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Assessment Of Bioactive Compounds and Antioxidant Activity In Wasted Parts of Citrus Fruits for Low-Cost Natural Antioxidants

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Abstract

The methanolic peel extracts from three Citrus varieties such as *C. sinensis, C. reticulata* and *C. limetta* were screened for bioactive contents (total phenolic content (TPC), Total Flavonoids (TF), Total Carotenoids, and Vitamin C), antioxidant potential (2,2 diphenyl-2- picryl hydrazyl (DPPH) radical scavenging activity, trolox equivalent antioxidant capacity (TEAC), β carotene bleaching, and reducing power) and antihemolytic activity. *C. sinensis* exhibited the significantly higher TPC (57.43 GAE/g.DM), TF (6.23 mg QE/g DW) and Vitamin C (1.42 mg/g DW) content. However, *C. reticulata* has higher total carotenoids (2.12 mg/g DW). Maximum antioxidant activity was observed in *C. sinensis*, verified by DPPH (54 %), reducing power (Absorbance 0.47), and β-carotene bleaching (73.4 %). Study found a strong correlation between the TPC and antioxidant activity for citrus peels ranging from 0.8211 to 0.8732. However, moderate correlation of 0.7231 to 0.7632 has been noted in between the total flavonoids and antioxidant activity. In contrast carotenoids and Vitamin C have shown weak correlations. Antihemolytic activity was enhanced in all peel extracts with an increase in methanolic extract concentration from 5 to 50mg/mL. Maximum 38.7% inhibition was observed in *C. sinensis* at 50mg/mL. Present study suggested the abundant wasted part of citrus fruits could be utilized as low-cost natural antioxidants in the food industry.

Keywords: Antioxidant, Fruit Peel, Phenolic Compound, Reducing Power Assay, β-Carotene

Introduction

The global production of citrus fruits has witnessed an impressive surge, with India standing as the second-largest producer following China. This thriving industry, primarily driven by the extraction of fruit juice, concurrently generates substantial byproducts in the form of peels, seeds, and pulp. Citrus peels, comprising over half of the original fresh fruit mass, are often disregarded, and discarded as waste, leading to significant environmental, economic, and nutritional losses. In developing countries like India, the lack of adequate infrastructure to manage this voluminous biomass exacerbates these challenges [1]. The disposal of citrus peels, an underutilized resource, represents a missed opportunity for harnessing valuable bioactive compounds. These compounds encompass diverse substances, including polyphenols, carotenoids, vitamins, enzymes, and other phytochemicals, each known for their potential health-promoting properties [2]. While studies have highlighted the antioxidant potential of citrus fruits' juice and edible portions, the extracts derived from citrus peels have yet to be fully explored [3,4]. This study is designed to systematically assess the bioactive compounds and antioxidant activity in the often-wasted parts of citrus fruits, specifically focusing on peels. The aim is to unlock the latent potential of these byproducts and establish them as a low-cost source of natural antioxidants. The extraction process involves the use of different solvents, such as hexane, ethyl acetate, chloroform, and methanol, to optimize conditions for obtaining extracts enriched with bioactive compounds [5].

The assessment encompasses several key parameters, including yield percentage, antioxidant and radical scavenging

capacities, and the produced extracts' reducing power ability. By employing a systematic and comprehensive approach, the study aims to provide insights into the most effective extraction methods and solvents to obtain high-quality extracts from citrus peels [6]. One of the pivotal aspects of this research is the exploration of the inhibitory effects of citrus peel extracts on the hemolysis of human erythrocytes induced by peroxidation [7]. Reactive oxygen species (ROS) generated within the human body, such as superoxide anion radicals, hydroxyl radicals, and hydrogen peroxide, can induce oxidative stress, contributing to various diseases, including cancer, cardiovascular diseases, aging, and neurodegenerative conditions [8]. Exogenous antioxidants derived from polyphenol-rich sources, such as fresh fruits and vegetables, are crucial in maintaining an optimal balance of ROS, thereby positively impacting human health.

In addition to addressing the potential health benefits of citrus peel extracts, the study also aims to tackle the broader issue of environmental sustainability. If strategically utilized, the abundance of citrus peels could mitigate the environmental impact of horticultural waste. By incorporating these byproducts into various industries, such as food, cosmetics, and pharmaceuticals, this research seeks to establish citrus peels as a valuable and low-cost resource [9]. The investigation aligns with the broader goals of sustainable consumption and waste reduction. It envisions a shift from viewing citrus peels as mere byproducts to recognizing them as a reservoir of bioactive compounds with diverse applications. The outcomes of this study could pave the way for the development of commercial units focused on extracting bioactive ingredients from fruit and vegetable residues, particularly in rural and village settings. Beyond economic benefits, such initiatives can contribute to environmental conservation by addressing the challenges posed by the improper disposal of agricultural residues [10].

In conclusion, the comprehensive assessment of bioactive compounds and antioxidant activity in the wasted parts of citrus fruits represents a crucial step toward unlocking the full potential of these byproducts. The study endeavors to position citrus peels as a valuable and low-cost natural antioxidant source, offering benefits to both human health and the environment. Through this interdisciplinary exploration, the research aims to bridge the gap between agricultural waste and sustainable practices, thereby contributing to the development of a more circular and ecologically responsible agricultural and industrial system [11].

Materials and Methods

Materials

The Citrus fruits, including *C. sinensis, C. reticulata*, and *C. limetta*, were obtained from the fruit market in Prayag raj, India. The fruits were thoroughly washed with distilled water. Before separating the non-edible portions such as peels and seeds from the fruits, a geomorphological description of the samples was recorded. The details of the sample preparation are covered in section 2.2 of the study. All solvents used in the research were of analytical grade purity. Various chemicals and standards, such as 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox), 1,1-diphenyl-2-picryl hydrazyl (DPPH), 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), Folin-Ciocalteu reagent, β-carotene, gallic acid, quercetin standards, L (+) ascorbic acid, and BHA, were procured from Himedia.

Samples preparation

The separated peels were cut into small pieces and spread on perforated trays to dry in a hot-air oven at 60 °C until complete drying. Depending on the moisture content in the samples, it took 12 - 18 h to complete drying of the samples. Thermal drying method was used in the determination of moisture content of the peels. 20 g of sample were placed in oven at 102.4 °C for 4h [12]. The moisture content was calculated by expressing weight loss upon drying gas, a fraction of the initial weight of the sample used. The moisture and ash contents of dried peels were determined by the gravimetric method [13].

$$MC(\%) = \frac{W_0}{W_1}$$

Where W_0 corresponds to the loss in weight (g) on drying and W_1 corresponds to the initial weight of sample (g). Dried peels were further pulverized into fine powder using a stainless-steel blender and passed through a 24-mesh sieve.

