

Comparative Effects of Ethanol Concentration on Phytochemical Profiles and *In Vitro* Bioactivities of Lime (*Citrus aurantifolia*) and Lemon (*Citrus limon*) Peel Extracts

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Abstract

This study comparatively evaluated the effects of ethanol concentration on the phytochemical characteristics and *in vitro* biological responses of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts. Extractions were performed using 50, 70, and 95% (v/v) ethanol. Extraction yield, total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity using DPPH and ABTS assays, nitric oxide (NO) production and cell viability in LPS-stimulated RAW 264.7 macrophages, and cellular metabolic activity in HeLa and A549 cancer cells were evaluated. Targeted phenolic and flavonoid profiles were also characterized using UHPLC–ESI–QTOF–MS. Lemon peel extracts consistently showed higher extraction yields than lime peel extracts across the tested ethanol concentrations, with the highest yield obtained using 50% ethanol (16.94% for lemon and 6.66% for lime). In contrast, lime peel extracts showed higher TPC values across all ethanol concentrations, with the highest TPC observed in the 95% ethanol lime peel extract (18.08 mg GAE/g extract), whereas the 95% ethanol lemon peel extract showed the highest TFC among all samples (27.82 mg QE/g extract). Among the tested extracts, the 95% ethanol lime peel extract exhibited the strongest antioxidant activity, with DPPH and ABTS IC₅₀ values of 1.04 and 0.53 mg/mL, respectively. The 95% ethanol lime peel extract also showed a lower NO level (55.36 µM) than the LPS control while maintaining moderate cell viability (65.39%). The 95% ethanol lemon peel extract showed a lower NO level (61.41 µM), but this was accompanied by lower cell viability (43.65%), indicating that reduced cellular metabolic activity may have contributed to the lower measured NO level. Targeted UHPLC–ESI–QTOF–MS analysis revealed species- and ethanol concentration-dependent differences in the measured phenolic and flavonoid profiles. Hesperidin and rutin were detected at higher concentrations in lime peel extracts, particularly in the 95% ethanol extract, whereas gallic acid was detected only in lemon peel extracts. Lime peel extracts also generally produced greater reductions in cellular metabolic activity than lemon peel extracts, particularly the 50 and 70% ethanol extracts under selected treatment conditions. Overall, both citrus species and ethanol concentration influenced the chemical composition, antioxidant activity, NO responses, and cellular metabolic activity of the peel extracts. These findings support further investigation of citrus peel extracts as sources of bioactive compounds, while additional analytical and biological studies are required to clarify the contributions of individual constituents and their potential applications.

Keywords: Lime and lemon peel extracts, Antioxidant activity, Nitric oxide production, UHPLC–ESI–QTOF–MS

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Introduction

Citrus fruits are among the most widely cultivated crops worldwide and are extensively processed for food and beverage applications (Andrade et al., 2023). Industrial processing generates substantial amounts of by-products, particularly fruit peels, which account for approximately 40–50% of the total

fruit mass (Andrade et al., 2023). Although these peels are often discarded or underutilized, they are increasingly recognized as valuable sources of bioactive phytochemicals, creating opportunities for their valorization as sustainable raw materials for higher-value applications (Ashraf et al., 2024; Saini et al., 2022). Citrus peels are rich in phenolic compounds, particularly flavonoids and phenolic acids, and generally contain higher concentrations of these constituents than the edible pulp (Zou et al., 2016; Saini et al., 2022). Citrus-derived phytochemicals, including flavanones, flavones, polymethoxylated flavones, phenolic acids, and limonoids, have been associated with a range of biological properties, including antioxidant, anti-inflammatory, antimicrobial, and cancer-related effects (Bocco et al., 1998; Dai & Mumper, 2010; Barreca et al., 2014; Saini et al., 2022). Many of these effects have been linked in previous studies to the radical-scavenging, metal-chelating, and redox-modulating properties of citrus phytochemicals, as well as their interactions with cellular signaling pathways involved in oxidative stress and inflammation (Benavente-García & Castillo, 2008; Russo et al., 2014). Citrus peel polyphenols have also been investigated for their effects on cancer-related cellular processes. Previous studies have reported that selected citrus-derived flavonoids can influence cell proliferation, apoptosis, mitochondrial function, and cell-cycle regulation in different experimental models (Barreca et al., 2014; Alaqeel, 2024). These reported biological effects provide a rationale for evaluating the responses of cancer cells to citrus peel extracts. However, because individual phytochemicals may act differently depending on concentration, matrix composition, and cell type, the biological effects of complex peel extracts cannot be attributed to any single constituent without further mechanistic evidence. Among citrus species, lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) are of particular interest because of their wide availability and distinct phytochemical compositions. Lime and lemon peels contain several flavonoids and phenolic acids, including hesperidin, rutin, naringin, eriocitrin, and phenolic acids, although their relative abundance varies among species, cultivars, and extraction conditions (Bocco et al., 1998; Nogata et al., 2006; Saini et al., 2022). Such compositional variability may contribute to differences in antioxidant and other *in vitro* biological responses and provides a rationale for direct comparison of these two citrus species under standardized extraction conditions. The extraction of phenolic and flavonoid compounds from plant matrices is strongly influenced by solvent composition. Ethanol is widely used as a relatively safe and food-compatible solvent for the recovery of plant-derived bioactive compounds. Adjustment of the ethanol–water ratio changes the overall solvent environment and can influence the solubility and recovery of phytochemicals according to their molecular structure, polarity, glycosylation, and interactions with the plant matrix (Nacz & Shahidi, 2004; Dai & Mumper, 2010). Consequently, extraction behavior cannot be predicted solely from nominal solvent polarity, and individual compounds may respond differently to changes in ethanol concentration. Changes in ethanol concentration may therefore alter both the total amount and the relative composition of phytochemicals recovered from citrus peels. Aqueous ethanol can facilitate extraction of more polar constituents, whereas ethanol-rich systems may favor the recovery of some less-polar compounds; however, these trends are compound- and matrix-dependent (Nacz & Shahidi, 2004; Dai & Mumper, 2010). Based on this rationale, ethanol concentrations of 50%, 70%, and 95% (v/v) were selected in the present study to compare extraction performance and phytochemical characteristics across solvent systems with different ethanol–water ratios. Despite extensive research on citrus peel phytochemicals, many studies have focused on individual citrus species, limited solvent conditions, or a single class of biological activity. Direct comparisons of *C. aurantifolia* and *C. limon* using the same extraction conditions together with targeted phytochemical analysis and multiple *in vitro* assays remain comparatively limited. Such an integrated approach may provide a more comprehensive understanding of the relationships among citrus species, extraction conditions, measured phytochemical profiles, and biological responses. From a sustainability perspective, the valorization of citrus peel by-products is consistent with the principles of circular resource utilization and the Bio-Circular-Green economy. Converting citrus processing residues into sources of bioactive compounds may help reduce agro-industrial waste while supporting further development of citrus peel-derived ingredients for future food, cosmetic, or related applications (Chemat et al., 2012). However, appropriate analytical characterization and biological evaluation are required before such applications can be established.

Therefore, this study aimed to comparatively investigate the effects of ethanol concentration (50, 70, and 95%, v/v) on the extraction yield, total phenolic and flavonoid contents, antioxidant activity determined by DPPH and ABTS assays, nitric oxide production and cell viability in LPS-stimulated RAW 264.7 macrophages, and cellular metabolic activity in HeLa and A549 cancer cells of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts. In addition, selected phenolic and flavonoid compounds were characterized using targeted UHPLC–ESI–QTOF–MS analysis to examine species- and ethanol concentration-dependent differences in their measured phytochemical profiles.

