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Original Research Article

Phytochemical Characterization, Antioxidant Activity and Cytotoxic Evaluation of *Citrus sinensis* Peel Extract Against MCF-7 Breast Cancer Cells

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ABSTRACT

The increasing demand for natural therapeutic agents has intensified the exploration of agro-waste materials as potential sources of bioactive compounds. The findings of this exploratory study indicate preliminary bioactive potential rather than direct therapeutic application. This study evaluated the preliminary phytochemical composition, antioxidant activity, and in vitro cytotoxic potential of the ethanolic extract of *Citrus sinensis* (orange) peel against the MCF-7 breast cancer cell line. Qualitative and quantitative phytochemical analyses indicated the presence of flavonoids, polyphenols, saponins, alkaloids, and tannins, with notably high total phenolic content. Antioxidant activity, assessed using the 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, showed a concentration-dependent scavenging effect with an IC_{50} of 15.40 mg/mL. Cytotoxicity was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, demonstrating a decrease in cell viability across tested concentrations with an estimated IC_{50} of 446 µg/mL. FTIR analysis identified prominent functional absorption bands, including hydroxyl, carbonyl, and aromatic groups, while GC-MS profiling revealed tentative library matches for several volatile constituents. These exploratory findings highlight the potential of orange peel as a sustainable source of phytochemical constituents, supporting further pharmacological investigation.

Keywords: Citrus sinensis, Orange peel extract, Phytochemical profiling, DPPH radical scavenging, MCF-7 cells, GC-MS, Cytotoxicity.

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Introduction

Cancer is the second leading cause of death globally, responsible for approximately 9.6 million deaths in 2018.[1] Breast cancer is one of the most common and deadly diseases affecting women worldwide, with high morbidity and mortality rates.[2] Natural products, particularly citrus fruits, are widely explored for their bioactive phytochemical constituents. *Citrus sinensis*, commonly known as sweet orange, yields substantial agricultural processing waste in the form of peels, which are rich in secondary metabolites such as flavonoids, phenolic acids, and carotenoids.[3] Citrus fruits, widely cultivated and consumed globally, have an approximate output of 158.5 million tonnes.[4] They have been extensively studied for their pharmaceutical properties. Phytochemicals present in citrus fruits, such as flavonoids, phenolic acids, dietary fibre, and carotenoids, have been found to possess anticancer, antioxidant, and anti-ageing properties.[5]

In many developing countries, indigenous natural products are extensively used for medicinal purposes, particularly in rural areas where access to modern medicine is limited. Natural products are often more accessible and affordable than orthodox medicine, and have been used for generations to treat various ailments.[6]

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An individual orange contains over one hundred and seventy phytonutrients and more than sixty flavonoids, which possess various properties, such as anti-tumour, anti-inflammatory, blood-clot-inhibiting, and antioxidant effects.[7]

The growing recognition among consumers regarding the potential health consequences of using synthetic treatments has prompted them to establish stringent criteria for their treatment. These criteria emphasise the importance of nutritional value, superior quality and the absence of chemical antioxidants. These demands come from concerns about safety and potential health risks. These reasons have driven individuals to adopt natural-based products for treatment. Consequently, using fruits, vegetables, herbs and various plant extracts or powders has been a prevalent practice in the treatment of various ailments. Also, *Citrus sinensis*, commonly known as sweet oranges, possesses significant utility in promoting human health. They are of significant therapeutic importance due to their rich bioactive profile, exhibiting potent antioxidative, anti-inflammatory, and anticarcinogenic activities that support their application in managing oxidative stress-related diseases and cancer.[8] The health benefits mentioned can be attributed to the presence of vitamins, particularly vitamin C, as well as several phytochemicals, including limonoids, hesperidin flavonoid, polyphenols, pectin, and others.[9]

Citrus fruits are predominantly utilised in many businesses. However, the peels are commonly discarded rather than used effectively. It is imperative to employ appropriate methodologies to convert orange peel and pulp into value-added goods. According to,[10] it has the potential to reduce environmental pollution. Recent analyses have shown that *Citrus sinensis* peel extracts have desirable pharmaceutical excipient attributes.[11] While consumer interest in natural-based products has grown, a significant knowledge gap remains regarding the comprehensive chemical profiling, precise antioxidant kinetics, and targeted *in vitro* cytotoxic mechanisms of ethanolic orange peel extracts against human breast carcinoma cell lines.

Therefore, the aim of this research was to extract, characterise via quantitative phytochemical screening, FTIR, and GC-MS, and

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evaluate both the DPPH radical scavenging antioxidant capacity and the *in vitro* cytotoxic effect of orange peel extract (OPE) on the MCF-7 breast cancer cell line.

Materials and Methods

Plant Material and Extraction
Sample Collection
The orange peels were collected in June 2025 at Engineer Junction, Mando Igabi, Kaduna, Nigeria, from a verified local fruit vendor and packaged in clean polyethene bags. The plant material was authenticated at the departmental herbarium.

Sample preparation
The orange peels were sorted, freed from extraneous matter, and spread evenly to dry at ambient room temperature for two weeks. The dried peels were pulverised using a clean mechanical mortar and pestle. The resulting powder was stored in a dry, well-ventilated container away from direct sunlight.

Extraction of the Orange Peel (Ethanol)
A mass of 200 g of the dried, pulverised orange peels was placed across Soxhlet thimbles, and extraction was carried out using 800 mL of analytical-grade ethanol (maintaining a 1:4 sample-to-solvent mass-to-volume ratio). The solvent was cycled approximately 7 times. The liquid extract was concentrated under reduced pressure using a rotary evaporator at 50 degrees Celsius and brought to complete dryness in a water bath. The mass of the recovered dried extract was 25.6 g, corresponding to a percentage yield of 12.8% relative to the initial dry starting mass of 200 g. The resulting extract was stored in a sterile container at 4°C for subsequent analysis.
The percentage yield of the extract was determined using the standard formula:

$$Percent\ (\%)\ of\ crude\ extract\ yield = \frac{(L_2 - L_1)}{L_0} \times 100$$

Equation (1)
Where:
L_2 = Mass of the extract + Mass of the beaker
L_1 = Mass of the empty beaker
L_0 = Mass of the pulverised orange peel sample

Qualitative Phytochemical Screening
Qualitative tests for flavonoids, polyphenols, cardiac glycosides, steroids, anthraquinones, tannins, alkaloids (using Dragendorff's reagent), saponins, and terpenoids were performed following standard established protocols.

