EXTRACTION, ISOLATION AND SEMI SYNTHESIS OF HESPERIDIN AS POTENT ANTIOXIDANT AND ANTIMICROBIAL AGENT

Kajal Tiwari[1*] and Abhinav Prasoon Mishra[2]

1. Student, department of Pharmaceutical Chemistry, Advance Institute of Bio Tech and Paramedical Sciences Kanpur, U.P
2. Professor, department of Pharmaceutical Chemistry, Advance Institute of Bio Tech and Paramedical Sciences Kanpur, U.P

ABSTRACT

The present study was undertaken to isolate hesperidin from peels of Citrus aurantium and evaluate the antioxidant and antimicrobial potential of hesperidin, hesperetin, and its brominated derivative. Pharmacognostical evaluation of the peels included macroscopic, microscopic, and physicochemical characterization according to WHO guidelines. Successive solvent extraction was performed using n-hexane, petroleum ether, chloroform, ethanol, and water. Hesperidin was isolated through methanolic extraction followed by acid precipitation and purified by recrystallization. The isolated compound was identified by melting point determination, TLC analysis, and solubility studies. Semi-synthesis of hesperetin and bromo-hesperetin derivative was carried out using acid hydrolysis and bromination reactions, respectively. Phytochemical screening confirmed the presence of flavonoids, terpenoids, tannins, carbohydrates, and alkaloids in different extracts. In vitro antioxidant activity was evaluated using DPPH, ABTS, FRAP, and hydrogen peroxide scavenging assays. Among all tested compounds, the bromo-hesperetin derivative exhibited the highest antioxidant activity, followed by hesperetin and hesperidin. Antimicrobial studies against Gram-positive, Gram-negative bacteria, and fungal strains also demonstrated superior activity of the brominated derivative. The study concludes that structural modification of hesperidin significantly enhances its biological activity and supports its potential as a promising natural therapeutic agent.

Keywords: *Citrus aurantium, Hesperidin, Hesperetin, Antioxidant activity.*

1. Introduction to Natural Products

1.1.1 Importance of Natural Products in Drug Discovery

Natural products have played a pivotal role in the field of drug discovery right from the early days of pharmaceutical research. These compounds obtained from plants, animals, marine life, and microbes have made contributions to over 50% of drugs approved by the FDA over the last few decades. Natural products' biological relevance and structure render them highly suitable as lead compounds for drugs due to their evolutionary optimization to interact with biological systems.[1]

The importance of natural products in contemporary pharmacology cannot be overemphasized. Examples in history include quinine from the bark of cinchona trees, penicillin from penicillium mold, and paclitaxel (Taxol) from the Pacific yew tree. These discoveries have transformed the treatment of conditions such as pain, malaria, infections, and cancers shared between discoveries based on natural products such as ivermectin (from Streptomyces avermitilis) and artemisinin (from Artemisia annua).[2]

With regard to drug discovery, natural products present various benefits: natural products contain built-in biological activity thanks to millions of years of evolution, they have higher chemical diversity than artificial compound libraries, and finally, they can target new biological targets that remain undiscovered by chemists. As per Newman and Cragg (2020), about 64% of all anticancer drugs approved during the period between 1981 and 2019 were based on natural products.[3]

The natural products drug market has experienced considerable development. In total, botanical medicine, drugs obtained from natural products, and nutraceuticals form an industry worth billions of dollars. The use of innovative technologies, including combinatorial biosynthesis, metabolomics, genomics, and artificial intelligence, significantly improves the discovery of new bioactive compounds.[4]

1.1.2 Role of Medicinal Plants in Therapeutics

The use of plants in medicinal therapy has existed across almost all civilizations throughout history, including those in ancient Egypt, Greece, China, Ayurvedic and Unani medicine. According to statistics from the WHO, an estimated 80% of people across the globe depend on herbal medicines for their health needs, especially in developing countries where advanced healthcare infrastructure may be lacking.[5]

The effectiveness of medicinal plants is attributed to secondary metabolites — chemicals that do not take part in the basic growth and development of the plant but are rather produced due to certain stresses imposed by their environment, animals, and microbes.[5]

In modern medicine, the uses of medicinal plants include acting as sources of drugs (for instance, atropine is obtained from Atropa belladonna while digoxin is obtained from Digitalis purpurea), as models of synthetic drugs (such as in the case of procaine which is modeled from cocaine), as crude drugs in herbal remedies and in food and health products.[6]

The scientific investigation of medicinal plants, which entails ethnobotanical and ethnopharmacological research, has become more popular because of the realization that the collective knowledge of medicinal plants accumulated over time offers important insights into the isolation of bioactive compounds. Medicinal plants utilized for traditional healing show a much greater frequency of pharmacological validation than random plant extracts.[12]

1.1.3 Phytoconstituents as Bioactive Agents

The chemical components of plants, phytoconstituents, can be subdivided into two broad categories: primary metabolites such as carbohydrates, proteins, lipids, and chlorophylls; and secondary metabolites. The secondary metabolites, however, are the most pharmacologically interesting phytoconstituents, among which the following have been described:[13]

- Alkaloids: These are nitrogen-containing organic compounds with powerful physiological activity, such as morphine (analgesic drug), quinine (antimalarial), vincristine (anticancer), and caffeine (a stimulant).
- Terpenoids/Terpenes: This is the largest class of phytoconstituents that comprises monoterpenes, sesquiterpenes, diterpenes, triterpenes, and tetraterpenes. Some examples include menthol, artemisinin, taxol, and carotenoids.
- Flavonoids: These are polyphenolic phytoconstituents characterized by a C6-C3-C6 carbon skeleton. They exhibit anti-inflammatory, antioxidant, antimicrobial, and anticancer activity. More than 8,000 natural flavonoids have been identified to date.
- Phenolic Acids: This category includes hydroxybenzoic and hydroxycinnamic acids. They exhibit antioxidant and anti-inflammatory activities.
- Glycosides: This group is characterized by a sugar moiety combined with an aglycone (non-sugar). This category includes cardiac glycosides, saponins, and anthraquinone glycosides.
- Tannins: These are high molecular weight polyphenols having the ability to precipitate proteins. They exhibit antimicrobial, antioxidant, and astringent activity.

1.2 Citrus Plants as a Source of Bioactive Compounds

1.2.1 Overview of Citrus Species

Citrus genus belongs to the Rutaceae family and is among the most commercially valuable fruit crops across the globe. The area of origin of Citrus is considered to be Southeast Asia, followed by its wide distribution to tropical and subtropical regions.

From a taxonomic point of view, the genus Citrus exhibits complexity owing to hybridization, mutation, and polyploidy. The production of world citrus is over 120 million metric tons per year, which makes it the largest fruit crop production in the world. The climatic conditions required by these plants have facilitated their growth mainly in areas such as the Mediterranean, South and North America, South Africa, and Asia.