Materials and methods

1. Chemicals

All chemicals used in this study were of analytical grade unless otherwise stated. Ethanol was obtained from RCI Labscan (RCI Labscan Ltd., Thailand). Folin–Ciocalteu reagent and anhydrous sodium carbonate (Na_2CO_3) were purchased from Loba Chemie (Loba Chemie Pvt. Ltd., India). Aluminum chloride (AlCl_3) and potassium acetate were obtained from Kemaus (Kemaus, Australia). Gallic acid, quercetin, 2,2-diphenyl-1-picrylhydrazyl (DPPH), Trolox [6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) were purchased from Sigma-Aldrich (Sigma-Aldrich Co. LLC, USA). Potassium persulfate was obtained from Ajax Finechem (Ajax Finechem Pty Ltd., Australia).

2. Plant material

Fresh lemon (*Citrus limon* (L.) Burm. f., cv. Eureka) fruits cultivated under organic conditions were obtained from a commercial lemon farm in Cha-am, Prachuap Khiri Khan Province, Thailand, in October 2024. Fresh lime (*Citrus aurantifolia* (Christm.) Swingle), local cultivar of “Manao Paen” fruits were purchased from Talaad Thai Market, Bangkok, Thailand, during September–October 2024. All fruits were harvested at commercial maturity.

The plant materials were identified based on morphological characteristics, including fruit shape, peel texture, and aroma, and were cross-checked with standard taxonomic descriptions reported for *Citrus aurantifolia* and *Citrus limon* in the literature. Voucher specimens were not deposited, as the samples were obtained from commercial sources; however, representative samples were documented and verified against published taxonomic references.

3. Sample preparation

Lime and lemon fruits were washed with tap water to remove surface contaminants and air-dried at ambient temperature. The peels were manually separated from the pulp and further air-dried at room temperature (25–30°C) until constant weight was achieved. The dried peels were coarsely ground using a laboratory blender and passed through a 40–60 mesh sieve to obtain uniform peel powders. The resulting powders were stored in airtight zip-lock bags under dry conditions at room temperature and protected from light until extraction.

4. Extraction procedure

Bioactive compounds from lime and lemon peels were extracted using 50, 70, and 95% (v/v) ethanol to evaluate the effects of ethanol concentration on extraction performance and phytochemical characteristics. These concentrations were selected to provide ethanol–water systems with different solvent compositions, which may influence the recovery of phenolic and flavonoid compounds according to their molecular characteristics and interactions with the plant matrix.

The sample-to-solvent ratio was fixed at 1:10 (w/v). Extraction was performed by maceration at ambient temperature (25±2°C) for 72 h with intermittent stirring (every 12 h). All extractions were conducted under light-protected conditions to minimize photodegradation of phenolic and flavonoid compounds.

After extraction, the mixtures were filtered through Whatman No. 1 filter paper, and the solvents were removed under reduced pressure using a rotary vacuum evaporator (Rotavapor R-205, Buchi Labortechnik AG, Switzerland) at 40°C. The resulting crude extracts were stored at 4°C until further analysis.

The extraction yield was determined gravimetrically after solvent removal and calculated as follows:

$$\text{Extraction yield (\%)} = \frac{\text{Weight of crude extract}}{\text{Weight of dried sample}} \times 100$$

5. Determination of total phenolic content (TPC)

Total phenolic content was determined using the Folin–Ciocalteu colorimetric method as described by Itsarasook et al. (2021), with minor modifications. Briefly, 100 µL of each extract was transferred into a well of a 96-well microplate, followed by the addition of 500 µL of deionized water and 50 µL of 50% (v/v) Folin–Ciocalteu reagent. The mixture was incubated at room temperature for 5 min, after which 100 µL of 5.0% (w/v) sodium carbonate (Na_2CO_3) solution was added. The reaction mixture was incubated in the dark at room temperature for 60 min. Absorbance was measured at 725 nm using a microplate reader (EZ Read 2000, Biochrom Ltd., UK). Gallic acid was used as the calibration standard, and results were expressed as mg of gallic acid equivalents/g of extract (mg GAE/g extract).

6. Determination of total flavonoid content (TFC)

Total flavonoid content was determined using the aluminum chloride colorimetric assay according to Itsarasook et al. (2021). Briefly, 125 µL of extract was mixed with 375 µL of ethanol, followed by the

addition of 25 μL of 10% (w/v) aluminum chloride (AlCl_3), 25 μL of 1.0 M potassium acetate, and 700 μL of deionized water. The mixture was incubated at room temperature for 30 min. Absorbance was measured at 415 nm using a microplate reader (EZ Read 2000, Biochrom Ltd., UK). Quercetin was used as the calibration standard, and results were expressed as mg of quercetin equivalents/g of extract (mg QE/g extract).

7. Antioxidant assay

7.1 DPPH radical scavenging assay

Antioxidant activity was evaluated using the DPPH radical scavenging assay following the method of Itsarasook et al. (2021). A 0.2 mM DPPH solution was prepared in ethanol. Extracts were dissolved in 99.8% (AR grade) ethanol, and Trolox was used as a reference standard. In a 96-well microplate, 75 μL of extract solution was mixed with 150 μL of DPPH solution and incubated in the dark at room temperature for 30 min. Absorbance was measured at 515 nm using a microplate reader (EZ Read 2000, Biochrom Ltd., UK). The percentage of DPPH radical scavenging activity was calculated using the following equation:

$$\% \text{ DPPH scavenging} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100$$

The half-maximal inhibitory concentration (IC_{50}) values were determined by nonlinear regression analysis using Microsoft Excel.

7.2 ABTS radical scavenging assay

The ABTS radical scavenging assay was conducted according to Itsarasook et al. (2021). A 7 mM ABTS solution was mixed with 2.45 mM potassium persulfate in a 2:1 (v/v) ratio and incubated in the dark at room temperature for 14–16 h to generate ABTS^+ radicals. The resulting ABTS^+ solution was diluted with ethanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm.

For the assay, 50 μL of extract solution was mixed with 150 μL of ABTS^+ solution in a 96-well microplate and incubated in the dark at room temperature for 6 min. Absorbance was measured at 734 nm using a microplate reader (EZ Read 2000, Biochrom Ltd., UK), with Trolox used as a positive control. The percentage of ABTS radical scavenging activity was calculated as follows:

$$\% \text{ ABTS scavenging} = \frac{\text{Abs}_{\text{blank}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{blank}}} \times 100$$

IC_{50} values were calculated by nonlinear regression using Microsoft Excel.

8. Identification and quantification of phenolic and flavonoid compounds

Phenolic and flavonoid compounds in lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts were analyzed using ultra-high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight mass spectrometry (UHPLC–ESI–QTOF–MS). The analytical method was adapted from Ledesma-Escobar et al. (2016) with modifications to the chromatographic and mass spectrometric conditions. Chromatographic separation was performed using a UHPLC system (UltiMate 3000, Thermo Fisher Scientific Inc., Germany) coupled to a QTOF mass spectrometer (microTOF-Q II, Bruker Daltonics GmbH & Co. KG, Germany) equipped with an electrospray ionization (ESI) source. Data acquisition was performed using Bruker Compass HyStar software (version 3.2, Bruker Daltonics GmbH & Co. KG, Germany), while chromatographic and mass spectral data were processed using Bruker Compass Data Analysis software (version 4.0, Bruker Daltonics GmbH & Co. KG, Germany). Separation was achieved on a Zorbax SB-C18 StableBond analytical column (4.6 $\times$ 250 mm, 3.5 μm particle size). The mobile phase consisted of (A) deionized water containing 0.1% (v/v) formic acid and (B) acetonitrile containing 0.1% (v/v) formic acid. The gradient elution program was as follows: 0–5 min, 10% B; 5–50 min, linear increase from 10 to 40% B; 50–65 min, linear increase from 40 to 80% B; 65–75 min, 80% B; 75–77 min, linear decrease from 80 to 10% B; and 77–80 min, 10% B. The flow rate was maintained at 0.8 mL/min throughout the analysis. Prior to analysis, extract solutions were filtered through 0.22 μm membrane filters.

