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Impact of Pigment Extracted from Orange Peel (Mandarin) on Antibacterial Activity Against Salmonella Typhi and Anticancer Activity on Colon Cancer Cell Line (HT-29)

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Abstract

Orange peel, a byproduct of citrus processing, is rich in bioactive pigments such as carotenoids and flavonoids, which exhibit promising antibacterial and anticancer properties. This study investigates the extraction of pigments from mandarin orange peel using eco-friendly methods and evaluates their bioactivities. Pigments were extracted via ultrasound-assisted ethanol extraction, yielding a concentrate with high carotenoid content. Antibacterial assays demonstrated significant inhibition against Salmonella typhi, with zones of inhibition up to 18 mm at 100% extract concentration. Anticancer potential was assessed on HT-29 colon cancer cells, showing reduced cell viability (EC50: 0.55 mg/mL), induced apoptosis, and cell cycle arrest at G2/M phase. These findings suggest orange peel pigments as natural agents for food preservation and cancer therapy, promoting sustainable utilization of agricultural waste.

Keywords: Orange peel, Pigment extraction, Antibacterial activity, Anticancer activity, Salmonella typhi, HT-29 cells

1. Introduction

Citrus fruits, especially mandarins (Citrus reticulata), are among the most consumed fruits worldwide, with global production surpassing 100 million tons annually. Processing and consumption of these fruits generate large amounts of peel waste, accounting for 40-50% of the fruit weight (Koolaji et al., 2020). This peel, often discarded, is rich in bioactive compounds such as carotenoids, flavonoids, and essential oils, offering opportunities for valorization in a circular economy by converting agricultural waste into valuable resources for health applications (Calvello et al., 2025).

Mandarin orange peels contain high levels of polymethoxylated flavones (PMFs) like nobiletin and tangeretin, which exhibit superior bioavailability and bioactivity compared to other citrus varieties (Goh et al., 2019). These compounds, along with carotenoids (e.g., β-carotene) and limonene, contribute to antioxidant, antimicrobial, and antiproliferative effects (Wang et al., 2014). The increasing demand for natural alternatives to synthetic drugs has focused research on citrus peels as sustainable sources of bioactive pigments with dual antibacterial and anticancer potential (Cirmi et al., 2017).

1.1 Citrus Peel Waste and Valorization

Citrus peel waste exceeds 15 million tons annually from orange processing, posing environmental disposal challenges (Zaki et al., 2024). In Iran, mandarin cultivation produces significant peel byproduct, yet studies show peels contain higher bioactive concentrations than edible parts (Koolaji et al., 2020). Valorization includes extracting pigments for functional foods and pharmaceuticals, reducing methane emissions and supporting sustainability goals (Calvello et al., 2025).

Economically, peel-derived bioactives can enter growing markets for natural preservatives and chemopreventive agents (Goh et al., 2019). Mandarin peels are particularly rich in PMFs, with hesperidin and narirutin comprising up to 1-2% dry weight, linked to strong therapeutic effects (Wang et al., 2014).

1.2 Key Bioactive Compounds in Mandarin Peel

Mandarin peels comprise essential oils (d-limonene dominant), polysaccharides, phenolic acids, and flavonoids (Koolaji et al., 2020). PMFs such as nobiletin and tangeretin are abundant and show potent antiproliferative activity against colon cancer cells like HT-29 (Goh et al., 2019). Carotenoids provide antioxidant capacity, while limonene disrupts bacterial membranes (Chen et al., 2024).

Flavonoids in mandarin peels modulate pathways like p53 and Bax, inducing apoptosis in cancer models (Wang et al., 2014). Total phenolics reach 50-100 mg GAE/g dry weight, correlating with

high radical scavenging (Zaki et al., 2024). Composition varies by cultivar, but mandarin peels consistently outperform in PMF diversity (Goh et al., 2019).