1.2.2 Medicinal Importance of Citrus Fruits

Medicinal importance of citrus fruits dates back several thousands of years. The use of dried tangerine peel (Chen pi) in treating gastrointestinal, respiratory, and cardiovascular disorders is a long-standing practice in traditional Chinese medicine. Likewise, the medicinal application of lemons and bitter oranges in the treatment of hepatitis, dyspepsia, and fever is well-documented by Ayurvedic practitioners.[15]

The significance of Citrus species in contemporary pharmacological studies ranges widely:

- Antioxidant activity: Citrus flavonoids such as hesperidin, naringenin, and nobiletin are effective antioxidants.
- Anti-inflammatory activity: Flavonoids suppress inflammation by inhibiting enzymes like COX, LOX, and NF-κB.
- Cardiovascular benefits: Hesperidin and other Citrus flavonoids enhance the lipid profile, mitigate arterial stiffness, and lower blood pressure.
- Anticancer activity: Limonoids, flavonoids, and essential oil constituents exert cytotoxicity against several types of cancer cells.
- Antimicrobial properties: Citrus essential oils, especially d-limonene and linalool, are effective against a wide array of bacteria and fungi.

- Neuroprotective activity: Flavonoids penetrate the blood-brain barrier and shield neurons from oxidative and inflammatory damage.

1.2.3 Phytochemical Constituents of Citrus Plants

The main phytochemical reservoir of Citrus is characterized by high diversity of biologically active substances. Flavonoids represent the most important group of Citrus phytochemicals with regard to biological activity. Flavonoid groups include flavanones (hesperidin, naringin, eriocitrin), flavones (nobiletin, tangeretin, sinensetin), and flavonols (rutin, quercetin). Peel and albedo are rich in flavonoids in comparison with the juice.[16]

Limonoids refer to highly oxygenated triterpenes unique to Citrus. Limonoids include limonin, nomilin, obacunone and their glucosides. Essential oils are represented by the aromatic volatile fraction in the peel, with d-limonene reaching up to 95% in some species. Carotenoids consisting of β-carotene, lycopene, zeaxanthin, and β-cryptoxanthin participate in orange coloration and exhibit antioxidant and vitamin A activity.[17]

1.3 Flavonoids

1.3.1 Definition and Classification of Flavonoids

Flavonoids represent an enormous and varied class of plant secondary metabolites. They perform significant biological functions in the form of providing UV protection, coloring, insect attraction, allelopathic activity, and resistance to pathogens. The name "flavonoid" is an adaptation of the Latin word "flavus", meaning yellow. All flavonoids share a common backbone comprising two aromatic rings (ring A and ring B) and a three-carbon unit forming the third ring (ring C). The classification of different types of flavonoids is based on the saturation level and oxidation state of ring C along with its orientation at the ring B junction.[18]

2. Materials and Methods

2.1 Chemicals and Reagents

Methanol, n-hexane, glacial acetic acid, sulfuric acid and silica gel were bought from the Avantor Performance Material India limited. Acetone and bromine were bought from the Central Drug House (P). Ltd., New Delhi. Distilled water was obtained from own institution.

2.2 Collection of Plant Material

Peels of *C. aurantium* were collected in the fruit market, Lucknow, U.P., India. This project was approved by HIPER/2021-22/118 and authenticated by NISCPR/RHMD/Consult/2022/4002-03.

2.3 Isolation of Crude Hesperidin

Air-dried orange peel was extracted in Soxhlet assembly with different solvents in a series of solvent extractions: n-hexane, petroleum ether, chloroform, 95% ethanol, and water.

2.4 Procedure for Isolation and Purification of Hesperidin from C. aurantium

100 g powdered air dried orange peels was extracted by Soxhlet apparatus for about 1 h with n-hexane (400 ml) on water bath. The extract was filtered while hot through a Buchner funnel and marc was dried at room temperature. The powder was extracted under refluxing for 4 h with 400 ml methanol. Filter while hot and wash the marc with 50 ml of hot methanol, add the washing to the filtrate. Concentrate the combined filtrate under reduced pressure to syrupy mass. The extract was acidified with dil. acetic acid and left for 3 to 4 h to yield crude hesperidin. Melting point was 240-242ºC. The IR, NMR and Rf value of product was found for structural conformation of compounds. The Rf value was found to be 0.61 using n-Butanol:water:Acetic acid (3:1:1) (Bharti *et al.,* 2021).

2.5 Procedure for Conversion of Hesperidin into Hesperetin

Take 9 g powder of hesperidin with methanol (250 ml) and concentrated sulphuric acid (9 ml) and stir under reflux for 8 h. The homogenous liquid extract was cooled, concentrated by evaporation, and then diluted with ethyl acetate (500 ml). The organic solution was washed with water and dried over magnesium sulphate. For purification, dissolve the crude product in a few ml of acetone and add to a vigorously stirred solution of water and acetic acid. Wash with water and cool the precipitated hesperetin in an ice bath. Purified hesperetin was obtained as a pure yellow powder with melting point 230-232ºC (Lahmer *et al.*, 2015).

2.6 Procedure for Synthesis of Hesperetin Derivatives

Take 1.0 g of hesperetin and dissolve it in 12 ml of glacial acetic acid. Carefully continue stirring in 2 ml of liquid bromine to the mixture. Allow the mixture to stand for 15 min or until the bromine colour persists. Add 80 ml of water. At the pump, remove the bromo compound using a filter before washing with a little cold water. The hesperidin derivative melting point was 140-142°C (Furniss et al., 2016).

2.7 In-vitro Pharmacological Activity

2.7.1 Antioxidant Activity

2.7.2 DPPH Radical Scavenging Assay

Prepare fresh 0.1 mM DPPH solution in methanol and prepare different concentrations of plant extract in methanol/ethanol. Take equal volumes of extract and DPPH solution and mix

properly. Incubate at room temperature in dark for 30 minutes. Prepare a control containing DPPH only and a positive control using Ascorbic acid/Trolox. Measure absorbance at 517 nm using UV–Vis spectrophotometer and calculate % DPPH inhibition.

Procedure:

Generate ABTS? radicals by mixing ABTS solution (7 mM) with potassium persulfate (2.45 mM) and incubating the mixture in the dark at room temperature for 12–16 hours. Dilute the ABTS? solution with ethanol or phosphate buffer to an absorbance of 0.70 ± 0.02 at 734 nm. Add 1 mL of extract solution (different concentrations) to 1 mL of ABTS? solution. After incubation for 6 minutes at room temperature, measure absorbance at 734 nm. Use a blank containing solvent only and a standard antioxidant for comparison.

Figure 1: DPPH Radical Scavenging Assay Procedure

2.7.3 ABTS Radical Cation Decolorization Assay

Procedure:

Prepare FRAP reagent by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 ratio. Warm the reagent to 37 °C before use. Add 100 µL of extract solution to 3 mL of FRAP reagent. Incubate at 37 °C for 30 minutes. Measure absorbance at 593 nm. Include a standard curve with $FeSO_4$ for quantification.

Figure 2: ABTS Radical Cation Decolorization Assay Procedure

2.7.4 Antimicrobial Activity

1. Agar Well Diffusion Method

The agar well diffusion method is widely used for preliminary screening of antimicrobial activity. In this technique, a suitable agar medium such as Mueller–Hinton agar is prepared and poured into sterile Petri plates, which are allowed to solidify. Test microorganisms are evenly spread over the agar surface using a sterile swab or spreader to form a uniform lawn. Wells of standard diameter (usually 6–8 mm) are punched into the agar using a sterile cork borer or pipette tip. Test samples (plant extracts, compounds, or formulations) are loaded into these wells in fixed volumes (50–100 µL). Plates are incubated at an optimum temperature (commonly 37°C for bacteria, 25–28°C for fungi) for 18–24 hours. The zone of inhibition around each well is measured in millimeters. Larger zones indicate stronger antimicrobial activity. This method is simple, cost-effective, and useful for comparing the relative potency of different samples but is limited to diffusible compounds.

