2026 Volume: 02 Issue: 03 www.ijrpa.com ISSN 2456-9995 Research Article

International Journal Research Publication Analysis

Page: 01-40

BIOACTIVE CONSTITUENT SCREENING AND IDENTIFICATION IN EUPHORBIA HIRTA (AMMAN PACHARISI, COMMONLY KNOWN AS ASTHMA PLANT.

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Article Received: 21 February 2026
Article Revised: 11 March 2026
Published on: 31 March 2026

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DOI: https://doi-doi.org/101555/ijrpa.1417

ABSTRACT

The present study investigated the phytochemical composition and antimicrobial potential of the ethanolic leaf extract of *Euphorbia hirta* L. (commonly known as Amman Pacharisi), collected from a pesticide-free medicinal garden in Salem, Tamil Nadu, India, during the peak flowering stage. The plant material was processed through careful shade drying, manual pulverization, and cold maceration in ethanol (1:50 w/v, 72 h at 25–30 °C) to preserve thermolabile bioactive constituents effectively.Gas chromatography–mass spectrometry (GC–MS) profiling of the crude extract identified 12 major and minor compounds, predominantly triterpenoids, sterols, fatty acids, and derivatives. Dominant constituents included lupeol (18.5%), 9,19-cyclolanost-23-ene-3,25-diol, 3-acetate (14.2%), an acetic acid derivative (11.7%), and β-sitosterol (9.8%), contributing substantially to the total ion chromatogram, with late-eluting compounds comprising approximately 65% of peak area. These phytochemicals are linked to mechanisms such as membrane disruption, enzyme inhibition, cytokine modulation, antioxidant activity, and apoptosis induction.In vitro disc diffusion assays demonstrated robust antibacterial activity against *Escherichia coli* (18 mm zone of inhibition) and *Staphylococcus aureus* (20 mm), surpassing amoxicillin (10 µg/disc) against *E. coli* and yielding comparable results against *S. aureus*. Ethanol solvent controls showed no inhibition, confirming the plant-derived efficacy.Literature correlations underscore strong therapeutic relevance against bacterial infections, inflammatory conditions, oxidative stress-related disorders, and emerging anticancer effects, aligning closely with traditional applications for respiratory, gastrointestinal, and infectious ailments.This project holds significant industrial importance in the herbal medicine and natural products sector. The

identified bioactive-rich extract, enriched with high-value compounds like lupeol, β-sitosterol, and phytol derivatives, supports applications in pharmaceutical formulations for antimicrobial, anti-inflammatory, and antioxidant products. Such natural extracts contribute to the growing demand for plant-based therapeutics, nutraceuticals, and functional ingredients in the herbal industry, offering sustainable, multi-target alternatives to synthetic agents and promoting ethnopharmacological validation for commercial herbal preparations.

KEYWORD: Active compound, Euphorbia hirta, *Staphylococcus aureus.*

1. INTRODUCTION

Euphorbia hirta Linn., a member of the Euphorbiaceae family, is a ubiquitous herbaceous plant that has garnered significant attention in ethnopharmacology and modern scientific research due to its diverse phytochemical profile and multifaceted biological activities. Commonly referred to as asthma weed, pill-bearing spurge, garden spurge, snakeweed, or tawa-tawa in various regions, this annual or perennial herb exhibits a prostrate to ascending growth habit, typically reaching heights of 15-50 cm, though it can vary based on environmental conditions (Ghosh et al., 2019; Kumar et al., 2010). The stems are slender, branched, and covered with yellowish or reddish multicellular hairs, giving the plant a somewhat pilose appearance. Leaves are opposite, simple, ovate-lanceolate to elliptical, with finely serrated margins, measuring 1-4 cm in length and 0.5-1.5 cm in width, and often display a reddish-purple tinge along the midrib or base, particularly under stress conditions (Al-Snafi, 2017; De and Sharma, 2023). The inflorescences are dense, axillary or terminal cyathia—characteristic of the Euphorbia genus—comprising minute unisexual flowers enclosed in a cup-like involucre with glandular appendages. Fruits are small, tricoccous capsules, approximately 1-2 mm in diameter, that dehisce explosively to release ovoid, reddish-brown seeds with a caruncle (Ghosh et al., 2019; Basu, 1998). This morphological adaptability allows E. hirta to thrive in a wide array of habitats, including disturbed soils, roadsides, agricultural fields, and urban waste areas across tropical and subtropical climates, from sea level to elevations of about 2000 meters (Council of Industrial and Scientific Research, 2005; Open Source for Weed Assessment in Lowland Paddy Fields, 2007). Its weedy nature, facilitated by prolific seed production and rapid germination, has led to its classification as an invasive species in some regions, yet this same resilience underscores its availability for traditional medicinal harvesting (Sharma, 2020; Samant et al., 2001).

The historical and ethnobotanical significance of E. hirta spans millennia, rooted in indigenous knowledge systems worldwide. In ancient Indian texts, such as those compiled in the Ayurvedic tradition, the plant is documented under names like "Dugdhika" or "Chhoti Dudhi," prized for its purported ability to alleviate respiratory disorders, including asthma—hence its common name "asthma weed"—as well as skin ailments, wounds, and gastrointestinal complaints (Kirtikar and Basu, 2003; Basu, 1998; Williamson, 2002). Traditional healers among the Van Gujjars of Uttarakhand, India, have utilized E. hirta specifically for treating warts, applying fresh latex directly to lesions, a practice that aligns with its antiviral and keratolytic properties observed in modern studies (Bhasker Joshi, 2011). In African ethnomedicine, particularly in Nigeria, Tanzania, and other sub-Saharan countries, E. hirta is integral to remedies for enteric infections, malaria, and microbial diseases, often administered as decoctions or infusions of the whole plant (Abubakar, 2009; Mbwale et al., 2025; Mohamed et al., 1996). The plant's latex and leaf extracts are employed topically for snakebites and scorpion stings, leveraging its analgesic and anti-inflammatory effects (Ogbulie et al., 2007; Sood et al., 2005). In Southeast Asia, including the Philippines and Malaysia, E. hirta is known as "gatas-gatas" or "tawa-tawa," and has gained prominence in folk treatments for dengue fever, where infusions are believed to increase platelet counts and mitigate hemorrhagic symptoms, a use supported by recent systematic reviews (Yam et al., 2018; Perera et al., 2018; Rajeh et al., 2010). Chinese integrative medicine incorporates E. hirta for urinary tract infections, conjunctivitis, and postpartum care, enhancing lactation and addressing reproductive health issues (Asgarpanah and Kazemivash, 2013; Nyeem et al., 2017). In Latin American traditions, such as those in Mexico, the plant is used for anti-inflammatory purposes, with hexane extracts yielding compounds active against edema (Martinez et al., 1999). These cross-cultural applications highlight E. hirta's versatility, often attributed to its milky latex containing bioactive euphorbol esters and other secondary metabolites (Kumar et al., 2010; Ghosh et al., 2019). However, while anecdotal evidence abounds, the transition from traditional lore to evidence-based medicine necessitates rigorous phytochemical characterization and bioactivity assays (Al-Snafi, 2017; Kausar et al., 2016).

phytochemical characterization of *Euphorbia hirta* Linn. begins with systematic extraction and screening protocols designed to isolate and identify the plant's diverse array of chemical constituents, particularly secondary metabolites that underpin its traditional medicinal uses and pharmacological potential. These protocols are foundational in natural products research, enabling the transition from ethnobotanical knowledge to evidence-based validation of bioactivity (Ghosh et al., 2019; Al-Snafi, 2017). The process typically commences with

careful collection and preparation of plant material, followed by extraction, qualitative screening, quantitative estimation, and advanced instrumental analysis. This multi-step approach ensures comprehensive profiling while minimizing degradation of sensitive compounds.

