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Characterization of Phenolic and Flavonoid Constituents from Citrus sinensis Peels with Antimicrobial Potentials: An Integrated In Vitro and In Silico Approach

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ABSTRACT

Introduction: Oranges (*Citrus sinensis*) are among the most widely cultivated citrus fruits, valued for their high vitamin C content and diverse bioactive compounds. While the edible portion is extensively used, the peel is often discarded as waste despite being a rich source of phytochemicals with potential therapeutic properties. This study focuses on the isolation and characterization of phenolics and flavonoids from orange peels and the evaluation of their antimicrobial potential through molecular docking and in vitro assays.

Materials and Methods : Methanolic extraction of orange peel was conducted using reflux condensation to obtain the methanolic extract of orange peel (ME-OP). Thin Layer Chromatography (TLC) and UV-visible spectroscopy were used for preliminary identification of phenolics and flavonoids. Structural elucidation was performed using Liquid Chromatography–Mass Spectrometry (LC-MS). Molecular docking studies were carried out to evaluate the interaction of isolated compounds with bacterial DNA gyrase (PDB ID: 3G75). Phytochemical screening and quantitative estimation of total phenolic and flavonoid content were also conducted, along with antimicrobial assays against selected Gram-positive and Gram-negative bacteria and fungi.

Results and Discussion : Phytochemical screening of ME-OP revealed the presence of carbohydrates, phytosterols, oils, proteins, amino acids, flavonoids, and abundant phenolics. TLC analysis showed flavonoids with Rf values of 0.17–0.23 and phenolics in the range of 0.33–0.63. Quantitative analysis indicated total phenolic content of 1.25 mg GAE/g and flavonoid content of 1.51 mg QE/g. LC-MS identified major phenolic acids such as p-coumaric, protocatechuic, caffeic, and ferulic acid, along with flavonoids including hesperidin and naringenin-7-O-glucoside. Molecular docking demonstrated that hesperidin and naringenin-7-O-glucoside had stronger binding affinities to bacterial DNA gyrase than the standard antibiotic ciprofloxacin. In vitro antimicrobial assays showed significant inhibition of Gram-positive bacteria (*Staphylococcus aureus, Bacillus subtilis*), moderate activity against Gram-negative bacteria, and minimal antifungal effects.

Conclusions: This study highlights orange peel as a valuable source of bioactive phenolics and flavonoids with promising antimicrobial activity. The strong binding affinity of key flavonoids to bacterial targets and their effective inhibition of Gram-positive bacteria indicates the potential of orange peel extract as a natural antimicrobial agent. These findings support the therapeutic potential of citrus peel waste and encourage its utilization in the development of plant-based pharmaceuticals or antimicrobial formulations..

Keywords: *Orange peel, phenolic compounds, flavonoids, spectral analysis, antimicrobial activity and molecular docking etc*

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1. . INTRODUCTION

Citrus sinensis L. Osbeck, commonly known as the orange plant, is an evergreen tree of small to medium stature belonging to the Rutaceae family. Originally native to Southeast Asia, it is now widely cultivated across tropical and subtropical regions due to its sweet, flavorful fruit. This plant prefers warm climates and well-drained soil, yielding globular fruits characterized by their vibrant orange peel and fragrant constituents. The fruit is highly appreciated not only for its nutritional richness especially its abundant vitamin C content but also for its diverse range of phytochemicals such as essential oils, flavonoids, and alkaloids, which are most concentrated in the peel and seeds [1]. Sweet orange (Citrus sinensis (L.) Osbeck) is an evergreen fruit tree valued for its edible, juicy berries. It is the most widely cultivated citrus, accounting for roughly 70% of global citrus production. Sweet orange trees grow to about 9–10 m in height with thorny branches, and they bear glossy, elliptic leaves (5–15 cm long) that exude a characteristic citrus aroma [2].

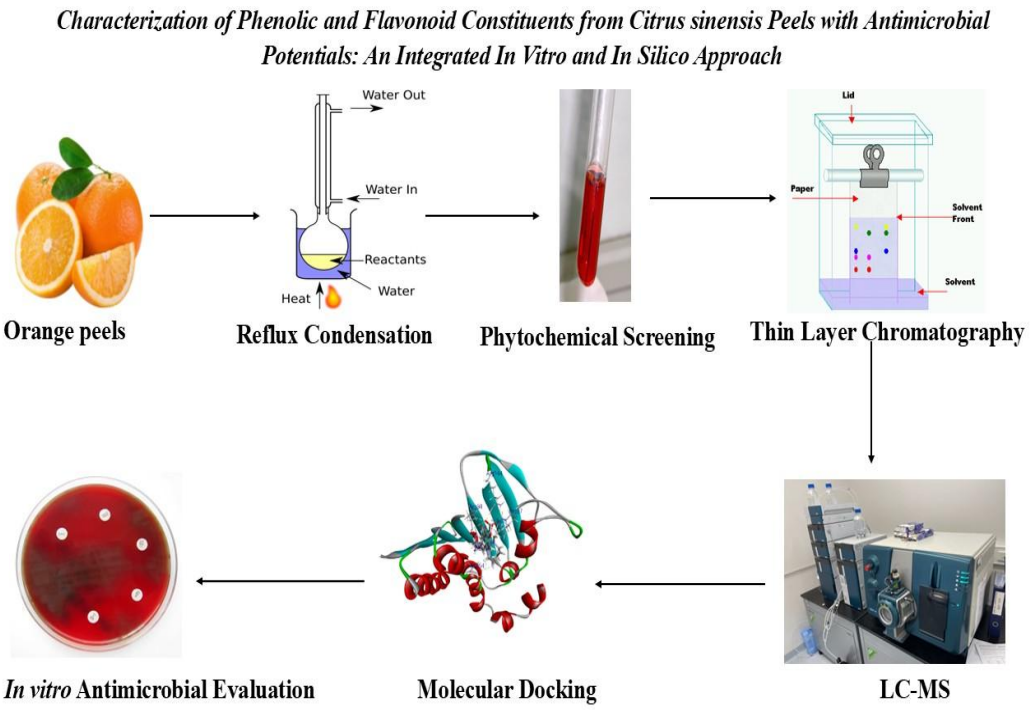


Fig-1: Graphical Abstract

Sweet orange trees are medium-sized evergreens. The trunk and branches often bear sharp axillary spines, especially on young growth. Leaves are alternately arranged; each leaf has a narrow-winged petiole and an elliptic-ovate blade (typically 6–15 cm long) with a faintly crenate margin. New leaves and shoots are usually tinted bronze before maturing to dark green. The white flowers are 2–3 cm in diameter with five petals; they occur singly or in small clusters (inflorescences) in the leaf axils. Fruit develops from the pollinated ovary: oranges are nearly round to slightly oval berries about 4–12 cm across. The rind of a mature fruit is smooth and glossy; microscopically it consists of an outer epicarp (flavedo) with a thick cuticle and innumerable oil glands, overlying a spongy mesocarp (albedo) of elongated cells. Inside the fruit are ~10–14 segments (carpels), each filled with sac-like juice vesicles. When fully ripe, the pulp is orange-colored and filled with flavorful juice. Typically each segment contains a few almond-shaped seeds (ranging from seedless to several per segment, depending on the variety), and *C. sinensis* exhibits polyembryonic seed development (multiple embryos per seed) in many cultivars [3].

Macroscopical Characteristics: Macroscopically, the sweet orange fruit is characterized by its round shape, orange colour, and segmented interior. The intact fruit measures about 4–12 cm in diameter and has a firm rind 4–6 mm thick. The flavedo (coloured surface) contains glands that appear as tiny dots and impart the strong citrus aroma when the peel is scratched or crushed. Upon cutting the fruit in cross-section, the multilocular structure is evident: each fruit typically has 10–14 wedge-shaped carpels radiating from the central axis. Each carpel is packed with numerous juice vesicles (elongated, fluid-filled cells) that give the segment its juicy texture [3]. The pulp colour ranges from bright orange to deep reddish-orange in pigmented varieties, reflecting carotenoid content. Seeds, when present, are located near the segment tips; they are smooth or slightly rough and whitish internally.

Fig-2: Visual appearance of Oranges

2. EXPERIMENTATION

2.1. Experimental Phytochemistry

Materials and instruments required

Chemicals

All chemicals and reagents used for extraction, phytochemical screening, and isolation of bioactive compounds were of Analytical Reagent (AR) and Laboratory Reagent (LR) grade. These were procured from certified commercial suppliers and used without further purification.

Instruments and Analytical Tools

The following instruments were employed for the analysis and characterization of phytoconstituents:

UV-Visible Spectrophotometer: This instrument was used for quantitative analysis of phenolics and flavonoids.

Thin Layer Chromatography (TLC) Apparatus: It was used for preliminary separation and identification of phytocompounds.

Liquid Chromatography–Mass Spectrometry (LC-MS): This is for advanced profiling and structural elucidation of bioactive constituents.