Figure 3: Agar Well Diffusion Method

2. Disc Diffusion Method (Kirby–Bauer Method)

The disc diffusion method is a standard antimicrobial susceptibility test recommended by CLSI (Clinical and Laboratory Standards Institute). Sterile filter paper discs (6 mm diameter) are impregnated with a fixed concentration of the test sample and allowed to dry. A microbial suspension, standardized to 0.5 McFarland turbidity, is swabbed uniformly over the agar plate surface. The impregnated discs are placed on the inoculated agar surface at suitable distances using sterile forceps. Plates are incubated under appropriate conditions, and inhibition zones are measured. This method is easy, reproducible, and allows comparison with standard antibiotics. It is particularly useful for screening plant extracts and comparing them to reference drugs.

Figure 4: Disc Diffusion Method (Kirby-Bauer Method)

3. Results and Discussion

3.1 Synthesis Scheme

The following scheme illustrates the structural transformation from hesperidin (HA1) to hesperetin (HA2) through acid hydrolysis and further to the novel brominated derivative (HA3) through electrophilic bromination.

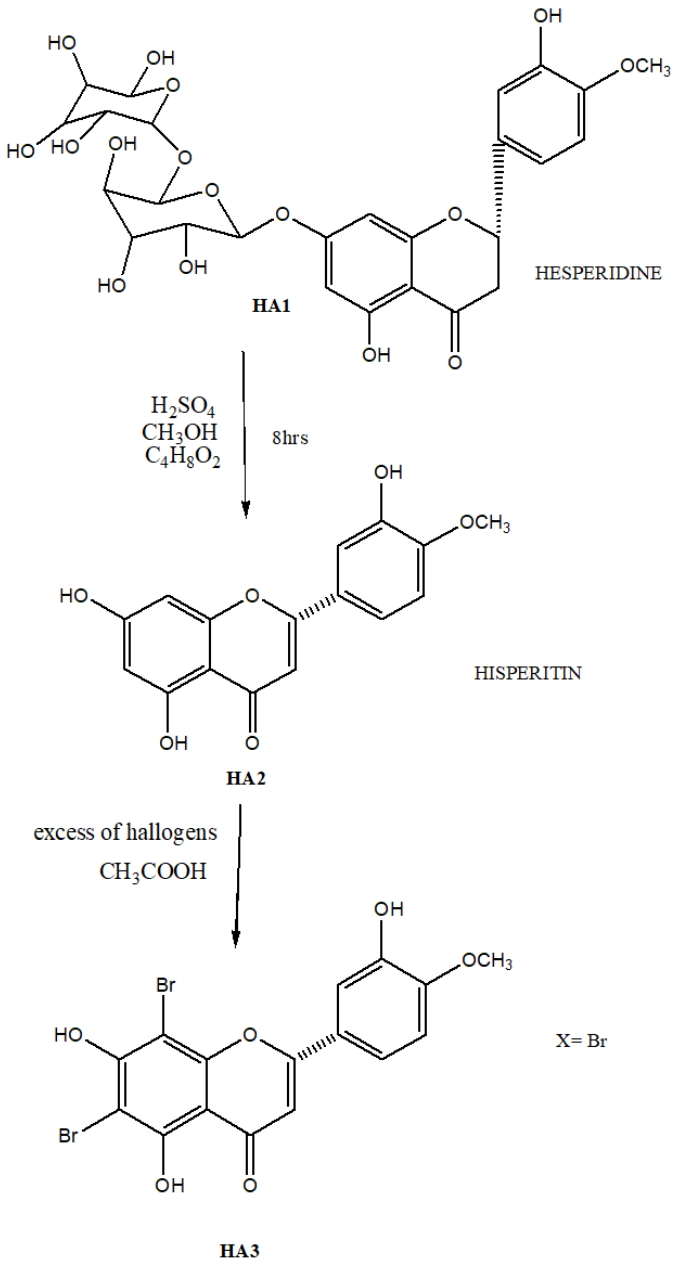

Figure 5: Chemical structures of isolated and semi-synthesized compounds

Figure 6: Isolation and purification of hesperidin from C. aurantium

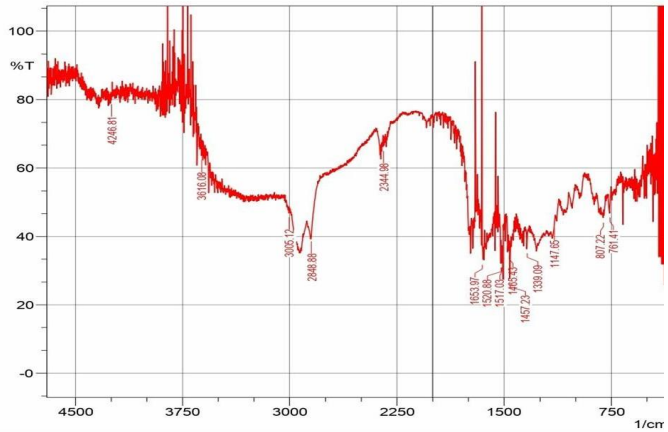

Figure 7: Structural characterization data of compounds HA1, HA2, and HA3

Table 1: Comparison between antioxidant activity of standard, isolated compound and its derivatives

Concentration (µg/ml)	200 µg	400 µg	600 µg	800 µg	1000 µg
Percent inhibition hesperidin (HA1)	42.2	50.3	62.9	67.37	78.48
Percent inhibition hesperitin (HA2)	32.38	39.35	45.19	48.41	54.8
Percent inhibition novel compound (HA3)	12.12	53.1	69.5	75.52	87.9
Percent inhibition standard	29.26	54.8	60.22	75.9	100

Table 2: Physicochemical properties of compounds

S.No	Compound code	Molecular weight	Molecular formula	TLC Rf value	Melting point	Percentage yield	Colour
1	Hesperidin (HA1)	610.6	C28H34O15	0.61	240-242	2%	Yellowish
2	Hesperitin (HA2)	302.28	C16H14O6	0.63	230-232	38%	Yellowish
3	Novel compound (HA3)	460.07	C16H12Br2O6	0.71	140-142	25%	Light brown

3.2 Spectral Characterization

3.2.1 Hesperidin (HA1)

Light yellow solid, Yield: 2%. M.P.: 240-241ºC, IR (KBr, ν/cm^{-1}) 3546 (C-OH), 2919 (C-H, Ar), 1601 (C=C, Ar), 1576 (C=O), 1180 (C-O). **^{1}H NMR (DMSO-d6, 400 MHz):** δ 12.80 (s, Ali-H, 1H), 8.01 (m, Ar-H, 2H), 7.11 (m, Ar-H, 2H), 6.90-6.82 (s, Ali-H, 4H), 6.40-5.00 (s, Ali-H, 3H), 4.51 (s, Ali-H, 2H), 3.80 (s, Ali-H, 3H), 3.21–3.62 (m, Ar-H, 7H), 3.22–3.61 (m, Ar-H, 5H), 2.50 (m, Ar-H, 2H), 1.12 (m, Ali-H, 3H). EIMS (m/z): 610.37 [M]$^{+}$, 611.09 [M+1]$^{+}$.

