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Original Research Article

Isolation and characterization of flavonoid fractions from orange peel with antioxidant properties

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ABSTRACT

Background: *Citrus sinensis* peel is an abundant agro-industrial byproduct rich in flavonoids and a potentially underexploited source of natural antioxidants. This study aimed to extract, characterize, and evaluate the antioxidant potential of flavonoid fractions from orange peel for food and pharmaceutical applications.
Methods: Dried *Citrus sinensis* peel underwent hydroalcoholic extraction (ethanol: water, 1:1 v/v) for 72 hours by column chromatography purification. Characterization utilized HPLC, UV-Vis, and FTIR spectroscopy. Antioxidant activity was quantified via DPPH radical scavenging and FRAP assays.
Results: HPLC identified hesperidin as the predominant flavonoid (52.4 mg/g extract), followed by naringin (18.9 mg/g), rutin (13.7 mg/g), and quercetin (8.5 mg/g). Total flavonoid content was 4.29 mg quercetin equivalents/g extract. The extract demonstrated concentration-dependent DPPH scavenging (32.4–88.6% at 0.5–2.5 mg/ml; IC_{50}=1.21 mg/ml) and strong ferric reducing capacity (1655±18 µmol Fe(II)/g). FTIR confirmed characteristic flavonoid functional groups.
Conclusions: *Citrus sinensis* peel extract exhibits significant antioxidant activity, validating its potential as a sustainable natural antioxidant source for food preservation and pharmaceutical formulations.

Keywords: *Citrus sinensis*, Flavonoids, Hesperidin, Antioxidant activity, Waste valorization

INTRODUCTION

Citrus sinensis (L.) Osbeck, the sweet orange, is one of the most extensively cultivated and consumed fruits worldwide, with global production exceeding 79 million tonnes annually.[1] While the pulp and juice are commercially exploited, the peel constitutes approximately 40–50% of the total fruit weight and is largely discarded as agro-industrial waste. This accumulation poses significant environmental challenges, yet orange peel harbors a rich and diverse array of bioactive phytochemicals including flavonoids, phenolic acids, carotenoids, and essential oils.[2]

Among these phytochemicals, flavonoids particularly hesperidin, naringin, rutin, and quercetin have attracted considerable scientific interest for their documented antioxidant, anti-inflammatory, antimicrobial, and cardioprotective properties.[3] The antioxidant capacity of flavonoids arises primarily from their ability to donate hydrogen atoms to reactive oxygen species (ROS) and chelate transition metal ions, thereby interrupting oxidative chain reactions. Persistent oxidative stress, resulting from an imbalance between ROS generation and antioxidant defense mechanisms, is now recognized as a central contributor to the pathogenesis of cardiovascular disease, neurodegenerative disorders, type 2 diabetes, and several malignancies.[4]

Despite growing awareness of the risks associated with synthetic antioxidants such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) including

potential carcinogenicity and endocrine disruption many food and pharmaceutical formulations continue to rely on these compounds.[5] This has intensified the search for safe, effective, and renewable natural antioxidant alternatives. Citrus peel represents a particularly attractive candidate given its abundance, low cost, and well-documented phytochemical richness.[6]

Previous investigations have reported antioxidant activity in crude citrus peel extracts; however, most studies have not systematically isolated, purified, and spectroscopically characterized distinct flavonoid fractions.[7] Comprehensive evaluation combining chromatographic separation, multi-spectral characterization, and complementary in vitro antioxidant assays remains limited. Such an integrative approach is essential to establish structure–activity relationships and to reliably assess the functional potential of orange peel-derived flavonoids.[8]

The present study therefore aimed to: extract flavonoid fractions from *Citrus sinensis* peel using a hydroalcoholic solvent system; purify fractions by column chromatography; characterize the isolated fractions using HPLC, UV–Vis spectroscopy, and FTIR; and evaluate antioxidant potential through DPPH radical scavenging and FRAP assays. This work aims to support the valorization of citrus processing waste into functional bioactive materials for food, nutraceutical, and pharmaceutical applications.[9,10]

METHODS

Study design, setting, and period

This was an experimental laboratory-based study conducted at the Department of Pharmaceutics and Pharmacology, JRSET College of Pharmacy, Panchpota, Chakdaha, Nadia, West Bengal, India (September 2024 to November 2024). The study involved the extraction, isolation, characterization, and antioxidant evaluation of flavonoid fractions from Citrus sinensis peel under controlled laboratory conditions. No human subjects or animal models were used; hence, institutional ethical approval was not required.