Test for flavonoids
About 5 mL of dilute ammonia was added to 2 mL of the orange peel extract. This was followed by the addition of 1 mL of concentrated sulphuric acid (H_2SO_4). The presence of flavonoids was confirmed by the appearance of a yellow colouration that disappears on standing. Another portion of the extract was taken, and a few drops of 10 % aluminium was added. A yellow colouration confirmed the presence of flavonoids.[12]

Test for Polyphenol
To test for polyphenols, 200 mg of the sample extract was dissolved in 2 mL of distilled water and filtered. To 2 mL of the filtrate, a few drops of 10 % ferric chloride were added. Formation of blue-green colour indicated the presence of phenolic compounds.[13]

Test for cardiac glycosides
About 500 mg of the extract was mixed with 5 mL of distilled water. Then 2 mL of glacial acetic acid was added, followed by one drop of ferric chloride ($FeCl_3$) solution. Subsequently, 1ml of concentrated sulphuric acid (H_2SO_4) was added along the inner walls. Formation of a brown ring at the interface was an indicator of a positive test result.[14]

Test for steroids
Crude extract (2 mg) was mixed with 2 mL of chloroform, and concentrated sulphuric acid was added dropwise. A red colour produced in the lower chloroform layer indicated the presence of steroids.[14]

Test for Anthraquinones
About 500 mg of the extract was heated with 10 mL of sulphuric acid, then filtered while hot. The filtrate was shaken with 5 mL of chloroform, and the chloroform layer was pipetted into another test tube, and 1 mL of dilute ammonia was added. The presence of anthraquinones was indicated by the development of a yellow colour.[13]

Test for tannins
About 500 mg of the sample extract was heated in 10 mL of water in a clean test tube, then filtered. A few drops of 5% ferric chloride were carefully added, and the solution was observed for brownish-green or blue-black colouration, indicating the presence of tannins.[13]

Test for alkaloids (Dragendorff test)
Testing for alkaloids was performed using 500 mg of the extract dissolved in sufficient distilled water. 5 mL of concentrated hydrochloric acid was added to the solution. The resultant mixture was filtered. 2 mL of the collected filtrate was placed in a test tube, followed by the addition of 1 mL of Dragendorff's reagent along the inner wall of the test tube. The formation of a reddish-brown precipitate indicated the presence of alkaloids.[13]

Test for saponins
Saponin testing was performed using 500 mg of the extract in a test tube. To the extract, 5 mL of distilled water was added. The mixture was vigorously agitated to achieve a stable, persistent froth. The formation of an emulsion after adding three drops of olive oil confirmed the presence of saponins.[13]

Test for terpenoids
To test for terpenoids, 2 mL of chloroform was added to 0.5 g of the extract. This was followed by the careful addition of 3 mL of concentrated sulphuric acid to the mixture, forming a layer. The presence of terpenoids was indicated by reddish-brown colouration at the interface.[13]

Quantification of Phytochemicals Using UV-Visible Spectrophotometry
Total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC), total alkaloid content (using Bromocresol Green dye), and total saponin content were determined in triplicate using established spectrophotometric assays.

Determination of total phenolic content (TPC) of OPE
This method by Al-Owaisi *et al*.[15] was employed to determine the total phenolic content (TPC) of the extracts involved using the Folin-Ciocalteu (FC) reagent method. During the experimental procedure, 1 millilitre of ethanolic extract was combined with 1.5 millilitres of FC reagent within a Falcon tube. The resulting mixture was then incubated at ambient temperature for three minutes. Subsequently, 1.5 millilitres of a 7.5% sodium carbonate (Na_2CO_3) solution was added, and the mixture was left undisturbed for 60 minutes. The absorbance was measured at 765 nm using a UV-Vis spectrophotometer (UV-2600, Shimadzu Corporation, USA). The blank reference for this measurement was ethanol. The extracts' total phenolic content (TPC) was quantified using a mathematical method that relates TPC to milligrams of gallic acid equivalents (GAE) per gram of extract. The measurements were repeated three times to determine the mean and

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standard deviation, thereby improving the precision and reliability of the results.

Determination of total flavonoid content (TFC) of OPE
The total flavonoid content of the samples was determined using the aluminium chloride colourimetric method, as outlined in the study by Gulo *et al*.[16] To create the extract stock solution at 1 mg/ml, 1.5 mL of 95% ethanol was placed in a test tube. Subsequently, this solution was diluted in increments of 0.5 mL aliquots. Then, 0.1 mL of a 10% aluminium chloride ($AlCl_3$) solution, 0.1 mL of a 1 mol/L potassium acetate solution, and 2.8 mL of distilled water were introduced into a test tube. The solution was allowed to stand at ambient temperature for 30 minutes. A proportional quantity of distilled water containing 10% aluminium chloride was employed in place of the void in the position of the hole. The absorbance was measured using a UV-visible spectrophotometer (UV-2600, Shimadzu Corporation, USA) at 415 nm. The overall flavonoid content of the extracted samples was quantified by comparing their absorbance values with a standard curve constructed from quercetin. The outcome was quantified in terms of quercetin equivalents (QE) per gram of extract (mg QE/g) or total flavonoid content (TFC). The measurements were reproduced three times to compute the mean and standard deviation, enhancing accuracy and reliability.

Determination of total tannin content (TTC) of OPE
The tannin content of OPE was determined using the method described in Ogo *et al*.[17] A total of 500 µL of the extract was taken in a test tube separately and treated with 100mg of polyvinylpolypyrrolidone and 500 µL of distilled water. This solution was incubated at 4°C for 4 h. The sample was then centrifuged at 5000 rpm for 5 min, and 20 µL of the supernatant was collected. This supernatant contains only simple phenolics free of tannins (the tannins would have been precipitated along with the polyvinylpolypyrrolidone). The phenolic content of the supernatant absorbance was measured using the UV-Visible spectrophotometer at a wavelength of 725 nm and expressed as the content of free phenolics on a dry matter basis. The total tannin content is calculated as;
Tannins (mg GAE/g extract) = Total phenolics (mg GAE/g extract)-Free phenolics (mg GAE/g extract).