Extraction of bioactive compounds

The process of extracting bioactive compounds from the finely powdered, dried peels involved a systematic approach. The lyophilized peel powder, amounting to 100 grams, underwent fractionation for the polar fraction using solvents with increasing relative polarity (Hexane < Ethyl acetate < Chloroform < Methanol) in a Soxhlet apparatus for 6 hours. During this process, individual mixtures were manually swirled for 15 minutes and subjected to ultrasonic treatment (Grant XB3, UK) for 60 minutes. Subsequently, the mixtures were filtered through Whatman No. 1 filter paper (GE Healthcare) to eliminate peel particles. This extraction procedure was repeated twice on the residue obtained from the filtration to ensure thorough extraction. The resulting filtrates were pooled, and the solvent was evaporated to dryness from the filtrates under reduced pressure at 60 °C using a rotary evaporator (Rotavapor R210-Buchi, Canada) [14]. The citrus peel extracts were then cooled in a desiccator for 30 minutes before calculating the yield of each extract. The yield percentage was determined using the formula

$$\%\text{yield} = \frac{\text{Final yield(g)}}{\text{Initial sample weight(g)}} x100$$

To measure the extracts, each 10 mL was dispensed into a pre-weighed aluminum dish, which was then placed in an oven at 85 °C for 24 hours and subsequently in a desiccator for 12 hours.

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The weight difference was utilized to calculate the percentage yield, expressed in mg/10 mL. The extracts were stored in dark bottles in a refrigerator at 4 ℃ until use [15]. For the isolation of the volatile fraction, including essential oils, approximately 100 grams of fresh peels mixed with 500 mL of distilled ultra-pure water underwent hydro-distillation for 3 hours using a Clevenger-type apparatus. The extracts were further processed by drying with anhydrous sodium sulfate (Na2SO4) and concentration using a rotary evaporator. The extraction yield was quantified in grams (g) of oil per 100 grams of fresh material. The collected oil was weighed, dissolved in methanol, and stored in sealed vials at -20 ºC for subsequent use. It's worth noting that all chemicals and solvents used in this study were of analytical grade and procured from Fisher Scientific (Mumbai, India).

Analysis of bioactive content in citrus peel extracts

Total phenols (TP) content

The determination of the total phenolic content in the methanolic extracts was conducted using the Folin-Ciocalteu colorimetric method, with some modifications as described in [16]. In brief, 0.5 mL of the extract at a 1 mg/mL concentration was combined with 0.5 mL of Folin-Ciocalteu reagent (2 N) through manual shaking for 15–20 seconds. After a 3-minute incubation period, 0.50 mL of a 7% (w/v) saturated sodium carbonate solution was introduced to the solution, followed by further dilution to 5 mL with deionized water. The reaction mixture was then incubated for 2 hours at room temperature (RT) in dark conditions. Subsequently, the samples underwent centrifugation, and the blue color absorbance of each sample was measured at 765 nm against deionized water, utilizing a dual-beam UV-Vis spectrophotometer.

The total content of phenolic compounds in the extracts, expressed in gallic acid equivalents (GAE), was calculated using the formula:

$$C = \frac{c.V}{m}$$

Where, C = Total content of phenolic compounds, expressed as milligrams of GAE per gram dry weight (g-dw) of residues; c = The concentration of gallic acid established from the calibration curve, milligrams per milliliter; V = The volume of extract, milliliters; M The weight of pure plant methanolic extract (grams).

Total Flavonoids (TF) Content

The determination of flavonoid content followed the method outlined by Chang et al., 2002 [17]. In this procedure, 500 µL of the extracted sample was combined with 1.5 mL of methanol (85%), 100 µL of a 10% aluminum chloride methanolic solution, 100 µL of a 1 M potassium acetate solution, and equilibrated with 2.8 mL of deionized water. Following an incubation period at room temperature (RT) for 40 minutes, the absorbance of the reaction mixture was measured at 415 nm using a UV–Vis spectrophotometer. This method provides a quantitative assessment of the flavonoid content in the sample based on the absorbance values obtained.

Total Carotenoids

For the analysis of total carotenoids, 5 grams of dried citrus peels were meticulously mixed with 50 mL of n-hexane–acetone–ethanol (50:25:25; v/v) in a flask. The mixture was placed on a shaker at 200 rpm for 10 minutes at room temperature (RT). Subsequently, the mixture underwent centrifugation at 6500 rpm for 5 minutes at 4 ºC to separate the two phases. Once the phases were clearly separated, an aliquot was collected from the upper phase and adjusted to 50 mL with the extraction solvent. The absorbance of this solution was measured at 450 nm [18], and the total carotenoid content was expressed in terms of β-carotene equivalents. This method quantitatively assesses the overall carotenoid content in the citrus peels.

Vitamin C

The determination of vitamin C utilized the titrimetric method outlined by Hughes in 1983 [19], with 2,6 di-chloro-phenol indophenol reagents, incorporating some modifications. Freeze-dried peel extracts (1 g) were blended with 4% oxalic acid (100 mL) and homogenized before filtration through the whatman paper. Subsequently, 5 mL of the filtered solution was diluted with a 4% oxalic acid solution (10 mL) prior to titration with 0.01% of the 2,6-di-chlorophenol indophenol solution. The endpoint of the titration was considered reached when the solution attained a pink color that persisted for 15 seconds. The calibration of the 2,6-di-chloro-phenolindophenol solution was carried out using a 0.05% ascorbic acid solution, allowing for the accurate determination of the vitamin C content in the citrus peel extracts. This method provides a reliable means of quantifying vitamin C using titration.

Antioxidant properties of Citrus fruit peels

DPPH Radical-Scavenging Activity

The DPPH (2,20-diphenyl-1-picrylhydrazyl) assay was conducted in triplicate, following the methods described by Brand-Williams et al., 1995, with slight modifications [20]. The stock solution was prepared by combining 2.5 mg of DPPH radical with 100 mL of methanol. In a test tube, 3.9 mL of the DPPH radical solution was added, and 100 µL of the antioxidant extract or standard was introduced (methanol served as the blank). The solution was mixed and allowed to incubate for 15 minutes at room temperature (RT) in a dark environment. The reduction in absorbance at 515 nm was measured at 1-minute intervals for the first 10 minutes and then at 5-minute intervals until equilibrium. A DPPH solution without extract served as a control, and two calibration curves were prepared using Trolox and ascorbic acid as standards.

The results were expressed as µM Trolox equivalents/100 g of DW and ascorbic acid equivalents in mg/100 g of DW. The DPPH

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scavenging activity of extracts was calculated using the following equation:

$$Radical\ Scavanging\ activity(\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] x100$$

Ascorbic acid and α-tocopherol were used as standards.