3. METHODOLOGY

Preparation of Methanolic Extract of Orange Peel (ME-OP)

To prepare the methanolic extract, 150 g of dried orange peel powder was initially defatted by refluxing with 750 mL of petroleum ether for 1 hour in a round-bottom flask. The hot mixture was filtered through a Buchner funnel using vacuum filtration, and the defatted powder was allowed to dry at room temperature. The dried material was then subjected to methanolic extraction by refluxing with 750 mL of methanol for 2 hours. The resulting mixture was again filtered while hot to separate the methanolic extract from the solid residue. The filtrate was concentrated under reduced pressure using a rotary evaporator until a thick, syrupy methanolic extract of orange peel (ME-OP) was obtained [4].

Purpose of Using Solvents

Petroleum ether was employed in the initial defatting step to remove non-polar compounds such as fats, waxes, chlorophyll, essential oils, and other lipophilic pigments, which could interfere with the extraction and analysis of target phytochemicals. This step enhances the efficiency of subsequent extraction using methanol a polar solvent capable of dissolving and extracting polar bioactive constituents including flavonoids (e.g., hesperidin), phenolic acids, ascorbic acid (vitamin C), tannins, and potentially alkaloids and glycosides, thereby ensuring a more selective and effective phytochemical profiling of the orange peel extract.

Phytochemical Screening

Qualitative phytochemical screening of the methanolic extract of orange peel (ME-OP) was carried out to identify the presence of various bioactive groups. Carbohydrate tests confirmed the presence of reducing sugars, ketohexoses, mono- and disaccharides, and starch [35]. Amino acid analysis indicated the presence of aromatic amino acids (tyrosine and tryptophan), arginine, sulfur-containing amino acids, and α-amino groups [5,6]. Phytosterol identification tests such as

Salkowski, Liebermann-Burchard, and Zak's confirmed sterol presence [5, 6]. Carotenoids were detected through solubility behaviour, color reactions, and UV absorbance at 470 nm [7]. Polyphenols were confirmed by reactions with lead acetate, $FeCl_3$, NH_4OH, and Tollen's reagent. Alkaloid tests showed negative results, confirming the absence of quinazoline alkaloids [8-12].

Isolation of bioactive compounds

Thin Layer Chromatography (TLC) was employed for the identification of flavonoids and phenolic compounds using silica gel 60 F_{254} precoated plates as the stationary phase. Sample extracts (~10 µL) were applied on the baseline and developed in pre-saturated chambers using solvent systems such as ethyl acetate: formic acid: acetic acid: water (100:11:11:27), butanol: acetic acid: water (4:1:5), or toluene: ethyl acetate: formic acid (5:4:1). Plates were visualized under UV light (254 and 366 nm), and sprayed with $AlCl_3$ or Naturstoff reagent A followed by PEG 4000 to enhance fluorescence. Rf values were calculated and compared to standards for compound identification [13].

Determination of Total Phenolic Content (TPC)

The total phenolic content of the extract was determined using the Folin–Ciocalteu method with gallic acid as the standard. A 1000 µg/mL gallic acid stock solution was prepared and serially diluted to obtain standards of 0–100 µg/mL. For each standard and sample, 0.5 mL was mixed with 2.5 mL of 1:10 diluted Folin–Ciocalteu reagent, incubated for 5 minutes, followed by the addition of 2.0 mL of 7.5% sodium carbonate solution. After 30 minutes of incubation in the dark at room temperature, absorbance was measured at 765 nm. A standard curve was generated by plotting absorbance against gallic acid concentrations to quantify phenolics in the sample [14-15].

Calculation

Perform linear regression to get the equation of the line, usually in this form:

$$\boldsymbol{Y = mx + c}$$

Where:

y = Absorbance of sample (A).

m = Slope.

x = Concentration of unknown sample.

c = Intercept

Calculate TPC: The total phenolic content is determined by using the calibration curve of gallic acid, expressed as mg gallic acid equivalents per gram of dry extract (mg GAE/g).

$$\textbf{Total Phenolic content (mg GAE/ g sample)} = \frac{\boldsymbol{C \times V}}{\boldsymbol{M}}$$

Where:

C = concentration from standard curve (µg/mL)

V = volume of extract used in reaction (mL)

M = mass of the dry sample extracted (g)

Determination of Total Flavanoids Content (TFC)

The total flavonoid content was estimated based on the formation of a yellow complex between flavonoids and aluminum chloride ($AlCl_3$) in the presence of potassium acetate, which is measurable at 415 nm using a UV-visible spectrophotometer. A quercetin stock solution (1 mg/mL in methanol) was serially diluted to obtain standard concentrations (10–100 µg/mL). For each standard and sample, 1 mL of solution was mixed with 0.3 mL of 10% $AlCl_3$, 0.3 mL of 1M potassium acetate, and 2.7 mL of methanol. After incubating for 30 minutes at room temperature, absorbance was measured at 415 nm against a reagent blank (without quercetin or extract). A calibration curve was plotted to quantify flavonoids in the extract [14-15].

Calculation

Plot a calibration curve of absorbance vs concentration using quercetin standards.

Determine the equation of the line: $y = mx + c$

Use the absorbance of your sample to calculate the flavonoid content from the curve:

$$\mathbf{x} = \frac{\mathbf{(y - c)}}{\mathbf{m}}$$

Where:

A (y) = absorbance of the sample.

m = slope of the standard curve.

c = intercept of the standard curve.

Express the result as mg quercetin equivalent (QE) per gram of dry extract (mg QE/g DW):

$$\mathbf{TFC}\left(\frac{\mathbf{mg\ QE}}{\mathbf{g}}\right) = \frac{(\mathbf{C} \times \mathbf{V})}{\mathbf{M}}$$

Where:

C = concentration from calibration curve (mg/mL)

V = volume of extract (mL)

M = mass of plant extract used (g).

Isolation of Bioactive molecules by LCMS

Materials

Materials required for the preparation of plant extracts for LC-MS analysis include fresh or dried plant material, HPLC-grade solvents such as methanol, water, or a methanol-water mixture, and equipment like a mortar and pestle or grinder for sample preparation. Additionally, a centrifuge is needed for separation, along with 0.22 µm PTFE or nylon syringe filters for filtration. Volumetric flasks and an analytical balance are essential for precise measurement and dilution, while a vortex mixer ensures thorough mixing. Finally, LC-MS vials are required for sample storage and injection into the instrument.

Preparation of plant extract

For LC-MS analysis, a 100-ppm plant extract was prepared using either powdered or liquid samples. Dried plant material was ground and extracted with 80% methanol, followed by centrifugation and filtration through a 0.22 µm syringe filter. For liquid extracts (juice or decoction), clarification was done by centrifugation and filtration. If the concentration was unknown, 1 mL of extract was evaporated and the dry residue weighed to estimate ppm. The extract was diluted accordingly to 100 µg/mL using LC-MS grade methanol or methanol: water (1:1), vortexed, filtered if needed, and transferred into LC-MS vials for analysis and stored at 4°C or –20°C [16-21].

Dilution to 100 ppm

To prepare 100 ppm the following formula is used: $\boldsymbol{C1V1 = C2V2}$

Where:

C1 **=** Concentration in per ml (10000 ppm).

C2 **=** Concentration of sample to be prepared (100 ppm).

V1 **=** Volume of sample to be prepared (ml or µ/L)

V2 **=** Desired volume (10 mg/ml).

$$V\mathbf{1} = \frac{\mathbf{C2} \times \mathbf{V2}}{\mathbf{C1}}$$

$$V\mathbf{1} = \frac{\mathbf{100} \times \mathbf{10}}{\mathbf{10,000}}$$

$$\boldsymbol{V1 = 0.1mL\ =\ 100\ \mu/L}$$

Methodology

The methodology involved the use of an LC-MS system equipped with an Ion-Trap Agilent model for compound identification and analysis. Liquid chromatography was performed using a reverse-phase (RP) column, allowing separation of compounds based on hydrophobicity. The injected sample (standard or plant extract) underwent ionization as it eluted from the column, and the mass spectrometer measured the mass-to-charge ratio (m/z) of the ions, revealing molecular weight data. Further, tandem mass spectrometry (MS/MS) was employed, where specific precursor ions such as $[M+H]^+$ were selected and fragmented, enabling detailed structural elucidation through analysis of the resulting fragment ions.

2.2. Computational Chemistry

Molecular docking

Molecular docking is defined as an optimization problem, which would describe the “best-fit” orientation of a ligand that binds to a particular protein of interest. During the course of the process, the ligand and the protein adjust their conformation to achieve an overall “best-fit” and this kind of conformational adjustment resulting in the overall binding is referred to as “induced fit [22].