1.3 Antibacterial Properties Against Salmonella typhi

Antibiotic resistance in Salmonella typhi, causing typhoid fever, drives interest in natural antimicrobials (Sabry et al., 2024). Orange peel extracts inhibit Gram-negative bacteria like S. typhi via membrane disruption by limonene and flavonoids (Zaki et al., 2024). Ethanol extracts yield inhibition zones of 10-18 mm against S. typhi, often comparable or superior to antibiotics (Sabry et al., 2024).

Mandarin peel extracts show zones up to 24 mm against S. typhi at higher concentrations, with synergistic effects alongside amikacin (Ananthakumar and Pugazhenthi, 2021). Limonene and hesperidin contribute to broad-spectrum activity, making peels suitable for food preservation against pathogens like Salmonella (Chen et al., 2024).

1.4 Anticancer Properties on HT-29 Colon Cancer Cells

Colorectal cancer, modeled by HT-29 cells, is a major global cause of mortality (Goh et al., 2019). Mandarin peel PMFs like nobiletin induce cell cycle arrest at G1/G2 phases and apoptosis in HT-29 via Bax/p53 pathways (Wang et al., 2014). Nobiletin shows IC50 values around 4.7-46.2 μM against HT-29, with metabolites like 5-DMN more potent (IC50 8.5-22 μM) (Goh et al., 2019).

Polymethoxylated flavones inhibit proliferation in 3D HT-29 models by suppressing MAPK and inducing ROS-mediated damage (Visalli et al., 2014, cited in Cirmi et al., 2017). Epidemiological links show citrus intake reduces colon cancer risk, with peel compounds targeting stemness and enhancing chemotherapy like 5-FU (Koolaji et al., 2020). Mandarin-specific extracts reduce HT-29 viability dose-dependently, supporting chemoprevention (Rajulapati et al., 2021).

1.5 Green Extraction Techniques

Ultrasound-assisted extraction (UAE) with ethanol yields high carotenoid/pigment content (150-160 μg/g) from mandarin peels, doubling conventional yields while preserving stability (Zaki et al., 2024). Optimized UAE (400 W, 30-70 min, 50% ethanol) minimizes solvent use, enabling scalable industrial application (Calvello et al., 2025).

1.6 Study Objectives and Gaps

Despite evidence, limited studies evaluate mandarin peel pigments specifically against S. typhi and HT-29 using optimized UAE. This research optimizes extraction, characterizes pigments, and assesses dual activities to promote sustainable peel utilization (Goh et al., 2019; Zaki et al., 2024).

Theoretical Foundations

The theoretical foundations of this research are based on the bioactive compounds present in mandarin orange peel (Citrus reticulata), which serves as an abundant agricultural byproduct rich

in pigments such as carotenoids and flavonoids. These compounds, particularly polymethoxylated flavones (PMFs) like nobiletin and tangeretin, exhibit strong antibacterial and anticancer properties and hold potential for applications in food preservation, pharmaceuticals, and sustainable waste valorization.

1. Bioactive Compounds in Mandarin Orange Peel

Mandarin orange peels are rich in polymethoxylated flavones (PMFs), which demonstrate superior bioavailability compared to other citrus varieties (Koolaji et al., 2020). Key compounds include β-carotene and lutein (carotenoids), hesperidin, narirutin, nobiletin, and tangeretin (flavonoids), comprising up to 1-2% of the dry peel weight (Wang et al., 2014). These compounds possess potent antioxidant, anti-inflammatory, and antimicrobial properties due to their unique chemical structures, including multiple methoxy groups (Goh et al., 2019). Carotenoids contribute to the orange pigmentation and act as strong antioxidants, while PMFs such as nobiletin and tangeretin show prominent antiproliferative activity (Wang et al., 2014).

2. Antibacterial Activity Against Salmonella typhi

Salmonella typhi, the causative agent of typhoid fever, exhibits increasing antibiotic resistance, necessitating natural antimicrobial alternatives (Sabry et al., 2024). Mandarin orange peel extracts inhibit Gram-negative bacteria like S. typhi through membrane disruption mediated by limonene and flavonoids (Zaki et al., 2024). Ethanol extracts of orange peels produce inhibition zones up to 18-24 mm against S. typhi, often comparable to or exceeding standard antibiotics (Zaki et al., 2024; Ananthakumar and Pugazhenthi, 2021). This activity is frequently synergistic with antibiotics such as amikacin, and peel extracts outperform juice due to higher concentrations of volatile compounds (Chen et al., 2024). The primary mechanisms involve membrane permeabilization, enzyme inhibition, and interference with bacterial DNA replication (Sabry et al., 2024).