The initial stage involves harvesting fresh *E. hirta* plants, often focusing on aerial parts (leaves, stems, whole plant) due to their higher metabolite concentration, though roots and latex are also studied (Basma et al., 2011; Baba et al., 2025). Collection is preferably done during optimal seasons (e.g., flowering or post-monsoon) to maximize secondary metabolite yield, as environmental factors like soil, altitude, and climate influence composition (Sharma et al., 2023; De and Sharma, 2023). Plants are authenticated by botanists or herbaria to avoid misidentification, a common issue with weedy Euphorbiaceae species.

Preparation starts with thorough washing to remove soil, dust, and contaminants, using distilled water or mild detergents if needed. Air-drying at room temperature (25–35°C) in shade is the preferred method to preserve thermolabile compounds such as flavonoids, volatile terpenoids, and glycosides, which can degrade under heat or direct sunlight (Basma et al., 2011; Igwe et al., 2016; Sudhan et al., 2021). Shade drying typically takes 7–14 days until constant weight is achieved, with moisture content reduced to <10% to prevent microbial growth. Oven drying at low temperatures (40–50°C) is sometimes used for faster processing but risks partial loss of volatiles (Redfern et al., 2014). Dried material is coarsely powdered using a mechanical grinder or mortar to increase surface area for solvent penetration, with particle size ideally 0.5–1 mm for optimal extraction efficiency (Jensen, 2007). Powdered samples are stored in airtight, light-protected containers at cool temperatures to maintain stability.

Extraction is the critical step for solubilizing phytochemicals, with choice of method and solvent dictating yield and metabolite profile. Traditional and modern techniques aim to mimic folk preparations while maximizing recovery of bioactive classes.

Soxhlet Extraction remains a gold standard for exhaustive extraction, invented in the late 19th century by Franz von Soxhlet. It involves continuous solvent recycling through a thimble containing the powdered plant, allowing repeated contact and high efficiency for non-polar to mid-polar compounds (Jensen, 2007; Redfern et al., 2014). Solvents are selected by polarity gradient: hexane or petroleum ether for lipids, sterols, and terpenoids; chloroform or dichloromethane for mid-polar terpenoids and alkaloids; ethyl acetate for flavonoids and phenolics; and methanol or ethanol for polar glycosides, tannins, and saponins (Sudhan et al.,

2021; Baba et al., 2025). For example, Basma et al. (2011) used methanol Soxhlet extraction on *E. hirta* leaves, achieving an 18.5% yield, rich in phenolics and flavonoids. Baba et al. (2025) applied similar methods to whole plants, yielding comprehensive profiles via GC-MS, including fatty acids and terpenoids. Yields vary: ethanol often gives 14–32%, methanol 9–20%, aqueous 7–58%, hexane 2–15%, and ethyl acetate 3–10%, depending on plant part and conditions (Asha, 2015; Suarjana, 2025; various studies 2015–2025). Soxhlet runs for 24–72 hours until exhaustion, with extracts concentrated under reduced pressure using rotary evaporators to remove solvent.

Maceration (cold extraction) simulates traditional decoctions/infusions, involving soaking powdered material in solvent at room temperature with occasional shaking for 24–72 hours (De and Sharma, 2023; Basyal et al., 2021). It preserves heat-sensitive compounds better than Soxhlet and is used for aqueous or hydroalcoholic extracts capturing water-soluble glycosides and phenolics. Yields are generally lower but more representative of folk uses.

Other Methods include reflux (hot solvent extraction), ultrasonic-assisted extraction (faster, higher yields via cavitation), microwave-assisted (rapid heating), and supercritical fluid extraction (CO_2 for green, selective non-polar extracts). Recent studies (2020–2026) favor sequential extraction (non-polar to polar) to fractionate metabolites (Tran et al., 2020; Waheed et al., 2025). Aqueous extracts are common for simulating herbal teas, yielding glycosides, tannins, and some phenolics (Basyal et al., 2021).

Extracts are filtered (Whatman No. 1), concentrated, and stored at 4°C or lyophilized for stability. Percentage yield is calculated as (weight of extract / weight of dry powder) × 100, providing an initial efficiency indicator.

Qualitative screening detects presence/absence of major secondary metabolite classes using color/precipitate-based tests (standardized protocols from Harborne, Trease & Evans, Sofowora). These are rapid, cost-effective preliminaries before quantification or isolation.

Alkaloids: Dragendorff's reagent (orange precipitate) or Mayer's (creamy precipitate); Wagner's (reddish-brown). *E. hirta* consistently tests positive in leaves/stems (Sharma et al., 2023; Waheed et al., 2025; Subramani, 2025; Baba et al., 2025). Reducing Sugars/Carbohydrates: Fehling's (red brick precipitate) or Benedict's (red/orange); Molisch's (violet ring). Present in most extracts (Ghosh et al., 2019). Saponins: Foam test (stable froth >1 cm on shaking); hemolysis test. Positive across polar extracts (Basyal et al., 2021; Sudhan et al., 2021). Steroids/Terpenoids: Liebermann-Burchard (violet ring/color change); Salkowski (red-brown). Ubiquitous in non-polar/hexane fractions (Rautela et al., 2020; Al Abboud et al., 2023). Phenols/Tannins: Ferric chloride (blue-green/black); lead acetate (white

precipitate). High in leaves/methanol extracts (Sharma et al., 2023; Tran et al., 2020). Flavonoids: Shinoda test (pink/red); alkaline reagent (yellow). Dominant in leaves (Basma et al., 2011; Singh et al., 2013). Glycosides: Keller-Kiliani (brown ring); Borntrager's (pink/red for anthraquinones). Variable, often in polar extracts. Coumarins, Quinones, Anthraquinones: Specific tests (e.g., UV fluorescence for coumarins). Frequently detected (De and Sharma, 2023; Subramani, 2025).

Studies (2019–2026) confirm alkaloids, flavonoids, tannins, saponins, steroids, terpenoids, glycosides, phenols, coumarins, quinones, and anthraquinones in *E. hirta*, with organ-specific variations: leaves richest in flavonoids/phenols (antioxidant sources), stems in tannins (astringent), roots in steroids/terpenoids (Ghosh et al., 2019; Rautela et al., 2020; Al Abboud et al., 2023; Baba et al., 2025; Shaikh, 2023

Quantitative estimation further refines this profile. Total phenolic content (TPC) is determined via Folin-Ciocalteu assay, expressed as gallic acid equivalents (GAE), with values ranging from 83.4-254.6 mg GAE/g in leaf extracts, highest in ethyl acetate fractions (Basma et al., 2011; Tran et al., 2020; Sharma et al., 2023). Total flavonoid content (TFC) uses aluminum chloride colorimetric method, yielding 18.2-37.5 mg quercetin equivalents (QE)/g, correlating with antioxidant potential (Basyal et al., 2021; Sharma et al., 2014). Tannin content, via vanillin-HCl assay, reaches 12-45 mg catechin equivalents/g, while alkaloid yields are 0.5-2.8% (De and Sharma, 2023; Waheed et al., 2025). Saponins and steroids are quantified gravimetrically or spectrophotometrically, showing 1-5% and 0.2-1.5%, respectively (Silwal, 2023; Baba et al., 2025). These quantifications vary with geographical origin, season, and extraction solvent; for example, plants from Northern Punjab exhibit higher TPC (254.6 mg GAE/g) than those from Nepal (83.4 mg GAE/g), possibly due to soil nutrients or altitude (Sharma et al., 2023; Basyal et al., 2021).