Posing and scoring function of docking

Molecular docking involves two critical steps: posing and scoring. Posing determines the possible conformations and orientations a ligand can adopt within a protein’s active site. Scoring functions evaluate these poses by estimating binding affinity through calculations involving van der Waals interactions, hydrogen bonds, desolvation energy, and electrostatic energy. The goal is to identify the most stable complex with the lowest binding free energy. Böhm introduced one of the earliest empirical scoring functions to predict ligand-receptor binding energy, aiding in drug discovery. This approach enables high-throughput virtual screening of compounds and helps in optimizing lead molecules for better potency and selectivity [23].

$$\Delta G_{bind} = \Delta G_0 + \Delta G_{hb} \sum n - bonds + \Delta G_{ionic} \sum_{ionic-int} + \Delta G\ lipophilic\ |A| + \Delta G_{rot} NROT$$

A more general thermodynamic "master" equation is as follows:

$$G_{bind} = -RTlnK_d$$

$$K_d = \frac{[Ligand][Receptor]}{[Complex]}$$

$$\Delta G_{bind} = \Delta G_{desolvation} + \Delta G_{motion} + \Delta G_{configuration} + \Delta G_{interaction}$$

4. MATERIALS AND METHODS

For the computational analysis, the target protein selected was bacterial DNA gyrase (Topoisomerase-II), identified by the PDB code 3G75. The molecular docking and related studies were performed using Discovery Studio Client software, which allows visualization, preparation, and analysis of protein-ligand interactions. This setup enabled accurate assessment of binding affinities and interaction patterns between the target and test compounds.

Crystalline structure of the target protein Topoisomerase-II (Crystal structure of Staphylococcus aureus Gyrase B: bacterial DNA gyrase) with PDB id 3G75 was retrieved from protein data bank and protein clean-up process was done and essential missing hydrogen atom were been added. Different orientation of the bioactive molecules along with standard drug ciprofloxacin with respect to the target protein was evaluated by Discovery studio program and the best dock pose was selected based on the interaction study analysis.

Fig-3: Structure of bacterial DNA gyrase

2.3. Experimental Microbiology

Requirements

Standard bacterial and fungal strains were obtained from the Microbial Type Culture Collection and Gene Bank (MTCC),

Chandigarh: *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 1133), *Escherichia coli* (MTCC 452), and *Aspergillus niger* (MTCC 281). Mueller-Hinton Agar (MHA; SRL Chemicals, Cat. No. 24756) was used for antibacterial assays, while Sabouraud Dextrose Agar (SDA; SRL Chemicals, Cat. No. 19427) was used for antifungal testing. Whatman No. 1 filter paper discs (5 mm diameter) were employed for sample loading. Dimethyl Sulfoxide (DMSO; SRL Chemicals, Cat. No. 28580) served as the vehicle control. Ciprofloxacin (2 mg/mL; SRL Chemicals, Cat. No. 78079) and Amphotericin B (10 mg/mL; Amphocare) were used as positive controls for antibacterial and antifungal activity, respectively. All discs were loaded with 10 µL of test sample or control solution.

Preparation of Culture Media

Nutrient agar and Sabouraud dextrose agar are widely used media for routine bacterial and fungal cultivation, respectively. The nutrient agar medium was prepared by dissolving peptone, beef extract, sodium chloride, and agar in 1000 mL of distilled water (pH 7.0), followed by boiling and sterilization at 15 lbs pressure and 121°C for 15 minutes [24–25]. Similarly, Sabouraud dextrose agar medium was prepared by dissolving glucose, peptone, and agar in 1000 mL of distilled water, adjusting the pH to 5.4, and sterilizing under the same conditions. Both media were cooled and poured into sterile Petri dishes for further use.

Table-1: Composition of Nutrient Agar Medium

Sl. No.	Ingredients	Quantity (g/L)
1.	Peptone	10 g
2.	Beef Extract	10 g
3.	Agar	20 g
4.	Sodium Chloride	5 g
5.	Distilled Water	1000 mL
6.	pH	7.0

Table-2: Composition of Sabouraud Dextrose Agar Medium

S. No.	Ingredients	Quantity (g/L)
1.	Glucose	20 g
2.	Peptone	10 g
3.	Agar	20 g
4.	Distilled Water	1000 mL
5.	pH	5.4

Method of Evaluation of In Vitro Antibacterial and Antifungal Activity

The antimicrobial activity was evaluated using the Zone of Inhibition Method (Kirby-Bauer method). For antibacterial testing, Mueller-Hinton Agar (MHA) plates were inoculated with 100 µL of *Staphylococcus aureus* culture prepared in Mueller-Hinton Broth and adjusted to 0.5 McFarland standard (≈1.5 × 10^8 CFU/mL). Sterile Whatman No.1 discs were loaded with 10 µL of test solutions (0–100%), placed on the inoculated plates, along with one disc containing only DMSO as vehicle control and a Ciprofloxacin disc (3 µg) as positive control. Plates were incubated at 37°C for 24 hours (Basil Scientific Corp. India), and zones of inhibition were measured in millimeters. For antifungal activity, Sabouraud Dextrose Agar (SDA) plates were similarly inoculated with 100 µL of *Aspergillus niger* culture prepared in Sabouraud Dextrose Broth and adjusted to 0.5 McFarland standard (≈1.5 × 10^8 CFU/mL). Discs loaded with 10 µL of different test

concentrations were placed on the surface along with DMSO (control) and Amphotericin B (100 µg) as the positive control. Plates were incubated at 37°C for 48 hours, and the zones of inhibition were measured and recorded [26–27].

5. RESULTS AND DISCUSSIONS

3.1. Phytochemical Screening

Table-3: Phytoconstituents present in ME-OP

Sl. No.	Types of phytoconstituents	Visibility
1.	Carbohydrates	++
2.	Reducing sugar group	+
3.	Proteins and amino acids	++
4.	Oils and Fats	+
5.	Steroids	++
6.	Alkaloids	-
7.	Phenolic compounds	+++
8.	Flavanoids	++
9.	Tannins	+
10.	Saponins	+
11	Cardiac glycoside	-
12.	Anthocyanidin	-

Note: ++ = Present (Clearly Visible), + = Present (Dark), - = Absent

Summary and Findings

Preliminary phytochemical screening was carried out to detect the presence of various primary and secondary metabolites such as reducing sugars, pentoses, disaccharides, polysaccharides, proteins and amino acids, polyphenols, carotenoids, and others. The methanolic extract of orange peel (ME-OP) tested positive for several bioactive phytoconstituents. The analysis revealed the presence of carbohydrates, phytosterols, oils and fats, proteins and amino acids, and polyphenols, indicating the therapeutic potential of the extract (Table 1).

3. 2. Isolation of Bioactive molecules phenolics and flavonoids in ME-OP

Table-4: R_f value of unknow constituents

Standard Flavanoids	R_f Range	Observed value	Phytoconstituents present in ME-OP
Quercetin	0.20 – 0.60	0.23	Flavanoids
		0.17	Flavanoids
Gallic acid	0.15 – 0.20	0.33	Phenolic compounds
		0.39	Phenolic compounds
		0.55	Phenolic compounds
		0.63	Phenolic compounds

Summary and Findings

Thin Layer Chromatography (TLC) analysis of the methanolic extract of orange peel (ME-OP) revealed the presence of both flavonoids and phenolic compounds, as indicated by their respective Rf values (Table-4). The observed Rf value of

0.23 aligns with the standard range for quercetin (0.20–0.60), confirming the presence of flavonoids, while a second spot at 0.17, though slightly below the standard range, may indicate a structurally related flavonoid or degradation product. For phenolic compounds, the standard Rf range for gallic acid (0.15–0.20) did not match any observed spots; however, multiple distinct Rf values (0.33, 0.39, 0.55, and 0.63) suggest the presence of other phenolic constituents in ME-OP. This variation highlights the phytochemical diversity of the extract, indicating the presence of several phenolic compounds beyond gallic acid.

3. 3. Total phenolic content (TPC) and Total Flavonoids Content (TFC) in ME-OP

Table-5: Absorbance of Standard drug gallic acid

Concentration	Absorbance
0	0.00
10	0.036
20	0.051
40	0.066
60	0.079
80	0.085
100	0.097
10 (ME-OP)	0.0227