Sample collection and selection criteria

Fresh, mature *Citrus sinensis* fruits were procured from certified local markets in Nadia district, West Bengal. Inclusion criteria for sample selection were: fully ripened fruits free from visible fungal infection, mechanical damage, or surface pesticide residue; uniform orange color of the peel; and fruits from a single commercially available variety to ensure phytochemical consistency. Fruits showing visible mold, cuts, or discoloration were excluded. Upon arrival, peels were manually separated from the pulp using sterile instruments, thoroughly washed under running water, and surface-dried.[11]

Sample preparation

Peels were oven-dried at 50°C to constant weight, cut into uniform pieces (~1×1 cm), and finely ground using a laboratory mill to obtain a homogeneous powder. Powdered samples were stored in airtight, light-resistant containers at room temperature until extraction.[12]

Extraction procedure

Cold maceration was employed to minimize thermal degradation of thermolabile flavonoids. One hundred grams of powdered peel was immersed in 1 litre of hydroalcoholic solvent (ethanol: water, 1:1 v/v) in an amber glass container at 25±2 °C for 72 hours with intermittent stirring every 8–10 hours. The macerate was filtered through muslin cloth followed by Whatman no. 1 filter paper. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator at 40°C and dried in a desiccator over anhydrous silica gel. The crude extract was stored at 4 °C until purification.[13]

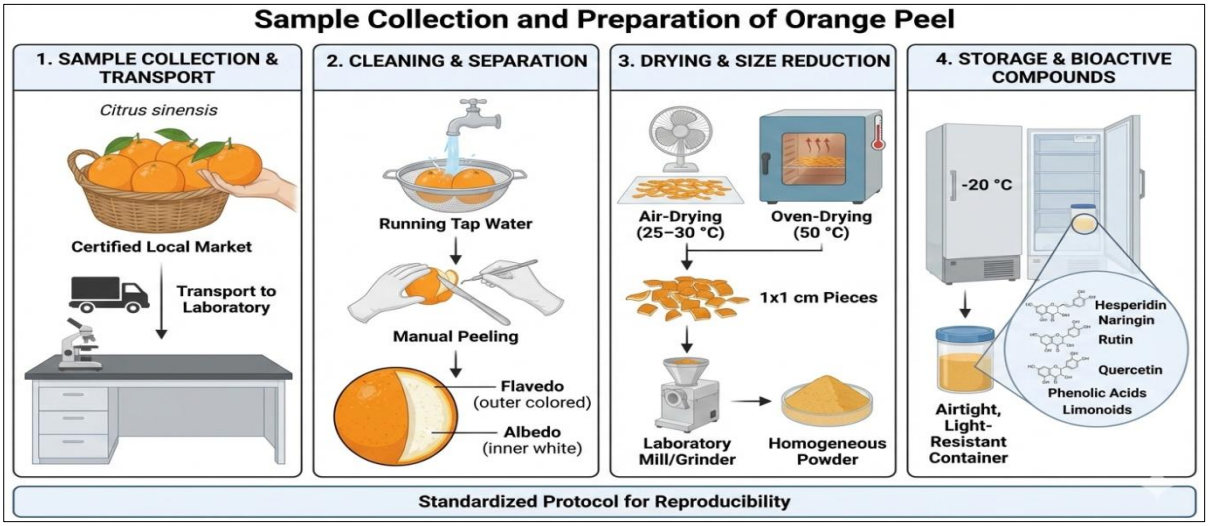

Figure 1: Sample collection and preparation from *Citrus sinensis* sweet orange.