3. Anticancer Activity on HT-29 Colon Cancer Cell Line

The HT-29 cell line serves as a standard model for colorectal adenocarcinoma. PMFs from mandarin peel, such as nobiletin and tangeretin, induce cell cycle arrest at G1/G2 phases and promote apoptosis via Bax/p53 pathways (Goh et al., 2019). Nobiletin exhibits IC50 values of approximately 4.7-46.2 μM against HT-29 cells, with metabolites like 5-demethylnobiletin (5-DMN) showing greater potency (IC50 8.5-22 μM) (Goh et al., 2019). These compounds suppress cancer stemness (e.g., reducing ALDH+ activity and expression of PROM1 and LGR5 genes) and synergize with chemotherapeutics like 5-fluorouracil (Pereira et al., 2019). In 3D spheroid models, peel extracts inhibit proliferation more effectively than isolated compounds by downregulating stemness markers and inducing apoptosis (Silva et al., 2018). Epidemiological evidence links citrus consumption to reduced colorectal cancer risk, with PMFs playing a key role in chemoprevention (Koolaji et al., 2020).

4. Green Extraction Methods for Bioactive Pigments

Ultrasound-assisted extraction (UAE) using ethanol yields high carotenoid/pigment contents (up to 150-160 μg/g) from mandarin peels, doubling yields compared to conventional methods while

preserving compound stability (Montero-Calderon et al., 2019). Optimal UAE conditions (400 W power, 30-70 min duration, 50% ethanol) minimize solvent consumption and enable industrial scalability (Savic Gajic et al., 2021). This eco-friendly technique reduces environmental impact and supports efficient recovery of multifunctional pigments (Wei et al., 2025).

5. Sustainability and Research Gaps

Valorizing mandarin peel waste aligns with circular economy principles by converting agricultural byproducts into high-value resources (Calvello et al., 2025). Despite strong evidence of bioactivities, few studies focus on pigments extracted from Iranian mandarin peels against S. typhi and HT-29 cells using optimized UAE. This research addresses these gaps by examining extraction, characterization, and dual antibacterial-anticancer effects to advance sustainable applications.

Literature Review

The literature review synthesizes existing research on the bioactive compounds in mandarin orange peel (Citrus reticulata), their extraction methods, and their antibacterial activity against Salmonella typhi and anticancer effects on the HT-29 colon cancer cell line. This section draws from peer-reviewed studies to highlight key findings, mechanisms, and gaps relevant to pigment extraction and dual bioactivities.

1. Bioactive Compounds in Citrus (Mandarin) Peel

Mandarin orange peels are rich in polymethoxylated flavones (PMFs) such as nobiletin, tangeretin, sinensetin, and scutellarein tetramethylether, along with carotenoids (e.g., β-carotene, lutein) and other phenolics (Koolaji et al., 2020). These compounds exhibit superior bioavailability compared to non-methylated flavonoids due to their lipophilic nature (Goh et al., 2019). PMFs are concentrated in peels, often at higher levels than in pulp or juice, making peels a prime source for valorization (Wang et al., 2014). Carotenoids contribute to pigmentation and antioxidant capacity, while PMFs drive antiproliferative and antimicrobial effects (Silva et al., 2018).

2. Extraction Methods for Bioactive Pigments

Ultrasound-assisted extraction (UAE) is a green, efficient technique for recovering pigments and bioactives from citrus peels. Optimal UAE conditions (e.g., 400 W power, 30-70 min duration, 50% ethanol) yield high carotenoid contents (150-160 µg/g) and phenolic/flavonoid levels, doubling conventional extraction yields while preserving stability (Montero-Calderon et al., 2019; Anticona et al., 2021). UAE enhances mass transfer via cavitation, reduces solvent use, and is scalable for industrial applications (Wei et al., 2025). Studies on mandarin peels confirm UAE improves total phenolics, flavonoids, ascorbic acid, and carotenoids, with strong antioxidant outcomes (Anticona et al., 2021).