The mass spectrometer was operated in negative ion mode because phenolic and flavonoid compounds commonly form deprotonated ions ($[\text{M}-\text{H}]^-$) under ESI conditions. The operating conditions were as follows: capillary voltage, 4500 V; nebulizer pressure, 2.0 bar; drying gas temperature, 180°C; and drying gas flow rate, 8.0 L/min. Full-scan mass spectra were acquired over an m/z range of 50–1500. Tandem MS/MS spectra were not acquired in the present study.

A targeted analytical approach was applied to gallic acid, catechin, rutin, quercetin, hesperidin, naringin, and naringenin, which were selected based on their reported occurrence in citrus matrices and the

availability of authentic standards. Compound assignment was based on comparison of chromatographic retention times and accurate mass-to-charge ratios with those of the corresponding authentic standards. Chromatographic peaks corresponding to the targeted compounds were evaluated based on retention-time matching with authentic standards, and their peak areas were used for quantification. Quantification was performed using external calibration curves constructed from the corresponding authentic standards. Each extract was analyzed using a single UHPLC–ESI–QTOF–MS injection; therefore, the reported concentrations represent individual analytical measurements and are not expressed as mean±SD. The concentrations of individual compounds were expressed as µg/mg extract.

9. Determination of nitric oxide production

Nitric oxide (NO) production was determined using a protocol adapted from Tabtimmai et al. (2025) with minor modifications. RAW 264.7 murine macrophages (passages 2–5) were seeded into 96-well plates at a density of 1×10^5 cells per well and incubated for 24 h to allow cell attachment. Cells were pretreated with different concentrations of each extract (3.125, 6.25, 12.5, 25, 50, and 100 µg/mL) for 1 h, followed by stimulation with lipopolysaccharide (LPS, 1 µg/mL) for an additional 24 h to induce an inflammatory response. Diclofenac sodium (10 µg/mL) was used as a positive anti-inflammatory control. Untreated cells (medium only) and LPS-stimulated cells served as negative and positive controls, respectively. The vehicle control (0.1% DMSO) was included to exclude solvent effects. After incubation, culture supernatants were collected for the determination of nitric oxide production. NO levels were quantified using Griess reagent according to the manufacturer's instructions and calculated based on a sodium nitrite standard curve. Absorbance was measured at 540 nm using a multimode microplate reader (Synergy HTX, BioTek Instruments, Inc., USA). Data analysis was performed using Prism 10.

Following NO determination, the remaining RAW 264.7 cells were assessed for cell viability using the MTT procedure described in Section 10.2, while the treatment conditions were those described above for the NO assay.

10. Cellular metabolic activity assessment using the MTT assay

10.1 Cell culture

Human lung adenocarcinoma cells (A549) and human cervical cancer cells (HeLa) were obtained from the American type culture collection (ATCC, USA). Both cell lines were used at passage numbers 13–15 throughout the study. Cells were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific Inc., USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Cytiva, Global Life Sciences Solutions USA LLC, USA) and 1% (v/v) penicillin–streptomycin solution (Gibco, Thermo Fisher Scientific Inc., USA). Cells were cultured at 37°C in a humidified incubator under a 5% CO₂ atmosphere. The culture medium was replaced every 2–3 days, and cells were sub-cultured upon reaching approximately 80–90% confluence.

10.2 MTT assay for cellular metabolic activity

The effects of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts on cellular metabolic activity were evaluated using the MTT assay, following a protocol adapted from Tabtimmai et al. (2025) with minor modifications. A549 cells were seeded into 96-well plates at a density of 5×10^3 cells/well, whereas HeLa cells were seeded at 1×10^3 cells/well. The cells were incubated for 16–18 h to allow attachment. The culture medium was then replaced with fresh medium containing each extract at concentrations of 3.125, 6.25, 12.5, 25, 50, and 100 µg/mL, followed by incubation for 72 h. Untreated cells served as the negative control, while cells treated with 0.1% DMSO served as the vehicle control. After treatment, the culture medium was removed and replaced with fresh medium containing MTT at a final concentration of 0.5 mg/mL. The cells were incubated for 3 h at 37°C. The medium was then discarded, and the resulting formazan crystals were dissolved in 50 µL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm using a reference wavelength of 630 nm with a multimode microplate reader (Synergy™ HTX, BioTek Instruments, USA). Cellular metabolic activity was expressed as a percentage relative to untreated control cells. Data analysis was performed using Prism 10.

11. Statistical analysis

Results are presented as mean±standard deviation (SD), where applicable. Statistical analyses were conducted using IBM SPSS Statistics (version 23, IBM Corp., USA). One-way analysis of variance (ANOVA), followed by Duncan's new multiple range test, was used to determine significant differences among means. Differences were considered statistically significant at $p < 0.05$. For UHPLC–ESI–QTOF–MS analysis, each extract was analyzed using a single injection; therefore, the concentrations of individual compounds are reported as individual analytical measurements without mean±SD.

Results and discussion

1. Extraction yield of lime and lemon peel extracts

The extraction efficiency of ethanol at different concentrations (50, 70, and 95%, v/v) for recovering constituents from lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peels was evaluated. The extraction yields obtained under each condition are presented in Table 1, together with the corresponding total phenolic content (TPC) and total flavonoid content (TFC).

Table 1 Extraction yield (%), total phenolic content (TPC), and total flavonoid content (TFC) of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts obtained using different ethanol concentrations

Extract	Yield (%)	Total Phenolic Content (mg GAE/g extract)	Total Flavonoid Content (mg QE/g extract)
Lime peel (EtOH 50%)	6.66	8.13±0.94 ^b	4.08±0.67 ^d
Lime peel (EtOH 70%)	6.19	8.16±1.27 ^b	6.83±0.50 ^c
Lime peel (EtOH 95%)	3.91	18.08±0.69 ^a	20.42±2.43 ^b
Lemon peel (EtOH 50%)	16.94	2.59±0.32 ^c	2.45±0.61 ^d
Lemon peel (EtOH 70%)	12.44	3.58±0.21 ^c	2.90±0.52 ^d
Lemon peel (EtOH 95%)	10.18	7.98±0.28 ^b	27.82±2.95 ^a

Remark: TPC and TFC values are expressed as mean±SD (n=3). Different superscript letters within each column indicate significant differences (p<0.05).

Extraction yield varied with ethanol concentration in both citrus species. In general, lower ethanol concentrations produced higher extraction yields. The highest yields were obtained using 50% ethanol, reaching 6.66% for lime peel and 16.94% for lemon peel, whereas the lowest yields were obtained using 95% ethanol, at 3.91 and 10.18%, respectively. Across all ethanol concentrations, lemon peel consistently produced higher extraction yields than lime peel. The higher extraction yield obtained with aqueous ethanol does not necessarily indicate a greater recovery of phenolic or flavonoid compounds. Extraction yield represents the total mass of soluble material recovered from the plant matrix and may include sugars, organic acids, pectins, and other non-phenolic constituents in addition to bioactive compounds. Water-rich ethanol systems may therefore recover a broader range of polar and water-soluble constituents, resulting in greater total extract mass. Conversely, higher ethanol concentrations may favor the recovery of certain less-polar constituents, although extraction behavior depends on compound structure, solvent composition, and interactions with the plant matrix. Accordingly, extraction yield and phytochemical content should be considered as different aspects of extraction performance, reflecting total mass recovery and the relative enrichment of specific compound classes, respectively.

