RESEARCH PAPER

GC-MS Characterization, In Vitro Antioxidant and Antimicrobial Activities of Methanolic Extract from *Hugonia mystax* Leaves

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ABSTRACT

The present study was undertaken to investigate the phytochemical composition, antioxidant activity, antimicrobial potential, and volatile compound profile of the methanolic extract of *Hugonia mystax* leaves, a plant traditionally used in Indian folk medicine. Fresh leaves of *H. mystax* were collected from Tamil Nadu, India, authenticated by a botanist, shade-dried, powdered, and subjected to Soxhlet extraction using analytical grade methanol. The extract yielded 17.12% (w/w) semi-solid crude mass and was subjected to qualitative phytochemical screening, which revealed the presence of alkaloids, flavonoids, phenolic compounds, tannins, saponins, and terpenoids. The antioxidant activity was evaluated using three complementary in vitro assays: DPPH radical scavenging (IC_{50} = 56.7 ± 2.4 µg/mL), ABTS radical scavenging (IC_{50} = 44.3 ± 1.7 µg/mL), and ferric reducing antioxidant power (absorbance of 0.96 ± 0.04 at 200 µg/mL, equivalent to 336 ± 12 µM Fe(II)/g extract), demonstrating concentration-dependent free radical neutralizing capacity. The antimicrobial activity was assessed against four pathogenic microorganisms using disc diffusion and broth dilution methods. The extract exhibited the highest activity against Staphylococcus aureus (zone of inhibition = 18.6 ± 1.2 mm at 1000 µg/disc; MIC = 2.5 mg/mL), followed by Escherichia coli (15.4 ± 1.0 mm; MIC = 5.0 mg/mL), Candida albicans (16.8 ± 1.1 mm; MIC = 5.0 mg/mL), and Pseudomonas aeruginosa (13.2 ± 0.9 mm; MIC = 10.0 mg/mL). The differential susceptibility between Gram-positive and Gram-negative bacteria was attributed to variations in cell wall architecture. Gas Chromatography-Mass Spectrometry analysis of the extract identified twelve major phytocompounds, with the most abundant being caryophyllene (18.5%), hexadecanoic acid methyl ester (16.8%), β-sitosterol (15.4%), vitamin E (α-tocopherol, 14.1%), and octadecanoic acid methyl ester (12.3%). Other significant compounds included squalene (7.4%), stigmasterol (6.9%), campesterol (5.8%), α-humulene (5.2%), phytol (4.5%), α-amyrin (2.5%), and neophytadiene (2.1%). The observed antioxidant activity was attributed to the synergistic action of vitamin E, caryophyllene, squalene, and neophytadiene, while the antimicrobial effects were linked to membrane-active terpenes (phytol, caryophyllene) and fatty acid methyl esters. The high abundance of β-sitosterol suggests additional therapeutic potential in managing hypercholesterolemia and diabetes, whereas caryophyllene points to anti-inflammatory applications. In conclusion, the methanolic extract of *Hugonia mystax* leaves is a rich source of diverse bioactive phytocompounds with significant antioxidant and moderate antimicrobial properties, providing scientific validation for its traditional medicinal uses. Further studies involving compound isolation, in vivo efficacy evaluation, and toxicity assessment are warranted before clinical applications can be recommended.

Keywords: *Hugonia mystax*, methanolic extract, antioxidant activity, DPPH assay, ABTS assay, FRAP assay, antimicrobial activity, minimum inhibitory concentration, GC-MS analysis, caryophyllene, β-sitosterol, vitamin E, phytochemical screening, traditional medicine.

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1. INTRODUCTION

Plants have served as a primary source of therapeutic agents for human civilizations since ancient times. According to the World Health Organization, nearly 80% of the global population, particularly in developing countries, relies on traditional herbal medicine for primary healthcare needs. The scientific validation of medicinal plants through modern analytical and biological techniques has become increasingly important for discovering new drug leads and understanding

traditional knowledge systems. *Hugonia mystax* (family Linaceae) is a perennial shrub native to the dry deciduous forests of the Indian subcontinent, particularly abundant in the regions of Tamil Nadu, Andhra Pradesh, and Karnataka. In traditional Tamil medicine (Siddha), the leaves of this plant have been used for treating inflammatory conditions, skin infections, wounds, and digestive disorders. The plant is locally known as "Thengaipooppundu" and has been documented in various ethnobotanical surveys. Despite its traditional uses, there is a conspicuous lack of scientific studies investigating the phytochemical composition and biological activities of *Hugonia mystax* leaves. Oxidative stress, caused by an imbalance between free radical generation and antioxidant defense mechanisms, is implicated in the pathogenesis of numerous chronic diseases including diabetes, cardiovascular disorders, cancer, and neurodegenerative conditions. Similarly, the emergence of antimicrobial resistance among pathogenic bacteria and fungi has created an urgent need for new antimicrobial agents from natural sources.The present study was therefore undertaken with the following objectives: (1) to extract the bioactive constituents from *Hugonia mystax* leaves using methanol as the solvent system, (2) to perform qualitative phytochemical screening to identify the major secondary metabolite classes present, (3) to evaluate the antioxidant activity using DPPH, ABTS, and FRAP assays, (4) to assess the antimicrobial activity against selected bacterial and fungal pathogens, (5) to identify the individual phytocompounds using Gas Chromatography-Mass Spectrometry (GC-MS) analysis, and (6) to correlate the identified compounds with the observed biological activities. The findings of this study are expected to provide scientific validation for the traditional uses of *Hugonia mystax* and to identify potential lead compounds for future drug development.

