Journal of Chemical Health Risks

www.jchr.org

JCHR (2025) 15(5), 731-745 | ISSN:2251-6727

Isolation, Characterization and Antimicrobial Assessment of Apple-Derived Polyphenols with Molecular Docking Insights

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(Received: 16 July 2025 Revised: 20 August 2025 Accepted: 02 September 2025)

KEYWORDS

Polyphenols, Flavonoids, Antimicrobial activity, LC-MS, Molecular docking, DNA gyrase, Procyanidins

ABSTRACT:

Introduction

Polyphenols are a diverse class of phytochemicals widely distributed in fruits and vegetables, valued for their antioxidant, anti-inflammatory, and antimicrobial properties. Apples are particularly rich in flavonoids and phenolic acids, making them a promising source for natural antimicrobial agents. In light of rising antibiotic resistance, plant-derived compounds like polyphenols offer potential alternatives to synthetic drugs. This study focused on the extraction, identification, and evaluation of polyphenolic compounds from apples, with an emphasis on their antimicrobial activity against common pathogens.

Materials and Methods

Methanolic extraction of fresh apple samples was conducted using reflux condensation to obtain a crude extract (ME-A). Compound separation was performed via thin-layer chromatography (TLC), and bioactive constituents were identified using liquid chromatography–mass spectrometry (LC-MS). Total phenolic and flavonoid contents were quantified as 0.66 mg GAE/g and 1.976 mg QE/g, respectively. Molecular docking studies using Discovery Studio software assessed interactions between key polyphenols and bacterial DNA gyrase (PDB ID: 3G75). Antimicrobial efficacy of ME-A was tested through agar diffusion against Staphylococcus aureus, Bacillus subtilis, Escherichia coli, Pseudomonas aeruginosa, and Candida albicans.

Results and Discussion

LC-MS analysis identified major polyphenols, including protocatechuic acid, vanillic acid, and procyanidins B1–B3. Docking studies showed strong binding affinities of procyanidins and quercetin-3-O-glucoside to bacterial DNA gyrase, surpassing ciprofloxacin. In vitro antimicrobial testing revealed significant zones of inhibition for S. aureus (27 mm) and B. subtilis (23 mm) at 6.25% ME-A, indicating potent Gram-positive activity. Minimal effects were observed against E. coli and P. aeruginosa, with no antifungal activity detected.

Conclusion

The findings confirm that apples are a valuable source of antimicrobial polyphenols, especially effective against Gram-positive bacteria. The in silico and in vitro results support their potential as natural therapeutic agents. These bioactive compounds demonstrated stronger binding affinity to bacterial targets than standard antibiotics, highlighting their mechanistic relevance.

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JCHR (2025) 15(5), 731-745 | ISSN:2251-6727

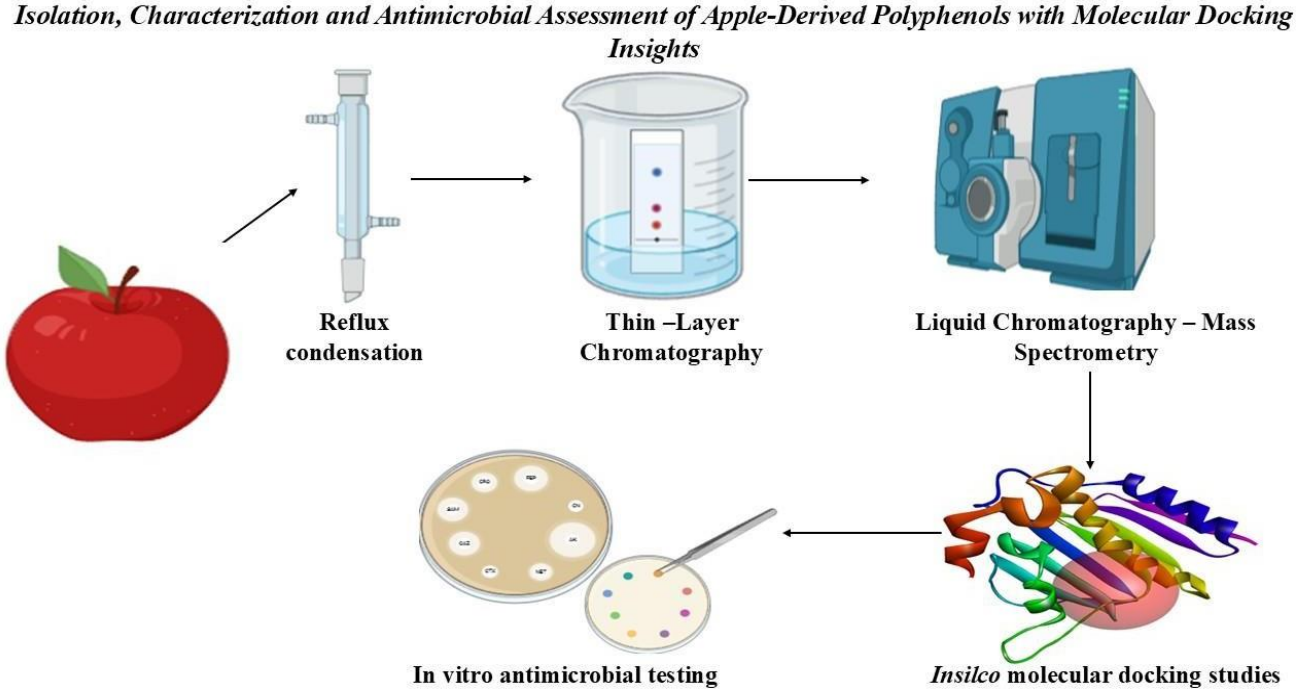

Fig-1: Graphical Abstract

INTRODUCTION

Apple (Malus domestica Borkh.), belonging to the Rosaceae family, is one of the most widely consumed and cultivated fruits globally. It is highly valued for its nutritional content, medicinal properties, and pleasant taste. Apples are rich in polyphenols, including flavonoids like quercetin and phenolic acids such as chlorogenic and caffeic acid. These compounds exhibit strong antioxidant, anti-inflammatory, cardioprotective, and antimicrobial activities (Boyer & Liu, 2004; Hyson, 2011). Grown mainly in temperate regions, major producers include China, the United States, and Poland (FAOSTAT, 2022). Apples are composed of around 84% water, carbohydrates, fibre, vitamin C, and phytochemicals mainly found in the peel (Wolfe et al., 2003). Their polyphenolic content has attracted attention for pharmaceutical and nutraceutical uses, especially for natural antimicrobial therapies.