2. Total phenolic content (TPC)

The total phenolic content of lime and lemon peel extracts, determined using the Folin–Ciocalteu method, is summarized in Table 1. Significant differences in TPC were observed among the extracts (p<0.05), as determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Among all samples, the 95% ethanol lime peel extract exhibited the highest TPC (18.08±0.69 mg GAE/g extract), which was more than twofold higher than those of the corresponding 50% and 70% ethanol lime peel extracts (8.13±0.94 and 8.16±1.27 mg GAE/g extract, respectively). Lime peel extracts also showed higher TPC values than the corresponding lemon peel extracts across the tested ethanol concentrations. Differences in TPC among the extracts may reflect variations in peel composition, phenolic profiles, and solvent–matrix interactions. Citrus peels are recognized as rich sources of polyphenolic compounds, including phenolic acids, flavanones, flavones, and related constituents, although their relative abundance varies among citrus species, cultivars, plant tissues, and extraction conditions (Singh et al., 2020; Ballistreri et al., 2019). Solvent composition can influence both the solubility of phenolic compounds and their release from plant tissues, thereby affecting the relative recovery of different phenolic fractions (Naczka & Shahidi, 2004; Dai & Mumper, 2010). The higher TPC observed in the 95% ethanol lime peel extract was accompanied by higher measured concentrations of hesperidin and rutin in the targeted UHPLC–ESI–QTOF–MS analysis. This compositional pattern may contribute, at least in part, to the stronger antioxidant activity observed in this extract; however, a direct causal relationship cannot be established from the present data. Importantly, the targeted UHPLC–ESI–QTOF–MS analysis did not comprehensively characterize all

phenolic constituents present in the extracts. The solvent-dependent trends observed in this study are generally consistent with previous reports showing that ethanol–water ratio plays an important role in phenolic recovery from citrus and other plant matrices. Ethanol-rich systems may enhance the recovery of some less-polar constituents, whereas aqueous ethanol may favor more polar compounds, depending on the chemical structure of the analytes and the characteristics of the plant matrix (Naczki & Shahidi, 2004; Dai & Mumper, 2010; Khan et al., 2010; Lagha-Benamrouche & Madani, 2013). The TPC values observed in the present study are generally comparable with values reported for citrus peel extracts; however, direct comparisons should be made cautiously because extraction procedures, citrus species, sample preparation, and expression bases differ among studies.

Overall, the results indicate that the 95% ethanol lime peel extract contained the highest TPC among the tested extracts. The contrasting relationship between extraction yield and TPC further illustrates that greater total extract recovery does not necessarily correspond to greater enrichment of phenolic constituents.

3. Total flavonoid content (TFC)

Total flavonoid content differed significantly among the citrus peel extracts obtained under different ethanol concentrations ($p < 0.05$), as determined by one-way ANOVA followed by Duncan's multiple range test. Among all samples, the 95% ethanol lemon peel extract exhibited the highest TFC (27.82 ± 2.95 mg QE/g extract), followed by the 95% ethanol lime peel extract (20.42 ± 2.43 mg QE/g extract). At lower ethanol concentrations, lime peel extracts showed higher TFC values than the corresponding lemon peel extracts. The observed variation in TFC may reflect differences in flavonoid composition, solvent composition, and interactions between flavonoids and the plant matrix. Flavonoid glycosides contain sugar moieties that affect their polarity and solubility, whereas other flavonoid subclasses may exhibit lower polarity because of differences in hydroxylation, methoxylation, and glycosylation patterns. Consequently, the extraction of individual flavonoids cannot be predicted solely from bulk solvent polarity (Peng et al., 2021; Zhang et al., 2019).

The targeted UHPLC–ESI–QTOF–MS results showed solvent-dependent differences in the measured concentrations of selected flavonoids. In lime peel extracts, for example, the measured concentrations of hesperidin and rutin increased with ethanol concentration. These findings indicate compound-specific extraction behavior rather than a simple relationship between flavonoid polarity and solvent composition. Because the UHPLC–ESI–QTOF–MS analysis was targeted to selected compounds, it should not be interpreted as a comprehensive profile of all flavonoid subclasses present in the extracts.

Citrus peels are known to contain diverse flavonoid classes, including flavanone glycosides, flavones, and polymethoxylated flavones, and their abundance varies according to species, cultivar, maturity, environmental conditions, and peel tissue (Peng et al., 2021; Multari et al., 2020; Seoka et al., 2020). These factors may partly explain the contrasting flavonoid recovery patterns observed between lime and lemon peel extracts.

Flavonoids have been widely reported to possess antioxidant and other biological activities, and their activities are influenced by structural characteristics such as hydroxylation and methoxylation patterns (Pietta, 2000; Fraga et al., 2010). However, the biological activity of a complex plant extract cannot be attributed directly to total flavonoid content alone. In the present study, some extracts with higher TFC also showed stronger antioxidant activity, but this relationship was not consistent across all extracts or biological assays. It should also be noted that colorimetric TFC assays may respond differently to different flavonoid subclasses depending on their chemical structures. Therefore, the reported TFC values should be interpreted as relative estimates of flavonoid content rather than absolute quantification of all individual flavonoids.

Overall, the differences in TFC across ethanol concentrations and citrus species indicate that flavonoid recovery was influenced by both solvent composition and plant matrix. The contrasting patterns observed between lime and lemon further suggest that extraction behavior is compound- and species-dependent. These findings emphasize the importance of considering both citrus species and extraction conditions when selecting solvent systems for the recovery of flavonoid-rich citrus peel extracts.

4. Antioxidant activity determined by DPPH and ABTS assays

The antioxidant activities of lime and lemon peel extracts were evaluated using DPPH and ABTS radical scavenging assays. The results are expressed as IC_{50} values in Table 2, where lower IC_{50} values indicate stronger antioxidant activity. Significant differences in antioxidant activity were observed among the peel extracts ($p < 0.05$), as determined by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test.

Table 2 Antioxidant activity (IC₅₀, mg/mL) of lime and lemon peel extracts determined by DPPH and ABTS assays

Extract	DPPH assay (IC ₅₀ , mg/mL)	ABTS assay (IC ₅₀ , mg/mL)
Lime peel (EtOH 50%)	2.52±0.29 ^d	1.93±0.11 ^c
Lime peel (EtOH 70%)	2.06±0.12 ^d	1.07±0.04 ^c
Lime peel (EtOH 95%)	1.04±0.06 ^e	0.53±0.01 ^f
Lemon peel (EtOH 50%)	9.95±0.79 ^a	5.82±0.28 ^a
Lemon peel (EtOH 70%)	7.26±0.15 ^b	3.92±0.04 ^b
Lemon peel (EtOH 95%)	3.87±0.01 ^c	1.61±0.03 ^d
Trolox (standard)	0.020±0.00008	0.016±0.0002

Remark: Values are expressed as mean±SD (n=3). Different superscript letters within each column indicate significant differences among the peel extracts (p<0.05).

Among the tested peel extracts, the 95% ethanol lime peel extract exhibited the strongest antioxidant activity, as indicated by the lowest IC₅₀ values in both the DPPH (1.04±0.06 mg/mL) and ABTS (0.53±0.01 mg/mL) assays. Within the lime peel extracts, antioxidant activity generally increased with increasing ethanol concentration, as reflected by progressively lower IC₅₀ values. A similar trend was observed for the lemon peel extracts, although their IC₅₀ values were generally higher than those of the corresponding lime peel extracts. The 50% ethanol lemon peel extract exhibited the weakest antioxidant activity, with the highest IC₅₀ values in both the DPPH (9.95±0.79 mg/mL) and ABTS (5.82±0.28 mg/mL) assays. These findings demonstrate extract-dependent differences in antioxidant activity across the tested citrus species and ethanol concentrations.