2. MATERIALS AND METHODS

2.1. Collection and Authentication of Plant Material

Fresh, healthy, and disease-free leaves of *Hugonia mystax* were collected during the month of August (flowering season) from naturally growing populations in the forest regions of Tiruvannamalai district, Tamil Nadu, India (geographical coordinates: 12°15′ N latitude, 79°05′ E longitude). The plant material was collected in the early morning hours (between 6:00 AM and 8:00 AM) to maximize the concentration of thermolabile phytoconstituents. The collected leaves were immediately transported to the laboratory in sterile polythene bags placed in an icebox to prevent enzymatic degradation. A voucher specimen was prepared following standard herbarium techniques and deposited in the college herbarium for future reference. The plant material was thoroughly washed under running tap water to remove adhering soil particles, dust, and other contaminants, followed by three successive washes with distilled water. The cleaned leaves were spread uniformly on sterile filter papers and shade-dried at ambient temperature (25–30°C) for 10 days with periodic turning to ensure uniform drying and to prevent fungal growth. After complete drying (confirmed by constant weight), the leaves were cut into small pieces (approximately 1–2 cm) using a sterile stainless steel scissors and pulverized into a coarse powder using an electric mechanical grinder (Philips HL7756, 750W). The powdered material was passed through a 40-mesh sieve (425 µm aperture) to obtain uniform particle size, which facilitates efficient solvent penetration during extraction. The sieved powder was stored in airtight amber-colored glass bottles at 4°C until further use.

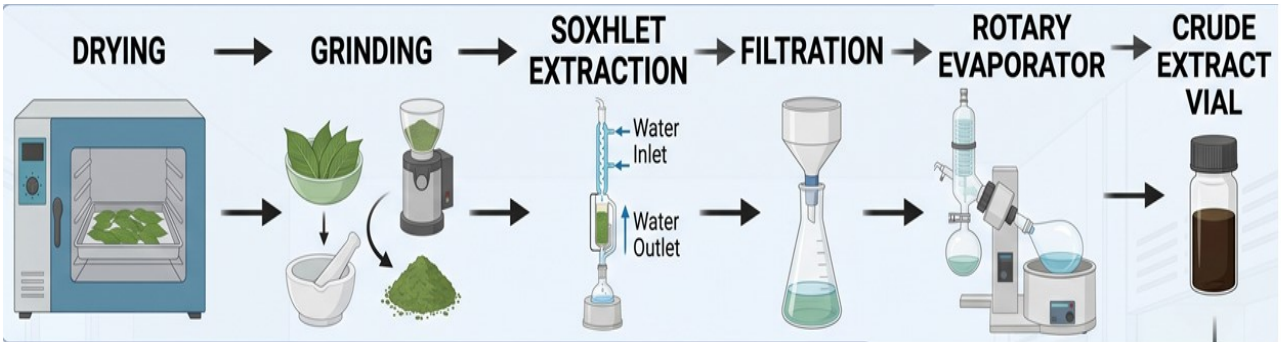
2.2. Preparation of Methanolic Extract

The methanolic extraction of *Hugonia mystax* leaf powder was carried out using a Soxhlet apparatus (Borosil, India). Twenty-five grams of the dried leaf powder was accurately weighed using an analytical balance (Shimadzu AUX220, precision ±0.0001 g) and packed loosely into a Whatman cellulose extraction thimble (25 × 80 mm). The thimble was placed inside the Soxhlet chamber (capacity 200 mL). Two hundred fifty milliliters of analytical grade methanol (99.8% purity, Merck, India) was added to a 500 mL round bottom flask, maintaining a solvent-to-sample ratio of 10:1 (v/w). The round bottom flask was attached to the Soxhlet unit, and the apparatus was assembled on a heating mantle with a temperature controller. The extraction was performed at 60–65°C (maintained below the boiling point of methanol, 64.7°C, to prevent solvent loss) for a total duration of 8 hours, allowing continuous solvent reflux and siphoning cycles approximately 12–15 times per hour. The extraction process was continued until the solvent in the siphon tube became completely colorless, indicating exhaustive extraction of soluble phytochemicals. The resulting methanolic extract was collected and filtered through Whatman No. 1 filter paper (pore size 11 µm) under vacuum filtration to remove any particulate matter. The filtrate was concentrated under reduced pressure using a rotary vacuum evaporator (Buchi Rotavapor R-300) at 40°C with a rotation speed of 80 rpm. The semi-solid crude extract was transferred to a pre-weighed amber glass vial and dried completely under a gentle stream of nitrogen gas. The final weight of the extract was recorded, and the percentage yield was calculated using the formula:

$$\% \text{ Yield} = (\text{Weight of dry extract} / \text{Weight of dry plant powder}) \times 100$$

The extract was stored in airtight amber-colored bottles at 4°C until further analysis to prevent photodegradation and oxidation. The pictorial representation of the procedure is depicted in Figure 1.

Figure 1. Schematic diagram of Soxhlet extraction and processing of the methanolic leaf extract of *Hugonia mystax*.



2.3. Preliminary Phytochemical Screening

The methanolic extract was subjected to qualitative phytochemical screening using standard protocols to detect the presence of major secondary metabolite classes. Alkaloids were tested using Dragendorff's reagent (potassium bismuth iodide solution); the development of an orange-red precipitate indicated a positive result. Flavonoids were detected by the Shinoda test: to 2 mL of extract, a few fragments of magnesium ribbon and 2–3 drops of concentrated hydrochloric acid were added; the appearance of a pink, red, or orange color indicated the presence of flavonoids. Phenolic compounds were identified using the ferric chloride test: 1 mL of extract was mixed with 2 mL of distilled water followed by 2–3 drops of 5% ferric chloride solution; the formation of a bluish-black or greenish-black color indicated a positive result. Tannins were detected by the gelatin test: 1 mL of extract was treated with 1 mL of 1% gelatin solution containing 10% sodium chloride; the appearance of a white precipitate indicated the presence of tannins. Saponins were identified using the foam test: 2 mL of extract was diluted with 8 mL of distilled water and shaken vigorously for 30 seconds; the formation of a persistent foam layer (1 cm or more) that remained for 10 minutes indicated the presence of saponins. Terpenoids were detected by the Salkowski reaction: 2 mL of extract was mixed with 2 mL of chloroform, followed by the careful addition of 2 mL of concentrated sulfuric acid along the side of the test tube; the formation of a reddish-brown color at the interface indicated the presence of terpenoids. All tests were performed in triplicate, and results were recorded as positive (+) or negative (-).