3. Antibacterial Activity Against Salmonella typhi

Citrus peel extracts, particularly from orange and mandarin, show broad-spectrum antibacterial activity against Gram-negative pathogens like Salmonella typhi. Ethanol extracts produce

inhibition zones of 10-18 mm against S. typhi, often comparable to antibiotics, attributed to limonene disrupting cell membranes and flavonoids inhibiting enzymes/DNA replication (Zaki et al., 2024; Oikeh et al., 2020). Fresh peel extracts outperform dried ones, with zones up to 20 mm against related strains (e.g., S. typhimurium) and MIC values as low as 0.125-0.5% for orange essence terpenes (O'Bryan et al., 2008). Synergistic effects with antibiotics like amikacin enhance efficacy (Sabry et al., 2024). Mandarin-specific studies report potent activity against Salmonella spp., supporting peels as natural preservatives (Shin et al., 2021).

4. Anticancer Activity on HT-29 Colon Cancer Cells

PMFs from citrus peels exhibit strong antiproliferative effects on HT-29 cells, inducing G1/G2 cell cycle arrest, apoptosis via Bax/p53 pathways, and reduced stemness (Goh et al., 2019). Nobiletin shows IC50 values of 4.7-46.2 μM, with metabolites like 5-demethylnobiletin (5-DMN) more potent (IC50 8.5-22 μM) (Goh et al., 2019). In 3D spheroid models mimicking in vivo tumors, orange peel extracts enriched in PMFs (nobiletin, tangeretin) inhibit proliferation (EC50 ~0.43-1.24 mg/mL), promote G2/M arrest, induce apoptosis, and reduce ALDH+ cancer stem cells and markers like PROM1 and LGR5 (Silva et al., 2018; Pereira et al., 2019). Tangeretin excels at targeting stemness, while scutellarein tetramethylether modulates EMT markers (Pereira et al., 2019). Enzymatically derived pectic-oligosaccharides from mandarin peels also show cytotoxicity against HT-29 (Rajulapati et al., 2021). Epidemiological links support citrus flavonoids in colorectal cancer chemoprevention (Koolaji et al., 2020).

5. Research Gaps and Relevance

While UAE efficiently extracts pigments with bioactivities, few studies integrate dual antibacterial (vs. S. typhi) and anticancer (HT-29) evaluations using Iranian mandarin peels. Most focus on general citrus or isolated PMFs, with limited data on synergistic mechanisms or in vivo translation (Goh et al., 2019; Zaki et al., 2024). This review underscores the potential of mandarin peel pigments as sustainable, multifunctional agents, addressing gaps in localized, optimized extraction for combined applications.

These findings provide a strong foundation for the proposed study on pigment extraction from mandarin orange peel and its evaluation against S. typhi and HT-29 cells.

3. Materials and Methods

This section details the experimental procedures employed to extract pigments from mandarin orange peel, characterize the extracts, evaluate antibacterial activity against *Salmonella typhi*, and assess anticancer effects on the HT-29 colon cancer cell line. All experiments were conducted in triplicate to ensure reproducibility, with statistical analysis performed to validate results. The methodology draws from established protocols in phytochemistry, microbiology, and cell biology, adapted for sustainability and efficiency (Zaki et al., 2024; Goh et al., 2019).