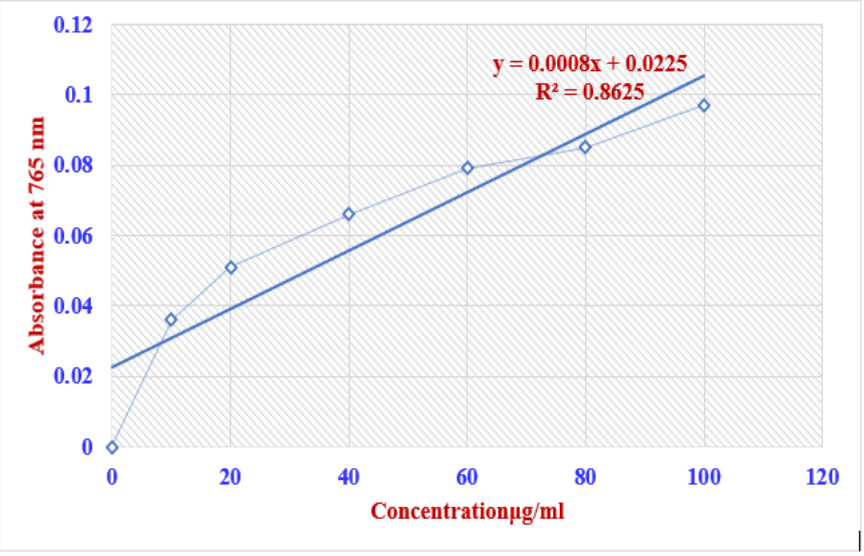


Fig-4: Standard graph of gallic acid

Table-6: Absorbance of Quercetin

Concentration	Absorbance
0	0.00
10	0.031
20	0.042
40	0.056

60	0.061
80	0.079
100	0.091
10 (ME-OP)	0.029

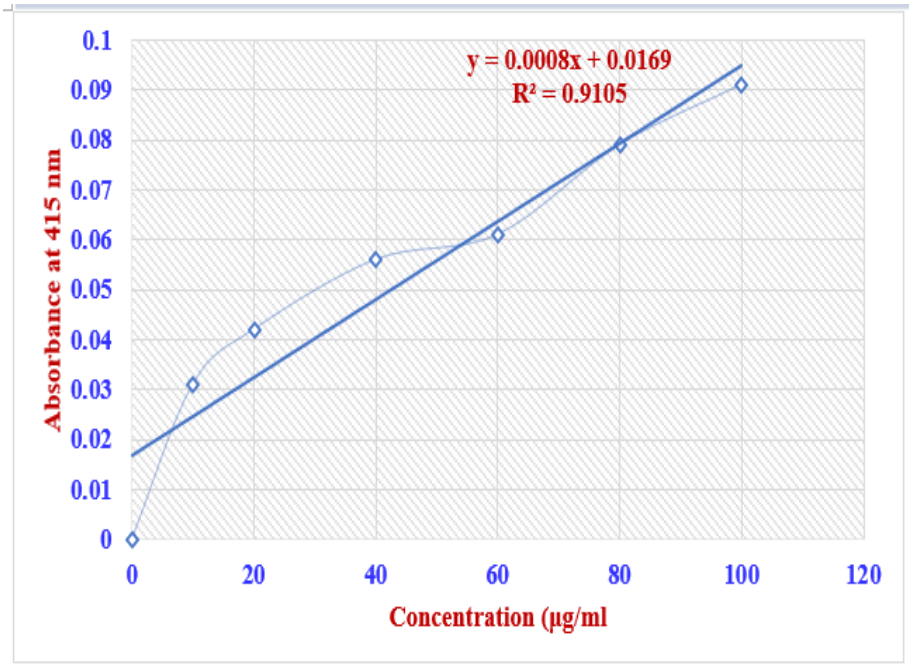


Fig-5: Standard graph of Quercetin

6. SUMMARY AND FINDINGS

The total phenolic content (TPC) of the methanolic extract of orange peel (ME-OP) was quantitatively determined using the Folin–Ciocalteu method, which is based on the formation of a blue-colored complex between phenolic compounds and the reagent under alkaline conditions. The absorbance of this complex was measured at 765 nm, and phenolic content was calculated using a gallic acid standard curve. With an absorbance of 0.0227 for ME-OP and using the standard curve equation ($y = 0.0008x + 0.0225$), the calculated concentration was 0.25 µg/mL, resulting in a TPC value of 1.25 mg gallic acid equivalents (GAE)/g of dry extract.

Similarly, the total flavonoid content (TFC) was measured by preparing a quercetin standard curve and measuring the absorbance of ME-OP at 510 nm. With a sample absorbance of 0.029 and using the standard curve equation ($y = 0.0008x + 0.0169$), the concentration was found to be 15.12 µg/mL. Using the formula for TFC calculation, the extract showed a total flavonoid content of 1.51 mg quercetin equivalents (QE)/g of extract.

3. 4. Interpretation of LCMS data and Characterization of phenolic and flavanoid compounds.

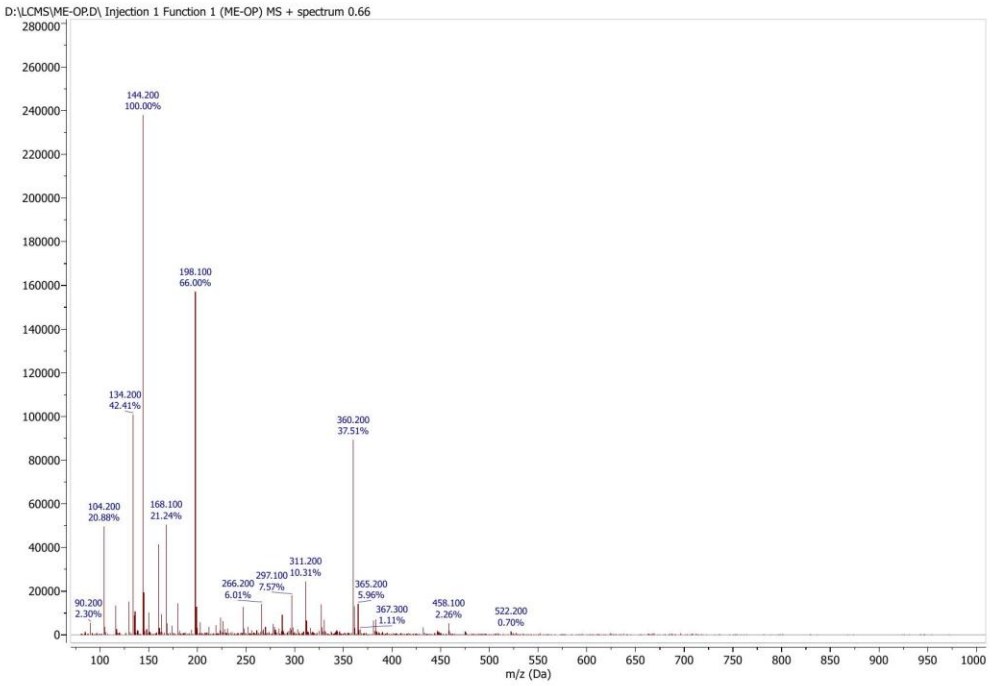


Fig-6: LCMS of ME-OP

Table-7: LC-MS Peaks of ME-OP and Fragmentation

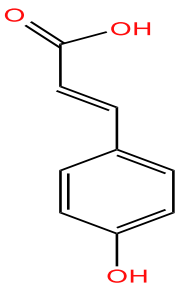
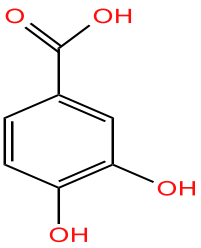
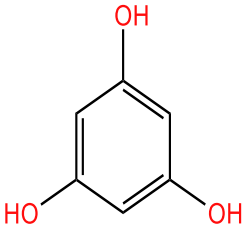
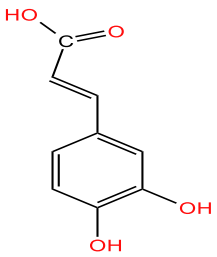
m/z	Ion Type	Neutral Mass (M)	Molecular Formula (Possible)	Probable Compound	Fragmentation Insight
104.2	Fragment ion	~104	C_7H_6O	p-Coumaric acid	Likely from hydroxycinnamic acids; fragment results from loss of CO_2 or CH_3.
134.2	Fragment ion	~134	$C_7H_6O_3$	Protocatechuic acid	A hydroxybenzoic acid derivative; loss of CO or water in fragmentation.
144.2	Fragment or parent ion	~144	$C_6H_6O_3$	Phloroglucinol	A simple polyphenol found in plants; may also be a fragment from dihydrochalcones or other phenolics.
168.1	$[M-H]^-$	~168	$C_9H_8O_4$	Caffeic acid	A hydroxycinnamic acid found in fruits including apples; can fragment via loss of CO_2 (→ m/z ~124).
198.1	$[M-H]^-$	~198	$C_{10}H_{10}O_4$	Ferulic acid	A methoxylated hydroxycinnamic acid; fragments via loss of CH_3 or CO_2.
311.1	$[M+H]+$ or $[M-H]-$	~310	$C_{21}H_{22}O_{10}$	Naringenin-7-O-glucoside	A flavanone glycoside; Loss of glucose (~162

