

COMPARATIVE PHYTOCHEMICAL PROFILE OF TWO CITRUS FRUITS: *CITRUS RETICULATA* AND *CITRUS SINENSIS* PEELS

Atiya Imran¹, Fatma Hussain^{1*}, Muniba Karamat¹, Fatima Imtiaz², Ajwa¹ and Iqra Saleem¹

¹Department of Biochemistry, Faculty of Sciences, University of Agriculture, Faisalabad, Pakistan

²National Institute of Food Science and Technology, Faculty of Food, Nutrition and Home Sciences, University of Agriculture, Faisalabad, Pakistan

*Corresponding author's email: fatmah@uaf.edu.pk

ABSTRACT

Citrus fruits have significant medicinal importance due to their antidiabetic, antivenom, antimicrobial, and antihyperlipidemic properties. The purpose of the current study was to evaluate and compare the bactericidal, antioxidant, enzyme-inhibitory, and cytotoxic effects of indigenous *Citrus sinensis* and *Citrus reticulata* peels collected from local market, Faisalabad. The extracts yield was 49.79% for *Citrus sinensis* and 10.65% for *Citrus reticulata* that may be due to genetic difference that affect peel composition and extractability. The total phenolic contents were 16.30 and 38.04 mg GAE/100 g, and total flavonoid contents were 3.90 and 9.92 mg CE/100 g and DPPH free radical scavenging activities were 20% and 77 % in *Citrus sinensis* and *Citrus reticulata* peels respectively. Aqueous extract of both varieties of peels exhibited maximal inhibitions of alpha-amylase (70.92 and 42.71%). For antibacterial activity, the zones of growth inhibition by *Citrus sinensis* were 12 mm and 14 mm against *E.coli* and *S. aureus*. While *Citrus reticulata* had 10 mm and 12 mm zones of inhibition against *E.coli* and *S. aureus*. The % hemolysis of *Citrus sinensis* and *Citrus reticulata* were 2.22 and 18.18%. Phytochemistry revealed the presence of phenols, carboxylic acids, alcohols, esters, sulphonyl compounds, and various nitrogenous compounds. The peels of *Citrus sinensis* and *Citrus reticulata* show promising bio-efficacy for eco-friendly usage in herbal medicine to treat different health conditions.

Key-words: *Citrus reticulata*, Antioxidants, Aqueous extract, Anti-microbial, Peels

INTRODUCTION

Throughout the world, plants are utilized due to their nutritional qualities and therapeutic benefits. These are sources of vitamins and minerals that support health and well-being (Salmerón-Manzano *et al.*, 2020). This encourages people to practice self-care and explore natural options for a healthier life. The study of medicinal plants and their active compounds continues to contribute to the development of novel pharmaceuticals (Jamil *et al.*, 2022; Khattak *et al.*, 2022a; Vitale *et al.*, 2022).

Citrus fruits belong to the family *Rutaceae*, which consists of 150 genera and 1600 species and are among the most common fruits in the world, with a significant amount of secondary metabolites. *Citrus sinensis* and *Citrus reticulata* peels, which make up the majority of the residue, contain more bioactive substances than citrus liquids and are an appropriate source of pectin and other important organic compounds (Kumari *et al.*, 2023). Citrus fruits rich in flavonoids and vitamin C, have been shown to modulate lipid metabolism, adipocyte differentiation, and reduce oxidative stress, while also enhancing glucose tolerance. Some are genus-specific chemicals, such as polymethoxyflavones, whereas, others may be found only in certain varieties (Gandhi *et al.*, 2020).

Citrus sinensis and *Citrus reticulata* have great medicinal properties. Due to their powerful antibacterial, antioxidant, anticancer, anti-diabetic, and anti-inflammatory properties, they also have a protective effect against the development of coronary heart disease by lowering plasma cholesterol levels (Alaqeel, 2023).