TEAC (Trolox equivalent antioxidant capacity) or ABTS assay

The ABTS (2,20-Azinobis-3-ethylbenzotiazoline-6-sulphonic acid) radical quenching assay is based on the ability of antioxidant molecules to neutralize the ABTS radical, a blue-green chromophore with characteristic absorption at 734 nm, in comparison to Trolox, a water-soluble analog of vitamin E. The ABTS+ cation is generated by the interaction of a 7 mmol. L-1 aqueous solution of ABTS with 2.45 mmol. L-1 potassium persulfate (K2S2O8). This cation is incubated in the dark at room temperature (RT) for 16 hours before use [21]. To prepare the ABTS activated radical, the stock solution is diluted in ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm. In the assay, 30 µL of the antioxidant extract or standard is added to 2.970 mL of the diluted ABTS solution, and absorbances are recorded 6 minutes after mixing. The percentage inhibition is then calculated against calibration curves of Trolox (ranging from 0 to 2.5 mM) as a standard. The results are expressed as milligrams of Trolox equivalent (TE) per gram of dry weight (g-dW), providing a quantitative measure of the antioxidant activity of the extracts.

Determination of reducing power

The determination of the reducing power of methanolic extracts followed the method described by Oyaizu in 1986 [22]. The concentrations of both the peel extracts and the synthetic antioxidant butylated hydroxytoluene (BHT) ranged from 5 to 30 mg/mL. To initiate the assay, adding distilled water added 0.5 mL of peel extract or standard was adjusted to 1.0 mL. Subsequently, 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferricyanide [K3Fe (CN)6] were added to the mixture. The combined solution was then incubated at 50 °C for 20 minutes and subjected to centrifugation at 5000×g after the addition of 2.5 mL of 10% trichloroacetic acid. Following centrifugation, a 2.5 mL aliquot of the upper layer (supernatant) was collected and mixed with 0.5 mL of 0.1% $Fecl_3$. The increase in absorbance was measured at 700 nm against a blank using a UV-Vis spectrophotometer, directly correlating to the increase in reducing power. This method provides insight into the ability of the peel extracts to exhibit reducing power, a key indicator of their antioxidant potential.

Lipid peroxidation inhibition by β carotene bleaching

The β-carotene bleaching assay of citrus peel extracts was conducted following the method outlined by Ismail and Hong in 2002 [23] with minor modifications. Initially, a β-carotene-linoleic acid emulsion was prepared by adding 3 mL of β-carotene solution (5 mg β-carotene/50 mL chloroform) to 40 mg of linoleic acid and 400 mg of Tween 20. The mixture was then mixed and dried under a stream of nitrogen gas. Subsequently, 100 mL of distilled water was added to the dried mixture to form a β-carotene-linoleic acid emulsion.

To assess the β-carotene bleaching activity of the extract, 20 µL of citrus peel extracts (1 mg/mL) was added to 1.5 mL of the β-carotene/linoleic acid emulsion. The mixture was incubated at 50 ºC for 60 minutes in a water bath. Finally, the absorbance of the samples was read at 470 nm. The measurements were taken at 15-minute intervals for 120 minutes. The synthetic antioxidant butylated hydroxyanisole (BHA) served as a positive control in this experiment, while 0.2 mL of methanol in 5 mL of the β-carotene/linoleic acid emulsion was used as the negative control. The antioxidant activity of citrus peel extracts was calculated using the following equation:

$$BleAntioxidant\ activity(\%) = \left[\frac{1 - BR_s}{BR_c}\right] x100$$

where A_i and A_f are absorbance of the β-carotene/linoleic acid emulsion before and after 2 h incubation, and BR_s and BR_c are bleaching rates of the sample and negative control, respectively.

Scavenging effect on H2O2-induced human blood hemolysis

Human blood samples from male and female volunteers aged 20-25 years were collected using the method described in a previous study [23,24]. The inhibition of human erythrocyte hemolysis was investigated following the procedure outlined by Rafat et al. [25]. Erythrocyte hemolysis was induced with H_2O_2 as a free radical initiator in human blood. A suspension of erythrocytes (500 µL) in isotonic phosphate buffer solution at pH 7.4 (IPB) was mixed with 1 mL of methanolic extract of citrus peel extracts (5-50 mg/mL), prepared in 5% DMSO and dissolved in isotonic phosphate buffer (IPB) at pH 7.4. The reaction mixture was gently shaken on an incubator shaker while being incubated at 37 °C for 3 hours. The inhibitory effect of the extract on the erythrocyte suspension was compared with a positive control, the standard antioxidant vitamin C (10 mg/mL), while non-pretreated erythrocyte suspension served as the negative control. Oxidative stress was induced on erythrocyte suspensions by adding 1 mL of 10 mM hydrogen peroxide (H_2O_2) and incubating at 37 °C for 150 minutes. After incubation, the volume of all pretreated and non-pretreated erythrocyte suspensions was adjusted to 9 mL by adding IPB. The released hemoglobin in the supernatant of the mixtures was measured using a spectrophotometer at 540 nm. The percentage inhibition was calculated using the following equation:

$$Percentage\ Inhibition(\%) = \left[\frac{1 - A_{antioxidant}}{A_{H_3O_3}}\right] x100$$

Where $A_{H_2O_2}$ is the absorbance of a sample containing no

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extract and $A_{antioxidant}$ is the absorbance of a sample containing extract.

Statistical Analysis

All extraction assays were conducted in three replicate determinations and expressed as Means ± Standard Deviation (SD). The data were statistically analyzed using PSPP (GNU Software). One-way analysis of variance (ANOVA) and least significant difference (LSD) were employed at a significant level of 5%. P values less than 0.05 were considered significant. Pearson's correlation analysis was performed to analyze the correlation values between antioxidant activity (DPPH Assay) and the levels of total phenolic content, total flavonoids, carotenoids, and vitamin C in methanolic extracts (1000 µg/mL) of citrus peel. This analysis helps to understand the relationship between antioxidant activity and the various bioactive compounds present in the citrus peel extracts.

Results and discussion

Moisture and ash content of peels

The moisture content of peel samples exhibited variation, ranging from 69.9 ± 4.2% in *C. limetta* to 78.2 ± 5.4% in *C. sinensis*, with an intermediate value of 74.5 ± 6.3% in *C. reticulata*. These values indicate a relatively higher percentage of moisture in all peel samples. The ash content also displayed variability, ranging from 4.43 ± 0.05% in *C. limetta* to 3.36 ± 0.03% in *C. sinensis*. The differences in ash contents can be influenced by factors such as plant species, geographical origins, the method of mineralization, and the impact of food processing, particularly drying Figure 1. Furthermore, the extraction yield of each sample was recorded in the range of 8.4% to 13.35% on a dry basis of the sample weight, with *C. sinensis* and *C. reticulata* exhibiting the minimum and maximum yields, respectively.

