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COMPUTATIONAL ANALYSIS OF BIOACTIVE COMPOUNDS FROM *CITRUS SINENSIS* FRUIT PEELS TARGETING GYRASE B PROTEIN: PHYTOCHEMICAL PROFILING AND *IN VITRO* ANTIBACTERIAL ACTIVITY

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ABSTRACT

Citrus sinensis fruit peel, commonly regarded as agricultural waste, represents a rich source of bioactive phytochemicals with potential antibacterial applications. In this study, an integrated computational and experimental approach was employed to evaluate the antibacterial potential of *C. sinensis* peel extracts. Preliminary phytochemical screening revealed the presence of alkaloids, flavonoids, phytosteroids, terpenoids, quinones, and glycosides, while saponins were confined to methanolic and ethanolic extracts. Gas Chromatography-Mass Spectrometry (GC-MS) examination of the methanolic extract identified sixteen bioactive compounds. *In silico* ADME profiling showed that all compounds complied with Lipinski's Rule of Five. Molecular docking against bacterial Gyrase B proteins (PDB IDs: 4URN and 1KZN) demonstrated strong binding energies, particularly for 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one against the Gram-positive (G^{+ve}) target (−5.5 kcal/mol) and (+)-limonene against the Gram-negative (G^{-ve}) target (−5.8 kcal/mol). The Methanol Extract of *Citrus sinensis* fruit peel (ME-CSFP) exhibited a concentration-dependent Antibacterial (AB) effect (30–100 µl). Greater inhibitory activity was observed against *B. subtilis* (6.0 ± 1.0 to 9.0 ± 1.2 mm) and *S. aureus* (5.0 ± 1.0 to 10.0 ± 1.0 mm), whereas comparatively lower susceptibility was noted in *E. coli* (4.33 ± 0.58 to 6.67 ± 2.52 mm) and *K. pneumoniae* (5.0 ± 1.0 to 6.67 ± 1.53 mm). The minimum inhibitory concentrations (MIC) of orange peel extract demonstrated stronger antibacterial activity against Gram-positive bacteria (*Staphylococcus aureus* 31.2 µg/ml; *Bacillus subtilis* 62.5 µg/ml) than against Gram-negative bacteria (*Escherichia coli* 250 µg/ml; *Klebsiella pneumoniae* 250 µg/ml). Overall, this study highlights the significant role of computational biology in prioritizing bioactive compounds and demonstrates that *Citrus sinensis* peel can serve as a promising, sustainable source and support its potential valorization in drug discovery.

Keywords: *Citrus sinensis,* Orange Fruit Peels, Molecular docking, Gyrase Enzymes, Drug-likeness

INTRODUCTION

Infectious disorders because of pathogenic micro-organisms have emerged as a significant international public health issue. The efficacy of currently available antibiotics is progressively declining due to the accumulation of genetic mutations in bacterial species and the emergence of antimicrobial resistance (**Yadav and Kapley, 2021**). Many human illnesses are linked with unstable free radicals in the body, which can damage cellular membranes and other biomolecules, including proteins, lipoproteins, and DNA (**Pham-Huy et al., 2008**). Additionally, chronic diseases such as cancer have contributed substantially to global mortality and continue to pose a major health challenge (**Kausar et al., 2022**). Plants are used as medicine and are considered safer alternatives to synthetic pharmaceuticals. Phytomedicines derived from various plant parts exhibit a broad spectrum of biological activities (**Soheilifar et al., 2024**).

Citrus sinensis fruit peel (CSFP) has been extensively utilized in various applications (**Kaviya et al., 2011; Bagyalakshmi and Prathiksha, 2025**). *C. sinensis* is the most highly valued and widely consumed fruit worldwide. From childhood to old age, the consumption of ripe sweet oranges is highly popular, leading to increased market demand and enhanced commodity value. An estimated 115 million tonnes of *C. sinensis* will be produced worldwide each year (**Mojo et al., 2024**). The bioactive constituents of *C. sinensis*, including anthraquinones, tannins, terpenoids, flavonoids, and saponins, significantly contribute to its high production and economic importance (**Gotmare and Gade, 2018**). The peels are rich in phytochemicals and nutrients and have been widely employed in difference pharmacological and food applications (**Abou et al., 2022**). Plant extracts are frequently utilized for their medicinal properties (**Favela-Hernández et al., 2016**). *C. sinensis* belongs to the family Rutaceae and typically grows to a height of three to ten meters. The plant bears small thorns and leaf stalks measuring 0.5–3.5 cm in length. The fruits occur in various shapes, including ovoid, elliptic, and elongated forms with pointed ends; they are unripe, green; when they mature, they become yellow to orange.

Secondary metabolites derived from medicinal plants represent a major source of novel drug candidates. These metabolites include diverse classes of chemicals (**Yang et al., 2020**). Advanced analytical techniques, particularly GC–MS, have proven to be rapid, sensitive, and reliable tools for the identification of bioactive constituents (**Kashyap et al., 2025**). Investigating the chemical composition of plant-derived chemicals to understand their biological roles remains an important area of research. Molecular docking has become popular in this setting as a valuable approach in modern drug discovery for predicting ligand–protein interactions (**Jorgensen, 2004**). Although phytochemical screening of plant extracts and essential oils helps elucidate their biological activities, the specific phytoconstituents responsible for these effects often remain unclear (**Bajorath, 2005**). Therefore, *in silico* studies are important for evaluating the compatibility and interaction of compounds with target receptors, thereby supporting their potential therapeutic relevance (**Badraoui et al., 2022; Khatoon et al., 2024**).