Citrus peels can be used in cooking due to their aroma, used to make tea and incorporated into skincare products due to their antioxidant properties, and can facilitate the composting process. Such practices reduce the peel waste. Even then, due to a lack of awareness about their potential benefits, as well as cultural practices, common food preparation habits, and a perceived lack of value, a large quantity of citrus peels is discarded daily. Therefore, the current study was designed to determine the phytochemistry of *Citrus reticulata* and *Citrus sinensis* peels as well as a comparative investigation of their bioactivities.

MATERIALS AND METHODS

Sample Preparation

Citrus reticulata peels (CRP) and *Citrus sinensis* peels (CSP) were collected from local juice- making vendors. Shade-dried peels were used for aqueous extraction by the maceration process. The sample (50 g) was mixed in 500

mL distilled water (1:10 ratio) and placed in shaking water bath (50 rpm at 58°C). After three days, the mixture was filtered and the filtrate was left at room temperature until it formed a semisolid consistency (Hussain *et al.*, 2021).

Antioxidant Profile

For the estimation of total phenolic content (TPC), the test sample (120 µL) and Na₂CO₃ (95 µL) were mixed with 10% Folin-Ciocalteu reagent (20 µL). The absorbance was measured at 765 nm after two hours of incubation. The TPC were expressed as mg gallic acid equivalents (GAE)/100 g dry weight. In total flavonoid content (TFC) evaluation, 35 µL test sample, 6.5 µL NaNO₂ and 150 µL distilled water were mixed and incubated (10 minutes). AlCl₃ (16 µL) was added, and absorbance (510 nm) was taken. TFC were expressed as mg catechin equivalents (CE)/100 g dry weight. Antioxidant activity was assessed by mixing DPPH solution (200 µL) and 2 µL of the sample (S) and incubating at ambient temperature for 35 minutes. Ascorbic acid was the positive control (C). Absorbance (A) was taken at 517 nm. % Antioxidant activity = $100 \times \{AC - AS / AC\}$ (Jabeen *et al.*, 2023).

Antimicrobial activity

The antimicrobial activity was evaluated against *Staphylococcus aureus* and *E. coli* by the well diffusion method. Strains were collected from the Institute of Microbiology, University of Agriculture, Faisalabad. In solidified agar plates with a uniform thickness of 3 mm, 40 µL (100 CFU/mL) of the inoculum was introduced into each well along with 40 µL test sample (3mg/mL). As a positive control, Ciprofloxacin was used instead of the plant extract. The DMSO (dimethyl sulphoxide) was used as negative control. After 48 hours of incubation, the diameters of the zone of inhibition (ZOI) were measured (Nawaz *et al.*, 2023).

Enzyme Inhibitory Effect: Glucose-lowering effect

Microbial (*Bacillus subtilis*) α- amylase (E.C. 3.2.1.1) was used, and acarbose (100µg/mL) was a synthetic inhibitor as a positive control (C). The enzyme (0.001 g) was added in 100mL of 20mM sodium phosphate buffer pH 6.9). The test sample (30 µL) and 30 µL of the acarbose (C) were incubated separately with 10 µL (0.5unit per mL) enzyme for 10 minutes. The mixture was incubated for 30 minutes after the addition of 1% starch (40 µL). Then, 1M HCl (20 µL) and iodine solution (40 µL) were incorporated, and absorbance (A) was read (580 nm) (Nawaz *et al.*, 2023). Enzyme inhibition (%): $100 \times [1 - AC/AS]$.

Cytotoxic Effect

The cytotoxic effect was determined by hemolytic assay (Nawaz *et al.*, 2023). The plasma was separated from 5 mL blood and the pellets were washed with 5 mL chilled phosphate buffer saline (PBS) three times. Erythrocytes (150 µL) and 30 µL of the test sample (TS) or 1% Triton X-100 (30 µL) were combined and incubated for 30 minutes at 37 °C. After centrifugation (1000rpm) for 10 minutes, the supernatant was reconstituted in 700 µL cold PBS. The absorbance (AB.) of samples was taken at 576 nm with PBS (-ve control: -C) and Triton X-100 (+ve control: +C). Percentage hemolysis was calculated by: $[AB.TS - AB. -C / AB. +C] \times 100$