Purification and isolation

The crude extract was re-dissolved in 50% ethanol and loaded onto a silica gel (60–120 mesh) column. Stepwise gradient elution was performed from chloroform: methanol (95:5 v/v) to ethyl acetate:ethanol: water (70:20:10 v/v/v). Fractions (~20 mL each) were monitored by TLC on silica gel GF254 plates (mobile phase: ethyl acetate: ethyl methyl ketone: glacial acetic acid:water, 5:0.5:0.5:0.5 v/v), visualized at 254 nm and with 5% ethanolic $FeCl_3$. Fractions with identical profiles were pooled, concentrated, and stored at 4 °C.[14]

Phytochemical screening

Qualitative tests were performed on the hydroalcoholic extract to identify major secondary metabolite classes, including the Shinoda test and alkaline reagent test for flavonoids, Dragendorff's test for alkaloids, Molisch's test for carbohydrates, Fehling's and Benedict's tests for reducing sugars, Biuret and Millon's tests for proteins, Liebermann's reaction for steroids, and Bornträger's test for anthraquinones.[15]

Characterization methods

Total flavonoid content (TFC)

It was determined by the aluminum chloride colorimetric method. Sample solutions (1.0 ml) were mixed with 2% $AlCl_3$ in methanol (1.0 ml), incubated 30 min, and absorbance read at 415 nm. Results expressed as mg quercetin equivalents/g extract (mg QE/g).[16]

HPLC analysis

Performed on a reversed-phase C18 column (250×4.6 mm, 5 µm) with mobile phase A (0.1% formic acid in water) and B (acetonitrile) at 1.0 ml/min; detection at 280 nm. Identification was based on retention times compared to authenticated standards.[17]

UV–Vis spectroscopy

Spectra of purified fractions were recorded in methanol over 200–600 nm to confirm characteristic flavonoid absorption maxima.[18]

FTIR spectroscopy

Spectra were recorded using the KBr pellet technique over 4000–400 cm^{-1} to identify characteristic functional groups.[19]

Scanning electron microscopy (SEM)

Surface morphology was examined at 15 kV accelerating voltage (200×–5000× magnification) after gold sputter-coating.[20]

Antioxidant assays

DPPH radical scavenging assay

DPPH solution (0.1 mM, 1.0 ml) was mixed with 1.0 ml of sample at 0.5–2.5 mg/ml, incubated 30 min in the dark, and absorbance recorded at 517 nm. Ascorbic acid served as reference.[21]

$$\%\ Inhibition = [(A_{ontrol} - A_{sample})/A_{control}] \times 100$$

FRAP assay

FRAP reagent (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ in 10:1:1 v/v) was incubated with sample (100 µl) at 37°C for 30 min; absorbance at 593 nm. Results expressed as µmol Fe(II) equivalents/g extract.[22]

Statistical analysis

All experiments were performed in triplicate. Results are expressed as mean±standard deviation (SD). IC_{50} values were determined by non-linear regression analysis using GraphPad Prism v9.0. Calibration curve linearity was assessed by Pearson's correlation coefficient (R^2).[23-25]

RESULTS

Sample preparation and extraction

Citrus sinensis peel was successfully dried and ground to a uniform pale orange powder (Figure 1). Cold maceration yielded a viscous, dark brown semi-solid crude extract with a characteristic citrus aroma (Figure 2), confirming effective extraction of phytoconstituents.

Figure 2: Dried and powdered *Citrus sinensis* peel used for extraction.

Phytochemical screening

Qualitative tests confirmed the presence of flavonoids (positive Shinoda and alkaline reagent tests) and alkaloids (positive Dragendorff's test). Carbohydrates, reducing sugars, proteins, steroids, glycosides, and anthraquinones were absent. Results are summarized in Table 1.

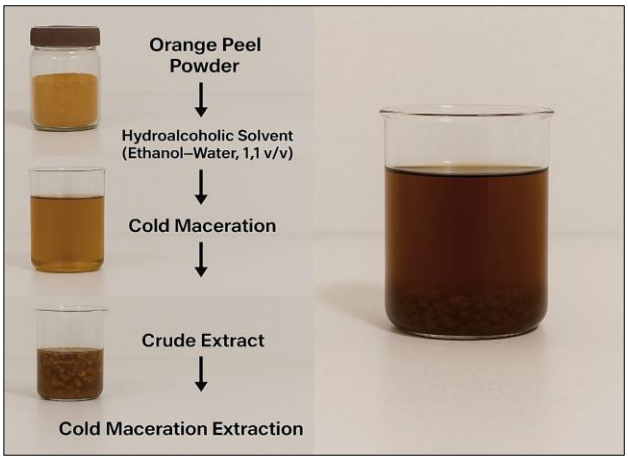

Figure 3: Crude hydroalcoholic extract obtained after cold maceration.