Fig-2: *Malus domestica* plant bearing apples

2. EXPERIMENTATION

2.1. Experimental Phytochemistry

Materials required

Fresh apple samples (or apple pomace), methanol (HPLC or analytical grade), distilled water, hydrochloric acid (HCl) or formic acid (for acidification), Whatman no. 1 filter paper, rotary evaporator, vortex mixer or shaker, centrifuge (optional), mortar and pestle or grinder and amber bottles (for storage).

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Methodology of Extraction

The sample preparation began by thoroughly washing fresh apples, followed by optional peeling, chopping, and drying at 40–50°C in a hot air oven or freeze-dryer. The dried or fresh samples were then ground into a fine powder using a grinder or mortar and pestle and stored in an airtight container or desiccator until use. For extraction, 80% methanol (v/v) was prepared in distilled water and acidified to pH 2–3 using HCl or formic acid to enhance the recovery of bound phenolics and anthocyanins. Approximately 5 g of apple powder was mixed with 50 mL of acidified methanol in a 100 mL conical flask, gently shaken, and incubated on an orbital shaker or water bath at room temperature (20–25°C) or 40°C for 1–2 hours, protected from light using aluminium foil due to the light sensitivity of polyphenols. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was collected in an amber container; centrifugation at 5000 rpm for 10 minutes was optionally performed for further clarification. The methanolic extract was then concentrated using a rotary evaporator at 40°C under reduced pressure to yield the methanolic extract of apple (ME-A), which could be re-dissolved in methanol if needed. Finally, the extract was stored at –20°C in amber vials to prevent degradation and preserve phytochemical integrity [5, 6].

Phytochemical Screening

Phytochemical screening of polyphenolic compounds in plant extracts is typically performed using various qualitative confirmatory chemical tests. These tests help identify the presence of major classes of phytochemicals such as flavonoids and phenolic acids and others based on characteristic colour changes or precipitate formation [7-9].

Isolation of Polyphenolic compounds

Thin Layer Chromatography (TLC) was employed for the analysis of flavonoids and phenolic acids using silica gel 60 F_{254} precoated plates as the stationary phase. Various mobile phase systems were used depending on the polarity of the flavonoids: Ethyl acetate: Formic acid: Glacial acetic acid: Water (100:11:11:27) for general flavonoid separation; Butanol: Acetic acid: Water (4:1:5) for flavones, flavanols, and glycosides; and Toluene: Ethyl acetate: Formic acid (5:4:1) for polar flavonoids, using the upper layer [10].

Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) of the apple extract was determined using the Folin–Ciocalteu reagent method. In this assay, 0.5 mL of methanolic extract was mixed with 2.5 mL of 10% Folin–Ciocalteu reagent and incubated for 5 minutes, followed by the addition of 2.0 mL of 7.5% Na_2CO_3. The mixture was kept in the dark for 30 minutes, and absorbance was measured at 765 nm using a UV-Vis spectrophotometer [11, 12].

Determination of Total Flavonoids Content (TFC)

Principle: Flavonoids form a yellow complex with aluminum chloride ($AlCl_3$) in the presence of potassium acetate or sodium nitrite, which can be measured spectrophotometrically at around 415 nm [11, 12].

Isolation of bioactive molecule flavonoids and phenolics by LCMS

Liquid Chromatography-Mass Spectrometry (LC-MS or LCMS) is a powerful analytical technique used to identify and quantify compounds in complex mixtures. The polyphenols present in ME-A is quantified by LC-MS.

2.2. Computational Chemistry

Computational chemistry is a branch of chemistry that uses computer simulation to assist in solving chemical problems. It uses methods of theoretical chemistry, incorporated into efficient computer programs, to calculate the structures and properties of molecules and solids [13].

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. The aim of the molecular docking to achieve an optimized conformation for both the protein and the ligand and to achieve relative orientation between protein and ligand such that free energy of overall system is minimized [13].

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JCHR (2025) 15(5), 731-745 | ISSN:2251-6727

Materials and Methods

Purpose: Computational Analysis.

Target Protein: Bacterial DNA gyrase (Topoisomerase-II).

PDB Code: 3G75.

Software used: Discovery Studio Client.

Methodology

Structure of bacterial DNA gyrase: 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.

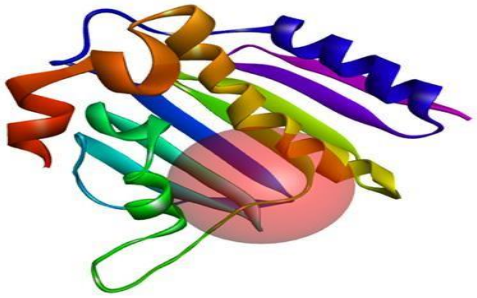

Fig-3: Structure of bacterial DNA gyrase

2.3. Experimental Microbiology

Requirements

The in vitro evaluation of antimicrobial activity involves the use of specific media and microbial cultures. For antibacterial testing, Mueller-Hinton Agar (MHA) plates are used along with bacterial strains such as *Staphylococcus aureus* (MTCC 96), *Bacillus subtilis* (MTCC 1133), and *Escherichia coli* (MTCC 452), all obtained from the Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. Whatman No. 1 filter paper discs (5 mm) are used for disc diffusion, with Dimethyl Sulfoxide (DMSO) serving as the solvent control, and Ciprofloxacin (2 mg/ml) as the standard antibacterial agent, with 10 µl loaded per disc. For antifungal activity, Sabouraud Dextrose Agar (SDA) plates are employed using *Aspergillus niger* (MTCC 281) as the test organism. Similar filter paper discs are used, with DMSO as the solvent control and Amphotericin B (10 mg/ml) as the standard antifungal agent, also loaded at 10 µl per disc.