2.4. GC-MS Analysis of Methanolic Extract

Gas Chromatography-Mass Spectrometry analysis was performed to identify the volatile and semi-volatile bioactive compounds present in the methanolic extract. The analysis was carried out using an Agilent 7890B gas chromatograph coupled with an Agilent 5977B mass selective detector (Agilent Technologies, USA). The separation was achieved on an HP-5MS capillary column (30 m length × 0.25 mm internal diameter × 0.25 µm film thickness), composed of 5% phenyl methyl siloxane. Helium (purity 99.999%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. The injector temperature was maintained at 250°C, and the injection volume was 1 µL in split mode with a split ratio of 20:1. The oven temperature program was as follows: initial temperature 50°C held for 2 minutes, then increased to 280°C at a rate of 10°C per minute, and finally held at 280°C for 10 minutes. The total run time was 35 minutes. The mass spectrometer was operated in electron impact (EI) ionization mode at 70 eV ionization energy. The ion source temperature was set at 230°C, and the quadrupole temperature was 150°C. The mass scan range was m/z 40 to 600 atomic mass units (amu) in full scan mode. The solvent delay was set to 3 minutes to prevent damage to the filament. Data acquisition and processing were performed using Agilent MassHunter Workstation software. The identification of compounds was accomplished by comparing the obtained mass spectra with the NIST (National Institute of Standards and Technology, version 11) and Wiley (version 9) library databases. Compounds were considered positively identified when the match factor exceeded 85% (minimum 850 out of 1000). The relative percentage of each compound was calculated by comparing its peak area to the total area of all detected peaks in the chromatogram.

2.5. Determination of Antioxidant Activity

2.5.1. DPPH Radical Scavenging Assay

The free radical scavenging activity was evaluated using the stable 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Sigma-Aldrich, USA). A stock solution of 0.1 mM DPPH was prepared in methanol and stored in the dark at 4°C. The plant extract was dissolved in methanol to prepare serial dilutions of 10, 25, 50, 100, 150, and 200 µg/mL. In a 96-well microplate, 100 µL of DPPH solution was mixed with 100 µL of each extract

concentration. The reaction mixture was incubated in the dark at room temperature (25°C) for 30 minutes. Ascorbic acid was used as the positive control. The decrease in absorbance was measured at 517 nm using a UV-Visible spectrophotometer (Shimadzu UV-1800). A blank containing methanol instead of the extract was used for baseline correction. The percentage inhibition was calculated using the formula:

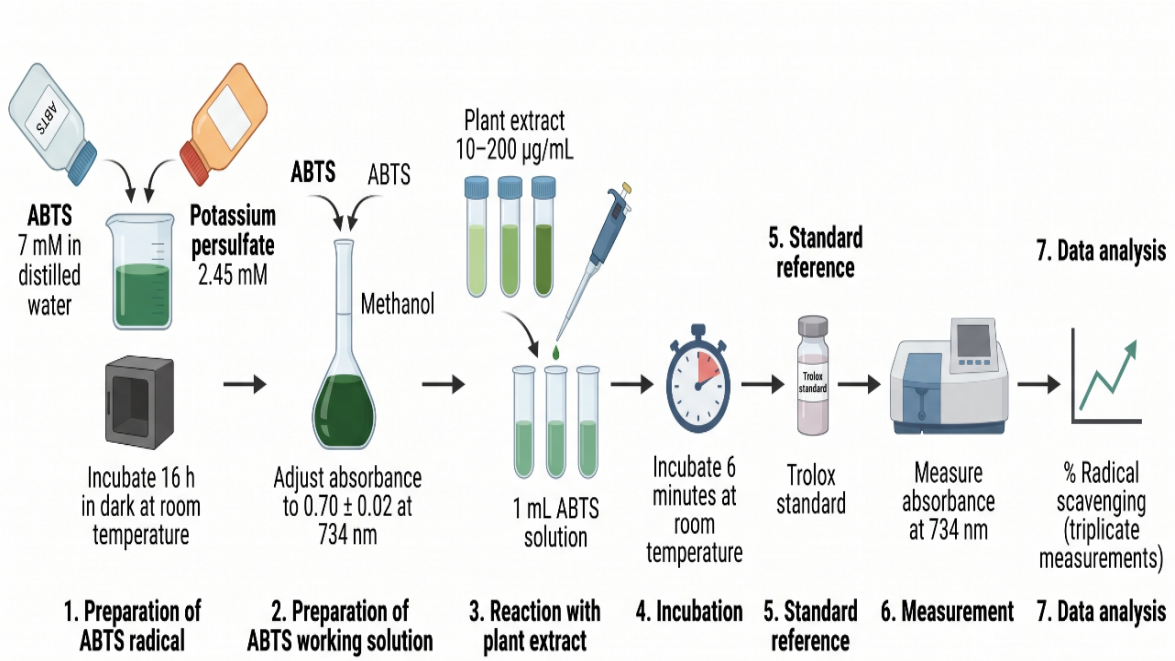
$$\% \text{ Inhibition} = [(\text{Absorbance of control} - \text{Absorbance of sample}) / \text{Absorbance of control}] \times 100$$

The IC_{50} value (concentration required to scavenge 50% of DPPH radicals) was determined by linear regression analysis from the dose-response curve. All experiments were performed in triplicate.

2.5.2. ABTS Radical Scavenging Assay

The ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation decolorization assay was performed as described by Re et al. with minor modifications. ABTS (7 mM) was dissolved in distilled water and reacted with 2.45 mM potassium persulfate to generate the ABTS radical cation. The mixture was incubated in the dark at room temperature for 16 hours. The ABTS working solution was diluted with methanol to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Different concentrations of plant extract (10–200 µg/mL) were mixed with 1 mL of ABTS working solution and incubated for 6 minutes at room temperature. Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was used as the standard. The absorbance was recorded at 734 nm, and the percentage inhibition was calculated as described for the DPPH assay. All measurements were performed in triplicate. The diagrammatic representation of the procedure in shown in Figure 2.