Purification and TLC

Column chromatography yielded well-resolved fractions with distinct colors and flavonoid concentrations. TLC of the crude extract showed multiple spots (R_f 0.28–0.72), while the purified fraction produced a single predominant band, confirming successful enrichment (Figure 4).

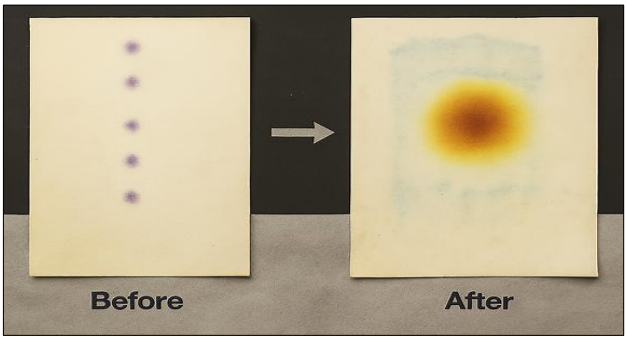

Figure 4: TLC of *Citrus sinensis* peel extract before and after column chromatography purification.

UV–Vis spectral analysis

The purified flavonoid fraction exhibited characteristic absorption at Band II (270 nm) and Band I (340 nm), corresponding to benzoyl and cinnamoyl ring systems of flavonoids, respectively (Figure 5 and Table 2).

Table 1: Phytochemical screening results of *Citrus sinensis* peel extract.

Phytochemical class	Test(s) applied	Observation	Inference
Flavonoids	Shinoda test; NaOH test; Acetate test	Positive (pink color; yellow color)	Present
Alkaloids	Dragendorff's; Mayer's; Hager's	Orange-brown ppt (Dragendorff's); others negative	Present (trace)
Carbohydrates	Molisch's test	Negative	Absent
Reducing sugars	Fehling's; Benedict's	Negative	Absent
Proteins	Biuret; Millon's	Negative	Absent
Steroids	Liebermann's reaction	Negative	Absent
Anthraquinones	Bornträger's test	Negative	Absent

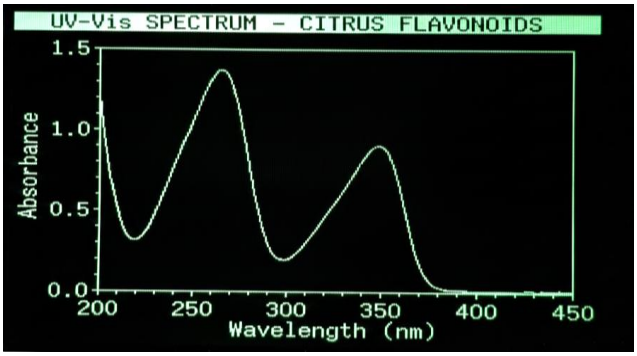

Figure 5: UV–visible absorption spectrum of the isolated flavonoid fraction.

Table 2: UV–visible absorption maxima of purified flavonoid fraction.

Sample	Band II (nm)	Band I (nm)	Inference
Purified fraction	270	340	Flavonoid confirmed

FTIR analysis

The FTIR spectrum (Figure 6) revealed: a broad O–H stretch at 3300–3400 cm^{-1} (phenolic hydroxyl groups); a C–H stretch at ~2920 cm^{-1}; a conjugated C=O stretch at ~1650 cm^{-1} confirming the flavonoid carbonyl; aromatic C=C stretches at 1500–1600 cm^{-1}; and C–O/C–O–C stretches at 1050–1270 cm^{-1} characteristic of flavonoid glycosides.

Scanning electron microscopy

SEM analysis at 100× magnification revealed distinct morphological differences between crude peel (heterogeneous, fibrous matrix with embedded bioactive compounds) and purified fraction (microcrystalline aggregates with sharp-edged flakes characteristic of high-purity hesperidin).

The increased surface area in the purified fraction is associated with improved dissolution rates (Figure 7).

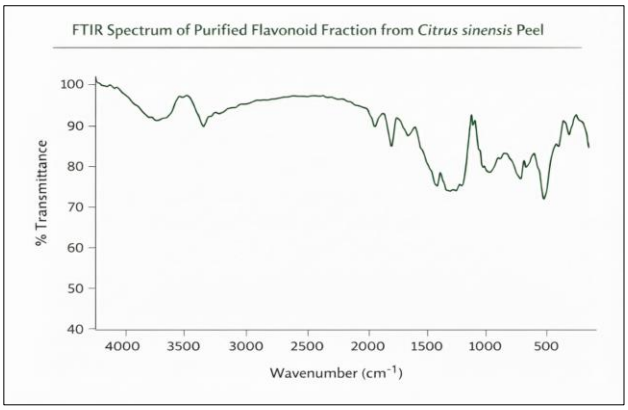

Figure 6: FTIR spectrum of purified flavonoid fraction from *Citrus sinensis* peel.