Advanced instrumental techniques enable precise identification of bioactive compounds. Gas chromatography-mass spectrometry (GC-MS) couples separation with mass fragmentation, referencing libraries like NIST or Wiley for compound matching (McLafferty, 1986; Stein, 1990; Igwe et al., 2016). In methanol leaf extracts, Igwe et al. (2016) identified 17 compounds, including niacin (nicotinic acid, C6H5NO2, MW 123, 31.70% area, retention time (RT) 7.85 min), known for vasodilatory effects (Bruckert et al., 2010; Duggal et al., 2010); S-methyl-L-cysteine (C4H9NO2S, MW 135, 2.94%, RT 3.45); 2-amino-3-sulfanylpropanoic acid (cysteine, C3H7NO2S, MW 121); chloromorpholin-4-ium (C4H9ClNO+, MW 122); 2,3,5-trimethyl-1H-pyrrole (C7H11N, MW 109); 4-amino-4-oxobut-2-enoic acid (maleamic acid, C4H5NO3, MW 115); and 17-carboxyheptadec-9-en-1-

ylium (oleic acid derivative, C18H33O2+, MW 281). Other studies report phytol (C20H40O, MW 296, 8.5-15%, acyclic diterpene alcohol with antimicrobial properties) (Inoue et al., 2005; McGinty et al., 2010; Barretto and Vootla, 2018); cycloartenol (C30H50O, MW 426, 34.7%, triterpenoid with membrane-stabilizing effects) (Rautela et al., 2020; Lozano-Grande et al., 2018); alpha-amyrin (C30H50O, MW 426, 10.7%, anti-inflammatory triterpene) (Panda et al., 2009); clionasterol (gamma-sitosterol, C29H50O, MW 414, 10.4%); linoleic acid (C18H32O2, MW 280, 7.7%, essential fatty acid); palmitic acid (C16H32O2, MW 256, 7.2%); squalene (C30H50, MW 410, antioxidant terpenoid) (Ghimire et al., 2016); megastigmatrienone (C13H18O, MW 190, flavor compound); heneicosane (C21H44, MW 296, alkane with microbicidal activity) (Vanitha et al., 2020); and brevifolincarboxylic acid derivatives (Yang et al., 2020). Baba et al. (2025) added fatty acid esters like methyl palmitate (C17H34O2, MW 270) and ethyl oleate (C20H38O2, MW 310), emphasizing lipid diversity.

High-performance liquid chromatography (HPLC) and liquid chromatography-mass spectrometry (LC-MS) target polar compounds. Flavonol glycosides isolated include afzelin (kaempferol-3-O-rhamnoside, C21H20O10, MW 432) (Liu et al., 2007); quercitrin (quercetin-3-O-rhamnoside, C21H20O11, MW 448, antidiarrheal) (Galvez et al., 1993); myricitrin (myricetin-3-O-rhamnoside, C21H20O12, MW 464); rutin (quercetin-3-O-rutinoside, C27H30O16, MW 610); quercetin (C15H10O7, MW 302, antioxidant) (Trinh and Le, 2014; Alam et al., 2022); kaempferol (C15H10O6, MW 286); isoquercitrin (quercetin-3-O-glucoside, C21H20O12, MW 464); hyperoside (quercetin-3-O-galactoside, C21H20O12, MW 464); and myricetin-3-O-rhamnoside (C21H20O12, MW 464) (Cayona and Creencia, 2022; Rao et al., 2017; Singh et al., 2013). Phenolic acids encompass gallic acid (C7H6O5, MW 170); protocatechuic acid (C7H6O4, MW 154); syringic acid (C9H10O5, MW 198); chlorogenic acid (C16H18O9, MW 354); caffeic acid (C9H8O4, MW 180); and galloylated tannins like euphorbin A-D, digallic acid (C14H10O9, MW 322), trigallic acid (C21H14O13, MW 474), and corilagin (C27H22O18, MW 634) (Yang et al., 2020; Meda et al., 2023). Triterpenes feature α-amyrin (pentacyclic, antihyperglycemic); β-amyrin; taraxerone (C30H48O, MW 424); taxerol; β-amyrin acetate; 11α,12α-oxidotaraxerol; 24-methylencycloartenol; and β-sitosterol (C29H50O, MW 414, cholesterol-lowering) (Kumar et al., 2010; Ghosh et al., 2019; Panda et al., 2009). Sterols include β-sitosterol-D-glucoside, cholesterol, brassicasterol, campesterol, and stigmasterol (Al-Snafi, 2017; Rautela et al., 2020).

Nuclear magnetic resonance (NMR) spectroscopy, including 1H-NMR, 13C-NMR, and 2D techniques like COSY, HSQC, and HMBC, confirms structures. For example, Yang et al. (2020) used NMR to elucidate chebulic acid (C14H12O11, MW 332) and brevifolincarboxylic acid (C13H8O8, MW 292) derivatives, assigning absolute configurations via CD spectroscopy. Fourier-transform infrared spectroscopy (FTIR) identifies functional groups: O-H stretch (3200-3600 cm^{-1} for phenols/alcohols), C=O (1700-1750 cm^{-1} for carbonyls), C-H (2800-3000 cm^{-1} for alkanes), and aromatic C=C (1450-1600 cm^{-1}) (Silwal, 2023; De and Sharma, 2023). Ultraviolet-visible (UV-Vis) spectroscopy detects chromophores, with flavonoids showing absorption at 250-280 nm (band II) and 300-400 nm (band I) (Basyal et al., 2021).

The antimicrobial activity of *Euphorbia hirta* Linn. is primarily attributed to its rich array of phytochemicals, including flavonoids (e.g., quercetin, kaempferol, rutin, quercitrin, myricitrin), phenolic acids (e.g., gallic acid, protocatechuic acid, chlorogenic acid), tannins (e.g., euphorbin A-D, corilagin), terpenoids (e.g., phytol, squalene, cycloartenol, α-amyrin), and other compounds like fatty acids and sterols. These metabolites manifest broad-spectrum effects as bacteriostatic (inhibiting growth), bactericidal (killing bacteria), fungistatic (slowing fungal growth), and virucidal (inactivating viruses), often through multiple synergistic mechanisms such as membrane disruption, enzyme inhibition, oxidative stress induction, and interference with quorum sensing or biofilm formation (Perumal et al., 2013; Yang et al., 2020; Tran et al., 2020; Al Abboud et al., 2023; Mbwale et al., 2025).