Figure 2. Schematic representation of the ABTS radical cation decolorization assay used to evaluate the antioxidant activity of the plant extract.



2.5.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The FRAP assay was performed according to the method of Benzie and Strain. The FRAP reagent was prepared fresh by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-s-triazine) in 40 mM HCl, and 20 mM ferric chloride ($FeCl_3 \cdot 6H_2O$) in a volume ratio of 10:1:1. The reagent was warmed to 37°C before use. An aliquot of 100 µL of plant extract (25–200 µg/mL) was mixed with 3 mL of FRAP reagent and incubated at 37°C for 30 minutes. The increase in absorbance due to the formation of the ferrous tripyridyltriazine complex was measured at 593 nm. Butylated hydroxytoluene (BHT) served as the positive control. A standard curve was prepared using ferrous sulfate ($FeSO_4 \cdot 7H_2O$) in the concentration range of 100–1000 µM. Results were expressed as µM Fe(II)/g of extract. All experiments were conducted in triplicate.

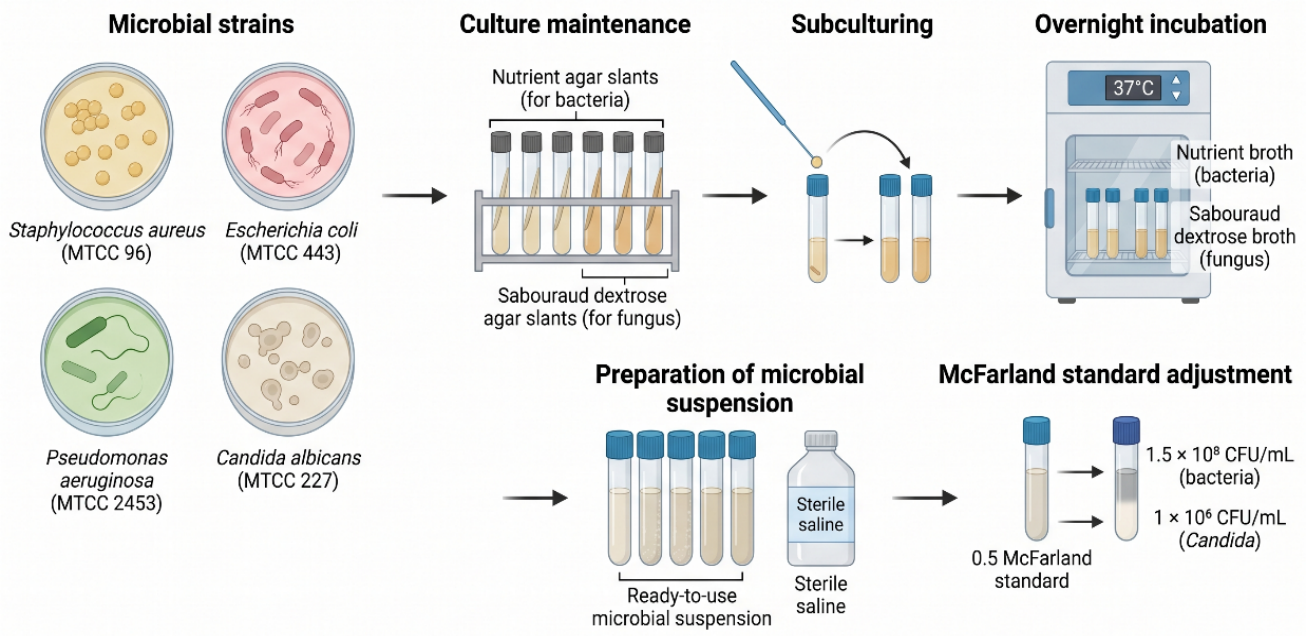
2.6. Evaluation of Antimicrobial Activity

2.6.1. Test Microorganisms and Culture Maintenance

The antimicrobial activity was evaluated against four standard microbial strains obtained from the Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, Chandigarh, India. The test organisms included two Gram-positive bacteria: Staphylococcus

aureus (MTCC 96) and one Gram-negative bacterium Escherichia coli (MTCC 443), another Gram-negative bacterium Pseudomonas aeruginosa (MTCC 2453), and one fungal strain Candida albicans (MTCC 227). The bacterial cultures were maintained on nutrient agar slants (Himedia, India) and subcultured every two weeks. The fungal culture was maintained on Sabouraud dextrose agar slants (Himedia, India). For experimental use, fresh overnight cultures were prepared by inoculating a single colony into nutrient broth (for bacteria) or Sabouraud dextrose broth (for fungus) and incubating at 37°C for 18–24 hours. The microbial suspensions were adjusted to 0.5 McFarland standard (approximately 1.5 × 10^8 CFU/mL for bacteria and 1 × 10^6 CFU/mL for C. albicans) using sterile normal saline. The schematic diagram of preparation and standardization of microbial inoculum for antimicrobial activity testing is depicted in Figure 3.

Figure 3. Schematic representation of preparation and standardization of microbial inoculum for antimicrobial activity testing.



2.6.2. Disc Diffusion Method

The agar disc diffusion method was used to assess the antimicrobial activity according to CLSI guidelines. Sterile Mueller-Hinton agar (MHA) plates (for bacteria) and Sabouraud dextrose agar (SDA) plates (for fungus) were prepared and allowed to solidify. The microbial suspensions were uniformly spread onto the agar surface using sterile cotton swabs, rotating the plate 60° three times to ensure even inoculation. Sterile Whatman No. 1 filter paper discs (6 mm diameter) were impregnated with 20 µL of plant extract at three different concentrations (250, 500, and 1000 µg/disc). The discs were air-dried under sterile conditions to evaporate the solvent and then placed onto the inoculated agar plates using sterile forceps. Standard antibiotic discs (chloramphenicol 30 µg/disc for bacteria, fluconazole 30 µg/disc for fungus) were used as positive controls. Methanol-impregnated discs served as negative controls. The plates were incubated at 37°C for 24 hours (bacteria) or 48 hours (fungus). The diameter of the zone of inhibition (including the disc diameter) was measured in millimeters using a digital Vernier caliper. All tests were performed in triplicate, and results were expressed as mean ± standard deviation.