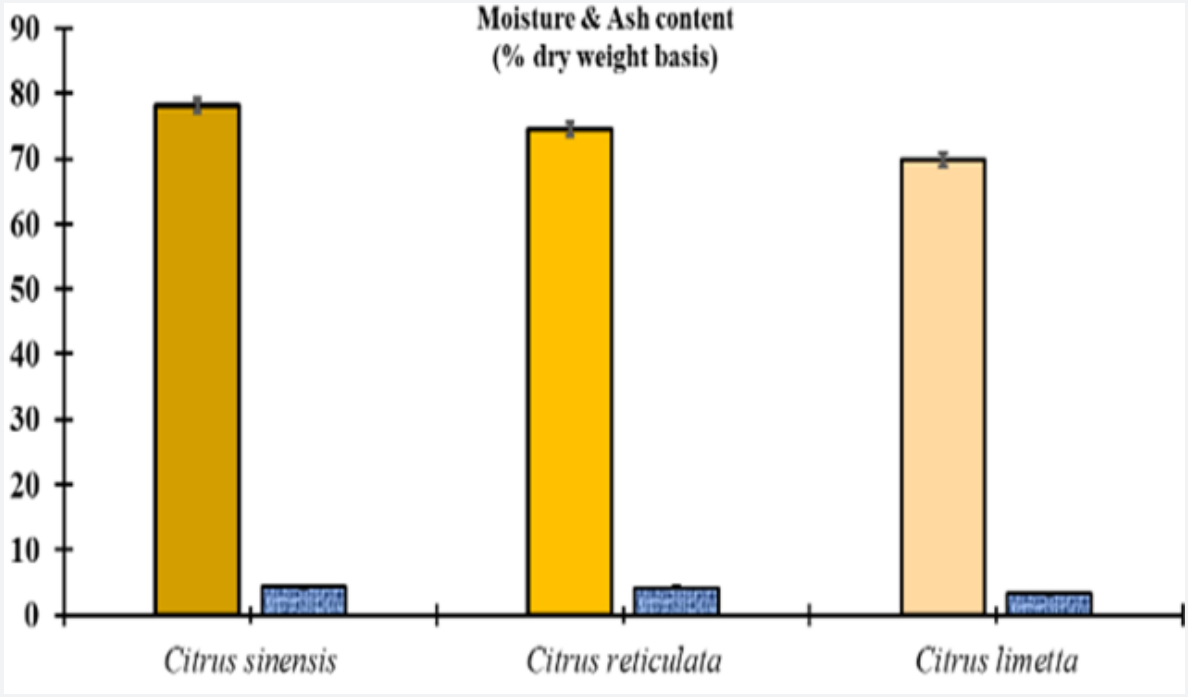

Figure 1: Moisture & Ash Content (% Dry Weight Basis) Of Fruit Peel Residues.

Chemical content of peels

Extraction conditions and the choice of an appropriate solvent play crucial roles in obtaining extracts with high accuracy and bioactive compound content. In the case of citrus peel powders, consecutive fractionation with organic solvents of increasing polarity was employed. The results of these extractions, including the yields and total phenolic contents, are presented in Figure 2. As anticipated, the yields obtained for the peels' polar fractions using organic solvents were significantly higher than those for the peels' volatile fractions in water. The pooled fraction of the extract with a polar solvent, specifically 70% methanol, yielded the highest percentages (ranging from 26.71% to 37.09%) from the citrus peel. In contrast, the yields obtained after fractionation with hexane were the lowest, ranging from 0.27% to 0.97%. Furthermore, the hexane extracts not only exhibited low yields but also contained the lowest levels of phenolic compounds, ranging from 0.13 to 0.24 mg. GAE/g-dw002E

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The protective properties of fruits are primarily attributed to the presence of phytonutrients or phytochemicals [26]. The extracts obtained from the intermediate polar solvent, ethyl acetate, contained substantially higher levels of phenolic compounds (ranging from 8.9 to 17.92 mg.GAE/g-dw) compared to the polar water fraction and were comparable to those found in the 70% chloroform extracts (Figure. 2). This suggests that the choice of solvent significantly influences the yield and phenolic content of the extracts, with polar solvents like ethyl acetate showing promise for extracting valuable bioactive compounds from citrus peels (Figure 2). The low recovery of phenols in the volatile water fraction could be attributed to the oxidation of phenolic compounds by polyphenol oxidase [27]. In contrast, the enzyme is inactivated in methanol, ethyl acetate, and chloroform. Furthermore, the citrus peels' methanolic extracts showed significantly higher phenolic compound levels ($p < 0.05$). It was evident that methanolic extracts of *C. sinensis* contained significantly higher phenolic compounds, followed by *C. reticulata* and *C. limetta* fruits. Specifically, the phenolic content was found to be 57.43 ± 1.9, 45.92 ± 2.5, and 29.3 ± 1.23 GAE/g.DM for *C. sinensis*, *C. reticulata*, and *C. limetta* peels, respectively.

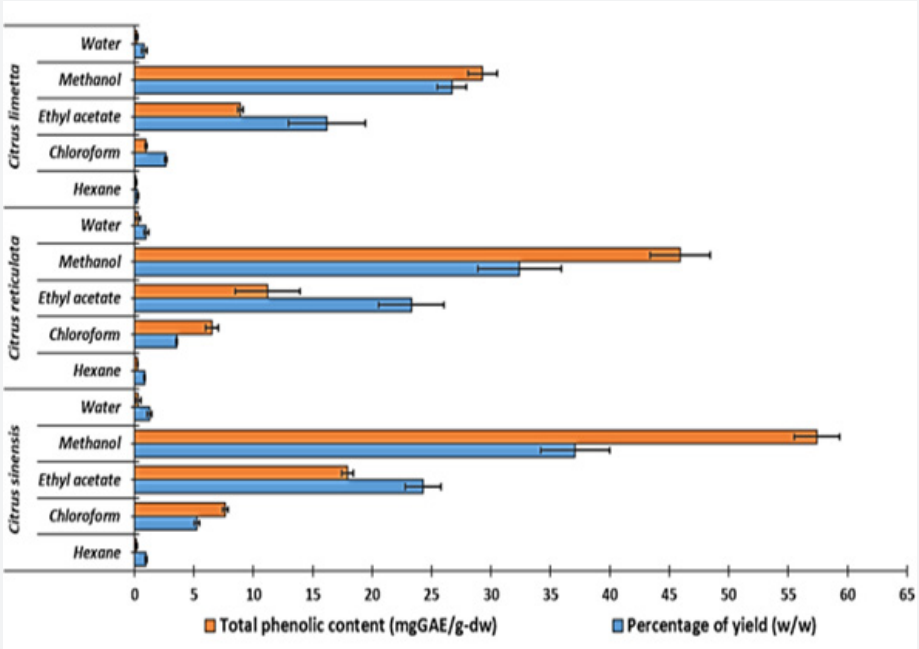

Figure 2: Yield and total phenolic content of fractionated dry extracts of citrus peels. Data represents the mean ± Sd (n = 3).

Our study's total phenol content range was 29.3 to 57.42 mg GAE/g DM, which is lower than the values reported by Kamran et al. in 2009 [28] (132.2 - 223.2 mg GAE/g DM). However, our results align with the findings of Casquete et al. in 2015 [29], who reported total phenolic contents of 22.3 mg GAE/g, 28.5 mg GAE/g, and 28.5 mg GAE/100 g in C. limon, *C. reticulata*, and *C. sinensis*, respectively. Additionally, Babbar et al. in 2011 [30] showed that peels of kinnow fruit, a hybrid of Citrus nobilis and Citrus deliciosa, have 17.5 mg GAE/gDW. These results highlight that phenolic compounds are not only present in the edible parts of citrus fruit but also in non-edible parts, such as citrus peels, making them an excellent source of phenolic compounds.