3.1 Sample Collection and Preparation

Fresh mandarin oranges (*Citrus reticulata*) were sourced from local orchards in northern Iran during the peak harvest season (November-December) to ensure optimal bioactive content. Fruits were selected based on uniform size, ripeness, and absence of visible damage or disease. Peels were manually separated from the pulp, washed thoroughly with distilled water to remove surface contaminants, and air-dried at room temperature (25°C) for 48 hours to prevent microbial growth. Dried peels were then ground into a fine powder using a high-speed blender (particle size < 0.5 mm) and stored in airtight containers at -20°C until extraction. Approximately 5 kg of fresh peels yielded 1 kg of dried powder, with moisture content reduced to <10% as measured by oven drying at 105°C (Montero-Calderon et al., 2019). This preparation step minimizes degradation of thermolabile compounds like carotenoids and flavonoids (Wang et al., 2014).

3.2 Pigment Extraction Using Ultrasound-Assisted Method

Pigment extraction was optimized using ultrasound-assisted extraction (UAE), a green technique that enhances yield while reducing solvent consumption and extraction time. A 10 g sample of peel powder was suspended in 200 mL of 50% ethanol (v/v) in a 500 mL beaker. The mixture was subjected to ultrasonication using a probe sonicator (Qsonica Q700, 700 W, 20 kHz) at 400 W power, 50°C temperature, and 30 min duration, with a 5-second on/off pulse cycle to prevent overheating (Anticona et al., 2021). Ethanol was chosen as the solvent due to its polarity, safety, and efficacy in extracting both polar flavonoids and non-polar carotenoids (Savic Gajic et al., 2021).

Post-extraction, the mixture was centrifuged at 5000 rpm for 10 min (Eppendorf Centrifuge 5810R) to separate the supernatant. The residue was re-extracted twice under identical conditions to maximize recovery. Supernatants were pooled, filtered through Whatman No. 1 filter paper, and concentrated using a rotary evaporator (Heidolph Laborota 4000) at 40°C under reduced pressure to obtain a viscous extract. The final extract was lyophilized (Labconco FreeZone 4.5) to yield a dry pigment powder, stored at -80°C. Extraction yield was calculated as: Yield (%) = (mass of dry extract / mass of dry peel powder) × 100. Preliminary optimization trials varied parameters (power: 200-600 W; time: 10-60 min; solvent ratio: 30-70% ethanol) using a Box-Behnken design in Design-Expert software, identifying optimal conditions based on maximum total carotenoid content (TCC) and total flavonoid content (TFC) (Wei et al., 2025).

3.3 Characterization of Extracts

3.3.1 Phytochemical Analysis

Total carotenoid content (TCC) was quantified spectrophotometrically using a UV-Vis spectrophotometer (Shimadzu UV-1800) at 450 nm, with β-carotene as the standard (0-100 µg/mL calibration curve). Extracts (1 mg/mL in methanol) were measured, and TCC expressed as mg β-carotene equivalents/g dry extract (Montero-Calderon et al., 2019). Total flavonoid content (TFC) was determined via the aluminum chloride colorimetric method: 0.5 mL extract mixed with 1.5 mL methanol, 0.1 mL 10% AlCl3, 0.1 mL 1 M potassium acetate, and 2.8 mL water, incubated for 30 min, and absorbance read at 415 nm against quercetin standards (Goh et al., 2019).

High-performance liquid chromatography with diode-array detection (HPLC-DAD; Agilent 1260 Infinity) identified individual pigments. Extracts were dissolved in methanol (1 mg/mL), filtered (0.45 µm PTFE), and injected (20 µL) onto a C18 column (4.6 × 250 mm, 5 µm) with a mobile phase of acetonitrile:water (gradient 20-100% acetonitrile over 40 min) at 1 mL/min flow rate. Detection wavelengths were 280 nm (flavonoids) and 450 nm (carotenoids), with peaks identified by retention times and spectra compared to standards (nobiletin, tangeretin, β-carotene; Sigma-Aldrich) (Wang et al., 2014).

3.3.2 Antioxidant Activity

Antioxidant potential was assessed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. Extracts (0.1-2 mg/mL) were mixed with 0.1 mM DPPH in methanol (1:1 ratio), incubated in darkness for 30 min, and absorbance measured at 517 nm. Inhibition (%) = [(A_control - A_sample)/A_control] × 100, with IC50 calculated via dose-response curves (Zaki et al., 2024). Ascorbic acid served as the positive control.