The ATP binding domain of DNA gyrase, which is important for DNA replication in bacteria, is carried out by the B subunit of DNA gyrase. Gyrase B hydrolyzes ATP, providing the energy required by Gyrase A to ligate and pass DNA strands (**Skok et al., 2020; Tiz et al., 2019**). Owing to its indispensable role in bacterial proliferation, Gyrase B represents a key target for AB medication development. Repression of its ATPase activity disrupts DNA supercoiling, an effect analogous to the mechanism of action of fluoroquinolone antibiotics (**Henderson et al., 2020**).

Given the rich phytochemical composition of *Citrus sinensis* fruit peel and the essential role of DNA gyrase B in bacterial survival, we hypothesize that bioactive compounds present in the peel can effectively bind to and inhibit the ATP-binding domain of bacterial DNA gyrase B, thereby exerting antibacterial activity. To test this hypothesis, preliminary phytochemical screening and GC–MS analysis were

performed to identify the bioactive compounds present in CSFP. Subsequently, drug-likeness evaluation, *in vitro* antibacterial assays, and molecular docking studies targeting DNA gyrase B were carried out.

MATERIALS AND METHODS

Plant Sample Collection

Oranges were acquired from a local fruit store in Pudukkottai District, Tamil Nadu, India. The sample were identified from PG and Research Department of Botany, J.J. College of Arts and Science (Autonomous), Pudukkottai, Tamil Nadu, India. The fruits were cleansed with water, and the peels were excised using a sharp knife. Subsequently, the peels were desiccated at 40 ºC for a duration of 8 days. The plant was verified by PG and Research Department of Botany. Subsequently, 20 grams of desiccated citrus peels were pulverized. The desiccated orange peels were gathered in a sealed container.

Preparation of Sample Extract

The extract was made with a crude extraction method, which involved dissolving 10 g of dried and powdered citrus peel in 100 mL of each solvent and allowing it to stand at room temperature for 24 hours. Methanol, acetone, ethanol, and ethyl acetate were used as solvents for the investigation. The samples were filtered and then evaporated to dryness. Subsequently, each individual extract was stored for further use.

Phytochemical Analysis

The dried extracts of orange fruit peels provide a preliminary assessment of phytochemicals according to conventional procedures (**Harborne, 1998; Balachandran et al., 2024**).

GC-MS Analysis of ME-CSFP

The natural compounds present in ME-CSFP were identified using GC–MS (Shimadzu GC-2030 coupled with MS-QP2020). The analysis was performed using electron ionisation (EI) at 70 eV and complementary chemical ionisation (CI) techniques. The temperature of the column was raised from 45°C to 280°C at a rate of 10°C per minute. A 1µL sample was injected. The mass scan range was 50–800 amu, and the total run time was 55 minutes. The obtained mass spectra were compared with those of standard compounds using the NIST Mass Spectral Library for compound identification **(Kathirvel et al., 2025; Singh et al., 2023; Petrova et al., 2023).**

Pharmacokinetic Studies of the identified Compounds

SwissADME was used to predict the test compounds' drug-likeness qualities, including Veber rule, LROF, and pharmacokinetic (ADME) properties (**Lipinski et al., 2012; Daina et al., 2017**).

Molecular Docking Studies

In this study, bacterial target protein gyrase B (4URN and 1KZN), from G^{+ve} *S. aureus* and G^{-ve} *E. coli* were downloaded from the Protein Data Bank (rcsb.org). According to recent studies, these proteins are essential for bacterial survival (**Hossen et al., 2024**). The co-crystallized ligands were removed, and the proteins were processed to remove water molecules, extra chains or heteroatoms, add hydrogen, estimate Kollman charges, and convert them to a pdbqt file. Identified bioactive compounds were retrieved from the PubChem database, and docking study using AutoDock Tool 4.2.6. Interacted amino acids analyzed using Biovia Discovery studio.

AB activity of ME-CSFP

The AB activity of the ME-CSFP was evaluated against selected Gram-positive and Gram-negative pathogenic bacteria, namely *Staphylococcus aureus, Bacillus subtilis, Escherichia coli,* and *Klebsiella pneumoniae*. The bacterial strains were obtained from the PG and Research Department of Microbiology, J.J. College of Arts and Science (Autonomous), Pudukkottai, Tamil Nadu, India. The antibacterial activity was assessed using the agar well diffusion method as described by **Lalitha et al. (2021)** with slight modifications. Briefly, overnight-grown bacterial cultures were uniformly mixed with 20 mL of sterile nutrient agar maintained at 45 °C and poured into sterile Petri plates. After solidification, wells of 5 mm diameter were punched aseptically using a sterile cork borer. The ME-CSFP extract was prepared at concentrations of 30, 50, and 100 µg/mL. A fixed volume of 50 µg/mL from each concentration was introduced into the respective wells. Methanol was used as the negative control to verify that the solvent had no inhibitory effect, while tetracycline served as the positive control. The plates were kept at 4 °C for 30 min to allow diffusion of the extract into the agar, followed by incubation at 36 °C for 24 h. After incubation, the zones of inhibition were measured in millimeters. All experiments were performed in triplicate, and results are expressed as mean ± SD. Data analysis and graph preparation were carried out using OriginPro 8.5. Statistical analysis was performed using SPSS (ver. 20) with One-way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test. Differences were considered significant at $P < 0.05$.