Determination of total alkaloid content (TAC) of OPE
Bromocresol green (BCG, Aldrich chemicals) dye was used to estimate total alkaloids.[18] The BCG solution was prepared by heating 69.8 mg BCG with 3 mL NaOH (2N) and 5 mL of distilled water until completely dissolved. The solution was then completed to 1 L with distilled water. Ten microliters of the crude extract were thoroughly mixed with 3 mL of BCG solution. After 30 min, 5 mL of chloroform was added, and the mixture was shaken for 2 min. The lower layer was separated after 30 min. The extraction was continued three times. A set of reference standard solutions of 0.1% atropine (Merck, Darmstadt) was prepared, and the steps described above were followed. The absorbance of colour was read at 725 nm. The total alkaloid content was expressed as Atropine equivalent (AE)/g of crude extract.

Determination of total saponin content (TSC) of OPE
The total saponin content was determined according to the method described in Udinyiwe and Salami[19]. It was based on the vanillin-sulphuric acid colourimetric reaction, with some modifications. About 50 µL of plant extract was added to 250 µL of distilled water. To this, about 250 µL of vanillin reagent (800 mg of vanillin in 10 mL of 99.5% ethanol) was added. Then 2.5 mL of 72% H_2SO_4 was added, and the mixture was mixed well. This solution was kept in a water bath at 60 degrees Celsius for 10 min. After 10 min, it was cooled in ice-cold water, and the absorbance was read at 544 nm. The values were expressed as diosgenin equivalents (mg DE/g extract) derived from a standard curve.

DPPH radical scavenging capacity of OPE
The antioxidant capacity of OPE was assessed using the methodology described in a previous study by Yang *et al*.[20] The stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) was used to test free radical scavenging activity. Diluted extract working solutions prepared in ethanol were mixed with freshly prepared DPPH ethanolic solution to achieve defined final reaction concentrations (4 mg/mL, 8 mg/mL, 12 mg/mL, 16 mg/mL, and 20 mg/mL of diluted OPE). After dark incubation, absorbance was measured at 517 nm against a blank, and percentage inhibition was calculated. Radical scavenging activity was expressed as a percentage of inhibition and calculated using the following equation.

$$DPPH\ radical\ scavenging\ activity\ (\%) = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \times 100$$

Equation (2)

Where:

$Abs_{control}$ is the absorbance of the control
Abs_{sample} is the absorbance of the sample

In Vitro Cytotoxicity Evaluation
The cytotoxic activity of the OPE against breast cancer cells was evaluated following a slightly modified method described by Zulkipli *et al*.[21] The human estrogen receptor-positive breast cancer cell line (MCF-7) was employed to assess the cytotoxic potential of the extract. The half-maximal inhibitory concentration (IC_{50}) value for the MCF-7 cell line was determined from the dose–response curve. The cytotoxic effect of the extract was further compared with that of the untreated control cells to evaluate the efficacy of OPE against breast cancer cells.

Cell Culture Conditions
MCF-7 cells (HTB-22, ATCC) were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 1% L-glutamine, and 1% antibiotics (penicillin−streptomycin) at 37 °C in a humidified 5% CO_2 incubator.[21]

Cell Survival Assay
The cytotoxic activity of the extract was evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.[21] MCF-7 human breast cancer cells (obtained from ATCC, authenticated and maintained at low passage) were seeded at 1×10^4 cells/well in 96-well plates and incubated at 37°C for 24 h in a humidified incubator with 5% CO_2. Cells were treated with varying concentrations (100–500 µg/mL) of the extract for 48 h. Untreated cells served as negative controls, vehicle-treated wells (Dimethyl Sulfoxide, DMSO≤0.1%) served as vehicle controls, and doxorubicin (10 µM) was included as a positive control to validate assay sensitivity and establish comparative cytotoxicity. Following 48 h of treatment, 10 µL of MTT reagent (5 mg/mL in phosphate-buffered saline) was added to each well. The plates were incubated for an additional 4 h at 37°C in a humidified 5% CO_2 incubator. Thereafter, the culture medium was carefully aspirated, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the purple formazan crystals. The plates were gently shaken on a microplate shaker for 10 minutes to ensure complete dissolution, and the absorbance was read at 570 nm using a microplate reader. All assays were performed across three independent biological replicates (n = 3). Cell viability was expressed as a percentage relative to the untreated control across independent triplicate runs using Equation 3.

$$\%Cell\ Viability = = \frac{Abs\ of\ sample - Abs\ of\ blank}{Abs\ of\ control - Abs\ of\ blank} \times 100$$

Equation (3)

Instrumental Characterisation (FTIR and GC-MS)
FTIR analysis of the extract was conducted using an FT-IR-8400S spectrometer to record characteristic functional group vibration bands. Gas chromatography-mass spectrometry (GC-MS) profiling was

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performed using a Shimadzu QP2010 Plus system equipped with a VF-5ms capillary column (30 m × 0.25 mm ID × 0.25 µm film thickness). Helium was employed as the carrier gas at a constant flow rate. The oven temperature program was optimised to ensure full elution of compounds over an extended run time, and constituents were tentatively identified by matching mass spectra against the NIST mass spectral library.

Fourier Transform Infrared (FT-IR) spectroscopic analysis of extract
10 mg of the extract was dissolved in 50 mL of an appropriate solvent, then centrifuged at 3000 rpm for 10 minutes. The supernatant was filtered through a Whatman No. 4 filter paper under vacuum. The resulting solution was further diluted 1:10 with the solvent. The diluted extract was examined using an FT-IR-8400S spectrometer, and the characteristic peaks were documented (Figure 3).

Gas Chromatography-Mass Spectrometry (GC-MS) analysis of extract
The dried ethanolic extract was re-dissolved in the appropriate solvent, vortexed, and then filtered through a 0.45 µm syringe filter. 1 µL aliquot of each filtered solution was subsequently injected into the GC-MS system for analysis.

Instrumentation and chromatographic conditions
GC-MS analysis was conducted using a Shimadzu QP2010 (Tokyo, Japan) Plus system equipped with an AOC-20i auto-sampler. The analysis utilised a VF-5ms fused silica capillary column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness) and employed electron ionisation at 70 eV. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1.58 mL/min. The injector and interface temperatures were set to 250°C and 200°C, respectively, with a 1 µL injection volume at a split ratio of 10:1. The oven temperature program began at 80°C (isothermal for 1 min), increased by 10°C/min to 200°C (held for 4 min), then rose by 10°C/min to 280°C, and ended with an isothermal hold to allow full elution up to 55 minutes, accommodating late-eluting compounds as shown in Table 7. The MS parameters included an ionisation energy of 70 eV, an ion source temperature of 200°C, a solvent cut time of 2.5 min, a scan speed of 1666 amu/sec, a mass scan range of 40-800 m/z, and an interface temperature of 250°C. The GC-MS analysis took 30 minutes, and the relative percentages of each compound in the extract were determined by normalising peak areas.