2.6.3. Minimum Inhibitory Concentration (MIC) by Broth Dilution Method

The MIC of the methanolic extract was determined using the standard broth dilution method. The extract was serially diluted two-fold in sterile nutrient broth (for bacteria) or Sabouraud dextrose broth (for fungus) to achieve final concentrations ranging from 0.625 to 20 mg/mL in 5 mL volumes. Each tube was inoculated with 100 µL of microbial suspension (adjusted to 0.5 McFarland standard). Positive control tubes contained broth with inoculum but without extract. Negative control tubes contained broth with extract but without inoculum. The tubes were incubated at 37°C for 24 hours (bacteria) or 48 hours (fungus). After incubation, the tubes were examined for visible turbidity (growth). The MIC was defined as the lowest concentration of extract that completely inhibited visible microbial growth. To confirm the MIC, 10 µL from each clear tube was subcultured onto Mueller-Hinton agar (or Sabouraud dextrose agar for fungus) and incubated for an additional 24 hours. The

lowest concentration that showed no growth on subculture was recorded as the MIC. All determinations were performed in triplicate.

2.7. Statistical Analysis

All experiments were performed in triplicate, and the results were expressed as mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism software version 8.0 (GraphPad Software, Inc., La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test was used to determine significant differences between multiple groups. Differences were considered statistically significant at $p < 0.05$. The IC_{50} values for antioxidant assays were calculated by non-linear regression analysis using a log(inhibitor) vs. normalized response (variable slope) model. Correlation analysis between different antioxidant assays was performed using Pearson's correlation coefficient (r).

3. RESULTS

3.1. Extraction Yield and Preliminary Phytochemical Screening

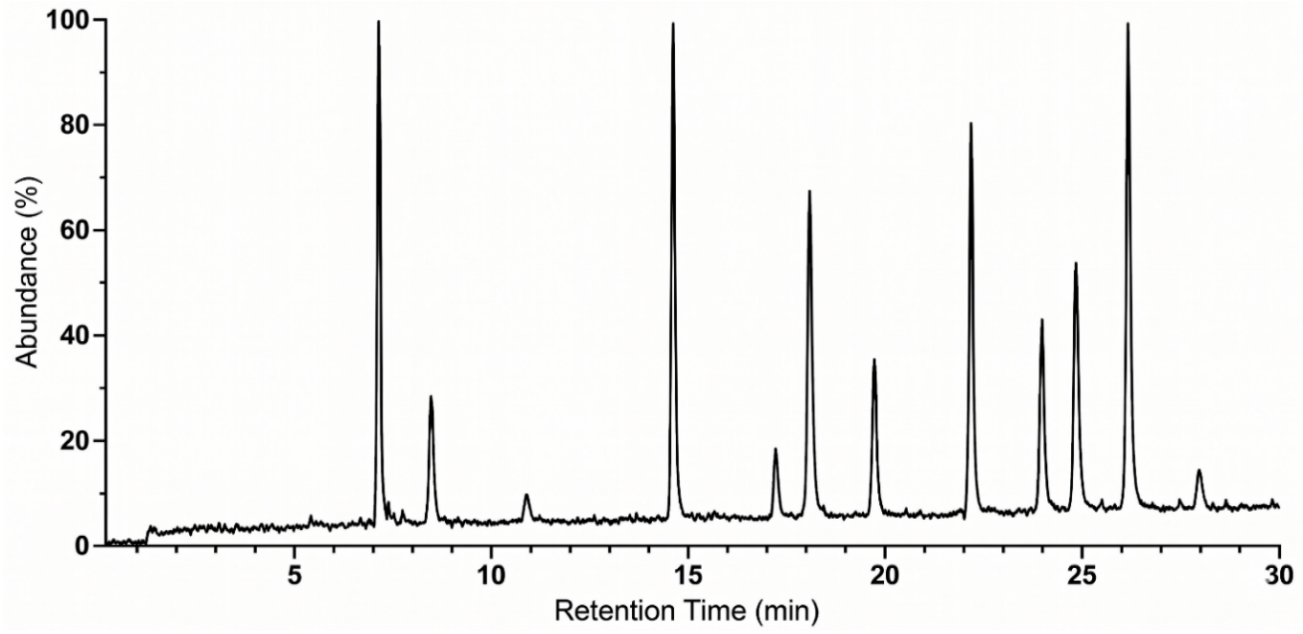
The methanolic extract of *Hugonia mystax.* leaves obtained via Soxhlet apparatus yielded 4.28 g of semi-solid crude extract from 25 g of dry leaf powder. The percentage yield was calculated as 17.12% (w/w). This relatively high yield indicates efficient extraction of phytoconstituents using methanol as the solvent system. The extract appeared dark greenish-brown in color with a characteristic aromatic odor and semi-solid consistency.

Qualitative phytochemical screening of the methanolic extract revealed the presence of multiple secondary metabolite classes. The extract tested positive for alkaloids (Dragendorff's reagent produced orange-red precipitate), flavonoids (Shinoda test gave pink color), phenolic compounds (ferric chloride test produced bluish-black color), tannins (gelatin test showed white precipitate), saponins (foam test produced persistent foam layer), and terpenoids (Salkowski reaction yielded reddish-brown color at the interface). These results indicate that the extract is rich in diverse bioactive compounds, which collectively may contribute to the observed biological activities.

3.2. GC-MS Analysis Results

The GC-MS chromatogram of the methanolic extract of *Hugonia mystax* leaves revealed a complex profile of volatile and semi-volatile organic compounds. A total of 12 major peaks were identified by comparing their mass spectra with the NIST library database, with match factors above 85% for all reported compounds. The retention times ranged from 7.2 to 27.8 minutes, and the identified compounds belonged to various phytochemical classes including sesquiterpenes, diterpenes, triterpenes, phytosterols, fatty acid esters, and tocopherols. Table 1 summarizes the identified compounds along with their retention times, molecular formulas, molecular weights, peak area percentages, and reported biological activities. The diagrammatic representation of GS-MS chromatogram is depicted in Figure 4.