The diversity in Citrus species complicates the extraction of phenolic compounds from their natural matrix, as the contents in fruit peels are susceptible to oxidation and hydrolysis. Many researchers have shown that apart from Citrus peel, the pulp fraction also contains phenolic compounds, although in comparatively lesser quantities. Guimarães et al. in 2010 [31] revealed that peels' polar fractions have the highest contents of phenolics, flavonoids, ascorbic acid, carotenoids, and reducing sugars, contributing to the highest antioxidant potential found in these fractions. Flavonoid, another polyphenolic compound, was also analyzed in the peels of citrus fruits. The total flavonoid content ranged from 3.64 to 6.23 mg QE/g DW, with the highest levels present in *C. sinensis* and *C. reticulata* peels (6.28 ± 0.09 and 4.3 ± 0.08 mg QE/g.DW, respectively), followed by *C. limetta* peel (3.64 ± 0.05 mg QE/g DW) ($p < 0.05$)(Figure 3).

Our study's total flavonoid content values in *C. sinensis* are higher than those reported in the reference [32], which were 1.29 mg QE/g DW and 1.28 mg QE/g DW. Similarly, Wang et al. (2008) also demonstrated a flavonoid content of 35.5 ± 1.04 mg QE/100g DM in orange peel. Flavonoid contents in peels may vary with the maturation of citrus fruits. Numerous studies have indicated that citrus peels tend to contain a greater flavonoid content than the

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pulp and seeds. However, it's important to note that flavonoids in peels are often in the form of less polar flavanones, flavone aglycones, and polymethoxyflavones, while the pulp fraction is primarily abundant in glycosides-based flavonoids Table 1.

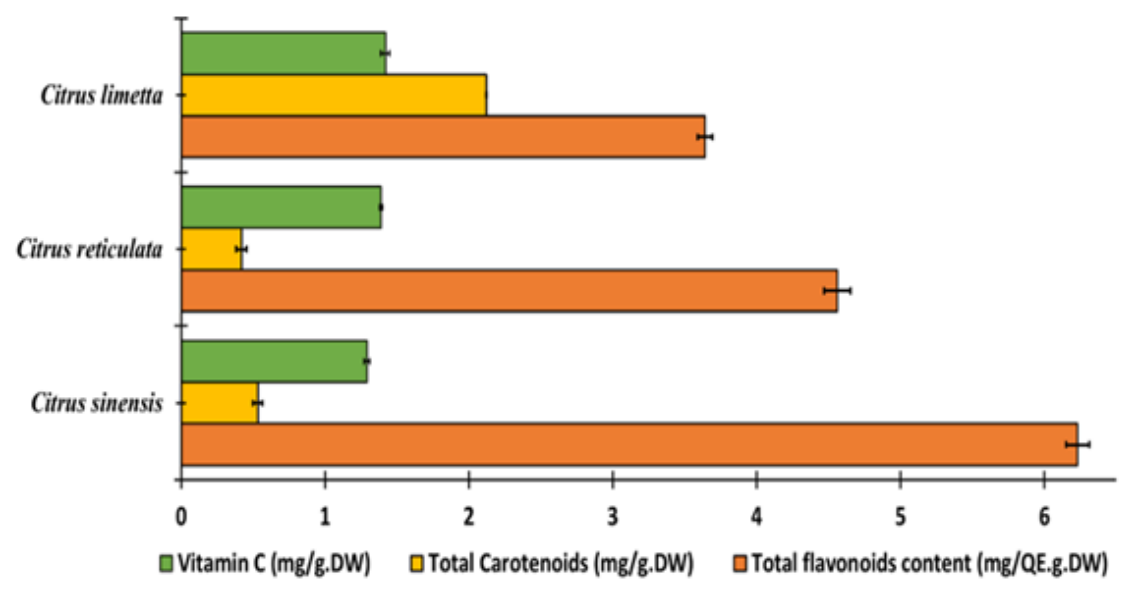

Figure 3: Total Flavonoid, Total Carotenoids, and Vitamin C content in citrus fruit cultivars.

Table 1: Total phenolic and flavonoid content reported and studied in fruit peel of different citrus species.

Peel source	Botanical Name		Phenolic content	Flavonoid content	References
Sweet orange	*C. sinensis*	EAE	66.9 mg GAE/100 g		[43]
Lemon	*C. limon*	AE	222.76 mg GAE/100 g		[29]
			530.05 mg GAE/100 g		[29]
Mandarin	*C. reticulata*		284.19 mg GAE/100 g		
Sweet orange	*C. sinensis*				
Orange (fresh peel)	*C. sinensis*	ME	39.45 mg GAE/g DW	12.95 mg CE/g DW	[44]
Orange (peel dried at 100 °C)			65.72 mg GAE/g DW	13.79 mg CE/g DW	
Orange (California)	*C. reticulata*	AE	51.8 mg GAE/g	31.9 mg/g	[45]
Orange (Guangxi)			42.0 mg GAE/g	26.0 mg/g	
Orange (Zhejiang)			46.3 mg GAE/g	23.2 mg/g	
Orange (Sichuan)			43.8 mg GAE/g	14.0 mg/g	
Orange (Xinhui)			50.2 mg GAE/g	25.0 mg/g	
Lemon	*C. limon*	ME	87.77 mg GAE/g	15.96 mg CE/g	[31]
Sweet orange	*C. sinensis*	ME	79.75 mg GAE/g	3.97 mg CE/g	
Sweet orange (Washington Navel)	*C. sinensis*	MWE	9.61 mg GAE/g DW	1.29 mg QE/g DW	[32]
Sweet orange (Thomson Navel)			25.60 mg GAE/g DW	1.28 mg QE/g DW	
	C. sinensis	ME	57.43 mgGAE/g-dw	6.23 mg/QE.g.DW	In this study
	C. reticulata		45.92 mgGAE/g-dw	4.56 mg/QE.g.DW	
	C. limetta		29.3 mgGAE/g-dw	3.64 mg/QE.g.DW	

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The evaluation of carotenoids, a significant phytochemical with potential health benefits, was conducted in the citrus peel extracts of the present study. Consistent with the findings of Wang et al. (2008), citrus peels in our study exhibited much lower levels of total carotenoids compared to total flavonoids, ranging from 0.14 ± 0.01 to 2.12 ± 0.04 mg/g DW (β-carotene equivalents). As illustrated in the figure, *C. reticulata* (2.12 ± 0.04 mg/g DW) had the highest total carotenoid content, followed by *C. sinensis* (0.53 ± 0.03 mg/g DW) and *C. limetta* (0.14 ± 0.01 mg/g DW). Furthermore, Wang et al. (2008) also reported that citrus peel extracts contained much lower total carotenoids than total flavonoids, ranging from 0.021 ± 0.0004 to 2.04 ± 0.034 mg/g db. (β-carotene equivalents).