Identification of components
The relative percentage of each phytochemical component was calculated by dividing its average peak area by the total peak area. Detection was performed using the NIST (National Institute of Standards and Technology) Version 2.0, Year 2005 library.

Data Analysis
All quantitative experiments and assays (including phytochemical quantification, DPPH scavenging, and MTT cell viability assays) were performed in independent triplicate determinations (n = 3). Experimental data are presented as mean ± standard deviation (SD). Statistical evaluation of differences between groups was carried out using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test using statistical software (SPSS Statistics version 26.0). A probability value of $p < 0.05$ was considered statistically significant.

Results and Discussion

Extraction yield of ethanolic extract from orange peel
The ethanolic extraction of 200 g of orange peel yielded 12.8% of dark-brown extract. The characteristics of the extract are detailed in Table 1.

Table 1: Percentage yield and appearance of Ethanolic OPE

Reagents	Weight Taken (g)	Amount after drying (g)	Percentage yield (g)	Appearance of the extract
Ethanol	200	25.6	12.8	Very dark-brown

Phytochemical composition of ethanolic OPE
The phytochemical constituents of the extract are listed in Tables 2 and 3. Qualitative screening confirmed the presence of flavonoids, polyphenols, tannins, saponins, alkaloids, and terpenoids. Quantitative evaluation revealed that total polyphenols were the most abundant (944 ± 1.11 mg GAE/g), followed by flavonoids (296.83 ± 0.31 mg/g) and saponins (175.05 ± 0.002 mg/g), with lower concentrations of tannins and alkaloids (0.219 ± 0.0011 mg/g). These high phenolic and flavonoid levels serve as primary contributors to the extract's hydrogen-donating capacity, aligning with established literature regarding the phytochemical richness of citrus processing sub-products.

Table 2: Phytochemical Constituents of Ethanolic OPE

Phytochemical constituents	Test	Observations	Inferences
Terpenoids	Salkowski Test	Reddish-brown coloration	+
Alkaloids	Dragendorff's Test	Reddish-brown precipitate	+
Anthraquinone	Borntrager's Test	Red coloration	+
Flavonoids	Alkaline Reagent Test	Yellow/reddish-brown coloration	+
Cardiac glycoside	Keller-Kiliani Test	Brown ring at interface	-
Saponins	Froth Test	Presence of foam precipitate	+
Tannins	Ferric Chloride Test	Reddish-brown coloration	+
Polyphenols			+

Key: +: presence of components -: Absence of components

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Table 3: Quantitative Phytochemical Content of Ethanolic OPE

Phytochemical Class	Quantified Equivalent Standard	Total Content (mg/g extract ± SD)
Alkaloids	Atropine Equivalent (AE)	0.219 ± 0.0011
Flavonoids	Quercetin Equivalent (QE)	296.83 ± 0.3065
Saponins	Diosgenin Equivalent (DE)	175.05 ± 0.0023
Polyphenols	Gallic Acid Equivalent (GAE)	944.00 ± 1.1100
Tannins	Gallic Acid Equivalent (GAE)	12.52 ± 0.0036

Estimation of total phytochemical content in the OPE

Quantitative phytochemical screening was performed to determine the levels of key bioactive compounds in the OPE. This analysis included Total flavonoid content (TFC), total phenol content (TPC), total saponin content (TSC), total alkaloid content (TAC), and total tannin content (TTC). These compounds are well known for their therapeutic potential, especially in the management of oxidative stress-related diseases.

A previous study has shown that orange peel extract (OPE) mitigates oxidative stress induced by paraquat.[32] In this study, the cytotoxicity of OPE was evaluated in the MCF-7 breast cancer cell line. Since OPE has been shown to contain various phytochemicals, we evaluated and quantified those present in this OPE. The total polyphenol content (TPC) was the highest among the quantified phytochemicals, at 944.00 ± 1.11 mg/g. Research has shown that phenolic compounds act as excellent hydrogen donors and radical scavengers, contributing significantly to the antioxidant capacity of the orange peel extract. Their abundance in the orange peel suggests that the extract may possess strong antioxidant, anti-inflammatory, anticancer and antimicrobial activities, which align with the traditional use of orange peels in herbal medicine. Recent studies indicate that certain phenolic subgroups can inhibit the growth of various cancer cells.[33] Flavonoids, the second most abundant in the ethanolic orange peel extract, are widely studied for their role in protecting cells against oxidative damage, supporting cardiovascular and immune health.[34] The TFC was found to be 296.83 ± 0.3065 mg/g, consistent with the literature, which reports that citrus fruits are rich in flavonoids, particularly hesperidin and naringenin, which are found in the peels and exhibit strong cytotoxicity. Saponins are present in moderate concentrations. The total saponin content of the ethanolic orange peel extract was 175.05 ± 0.0023 mg/g. These compounds can increase cell membrane permeability[35] and often exhibit surface-active properties, binding cholesterol to form insoluble complexes that are excreted in bile. Saponins are known for their diverse biological and pharmacological activities, including anti-allergic, cytotoxic, antiviral, antihepatotoxic, antibacterial, anticancer, antiparasitic, antifungal and immunoadjuvant effects.[36] Orange peel extract contains small amounts of alkaloids and tannins. TAC and TTC were 0.219 ± 0.0011 mg/g and 12.52 ± 0.0036 mg/g, respectively. These compounds are identified for their pharmacological activities, including anticancer, antimalarial and analgesic effects. Their presence in small quantities may result from orange ripeness, as fully ripe oranges show higher alkaloid and tannin levels, and from the orange source, which influences alkaloid and tannin levels.[37]

DPPH antioxidant activity of the ethanolic OPE

This research assesses the extract's ability to donate hydrogen atoms or electrons to neutralise DPPH radicals, a property indicative of antioxidant potential. Table 4 shows the antioxidant activity of the ethanolic extract of orange peel using the DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging assay. The DPPH radical scavenging assay demonstrated a concentration-dependent increase in antioxidant inhibition (Figure 1), rising from 20.43 ± 0.85% at 4 mg/mL to 58.16 ± 1.05% at 20 mg/mL. One-way ANOVA revealed that increase in radical scavenging capacity across concentrations was statistically significant ($F = 512.4$, $p<0.001$). Tukey's post-hoc pairwise comparisons showed significant differences ($p<0.05$) between most concentration groups, except between 12 mg/mL and 16 mg/mL ($p>0.05$; Table 4). Regression analysis yielded an estimated IC_{50} value of 15.40 mg/mL. This moderate radical scavenging behaviour is directly attributable to the hydroxyl-bearing phenolic and flavonoid constituents capable of neutralising reactive free radicals. These compounds are known to contribute to radical neutralisation by acting as hydrogen donors.[22] This finding aligns with the report of Hegazy & Ibrahim[22] on citrus peel extracts exhibiting antioxidant properties, and supports the potential utilisation of orange peel in the development of natural antioxidant agents.