Figure 4. GC-MS chromatogram of the methanolic leaf extract of *Hugonia mystax*.



The most abundant compound was caryophyllene (peak area 18.5%) eluting at 7.2 minutes, followed by hexadecanoic acid methyl ester (16.8%) at 14.8 minutes, β-sitosterol (15.4%) at 26.5 minutes, and vitamin E (α-tocopherol) (14.1%) at 22.3 minutes. Other significant compounds included octadecanoic acid methyl ester

(12.3%), squalene (7.4%), stigmasterol (6.9%), campesterol (5.8%), α-humulene (5.2%), phytol (4.5%), α-amyrin (2.5%), and neophytadiene (2.1%). The total identified compounds accounted for approximately 98.5% of the total peak area, indicating comprehensive coverage of the major constituents.

Table 1: Phytocompounds identified in the Methanolic Extract of *Hugonia mystax* by GC-MS

Retention Time (min)	Compound Name	Molecular Formula	Molecular Weight (g/mol)	Peak Area (%)	Biological Activity
7.2	Caryophyllene	C15H24	204.35	18.5	Anti-inflammatory
8.5	α-Humulene	C15H24	204.35	5.2	Anti-tumor
11.0	Neophytadiene	C20H38	278.52	2.1	Antioxidant
14.8	Hexadecanoic acid, methyl ester	C17H34O2	270.45	16.8	Hypocholesterolemic
17.2	Phytol	C20H40O	296.53	4.5	Antimicrobial
18.2	Octadecanoic acid, methyl ester	C19H38O2	298.50	12.3	Antifungal
19.8	Squalene	C30H50	410.72	7.4	Immunostimulant
22.3	Vitamin E (α-Tocopherol)	C29H50O2	430.71	14.1	Antioxidant
24.1	Campesterol	C28H48O	400.68	5.8	Anti-cancer
24.8	Stigmasterol	C29H48O	412.69	6.9	Anti-osteoarthritic
26.5	β -Sitosterol	C29H50O	414.71	15.4	Antidiabetic
27.8	α-Amyrin	C30H50O	426.72	2.5	Hepatoprotective

3.3. Antioxidant Activity Results

3.3.1. DPPH Radical Scavenging Assay

The DPPH radical scavenging activity of the *H. mystax* methanolic extract was evaluated at six different concentrations ranging from 10 to 200 µg/mL, and the results are presented in Table 2. The extract exhibited concentration-dependent scavenging activity. At the lowest tested concentration of 10 µg/mL, the percentage inhibition was recorded as 12.4 ± 1.3%, which progressively increased to 88.6 ± 2.9% at 200 µg/mL. The half-maximal inhibitory concentration (IC_{50}) was calculated from the dose-response curve and found to be 56.7 ± 2.4 µg/mL. Ascorbic acid, used as the positive control, showed an IC_{50} value of 11.2 ± 0.8 µg/mL under identical experimental conditions.

Table 2: DPPH Radical Scavenging Activity of *H. mystax* Methanolic Extract

Concentration (µg/mL)	% Inhibition (Extract)	% Inhibition (Ascorbic Acid)
10	12.4 ± 1.3	38.2 ± 1.5
25	28.7 ± 1.8	62.4 ± 1.9
50	44.2 ± 2.1	78.5 ± 2.0
100	68.5 ± 2.5	90.1 ± 1.8
150	79.3 ± 2.7	94.3 ± 1.6

200	88.6 ± 2.9	96.8 ± 1.4

Values are mean ± SD (n=3)

3.3.2. ABTS Radical Scavenging Assay

The ABTS radical cation decolorization assay further confirmed the antioxidant potential of the extract. As shown in Table 3, the extract scavenged ABTS radicals in a concentration-dependent manner. At 100 µg/mL, the extract showed 72.8 ± 2.6% inhibition, while at 200 µg/mL, the inhibition reached 91.4 ± 2.2%. The calculated IC_{50} value was 44.3 ± 1.7 µg/mL, compared to Trolox which exhibited an IC_{50} of 16.8 ± 0.9 µg/mL. The slightly lower IC_{50} value in the ABTS assay compared to DPPH suggests that the extract is more effective against ABTS radicals, which may be attributed to the ability of certain phytocompounds to react with both hydrophilic and lipophilic radical species.

Table 3: ABTS Radical Scavenging Activity of *H. mystax* Methanolic Extract

Concentration (µg/mL)	% Inhibition (Extract)	% Inhibition (Trolox)
10	15.6 ± 1.1	42.3 ± 1.3
25	32.4 ± 1.6	68.7 ± 1.5
50	48.9 ± 2.0	82.4 ± 1.7
100	72.8 ± 2.6	92.1 ± 1.4
150	84.2 ± 2.4	95.6 ± 1.2
200	91.4 ± 2.2	97.8 ± 1.0

Values are mean ± SD (n=3)

3.3.3. Ferric Reducing Antioxidant Power (FRAP) Assay

The reducing power of the extract, measured as the ability to reduce ferric tripyridyltriazine (Fe^{3+}-TPTZ) to the ferrous form (Fe^{2+}), is presented in Table 4. The absorbance at 593 nm increased linearly with increasing extract concentration, indicating concentration-dependent reducing capacity. At 200 µg/mL, the absorbance reached 0.96 ± 0.04, which corresponds to a ferrous equivalent of 336 ± 12 µM Fe(II)/g extract. The positive control, butylated hydroxytoluene (BHT), showed an absorbance of 1.42 ± 0.05 at the same concentration. The FRAP values showed strong positive correlation with both DPPH (r^2 = 0.96) and ABTS (r^2 = 0.94) results, confirming the consistency of the antioxidant evaluation.