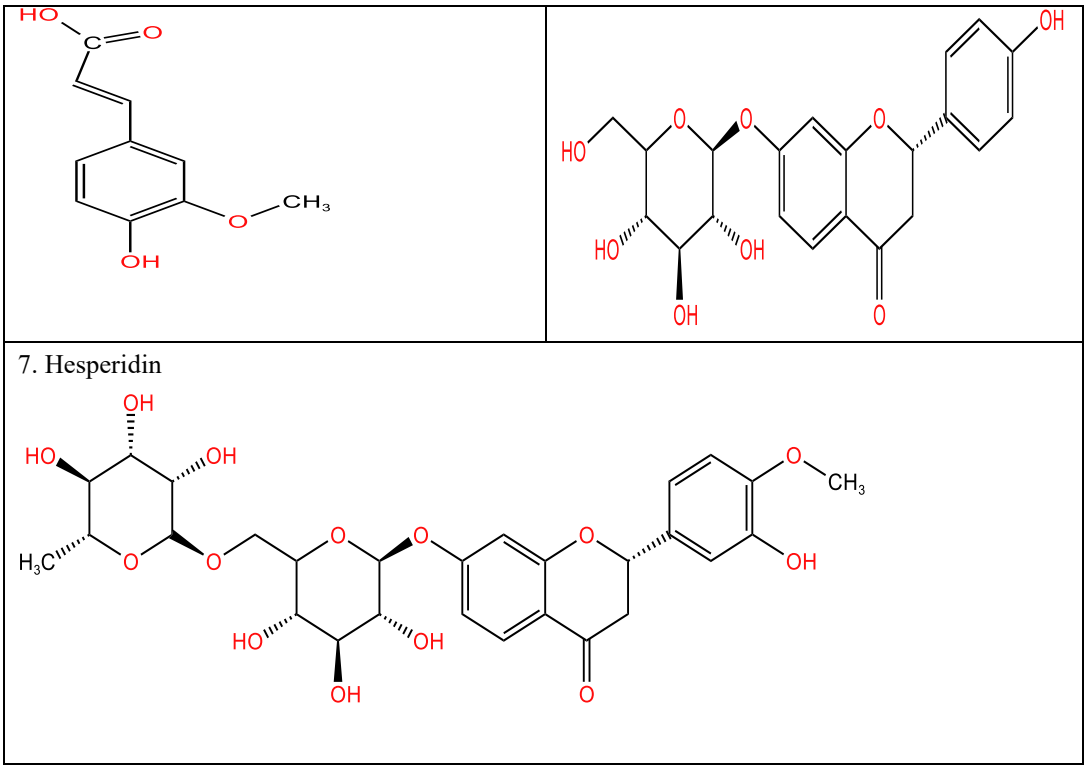
					Da) gives naringenin (m/z ~271).
360.2	Positive ion mode ($[M+H]^+$	611.1887 Da (parent)	$C_{16}H_{16}O_7$ (361.0863 Da)	Parent compound: Hesperidin ($C_{28}H_{34}O_{15}$) .Hesperetin with partial sugar (mono-hexose adduct or hydrated flavonoid)	Glycosidic bond cleavage — loss of disaccharide rutinose (~250 Da)

Discussion and Summary (m/z-based Identification of Phenolic Compounds)

Mass spectrometric analysis of the methanolic extract of orange peel (ME-OP) revealed a diverse profile of phenolic and flavonoid constituents based on their m/z (mass-to-charge ratio) values. A fragment at m/z 104.2 suggests a breakdown product of hydroxy-p-coumaric acid, indicating typical fragmentation of hydroxycinnamic acid derivatives via CO_2 loss. The ion at m/z 134.2 matches protocatechuic acid ($C_7H_6O_4$), a common benzoic acid derivative formed through phenolic degradation. Phloroglucinol ($C_6H_6O_3$), a core structure in many polyphenols, was indicated by the m/z 144.2 peak. The peaks at m/z 168.1 and 198.1 correspond to caffeic acid and ferulic acid, respectively, both hydroxycinnamic acids involved in the phenylpropanoid biosynthetic pathway. A higher m/z value at 311.1 is attributed to naringenin-7-O-glucoside, a glycosylated flavanone, evidenced by a neutral glucose loss (162 Da), indicating the presence of both glycoside and aglycone forms. Lastly, the m/z 360.2 peak likely represents a hesperetin derivative, supporting the citrus origin of the extract. Together, these ions confirm the rich phenolic and flavonoid composition of ME-OP, reflecting its potential antioxidant and therapeutic value.

Table-8: List of probable compounds present in ME-OP

1. P-coumaric acid 	2. Protocatechuic acid 
3. Phloroglucinol 	4. Caffeic acid 
5. Ferulic acid	6. Naringenin-7-O-glucoside

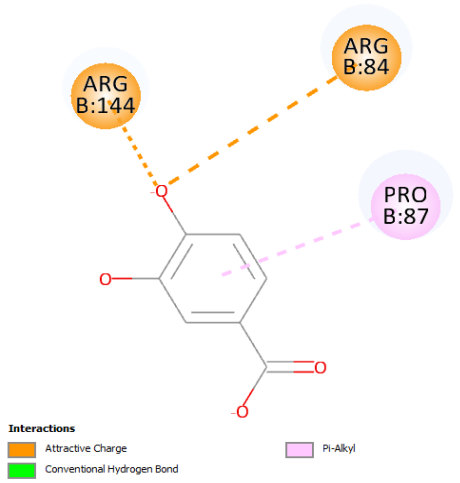
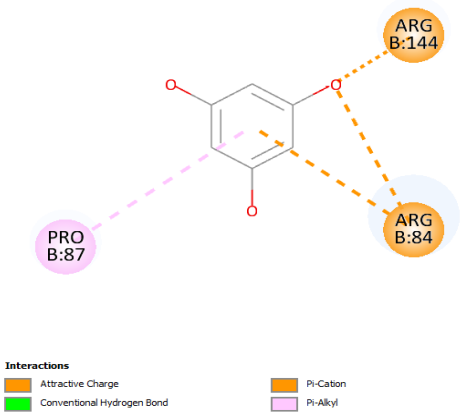
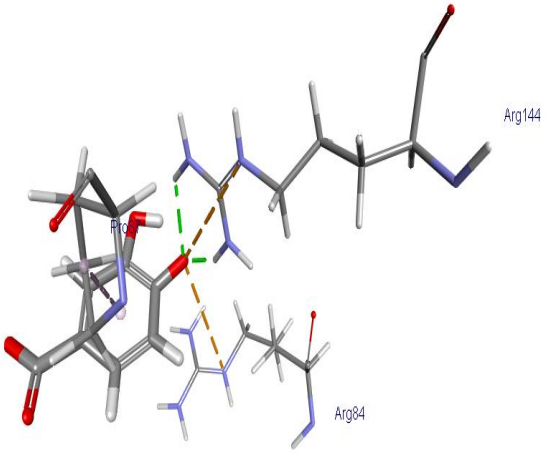
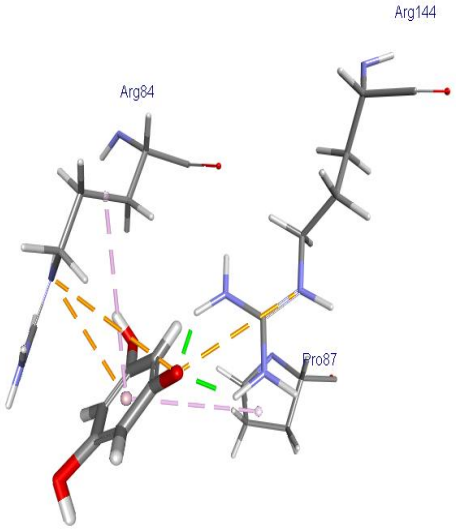
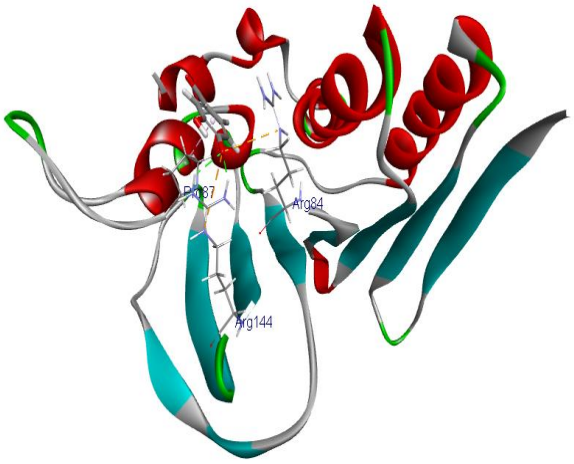
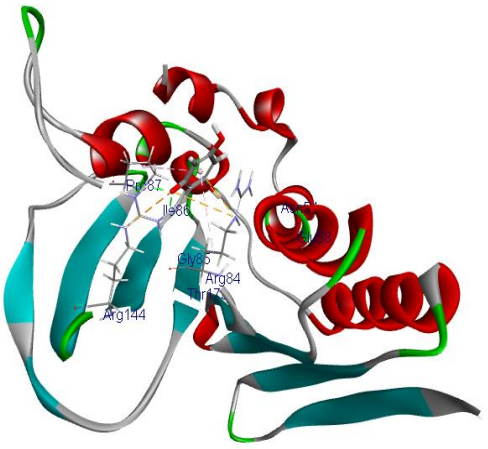