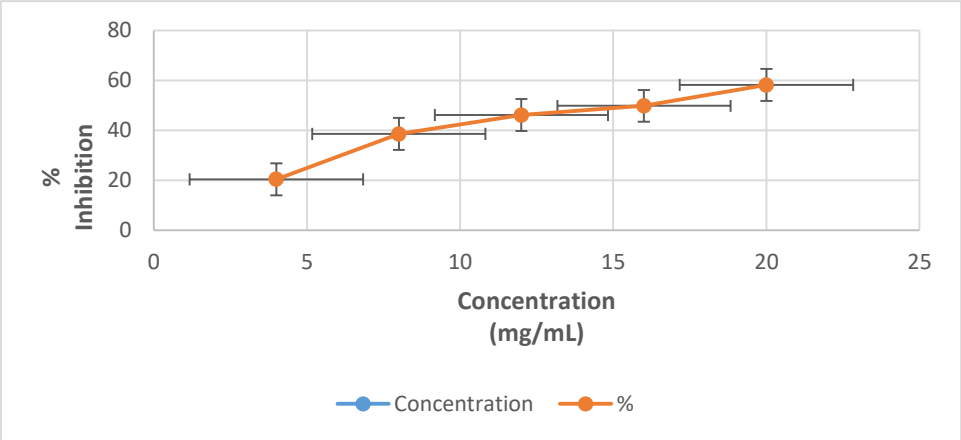

Figure 1: DPPH radical scavenging activity (% inhibition) of ethanolic orange peel extract across concentrations (4-20 mg/mL). Data points represent the mean ± standard deviation of independent triplicate determinations (n = 3). The solid line indicates the linear regression fit (y = 2.1675x + 16.616, R^2 = 0.923) from which the half-maximal inhibitory concentration (IC_{50}) of 15.40 mg/mL was derived.

Table 4: DPPH Radical Scavenging Activity of Ethanolic OPE

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Concentration (mg/mL)	Absorbance (517 nm, Mean ± SD)	Inhibition Percentage (% Inhibition, Mean ± SD
4	0.9700 ± 0.015	20.43 ± 0.85[a]
8	0.7487 ± 0.012	38.58 ± 1.10[b]
12	0.6565 ± 0.009	46.14 ± 0.95[c]
16	0.6117 ± 0.011	49.82 ± 1.20[c]
20	0.5100 ± 0.008	58.16 ± 1.05[d]

Note: Values represent the mean ± standard deviation of independent triplicate determinations (n = 3). Means with different superscript letters ([a-d]) within the column are significantly different from each other based on one-way ANOVA followed by Tukey's post-hoc test ($p<0.05$). Percentage inhibition (% Inhibition) was calculated relative to the untreated DPPH control blank. The half-maximal scavenging concentration (IC_{50}) of 15.40 mg/mL was determined via linear regression analysis (y = 2.1675x + 16.616, R^2 = 0.923).

Cytotoxicity effect of OPE on MCF-7 cells

The cytotoxic potential of the OPE evaluated using the MTT assay against the MCF-7 breast cancer cell line is presented in Table 5 as mean ± standard deviation (SD) of triplicate determinations (n = 3). Exposure to varying concentrations of the extract resulted in a statistically significant decrease in cell viability (One-way ANOVA, F = 42.8, $p<0.05$) at higher doses, although a minor, non-significant variation in cell survival was observed between 100 µg/mL (84.44 ± 2.15%) and 200 µg/mL (84.89 ± 1.92%) before dropping progressively to 35.56 ± 1.48% at 500 µg/mL (Table 5 and Figure 2).

Table 5: Cytotoxic Effect of Ethanolic OPE on MCF-7 Cancer Cell Line

Concentration (µg/mL)	Absorbance (570 nm, Mean ± SD)	Cell Viability (% Viability, Mean ± SD)
Control (0)	0.530 ± 0.012	100.00 ± 0.00
100	0.448 ± 0.010	84.44 ± 2.15[ns]
200	0.432 ± 0.009	84.89 ± 1.92[ns]
300	0.350 ± 0.011	66.67 ± 2.40[*]
400	0.330 ± 0.008	62.22 ± 1.85[**]
500	0.210 ± 0.007	35.56 ± 1.48[***]
Positive Control (Doxorubicin, 10 µM)	0.120 ± 0.005	18.20 ± 1.10[***]

Note: Values represent the mean ± standard deviation of independent triplicate determinations (n = 3). Means sharing symbols differ significantly from the untreated control based on one-way ANOVA followed by Tukey's post-hoc test ([ns] $p>0.05$, [*] $p<0.05$, [**] $p<0.01$, [***] $p<0.001$). The half-maximal inhibitory concentration (IC_{50}) of 446 µg/mL was derived via linear regression interpolation across the concentration response curve.