Correlation analysis showed negative relationships between total phenolic content (TPC) and IC₅₀ values, with Pearson correlation coefficients of $r = -0.82$ for DPPH and $r = -0.81$ for ABTS. Total flavonoid content (TFC) also showed negative relationships with IC₅₀ values, although these associations were weaker, with $r = -0.48$ for DPPH and $r = -0.59$ for ABTS. Because lower IC₅₀ values indicate stronger antioxidant activity, these negative correlations suggest that extracts with higher TPC tended to exhibit stronger antioxidant activity in both assays. The associations with TFC were less pronounced, indicating that total flavonoid content alone did not fully account for the observed antioxidant responses.

The relatively strong negative associations between TPC and both DPPH and ABTS IC₅₀ values are consistent with a contribution of phenolic constituents to the antioxidant properties of the citrus peel extracts. However, these relationships should be interpreted cautiously because antioxidant activity may reflect the combined contributions of multiple phytochemical constituents rather than any single compound class. In addition, the correlation analysis was based on a limited number of extract groups and should therefore be considered descriptive rather than evidence of a causal relationship.

Differences between the DPPH and ABTS responses may partly reflect differences in assay chemistry and solvent compatibility. DPPH is commonly applied in organic media, whereas ABTS can be used in both aqueous and organic systems and can respond to a broader range of hydrophilic and lipophilic antioxidants (Re et al., 1999; Prior et al., 2005). Differences in radical chemistry, reaction kinetics, and interactions with individual antioxidants may therefore contribute to variations in IC₅₀ values between the two assays.

The association between phenolic and flavonoid composition and antioxidant activity observed in the present study is generally consistent with previous reports showing that phenolic compounds can contribute to antioxidant capacity through electron- or hydrogen-donating properties and radical stabilization (Naczki & Shahidi, 2004; Dai & Mumper, 2010). However, extraction efficiency depends on several factors, including solvent composition, compound structure, and interactions with the plant matrix. Therefore, the stronger antioxidant activity observed in some ethanol-rich extracts should not be attributed solely to solvent polarity.

The targeted UHPLC–ESI–QTOF–MS results provide additional compositional context for the antioxidant findings. Among the targeted compounds detected, hesperidin and rutin were present at higher measured concentrations in lime peel extracts, particularly in the 95% ethanol extract. Both compounds have been reported to possess antioxidant properties (Pietta, 2000; Fraga et al., 2010). The 95% ethanol lime peel extract, which contained the highest measured concentrations of hesperidin and rutin among the lime extracts, also exhibited the lowest IC₅₀ values in both antioxidant assays. Nevertheless, the present

data do not establish that these individual compounds were directly responsible for the antioxidant activity because the extracts contain multiple constituents that may contribute collectively to the overall response.

Species-dependent differences in citrus phytochemical composition have also been reported by García-Nicolás et al. (2023), who demonstrated variation in the distribution and composition of flavonoids and other antioxidant-related constituents among citrus tissues. Such compositional variability may contribute to the differences in antioxidant activity observed between lime and lemon peel extracts in the present study.

Furthermore, the antioxidant activity of phenolic compounds is influenced by structural characteristics, including the number and position of hydroxyl groups, which can affect radical scavenging, metal chelation, and electron-donating capacity (Pietta, 2000; Fraga et al., 2010). Therefore, total phenolic or flavonoid content alone may not fully predict antioxidant activity without considering the qualitative composition and relative abundance of individual constituents.

Overall, the 95% ethanol lime peel extract exhibited the strongest antioxidant activity among the tested extracts. Both lime and lemon peel extracts showed a general tendency toward stronger antioxidant activity at higher ethanol concentrations, although the magnitude of the response differed between species. These findings highlight the importance of considering both citrus species and extraction conditions when evaluating the antioxidant properties of citrus peel extracts.

5. Phenolic and flavonoid profiles of lime and lemon peel extracts

To further characterize the phytochemical composition of the peel extracts in relation to the antioxidant activities described in the previous section, targeted phenolic and flavonoid profiles of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts obtained using 50, 70, and 95% (v/v) ethanol were analyzed by UHPLC–ESI–QTOF–MS. A literature-guided targeted analytical approach was applied to selected phenolic acids and flavonoids based on their reported occurrence in citrus matrices and the availability of authentic standards. The targeted compounds included gallic acid, catechin, rutin, quercetin, hesperidin, naringin, and naringenin. Compound assignments were based on comparison of chromatographic retention times and accurate mass-to-charge ratios with those of authentic standards, while quantification was performed using external calibration curves constructed from the corresponding standards. Representative base-peak chromatograms (BPCs) and extracted-ion chromatograms (EICs) of the mixed authentic standards and the 95% ethanol extracts of lime and lemon peels are shown in Fig. 1. Because tandem MS/MS spectra were not acquired, the assignments of structurally related flavonoids should be interpreted with caution. The quantitative profiles are presented in Table 3.

Table 3 Targeted phenolic and flavonoid profiles of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts determined by UHPLC–ESI–QTOF–MS

Extract	Gallic acid ($\mu\text{g}/\text{mg}$)	Catechin ($\mu\text{g}/\text{mg}$)	Rutin ($\mu\text{g}/\text{mg}$)	Quercetin ($\mu\text{g}/\text{mg}$)	Hesperidin ($\mu\text{g}/\text{mg}$)	Naringin ($\mu\text{g}/\text{mg}$)	Naringenin ($\mu\text{g}/\text{mg}$)
Lime peel (EtOH 50%)	ND	ND	0.15	ND	5.56	0.010	ND
Lime peel (EtOH 70%)	ND	ND	0.22	ND	7.75	0.010	ND
Lime peel (EtOH 95%)	ND	ND	0.25	ND	9.31	0.010	ND
Lemon peel (EtOH 50%)	0.03	ND	0.05	ND	0.89	0.005	ND
Lemon peel (EtOH 70%)	0.04	ND	0.05	ND	0.94	0.003	ND
Lemon peel (EtOH 95%)	0.06	ND	0.06	ND	1.48	0.006	ND

Remark: Concentrations are expressed as $\mu\text{g}/\text{mg}$ extract. Each extract was analyzed using a single UHPLC–ESI–QTOF–MS injection; therefore, the values represent individual analytical measurements. ND, not detected under the applied analytical conditions