Methodology

Nutrient agar and Sabouraud dextrose agar media were prepared using standard compositions for bacterial and fungal growth, respectively, and sterilized by autoclaving at 121°C for 15 minutes. Antibacterial activity was evaluated using the Kirby-Bauer disc diffusion method on MHA plates inoculated with *Staphylococcus aureus*, and antifungal activity was assessed similarly on SDA plates inoculated with *Aspergillus niger*. Discs were loaded with test samples (10 µl, 0–100% concentration), with Ciprofloxacin (3 µg) and Amphotericin B (100 µg) serving as positive controls, and DMSO as the vehicle control. Plates were incubated at 37°C for 24 hours (bacteria) and 48 hours (fungi). Zones of inhibition were measured to determine antimicrobial efficacy [13-16].

3. RESULTS AND DISCUSSION

3.1. Phytochemical Screening

Preliminary phytochemical screening was conducted to identify the presence of reducing sugars, pentoses, disaccharides, polysaccharides, proteins and amino acids, polyphenols, carotenoids etc. The methanolic extract of orange peel (ME-A) revealed the presence of various bioactive phytoconstituents, including carbohydrates, phytosterols, oil and fats, proteins and amino acids and polyphenols.

Table-1: Phytoconstituents present in ME-A

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	+

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11	Cardiac glycoside	-
12.	Anthocyanidin	-

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

2. 2. Isolation of flavonoids and phenolic compounds

Table-2: R_f value of unknow constituents

Standard Flavanoids	R_f Range	Observed value	Phytoconstituents present in ME-A
Quercetin	0.20 – 0.60	0.26	Flavonoids
		0.19	Flavonoids
Gallic acid	0.15 – 0.20	0.24	Phenolic compounds
		0.85	Phenolic compounds
		0.55	Phenolic compounds
		0.74	Phenolic compounds

Summary and findings

The findings confirm the presence of bioactive phytochemicals in the extract. Rf values at 0.19 and 0.26 indicate the presence of quercetin-like flavonoids, aligning well with the standard Rf range of quercetin (0.20–0.60), thus supporting its identification. Additionally, the detection of multiple Rf values such as 0.24, 0.55, 0.74, and 0.85 suggests the presence of various phenolic compounds. While gallic acid typically appears in the range of 0.15–0.20, the observed values indicate the potential presence of other phenolics like caffeic acid, ferulic acid, or structurally related compounds, highlighting the extract's rich polyphenolic profile.

2. 3. Determination of total phenolic content (TPC) in ME-A

Table-3: Absorbance Standard phenolic compound (gallic acid)

Sl. No	Concentration Gallic acid (µg/ml)	Absorbance
1.	0	0.00
2.	10	0.151
3.	20	0.168
4.	40	0.182
5.	60	0.212
6.	80	0.230
7.	100	0.298
8.	10 (ME-A)	0.131

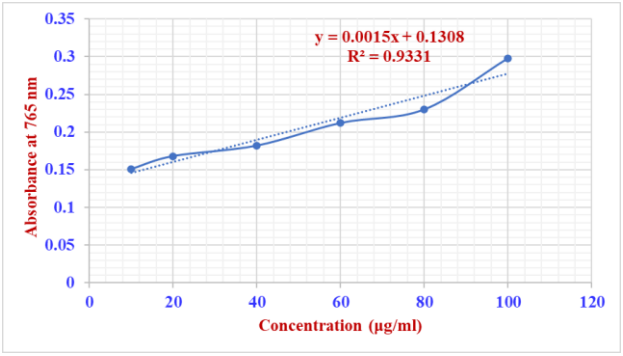

Fig-4: Standard graph of gallic acid

2. 4. Determination of Total Flavonoids Content (TFC) in ME-A

Table-4: Absorbance Standard phenolic compound (Quercetin)

Sl. No	Concentration Gallic acid (µg/ml)	Absorbance
1.	0	0.00
2.	10	0.142
3.	20	0.155
4.	40	0.169
5.	60	0.188
6.	80	0.212
7.	100	0.231
8.	10 (ME-A)	0.116

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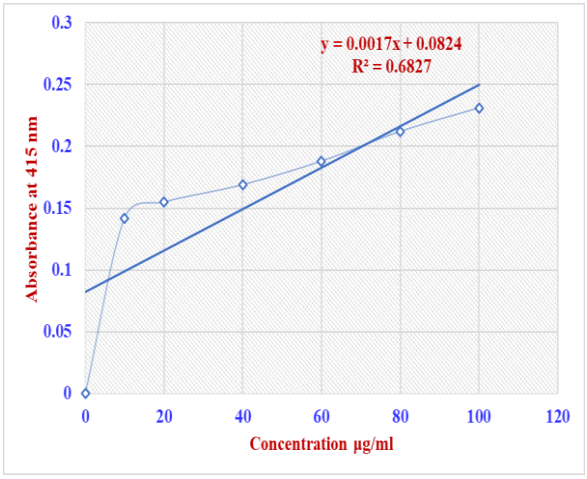