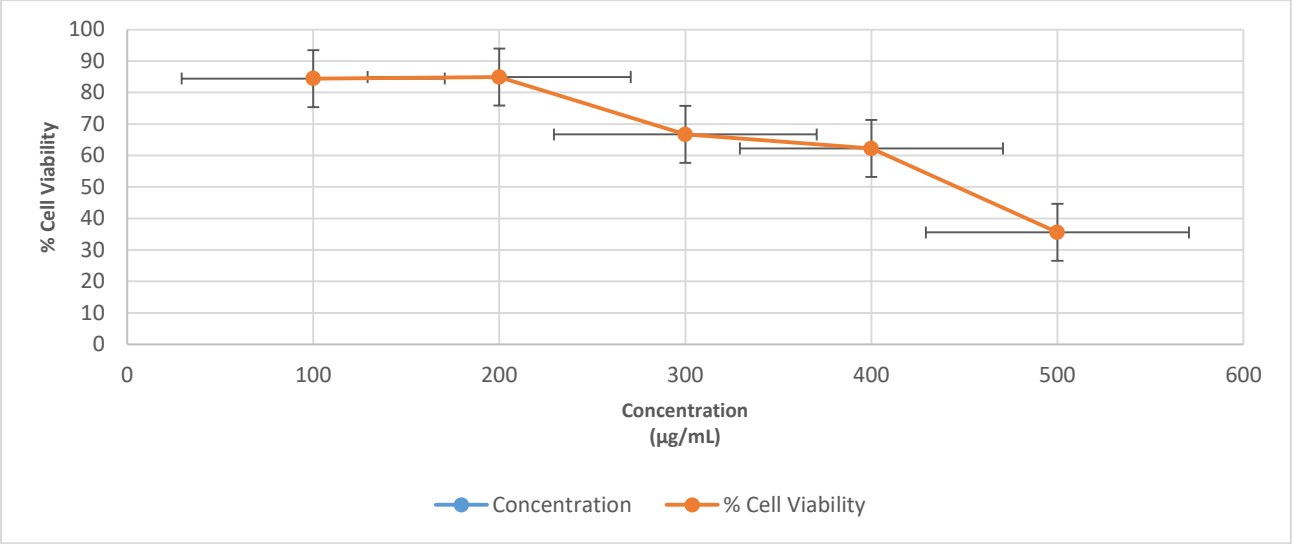

Figure 2: Percentage cell viability of MCF-7 human breast cancer cells following 48-hour exposure to varying concentrations (100-500 µg/mL) of ethanolic orange peel extract (OPE) measured via the MTT assay. Data points represent the mean ± standard deviation of independent triplicate determinations (n = 3). The half-maximal inhibitory concentration (IC_{50}) of 446 µg/mL was derived by linear regression interpolation.

The half-maximal inhibitory concentration (IC_{50}) was determined by linear regression analysis from the concentration–response curve plotting percentage cell inhibition against extract concentrations (100-500 µg/mL). Based on the linear interpolation between adjacent data points spanning the 50% effect threshold (300 µg/mL at 66.67% viability / 33.33% inhibition and 500 µg/mL at 35.56% viability / 64.44% inhibition), the calculated IC_{50} value is 446 µg/mL (y = 0.0778x + 15.33, R^2 = 0.941), confirming an estimated inhibitory potency of 446 µg/mL).

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Evaluation of MCF-7 cell viability via the MTT assay revealed a reduction in metabolic survival at higher concentrations, yielding an estimated half-maximal inhibitory concentration (IC_{50}) of 446 µg/mL determined via linear regression interpolation between 300 and 500 µg/mL. The percentage of viable cells was calculated relative to the untreated control. A dose-dependent decrease in cell viability was observed with increasing extract concentration, indicating enhanced cytotoxic activity at higher doses (Table 5 and Figure 2). While crude extracts exhibit preliminary in vitro cytotoxic activity, further bioassay-guided fractionation is required to isolate active principles before claiming definitive anticancer efficacy. However, this pattern suggests that the extracts may contain bioactive phytochemicals capable of disrupting cancer cell growth. The observed cytotoxicity may be attributed to bioactive compounds such as limonene, squalene, benzoic acid, and flavonoids, which research has previously reported to possess anticancer and antioxidant activity, as confirmed by the GC-MS and phytochemical analyses conducted. This result is consistent with other studies, including Wallace *et al.*[3], which supported the anticancer efficacy of citrus peel extract against various cancer cell lines. The moderate cytotoxic activity observed in this study (IC_{50} 446 µg/mL) suggests that the crude extract exhibits biological activity, although it is less potent compared to standard chemotherapeutic agents. This may be attributed to the crude nature of the extract, in which active compounds are present at low concentrations. Evaluation of MCF-7 cell viability via the MTT assay demonstrated a concentration-dependent reduction in metabolic survival. While these in vitro findings indicate that the crude extract exhibits preliminary suppressive activity against the tested breast cancer cell line, such reduction in cell viability observed in an exploratory screening assay cannot be equated with definitive in vivo anticancer efficacy, nor does it confirm specific mechanisms of cellular disruption or comparative potency against standard chemotherapeutic agents. Furthermore, while bioassay-guided fractionation is commonly hypothesised to concentrate active constituents, assuming that purification will automatically enhance pharmacological activity requires rigorous validation through targeted isolation and mechanism-specific biological assays.

Characterisation of ethanolic OPE

FT-IR Analysis

FTIR spectroscopic analysis identified key absorption bands, including a strong broad band at 3268.9 cm^{-1} (O-H stretch for hydroxyl/phenolic groups), 2927.8 cm^{-1} (C-H aliphatic stretch), 1718.3 cm^{-1} (C = O carbonyl stretch), and aromatic ring (C=C) stretches around 1515.2 cm^{-1} and 1407.1 cm^{-1} (Table 6 and Figure 3). While these vibrational assignments point to the presence of broad chemical classes such as phenolics and aliphatics, attributing specific biological activities such as antimicrobial, anti-inflammatory, antidiabetic, or immunomodulatory effects directly from unisolated, nonspecific infrared absorption bands represent an unverified progression from functional group identification to therapeutic mechanism; thus, these spectral bands are interpreted strictly as structural vibrational evidence.