Structural Elucidation

A Bruker Tensor 27 FTIR spectrometer was used for Fourier transform infrared spectroscopy analysis to facilitate structural elucidation (Nawaz *et al.*, 2023). The powdered sample (2mg) was mixed with KBr (1:100 ratio), compressed into a thin pellet, and the IR spectra, along with peak values were noted in the range between 400 to 4000 cm⁻¹. The spectrophotometer has a 7,500 to 370 cm⁻¹ spectral range and a KBr beamsplitter. Its function can be expanded by beamsplitters (optional) and optics that can cover a vast range. With 0.5 cm⁻¹ resolution options, vital features are RockSolid interferometer, high-sensitivity detectors and converters.

Statistical examination

Results were summarized as mean ± Standard deviation. Minitab statistical software version 17 was used for data analysis by ANOVA.

RESULTS

Measurement of yield in extraction not only indicates the efficiency of the solvent but also highlights the quantity of the analyte lost during the extraction process. The yield of aqueous extract of CSP was 49.792 % and that of CRP was 10.654 %.

Antioxidant Profile

Antioxidant contents and activity were assessed by TPC, TFC and DPPH radical scavenging assay, as shown in Fig. 1. It was observed that the aqueous extract of CRP had a higher antioxidant potential as compared to CSP.

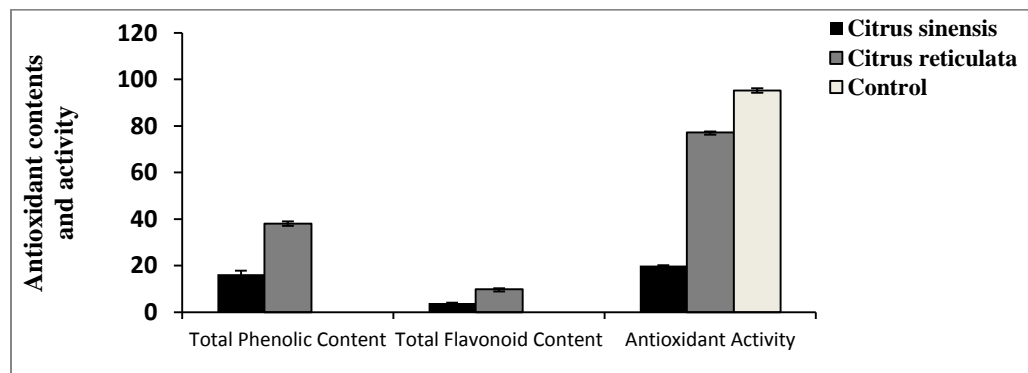


Fig. 1. Antioxidant profile.

Data is presented as mean ± standard deviation for the triplicate measurements. Antioxidant activity: DPPH free radical scavenging activity. Control: Ascorbic acid (antioxidant activity)

Antimicrobial Activity

The antibacterial activity was performed to assess the potential of the samples to combat microbes. Two strains of *Escherichia coli* and *Staphylococcus aureus* were used in this study. Among both extracts, CSP exhibited a higher zone of inhibition against *Escherichia coli* (12 mm) and *Staphylococcus aureus* (14 mm). The positive control (Ciprofloxacin) had 26 mm inhibition zones against both microbial strains (Fig. 2 and 3).