Minimum Inhibitory Concentration assay by Resazurin method

The minimum inhibitory concentration (MIC) values for orange peel extract against test organisms *S. aureus, B. subtilis E. coli,* and *K. pneumoniae* were assessed using Mueller-Hinton broth (MHB) by a broth microdilution method under sterile circumstances within microtiter plates. The Minimum Inhibitory Concentration (MIC) was determined using a twofold dilution of the stock solution, ranging from 1000 µg/ml to 2.5 µg/ml in sterile Mueller-Hinton Broth (MHB) medium. An equal volume of bacterial culture was included and incubated at 37°C for 24 hours. The same procedure was replicated for the stock solution of the control antibiotic chloramphenicol (1280 µg/ml). Following incubation, 10 µl (270 mg/ 40 ml) of resazurin dye was introduced and incubated for an additional 4 hours (Sundaram et al., 2021). Observable colour transitions from purple to pink signify the existence of growth. The results were analysed in accordance with CLSI recommendations, wherein observable growth suppression at the highest dilution was deemed the MIC of the antibiotic.

Results and Discussion

Phytochemical Analysis

The phytochemical screening of *C. sinensis* fruit peel extracts prepared using different solvents revealed the presence of several bioactive constituents. Alkaloids, flavonoids, phytosteroids, terpenoids, quinones, and glycosides were detected in all four solvent extracts, indicating their broad solubility across polar and semi-polar solvents. Saponins were observed in the methanolic and ethanolic extracts but were absent in the acetone and ethyl acetate extracts. Carbohydrates were identified in the methanol, ethanol, and acetone extracts, whereas the ethyl acetate extract tested negative for carbohydrates. In contrast, tannins and phenolic compounds were absent in any of the solvent extracts analyzed. The overall phytochemical profile demonstrates that methanol and ethanol were more effective solvents for extracting a wider range of phytoconstituents compared to acetone and ethyl acetate. The detailed phytochemical composition of each extract is summarized in Table 1**.** According to **Booker et al. (2008)**, terpenoids, alkaloids, and flavonoids are being used as medications or dietary supplements to treat or prevent a variety of illnesses.

Secondary metabolites present in *citrus sp*. are recognized for their therapeutic importance in the management of various diseases. Phytochemical investigations of *C. sinensis* have reported the presence of carbohydrates, flavonoids, glycosides, and coumarins (**Oikeh et al., 2013; Dhiman et al., 2012**). Among these constituents, flavonoids and saponins are considered key contributors to the antimicrobial activity of plants (**Rahman et al., 2011**). Flavonoids, in particular exhibit diverse biological properties. They also regulate enzymatic functions, inhibit cell proliferation, and serve as defensive compounds in plants against pathogenic invasion (**Górniak et al., 2019; Oikeh et al., 2020**).

Table 1 Preliminary phytochemical analysis of fruit peel extracts of *C. sinensis* using different solvents

S. No	Phytochemicals	Methanol	Ethanol	Acetone	Ethyl acetate
1.	Alkaloids	+	+	+	+
2.	Flavonoids	+	+	+	+
3.	Saponins	+	+	-	-
4.	Tannins	-	-	-	-
5.	Phenolic compounds	-	-	-	-
6.	Phyto steroids	+	+	+	+
7.	Terpenoids	+	+	+	+
8.	Carbohydrates	+	+	+	-
9.	Quinone	+	+	+	+
0.	Glycosides	+	+	+	+

Present (+) Absent (-)

GC-MS analysis of ME-CSFP

For the identification of phytocompounds, ME-CSFP was further subjected to GC–MS evaluate. The chromatogram revealed 16 distinct phytocompound peaks, indicating a diverse chemical composition. Compound identification was carried out by comparing the retention times, peak area percentages, and mass spectral fragmentation patterns with those of authenticated compounds available in the NIST mass spectral library**.** The GC–MS analysis chromatogram (Figure 1) showed 16 peaks and detected 16 phytocompounds derivatives (Table 2)**.** The 2D structures of all compounds are presented in Figure 2**.** Among the identified constituents, (Isobutoxymethyl)oxirane was the most abundant compound with a peak area of 38.02%, followed by methyl α-D-galactopyranoside (11.99%),

glycerol (10.03%), glyceraldehyde (5.51%), and 5-(hydroxymethyl)furfural (5.27%). Other notable compounds included 3-(acetylthio)-2-methylpropanoic acid (4.80%), (+)-limonene (4.68%), pentyl methacrylate (4.08%), 1,2-cyclopentanedione (2.98%), glyceryl acetate (2.57%), and 4-vinylguaiacol (2.53%). Several minor constituents such as 6-oxoheptanoic acid, 2-methyl-3-(1-oxopropoxy)-4H-pyran-4-one, 3-octyl acetate, and 3,6-dihydro-2H-pyran-2,6-dione were also detected in lower proportions. Previous studies have consistently reported the presence of limonene as a major phytoconstituent in *C. sinensis* (**Javed et al., 2014; Al-Sharifi and Alnomani, 2022**). Limonene is recognized as the predominant component of *Citrus* pericarp oil and has been shown to strong antioxidant activity on par with powerful conventional antioxidants (**Doughari and Bazza, 2020**). In a recent investigation, Stearic acid was the most prevalent compound in orange fruit and peel extracts, followed by ascorbic acid, limonene, linoleic acid, palmitic acid, linalool, and pentadecyclic acid, according to **Ogo et al. (2024)**. Furthermore, *C. sinensis* fruit has shown notable biological activity against a range of pathogenic microorganisms, which has been attributed to the synergistic effects of their diverse phytochemicals (**Shetty et al., 2016; Suriyaprom et al., 2022**).