Table 6: FTIR Spectral Absorption Bands and Functional Group Assignments for Ethanolic Orange Peel Extract

Frequency peak (cm^{-1})	Bond	Functional group	Peak details
3268.9	O-H	Alcohol/Phenol	Strong, broad (O-H stretching vibration)
2927.8	C-H	Alkane (Aliphatic)	Strong (C-H stretching of aliphatic chains)
2199.0	C≡C/Cumulated	Alkyne / Allene system	Weak to medium
1718.3	C=O	Carbonyl (Ketone, Ester, or Acid)	Strong (C=O stretching)
1627.0	C=C	Alkene / Aromatic ring	Medium
1515.2	C=C	Aromatic ring	Strong (Aromatic skeletal vibration)
1407.1	C-H/C=C	Alkane / Alkene	Weak (CH_2/CH_3bending or ring stretch)
1308.1	C-O	Alcohol / Ester / Ether linkage	Medium
1222.6	C-O	Alcohol / Ester / Ether linkage	Medium to strong
1030.6	C-O	Alcohol / Ether / Ester linkage	Strong (C-O stretching vibration)
989.6	C-H	Alkene	Medium (Out-of-plane bend)
922.5	O–H / Fingerprint	Carboxylic acid / Skeletal	Weak
814.4	C-H	Aromatic / Alkene	Medium (Out-of-plane bending / fingerprint region)

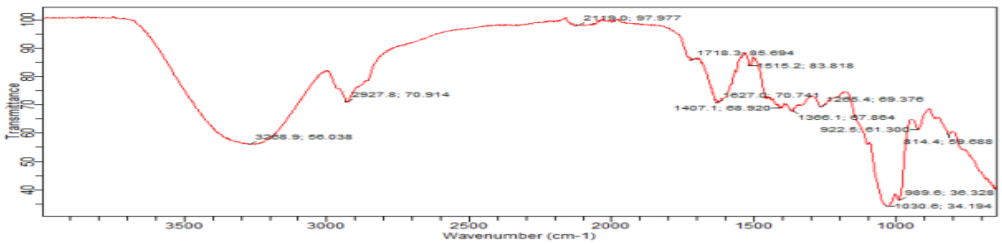

Figure 3: FTIR transmittance spectrum of the ethanolic OPE prepared as a thin film/KBr disc. Prominent labelled absorptions occur at approximately 3268.9, 2927.8, 2199.0, 1718.3, 1627.0, 1515.2, 1407.1, 1308.1, 1222.6, 1030.6, 989.6, 922.5, and 814.4 cm^{-1}. The broad band at 3268.9 cm^{-1} corresponds to O-H stretching, 2927.8 cm^{-1} to aliphatic C-H stretching, and 1718.3 cm^{-1} to carbonyl (C = O) stretching. Bands in the 1627.0-1407.1 cm^{-1} region reflect aromatic ring or alkene stretches, while absorptions between 1308.1 and 1030.6 cm^{-1} indicate C-O stretching (ethers/alcohols/esters rather than carbonyl). Peaks below 1000 cm^{-1} represent fingerprint-region vibrations.

The FT-IR spectrum of the ethanol extract of orange peel shows several notable absorbance bands corresponding to key functional groups, suggesting the presence of potential bioactive compounds. A strong and broad absorption around 3268.9 cm^{-1} suggests the presence

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of O–H stretching vibrations, typically associated with alcohols and phenolic compounds. These groups are known for their antioxidant and antimicrobial properties. Phenolic compounds, in particular, have been widely reported in citrus peels and are linked to free radical scavenging activity. The band at 2927.8 cm^{-1} corresponds to C–H stretching vibrations of aliphatic chains, indicating the presence of saturated hydrocarbons, possibly from fatty acids or terpenoids. These compounds often contribute to anti-inflammatory and lipid-lowering effects. A prominent peak at 1718.3 cm^{-1} is characteristic of C=O stretching vibrations, which are typically found in esters, aldehydes, or carboxylic acids. This suggests the presence of flavonoids or other carbonyl-containing compounds, which are known for their anti-cancer and anti-diabetic activities. The absorption bands at 1515.2 cm^{-1} and 1407.1 cm^{-1} are attributed to aromatic C=C stretching, supporting the presence of aromatic rings, likely flavonoids or polyphenols. These compounds are well-documented for their role in modulating oxidative stress and inflammation. Bands observed at 1030.6 cm^{-1} and 922.5 cm^{-1} are indicative of C–O stretching vibrations, which may arise from alcohols, ethers or glycosidic linkages, suggesting the presence of glycosides or polysaccharides with potential immunomodulatory effects.

These interpretations align with findings from,[23] which have consistently highlighted the phytochemical richness of plant extracts containing these notable absorbance bands and their corresponding phytocompounds, as shown in the citrus extract of this study. For instance, [23] identified phenolic acids and flavonoids in the ethanolic extracts of *Mucuna* species accessions as key contributors to antimicrobial activity. Phytochemical and proximate analyses of fruit mixtures (blend components) typically identify similar key bioactive compounds that have been demonstrated to enhance antioxidant potential and improve nutritional profiles.[24] In another study, they reported that the presence of carbonyl and hydroxyl functional groups in citrus peel extracts correlated with significant antioxidant potential. Furthermore, the role of terpenoids and glycosides in modulating inflammatory pathways *in vitro* was also emphasised.[25]

GC-MS Analysis

GC-MS chromatographic profiling revealed multiple chromatographic peaks corresponding to tentative library identifications, including D-limonene, squalene, and 2-methoxy-4-vinylphenol (Table 7 and Figure 4). These instrumental findings provide baseline compositional data for the exploratory extract. GC-MS chromatographic profiling of the ethanolic extract (clarified as a crude hydroalcoholic extract rather than an isolated essential oil) revealed multiple constituents whose mass spectra matched NIST library entries. These library matches are presented strictly as tentative compositional evidence rather than definitively confirmed single-compound structures, as authentic reference standard co-injection was not performed. Consequently, claims regarding absolute therapeutic value, solvent-induced specificity, synergistic multi-compound interactions, or commercial pharmaceutical suitability cannot be drawn from these tentative chromatographic matches alone without component-specific validation and bioactivity isolation.

Table 7: Tentative GC-MS Identification of Volatile Constituents in Ethanolic OPE