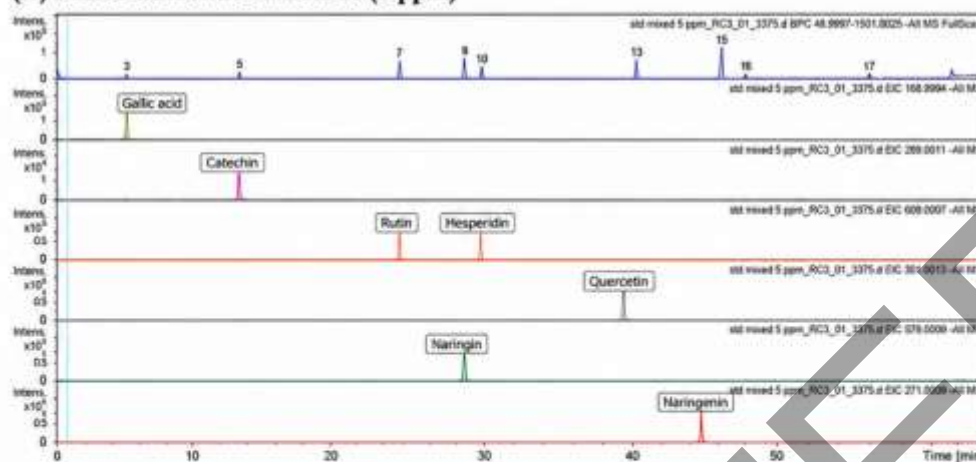
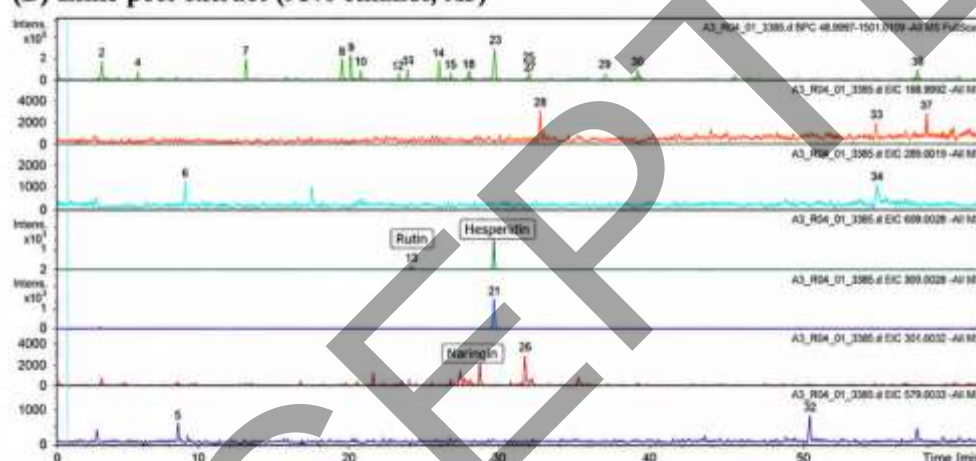
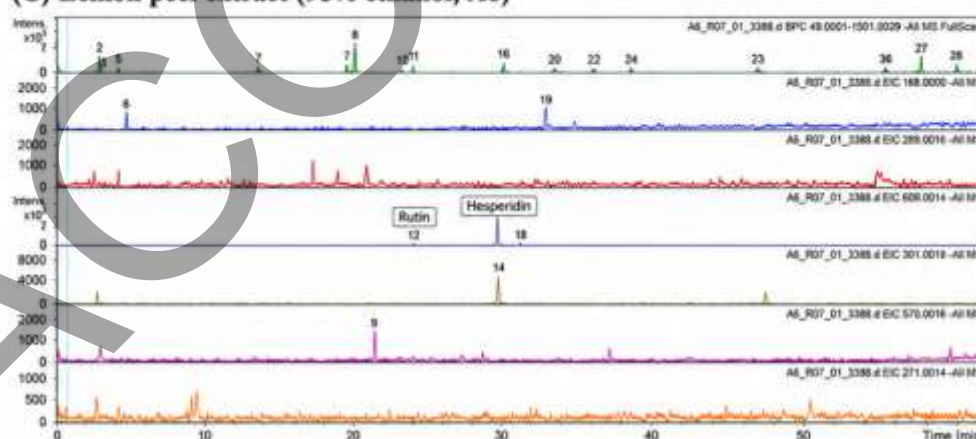
(A) Mixed authentic standards (5 ppm)**(B) Lime peel extract (95% ethanol; A3)****(C) Lemon peel extract (95% ethanol; A6)**

Fig. 1 Representative base-peak chromatograms (BPCs) and extracted-ion chromatograms (EICs) obtained by UHPLC–ESI–QTOF–MS in negative ion mode for (A) a mixed authentic standard solution (5 ppm), (B) 95% ethanol lime (*Citrus aurantifolia*) peel extract (A3), and (C) 95% ethanol lemon (*Citrus limon*) peel extract (A6)

Remark: The targeted compounds included gallic acid, catechin, rutin, hesperidin, quercetin, naringin, and naringenin. Compound assignments were based on comparison of chromatographic retention times and accurate mass-to-charge ratios with those of authentic standards.

As shown in Table 3, the targeted phenolic and flavonoid profiles varied according to citrus species and ethanol concentration. Among the targeted compounds detected, hesperidin was present at the highest measured concentration in both citrus species, followed by rutin, whereas gallic acid was detected only in lemon peel extracts at relatively low levels. Catechin, quercetin, and naringenin were not detected under the applied analytical conditions. The non-detection of these compounds may reflect differences in their abundance, extraction efficiency, and ethanol concentration-dependent recovery (Dai & Mumper, 2010; Nacz & Shahidi, 2004). Analytical factors, including compound-dependent ionization efficiency and matrix-related ion suppression in LC–ESI–MS analysis, may also influence detectability. Therefore, the absence of a detectable signal should not necessarily be interpreted as the complete absence of these compounds from the peel extracts.

Among the detected compounds, hesperidin was the predominant flavanone, particularly in lime peel extracts. Structurally, hesperidin is a flavanone glycoside consisting of the aglycone hesperetin linked to the disaccharide rutinose, resulting in both hydrophilic and hydrophobic characteristics. These structural features can influence its solubility and extraction behavior in ethanol–water systems (Hajialyani et al., 2019). In lime peel extracts, the measured hesperidin concentration increased from 5.56 $\mu\text{g}/\text{mg}$ in the 50% ethanol extract to 7.75 and 9.31 $\mu\text{g}/\text{mg}$ in the 70 and 95% ethanol extracts, respectively. Rutin showed a similar increasing trend, from 0.15 to 0.22 and 0.25 $\mu\text{g}/\text{mg}$ extract. These trends were consistent with the higher total flavonoid content and stronger antioxidant activity observed for the 95% ethanol extract among the lime peel extracts.

Although ethanol-rich solvent systems may favor the recovery of some less-polar constituents, the extraction of individual flavonoid glycosides is governed by multiple factors, including molecular structure, glycosylation, solvent composition, and interactions with the plant matrix (Dai & Mumper, 2010; Nacz & Shahidi, 2004). Accordingly, the higher measured concentrations of hesperidin and rutin in the 95% ethanol lime peel extract likely reflect compound-specific extraction behavior rather than a simple relationship with solvent polarity alone. Naringin was detected at very low concentrations in both citrus species, whereas naringenin was not detected under the applied analytical conditions. These findings may reflect differences in flavanone composition between the 2 citrus species as well as differences in extraction and ionization behavior.

In lemon peel extracts, the measured concentrations of the targeted flavonoids were generally lower than those observed in lime peel extracts. Hesperidin increased from 0.89 $\mu\text{g}/\text{mg}$ in the 50% ethanol extract to 0.94 and 1.48 $\mu\text{g}/\text{mg}$ in the 70% and 95% ethanol extracts, respectively, whereas rutin remained at relatively low levels of 0.05–0.06 $\mu\text{g}/\text{mg}$. Gallic acid was detected only in lemon peel extracts and increased from 0.03 to 0.06 $\mu\text{g}/\text{mg}$ across the tested ethanol concentrations. These findings indicate species- and ethanol concentration-dependent differences in the targeted phenolic and flavonoid profiles. However, because gallic acid was detected at relatively low concentrations, its contribution to the overall antioxidant activity cannot be established from the present data alone.

Comparison of the targeted phytochemical profiles with antioxidant activity showed that the 95% ethanol lime peel extract, which contained the highest measured concentrations of hesperidin and rutin among the lime extracts, also exhibited the lowest IC_{50} values in both the DPPH and ABTS assays. This pattern is consistent with a potential contribution of these flavonoids to the overall antioxidant activity. However, a direct causal relationship cannot be established because antioxidant activity may reflect the combined contributions of multiple phytochemical constituents (Rice-Evans et al., 1996; Prior et al., 2005). Moreover, the correlation analysis presented in Section 4 was based on total phenolic and total flavonoid contents rather than the concentrations of individual compounds.

Hesperidin has been widely reported to exhibit antioxidant, anti-inflammatory, and vascular-related biological activities (Papoutsis et al., 2017; Rangel-Huerta et al., 2015). Nevertheless, the present targeted compositional analysis does not establish that hesperidin was directly responsible for the biological responses observed in the peel extracts. Further studies involving individual-compound bioactivity testing, broader phytochemical characterization, and mechanistic investigations would be required to clarify the contributions of specific constituents.