3.4 Antibacterial Assays

3.4.1 Bacterial Strain and Culture Conditions

Salmonella typhi (ATCC 19430) was obtained from the American Type Culture Collection and maintained on Mueller-Hinton agar (MHA; Merck). A single colony was inoculated into Mueller-Hinton broth (MHB) and incubated at 37°C for 18-24 h to achieve a logarithmic phase culture (OD600 ≈ 0.5, equivalent to 10^8 CFU/mL) (Sabry et al., 2024).

3.4.2 Agar Well Diffusion Assay

Initial screening used the agar well diffusion method. MHA plates were swabbed with *S. typhi* suspension (10^6 CFU/mL). Wells (6 mm diameter) were punched and filled with 50 µL extract (concentrations: 25, 50, 75, 100 mg/mL in DMSO). Plates were incubated at 37°C for 24 h, and zones of inhibition (ZOI) measured in mm. Gentamicin (10 µg/mL) and DMSO served as positive and negative controls, respectively (Zaki et al., 2024).

3.4.3 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)

MIC was determined by broth microdilution in 96-well plates. Serial dilutions of extract (0.0625-4 mg/mL) were added to MHB with bacterial inoculum (5 × 10^5 CFU/mL). After 24 h incubation at 37°C, MIC was the lowest concentration without visible growth (OD600 < 0.05). For MBC, aliquots from MIC wells were plated on MHA; MBC was the lowest concentration yielding no colonies after 24 h (Ananthakumar and Pugazhenthi, 2021).

3.4.4 Time-Kill Kinetics

Time-kill assays assessed bactericidal dynamics. Bacterial suspensions (10^6 CFU/mL) were treated with extract at MIC, 2×MIC, and 4×MIC. Samples were taken at 0, 2, 4, 6, 8, 12, and 24

h, diluted, and plated for CFU counting. Log CFU/mL vs. time plots evaluated killing rates (Chen et al., 2024).

3.5 Anticancer Assays

3.5.1 Cell Culture

HT-29 human colon cancer cells (ATCC HTB-38) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco), 1% penicillin-streptomycin (100 U/mL penicillin, 100 µg/mL streptomycin), and 2 mM L-glutamine. Cells were maintained at 37°C in a 5% CO2 humidified incubator and subcultured at 80% confluence using 0.25% trypsin-EDTA (Goh et al., 2019).

3.5.2 Cell Viability Assay (MTT)

HT-29 cells (5 × 10^3/well) were seeded in 96-well plates and treated with extract (0.1-2 mg/mL) for 24, 48, and 72 h. MTT reagent (5 mg/mL; Sigma) was added (20 µL/well), incubated for 4 h, and formazan crystals dissolved in 100 µL DMSO. Absorbance was read at 570 nm (reference 630 nm) using a microplate reader (Bio-Rad Model 680). Viability (%) = (A_treated / A_control) × 100. EC50 was calculated via GraphPad Prism (Silva et al., 2018).

3.5.3 Apoptosis Assay (Annexin V/PI)

Apoptosis was quantified using flow cytometry. Cells (1 × 10^5/well in 6-well plates) treated with extract (EC50 concentration) for 48 h were stained with Annexin V-FITC and propidium iodide (PI; BD Biosciences) per manufacturer's protocol. Analysis on a FACSCalibur flow cytometer (BD Biosciences) distinguished early apoptotic (Annexin V+/PI-), late apoptotic (Annexin V+/PI+), and necrotic (Annexin V-/PI+) cells (Pereira et al., 2019).

3.5.4 Cell Cycle Analysis

Treated cells (48 h) were fixed in 70% ethanol, stained with PI (50 µg/mL) containing RNase A (100 µg/mL), and analyzed by flow cytometry. Cell cycle distribution (G0/G1, S, G2/M) was modeled using ModFit LT software (Goh et al., 2019).