3.2.2 Hesperetin (HA2)

Light yellow solid, Yield: 38%. M.P.: 230-231ºC, IR (KBr, ν/cm^{-1}) 3650 (C-OH), 2914 (C-H, Ali), 1653 (C=C, Ar), 1472 (C=O), 1107 (C-O). **^{1}H NMR (300 MHz, DMSO-d6):** δ 6.92-6.77 (m, Ar-H, 3H), 6.44 (s, Ali-H, 1H), 5.88 (m, Ar-H, 2H), 5.48-5.01 (m, Ar-OH, 3H), 3.76 (m, Ali-H, 3H). EIMS (m/z): 300.1 [M]$^{+}$, 301.01 [M+1]$^{+}$.

3.2.3 6,8-Dibromo-5,7-dihydroxy-2-(3-hydroxy-4-methoxyphenyl)-4H-chromen-4-one (HA3)

Light brown solid, Yield: 25%. M.P.: 140-141ºC. IR (KBr, ν/cm^{-1}) 3616 (C-OH), 3005 (C-H, Ali), 2848 (C-H, Ar), 1653 (C=C, Ar), 1520 (C=O), 1147 (C-O), 761 (C-Br, Ar).

3.3 Pharmacognostical Evaluation

Crushed orange peel produced approximately 100 g of fine powder from 200 g of fresh wet-weighed peel. Around 100 g of dried and crushed orange powder was weighed and extracted with 400 ml of methanol as the solvent. The obtained crude hesperidin extracted from C. aurantium was about 2% yield.

3.4 Physicochemical Standardization

The physicochemical standardization of the crude drug is critical for quality control and reproducibility. The obtained results are summarized in Table 5.3 together with the reference values according to the WHO guidelines on quality control of herbal substances.

Table 5.3: Physicochemical parameters of Citrus aurantium peels (n=3, Mean ± SD)

Parameter	Result (% w/w)	Limit	Status	Inference
Total Ash Value	5.82 ± 0.18	NMT 6.0%	PASS	Total mineral content; within acceptable limits
Acid-Insoluble Ash	0.74 ± 0.06	NMT 2.0%	PASS	Low siliceous impurity; confirms purity
Water-Soluble Ash	2.46 ± 0.12	NLT 1.5%	PASS	Water-soluble mineral content adequate
Moisture Content (LOD)	8.24 ± 0.32	NMT 10%	PASS	Acceptable; prevents microbial spoilage
Alcohol-Soluble Extractive	24.68 ± 0.84	NLT 20%	PASS	High flavonoid-rich alcohol-soluble fraction
Water-Soluble Extractive	18.46 ± 0.62	NLT 15%	PASS	Adequate polar metabolite content

NMT = Not more than; NLT = Not less than.

Phytochemical Test	Hex	PE	CHCl3	EtOH	MeOH	Aq.
Carbohydrates – Molisch Test	-	-	-	+	+	+
Carbohydrates – Anthrone Test	-	-	-	+	+	+
Carbohydrates – Fehling's Test	-	-	-	+	+	+
Proteins – Biuret Test	-	-	-	-	-	+
Proteins – Ninhydrin Test	-	-	-	-	-	+
Alkaloids – Mayer's Test	-	-	+	-	-	-
Alkaloids – Dragendorff's Test	-	-	+	-	-	-
Flavonoids – Alkaline Test	-	-	+	+	+	+
Saponins – Frothing Test	-	-	-	-	+	+
Tannins – Ferric Chloride Test	-	-	-	+	+	+
Tannins – Phenazone Test	-	-	-	+	+	+
Steroids – Salkowski	+	+	+	-	-	-
Terpenoids – Salkowski Test	+	+	+	-	-	-
Fixed Oils – Spot Test	+	+	-	-	-	-

Table 5.4: Qualitative phytochemical screening of Citrus aurantium peel extracts

3.5 Phytochemical Screening

Qualitative systematic analysis was carried out in successive extracts with different polarity for determining the pattern of distribution of secondary metabolites. From the results, it is evident that the distribution of secondary metabolites was significantly based on polarity. Flavonoids were highly concentrated in ethanolic and methanolic extracts (+), indicating that they are polar glycosides. The presence of hesperidin in high concentration in polar extracts is attributed to the high polarity of this compound, due to the presence of disaccharide rutinoside in its molecular structure. Steroids and terpenoids were only found in the non-polar fractions (n-hexane and petroleum ether).

3.6 Isolation and Purification of Hesperidin

Hesperidin separation from Citrus aurantium peels was achieved through a process of solvent extraction followed by acid precipitation. Results are presented in Table 5.5.

Table 5.5: Stepwise isolation and purification of hesperidin from Citrus aurantium peels

Step	Procedure / Condition	Observation / Result
Raw Material	Citrus aurantium peel (air-dried, powdered)	100 g
n-Hexane Extraction	Soxhlet, 400 mL n-hexane, 1 h, hot filtration	Marc retained; wax/fat removed
Methanol Extraction	Soxhlet, 400 mL methanol; marc washed 50 mL hot MeOH	Combined filtrates concentrated
Concentration	Rotary evaporation under reduced pressure	Syrupy brown mass obtained
Acid Treatment	Acidified with dilute acetic acid; left 3–4 h undisturbed	Crude hesperidin precipitated
Crude Yield	Yellow crystalline precipitate after filtration	4.82 g (4.82% w/w)
Purification	Recrystallization from hot methanol	Pale yellow needles
Pure Yield	After drying at 60°C	3.64 g (3.64% w/w)
Melting Point (Crude)	Open capillary method	241°C (lit. 242°C)
Melting Point (Pure)	Open capillary method	258–260°C (lit. 258–262°C)

N-hexane extraction acted as the defatting process as it removed waxes, chlorophylls, essential oils, and non-polar components. Methanol was used to solubilize the polar hesperidin component. After concentrating the methanol filtrate to get a brown syrupy mass, pale yellow crystalline precipitate was obtained by adding dilute acetic acid. The crude yield was found to be 4.82 g (4.82% w/w), whereas after recrystallization from methanol, the yield was 3.64 g (3.64% w/w). The melting point of isolated hesperidin was found to be in the range of 258-260°C, which matches well with the literature value (258-262°C).

3.7 Thin Layer Chromatographic Analysis

TLC was carried out on silica gel G plates pre-activated at 110°C with the mobile phase n-butanol:water:acetic acid (3:1:1 v/v/v), and detection was done under ultraviolet light (UV) at 340 nm. The Rf values and properties of all compounds are presented in Table 5.6.