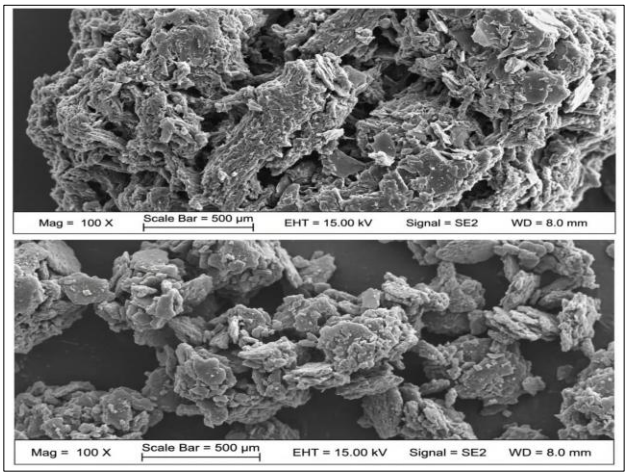

Figure 7: SEM images: (a) crude peel derivative showing heterogeneous fibrous matrix; and (b) purified flavonoid fraction showing microcrystalline morphology.

HPLC analysis

HPLC chromatographic analysis of the purified fraction identified four major flavonoid peaks. Hesperidin was the predominant constituent (Rt 8.15 min; 52.4 mg/g), followed by naringin (Rt 12.42 min; 18.9 mg/g), rutin (Rt 18.06 min; 13.7 mg/g), and quercetin (Rt 23.78 min; 8.5 mg/g) (Figure 8). All calibration curves showed excellent linearity (R^2 >0.995).

Total flavonoid content

The TFC of the orange peel extract was 4.29±0.08 mg QE/g extract (mean±SD, n=3), determined from the calibration equation y=0.0092x + 0.031 (R^2=0.998).

DPPH radical scavenging activity

The purified flavonoid fraction demonstrated clear concentration-dependent DPPH radical scavenging activity. Inhibition increased from 32.4±1.8% at 0.5 mg/ml to 88.6±1.2% at 2.5 mg/ml, yielding an IC_{50} of 1.21 mg/ml compared to ascorbic acid (IC_{50}=0.47 mg/ml) (Figure 9).

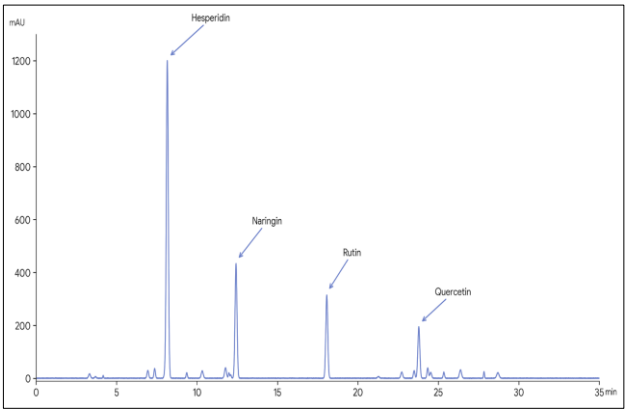

Figure 8: HPLC chromatogram of purified flavonoid fraction showing major constituents.

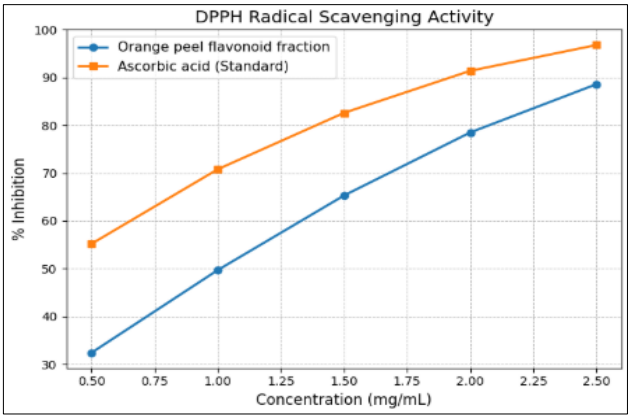

Figure 9: DPPH radical scavenging activity of flavonoid fraction versus ascorbic acid.