In vitro antimicrobial assays for *E. hirta* extracts typically employ standardized methods to quantify activity reliably and reproducibly. The disk diffusion (Kirby-Bauer) method involves impregnating sterile filter paper disks with extract concentrations (e.g., 10–100 mg/mL or µg/disk) and placing them on agar plates seeded with test organisms, followed by incubation at 37°C for 18–24 h. Zones of inhibition (ZOI) are measured in millimeters as clear areas around disks where microbial growth is absent. This qualitative/semi-quantitative technique correlates well with susceptibility (Abubakar, 2009; Ogbulie et al., 2007; Tran et al., 2020). The agar well diffusion variant creates wells (6–8 mm diameter) in seeded agar, fills them with extract, and measures ZOI similarly, offering advantages for viscous or high-concentration samples (Sudhan et al., 2021; Iskandar et al., 2021). For precise quantification, broth microdilution determines minimum inhibitory concentration (MIC)—the lowest concentration preventing visible growth—and minimum bactericidal/fungicidal concentration (MBC/MFC)—the lowest killing ≥99.9% of inoculum—using 96-well plates with serial dilutions (typically 0.031–100 mg/mL), turbidity monitoring via optical density (OD600),

and subculturing on agar for MBC (Perumal et al., 2013; Mbwale et al., 2025). Time-kill kinetics monitors viable counts over time (e.g., 0–48 h) at 1×, 2×, 4× MIC to distinguish bacteriostatic (≤3 log reduction) from bactericidal (>3 log reduction) effects, revealing concentration- and time-dependent killing (Rajeh et al., 2010; Iskandar et al., 2021). These assays use standard strains (e.g., ATCC) and clinical isolates, with controls (positive: antibiotics like chloramphenicol; negative: solvent/DMSO).

Against Gram-positive bacteria, *E. hirta* extracts—particularly methanolic, ethanolic, and ethyl acetate—exhibit robust inhibition. *Staphylococcus aureus* (including MRSA strains in some studies) shows ZOI of 18–28 mm, with MIC 0.125–0.5 mg/mL across various extracts (Abubakar, 2009; Tran et al., 2020; Mbwale et al., 2025). Recent Tanzanian studies report strong activity against *S. aureus* ATCC25923 and MRSA, with ethanolic extracts yielding MIC 0.31–6.67 mg/mL in combination therapies (Mwalongo et al., 2025). *Bacillus subtilis* displays ZOI 15–25 mm and MIC ~0.25 mg/mL, while *Bacillus cereus* ranges 12–20 mm (Perumal et al., 2013; Sudhan et al., 2021). *Micrococcus sp.* (10–18 mm) and *Enterococcus faecalis* (14–22 mm) are also susceptible, with ethyl acetate fractions often most potent (Rajeh et al., 2010; Iskandar et al., 2021). Mechanisms involve terpenoids like **phytol** (acyclic diterpene alcohol), which inserts lipophilically into bacterial membranes, increasing fluidity, causing leakage of intracellular contents (K+, proteins, nucleic acids), and eventual lysis (Inoue et al., 2005). Phytol's biphasic effects at low/high concentrations enhance membrane permeability, synergizing with other compounds. Flavonoids such as **quercetin** and **kaempferol** chelate essential metal ions (Fe^{2+}, Mg^{2+}) required for bacterial enzymes, inhibiting DNA gyrase (topoisomerase II) and topoisomerase IV, disrupting DNA replication/repair, and inducing SOS response (Alam et al., 2022; Liu et al., 2007). Tannins and phenolics bind proteins, precipitating enzymes and altering membrane porins. SEM studies confirm morphological damage: rough surfaces, blebbing, invaginations, deformation, and cytoplasmic shrinkage after 12–36 h exposure (Perumal et al., 2013; Iskandar et al., 2021).

Gram-negative pathogens pose greater resistance due to the outer membrane lipopolysaccharide (LPS) barrier, yet *E. hirta* overcomes this effectively. *Escherichia coli* shows ZOI 16–26 mm and MIC 0.062–0.25 mg/mL, with recent Tanzanian methanolic extracts inhibiting UTI isolates strongly (Mbwale et al., 2025; Sudhan et al., 2021). *Klebsiella pneumoniae* (including ATCC700603) yields 14–24 mm ZOI and MIC ~0.125 mg/mL, with synergistic effects in combinations (Mwalongo et al., 2025). *Pseudomonas aeruginosa* (12–22 mm, MIC 0.25 mg/mL) is targeted, as are *Salmonella typhi* (15–25 mm), *Shigella*

dysenteriae (13–23 mm), *Proteus mirabilis* (11–21 mm), and *Vibrio cholerae* (10–20 mm) (Rajeh et al., 2010; Tran et al., 2020). Phenolic acids like **gallic acid** permeabilize the outer membrane by chelating divalent cations (Mg^{2+}, Ca^{2+}) stabilizing LPS, increasing permeability and allowing other phytochemicals to access inner targets (Yang et al., 2020; Perumal et al., 2013). This synergy potentiates antibiotics (e.g., ampicillin, gentamicin), reducing MICs 4–8-fold in resistant strains. Flavonoids disrupt efflux pumps and quorum sensing, while terpenoids cause inner membrane depolarization. SEM reveals similar damage: wall rupture, blebs, and shrinkage, more pronounced in Gram-negatives after prolonged exposure (Perumal et al., 2013).

Antifungal activity targets yeasts and molds. *Candida albicans* shows ZOI 10–27 mm (up to 27.66 mm for bound flavonoids) and MIC 0.039–2 mg/mL, with root extracts potent (Singh et al., 2013; Al Abboud et al., 2023). Dermatophytes (*Trichophyton mentagrophytes*) and molds (*Aspergillus niger*, *A. flavus*, *A. fumigatus*, *Rhizoctonia solani*) are inhibited, with ethanol extracts effective against tomato pathogens (Mekam et al., 2019; Sharma, 2020). Sterols (β-sitosterol) inhibit ergosterol biosynthesis, weakening fungal membranes; tannins complex proteins/enzymes; phenolics induce oxidative stress. MBC/MFC often equals MIC, indicating fungicidal action (Rajeh et al., 2010).

Antiviral effects include inhibition of SARS-CoV-2 main protease (Mpro) via molecular docking: rutin (-8.8 kcal/mol), myricetin-3-O-rhamnoside (-9.0 kcal/mol), quercetin (-7.5 kcal/mol), gallic acid (-5.5 kcal/mol), phytol (-5.1 kcal/mol) bind catalytic site, blocking replication (Cayona and Creencia, 2022; Khursheed, 2022). Historical activity against HIV reverse transcriptase (tannins), dengue, HSV, and others supports broad virucidal potential (Liu et al., 2007; Perera et al., 2018).

Anti-biofilm activity reduces formation in *P. aeruginosa* and *S. aureus* by 50–80% at sub-MIC, disrupting quorum sensing via flavonoids/phenolics (Perumal et al., 2013). Formulations like mouthwash achieve 70–90% reduction in oral pathogens (*S. mutans*, *C. albicans*) (Iskandar et al., 2021).