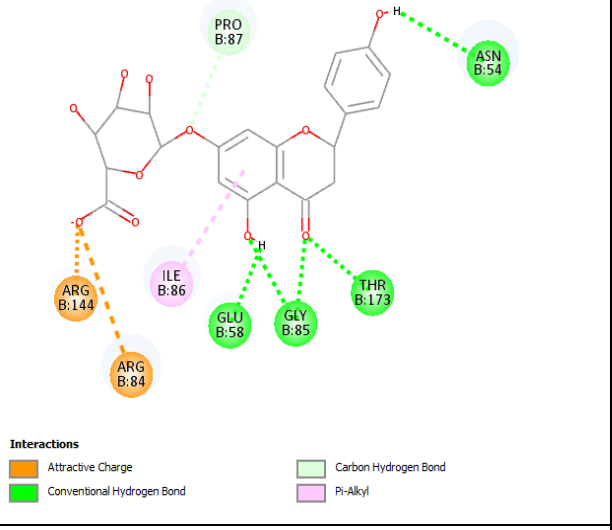
3. 5. In silico docking studies

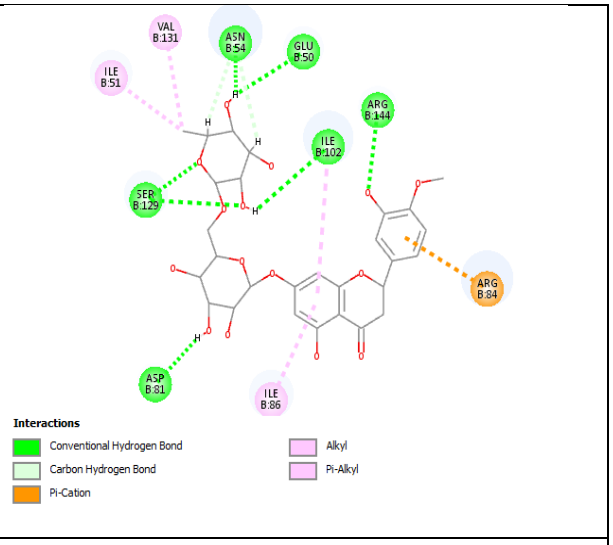
Table-9: Docking results analysis

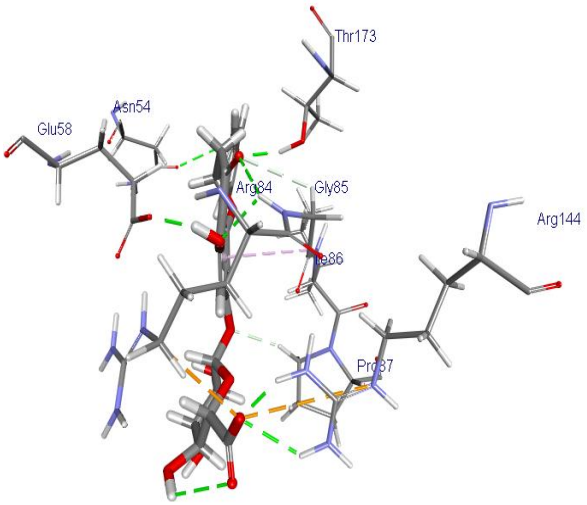
Sl. No.	Name of the compounds	Cdocker Energy (K. Cal/mol)	Cdocker Interaction Energy (K. Cal/mol)	Binding Energy (K. Cal/mol)
1.	P-coumaric acid	28.6907	31.8558	-140.493
2.	Protocatechuic acid	37.6785	34.3245	-225.958
3.	Phloroglucinol	30.5854	30.8059	-185.943
4.	Caffeic acid	31.5287	32.4082	-173.386
5.	Ferulic acid	29.0108	32.7679	-154.778
6.	Naringenin-7-O glucoside	23.6044	55.1704	-271.164
7.	Hesperidin	4.06315	63.0783	-307.262
8.	Ciprofloxacin	23.3649	49.2324	-288.719

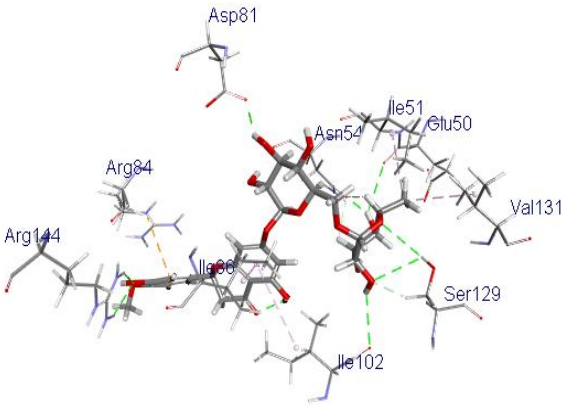
Table-10: 2D interaction study, 3D structures and 3D ligand-receptor complex with Bacterial DNA gyrase (3G75)

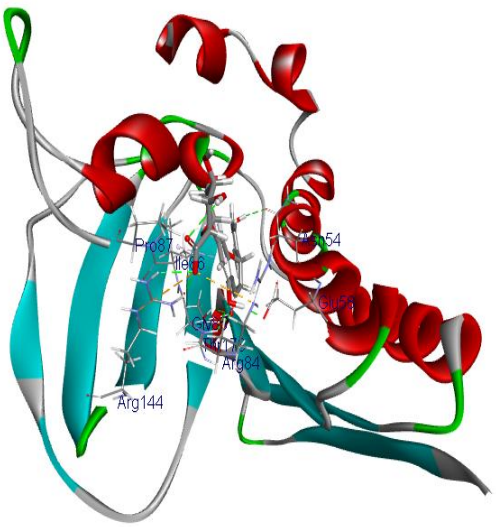
Protocatechuic acid	Phloroglucinol
	
	
	