Additionally, another natural antioxidant compound, vitamin C, was assessed in citrus peel extracts. The results indicated that *C. sinensis* (1.42 ± 0.1 mg/g DW) had slightly higher vitamin C content than *C. reticulata* (1.39 ± 0.08 mg/g DW). However, *C. limetta* presented the lowest vitamin C content of 1.31 ± 0.1 mg/g DW. Sir Elkhatim et al. also demonstrated similar findings in wasted parts of Sudanese citrus cultivars (orange, lemon, and grapefruits). They reported that the vitamin C content of grapefruit, orange, and lemon peels was found to be 1.13, 1.10, and 0.59 mg/g DW, respectively.

Antioxidant activity

The antioxidant properties of citrus peels were assessed by considering the separate contributions of the peels' volatile fraction (including essential oils) and polar fraction (containing antioxidants such as phenolics, flavonoids, ascorbic acid, and carotenoids). Different in vitro antioxidant assays were conducted in this study to determine the antioxidant capacity of citrus peels through various mechanisms. The DPPH assay, among all the tested methods, is considered a precise, easy, and economical method for assessing antioxidant activity in a short period. This method is based on a single electron transfer (SET) reaction, where sample antioxidants are oxidized by synthetic oxidants through hydrogen donation. The deep purple color of freshly prepared DPPH solution gradually diminishes in the presence of a good hydrogen donor, and the decrease in absorbance at 517 nm indicates the antioxidant activity.

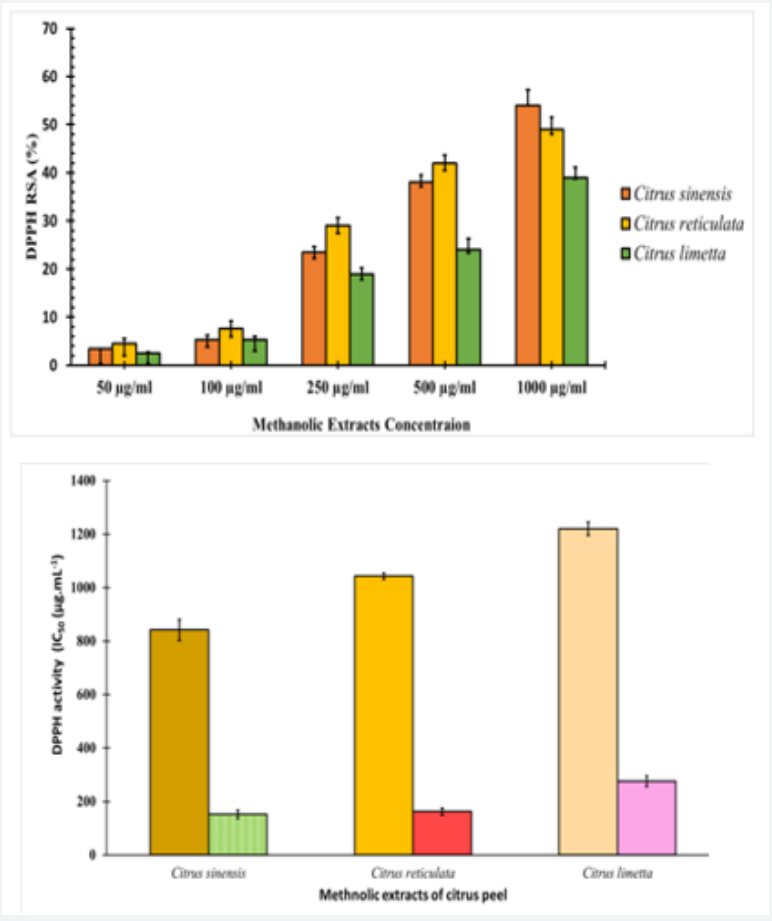

Figure: 4 DPPH radical scavenging activity (a) and IC50 (b) of citrus peel extracts. Values are mean with standard deviation.

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In Figure 4, a significant ($p < 0.05$) decrease in the concentration of DPPH is observed due to the scavenging activity of methanolic extracts of orange peel in a dose-dependent manner. The percentage of radical scavenging activity for citrus peel extracts varied between 2.5% to 54%. *C. sinensis* exhibited the maximum scavenging activity (54% ± 3.2%), followed by *C. reticulata* (49% ± 2.5%) and *C. limetta* (39% ± 2.2%) at a concentration of 1000 µg/ml methanolic extracts. The minimum scavenging activity (2.5% ± 0.2%) was observed for *C. limetta*, followed by *C. sinensis* (3.5% ± 0%) and *C. reticulata* (4.5% ± 1%) at a concentration of 50 µg/ml. Comparing IC50 values (the concentration of the sample required to scavenge 50% of the free radical content), the lowest IC50 values were observed in the methanolic extracts of *C. sinensis* (841.6 ± 30 µg/mL), followed by *C. reticulata* (1042.9 ± 12 µg/mL) and *C. limetta* (1220.23 ± 24 µg/mL) ($p < 0.05$). A lower IC50 corresponds to a stronger inhibitory capacity against the DPPH radical.

In addition to the DPPH assay, the Trolox equivalent antioxidant capacity (TEAC) assay was also conducted to assess antioxidant activity. The IC50 values of TEAC activity ranged from 312 to 423 mg Trolox/100g. *C. limetta* peel extract exhibited the highest ABTS radical scavenging activity, with an IC50 value of 423 mg Trolox/100g, among the analyzed residues in this study. It is noteworthy that the Total Phenolic Content in *C. limetta* peel was lower than those of *C. sinensis* and *C. reticulata*. This suggests that certain non-phenolic compounds, such as ascorbates, carotenoids, and terpenes present in *C. limetta*, could also contribute to the total antioxidant activity. The antioxidant activities assessed by both methods (DPPH and ABTS) showed similar correlation trends with high R2 values, i.e., 0.958, 0.923, and 0.942 with *C. sinensis*, *C. reticulata,* and *C. limetta*, respectively. These high correlation values indicate a consistent relationship between the antioxidant activities measured by the two different assays across the different citrus peel samples Figure 5.

