extracts may include distinct antibacterial agents with distinct modes of action, or the bacterium may develop a specific metabolism to overcome or adapt to its activity (Kumar *et al*., 2011). The presence of more phytochemicals in ethanol extract (alkaloids, tannins, flavonoids, steroids), when compared to extract of other solvents, might have induced increased bacterial activity. The antibacterial effects found in this study might be attributed to the synergistic activity of the phytochemical group (Oikeh *et al*., 2020).

Table 1. Zone of inhibition of *Citrus reticulata* peel extract for various bacterial strains (Well concentration- 100mg/ml)

Bacteria	Zone of inhibition (mm)			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	11	12	11.5	10.5
S. aureus	13	11.5	10.5	10
S. typhi	10	12	10	11.5

The inhibition area obtained for ethanol, acetone, and ethyl acetate extract of *Citrus japonica* peel contrary to *S. aureus* bacteria is 21.4 mm, 11.6 mm, and 13.8 mm respectively (Al *et al*., 2019). Comparing to the previous studies, the test organisms and solvents used in this study demonstrated the following zone of inhibition: *E. faecalis* (acetone extract- 11mm, ethanol extract- 12 mm, ethyl acetate extract- 11.5 mm, petroleum ether extract- 10.5 mm); *S. aureus* (acetone extract- 13 mm, ethanol extract- 11.5 mm, ethyl acetate extract- 10.5 mm, petroleum ether extract- 10 mm); *S. Typhi* (acetone extract- 10 mm, ethanol extract- 12 mm, ethyl acetate extract- 10 mm, petroleum ether extract- 11.5 mm). The positive control ciprofloxacin indicated a complex inhibition area. The Gram-negative bacteria were less sensitive to the solvent extraction of the peels when compared to the Gram-positive bacteria. The differences observed in the insensitivity of various test organisms to the studied solvent extracts were due to differences in the cell wall configuration and edifice of the Gram-positive and Gram-negative bacteria (Teneva *et al*., 2019). Less susceptibility of Gram-negative bacteria to the antibacterial agent is because the cell wall is surrounded by an outer membrane, that prevents hydrophobic compounds from diffusing through the lipopolysaccharide covering (Hsouna *et al*., 2017). However, cell wall of Gram-positive bacteria is mostly made up of peptidoglycan and hydrophobic molecules that make it easier for them to pass through (Gorski *et al*., 2022) The extracts antimicrobial activity acquired using different solvents revealed that not every phytochemical liable for antimicrobial activity is miscible in a single solvent (Saleem & Saeed, 2020).

The MIC and MBC of various solvent extracts of *Citrus reticulata* is demonstrated in **(Tables 2 and 3)**. The extracts demonstrated substantial activity against the bacteria. MIC was performed using broth dilution method. The broth technique, which is performed in microtiter trays, has the benefit of lower workloads for a greater number of replicates as well as the use of tiny amounts of the test material and growth medium. The results of MIC and MBC also shows that ethanol extracts are more efficient than extracts of other solvents. MIC values if various solvent extract against different bacteria is as follows: *E. faecalis* (acetone extract- 25 mg/ml, ethanol extract- 12.5 mg/ml, ethyl acetate extract- 6.5 mg/ml, petroleum ether extract- 25 mg/ml); *S. aureus* (acetone extract- 12.5 mg/ml, ethanol extract- 25 mg/ml, ethyl acetate extract- 25 mg/ml, petroleum ether extract- 6.25 mg/ml); *S. typhi* (acetone extract- 50 mg/ml, ethanol extract- 12.5mg/ml, ethyl acetate extract- 25mg/ml, petroleum ether extract- 50 mg/ml). MBC values if various solvent extract against different bacteria is as follows: *E. faecalis* (acetone extract- 50 mg/ml, ethanol extract- 25 mg/ml, ethyl acetate extract- 25mg/ml, petroleum ether extract- 50mg/ml); *S. aureus* (acetone extract- 25mg/ml, ethanol extract- 50mg/ml, petroleum ether extract- 25mg/ml); *S. typhi* (ethanol extract- 25 mg/ml, ethyl acetate extract- 50 mg/ml). Less activity at low concentration of extract could indicate that the active constituent concentration present in the extracts are too less to have any antibacterial effect (Musa *et al*., 2019). Ethanol extract of the peel can be preferred over extract of other solvent to combat diseases associated with *E. faecalis, S. typhi* and *S. aureus* (Egbuonu & Osuji, 2016).

Table 2. MIC value of *Citrus reticulata* peel extract for various bacterial strains

Bacteria	Extract *Citrus reticulata* peel (mg/ml)			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	25	12.5	6.25	25
S. aureus	12.5	25	25	6.25
S. typhi	50	12.5	25	50

Table 3. MBC values of *Citrus reticulata* peel extract for various bacterial strains

Bacteria	Extract *Citrus reticulata* peel (mg/ml)			
	Acetone	Ethanol	Ethyl acetate	Petroleum ether
E. faecalis	50	25	25	50
S. aureus	25	50	-	25
S. typhi	-	25	50	-

The presence of various components of orange peels was discovered during phytochemical analysis. As no single can reliably extract all of the phytochemical and antioxidant compounds present in plant material, diverse phytochemicals are extracted in solvents of varying polarity (Nawaz *et al*., 2020). The orange peel antimicrobial activity is associated with the manifestation of various phytochemicals like tannins, saponins, essential oils, flavonoids, and phenolic compounds (Mostafa & Essawy, 2021). Phytochemicals of various classes were found in extracts of different solvents **(Table 4)**. Steroids, flavonoids, and tannins were majorly found in all extracts. This shows that all solvents were able to dissolve flavonoids, tannins, and steroids. Amino acids were completely absent in the extracts of different solvents. The presence and absence of various

compounds in the extracts could be due to the polarity of the compound and the solvent. Tannins, steroids, flavonoids, and alkaloids are secondary active metabolites present in the plants that play a key part in the antibacterial efficacy of citrus peel (Rehab *et al*., 2018). The presence of flavonoids and tannins in the peel of *Citrus reticulata* indicates the antioxidant properties of the peel. Tannins are also said to have antibacterial properties. Alkaloids, in addition to having antifungal and antibacterial properties, have the potential to be cytotoxic (Silva *et al*., 2020).

Table 4. Phytochemical analysis (qualitative analysis) of *Citrus reticulata* peel extracts

Phytochemicals	Solvents			
	Acetone	*Ethanol*	Ethyl acetate	Petroleum ether
Alkaloids	-	+	-	-
Amino acids	-	-	-	-
Flavonoids	-	+	+	+
Steroids	-	+	+	+
Tannins	+	+	+	+

- indicates the absence of phytochemical; + indicates the presence of phytochemical

CONCLUSION

This study identifies the antimicrobial activity of *Citrus reticulata* which is commonly known as mandarin orange and distributed widely in South-Eastern parts of Asia. The phytochemical analysis also shows the presence of various bioactive compounds in different solvent extractions. The antimicrobial activity of the extract against various test organisms used in this study can be hypothesized to be the manifestation of numerous bioactive compounds in the extract. Ethanol extract showed the highest percentage yield and inhibition against the area of the test bacteria when equated to other solvent extraction. The antimicrobial activity of the extracts acquired using different solvents revealed that not every phytochemical liable for antimicrobial activity is miscible in a single solvent. However, further studies need to be performed to know how exactly the bioactive compound confers antimicrobial properties to the orange peel extract and in the utilization of these extracts to treat infectious bacteria like *S. Typhi*.

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