Table 5.6: TLC data for isolated and semi-synthesized compounds from Citrus aurantium peels

Compound	Rf Value	Colour (UV 340 nm)	Inference
Hesperidin (crude)	0.57	Yellow-brown	Broad spot; minor impurities visible
Hesperidin (pure)	0.58	Bright yellow	Single, sharp spot; confirmed identity

Hesperetin (semi-synth.)	0.72	Yellow-orange	Higher Rf – loss of sugar moiety increases mobility
Bromo-hesperetin deriv.	0.78	Dark orange-brown	Further increased Rf; bromine substitution
Hesperidin (std. reference)	0.58	Yellow	Co-migration with isolated compound confirms identity

The consistent Rf value of 0.58 for both crude and pure hesperidin, as well as the authentic standard, validates the identification of the isolated compound. The increase in Rf values from hesperidin (0.58) to hesperetin (0.72) to the brominated derivative (0.78) makes chemical sense since removing the disaccharide group and adding bromine reduces the polarity of the compounds, increasing their mobility in the butanol-acetic acid solvent system.

3.8 Solubility Studies

Solvent	Hesperidin	Hesperetin	Bromo-derivative
Water	Practically insoluble	Insoluble	Practically insolub
Methanol	Slightly soluble	Sparingly soluble	Practically insolub
Ethanol (95%)	Slightly soluble	Sparingly soluble	Slightly soluble
Acetone	Slightly soluble	Soluble	Freely soluble
Ethyl Acetate	Practically insoluble	Sparingly soluble	Freely soluble
Chloroform	Practically insoluble	Practically insoluble	Sparingly soluble
Glacial Acetic Acid	Slightly soluble	Soluble	Freely soluble
Dimethyl Sulphoxide (DMSO)	Freely soluble	Freely soluble	Freely soluble
n-Hexane	Practically insoluble	Practically insoluble	Practically insolub
Benzene	Practically insoluble	Practically insoluble	Sparingly soluble

Table 5.7: Solubility profile of hesperidin, hesperetin, and bromo-hesperetin derivative in various solvents

Hesperidin had poor solubility in organic solvents and had almost no solubility in water, which indicated a large molecule with many functional groups and intramolecular hydrogen bonding. Hesperetin was more soluble in acetone and glacial acetic acid due to the removal of the sugar portion. Brominated hesperetin was significantly more soluble in organic solvents since its OH groups were substituted with Br atoms. DMSO had excellent solubilization properties for all three substances.

3.9 Semi-Synthesis of Hesperidin Derivatives

3.9.1 Synthesis of Hesperetin

The reaction of hesperidin (9 g) with methanol (250 mL) under reflux with concentrated sulfuric acid (9 mL) for 8 hours brought about the selective hydrolysis of the glycoside portion at position 7 of the structure to give hesperetin as a pale yellow solid. Purification by crystallization using acetone-water-acetic acid system gave hesperetin having a melting point of 226–228°C (lit. 226–228°C). Structural identification was carried out by TLC using standard hesperetin (Sigma-Aldrich).

3.9.2 Synthesis of Bromo-Hesperetin Derivative

Bromination by electrophilic substitution of hesperetin (1 g) with liquid bromine (3-4 ml) in glacial acetic acid yielded an orange-brownish crystalline product after recrystallization from dilute ethanol. The persistence of bromine colour for 15-20 minutes confirmed stoichiometric consumption of bromine via electrophilic aromatic substitution rather than addition to double bonds. Electrophilic bromine preferentially reacted with the electron-rich aromatic ring at positions ortho/para to the activating -OH groups. According to the reactivity pattern of electrophilic substitution on the A-ring of flavanone, the substitution occurred at C-6 and C-8. The melting point of the compound was 198-200°C.

3.10 In Vitro Antioxidant Activity

Antioxidant property of isolated hesperidin, semi-synthesized hesperetin, hesperetin bromide, and ethanolic extract of C. aurantium peel was studied by employing four different assays — DPPH, ABTS, FRAP, and hydrogen peroxide (H_2O_2) scavenging assay — to assess radical scavenging activity and reducing power.

3.10.1 DPPH Radical Scavenging Activity

The capacity of the test compounds to scavenge the stable DPPH radical was evaluated at four concentrations (25–200 µg/mL) and IC_{50} values were calculated. Results are presented in Table 5.8.

Table 5.8: DPPH radical scavenging activity — % inhibition (Mean ± SD, n=3) and IC_{50} values

Compound / Extract	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Hesperidin (isolated)	28.42±1.14	46.82±1.38	68.46±1.62	84.28±1.84
Hesperetin (semi-synth.)	34.16±1.22	54.28±1.46	74.82±1.74	90.46±1.92
Bromo-hesperetin deriv.	38.64±1.32	60.14±1.54	80.28±1.86	93.62±2.04
Ethanolic extract	18.46±0.92	32.84±1.18	52.16±1.36	72.48±1.58
Ascorbic acid (std.)	56.82±1.64	74.46±1.78	88.64±1.94	96.28±2.12

Values expressed as % inhibition ± SD (n=3). IC_{50} = concentration producing 50% inhibition.

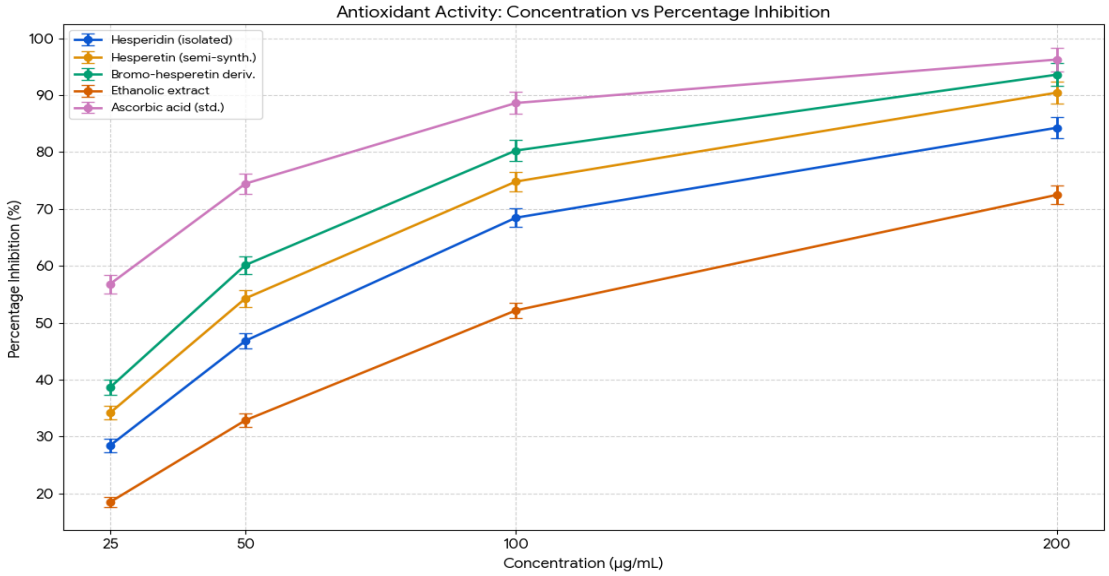

Figure 5.1: DPPH radical scavenging activity — % inhibition (Mean ± SD, n=3) and IC_{50} values

Scavenging activity of the DPPH radical was in the order: Ascorbic acid (IC_{50} 28.64) > Bromo-hesperetin derivative (IC_{50} 46.18) > Hesperetin (IC_{50} 52.36) > Hesperidin (IC_{50} 68.24) > Ethanolic extract (IC_{50} 96.42 µg/mL). The higher efficiency of scavenging activity for the bromo-hesperetin derivative was due to the ability of free hydroxyl groups in the aglycone to perform hydrogen atom transfer to the DPPH radical.