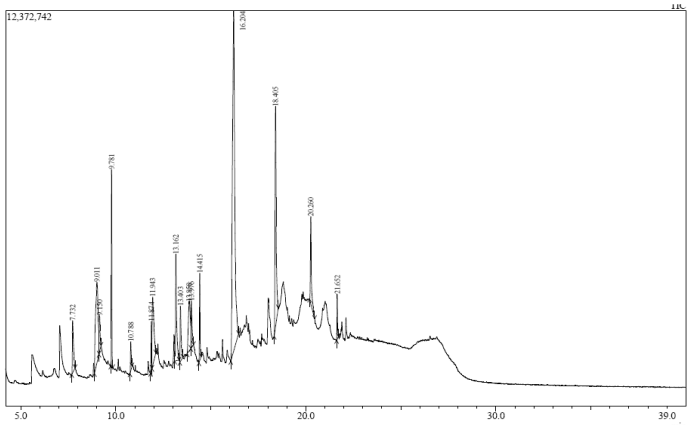

Figure 1 Chromatogram in GC-MS analysis from ME-CSFP

Table 2 GC-MS analysis of ME-CSFP

P. No	R.T	Compound Name	M.W (g/mol)	M.F	Peak %
1.	7.732	1,2-Cyclopentanedione	98.10	$C_5H_6O_2$	2.98
2.	9.011	Glycerol	92.09	$C_3H_8O_3$	10.03
3.	9.150	3,6-dihydro-2H-pyran-2,6-dione	112.08	$C_5H_4O_3$	1.52
4.	9.781	(+) -Limonene	136.23	$C_{10}H_{16}$	4.68
5.	10.788	2-methyl-3-(1-oxopropoxy)-4H-pyran-4-one	182.17	$C_9H_{10}O_4$	1.03
6.	11.874	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	144.12	$C_6H_8O_4$	1.15
7.	11.943	Glyceraldehyde	90.08	$C_3H_6O_3$	5.51
8.	13.162	5-(Hydroxymethyl)furfural	126.11	$C_6H_6O_3$	5.27
9.	13.403	Glyceryl Acetate	134.13	$C_5H_{10}O_4$	2.57
10.	13.858	3-(Acetylthio)-2-methylpropanoic acid	162.21	$C_6H_{10}O_3S$	4.80
11.	13.976	6-Oxoheptanoic acid	144.17	$C_7H_{12}O_3$	2.19
12.	14.415	4-Vinylguaiacol	150.17	$C_9H_{10}O_2$	2.53
13.	16.204	(Isobutoxymethyl)oxirane	130.18	$C_7H_{14}O_2$	38.02
14.	18.405	methyl alpha-D-galactopyranoside	194.18	$C_7H_{14}O_6$	11.99
15.	20.260	Pentyl methacrylate	156.22	$C_9H_{16}O_2$	4.08
16.	21.652	3-Octyl acetate	172.26	$C_{10}H_{20}O_2$	1.24

P. No – Peak Number; R.T- Retention Time; M.W – Molecular Weight; M.F – Molecular Formula

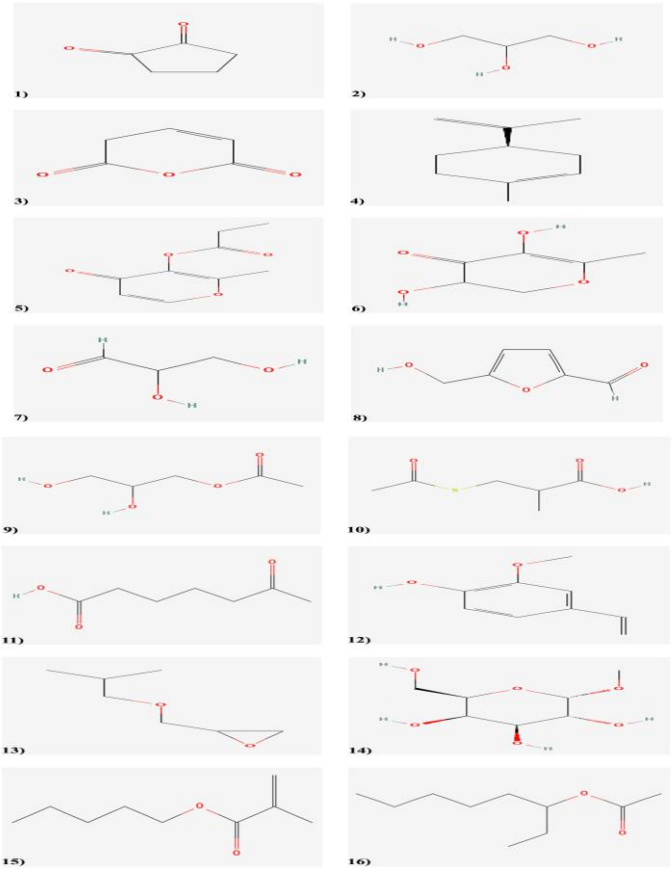

Figure 2 2D Structure of identified bioactive compounds from ME-CSFP using GC-MS 1) 1,2-Cyclopentanedione; 2) Glycerol; 3) 3,6-dihydro-2H-pyran-2,6-dione; 4) (+) –Limonene; 5) 2-methyl-3-(1-oxopropoxy)-4H-pyran-4-one; 6) 2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one; 7) Glyceraldehyde; 8) 5-(Hydroxymethyl)furfural; 9) Glyceryl Acetate; 10) 3-(Acetylthio)-2-methylpropanoic acid; 11) 6-Oxoheptanoic acid; 12) 4-Vinylguaiacol; 13) (Isobutoxymethyl)oxirane; 14) methyl alpha-D-galactopyranoside; 15) Pentyl methacrylate; and 16) 3-Octyl acetate.