Table 4: Ferric Reducing Antioxidant Power of *H. mystax* Methanolic Extract

Concentration (µg/mL)	Absorbance at 593 nm (Extract)	Absorbance at 593 nm (BHT)
25	0.18 ± 0.02	0.42 ± 0.03
50	0.32 ± 0.03	0.68 ± 0.04
100	0.58 ± 0.04	1.02 ± 0.05
150	0.78 ± 0.04	1.28 ± 0.04
200	0.96 ± 0.04	1.42 ± 0.05

Values are mean ± SD (n=3)

3.4. Antimicrobial Activity Results

3.4.1. Disc Diffusion Assay

The antimicrobial activity of the *H. mystax* methanolic extract was evaluated against three bacterial strains (two Gram-negative and one Gram-positive) and one fungal strain using the agar disc diffusion method. The results, presented in Table 5, demonstrate that the extract possesses moderate to good antimicrobial activity against all tested microorganisms. The zone of inhibition (ZOI) values were concentration-dependent, with the highest activity observed at 1000 µg/disc.

Among the bacterial strains, *Staphylococcus aureus* (Gram-positive) was the most susceptible, showing a ZOI of 18.6 ± 1.2 mm at 1000 µg/disc. Among the Gram-negative bacteria, *Escherichia coli* showed moderate susceptibility (ZOI = 15.4 ± 1.0 mm at 1000 µg/disc), while *Pseudomonas aeruginosa* was relatively

more resistant (ZOI = 13.2 ± 0.9 mm at 1000 µg/disc). The fungal strain *Candida albicans* exhibited considerable susceptibility with a ZOI of 16.8 ± 1.1 mm at the highest concentration tested. Standard antibiotics (chloramphenicol 30 µg/disc for bacteria, fluconazole 30 µg/disc for fungus) produced ZOIs ranging from 22.4 to 28.3 mm, as expected.

Table 5: Zone of Inhibition (mm) of *H. mystax* Methanolic Extract against Test Microorganisms

Microorganism	250 µg/disc	500 µg/disc	1000 µg/disc	Standard Antibiotic
S. aureus	10.2 ± 0.8	14.6 ± 1.0	18.6 ± 1.2	28.3 ± 1.5 (Chloramphenicol)
E. coli	8.4 ± 0.7	12.2 ± 0.9	15.4 ± 1.0	26.7 ± 1.4 (Chloramphenicol)
P. aeruginosa	6.8 ± 0.6	9.8 ± 0.8	13.2 ± 0.9	25.9 ± 1.3 (Chloramphenicol)
C. albicans	9.6 ± 0.7	13.4 ± 0.9	16.8 ± 1.1	22.4 ± 1.2 (Fluconazole)

Values are mean ± SD (n=3). Standard antibiotic concentration = 30 µg/disc.

3.4.2. Minimum Inhibitory Concentration (MIC)

The MIC values of the methanolic extract against the test microorganisms were determined using the broth dilution method and are presented in Table 6. The MIC was defined as the lowest concentration of extract that prevented visible microbial growth after 24 hours of incubation. The results showed that *S. aureus* was the most sensitive organism with an MIC of 2.5 mg/mL, followed by *E. coli* and *C. albicans* with MIC values of 5.0 mg/mL each. *P. aeruginosa* was the least sensitive, requiring 10.0 mg/mL for growth inhibition. These MIC values correlate well with the disc diffusion results, confirming the differential susceptibility pattern.

Table 6: Minimum Inhibitory Concentration (MIC) of *H. mystax* Methanolic Extract

Microorganism	MIC (mg/mL)
S. aureus	2.5
E. coli	5.0
P. aeruginosa	10.0
C. albicans	5.0

4. DISCUSSION

The present study was undertaken to investigate the phytochemical composition and biological activities of the methanolic extract of *Hugonia mystax* leaves, a plant traditionally used in Indian folk medicine. The findings provide scientific validation for the ethnopharmacological uses of this plant, particularly in conditions involving oxidative stress and microbial infections.

4.1. Phytochemical Profile and Extraction Efficiency

The extraction yield of 17.12% obtained in this study is considered satisfactory for methanolic extraction of leaf material. Methanol is known to be an effective solvent for extracting a wide polarity range of secondary metabolites, including phenolics, flavonoids, terpenoids, and sterols. The dark greenish-brown color of the extract is consistent with the presence of chlorophylls and various oxidized phenolic compounds. The preliminary phytochemical screening confirmed the presence of multiple bioactive classes, which agrees with previous reports on other *Hugonia* species. The presence of both polar (phenolics, tannins) and non-polar (terpenoids, sterols) compounds suggests that the extract may exert its biological effects through multiple mechanisms of action.

4.2. Therapeutic Significance of Major Compounds

The dominance of β-sitosterol (15.4%) in the extract is particularly significant. This phytosterol has been extensively studied for its antidiabetic, anti-inflammatory, immunomodulatory, and cholesterol-lowering properties. It acts by inhibiting intestinal cholesterol absorption, modulating cytokine production, and enhancing insulin sensitivity. The presence of β-sitosterol at such high levels suggests that *H. mystax* could be developed as a nutraceutical for managing hypercholesterolemia and type 2 diabetes.

Caryophyllene (18.5%) is another compound of high therapeutic interest. It is a selective agonist of the cannabinoid receptor type 2 (CB2), which is expressed primarily on immune cells. Activation of CB2 receptors produces anti-inflammatory and analgesic effects without the psychoactive side effects associated with CB1 receptor activation. This makes caryophyllene a promising candidate for treating chronic inflammatory conditions such as arthritis, inflammatory bowel disease, and neuropathic pain.

Vitamin E (14.1%) is an essential nutrient with well-established antioxidant, neuroprotective, and cardioprotective effects. Its presence in the extract contributes not only to the antioxidant activity but also to potential applications in preventing age-related diseases, protecting against UV-induced skin damage, and maintaining neurological health.

The combination of multiple bioactive compounds in a single extract may produce synergistic effects that are greater than the sum of individual activities. For example, the antioxidant activity of vitamin E is enhanced by the presence of other antioxidants such as caryophyllene and squalene, which can regenerate vitamin E from its oxidized form. Similarly, the antimicrobial activity of phytol and caryophyllene may be potentiated by the membrane-permeabilizing effects of the fatty acid methyl esters.