Fig-5: Standard graph of Quercetin

2.5. Interpretation of LCMS data and Characterization of phenolic and flavonoid compounds in ME-A.

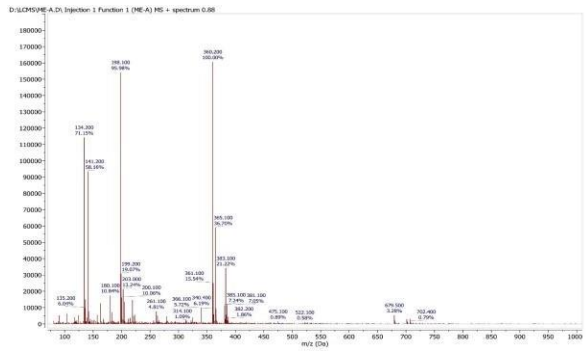

Fig-6: LCMS of ME-A

Table-5: LC-MS Peaks of ME-A and Fragmentation

m/z (Observed)	Ion Type	Neutral Mass (M)	Molecular Formula (Possible)	Probable Compound	Fragmentation
134.2	$[M+H]^+$	~133.2	$C_7H_6O_3$	Protocatechuic acid	Loss of H_2O (–18 Da) or OH group from parent ion
141.2	$[M+H]^+$	140.2	$C_7H_8O_3$	Vanillic acid	Phenolic compound, $[M+H]^+$ ion
198.1	$[M+H]^+$	197.1 Da	$C_9H_8O_4$ (neutral) or $C_{10}H_{12}O_4^+$ ($[M+H]^+$)	Caffeic acid	- Loss of H_2O (−18 Da) → m/z 180.1
199.2	$[M+H]^+$		198.2 $C_{11}H_{14}NO_3$	Ferulic acid, caffeic acid methyl ester	Aromatic acid; fragmentation may involve CO_2 or H_2O loss
361.1	$[M+H]^+$	360.1 Da	$C_{18}H_{18}O_8$	Procyanidin B2	- Retro-Diels–Alder (RDA) cleavage
360.2	$[M+H]^+$	359.2 Da	$C_{18}H_{18}O7$	Procyanidin B1	-Loss of H_2O (−18 Da), - Loss of a galloyl group (−152 Da)
365.1	$[M+H]^+$	364.1 Da	$C_{18}H_{18}O_8$	Procyanidin B1	Loss of H_2O (−18 Da) - Loss of a galloyl group (−152 Da) - Catechin or epicatechin monomer release (−288 Da)
383.1	$[M+H]^+$	382.1 Da	$C_{19}H_{20}O_8$	Quercetin-3-O-glucoside	Loss of glucose (−162 Da) → m/z 221.1 - Loss of H_2O (−18 Da) → m/z 365.1 - Further loss of H_2O or glucose, producing ions at m/z 179.1, 151.1, etc.

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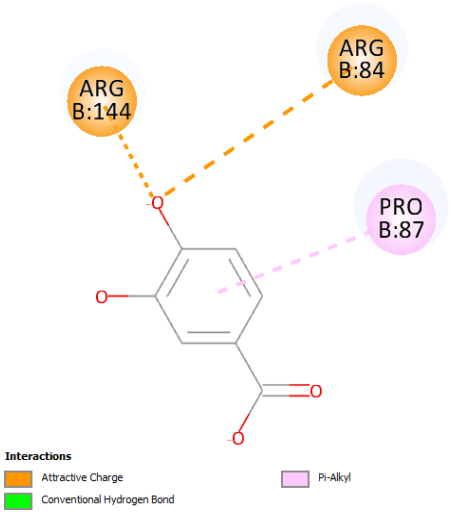
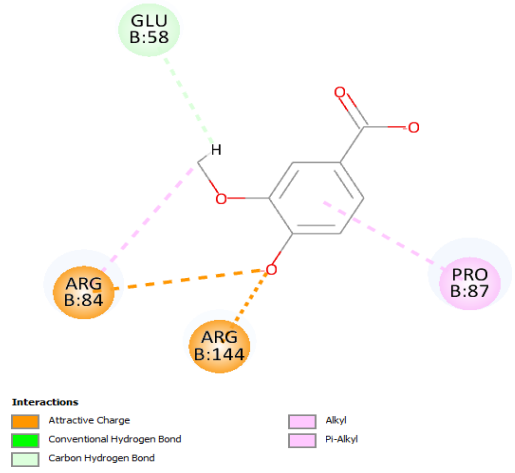
Table-6: Docking results analysis

Sl. No.	Name of the compounds	Cdocker Energy (K. Cal/mol)	Cdocker Interaction Energy (K. Cal/mol)	Binding Energy (K. Cal/mol)
1.	Protocatechuic acid	37.6785	34.3245	-225.958
2.	Vanillic acid	28.2384	36.7449	-238.617
3.	Caffeic acid	31.8659	34.289	-168.905
4.	Ferulic acid	29.0108	32.7679	-154.778
5.	Procyanidin B2	44.3688	60.913	-368.436
6.	Procyanidin B1	39.447	52.1554	-371.93
7.	Procyanidin B3	38.7011	49.647	-340.198
8.	Quercetin-3-o-glucoside	7.05197	48.2284	-314.554
9.	Ciprofloxacin	23.3649	49.2324	-288.719

Table-7: 2D interaction study, 3D structures and 3D ligand-receptor complex with Bacterial DNA gyrase (3G75)

Protocatechuic acid	Vanilic acid
	

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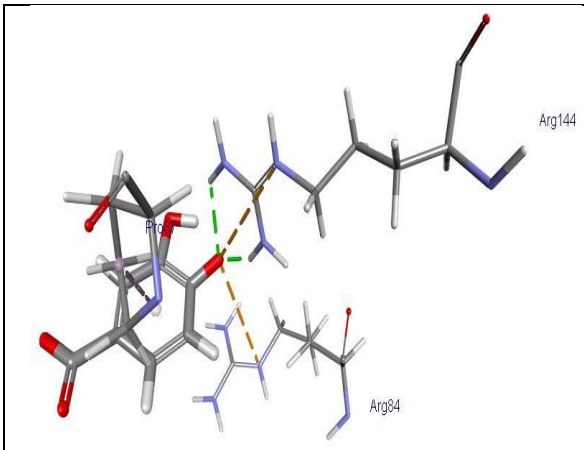
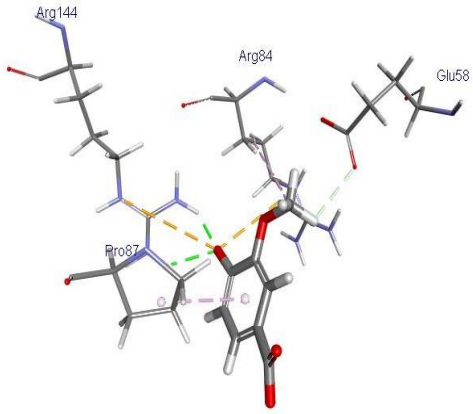
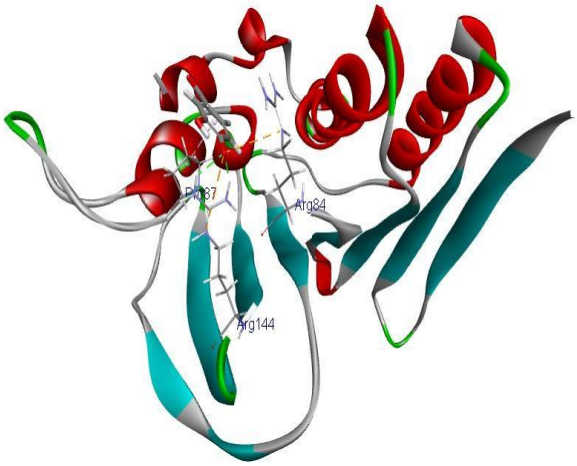
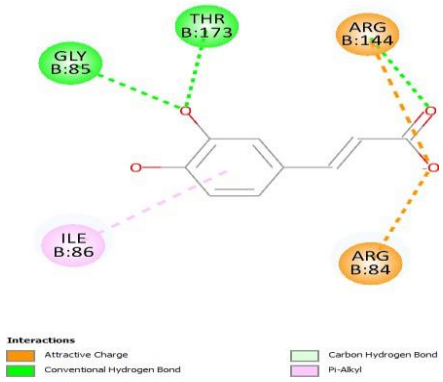