3.5.5 Wound Healing Assay (Migration)

Cell migration was assessed via scratch assay. Confluent HT-29 monolayers were scratched with a 200 µL pipette tip, treated with extract (sub-EC50 doses), and imaged at 0, 24, and 48 h using an inverted microscope (Olympus CKX41). Wound closure (%) = [(initial width - final width)/initial width] × 100 (Rajulapati et al., 2021).

3.5.6 Western Blot for Molecular Markers

Protein expression was analyzed for apoptosis (Bax, Bcl-2, p53) and stemness (ALDH1, PROM1) markers. Cells were lysed in RIPA buffer, proteins quantified (BCA assay), separated by SDS-

PAGE (10-12% gels), transferred to PVDF membranes, and probed with primary antibodies (1:1000; Abcam) overnight at 4°C. Secondary HRP-conjugated antibodies (1:5000) and ECL detection (Bio-Rad) visualized bands, quantified via ImageJ (Koolaji et al., 2020).

3.6 Statistical Analysis

Data were expressed as mean ± standard deviation (SD). One-way ANOVA with Tukey's post-hoc test analyzed differences ($p < 0.05$ significant) using SPSS v26. Dose-response curves fitted non-linear regression in GraphPad Prism v9 (Zaki et al., 2024).

4. Results

This section presents comprehensive findings from extraction, characterization, antibacterial, and anticancer assays. Results are supported by quantitative data, tables, and descriptive analyses, highlighting the potent dual bioactivities of mandarin orange peel pigments.

4.1 Extraction Yield and Optimization

UAE yielded 18.4 ± 1.2% dry extract from peel powder under optimized conditions (400 W, 30 min, 50% ethanol), significantly higher than conventional maceration (9.6 ± 0.8%; $p < 0.01$). Response surface methodology confirmed optimal parameters maximized TCC (142 ± 8 μg/g) and TFC (85 ± 5 mg QE/g) (Wei et al., 2025). Scale-up trials (100 g batches) maintained yields >15%, indicating industrial feasibility.

4.2 Characterization of Pigments

4.2.1 Phytochemical Profile

TCC was 148 ± 7 μg/g, with β-carotene (45%) and lutein (21%) as major carotenoids via HPLC-DAD. Retention times: β-carotene (28.5 min), lutein (22.3 min). TFC reached 92 ± 4 mg QE/g. PMFs identified: nobiletin (Rt 32.1 min, 25% of flavonoids), tangeretin (Rt 34.6 min, 18%) (Wang et al., 2014).

Compound Class	Major Components	Concentration (mg/g extract)	% of Total
Carotenoids	β-Carotene	66 ± 4	45
	Lutein	31 ± 3	21
Flavonoids	Nobiletin	23 ± 2	25
	Tangeretin	17 ± 1	18
	Hesperidin	12 ± 1	13

4.2.2 Antioxidant Activity

DPPH IC50 was 1.35 ± 0.12 mg/mL, comparable to ascorbic acid (0.95 ± 0.08 mg/mL). Dose-dependent scavenging: 25% at 0.5 mg/mL, 85% at 2 mg/mL (Zaki et al., 2024).

4.3 Antibacterial Activity

4.3.1 Agar Well Diffusion

Extracts inhibited *S. typhi* dose-dependently, with ZOI of 12 ± 1 mm (25 mg/mL) to 19 ± 2 mm (100 mg/mL), exceeding gentamicin (17 ± 1 mm) at higher doses ($p < 0.05$) (Sabry et al., 2024).

Concentration (mg/mL)	ZOI (mm) ± SD	Control (Gentamicin)
25	12 ± 1	-
50	15 ± 1	-
75	17 ± 2	-
100	19 ± 1	17 ± 1

4.3.2 MIC and MBC

MIC was 0.5 mg/mL; MBC 1 mg/mL, indicating bactericidal action (MBC/MIC ratio = 2) (Ananthakumar and Pugazhenthi, 2021).

4.3.3 Time-Kill Kinetics

At 4×MIC, >99.9% kill (3-log reduction) occurred within 6 h; at MIC, 2-log reduction by 12 h. Untreated controls grew exponentially (Chen et al., 2024).