Pharmacokinetics of the identified Compounds

LRO5 is widely used to assess the drug-likeness of new chemical entities (**Lipinski et al., 2012**). Based on the present analysis, all the investigated compounds complied with Lipinski's criteria. All compounds showed MW below 500 g/mol, acceptable lipophilicity (cLogP < 5), and no violations of hydrogen bond donor or acceptor limits (Table 3). Each molecule exhibited a MW ranging from 90.08 to 194.18 g/mol, which is well below the 500 g/mol threshold specified by LROF, indicating favorable oral bioavailability. Most compounds demonstrated balanced hydrophilic–lipophilic properties, with Log P values between −1.73 and 3.37, suggesting adequate membrane permeability. The number of HBD (0–4) and HBA (0–13) was within the acceptable range for drug-like molecules. Notably, none of the compounds violated LROF, confirming strong drug-likeness. The TPSA values ranged from 0.00 to 116.73 Å². Methyl α-D-galactopyranoside exhibited higher TPSA values, reflecting greater polarity, whereas (+)-limonene showed a TPSA of 0.00 Å², indicating a non-polar nature. The number of rotatable bonds (0–7) suggests moderate molecular flexibility among the compounds, which may influence binding interactions with biological targets. Overall, physicochemical profiling indicates that all identified bioactive compounds possess favorable drug-like characteristics without Lipinski violations, supporting their potential oral bioavailability and suitability for molecular docking and further pharmacological investigations.

Table 3 Drug likeness of identified bioactive compounds from ME-CSFP

S. No	Compound Name	Formula	MW (g/mol)	NHD	NHA	*Lop P*	LROF	NRB	TPSA (A^2)
1.	1,2-Cyclopentanedione	$C_5H_6O_2$	98.10	0	7	0.34	0	0	34.14
2.	Glycerol	$C_3H_8O_3$	92.09	0	6	-1.09	0	2	60.69
3.	3,6-dihydrate-2H-pyran-2,6-dione	$C_5H_4O_3$	112.08	0	8	0.41	0	0	43.37
4.	(+)-Limonene	$C_{10}H_{16}$	136.23	0	0	3.37	0	1	0.00
5.	2-methyl-3-(1-oxopropoxy)-4H-pyran-4-one	$C_9H_{10}O_4$	182.17	0	4	1.26	0	3	56.51
6.	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	$C_6H_8O_4$	144.13	2	10	-0.22	0	0	66.76
7.	Glyceraldehyde	$C_3H_6O_3$	90.08	2	6	-0.65	0	2	57.53
8.	5-(Hydroxymethyl)furfural	$C_6H_6O_3$	126.11	1	3	0.19	0	2	50.44
9.	Glyceryl Acetate	$C_5H_{10}O_4$	134.13	2	9	-0.46	0	4	66.76
0.	3-(Acetylthio)-2-methylpropanoic acid	$C_6H_{10}O_3S$	162.21	1	10	0.80	0	4	79.67
1.	6-Oxoheptanoic acid	$C_7H_{12}O_3$	144.17	1	10	0.87	0	5	54.37
2.	4-Vinylguaiacol	$C_9H_{10}O_2$	150.17	1	11	2.14	0	2	29.46
3.	(Isobutoxymethyl)oxirane	$C_7H1_4O_2$	130.18	0	9	1.41	0	4	21.76
4.	Methyl alpha-D-galactopyranoside	$C_7H_{14}O_6$	194.18	4	13	-1.73	0	2	99.38
5.	Pentyl methacrylate	$C_9H_{16}O_2$	156.22	0	11	2.58	0	6	26.30
6.	3-octyl acetate	$C_{10}H_{20}O_2$	172.26	0	2	2.85	0	7	26.30

MW- Molecular Weight; NHD – Number of Hydrogen Bond Donor; Number of Hydrogen Bond Acceptor; Lipinski Rule of Five; NRB – Number of Rotatable Bond; TPSA - Topological Polar Surface Area

Molecular Docking Studies

The rising incidence of microbial resistance to existing antibiotics has intensified the search for novel antimicrobial agents. Consequently, the screening of plants for new and potent antimicrobial compounds has become an important field of study in science. In modern drug discovery and design, plant-derived compounds play a crucial role due to their therapeutic potential (**Saleem et al., 2023**). Bacterial gyrase plays a pivotal role in AB drug discovery, as it introduces negative supercoils into double-stranded DNA, a process essential for DNA replication, transcription, and recombination (**Amer et al., 2021**). Variations in antibacterial inhibition can be ascribed to structural differences between bacterial cell walls. G+ve bacteria have large peptidoglycan layers and no outer membrane, whereas G-ve have thin ones. Sugars and amino acids form peptididoglycan, a mesh-like polymer that strengthens cell walls. This layer serves as a protective barrier, shielding microorganisms from AB agents, including antibiotics, toxins, chemicals, and degradative enzymes (**Alotaibi and Amer, 2020**).

Table 4 Binding affinities of target proteins and ligand interactions

S. No	Compound Name	Binding Affinities (kcal/mol)	
		4URN	**1KZN**
1.	1,2-Cyclopentanedione	-4.1	-3.3
2.	Glycerol	-3.8	-3.8
3.	3,6-dihydrate-2H-pyran-2,6-dione	-4.8	-4.3
4.	(+)-Limonene	**-4.9**	**-5.8**
5.	2-methyl-3-(1-oxopropoxy)-4H-pyran-4-one	-4.0	-3.8
6.	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	**-5.5**	-4.5
7.	Glyceraldehyde	-3.7	-3.6
8.	5-(Hydroxymethyl)furfural	**-5.3**	**-4.8**
9.	Glyceryl Acetate	-4.6	-4.2
10.	3-(Acetylthio)-2-methylpropanoic acid	**-4.7**	-4.3
11.	6-Oxoheptanoic acid	-4.6	-4.0
12.	4-Vinylguaiacol	**-5.3**	**-5.5**
13.	(Isobutoxymethyl)oxirane	-4.2	-3.6
14.	Methyl alpha-D-galactopyranoside	-4.4	**-4.8**
15.	Pentyl methacrylate	-4.2	**-4.6**
16.	3-octyl acetate	-4.1	-4.0
	Tetracycline (Standard)	-6.6	-7.2