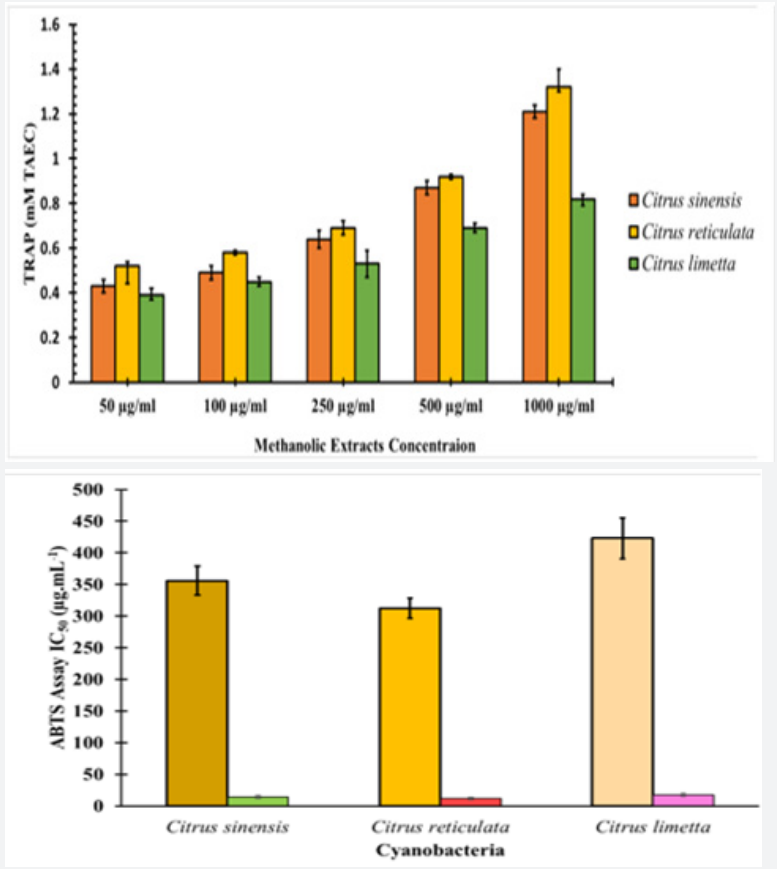

Figure: 5 TEAC (Trolox equivalent antioxidant capacity) or ABTS assay (a) and IC50 (b) of citrus peel extracts. Values are mean with standard deviation.

The antioxidant potential of citrus peel extracts was further evaluated using the potassium ferricyanide reduction method. This method assesses the reducing power of the extracts based on their ability to reduce ferric ions (Fe+3) to ferrous ions (Fe+2). The presence of reducing agents in the extracts induces this reduction, and the intensity of the resulting blue-green color is

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measured at a wavelength of 700 nm. In the analysis of reducing power across concentrations ranging from 50 to 1000 µg/mL, a similarity in reducing power ($P > 0.05$) was observed among peels of different citrus fruits. At a concentration of 1000 µg/mL, *C. sinensis* peels exhibited the highest absorbance of 0.47 ± 0.08, indicating the most pronounced reducing power overall. On the other hand, *C. limetta* showed an absorbance of 0.123 ± 0.02 at 50 µg/mL concentration. In contrast to reducing power values, the IC50 values were lowest in the methanolic extracts of *C. sinensis* (845 ± 32 µg/mL), followed by *C. reticulata* (1123 ± 36 µg/mL) and *C. limetta* (1523 ± 22 µg/mL) ($p < 0.05$). This suggests that *C. sinensis* had the strongest reducing power among the citrus peels analyzed. The results are consistent with the understanding that different citrus fruits contain a diverse array of phenolic compounds, contributing to their varying antioxidant properties Figure 6.

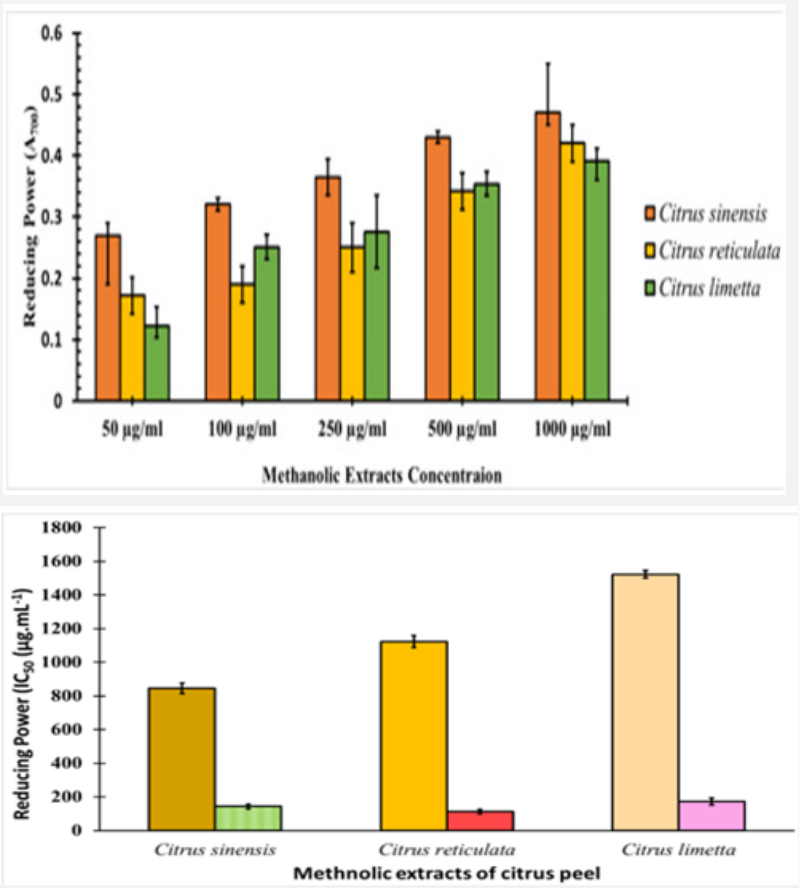

Figure: 6 Reducing power assay (a) and IC50 (b) of citrus peel extracts. Values are mean with standard deviation.

The antioxidant ability of citrus peel extracts to inhibit the bleaching of β-carotene was assessed over a concentration range of 50 to 1000 µg/mL. The bleaching activity of peel extracts was compared with that of the positive control (BHA at 100 µg/mL) using a linear BHA standard curve for interpolation. The negative control contained no antioxidant component. The results, as illustrated in Figure 7, revealed that *C. sinensis* peel extract at 1000 µg/mL exhibited the highest antioxidant activity (73.4%), while *C. limetta* at 50 µg/mL showed the least antioxidant activity (4.3%) in terms of inhibiting the bleaching of β-carotene. Therefore, the descending order of relative antioxidant activity among the tested extracts is *C. sinensis* > *C. reticulata* > *C. limetta*. ANOVA analysis indicated that the antioxidant activity of peel extracts is significantly lower ($p < 0.05$) than that of the standard [33-37]. Additionally, there was a correlation, with moderate coefficients, between the inhibition of the bleaching of β-carotene and total phenol, with R2 values of 0.743, 0.79, and 0.752 for *C. sinensis*, C. reticulata, and C. limetta, respectively. This suggests that the total phenolic content contributes to the antioxidant activity observed in the β-carotene bleaching assay for these citrus peel extracts Figure 7.