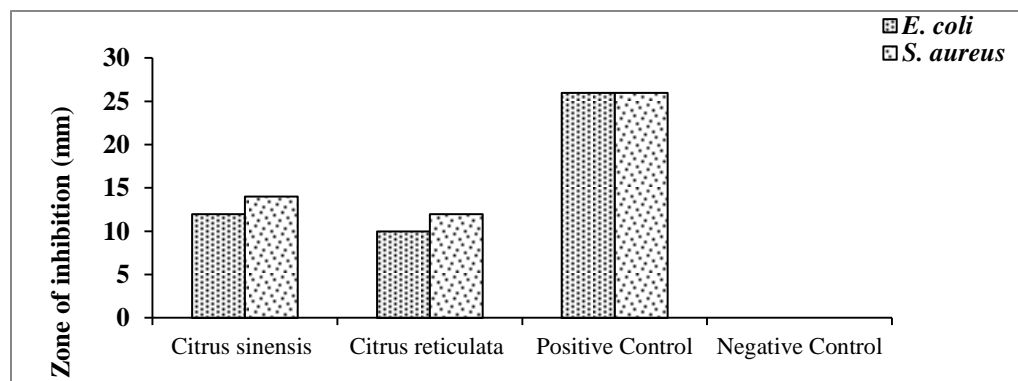


Fig.2. Antibacterial activity.

Positive Control: Ciprofloxacin, Negative Control: DMSO.



Fig. 3. Antibacterial activity with zones of growth inhibition (a) *E. coli*, (b) *S. aureus*.

Antidiabetic potential

The anti-diabetic activity was determined using the alpha-amylase inhibition assay. The results showed significantly higher ($P < 0.05$) alpha amylase inhibition by CSP ($70.92 \pm 0.08\%$) as compared to CRP ($42.71 \pm 0.75\%$). At the same time, control had the highest enzyme restriction potential ($81.47 \pm 0.03\%$).

Cytotoxic activity

Hemolytic activity was performed to check whether the toxicity of the sample destroys red blood cells. It was observed that both plant extracts are safer to use and have lower cytotoxicity. The lowest hemolysis was shown by CSP, which was $2.22 \pm 0.22\%$ and CRP exhibited $18.18 \pm 0.34\%$ hemolysis as compared to the control value ($94.87 \pm 0.00\%$).

Structural characterization

Fourier Transform Infrared Spectroscopy (FTIR) was used for the structural characterization and identification of bioactive functional groups of the peels of both plant samples. Results are presented in Fig. 4 and 5.

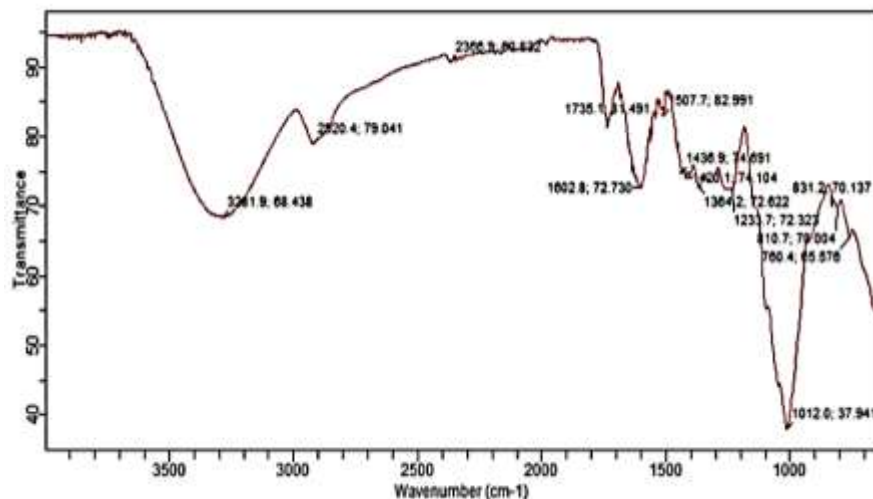


Fig. 4: FTIR spectra of *Citrus sinensis*.