Naringenin-7-O glucoside	Hesperidin

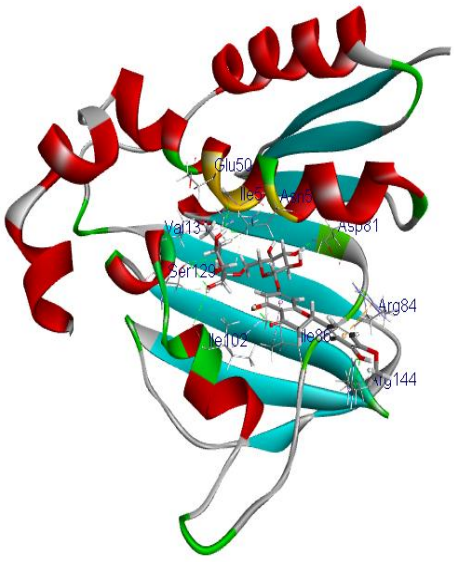




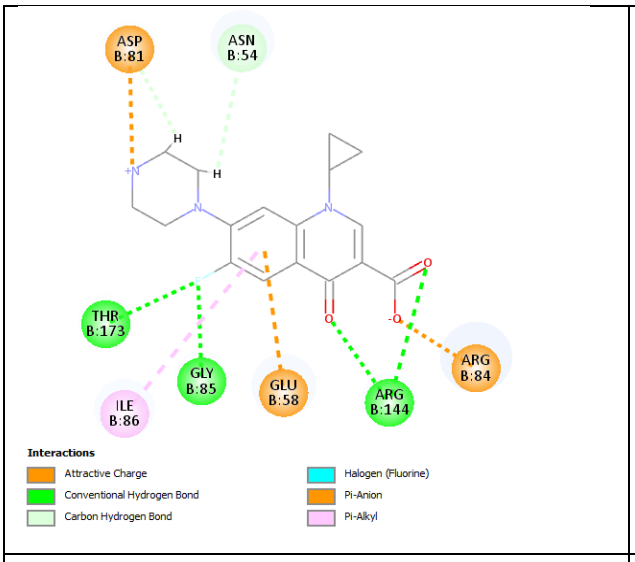


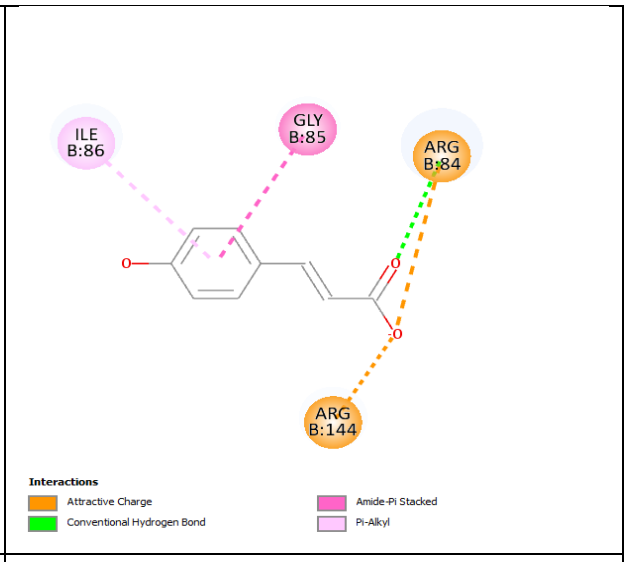


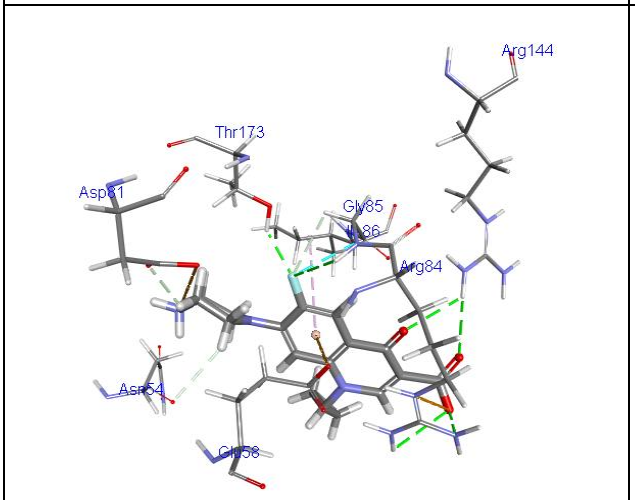


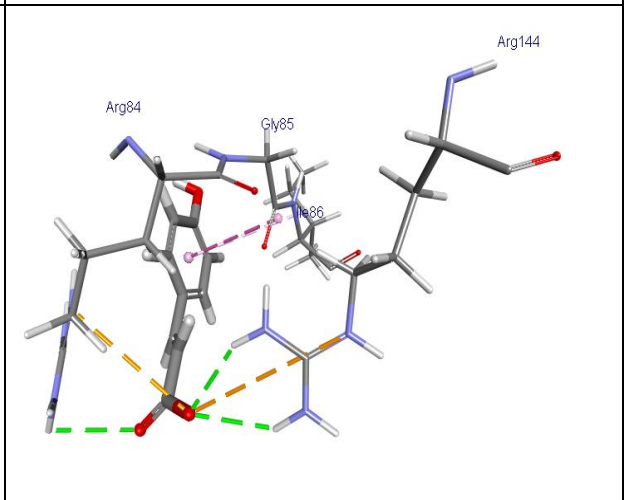


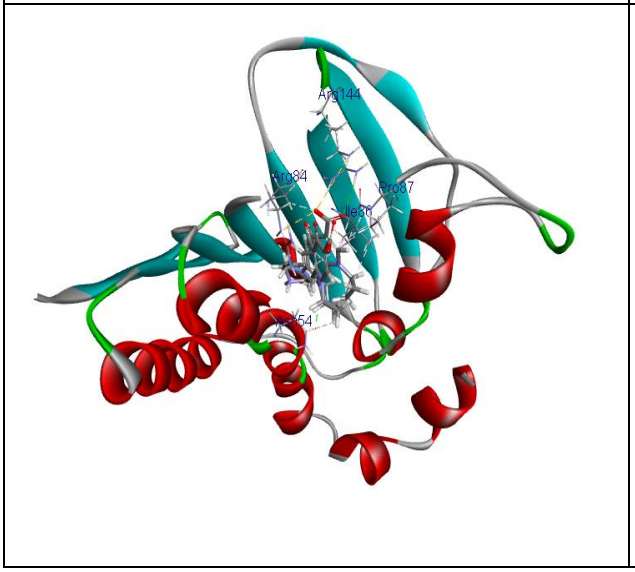
Ciprofloxacin	P-Coumaric acid



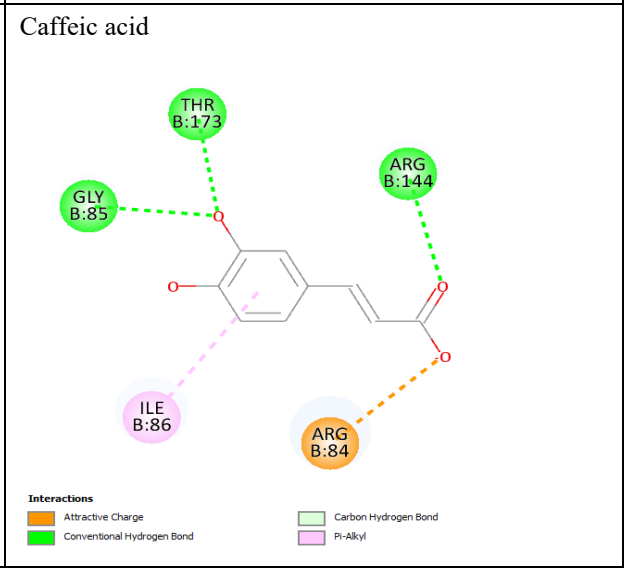


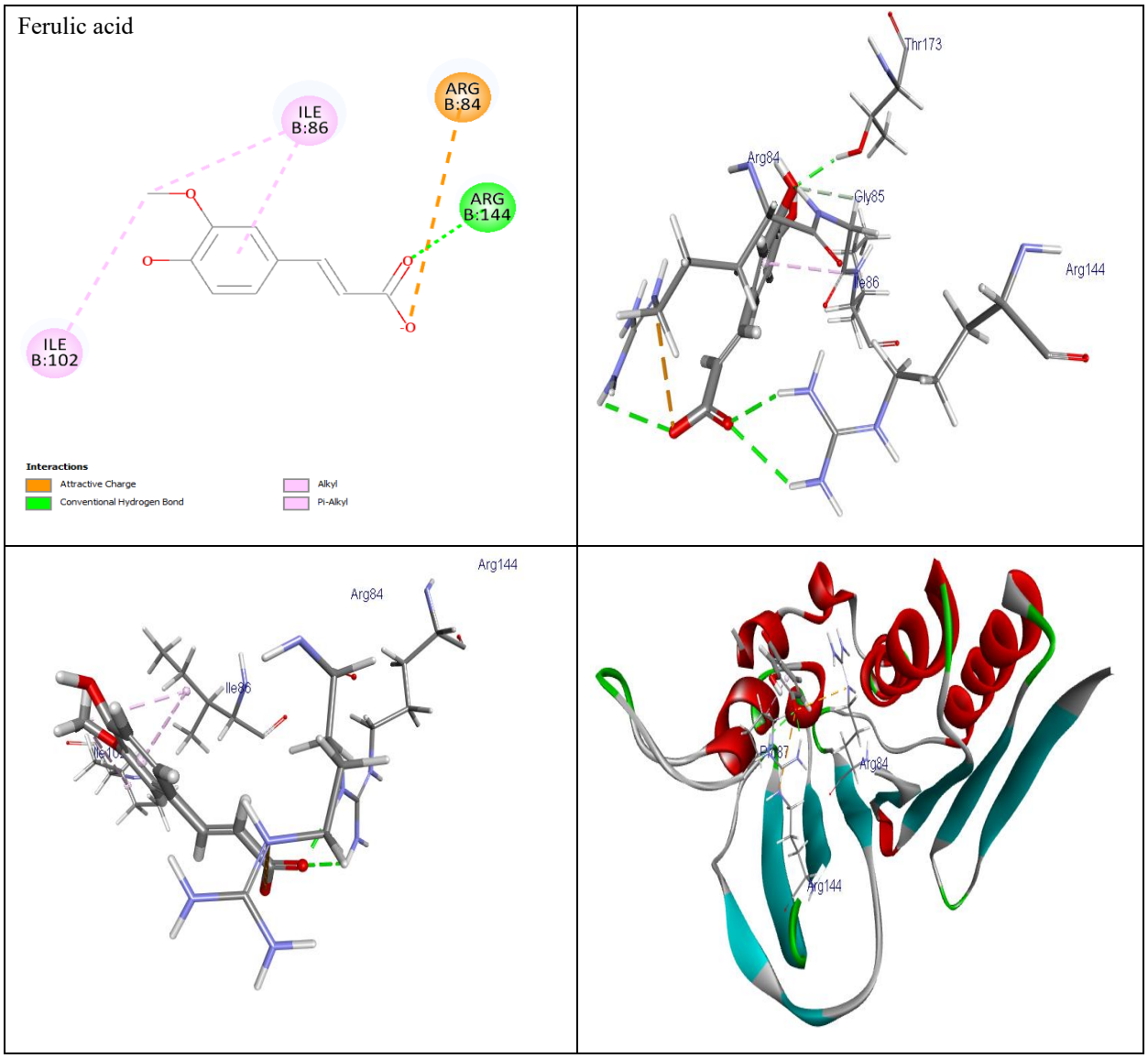






Caffeic acid





7. SUMMARY AND FINDINGS

The molecular docking analysis of bioactive compounds from the methanolic extract of orange peel (ME-OP) revealed significant variation in binding affinities and interaction profiles with the target protein. Among the tested phytoconstituents, Hesperidin demonstrated the most promising results with the highest Cdocker Interaction Energy (63.08) and the most negative Binding Energy (–307.26), suggesting strong and stable ligand–protein interactions. Naringenin-7-O-glucoside also performed exceptionally well, with high interaction energy (55.17) and a binding energy of –271.16, indicating excellent affinity. These two compounds outperformed the standard drug Ciprofloxacin, which showed a binding energy of –288.72 and interaction energy of 49.23, underscoring the potential of citrus-derived flavonoids as potent bioactive agents.

Moderate docking performance was observed for Protocatechuic acid and Phloroglucinol, with reasonably strong interaction and binding energies (–225.96 and –185.94, respectively), indicating their potential for further investigation. Caffeic acid and Ferulic acid showed moderate interaction but relatively lower binding affinity, while P-coumaric acid exhibited the weakest performance overall. The comparative docking results highlight Hesperidin as the most promising lead compound, followed by Naringenin-7-O-glucoside, due to their superior binding characteristics, supporting their potential use in drug development or therapeutic applications.