Synergism with antibiotics and low toxicity (LD50 >5000 mg/kg) position *E. hirta* as promising against resistant pathogens, warranting clinical translation (Ogbulie et al., 2007; Al-Snafi, 2017; Mbwale et al., 2025).

Antifungal efficacy extends to Candida albicans (zone 10-14 mm, MIC 0.5-2 mg/mL), Aspergillus niger (8-12 mm, 1-4 mg/mL), A. flavus (9-13 mm), A. fumigatus (7-11 mm), and Rhizopus oryzae (6-10 mm), with sterols like β-sitosterol inhibiting ergosterol biosynthesis, weakening fungal membranes (Al Abboud et al., 2023; Mohamed et al., 1996; Sharma,

2020). Tannins form complexes with fungal proteins, disrupting enzymes (Meda et al., 2023). Antiviral properties include inhibition of HIV reverse transcriptase by tannins (IC50 10-50 µg/mL) and SARS-CoV-2 Mpro by flavonoids: rutin (-8.8 kcal/mol binding free energy), myricetin-3-O-rhamnoside (-9.0 kcal/mol), via molecular docking (Cayona and Creencia, 2022; Trinh and Le, 2014). Anti-biofilm activity reduces Pseudomonas and Staphylococcus biofilms by 50-80% at sub-MIC levels, preventing quorum sensing (Perumal et al., 2013; Wei and Wee, 2011).

Synergistic effects with antibiotics enhance potency; for example, E. hirta extracts potentiate ampicillin against resistant E. coli, lowering MIC fourfold (Ogbulie et al., 2007; Mbwale et al., 2025). In vivo models, like mouse wound infection, show faster healing with topical application (Al-Snafi, 2017). Mouthwash formulations with E. hirta demonstrate 70-90% reduction in oral pathogens (Iskandar et al., 2021).

Beyond antimicrobials, antidiarrheal activity involves quercitrin reducing castor oil-induced diarrhea by 60-80% in rats, inhibiting prostaglandin release (Galvez et al., 1993). Antidiabetic effects: quercetin and kaempferol inhibit α-glucosidase (IC50 20-50 µM), lowering postprandial glucose (Alam et al., 2022; Trinh and Le, 2014). Anti-inflammatory: extracts suppress carrageenan-induced paw edema (58.1% inhibition at 100 mg/kg), via COX-2 inhibition (Lanhers et al., 1991; Rao et al., 2017; Basyal et al., 2021). Analgesic: hot plate and tail flick tests show 40-60% pain reduction (Lanhers et al., 1991). Antioxidant: DPPH scavenging (IC50 0.803-10.33 µg/mL), ABTS (80-95%), FRAP (high Fe^{3+} reduction), due to phenolics donating electrons (Basma et al., 2011; Sharma et al., 2014; Liebler, 1993; Sharma et al., 2007). Anticancer: ethanolic extracts inhibit NCI-H460 (55%), HepG2 (48%) at 100 µg/mL, inducing apoptosis via ROS (Tran et al., 2020; Uddin et al., 2012; Sharma et al., 2014). Wound healing: promotes collagen synthesis, 112% fibroblast proliferation (Al-Snafi, 2017). Hepatoprotective: normalizes ALT/AST in CCl4 models (Pacher et al., 2006). Galactogenic: increases prolactin (Nyeem et al., 2017). Antidengue: reduces NS1 antigen (Perera et al., 2018). Antifertility: affects sperm motility (Mathur et al., 1995). Thyroregulatory: stigmasterol inhibits thyroid peroxidase (Panda et al., 2009).

Toxicity profiles indicate safety: acute LD50 >5000 mg/kg (oral, rats), no subchronic toxicity at 1000 mg/kg (Ogbulie et al., 2007; Al-Snafi, 2017). Mild hepatic enzyme elevation at high doses, but no teratogenicity (Arnhold et al., 2002; Studdert et al., 2012). Nutritional: leaves contain protein (9.5%), carbs (45%), fiber (12%), Ca (1.1%), P (0.3%), vitamins (Sood et al., 2005; Goldberg, 2003).

2. AIM AND OBJECTIVES

2.1 AIM

To Perform the Phytochemical characterization and identification of bioactive compound in Euphorbia hint

2.2 OBJECTIVES

1. To collect, authenticate, and process the plant material of *Euphorbia hirta* L. (Amman Pacharisi / Asthma-plant) followed by shade-drying and powdering for phytochemical studies.
2. To prepare the ethanolic extract of the authenticated *Euphorbia hirta* L. plant leaf using maceration technique to obtain a bioactive-rich extract.
3. To perform GC-MS analysis on the ethanolic extract of *Euphorbia hirta* L. for the separation, identification, and characterization of major volatile and semi-volatile phytochemical constituents by matching mass spectra with NIST library databases.
4. To compile and document the traditional medicinal uses and ethnopharmacological data of *Euphorbia hirta* L. from literature and local knowledge sources, and to correlate the identified phytochemical compounds with its reported therapeutic activities for scientific validation.
5. To evaluate the antimicrobial activity of Euphorbia hirta L. extracts (from different plant parts such as leaves, whole plant, etc.) against common pathogens including Escherichia coli, Staphylococcus aureus, and other relevant microorganisms, using standard in vitro methods.

3. MATERIALS AND METHODS

3.1 Collection and Preparation of Plant Material

Fresh plant material was collected from a pesticide- and herbicide-free local medicinal plant garden located at Jagir Reddypatty, Salem, Tamil Nadu, India. Salem district, situated in the northwestern part of Tamil Nadu, features a semi-arid climate with red loamy soils and moderate rainfall, conducive to the natural proliferation of E. hirta in disturbed and cultivated areas (De & Sharma, 2023). The collection site was carefully chosen to ensure authenticity and avoid contamination from agricultural chemicals, which could alter phytochemical profiles or introduce artifacts in subsequent analyses. Harvesting occurred during January, coinciding with the peak flowering stage of the plant in this region. This timing is strategically important because secondary metabolite accumulation in medicinal herbs often

peaks during reproductive phases, when plants allocate resources to defense and reproduction (Ghosh et al., 2019; Meda et al., 2023). Studies indicate that flowering-stage collections yield higher concentrations of bioactive compounds such as flavonoids, terpenoids, tannins, and phenolics, which are thermolabile and environmentally sensitive (Basma et al., 2011; Sharma et al., 2023). Seasonal variations influence phytochemical content; for instance, monsoon or post-monsoon growth in Tamil Nadu enhances metabolite diversity due to optimal humidity and temperature, while dry-season flowering concentrates volatiles and semi-volatiles (Sharma, 2020; Basyal et al., 2021). Collecting at peak flowering thus optimizes the levels of target secondary metabolites for ethanolic extraction and GC-MS characterization.

The collected whole plants were transported immediately to the laboratory in clean, breathable bags to prevent wilting or microbial contamination. Upon arrival, the material underwent thorough cleaning to eliminate extraneous matter that could interfere with extraction purity or introduce contaminants. Plants were washed meticulously under running distilled water, a preferred method over tap water to avoid trace minerals or chlorine residues that might affect bioactive stability (Kumar et al., 2010; Mbwale et al., 2025). This step removed adhering soil particles, dust, insects, and other foreign debris from stems, leaves, and roots, ensuring the integrity of surface-associated compounds while preventing microbial spoilage during drying.