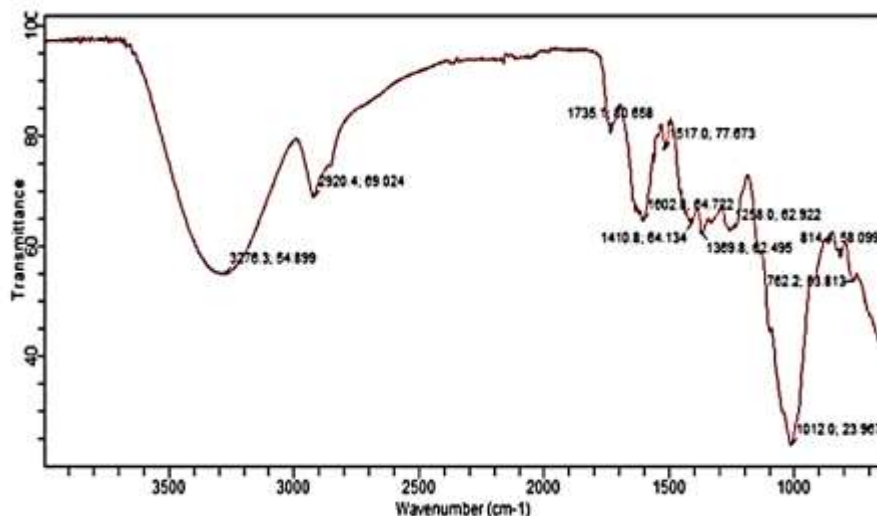


Fig. 5: FTIR spectra of *Citrus reticulata*.

FTIR analysis of *Citrus sinensis* and *Citrus reticulata* revealed various functional groups. In CRP, an alcohol was present as indicated by the medium peaks measured at 3276cm^{-1} and 3281cm^{-1} . A weak peak of aldehyde is present at 2820.4cm^{-1} . Carbon dioxide exhibits a strong peak at 2366.9cm^{-1} . Ester was present as a strong band at

1735 cm^{-1} . Amine was detected as a medium band at 1602.8 cm^{-1} . Primary and secondary amines and amides were observed. Nitro compound was observed as a strong band at 15307 cm^{-1} .

In CSP, alcohol and carboxylic acid were observed as a sharp peak and a strong band at 1436.9 cm^{-1} and 1420 cm^{-1} , respectively. A prominent band at 1364 cm^{-1} demonstrated the presence of sulfonate and sulfonamides. A strong peak for alkyl ethers and esters was observed at 1233 cm^{-1} . Carboxylic acid and fluoro compound were detected as a peak at 1012 cm^{-1} . A medium peak of alkenes was detected at 831 cm^{-1} and 810 cm^{-1} . Alkene was measured as a strong peak at 760 cm^{-1} .

DISCUSSION

In the realm of natural medicine, phytoconstituents that can enhance acquired immunity are plant-based vaccines. These are the focus of research as an alternate therapeutic option worldwide (Saleem *et al.*, 2024).

The percentage yields of CSP and CRP extracts had vast variation. Such variations can be attributed to genetic differences, variations in peel thickness, and differences in cell structure that impact extraction yields. In the current research, *Citrus reticulata* variety showed higher flavonoid content compared to *Citrus sinensis*. According to El-Beltagi *et al.*, (2022), the TFC of CSP was $22.2 \pm 0.5\text{mg CE}/100\text{g}$, which was higher than those measured in the current study ($3.90 \pm 0.13\text{mg CE}/100\text{g}$). Similarly, the TFC value of CRP aqueous extract was $6.15 \pm 0.43\text{mg CE}/100\text{g}$ (Zhang *et al.* 2018), which is lower than the TFC value of $9.928 \pm 0.45\text{mg CE}/100\text{g}$ in the existing study. Regarding phenols, TPC in CSP was lower than that in CRP ($P < 0.05$). Earlier, Ashraf *et al.* (2024) reported 156.1 ± 4.2 to 189.6 ± 5.2 mg (GAE)/100 g TPC in different solvents. However, they compared different extraction techniques at various time intervals. According to El-Beltagi *et al.* (2022), the water extract of *Citrus sinensis* peel exhibited high antioxidant activity (82.2 %). Similarly, Liew *et al.* (2018) observed 54.87% DPPH radical scavenging activity of *Citrus reticulata*. In contrast, current outcomes indicate a significantly high ($P < 0.05$) antioxidant potential of CRP as compared to CSP. These inferences are partially based on the findings of Ashraf *et al.* (2024), who documented $60.55 \pm 2.4\%$ antioxidant activity in methanolic extracts of citrus peels.