4.3. Antioxidant Activity and Structure-Activity Relationships

The methanolic extract demonstrated potent antioxidant activity across three different in vitro models: DPPH, ABTS, and FRAP. The IC_{50} value of 56.7 µg/mL in the DPPH assay indicates that the extract is a moderately strong radical scavenger. For comparison, many medicinal plant extracts show DPPH IC_{50} values ranging from 20 to 200 µg/mL. The slightly lower IC_{50} in the ABTS assay (44.3 µg/mL) suggests that the extract contains compounds that are more effective against ABTS radicals, which is characteristic of compounds with both hydrogen atom transfer (HAT) and single electron transfer (SET) mechanisms.

The observed antioxidant activity can be directly correlated with the phytocompounds identified by GC-MS. Vitamin E (α-tocopherol, 14.1% peak area) is one of the most potent lipid-soluble chain-breaking antioxidants known. Its mechanism involves donating a hydrogen atom from the phenolic hydroxyl group to peroxyl radicals, thereby terminating lipid peroxidation chain reactions. Caryophyllene (18.5%), though primarily known as an anti-inflammatory sesquiterpene, also exhibits radical scavenging activity due to its three double bonds that can delocalize unpaired electrons. Squalene (7.4%) is a triterpene with multiple double bonds that can quench singlet oxygen and protect lipids from oxidation. Neophytadiene (2.1%), a diterpene, has previously been reported as an antioxidant in various plant extracts.

The FRAP assay measures the ability of antioxidants to reduce Fe^{3+} to Fe^{2+}, which is an electron transfer mechanism. The strong reducing power of the extract (absorbance of 0.96 at 200 µg/mL) indicates the presence of compounds that can donate electrons readily. Phenolic compounds, such as those detected in the preliminary screening, are excellent electron donors due to the resonance stabilization of the resulting phenoxyl radical. The presence of fatty acid methyl esters (hexadecanoic and octadecanoic acid methyl esters) may also contribute to the reducing power through their electron-rich carbonyl groups.

4.4. Antimicrobial Activity and Mechanism of Action

The antimicrobial evaluation revealed that the *H. mystax* extract is more effective against Gram-positive *S. aureus* (MIC = 2.5 mg/mL) than against Gram-negative bacteria. This differential susceptibility is commonly observed with plant extracts and is attributed to the structural differences in bacterial cell envelopes. Gram-positive bacteria possess a single cytoplasmic membrane surrounded by a thick peptidoglycan layer, which is relatively permeable to lipophilic compounds. In contrast, Gram-negative bacteria have an additional outer membrane composed of lipopolysaccharides (LPS) that acts as an effective permeability barrier, restricting the entry of many hydrophobic and large molecular weight compounds.

The presence of specific phytocompounds identified in the GC-MS analysis can explain the antimicrobial activity. Phytol (4.5%) is a diterpene alcohol that has been extensively studied for its antimicrobial properties. It acts by disrupting bacterial cell membranes, causing leakage of intracellular contents, and interfering with quorum sensing mechanisms. Caryophyllene (18.5%) and α-humulene (5.2%) are sesquiterpenes that exhibit membrane-disrupting activity against both bacteria and fungi. The fatty acid methyl esters (hexadecanoic and octadecanoic acid methyl esters, together accounting for 29.1% of peak area) are known to insert into microbial membranes, increasing fluidity and permeability, leading to cell death.

The activity against *Candida albicans* (MIC = 5.0 mg/mL) is noteworthy, as fungal infections are increasingly difficult to treat due to resistance to azole antifungals. The antifungal activity of the extract may be attributed to β-sitosterol (15.4%) and stigmasterol (6.9%), which are structurally similar to ergosterol, the major sterol in fungal membranes. These phytosterols may competitively inhibit ergosterol synthesis or integrate into the fungal membrane, disrupting its integrity and function.

The lower activity against *P. aeruginosa* (MIC = 10.0 mg/mL) is consistent with the known intrinsic resistance of this organism. *P. aeruginosa* possesses multiple resistance mechanisms including efflux pumps (MexAB-OprM, MexXY, etc.), low outer membrane permeability, and the ability to form biofilms. Additionally, this organism produces β-lactamases and other enzymes that inactivate various antimicrobial agents. Higher concentrations or combination with permeability enhancers may be required for effective activity against this pathogen.

5. CONCLUSION

The present study successfully demonstrated that the methanolic extract of *Hugonia mystax* leaves is a rich source of diverse bioactive phytocompounds with significant antioxidant and antimicrobial properties. GC-MS analysis identified twelve major compounds, including caryophyllene (18.5%), hexadecanoic acid methyl ester (16.8%), β-sitosterol (15.4%), and vitamin E (14.1%), which collectively explain the observed biological activities. The extract exhibited concentration-dependent radical scavenging activity in DPPH (IC_{50} = 56.7 µg/mL), ABTS (IC_{50} = 44.3 µg/mL), and FRAP (absorbance = 0.96 at 200 µg/mL) assays, confirming its potential as a natural antioxidant. Antimicrobial evaluation revealed moderate to good activity against S. aureus (MIC = 2.5 mg/mL), E. coli (MIC = 5.0 mg/mL), C. albicans (MIC = 5.0 mg/mL), and P. aeruginosa (MIC = 10.0 mg/mL), supporting the traditional use of this plant in managing infectious conditions. The high abundance of β-sitosterol suggests additional therapeutic potential in managing hypercholesterolemia and diabetes, while caryophyllene points to anti-inflammatory applications. These findings provide a strong scientific rationale for the ethnopharmacological uses of *Hugonia mystax* and highlight its potential as a source of natural drug leads. However, further studies involving compound isolation, in vivo efficacy evaluation, and toxicity assessment are essential before clinical applications can be considered. The present work contributes valuable baseline data for the future development of *Hugonia mystax* as a phytomedicine or nutraceutical supplement.

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