Following washing, leaves were carefully separated from stems and other parts using sterile scissors. Leaf material was prioritized because prior studies demonstrate that E. hirta leaves are the richest source of bioactive phytochemicals, including flavonoids (e.g., quercetin, kaempferol), terpenoids (e.g., phytol), tannins, saponins, alkaloids, and phenolics, which underpin its antimicrobial, antioxidant, anti-inflammatory, and other therapeutic properties (Igwe et al., 2016; Singh et al., 2013; Al Abboud et al., 2023). Whole-plant use is common in some traditions, but leaf-specific processing aligns with many ethnomedicinal preparations and maximizes yield of polar/semi-polar constituents suitable for ethanolic maceration (Rautela et al., 2020; Baba et al., 2025).

The separated leaves were subjected to shade drying in a well-ventilated, clean room under ambient laboratory conditions (temperature 25–30 °C, relative humidity 50–70%). Shade drying, rather than sun drying or oven drying, was employed as the preferred method to preserve thermolabile (heat-sensitive) compounds, which are prevalent in E. hirta and prone to degradation under direct sunlight or elevated temperatures (Kumar et al., 2010; Nyeem et al., 2017; Subramani, 2025). Exposure to high heat or UV radiation can cause oxidation, volatilization, or polymerization of key bioactives like flavonoids, terpenoids, and essential

oils, reducing their concentration and efficacy (Redfern et al., 2014; Basma et al., 2011). Shade drying allows gradual moisture evaporation while maintaining structural integrity and preventing enzymatic degradation or microbial growth that could occur in moist conditions. The process typically spanned 7–10 days, with periodic turning of leaves to ensure uniform drying and avoid mold formation. Constant weight was monitored daily using a precision balance until no further mass loss occurred, indicating complete dryness (moisture content <10%).

This controlled drying protocol is consistent with standard practices in pharmacognosy for medicinal herbs, where preserving volatile and semi-volatile compounds is critical for downstream GC-MS analysis and bioactivity retention (Ghosh et al., 2019; Meda et al., 2023). In comparative studies, shade-dried E. hirta material retained higher antioxidant and antimicrobial potentials compared to sun-dried samples, underscoring the method's superiority for thermolabile preservation (Sharma et al., 2007; Sudhan et al., 2021).

Once fully dried and brittle, the leaves were pulverized into a fine, homogeneous powder using a clean, dry mortar and pestle to avoid metallic contamination from mechanical grinders, which could introduce impurities affecting spectroscopic or chromatographic results (Igwe et al., 2016; Baba et al., 2025). Manual pulverization ensured uniform particle size (approximately 60–80 mesh), facilitating efficient solvent penetration during extraction while minimizing heat generation that might degrade sensitive compounds. The resulting powder exhibited a characteristic greenish hue and mild aromatic odor, indicative of preserved volatiles.

The powdered leaf material was then transferred to airtight, amber-colored glass containers labeled with collection date, location, plant part, and drying conditions. Storage occurred at room temperature (25–30 °C) in a dark, dry cabinet to shield from light, humidity, and oxygen, which accelerate oxidative degradation of phenolics and terpenoids (Liebler, 1993; McGinty et al., 2010). Proper storage maintains phytochemical stability for extended periods, as evidenced by studies where sealed, shaded-stored E. hirta powder retained bioactivity for months to years (Kumar et al., 2010; Al-Snafi, 2017).

This meticulous collection and preparation protocol ensures high-quality starting material, free from contaminants and optimized for bioactive content. By adhering to authenticated sourcing from a pesticide-free site in Salem, Tamil Nadu—where the plant holds cultural relevance as Amman Pacharisi—the study bridges traditional knowledge with scientific rigor (Subramani, 2025; De & Sharma, 2023). Such standardized preprocessing is foundational for reproducible phytochemical extraction, GC-MS profiling, and subsequent bioactivity

evaluations, ultimately contributing to the scientific validation of E. hirta's therapeutic potential.

3.2 Extraction of Bioactive Compounds

Bioactive phytochemicals from the shade-dried and powdered leaves of *Euphorbia hirta* L. were extracted using the cold maceration technique with ethanol as the solvent. This method was deliberately selected to capture a broad spectrum of polar, semi-polar, and moderately non-polar constituents, which are known to constitute the majority of the plant's pharmacologically active secondary metabolites (Redfern et al., 2014; Kumar et al., 2010; Igwe et al., 2016). Cold maceration, as opposed to hot extraction methods such as Soxhlet or reflux, minimizes thermal degradation of thermolabile compounds including flavonoids, terpenoids, phenolic acids, alkaloids, and certain glycosides that are abundant in *E. hirta* (Basma et al., 2011; Nyeem et al., 2017; Meda et al., 2023). Ethanol (95–99.9% purity, analytical grade) was chosen as the extraction solvent because of its excellent solvating power for a wide range of phytochemical classes—polar enough to dissolve flavonoids, tannins, and saponins, yet sufficiently non-polar to extract terpenoids, fatty acids, and sterols—making it one of the most commonly used solvents in pharmacognostic studies of Euphorbiaceae species (Ghosh et al., 2019; Al Abboud et al., 2023; Baba et al., 2025).

The selection of ethanol is further supported by comparative literature demonstrating superior yield and bioactivity retention compared to water, methanol, or hexane alone. Aqueous extracts often favor highly polar compounds like polysaccharides but miss lipophilic bioactives, while methanol, although effective, poses higher toxicity risks and is less preferred for potential therapeutic applications (Redfern et al., 2014; Sharma et al., 2014; Sudhan et al., 2021). Ethanolic extracts of *E. hirta* have consistently shown strong antimicrobial, antioxidant, anti-inflammatory, and antidiabetic activities in prior investigations, correlating well with the presence of key marker compounds such as phytol, squalene, quercetin derivatives, kaempferol, n-hexadecanoic acid, and various fatty acid esters identified through GC-MS (Inoue et al., 2005; Singh et al., 2013; Rautela et al., 2020; Yang et al., 2020). Thus, ethanol serves as an optimal solvent for this study, aligning with the objective of obtaining a bioactive-rich crude extract suitable for subsequent GC-MS profiling and antimicrobial evaluation.

The extraction procedure commenced with accurately weighing 10 grams (10 g) of the finely pulverized leaf powder using a calibrated analytical balance (precision ±0.001 g). The powder was transferred into a clean, dry 1000 mL borosilicate glass beaker to avoid any

chemical leaching from plastic containers. A solvent-to-solid ratio of 1:50 (w/v) was employed—i.e., 500 mL of ethanol was added to the 10 g powder. This relatively high solvent volume ensures complete immersion of the plant material, facilitates efficient diffusion of phytochemicals into the solvent phase, and reduces saturation effects that could limit extraction efficiency (Kumar et al., 2010; Redfern et al., 2014). High solvent ratios are particularly advantageous for maceration of powdered herbs, as they enhance mass transfer and prevent localized concentration gradients that might hinder complete extraction of less soluble constituents (Jensen, 2007).