According to Kaviya *et al.* (2011), both water and ethanol extracts of *Citrus sinensis* peels showed moderate antibacterial activity against *E. coli* (12.5 mm) and a 9.2 mm zone of inhibition against *S. aureus*. Analogous results were observed in the current study. Due to the presence of flavonoids and other secondary metabolites, both citrus varieties exhibited moderate bactericidal activity. Contrary to this, *Citrus reticulata* extract exhibited strong bactericidal potentials with 21.2 mm and 24.8 mm inhibitory zone diameters for *Staphylococcus aureus* and *E. coli*, respectively (Rafique *et al.*, 2021).

Starch contents vary among natural products and even within the same cultivar; ripening stages affect starch contents (Siddiqui *et al.*, 2017; Khattak *et al.*, 2022b). As a part of an anti-diabetic activity, an alpha-amylase inhibition assay was performed. Previously, the range of alpha amylase inhibition by CRP was 15-90% in different solvents (Ghauri *et al.*, 2021), and it was in agreement with current inferences (>70% inhibition). There are strong indications linking citrus peel flavonoids with antihyperglycemic modulations as evident in the current study (Sahnoun *et al.*, 2017).

In Azantsa *et al.* (2019) study, the % hemolytic potential of peel extract was 87.7% which means the *Citrus sinensis* peels extract was cytotoxic against red blood cells. According to Malleshappa *et al.* (2018), the hemolytic potential of *Citrus reticulata* was 80.32% while in our study, the percentage hemolysis of *Citrus sinensis* extract was 2.22% and *Citrus reticulata* extract was 18.18% which means both the plants are less toxic and safe for consumption. Results of structural elucidation by Adeniyi *et al.* (2020) described that the O-H bond type and functional group, such as alcohol, phenols and carboxylic acid, were observed at 3896 cm^{-1} , which was similar to our bond type and functional group observed at 3541 cm^{-1} . In the current study, the first band observed at 3281 cm^{-1} indicated the presence of the bond type O-H, which is a functional group of alcohol that did not exist in the previous study, while the last band observed at 760 cm^{-1} , which is related to 779 cm^{-1} in previous research. The strong peak at 1681 cm^{-1} was primary amides' C=O stretching and N-H bending vibrations, which was similar to the bond type and group we observed at 1608.1 cm^{-1} . The two peaks at 1527 cm^{-1} and 1411 cm^{-1} were attributed to the aromatic ring's COO-COO- stretching and nitro-bending vibrations; however, in the current study, the peaks were present at 1507 cm^{-1} , 1436 cm^{-1} and 1420 cm^{-1} , which were closest to the prior study. Kumari *et al.*, (2023) stated that the first band seen at 3285 cm^{-1} and the last band seen at 640 cm^{-1} represents O-H stretching vibrations in hydroxyl groups, while in our research, the first peak observed at 3276 cm^{-1} and the last at 762 cm^{-1} represents O-H stretching vibrations in hydroxyl groups. Peaks seen at 1590 cm^{-1} and 1600 cm^{-1} reflect the stretching of the aromatic rings. According to Kaur *et al.*, (2021), the extract of *Citrus reticulata* peels showed absorbing bands from 4000 to 600 cm^{-1} . The O-H bond stretching vibrations of the hydroxyl group showed at peak 3300 cm^{-1} , which is similar to our band observed at 3276.3 cm^{-1} . Absorption peaks at 1648.2, 1450.1, 668.2, 878.8 cm^{-1} are shown, in which 1648.3 cm^{-1} peaks may be

due to N–H bond, 1088.1 cm^{-1} , 1016 cm^{-1} peaks are possibly caused by alkoxy stretching vibrations. A peak at 1619 cm^{-1} was due to stretching vibrations of C = C, but the current study obtained a peak at 1602.8 cm^{-1} .

Conclusions

The medicinal advantages of citrus fruits are well known worldwide, as proven by accessible literature. The current study highlighted the diverse medicinal value of *Citrus sinensis* and *Citrus reticulata* peels, which are mostly wasted. This study exposed more or less equal *in vitro* therapeutic potentials that are eco-friendly.

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