FRAP assay

The extract exhibited strong ferric reducing capacity of 1655±18 µmol Fe(II) equivalents/g dried extract (mean±SD, n=3; R^2=0.998 for calibration curve), indicating substantial electron-donating capability attributable to its flavonoid content.

DISCUSSION

The present study provides an integrated account of the isolation, characterization, and antioxidant evaluation of flavonoid fractions from *Citrus sinensis* peel. Hydroalcoholic extraction combined with column chromatography proved effective in enriching the flavonoid fraction, a finding consistent with Xu et al who demonstrated that ethanol–water mixtures in the 50–70% v/v range maximize flavonoid and phenolic yields from citrus peel.[9] The simplification of TLC banding patterns and the emergence of microcrystalline morphology in SEM confirmed progressive enrichment and removal of structural polysaccharides.

Hesperidin was the predominant flavonoid at 52.4 mg/g extract, consistent with prior HPLC studies of *C. sinensis* peel. Ogo et al similarly reported hesperidin as the dominant flavanone glycoside across multiple sweet orange peel varieties, with concentrations ranging from 44.6 to 68.3 mg/g depending on variety and extraction method.[4] The co-identification of naringin, rutin, and quercetin reflects the characteristic polyphenolic diversity of citrus peel and is in agreement with the compositional profile described by Singh et al.[7]

The TFC of 4.29 mg QE/g is aligned with reported values for hydroalcoholic citrus peel extracts.[2] UV–Vis absorption bands at 270 nm and 340 nm correspond to the benzoyl (Band II) and cinnamoyl (Band I) chromophores of flavonoids and are consistent with spectral profiles reported for hesperidin and related citrus flavanones.[9] FTIR peaks at ~1650 cm^{-1} (conjugated C=O) and 1050–1270 cm^{-1} (C–O–C of glycosidic linkage) corroborate the structural identity of the isolated fraction and are in agreement with FTIR assignments reported for purified hesperidin.[10]

The IC_{50} of 1.21 mg/ml for DPPH radical scavenging, while higher than pure ascorbic acid (IC_{50}=0.47 mg/mL), falls within the range reported for flavonoid-rich plant extracts. Baliyan et al.[23] noted that IC_{50} values between 0.8 and 2.0 mg/ml are characteristic of purified polyphenolic fractions with meaningful antioxidant activity. The observed activity is attributed to the multiple phenolic hydroxyl groups of hesperidin and naringin, which donate hydrogen atoms to DPPH radicals, forming stable phenoxyl intermediates through resonance delocalization.

The FRAP value of 1655±18 µmol Fe(II)/g reflects strong electron-donating capacity consistent with the conjugated aromatic systems of flavanone glycosides. This value is comparable to those reported by Rafiq et al who found FRAP values of 1420–1890 µmol Fe(II)/g in hydroalcoholic citrus peel extracts.[2] Concordance between DPPH and FRAP results suggests that the antioxidant activity arises from genuine multi-mechanism redox functionality specifically both hydrogen atom transfer and single electron transfer—rather than assay-specific artifacts. Such multi-pathway activity enhances practical utility in both food preservation and pharmaceutical applications.

Collectively, these findings validate the potential of orange peel as a sustainable, low-cost natural antioxidant source. However, the current study is limited to in vitro evaluation. Future studies should investigate bioavailability, compound-specific contributions to antioxidant activity, synergistic interactions among identified flavonoids, and in vivo validation to support translational applications.

CONCLUSION

Hydroalcoholic extraction followed by column chromatography successfully isolated flavonoid-rich fractions from *Citrus sinensis* peel, with hesperidin, naringin, rutin, and quercetin identified as the principal constituents. Spectroscopic and chromatographic characterization confirmed flavonoid structural integrity. The purified fraction demonstrated significant antioxidant activity in both DPPH and FRAP assays through complementary redox mechanisms. These results establish orange peel as a potent and sustainable natural antioxidant source with promising potential in food preservation, nutraceutical, and pharmaceutical formulations. Further in vivo and bioavailability studies are warranted.

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