The phytochemical constituents identified from the peel extracts, along with the standard drug tetracycline, were subjected to molecular docking against the target proteins 4URN and 1KZN to evaluate their virtual BE. For *E. coli* gyrase B (PDB ID: 4URN), the BE of the identified compounds ranged from −3.7 to −5.5 kcal/mol, whereas tetracycline exhibited a stronger BE of −6.6 (Table 4). Among the screened phytocompounds, five showed comparatively higher BE (Table 5 and Figure 3). Notably, 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one demonstrated the highest BE (−5.5), forming interactions with GLY80, THR168, and PRO82. Both 5-(hydroxymethyl)furfural and 4-vinylguaiacol exhibited identical binding energies (−5.3); however, 5-(hydroxymethyl)furfural interacted with ASP76, GLY80, MET81, and THR168, while 4-vinylguaiacol interacted with PRO82, MET81, and ILE96. (+)-limonene displayed a BE of −4.9 and interacted with MET81 and PRO82, whereas 3-(Acetylthio)-2-methylpropanoic acid showed a BE of −4.7, interacting with GLU53, and THR168. In comparison, tetracycline exhibited the strongest binding energy and formed interactions with key residues including LYS165, THR163, THR166, ASP139, ARG138, ARG79, and TYR58. Recent study, **Hatif et al. (2024)** showed that Diospyrin, Isodiospyrin, and Pyranocoumarin had the highest binding affinities (−9.1 to −8.6 kcal/mol) against *S. aureus* DNA gyrase

Table 5 Identified compounds and standard drug interaction with 4URN

Compound name	Binding Affinity	Amino acid Interaction
(+)-Limonene	`-4.9	MET81, PRO82
3-(Acetylthio)-2-methylpropanoic acid	-4.7	GLU53, THR168
2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-5.5	GLY80, THR168, PRO82
5-(Hydroxymethyl)furfural	-5.3	ASP76, GLY80, MET81
4-Vinylguaiacol	-5.3	THR168, PRO82, MET81, ILE96
Tetracycline (Standard)	-6.6	LYS165, THR163, THR166, ASP139, ARG138, ARG79, TYR58

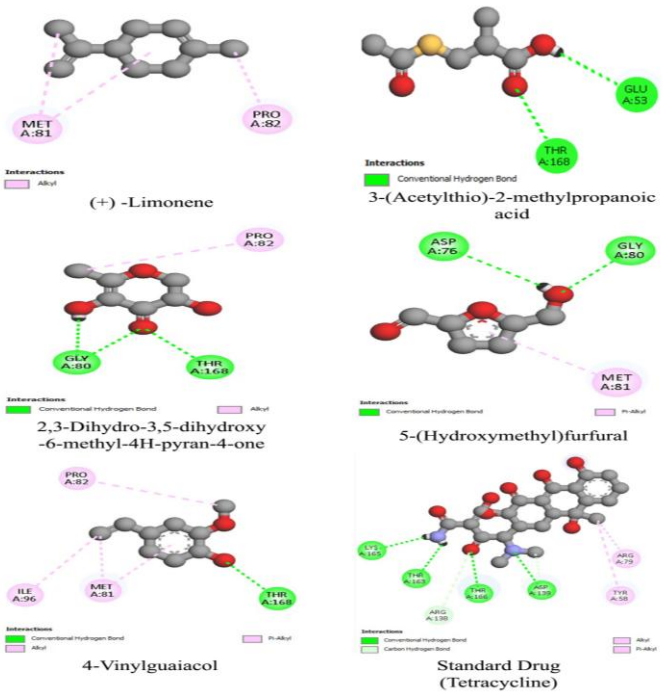

Figure 3 Interaction profile of DNA gyrase B (*S.aureus*) (4URN) with identified compounds and standard drug.

For the second target protein (PDB ID: 1KZN), the binding energies of the phytocompounds ranged from −3.3 to −5.8 kcal/mol, while tetracycline again

demonstrated superior binding with an BE of −7.2 (Table 4). Among the screened phytocompounds, five showed comparatively higher BE (Table 6 and Figure 4). Notably, (+)-limonene showed the highest BE (−5.8), interacting with VAL167, ALA47, VAL71, VAL43, and ILE78. 4-vinylguaiacol also exhibited strong BE (−5.5) and interacted with ASN46, THR165, ALA47, ILE78, and VAL71. 5-(hydroxymethyl)furfural and methyl α-D-galactopyranoside displayed identical binding energies (−5.3), interacting with THR165, VAL71, and ALA47, and ALA96, ASN46, VAL120, and GLY119, respectively. Additionally, Pentyl methacrylate showed a binding score of −4.6 and formed interactions with VAL71, ILE78, ILE90, and ALA47.Tetracycline demonstrated the strongest BE and interacted with key residues including ASP49, ASN46, ALA96, HIS95, SER121, GLY117, and VAL118 (Table 6 and Figure 4). **Hatif et al. (2024)** reported that Diospyrin, Yohimbine, and Ellagic acid showed the highest binding affinities against *E. coli* DNA gyrase. Importantly, it should be noted that molecular docking provides only a predictive assessment of ligand–protein interactions and does not directly confirm biological activity. Since the antibacterial assays in this study were performed using crude extracts, the observed activity cannot be attributed to any single compound. The antibacterial effect is more likely due to the combined or synergistic action of multiple phytoconstituents present in the extract. Therefore, the docking results should be interpreted as supportive evidence for potential activity rather than definitive proof. Further studies involving isolation and individual compound testing are required to validate their specific antibacterial roles.