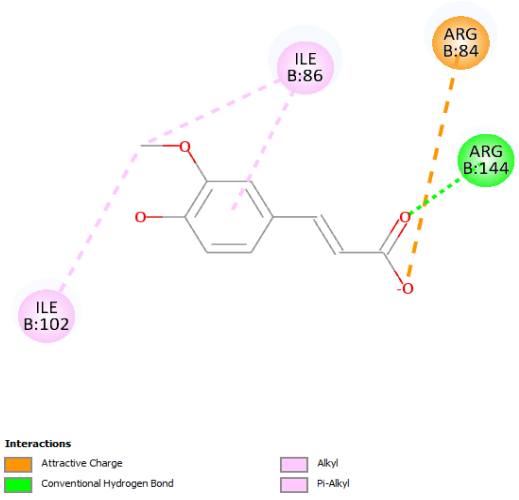


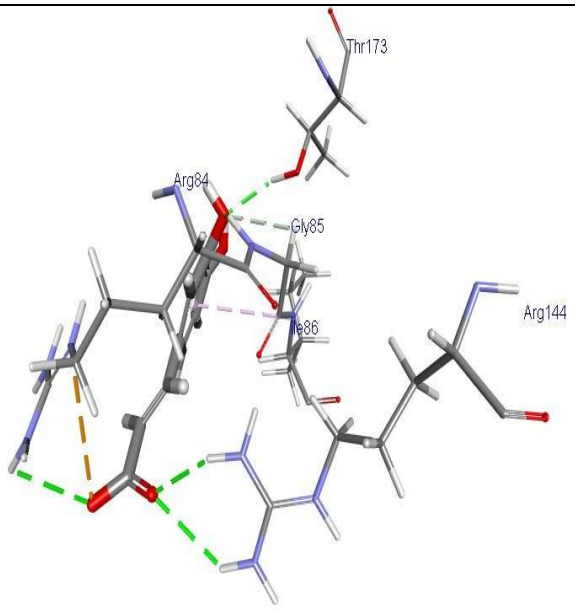


Caffeic acid



Ferulic acid





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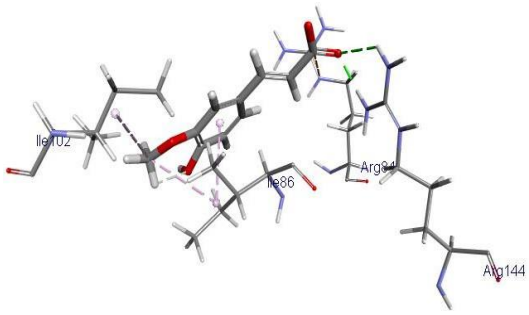
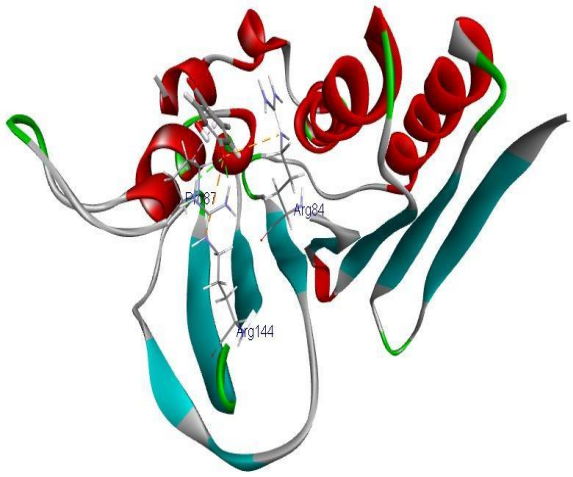
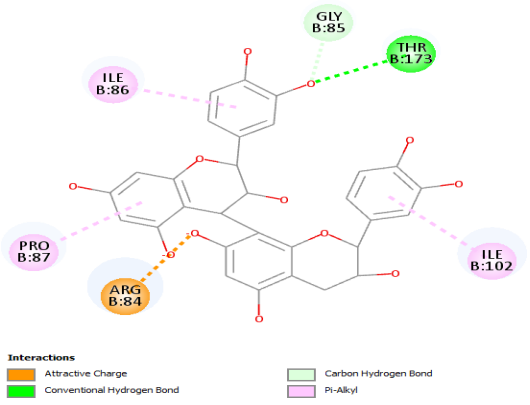
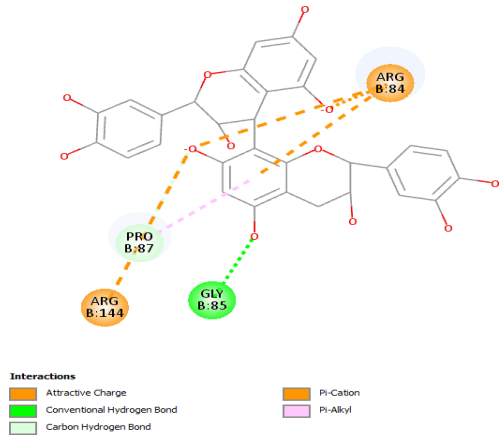