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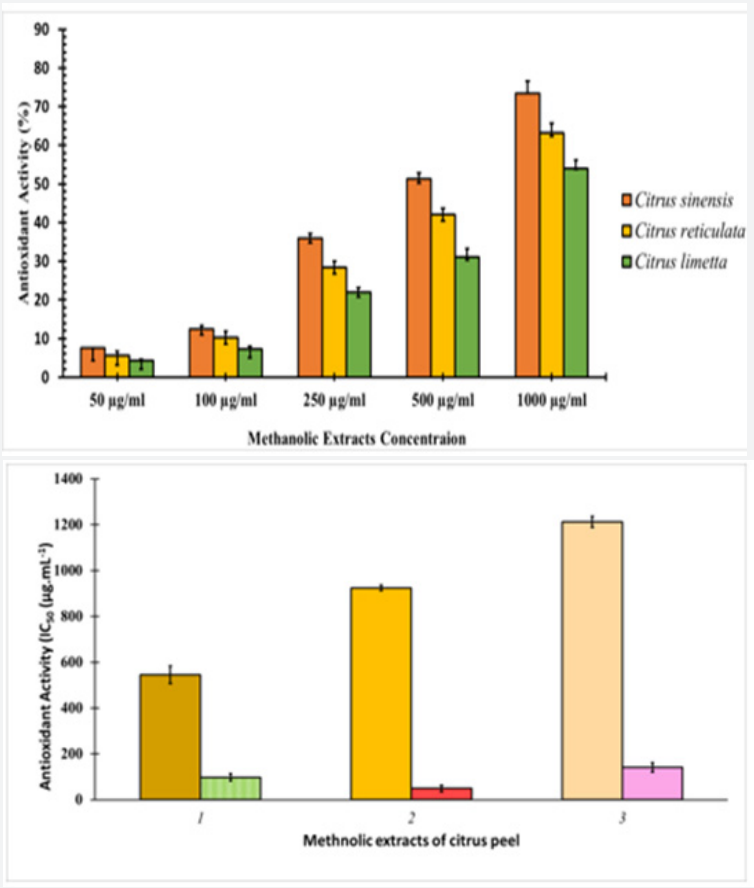

Figure 7: β carotene bleaching assay (a) and IC50 (b) of citrus peel extracts. Values are mean with standard deviation.

Comparison of antioxidant activities and total phenol

The results obtained in this study indicate that the wasted peel parts of citrus species contain appreciable amounts of phenolics, flavonoids, carotenoids, and vitamin C, along with high antioxidant activity. Extracts that inhibit oxidation are known as free radicals' scavengers or secondary antioxidants. The extracts in this study are complex fractions in composition and may contain different compounds acting independently or synergistically. As discussed earlier, the high antioxidant activity of citrus peel extracts in this study might be attributed to their high phenolic content, particularly flavonoids. Table 2 presents the correlation analysis between antioxidant activity (DPPH Assay) and the levels of total phenolic, total flavonoids, carotenoids, and vitamin C in methanolic extracts (1000 µg/mL) of citrus peel. It is evident that among the metabolites of peel extracts, total phenolic content has a higher correlation with antioxidant activity. A direct correlation between total phenolic content and antioxidant activity suggests that phenolics could be one of the main contributors to the antioxidant capacities of citrus fruit residues. Several studies have reported that the activity of antioxidants is largely governed by phenolic compounds, although some studies have found no such correlation [38-41]. In our study has revealed a strong correlation between total phenolic content (TPC) and antioxidant activity in citrus peels, with correlation coefficients ranging from 0.8211 to 0.8732. Additionally, a moderate correlation (0.7231 to 0.7632) has been observed between total flavonoids and antioxidant activity. On the other hand, weak correlations were noted between carotenoids, vitamin C, and antioxidant activity in all tested peel extracts.

This emphasizes the significant role of phenolic compounds, particularly flavonoids, in contributing to the antioxidant activity of citrus peels. It's noteworthy that antioxidant activity is not solely attributed to phenolic compounds; interactions between phenolic and non-phenolic compounds, such as reducing carbohydrates, vitamins, pigments, carotenoids, tocopherols, and terpenes, may also play a substantial role. Synergistic effects among these chemical constituents could contribute to the overall antioxidant activity of plant crude extracts. To gain

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a more detailed understanding, further research could focus on identifying specific phenolic constituents responsible for the higher antioxidant activity observed in citrus peel extracts. This could provide insights into the mechanisms underlying the antioxidant potential of these extracts.

Table: 2 Summarize correlation values in between antioxidant activity (DPPH Assay) and level of total phenolic, total flavonoids, Carotenoids, and vitamin C in methanolic extracts (1000 µg. mL^{-1}) of citrus peel.

Correlation	Coefficient of determination (R^2)		
	C. sinesis	*C. reticulata*	*C. limetta*
TPC vs. DPPH	0.8211	0.8732	0.8697
Total flavonoids vs. DPPH	0.7231	0.7453	0.7632
Carotenoids vs. DPPH	0.2140	0.2231	0.1001
Vitamin C vs. DPPH	0.2132	0.2423	0.2021

Scavenging effect on H2O2-induced human blood hemolysis

The study demonstrates the dose-dependent inhibition of H2O2-induced hemolysis of human erythrocytes by methanolic extracts of citrus peels (5-50 mg/mL). The inhibitory effect increased with the concentration of methanolic extract, ranging from 5 to 50 mg/mL. Among the citrus peel extracts, C. sinensis exhibited the maximum inhibition of 38.7 + 1.3% at 50 mg/mL. Notably, *C. limetta* showed slightly higher % inhibition (35.6 + 2.3) compared to *C. reticulata* (33.2 + 1.1), indicating potential variations in the hemolytic effects of peel extracts from different citrus fruits. This variability may be attributed to the synergistic actions of bioactive compounds present in the extracts [42]. The study aligns with previous research highlighting the protective role of polyphenolics in enhancing red blood cell resistance to oxidative stress. Polyphenolics, such as those found in citrus peel extracts, have shown the potential to mitigate oxidative stress both in vitro and in vivo. The findings contribute to the growing body of evidence supporting the antioxidant and cytoprotective properties of citrus peel extracts.

Conclusion

The study highlights the significant potential of extracts obtained from by-products of citrus fruits in both the food and pharmaceutical industries. Methanolic peel extracts, particularly from *C. sinensis*, were found to contain higher levels of phenolics and demonstrated potent antioxidant properties. *C. sinensis* exhibited the highest levels of total phenols and strong antioxidant activities, including reducing power, DPPH scavenging, and inhibition of β-carotene bleaching, compared to other varieties tested (*C. limetta* and *C. reticulata*). The results suggest a correlation between antioxidant activities and the total phenol content of the tested citrus varieties. The study further explored the application of polyphenols from citrus peel extracts in protecting human erythrocytes against hemolysis, with *C. sinensis* showing the maximum inhibition of 38.7 + 1.3% at 50 mg/mL. The findings support the idea that the wasted parts of citrus fruits could serve as abundant and low-cost natural antioxidants in the food industry. This not only has the potential to contribute to the development of bioactive ingredients but also addresses environmental pollution issues associated with the improper disposal of fruit or vegetable residues [43-46]. The study encourages the establishment of commercial units for the extraction of bioactive compounds from agricultural by-products, promoting sustainable practices and waste reduction in both rural and urban settings.

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Author contribution

S.G, and S.S. performed the experiment. S.G, S.S, P.S and R.K.J wrote the manuscript. P.S and R.K.J supervised the study.

Competing interests

All other authors declare no competing interests.

Ethical approval

Not required.

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