3. 6. Preliminary screening of antimicrobial activity

Table-11: Antibacterial Activity of ME-OP against *S. aureus*

Test Organism: *S. aureus*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: ME-OP						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	22	23	22.6667	0.57735	0.33333
6.25	27	29	30	28.6667	1.52753	0.88192
12.50	30	32	33	31.6667	1.52753	0.88192
25	34	32	34	33.3333	1.1547	0.66667
50	35	34	35	34.6667	0.57735	0.33333
100	35	35	36	35.3333	0.57735	0.33333

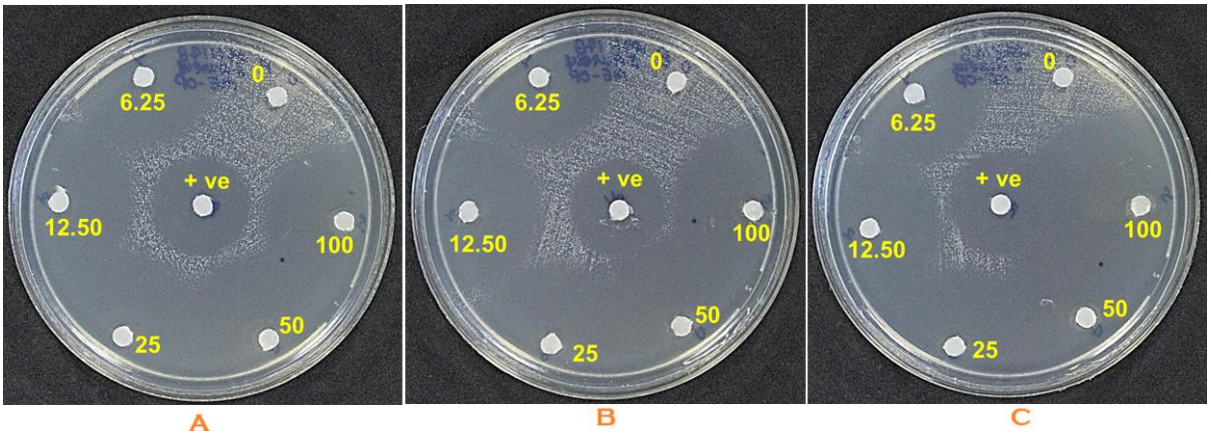


Fig-7: Zone of Inhibition of ME-OP against *S. aureus* at concentration present per disc in percentage (%).

Table-12: Antibacterial Activity of ME-OP against *B. subtilis*

Test Organism: *B. subtilis*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: ME-OP						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC)	23	22	22	22.3333	0.57735	0.33333
6.25	26	25	25	25.3333	0.57735	0.33333
12.50	27	27	26	26.6667	0.57735	0.33333
25	28	27	28	27.6667	0.57735	0.33333
50	31	30	31	30.6667	0.57735	0.33333
100	32	33	32	32.3333	0.57735	0.33333

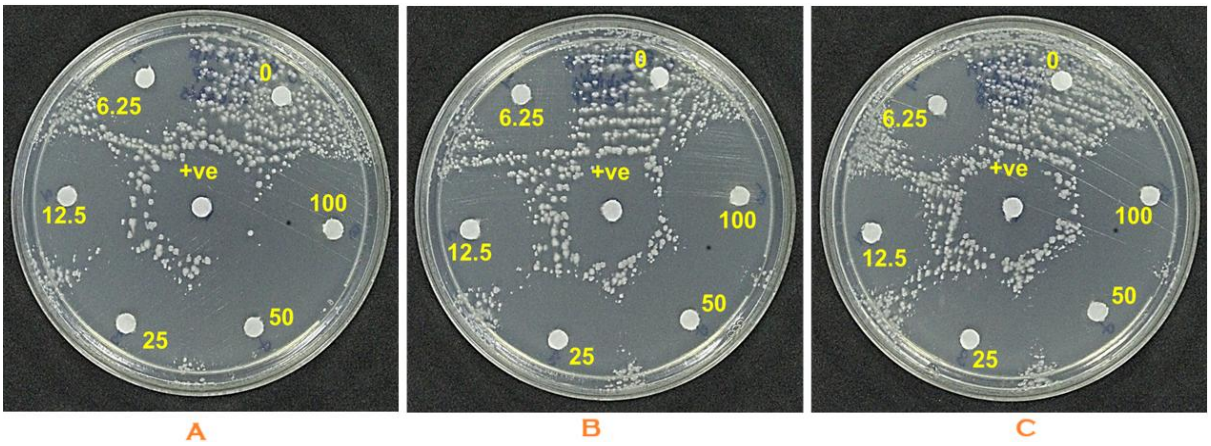


Fig-8: Zone of Inhibition of ME-OP against *B. subtilis* at concentration present per disc in percentage (%)

Table-13: Antibacterial Activity of ME-OP against *E. coli*

Test Organism: *E. coli*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: ME-OP						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC)	24	25	24	24.33333333	0.577350269	0.333333333
6.25	8	8	8	8	0	0
12.50	9	8	10	9	1	0.577350269
25	14	11	11	12	1.732050808	1
50	15	15	15	15	0	0
100	18	18	17	17.66666667	0.577350269	0.333333333

Table-14: Antibacterial Activity of ME-OP against *P. aeruginosa*

Test Organism: *S. aureus*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: ME-OP						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC)	25	25	25	25	0	0
6.25	8	9	8	8.3333333	0.57735	0.3333333
12.50	10	11	10	10.333333	0.57735	0.3333333
25	12	13	13	12.666667	0.57735	0.3333333
50	16	16	15	15.666667	0.57735	0.3333333
100	18	18	18	18	0	0

Table-15: Antifungal Activity of ME-OP against *A. niger*

Test Organism: *A. niger*						
X Axis: Amount (%/disc)						
Y Axis: *Zone Size (in mm)*						
Sample code: ME-OP						
Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
Amph-B (PC)	18	17	18	17.66666667	0.577350269	0.333333333
6.25	7	6	7	6.666666667	0.577350269	0.333333333
12.50	8	7	7	7.333333333	0.577350269	0.333333333
25	8	8	8	8	0	0
50	8	8	8	8	0	0
100	8	8	8	8	0	0

Table-16: Interpretation of Average Zone of Inhibition at effective concentration

Sample code	Test organisms	Effective concentration	Average Zone of Inhibition at effective amount (mm)
CF (Std.)	*S. aureus*	0.03%	23.00
ME-OP	*S. aureus*	6.25%	28.66
CF (Std.)	*B. subtilis*	0.03%	23.00
ME-OP	*B. subtilis*	6.25%	25.00
CF (Std.)	*E. coli*	0.03%	24.66
ME-OP	*E. coli*	6.25%	8.00
CF (Std.)	*P. aeruginosa*	0.03%	25
ME-OP	*P. aeruginosa*	6.25%	8.33
Amphotericin B	*A. niger*	0.03%	17.66
ME-OP	*A. niger*	6.25%	6.66

Summary of Findings (Compared to Standards)

The methanolic extract of orange peel (ME-OP) demonstrated notable antimicrobial activity when tested via the disc diffusion method. Against Staphylococcus aureus, ME-OP at 6.25% produced a larger zone of inhibition (28.66 mm) than Ciprofloxacin at 0.03% (23.00 mm), indicating strong antibacterial potential. For Bacillus subtilis, the extract also performed slightly better (25.00 mm) than the standard (23.00 mm), confirming good Gram-positive bacterial activity. However, against Escherichia coli and Pseudomonas aeruginosa, ME-OP was significantly less effective, producing inhibition zones of only 8.00 mm and 8.33 mm respectively, compared to 24.66 mm and 25.00 mm with the standard drug. Against Aspergillus niger, ME-OP showed a minimal antifungal effect (6.66 mm) versus 17.66 mm for Amphotericin B.

General Observations

Gram-Positive vs. Gram-Negative Sensitivity: ME-OP was more effective against Gram-positive bacteria like *S. aureus and B. subtilis*, and showed poor activity against Gram-negative strains such as *E. coli and P. aeruginosa*, likely due to the protective outer membrane of Gram-negative bacteria which limits compound penetration.

Antifungal Activity: The extract demonstrated weak antifungal activity against *A. niger,* indicating that its phytochemicals are not sufficiently potent to inhibit fungal growth at the tested concentration.

Effective Concentration: While ME-OP was tested at a higher concentration (6.25%) than the standards (0.01–0.03%), it still underperformed in some cases. This indicates moderate antimicrobial potency and suggests that higher doses are necessary to match or surpass standard drugs.

8. CONCLUSION

From the above experimentation and data, it was concluded that the methanolic extract of orange peel (ME-OP), prepared via reflux condensation, successfully yielded a rich phytochemical profile. Preliminary screening confirmed the presence of carbohydrates, phytosterols, oils, proteins, amino acids, and abundant polyphenols. TLC analysis revealed Rf values indicative of flavonoids (0.23, 0.17) and various phenolic compounds (0.33–0.63). Quantitatively, ME-OP showed significant Total Phenolic Content (1.25 mg GAE/g) and Flavonoid Content (1.51 mg QE/g). LC-MS identified major compounds including Hesperidin and Naringenin-7-O-glucoside, which showed superior binding affinity in docking studies. ME-OP demonstrated strong antibacterial effects against Gram-positive bacteria, but weaker antifungal and Gram-negative activity.

9. ACKNOWLEDGEMENT

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10. CONFLICT OF INTEREST

We declare that there is no conflict of interest regarding the publication of this research. All experiments were conducted independently without any financial or personal relationships that could influence the outcomes.

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