3.10.2 ABTS Radical Cation Decolorization Assay

The $ABTS^{+}$ radical cation scavenging activity was determined to assess the trolox-equivalent antioxidant capacity (TEAC). Results are presented in Table 5.9.

Table 5.9: ABTS radical cation scavenging activity — % inhibition (Mean ± SD, n=3) and IC_{50} values

Compound / Extract	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
Hesperidin (isolated)	30.18±1.18	50.42±1.44	72.84±1.66	87.64±1.88
Hesperetin (semi-synth.)	36.82±1.26	58.46±1.52	78.64±1.82	92.18±1.96
Bromo-hesperetin deriv.	42.14±1.36	64.28±1.58	84.46±1.92	95.82±2.08
Ethanolic extract	20.64±0.96	36.42±1.22	56.84±1.44	76.18±1.62
Trolox (std.)	58.46±1.68	76.82±1.84	90.28±1.98	97.42±2.16

Values expressed as % inhibition ± SD (n=3). Trolox used as positive standard.

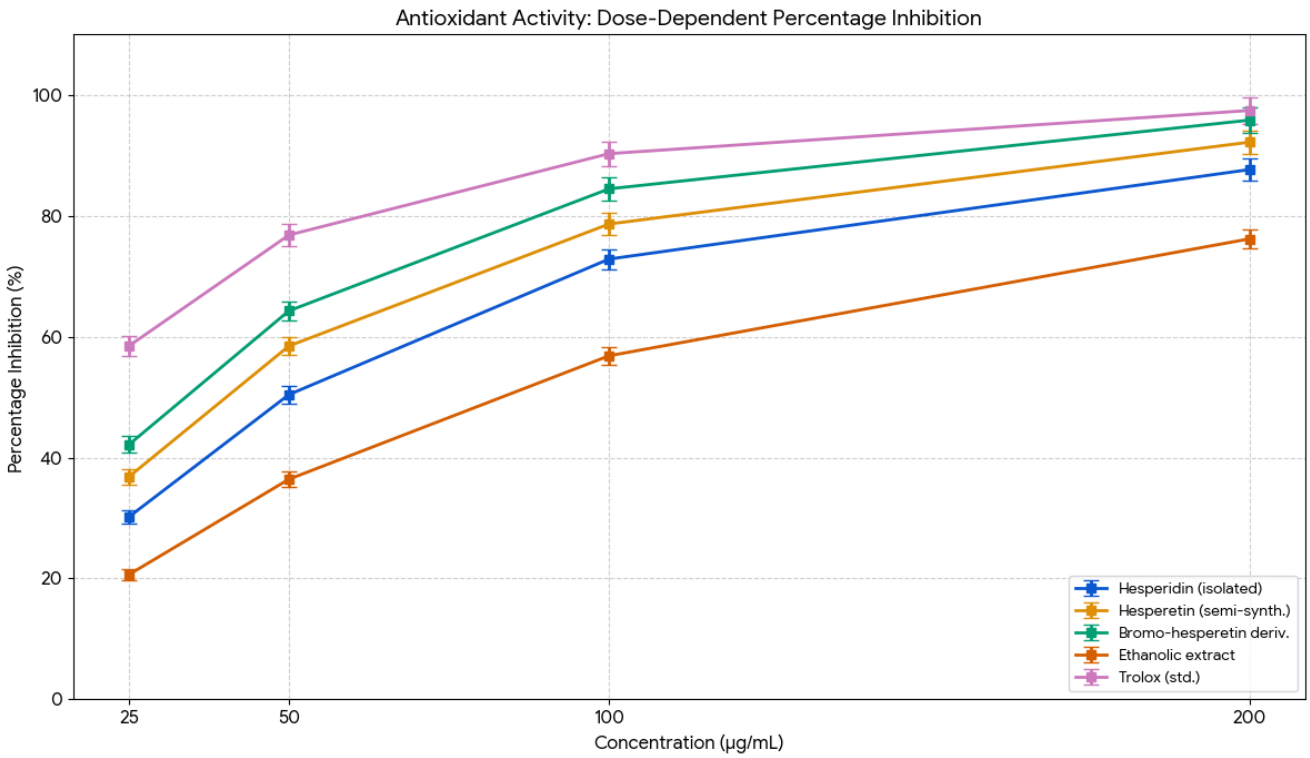

Figure 5.2: ABTS radical cation scavenging activity — % inhibition

The findings from the ABTS test were in line with the activity trend seen in the DPPH test, where the bromo-derivative showed the lowest IC_{50} value (42.36 µg/mL), followed by hesperetin (48.62 µg/mL) and hesperidin (62.84 µg/mL). The ABTS test is more versatile, covering a wider pH spectrum and measuring the antioxidant properties of both lipophilic and hydrophilic antioxidants.

3.10.3 Ferric Reducing Antioxidant Power (FRAP)

The reducing power of the compounds was assessed by their ability to reduce Fe^{3+} to Fe^{2+}. Results are presented in Table 5.10.

Table 5.10: FRAP values (µM Fe^{2+} equivalents per gram) at 100 and 200 µg/mL (Mean ± SD, n=3)

Compound / Extract	FRAP Value at 100 µg/mL (µM Fe^{2+}/g)	FRAP Value at 200 µg/mL (µM Fe^{2+}/g)	Relative Activity (%)
Hesperidin (isolated)	284.62 ± 8.42	482.36 ± 12.64	72.4
Hesperetin (semi-synth.)	326.18 ± 9.18	548.82 ± 13.86	82.4
Bromo-hesperetin deriv.	368.46 ± 10.24	614.28 ± 14.92	92.3
Ethanolic extract	196.84 ± 6.86	346.42 ± 10.28	52.1
Ascorbic acid (std.)	424.68 ± 11.46	665.84 ± 15.64	100.0