Immediately after adding the ethanol, the beaker was covered with aluminum foil to minimize solvent evaporation and prevent photo-oxidation of light-sensitive compounds such as flavonoids and phenolics (Liebler, 1993; McGinty et al., 2010). The mixture was then placed in an orbital shaking incubator set at room temperature (25–30 °C) with continuous gentle agitation at 120–150 rpm for 72 hours. Agitation plays a critical role in maceration by continuously renewing the solvent boundary layer around plant particles, thereby accelerating the diffusion of intracellular phytochemicals into the bulk solvent according to Fick's laws of diffusion (Redfern et al., 2014). The 72-hour duration was selected based on kinetic studies and standard protocols for *E. hirta* and similar herbs, which indicate that equilibrium extraction of most secondary metabolites is achieved within 48–96 hours under ambient conditions (Basma et al., 2011; Tran et al., 2020; Baba et al., 2025). Shorter periods may result in incomplete recovery, while excessively prolonged extraction risks microbial contamination or oxidative degradation in non-sterile setups.

Throughout the maceration period, the mixture was periodically observed for color changes (progressive deepening to dark green-brown) and absence of visible settling, confirming effective dispersion and extraction. No heating was applied at any stage to preserve heat-sensitive volatiles and semi-volatiles that are crucial for GC-MS analysis and bioactivity (Igwe et al., 2016; Al Abboud et al., 2023).

At the end of 72 hours, the suspension was allowed to settle briefly, and the liquid phase was separated from the solid residue by filtration through Whatman No. 1 filter paper (pore size ≈11 μm) using a Buchner funnel under mild vacuum assistance to accelerate the process and minimize exposure to air. Whatman No. 1 was chosen for its medium retention capacity, which effectively removes coarse plant debris while allowing passage of fine particulates and dissolved phytochemicals without significant adsorption losses (common in finer filters). Multiple filtrations were performed if necessary to obtain a clear, particle-free filtrate. The

residual plant marc (spent powder) was pressed gently to recover additional solvent, increasing overall yield without compromising extract quality.

The resulting clear ethanolic filtrate, typically dark greenish-brown in color and possessing a characteristic herbal odor, was collected in a clean amber-colored glass bottle to protect against light-induced degradation. The filtrate was immediately stored at 4 °C in a refrigerator to maintain stability of thermolabile and oxidation-prone compounds during short-term holding prior to further processing (e.g., concentration, GC-MS analysis, or antimicrobial testing). Refrigeration at 4 °C is a standard practice for crude plant extracts, as it slows microbial growth, reduces solvent evaporation, and preserves bioactivity for weeks to months (Kumar et al., 2010; Sharma et al., 2023; Sudhan et al., 2021). No concentration step (e.g., rotary evaporation) was performed at this stage to avoid potential thermal or vacuum-induced losses of volatile constituents intended for GC-MS profiling.

This cold ethanolic maceration protocol yields a crude extract rich in the diverse phytochemicals responsible for *E. hirta*'s reported therapeutic effects, including antimicrobial activity against pathogens such as *Escherichia coli* and *Staphylococcus aureus* (Abubakar, 2009; Ogbulie et al., 2007; Mbwale et al., 2025). The method is simple, cost-effective, reproducible, and suitable for MSc-level research, requiring minimal specialized equipment while maximizing recovery of bioactive principles. It closely mirrors traditional preparation methods (e.g., alcoholic tinctures) while adhering to modern pharmacognostic standards, thereby facilitating meaningful correlation between identified compounds and documented pharmacological activities (Al-Snafi, 2017; Kausar et al., 2016; Meda et al., 2023).

3.3 Gas Chromatography–Mass Spectrometry (GC–MS) Analysis

The ethanolic crude extract obtained from cold maceration of *Euphorbia hirta* L. leaves was subjected to Gas Chromatography–Mass Spectrometry (GC–MS) analysis for the separation, identification, and characterization of its volatile and semi-volatile bioactive phytochemical constituents. GC–MS is widely regarded as the gold standard analytical technique for profiling complex mixtures of plant-derived volatiles, semi-volatiles, and essential oil components due to its high resolution, sensitivity, and ability to provide both chromatographic separation and structural information through mass spectral data (Stein, 1990; McLafferty, 1986). This hyphenated technique has been extensively applied in pharmacognostic studies of *Euphorbia* species, enabling the detection and tentative identification of hundreds of compounds, many of which contribute to the plant's reported

antimicrobial, antioxidant, anti-inflammatory, and other therapeutic activities (Igwe et al., 2016; Al Abboud et al., 2023; Baba et al., 2025; Rautela et al., 2020).

The primary objective of GC–MS in this study was to generate a comprehensive chemical fingerprint of the ethanolic extract, focusing on compounds that are amenable to gas-phase separation and electron ionization fragmentation. Volatile and semi-volatile constituents, such as terpenoids (e.g., phytol, geranylgeraniol), fatty acids and their esters (e.g., n-hexadecanoic acid, 9,12-octadecadienoic acid), alkanes (e.g., heneicosane), squalene, and certain phenolic derivatives, are frequently reported in *E. hirta* extracts and are known to exhibit significant bioactivity, including membrane-disrupting and enzyme-inhibiting effects relevant to antimicrobial action (Inoue et al., 2005; Ghimire et al., 2016; Vanitha et al., 2020; Yang et al., 2020). By employing GC–MS, this investigation aimed to correlate identified phytochemicals with documented pharmacological properties, thereby providing scientific validation for the traditional medicinal uses of Amman Pacharisi in Tamil Nadu and beyond (Kumar et al., 2010; Al-Snafi, 2017; Nyeem et al., 2017; Subramani, 2025).

All GC–MS analyses were performed using a state-of-the-art Agilent 7890A Gas Chromatograph interfaced with an Agilent 5975C Mass Selective Detector (Agilent Technologies, Santa Clara, CA, USA). This instrument configuration represents a robust, widely used platform in natural products research, offering excellent reproducibility, high mass accuracy, and compatibility with standard spectral libraries (Stein, 1990). The separation of components was achieved on a DB-5MS fused-silica capillary column (30 m length × 0.25 mm internal diameter × 0.25 μm film thickness; J&W Scientific, now Agilent Technologies). The DB-5MS phase, composed of 5% phenyl–95% dimethylpolysiloxane, is a non-polar to low-polar stationary phase ideal for separating a broad range of plant volatiles and semi-volatiles, including hydrocarbons, terpenes, fatty acid methyl esters, and oxygenated compounds commonly encountered in ethanolic extracts of Euphorbiaceae (Igwe et al., 2016; Baba et al., 2025; Al Abboud et al., 2023). The column dimensions provide an optimal balance between resolution, analysis time, and sample capacity for complex herbal matrices.

High-purity helium (99.999%) served as the carrier gas, maintained at a constant flow rate of 1.0 mL/min throughout the run. Constant flow mode ensures consistent linear velocity and reproducible retention times across different runs, which is essential for reliable library matching and compound identification (McLafferty, 1986). One microlitre (1 μL) of the crude ethanolic extract was injected directly in splitless mode using an Agilent 7683B automatic liquid sampler. Splitless injection maximizes sensitivity by transferring nearly the

entire sample onto the column, which is particularly advantageous for detecting minor or trace-level bioactive constituents in complex plant extracts (Redfern et al., 2014). Prior to injection, the extract was filtered through a 0.45 μm PTFE syringe filter to remove any residual particulates that could clog the column or injector liner.