Peak	Tentative Compound Name	Molecular Formula	Molecular Weight (g/mol)	Retention Time (t_R, min)	Peak Area (%)	NIST Match Score (%)
1	D-Limonene	$C_{10}H_{16}$	136.24	9.96	0.11	91
2	Benzoic acid	$C_7H_6O_2$	122.12	11.41	0.06	88
3	2,4-Cycloheptadien-1-one,2,6,6-timethyl-	$C_{10}H_{14}O$	150.22	13.89	1.66	85
4	Benzofuran,2,3-dihydro-	C_8H_8O	120.15	14.24	1.10	89
5	Ocinol	$C_7H_8O_2$	124.14	14.62	0.64	86
6	1-Cyclohexane-1-methanol	$C_7H_{14}O$	114.19	22.50	0.19	83
7	n-Decanoic acid	$C_{10}H_{20}O_2$	172.26	28.34	4.08	92
8	Squalene	$C_{30}H_{50}$	410.72	53.44	0.42	95
9	Methyl stearate	$C_{19}H_{38}O_2$	298.50	39.55	0.25	89
10	Octadecenoic acid	$C_{18}H_{34}O_2$	282.46	40.48	1.93	93
11	2-Furanmethaneamine	C_5H_7NO	97.11	31.75	0.26	81
12	4-Chloro-3-n-butyltetrahydropyran	$C_9H_{17}ClO$	176.68	31.84	0.71	83
13	(E,Z)-.alpha.-Farnesene	$C_{15}H_{24}$	204.36	30.29	1.43	87
14	Phenol,2,6-bis(1,1-dimethyl)-	$C_{12}H_{18}O$	178.27	23.51	1.36	90
15	2-Isopropyl-6-methylaniline	$C_{10}H_{15}N$	149.23	19.65	0.31	82
16	1-Dodecanol,2-hexyl-	$C_{18}H_{38}O$	270.50	23.87	0.33	84
17	1-Butene,-2-chloro-3-methyl-3	C_5H_9Cl	92.58	22.23	0.09	80
18	Benzaldehye,-4-hydroxy-	$C_7H_6O_2$	122.12	19.36	0.15	
19	2-Methoxy-4-vinylphenol	$C_9H_{10}O_2$	150.17	17.12	2.16	94
20	Hexadecanoic, ethyl ester	$C_{18}H_{36}O_2$	284.48	36.45	0.36	91

Note: Major chromatographic peaks detected at retention times 35.89 min and 48.34 min (as well as minor signals) are omitted from the itemised list because their corresponding NIST mass spectral library match scores fell below the rigorous quality-control threshold of 80%. Match scores below 80% indicate low spectral confidence and insufficient structural matching; consequently, these peaks were excluded to maintain high analytical credibility and prevent false-positive or misidentified phytochemical reporting.

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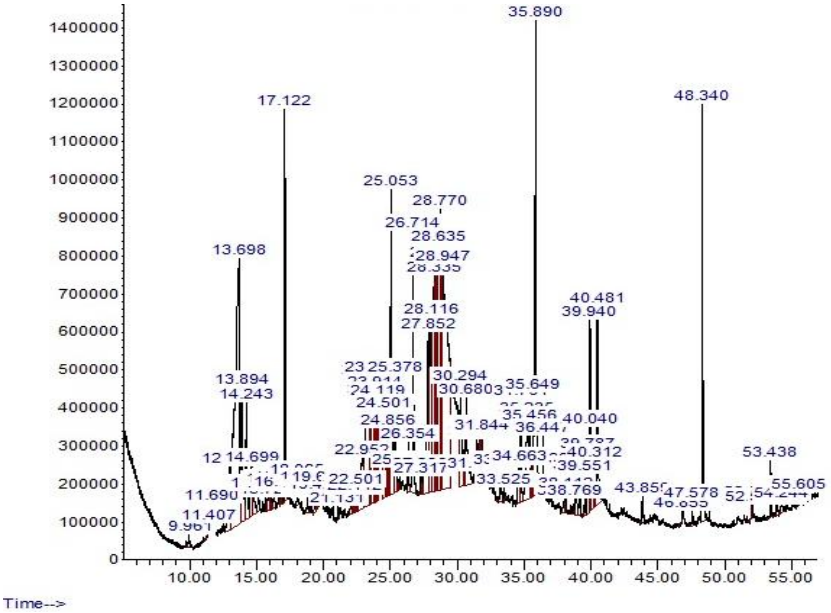

Figure 4: Total-ion chromatogram of the ethanolic OPE obtained by GC-MS. Retention time is shown in minutes on the *x*-axis, and detector response (abundance) is shown on the *y*-axis. The labelled values denote retention times of integrated chromatographic peaks. Chromatographic conditions are described in Section 2.6.2.1, and tentative library assignments are presented in Table 7.

The GC-MS analysis of the essential oil of the ethanolic extract of orange peel revealed a rich spectrum of bioactive phytochemicals, reinforcing its potential for various pharmacological applications. The analysis identified numerous compounds, including terpenes, fatty acids, esters, phenolics, hydrocarbons and alcohols, many of which have been linked to antioxidant, antimicrobial, anticancer and anti-inflammatory effects.[26] 2-Methoxy-4-vinylphenol, with the highest peak (Peak area: 2.1624 and RT: 17.12), is a phenolic compound known for its strong antioxidant activity and free-radical-scavenging properties. n- Decanoic acid (peak area: 4.0753 and RT: 28.34) is a saturated fatty acid recognised for its antimicrobial and antifungal properties. Its presence suggests potential roles in infection control or preservation. Octadecenoic acid (oleic acid) and Methyl stearate are fatty acid esters associated with anti-inflammatory and skin-protective properties. These compounds are often used in cosmetic and therapeutic formulations.[27] Squalene (peak area: 0.4175 and RT: 53.44), a naturally occurring triterpene, is known for its antioxidant, anticancer, and lipid-lowering effects. It enhances cellular defense mechanisms against oxidative stress.[28] D-Limonene (peak area: 0.1098 and RT: 9.9607), a major terpene in citrus peels, exhibits antimicrobial, anti-inflammatory, and chemopreventive activities. It also contributes to the characteristic citrus aroma.[29] Other compounds, such as Benzoic acid, Benzofuran, and Phenol-2,6-bis (1,1-dimethyl), further suggest antimicrobial and antioxidant synergy within the extract.[27] The chemical diversity detected confirms that orange peel, often discarded as waste, is a rich source of bioactive compounds with therapeutic value.[30] The use of ethanol as the extraction solvent also played a key role, as it effectively solubilises both polar and moderately nonpolar phytochemicals. This interpretation aligns with Egharevba *et al.*[31] and supports the medicinal and commercial value of citrus peel extracts. The combination of multiple compounds enhances the likelihood of synergistic biological effects, making orange peel extract a viable candidate for pharmaceutical applications.

Conclusion

This exploratory study showed that ethanolic *Citrus sinensis* peel extract contains diverse phytochemical constituents and exhibits moderate *in vitro* DPPH radical scavenging activity, along with preliminary suppressive effects on MCF-7 breast cancer cell viability at higher tested concentrations. While these findings outline the extract's baseline chemical profile, functional group characteristics, and antioxidant potential, further bioassay-guided fractionation, quantitative isolation, and mechanism-specific biological evaluations are needed to fully elucidate its pharmacological properties.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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