Table 6 Identified compounds and standard drug interaction with 1KZN

Compound name	Binding Affinity	Amino acid Interaction
(+)-Limonene	`-5.8	VAL167, ALA47, VAL71, VAL43, ILE78
Pentyl methacrylate	-4.6	VAL71, ILE78, ILE90, ALA47
5-(Hydroxymethyl)furfural	-4.8	THR165, VAL71, ALA47
4-Vinylguaiacol	-5.5	ASN46, THR165, ALA47, ILE78, VAL71
Methyl α-D-galactopyranoside	-4.8	ALA96, ASN46, VAL120, GLY119
Tetracycline (Standard)	-7.2	ASP49, ASN46, ALA96, HIS95, SER121, GLY117, VAL118

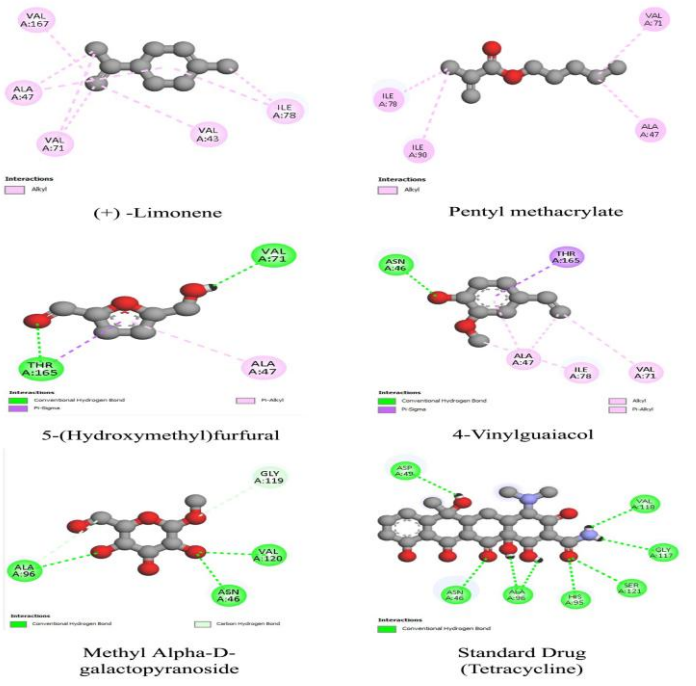

Figure 4 Interaction profile of DNA gyrase B (*E. coli*) (1KZN) with identified compounds and standard drug.

AB activity of ME-CSFP

AB active molecules are compounds that eliminate or suppress the growth of bacteria at a localized site without causing significant damage to surrounding tissues (Hasan et al., 2022). The present study evaluated the AB activity of the ME-CSFP was evaluated against four pathogenic bacterial strains at 30, 50, and 100 µg/mL concentration using the agar WDM. The results demonstrated a concentration-dependent increase in the zone of inhibition against all tested microorganisms (Figure 5). Among the tested bacteria, *B. subtilis* exhibited the highest susceptibility to ME-CSFP, showing zones of inhibition of 6.0 ± 1.0, 8.0 ± 1.0, and 9.0 ± 1.2mm at 30, 50, and 100 µg/mL, respectively. *S. aureus* also showed notable sensitivity, with inhibition zones increasing from 5.0 ± 1.0mm (30 µg/mL) to 10.0 ± 1.0mm (100 µg/mL).

In comparison, G^{-ve} bacteria demonstrated relatively lower susceptibility. *Escherichia coli* showed inhibition zones of 4.33 ± 0.58, 5.0 ± 0.50, and 6.67 ± 2.52mm at increasing concentrations, while *K. pneumoniae* exhibited zones of 5.0 ± 1.0, 5.67 ± 1.53, and 6.67 ± 1.53mm, respectively. The standard antibiotic (AB) produced higher zones of inhibition against all tested strains, with maximum activity observed against *B. subtilis* (13.67 ± 1.53), followed by *K. pneumoniae* (12.0 ± 1.0), *S. aureus* (10.67 ± 1.53), and *E. coli* (9.65 ± 1.53) (Figure 6 and Table 7). Overall, the methanolic *C. sinensis* fruit peel extract exhibited moderate AB activity. The antimicrobial activities observed in the citrus peel extract are mainly attributed to its high flavonoid content (**Oikeh et al. 2020**). In a study, **Jayaprakasha et al. (2000)** reported that although the acetone extract of citrus peels was comparatively less effective than other fractions, the ethanol-soluble fractions exhibited greater AB activity against the tested organisms. Previous research further demonstrated that *Bacillus cereus* showed a zone of inhibition of 22.33 ± 1.04 mm, indicating high sensitivity to orange peel extract (**Saleem et al., 2023**).

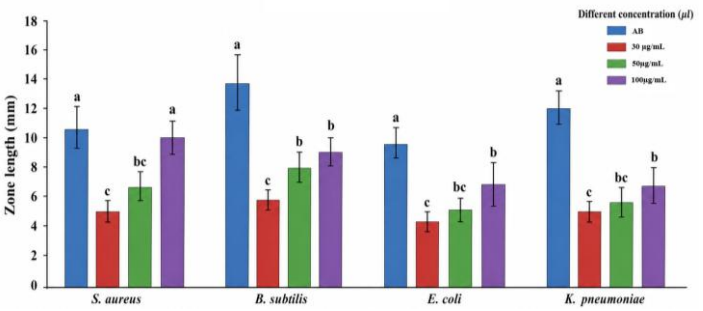

Figure 5 Antibacterial activity of ME-CSFP. Values are expressed as mean ± SD (n = 3). Means within the same row followed by different superscript letters are significantly different at P < 0.05 according to Duncan's Multiple Range Test after one-way ANOVA.