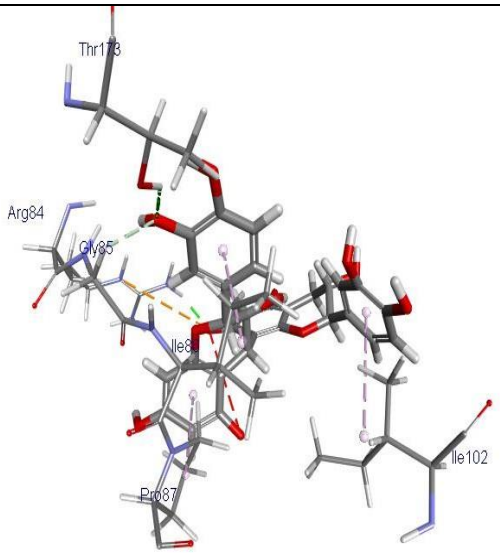


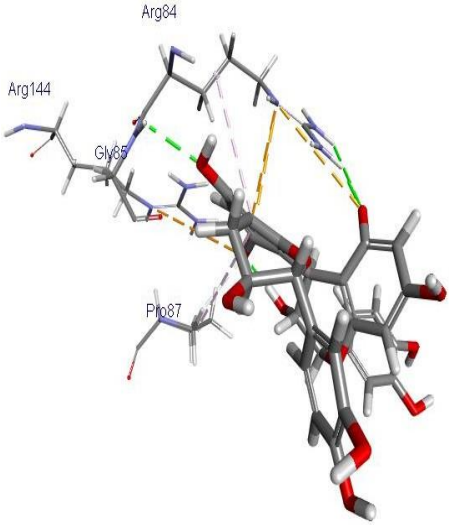
Procyanidin B2



Procyanidin B3







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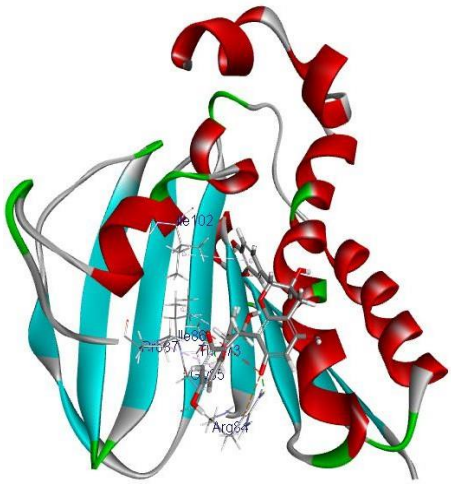
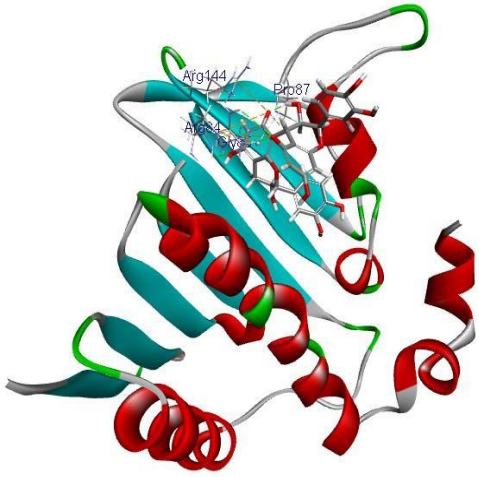
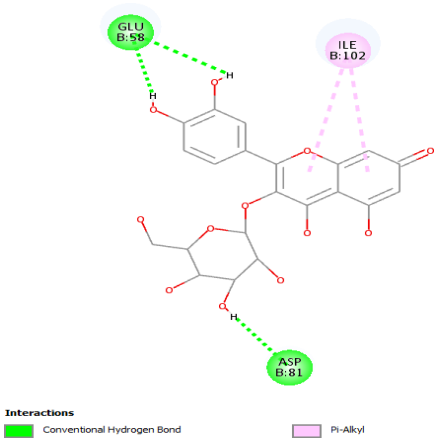
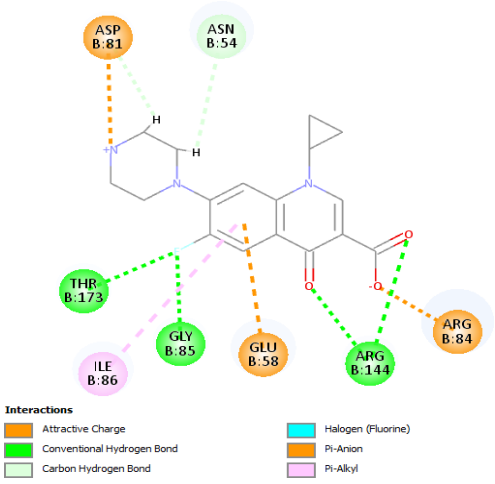