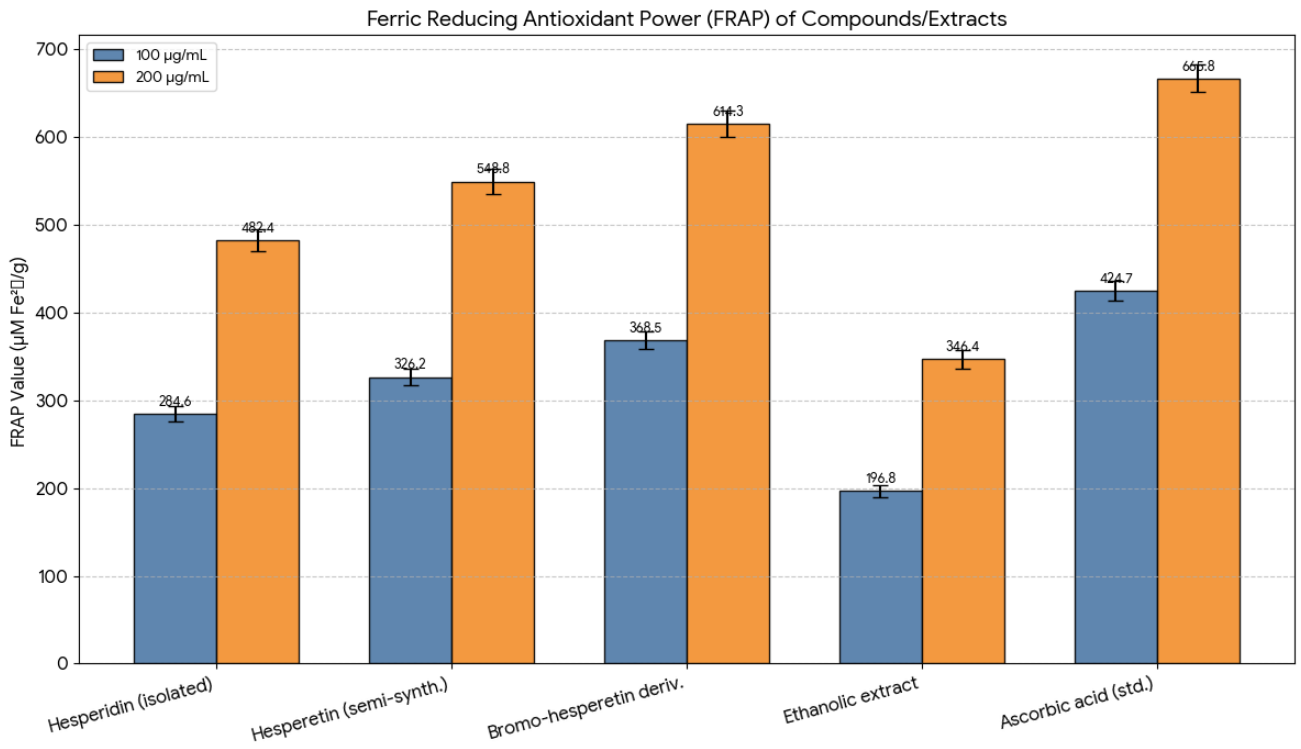

Figure 5.3: FRAP values

FRAP results verified the dose-dependent reducing potential of all tested agents. The bromo-hesperetin showed the highest reducing ability (614.28 ± 14.92 µM Fe^{2+}/g at 200 µg/mL), equal to 92.3% compared to ascorbic acid. Improved electron donating properties may be explained by increased polarizability due to the presence of bromine and easy access of phenolic groups in the aglycone.

3.10.4 Hydrogen Peroxide Scavenging Activity

The ability of the compounds to scavenge H_2O_2 was tested as an indicator of their efficacy in reducing a physiologically relevant reactive oxygen species. Results are summarized in Table 5.11.

Table 5.11: Hydrogen peroxide scavenging activity — % inhibition (Mean ± SD, n=3) and IC_{50} values

Compound / Extract	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL	IC_{50} (µg/mL)
Hesperidin (isolated)	22.46±0.92	40.18±1.26	62.84±1.54	80.46±1.78	78.42±1.62
Hesperetin (semi-synth.)	28.82±1.08	50.64±1.42	74.28±1.68	88.14±1.88	56.84±1.34
Bromo-hesperetin deriv.	34.16±1.18	58.42±1.48	80.64±1.78	92.84±1.96	48.26±1.18
Ethanolic extract	14.82±0.78	28.46±1.04	46.28±1.28	66.84±1.54	108.62±2.28
Ascorbic acid (std.)	52.64±1.56	72.48±1.72	86.82±1.88	94.64±2.04	32.46±0.88

Values expressed as % inhibition ± SD (n=3). Ascorbic acid used as positive standard.

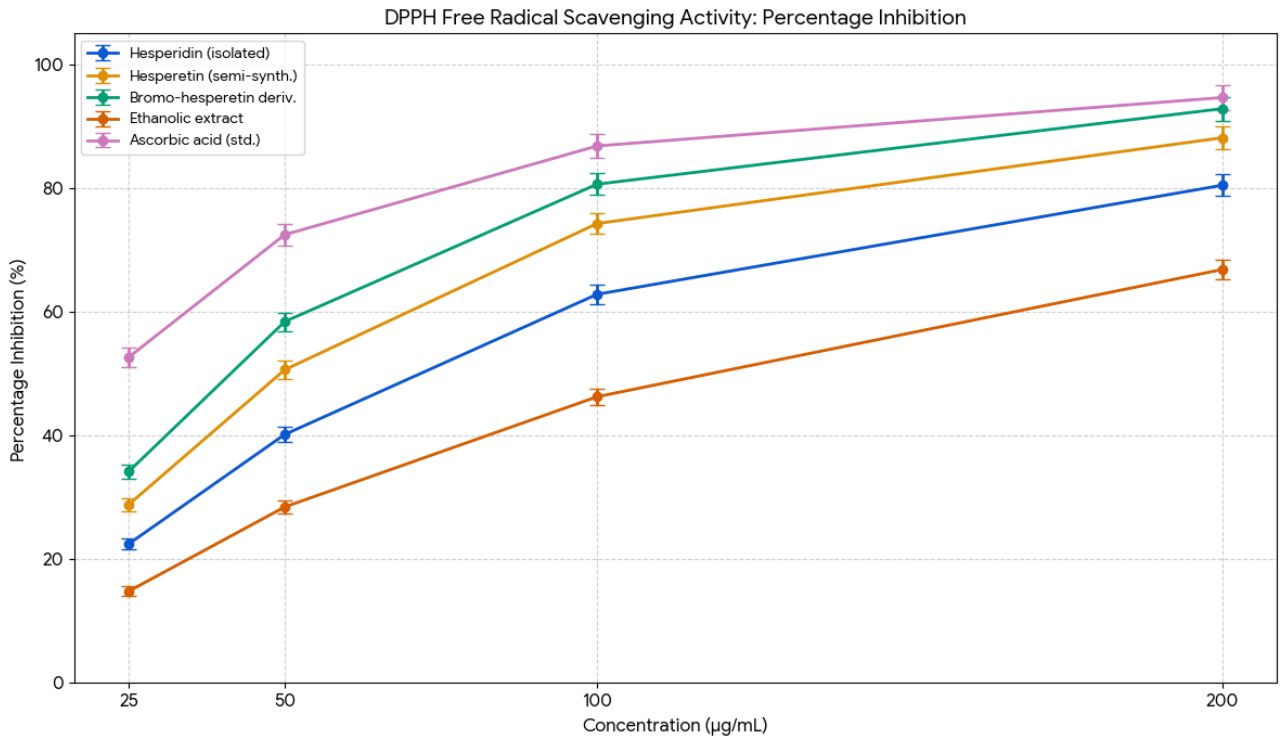

Figure 5.4: Hydrogen peroxide scavenging activity — % inhibition

H_2O_2 scavenging assay data showed the better performance of bromo-hesperetin (IC_{50} = 48.26 µg/mL) than hesperetin (IC_{50} = 56.84 µg/mL) and hesperidin (IC_{50} = 78.42 µg/mL). This trend holds true across all four antioxidant experiments, lending strong convergent evidence to the superior antioxidant property of the semi-synthesized brominated derivative.

3.11 In Vitro Antimicrobial Activity

The antimicrobial activity of the isolated and semi-synthesized compounds was evaluated by both the agar well diffusion and disc diffusion methods against two Gram-positive bacteria, two Gram-negative bacteria, and one pathogenic fungus. Zones of inhibition (mm) are reported in Table 5.12.