Overall, the targeted UHPLC–ESI–QTOF–MS analysis revealed species- and ethanol concentration-dependent differences in the measured phenolic and flavonoid profiles. Lime peel extracts, particularly the 95% ethanol extract, contained higher measured concentrations of hesperidin and rutin than the corresponding lemon peel extracts, whereas gallic acid was detected only in lemon peel extracts. These compositional differences provide additional context for the variation in antioxidant and cellular responses observed among the extracts; however, direct causal relationships between individual compounds and the measured biological responses cannot be established from the present data.

6. Effects of lime and lemon peel extracts on nitric oxide production in LPS-stimulated RAW 264.7 macrophages

The effects of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts on nitric oxide (NO) production were evaluated together with cell viability in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages (Table 4). LPS stimulation markedly increased NO production compared with untreated cells, indicating successful induction of an inflammatory response. Diclofenac reduced NO production relative to the LPS control while maintaining higher cell viability, supporting the responsiveness of the assay system.

Table 4 Effects of lime and lemon peel extracts on nitric oxide (NO) production and cell viability in LPS-stimulated RAW 264.7 macrophages

Sample	NO level (μM)	Cell viability (%)
Un	4.21 $\pm$ 0.52	100 $\pm$ 1.59
LPS	70.24 $\pm$ 5.48	61.17 $\pm$ 7.36
DIC (Diclofenac)	21.71 $\pm$ 1.08	74.64 $\pm$ 6.03
Lime peel (EtOH 50%)	70.91 $\pm$ 4.90 ^{bc}	57.00 $\pm$ 7.47 ^b
Lime peel (EtOH 70%)	74.18 $\pm$ 5.89 ^{ab}	49.08 $\pm$ 4.85 ^{bc}
Lime peel (EtOH 95%)	55.36 $\pm$ 1.56 ^e	65.39 $\pm$ 2.93 ^a
Lemon peel (EtOH 50%)	80.17 $\pm$ 1.80 ^a	67.75 $\pm$ 4.70 ^a
Lemon peel (EtOH 70%)	67.08 $\pm$ 1.30 ^{cd}	52.70 $\pm$ 3.20 ^b
Lemon peel (EtOH 95%)	61.41 $\pm$ 1.69 ^d	43.65 $\pm$ 1.54 ^c

Remark: Values are expressed as mean $\pm$ standard deviation (SD) (n = 3). Different superscript letters within the same column indicate statistically significant differences among extract-treated groups (p<0.05). Untreated (Un), LPS-stimulated, and diclofenac-treated groups served as controls and were excluded from post hoc multiple comparisons.

NO production was interpreted together with cell viability because reductions in cellular metabolic activity may influence the measured NO concentration. Among the tested extracts, the 95% ethanol lime peel extract showed the lowest NO level (55.36 $\pm$ 1.56 μM) among the lime extracts and maintained a cell viability of 65.39 $\pm$ 2.93%. This combination suggested a more favorable balance between reduced NO production and cell viability than that observed for the other lime peel extracts. In contrast, the 50% and 70% ethanol lime peel extracts showed NO levels close to or higher than the LPS control and were accompanied by lower cell viability.

For lemon peel extracts, the 95% ethanol extract showed a lower NO level (61.41 $\pm$ 1.69 μM) than the LPS control; however, this was accompanied by low cell viability (43.65 $\pm$ 1.54%). Therefore, the possibility that reduced cellular metabolic activity contributed to the lower measured NO level cannot be excluded. The 70% ethanol lemon peel extract showed an intermediate NO level together with reduced cell viability, whereas the 50% ethanol extract did not reduce NO production relative to the LPS control.

The differences in NO responses among the extracts may be related, at least in part, to variation in their phytochemical composition. LPS-induced NO production in macrophages is commonly associated with activation of inflammatory pathways involving inducible nitric oxide synthase (iNOS), and phenolic compounds and flavonoids have been reported to modulate NO production and related inflammatory signaling pathways in macrophage models (Aktan, 2004; Dai & Mumper, 2010; Middleton et al., 2000; Kim et al., 2004). However, the present study measured only NO production and cell viability and did not directly assess iNOS expression, NF- κ B activation, or cytokine production.

The targeted UHPLC–ESI–QTOF–MS analysis provides additional compositional context for these findings, as lime peel extracts contained measurable hesperidin and rutin, compounds that have been reported to possess anti-inflammatory properties (Li & Schluesener, 2017; Parhiz et al., 2015). Hesperidin and related flavonoids have been reported in previous studies to modulate inflammatory mediators, including NO production and iNOS-related pathways. Nevertheless, the present study does not establish that these individual compounds were directly responsible for the NO responses observed in the peel extracts.

The comparatively weaker cellular responses observed for some lemon peel extracts may reflect differences in phytochemical composition as well as differences in cellular effects at the tested concentration. These findings indicate that changes in NO production should be interpreted together with

cell viability to avoid attributing lower NO levels solely to anti-inflammatory effects when reduced cellular metabolic activity may also contribute.

Overall, the 95% ethanol lime peel extract showed the most favorable combination of lower NO production and moderate cell viability among the tested extracts. Together with the antioxidant findings, these results suggest that differences in phytochemical composition may contribute to the variation in cellular responses among lime and lemon peel extracts. However, further studies examining specific inflammatory mediators and signaling pathways are required to clarify the mechanisms involved.

7. Effects of lime and lemon peel extracts on cellular metabolic activity in HeLa and A549 cancer cells

The effects of lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts on cellular metabolic activity were evaluated in HeLa cervical cancer cells and A549 human lung cancer cells using the MTT assay. Cells were treated with extract concentrations ranging from 3.125 to 100 $\mu\text{g/mL}$, and the corresponding dose–response profiles are presented in Fig. 2.