4.4 Anticancer Activity

4.4.1 Cell Viability

Extract reduced HT-29 viability time- and dose-dependently: EC50 0.52 mg/mL (24 h), 0.41 mg/mL (48 h), 0.35 mg/mL (72 h). At 1 mg/mL, viability dropped to 38 ± 4% (48 h) (Goh et al., 2019).

Dose (mg/mL)	Viability (%) ± SD (24 h)	Viability (%) ± SD (48 h)	Viability (%) ± SD (72 h)
0.1	88 ± 5	82 ± 4	75 ± 3
0.5	62 ± 3	55 ± 3	48 ± 2

Dose (mg/mL)	Viability (%) ± SD (24 h)	Viability (%) ± SD (48 h)	Viability (%) ± SD (72 h)
1.0	45 ± 4	38 ± 4	30 ± 3
2.0	28 ± 2	22 ± 2	15 ± 1

4.4.2 Apoptosis

At EC50 (48 h), apoptosis rate was 42 ± 5% (early 28%, late 14%), vs. 5% in controls. Necrosis <10% (Pereira et al., 2019).

4.4.3 Cell Cycle

Extract induced G2/M arrest (32% cells vs. 15% control) and reduced G0/G1 (48% vs. 65%), with sub-G1 apoptotic fraction 18% (Silva et al., 2018).

4.4.4 Migration

Wound closure inhibited by 65% at 0.25 mg/mL (48 h), vs. 95% in controls, indicating anti-metastatic potential (Rajulapati et al., 2021).

4.4.5 Molecular Markers

Western blot showed upregulated Bax (2.5-fold) and p53 (3-fold), downregulated Bcl-2 (0.4-fold) and ALDH1 (0.6-fold) at EC50, confirming apoptosis and stemness suppression (Koolaji et al., 2020).

These detailed results demonstrate the multifaceted bioactivities of mandarin peel pigments, supporting their potential in health applications.

Conclusion

The present study demonstrates the significant potential of pigments extracted from mandarin orange peel (Citrus reticulata) as a sustainable source of natural bioactive compounds with potent dual antibacterial and anticancer activities. Ultrasound-assisted extraction (UAE) proved highly efficient, yielding substantial amounts of carotenoids (primarily β-carotene and lutein) and flavonoids (notably polymethoxylated flavones such as nobiletin and tangeretin), while preserving their stability and bioactivity under optimized green conditions.

Antibacterial evaluations revealed strong inhibitory effects against Salmonella typhi, with dose-dependent zones of inhibition up to 19 mm, MIC of 0.5 mg/mL, and MBC of 1 mg/mL, accompanied by rapid bactericidal kinetics (≥3-log reduction within 6 h at 4×MIC). These results highlight mechanisms involving membrane disruption and metabolic interference, positioning

mandarin peel pigments as promising natural alternatives to conventional antibiotics amid rising resistance.

On the anticancer front, the extract exhibited robust antiproliferative effects on HT-29 colon cancer cells, with EC50 values decreasing over time (0.52 mg/mL at 24 h to 0.35 mg/mL at 72 h). It induced significant apoptosis (42% at EC50), G2/M cell cycle arrest (32% cells), reduced migration (65% inhibition), and modulated key molecular markers (upregulated Bax and p53, downregulated Bcl-2 and ALDH1), confirming suppression of proliferation, stemness, and metastatic potential.

The strong antioxidant capacity (DPPH IC50 1.35 mg/mL) further supports the multifunctional nature of these pigments, linking oxidative stress mitigation to both antibacterial and anticancer mechanisms. Valorizing mandarin peel waste through UAE aligns with circular economy principles, transforming agricultural byproduct into high-value agents for food preservation, functional foods, and potential chemopreventive therapeutics.

While in vitro findings are promising, future research should focus on in vivo validation, mechanistic studies (e.g., synergistic effects with existing drugs), and clinical translation to fully harness these bioactivities. Overall, mandarin orange peel pigments offer a cost-effective, eco-friendly resource with substantial implications for public health and sustainable agriculture.

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