Table 5.12: Zone of inhibition (mm ± SD, n=3) — agar well diffusion at 100 µg/mL

Compound	S. aureus	B. subtilis	E. coli	P. aeruginosa	C. albicans	Conc. (µg/mL)
Hesperidin (isolated)	14.2±0.42	12.8±0.38	11.6±0.34	10.4±0.32	13.2±0.40	100
Hesperetin (semi-synth.)	16.8±0.48	15.4±0.44	14.2±0.40	12.8±0.36	15.6±0.46	100
Bromo-hesperetin deriv.	20.4±0.62	18.6±0.54	17.4±0.50	15.8±0.46	18.8±0.56	100
Ethanolic extract	12.6±0.36	10.4±0.30	9.8±0.28	8.6±0.26	11.4±0.34	100
Ciprofloxacin (std.)	28.6±0.86	26.4±0.78	24.8±0.72	22.6±0.66	—	5
Fluconazole (std.)	—	—	—	—	24.2±0.72	5

DMSO (−ve control)	–	–	–	–	–	—

Std. = Ciprofloxacin (5 µg/disc) for bacteria; Fluconazole (5 µg/disc) for fungi. −ve control: DMSO.

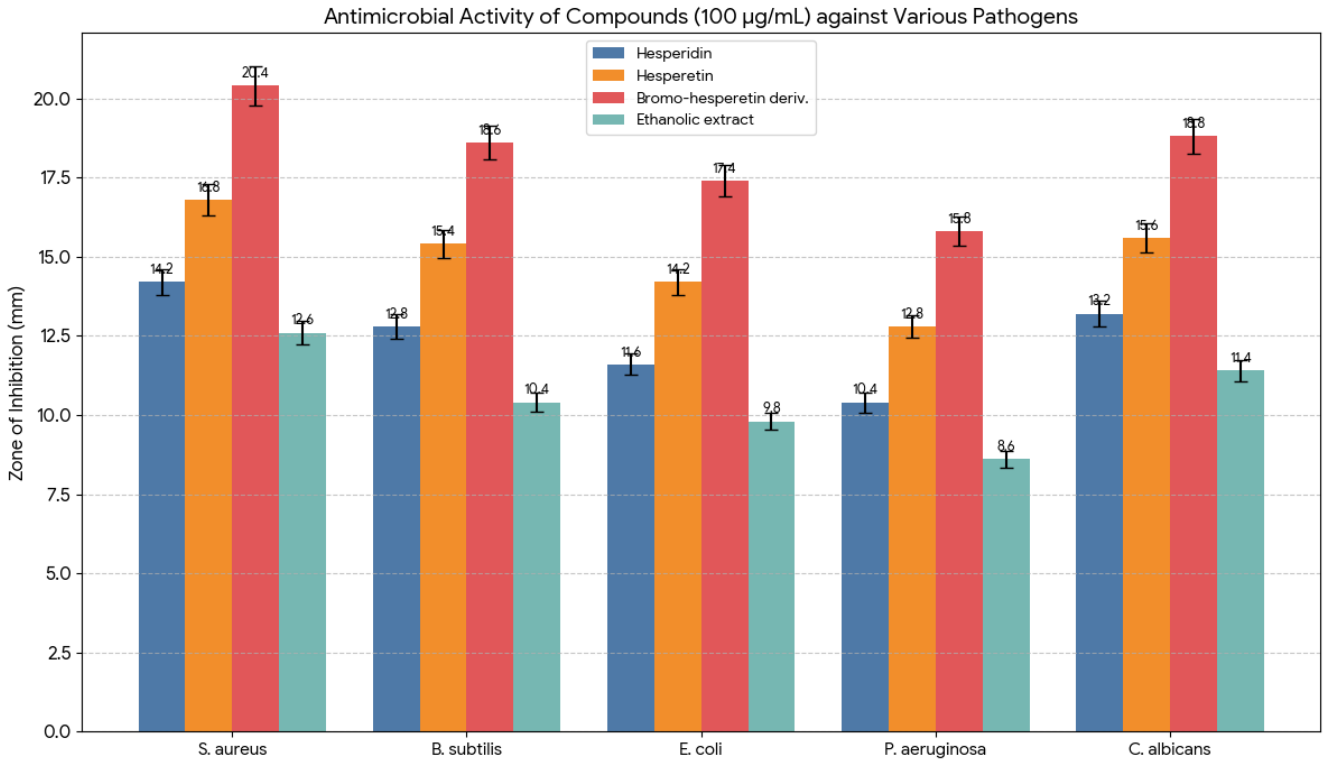

Figure 5.5: Zone of inhibition (mm ± SD, n=3) — agar well diffusion at 100 µg/mL

Concentration-dependent antimicrobial effects were observed for all three tested compounds against the target microorganisms. The brominated form of hesperetin showed the highest antimicrobial activity among all test compounds for all bacterial species. Zones of inhibition for the bromo-derivative ranged from 15.8 ± 0.46 mm (P. aeruginosa) to 20.4 ± 0.62 mm (S. aureus). Gram-positive bacteria proved more sensitive than Gram-negative microorganisms due to the additional permeability layer represented by the outer lipopolysaccharide membrane. Moderate sensitivity to all test compounds was found for C. albicans, with the brominated compound showing the strongest antifungal activity (18.8 ± 0.56 mm). No zone of inhibition was observed with DMSO as the negative control.

4. Summary and Conclusion

The results of the current study indicate that hesperidin was successfully isolated from Citrus aurantium peels in an amount of 3.64% w/w, followed by semi-synthesis into hesperetin and its novel brominated analog. Significant pharmacological activity was demonstrated for all three substances in both antioxidant and antimicrobial assays.

The stable order of efficacy — brominated derivative > hesperetin > hesperidin — throughout all performed experiments implies certain mechanistic consequences. Due to deprotonation of the hydroxyl group at C7 and excision of the hydrophilic disaccharide molecule, the catechol-type B-ring along with free phenolic hydroxyl groups responsible for flavonoid-based radical scavenging are unmasked in the structure of hesperetin. Increased efficacy of the synthesized

bromo-derivative can be explained by: (a) higher lipophilicity leading to enhanced permeability of biological membranes; (b) modification of the A-ring by an electrophile causing redistribution of electron density; and (c) possible charge transfer between organic molecules and biological macromolecules.

The IC_{50} values obtained for hesperidin (DPPH: 68.24 μg/mL; ABTS: 62.84 μg/mL) agree well with literature data for hesperidin isolated from citrus sources. The enhanced effect of hesperetin is in accordance with published findings that deglycosylation increases the antioxidative activity of all tested flavanone glycosides. Antimicrobial activity of hesperidin and hesperetin toward S. aureus and E. coli is in agreement with reported MIC values.

The current research was performed only in vitro, and further pharmacokinetic and pharmacodynamic studies in vivo are required. The brominated hesperetin derivative, which has shown significant in vitro activity, needs thorough spectroscopic analysis (IR, ^{1}H NMR, ^{13}C NMR, HRMS), as well as in vivo toxicity studies to confirm its potential pharmacological effects. Fractionation of the most active extracts may lead to isolation of new bioactive compounds.

5. References

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