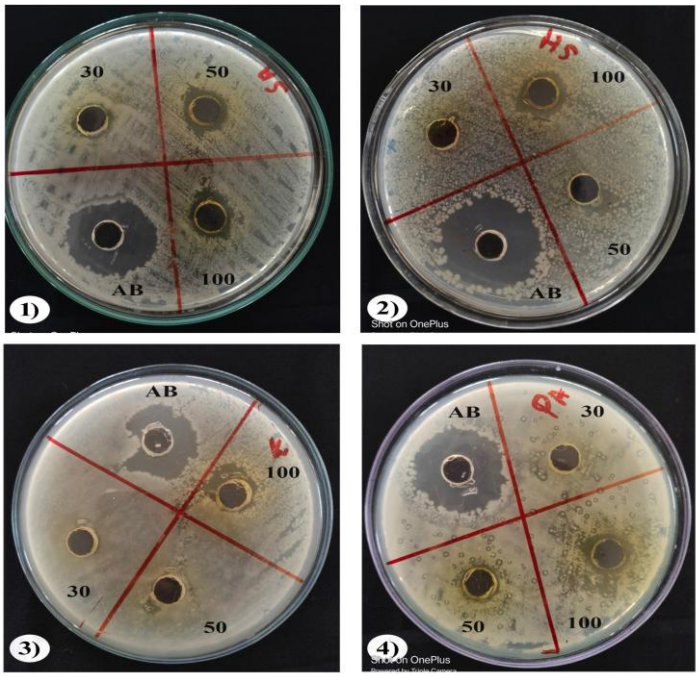

Figure 6 Antibacterial activity ME-CSFP by Agar well diffusion method 1) *Staphylococcus aureus,* 2) *Bacillus subtilis,* 3) *Escherichia coli* and 4) *Klebsiella pneumoniae*

MIC concentration assay

The MIC of orange peel extract demonstrated significant growth inhibition against the test organisms. The minimum inhibitory concentration was determined to be 31.2 µg/ml for *S. aureus*, 62.5 µg/ml for *B. subtilis*, and 250 µg/ml for both *E. coli* and *K. pneumoniae* (Figure 7). This study demonstrated that orange peel extract exhibited superior inhibitory efficacy against Gram-positive bacteria compared to Gram-negative bacteria. Orange peels contain essential oils and various

phytochemicals that readily penetrate and interact with the cell membranes of gram-positive bacteria, resulting in membrane destabilization and oxidative stress, thereby inhibiting bacterial growth. In contrast, gram-negative bacteria, which possess a robust lipopolysaccharide layer and active porin channels, restrict the entry of hydrophobic chemical molecules found in orange peel extract, leading to gradual damage at elevated concentrations (**Dhiman et al., 2012; Oikeh et al., 2020**). However, further studies are required to isolate and characterize the specific bioactive compounds responsible for the observed antibacterial activity.

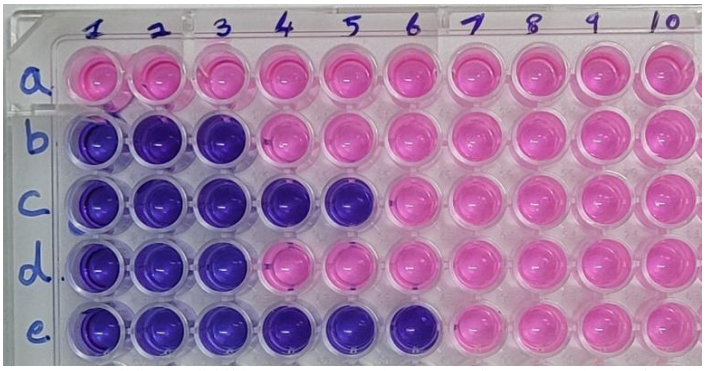

Figure 7 Minimum inhibitory concentration assay by Resazurin method in microtiter plate. Blue colour indicates absence of bacterial growth and pink colour indicates presence of bacterial growth. The change in colour is due to variation in pH caused by bacterial growth. (a) negative control without extract, (b) *E. coli*, (c) *B. subtilis*, (d) *K. pneumoniae*, (e) *S. aureus*.

Table 7 Antibacterial activity of the ME-CSFP

S.No	Microorganism	Different concentration (µg/mL) Zone length(mm)			
		AB	30	50	100
1.	*S. aureus*	10.67±1.53^{a}	5.0±1.0^{c}	6.67±1.53^{bc}	10.0±1.0^{a}
2.	*B. subtilis*	13.67±1.53^{a}	6.0±1.0^{c}	8.0±1.0^{b}	9.0±1.2^{b}
3.	*E. coli*	9.65±1.53^{a}	4.33±0.58^{c}	5.0±0.50^{bc}	6.67±2.52^{b}
4.	*K. pneumoniae*	12.0±1.0^{a}	5.0±1.0^{c}	5.67±1.53^{bc}	6.67±1.53^{b}

Values are expressed as mean ± SD (n = 3). Means within the same row followed by different superscript letters are significantly different at $P < 0.05$ according to Duncan's Multiple Range Test after one-way ANOVA.

CONCLUSION

The present study demonstrates that methanol extract of *Citrus sinensis* peel is a promising source of bioactive compounds with significant antibacterial potential. GC–MS analysis identified sixteen phytochemicals, many of which exhibited favorable drug-likeness properties. Computational studies, including ADMET prediction and molecular docking against bacterial Gyrase B, highlighted key compounds such as (+)-limonene and 2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one with strong binding affinities. These *in silico* results were corroborated by *in vitro* antibacterial assays, confirming their biological relevance. Overall, this study underscores the effective integration of computational and experimental approaches in identifying potential antibacterial agents and supports the valorization of *Citrus sinensis* peel as a sustainable candidate for future drug development.

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