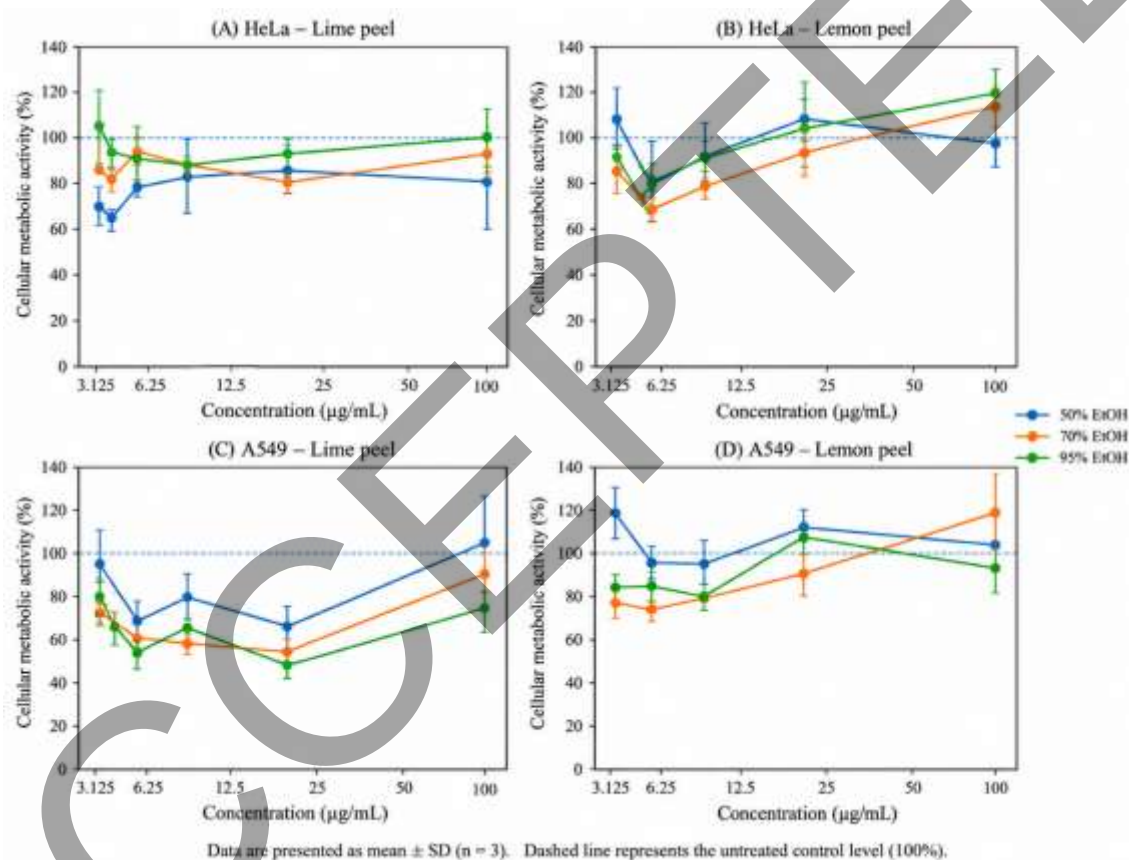


Fig. 2 Cellular metabolic activity of HeLa cervical cancer cells and A549 human lung cancer cells following treatment with lime and lemon peel extracts, as determined by the MTT assay

In HeLa cells, most extract treatments maintained relatively high metabolic activity across the tested concentration range, although concentration-dependent reductions were observed for some extracts. Lime peel extracts obtained with 50 and 70% ethanol generally produced greater reductions in MTT response than the 95% ethanol extract, particularly within the concentration range of 6.25–25 $\mu\text{g/mL}$. Lemon peel extracts showed comparatively smaller reductions in metabolic activity across most concentrations.

A similar pattern was observed in A549 cells, although some lime peel extracts produced greater reductions in metabolic activity than those observed in HeLa cells. In particular, the 50 and 70% ethanol lime peel extracts showed lower MTT responses at several concentrations, whereas lemon peel extracts generally maintained higher metabolic activity across the tested range. These findings indicate differences in cellular responses among the extracts; however, reductions in MTT response may reflect changes in cellular metabolism, cell number, growth rate, and/or cytotoxic effects and cannot be attributed to a single mechanism based on the present assay alone.

The extracts exhibited intrinsic coloration and variable solubility, which may have influenced absorbance-based MTT measurements. Greater variability was observed at the highest tested concentration (100 µg/mL), and the results at this concentration should therefore be interpreted with caution because optical interference and solubility-related effects cannot be excluded.

Among the lime peel extracts, those obtained with 50 and 70% ethanol showed more pronounced reductions in HeLa cell metabolic activity than the 95% ethanol extract, particularly at intermediate concentrations. In A549 cells, lime peel extracts also showed concentration-dependent reductions in MTT response, with some treatments approaching approximately 50% viability. However, reliable IC_{50} values could not be determined because the extracts did not produce sufficiently consistent dose–response relationships reaching 50% inhibition across the tested concentration range.

The distinction between IC_{50} and GI_{50} is also important when interpreting these findings. IC_{50} represents the concentration required to reduce the measured response by 50%, whereas GI_{50} reflects inhibition of net cell growth relative to the initial cell population. GI_{50} values were not determined in the present study because baseline (T_0) measurements required for growth-based calculations were not available. Therefore, the observed effects are described primarily in terms of concentration-dependent changes in cellular metabolic activity rather than calculated growth-inhibition parameters.

Differences in cellular responses among the extracts may reflect differences in their overall phytochemical composition and extraction behavior. The targeted UHPLC–ESI–QTOF–MS analysis demonstrated species- and ethanol concentration-dependent variation in selected phenolic and flavonoid compounds, including hesperidin and rutin. However, the specific constituents responsible for the observed reductions in cellular metabolic activity were not established in the present study, and no direct mechanistic relationship between individual compounds and the cellular responses can be inferred.

Previous studies have reported that citrus-derived phenolic compounds and flavonoids can influence cancer cell proliferation, apoptosis, mitochondrial function, and cell-cycle regulation (Benavente-García & Castillo, 2008; Khan et al., 2014; Cirimi et al., 2016). Nevertheless, these mechanisms were not examined in the present study and should be considered as literature context rather than direct explanations for the observed responses.

It should also be noted that the MTT assay is a single end-point metabolic assay that reflects cellular metabolic activity and may not directly correspond to cell number or cell death (Fotakis & Timbrell, 2006). Therefore, the distinction between growth inhibition and cytotoxicity cannot be established conclusively from MTT data alone. Further studies incorporating complementary methods, such as direct cell counting, apoptosis assays, membrane-integrity assays, and cell-cycle analysis, would be required to clarify the nature and mechanism of the observed effects.

Overall, lime peel extracts generally produced greater reductions in cellular metabolic activity than lemon peel extracts under the tested conditions, particularly for some 50 and 70% ethanol extracts. These findings suggest differences in cellular responses between lime and lemon peel extracts and indicate that both citrus species and extraction conditions may influence their *in vitro* biological effects. Further mechanistic and confirmatory studies are required before specific antiproliferative or cytotoxic activities can be established.

Conclusion

This study demonstrated differences in extraction yield, phytochemical characteristics, and *in vitro* biological responses among lime (*Citrus aurantifolia*) and lemon (*Citrus limon*) peel extracts obtained using different ethanol concentrations. Lemon peel extracts exhibited higher extraction yields than lime peel extracts across the tested ethanol concentrations, whereas lime peel extracts showed higher total phenolic contents. Total flavonoid content varied among the extracts, with the highest value observed in the 95% ethanol lemon peel extract.

Among the tested extracts, the 95% ethanol lime peel extract exhibited the strongest antioxidant activity, as indicated by the lowest IC_{50} values in both the DPPH and ABTS assays. Targeted UHPLC–ESI–QTOF–MS analysis revealed species- and ethanol concentration-dependent differences in the measured phenolic and flavonoid profiles. Hesperidin and rutin were present at higher measured concentrations in lime peel extracts, particularly in the 95% ethanol extract, whereas gallic acid was detected only in lemon peel extracts. These compositional patterns may help explain, at least in part, the differences in antioxidant activity among the extracts; however, direct causal relationships between individual compounds and biological responses cannot be established from the present data. The 95% ethanol lime peel extract also showed a lower nitric oxide level in LPS-stimulated RAW 264.7 macrophages while maintaining moderate cell viability. In contrast, some 50–70% ethanol extracts produced greater

reductions in cellular metabolic activity in the tested cancer cell lines, although these responses were generally modest and concentration dependent. Compound assignments were based on comparison of chromatographic retention times and accurate mass-to-charge ratios with those of authentic standards. Because tandem MS/MS spectra were not acquired, the assignments of structurally related flavonoids should be interpreted with appropriate caution.

The biological responses reported in this study were based on *in vitro* assays; therefore, direct extrapolation to *in vivo* systems is limited. Further studies should include broader analytical characterization, mechanistic evaluation, bioavailability assessment, and, where appropriate, *in vivo* investigation. Advanced formulation or delivery approaches, including encapsulation-based strategies, may also be explored in future studies to improve the solubility, stability, or bioaccessibility of flavonoid-rich citrus peel extracts.

Overall, the findings demonstrate clear differences among citrus peel extracts prepared using different ethanol concentrations. The 95% ethanol lime peel extract showed particular potential as a source of antioxidant-associated flavonoids; however, further analytical characterization and biological evaluation are required before specific product applications can be established.

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Conflicts of interest

The authors declare no conflicts of interest.

Declaration of Generative Artificial Intelligence in the Writing Process

During the preparation of this manuscript, the authors used ChatGPT and Gemini to assist with language editing, sentence restructuring, and improvement of clarity. The final content was conceived, verified, and critically reviewed by the authors. The authors take full responsibility for the accuracy and integrity of the manuscript.

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