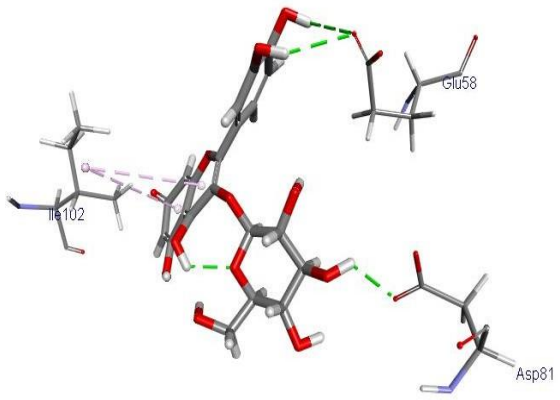


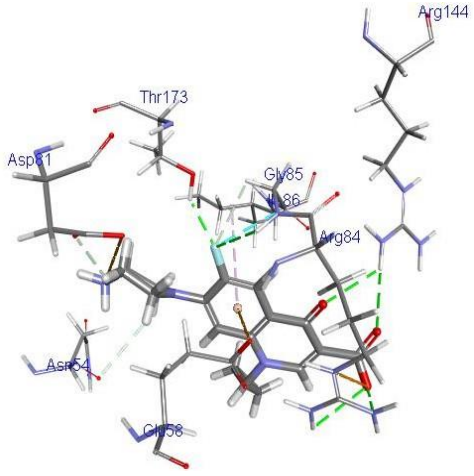
Quercetin-3-o-glucoside



Ciprofloxacin







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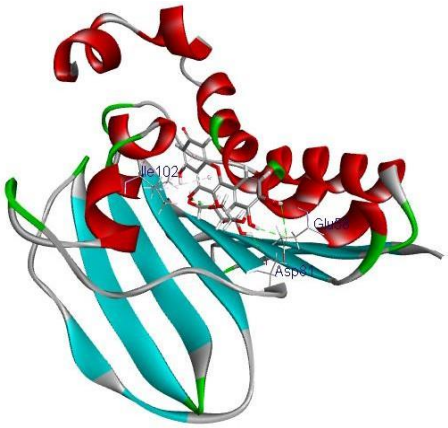
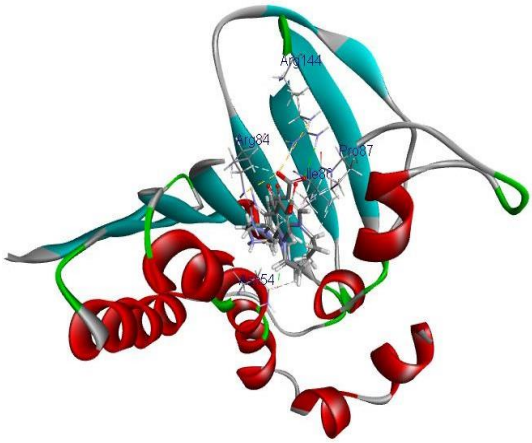
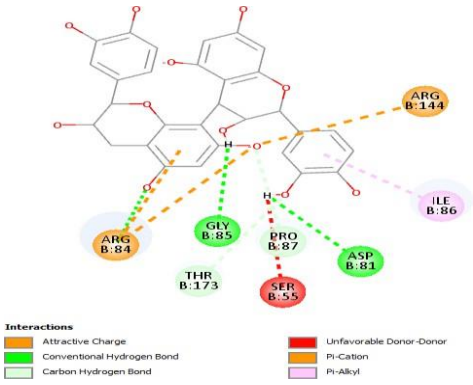
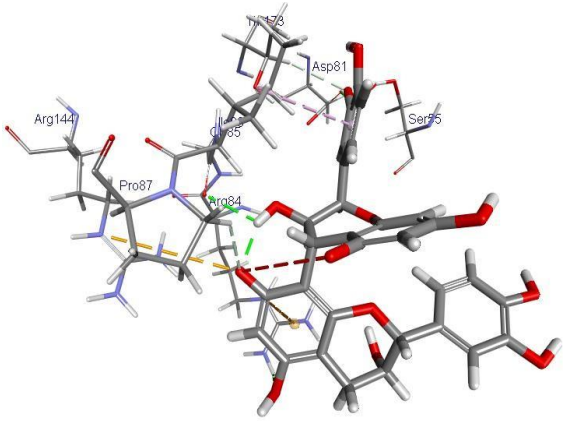


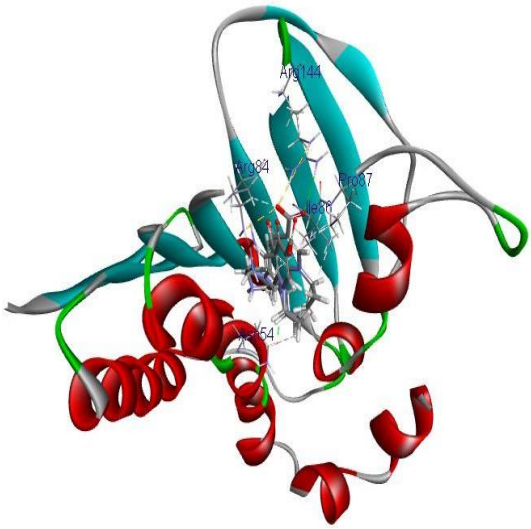


Procyanidin B1 (2D)



Procyanidin B1 (3D)





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Summary Findings

The molecular docking analysis highlights Procyanidin B1 as the top performer, exhibiting the strongest binding affinity and most favourable interaction among all tested compounds, followed closely by Procyanidin B2, B3, and Quercetin-3-O-glucoside. These polyphenolic compounds not only demonstrated superior binding compared to simple phenolic acids but also outperformed the standard antibiotic, ciprofloxacin, indicating their potential as potent bioactive agents. Among the simple phenolic acids, vanillic acid showed the most promising interaction, while ferulic acid had the weakest binding and lowest stability. The presence of multiple hydroxyl groups and complex structures in procyanidins and quercetin derivatives likely facilitates stronger multivalent binding, enhancing receptor affinity. Quercetin-3-O-glucoside, despite low docker energy, formed a stable and efficient complex, possibly aided by its sugar moiety. Overall, the findings suggest that polyphenolic structure plays a critical role in improving binding strength and biological potential.

2.6. Preliminary screening of antimicrobial activity

Table-8: Antibacterial Activity of ME-A against *S. aureus*

Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC:0.03)	23	23	23	0	0	0
6.25	27	27	27	27	0	0
12.50	28	28	28	28	0	0
25	29	29	29	29	0	0
50	31	30	30	30.333	0.577	0.333
100	32	32	32	32	0	0

Table-9: Antibacterial Activity of ME-A against *E. coli*

Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC)	24	24	25	24.333	0.577	0.333
6.25	6	6	7	6.333	0.576	0.333
12.50	8	8	8	8	0	0
25	11	12	13	12	1	0.577
50	14	14	15	14.333	0.577	0.333
100	16	16	16	16	0	0

Table-10: Antibacterial Activity of ME-A against *P. aeruginosa*

Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
CF (PC)	25	25	25	25	0	0
6.25	9	8	9	8.666	0.577	0.333

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12.50	12	11	12	11.666	0.577	0.333
25	14	13	14	13.666	0.577	0.333
50	16	15	15	15.333	0.577	0.333
100	19	18	19	18.666	0.577	0.333

Table-11: Antifungal Activity of ME-A against *A. niger*

Amount (%/disc)	Plate A	Plate B	Plate C	Average	SD	SEM
Amph-B (PC)	18	17	17	17.333	0.577	0.4082
6.25	0	0	0	0	0	0
12.50	0	0	0	0	0	0
25	0	0	0	0	0	0
50	0	0	0	0	0	0
100	0	0	0	0	0	0

Table-12: 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-A	*S. aureus*	6.25%	27.00
CF (Std.)	*B. subtilis*	0.03%	23.00
ME-A	*B. subtilis*	6.25%	23.00
CF (Std.)	*E. coli*	0.03%	24.66
ME-A	*E. coli*	6.25%	6.33
CF (Std.)	*P. aeruginosa*	0.03%	25
ME-OP	*P. aeruginosa*	6.25%	8.66
Amphotericin B	*A. niger*	0.03%	17.66
ME-A	*A. niger*	-	-

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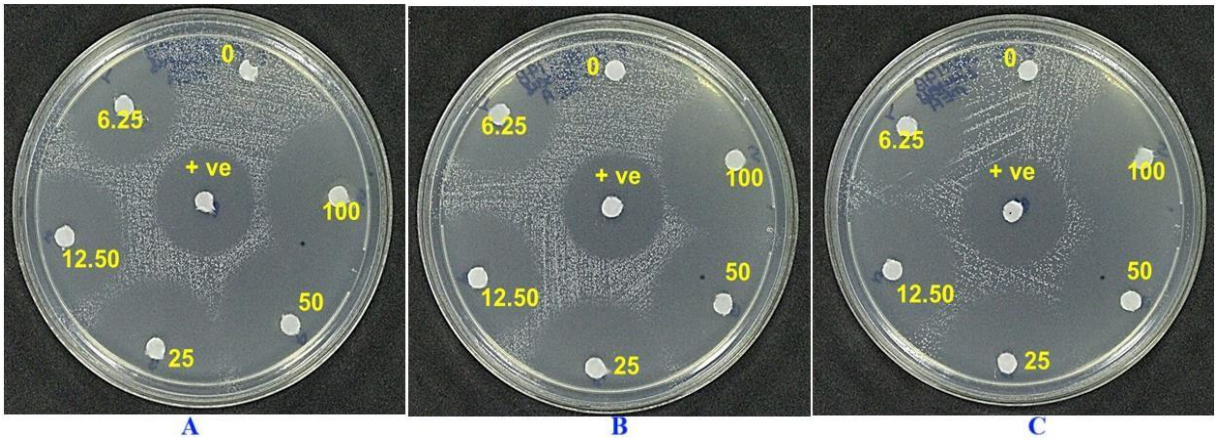

Fig-7-A: Zone of Inhibition of ME-A against *S. aureus* at concentration present per disc in percentage (%).

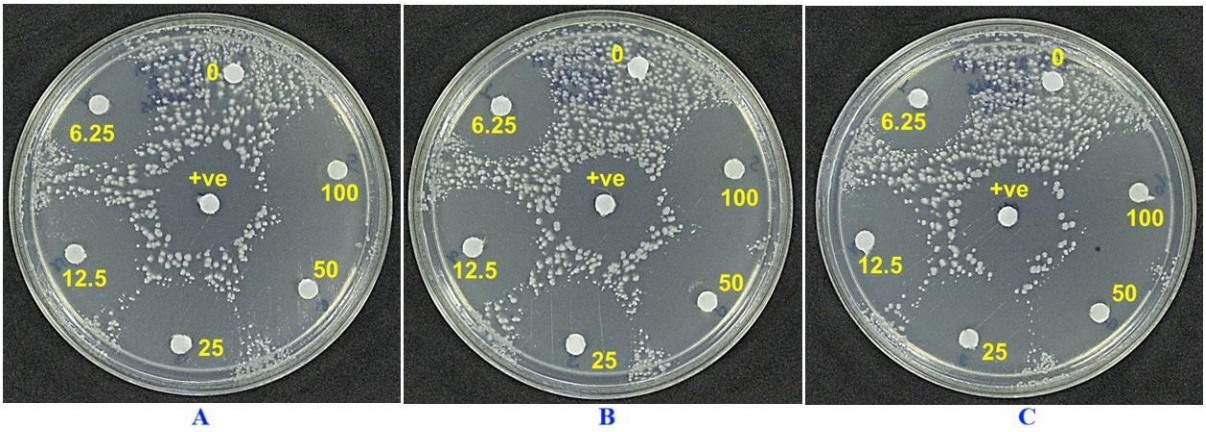

Fig-7-B: Zone of Inhibition of ME-A against *B. subtilis* at concentration present per disc in percentage (%)

Summary and findings

The methanolic extract of orange peel (ME-A) demonstrated selective antimicrobial activity, showing strong efficacy against Gram-positive bacteria, particularly *Staphylococcus aureus*, where it outperformed ciprofloxacin. It exhibited equal activity against *Bacillus subtilis* but was significantly less effective against Gram-negative strains like *E. coli* and *Pseudomonas aeruginosa*, likely due to their protective outer membrane. ME-A showed no detectable antifungal activity against *Aspergillus niger*. Overall, ME-A required higher concentrations to exhibit activity compared to standard drugs, suggesting moderate potency. The antimicrobial effects are attributed to the presence of flavonoids and phenolic compounds identified in the extract.

4. CONCLUSION

The methanolic extract of apple (ME-A) contains a diverse range of bioactive phytochemicals, including flavonoids, phenolics, carbohydrates, and steroids, with major constituents like quercetin derivatives, caffeic acid, ferulic acid, and procyanidins. TLC and LC-MS analyses confirmed these compounds, supported by Rf values and mass fragmentation data. Quantitative analysis revealed significant antioxidant content with 0.66 mg GAE/g of phenolics and 1.976 mg QE/g of flavonoids. Molecular docking highlighted strong binding affinities of Procyanidin B1–B3 and quercetin-3-O-glucoside, outperforming ciprofloxacin. ME-A exhibited potent antibacterial activity against Gram-positive bacteria but was less effective against Gram-negative strains and fungi. These findings suggest its potential for development as a natural antimicrobial agent.

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Future Perspective

Future work should explore advanced extraction techniques and assess the in vivo efficacy of apple polyphenols. Studies on synergistic effects with existing antibiotics could enhance antimicrobial outcomes. Additionally, incorporating apple-derived compounds into pharmaceutical and food-grade applications could provide sustainable and safe alternatives to synthetic antimicrobials.

Acknowledgement

We would like to thank Aakaar Biotechnologies Pvt. Ltd., Lucknow, for conducting the antimicrobial activity testing, which was crucial to this research. We are also grateful to the Head, Department of Chemical Sciences, Tezpur University, for providing the facilities for LC-MS analysis.

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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