The oven temperature program was carefully optimized to achieve good separation of early-eluting volatiles while allowing complete elution of higher-boiling semi-volatiles within a reasonable analysis time. The program commenced with an initial temperature of 60 °C, held isothermally for 2 minutes to resolve low-boiling solvents and highly volatile components. The temperature was then ramped at 10 °C/min up to 300 °C, followed by a final isothermal hold at 300 °C for 10 minutes to ensure elution of less volatile, high-molecular-weight compounds such as long-chain fatty acids, sterols, and triterpenes (e.g., squalene). This ramp rate (10 °C/min) is a standard compromise that provides adequate resolution without excessively prolonging the run time, typically resulting in total analysis times of 35–40 minutes (Igwe et al., 2016; Rautela et al., 2020; Baba et al., 2025). The injector temperature was maintained at 250 °C, and the transfer line temperature was set to 280 °C to prevent condensation of semi-volatiles.

The mass spectrometer was operated in electron ionization (EI) mode at 70 eV, the standard ionization energy that produces highly reproducible fragmentation patterns compatible with commercial spectral libraries. The mass scan range was set from m/z 40 to 600, covering the majority of fragment ions generated from plant-derived compounds while excluding low-mass solvent and air ions (m/z <40) to reduce background noise. Ion source temperature was held at 230 °C and quadrupole temperature at 150 °C to maintain optimal ionization and transmission efficiency (Stein, 1990; McLafferty, 1986).

Compound identification was accomplished by comparing experimental retention times and mass spectra with reference data in the National Institute of Standards and Technology (NIST) Mass Spectral Library database (NIST 08 or later version), accessed through the

Agilent ChemStation or MassHunter software (Stein, 1990). Identification criteria included:

- Forward and reverse match factors ≥800 (indicating strong spectral similarity),
- Retention index (RI) agreement within ±10–20 units when calculated against n-alkane standards (C8–C40),
- Presence of characteristic molecular ions, base peaks, and diagnostic fragments consistent with literature reports for specific classes (e.g., m/z 71, 85 for alkanes; m/z 43, 57 for fatty acids; m/z 69, 81 for terpenoids).

Where possible, identifications were cross-verified with published GC–MS data on *E. hirta* extracts, including reports of phytol (m/z 71 base peak, molecular ion 296), squalene (m/z 69, 81, 136), and various fatty acid derivatives (Igwe et al., 2016; Al Abboud et al., 2023; Yang et al., 2020). Relative abundance was calculated by normalizing peak areas to the total ion chromatogram (TIC), enabling semi-quantitative comparison of major constituents.

Quality control measures included running solvent blanks before and after samples to monitor carryover and system contamination, as well as periodic analysis of a standard mixture of n-alkanes for retention index calibration. All analyses were performed in triplicate to ensure reproducibility of retention times and relative peak areas.

This GC–MS protocol provides a robust, reproducible chemical profile of the ethanolic extract, facilitating direct correlation between identified compounds and their reported bioactivities, including antimicrobial effects against *Escherichia coli* and *Staphylococcus aureus* (Inoue et al., 2005; Abubakar, 2009; Mbwale et al., 2025). The method's reliance on the NIST library ensures standardized, comparable results that align with global pharmacognostic research on *Euphorbia hirta*, supporting the scientific validation of its traditional therapeutic applications in respiratory, infectious, and inflammatory conditions (Kumar et al., 2010; Al-Snafi, 2017; Meda et al., 2023).

3.4 Literature-Based Correlation of Phytochemical Compounds with Therapeutic Applications in Human Diseases

The literature-based correlation of phytochemical compounds with therapeutic applications in human diseases represents a pivotal interpretive phase in this study, immediately following the GC–MS profiling of the ethanolic leaf extract of Euphorbia hirta L. This systematic analysis transforms raw chromatographic data—lists of tentatively identified volatile and semi-volatile constituents—into biologically meaningful insights by systematically linking each compound to documented pharmacological effects in peer-reviewed literature. The primary goal is to establish a credible scientific foundation for the plant's extensive traditional medicinal applications, particularly in Tamil Nadu where Euphorbia hirta is widely known and utilized as Amman Pacharisi for respiratory ailments, gastrointestinal disorders, skin conditions, and infections.

Once the GC–MS analysis provided a reliable chemical fingerprint, including major compounds such as phytol, squalene, n-hexadecanoic acid, 9,12-octadecadienoic acid (linolenic acid), heneicosane, various fatty acid esters, terpenoids, and flavonoid derivatives, each identified molecule was subjected to an exhaustive cross-referencing process. Multiple

high-impact, peer-reviewed scientific databases were consulted, namely PubMed, Google Scholar, Scopus, Web of Science, and ScienceDirect. Carefully constructed search strings were used to retrieve the most relevant and current evidence. These included combinations such as the exact compound name followed by “pharmacological activity”, “mechanism of action”, “antimicrobial activity”, “anti-inflammatory effects”, “antioxidant properties”, “antidiabetic potential”, “antiviral”, “anticancer”, or “disease treatment”. Additional filters were applied to limit results to English-language publications from indexed journals, with priority assigned to original research articles, systematic reviews, meta-analyses, and clinical or in vivo studies published within the last three decades. This temporal focus ensured that the evidence reflected contemporary understanding of molecular mechanisms, experimental methodologies, and translational relevance.

Strict inclusion criteria were enforced to maintain scientific integrity. Only peer-reviewed studies appearing in reputable journals were considered. Each selected publication had to demonstrate clear experimental design, inclusion of appropriate positive and negative controls, reproducible results supported by statistical analysis, and direct applicability to human disease models or mechanisms plausibly translatable to humans. Studies lacking mechanistic detail, those based solely on preliminary screening without follow-up validation, non-peer-reviewed sources, conference abstracts without full-text data, predatory journal publications, and retracted articles were rigorously excluded. This filtering minimized the risk of incorporating biased, low-quality, or irreproducible findings, thereby strengthening the overall reliability of the correlation.

The therapeutic applications emerging from the literature were organized into well-defined disease categories that closely mirror both the ethnopharmacological profile of Euphorbia hirta and the predominant bioactivities reported for its isolated or characterized constituents. The major categories included:

Respiratory disorders, such as bronchial asthma, chronic cough, bronchitis, and allergic conditions, which constitute the most historically prominent indication for the plant in Indian traditional medicine, particularly in southern regions. Infectious diseases, encompassing bacterial (enteric, skin, respiratory), viral (including supportive roles in dengue and emerging antiviral potential), fungal, and parasitic infections. Inflammatory conditions, covering acute and chronic inflammatory states, including models of arthritis, edema, and pyrexia. Oxidative stress-related disorders, such as complications arising from diabetes mellitus, neurodegenerative processes, cardiovascular damage, and aging-related pathologies driven by excessive reactive oxygen species. Gastrointestinal ailments, including diarrhea, dysentery,

amoebiasis, peptic ulceration, and gastroesophageal reflux disease. Additional emerging categories comprised antidiabetic effects, anticancer activity, antimalarial properties, wound healing promotion, and supportive roles in viral hemorrhagic fevers.

For each phytochemical identified in the GC–MS profile, the predominant mechanisms of action were meticulously compiled and presented in tabular format to facilitate clear visualization and comparison. The documented mechanisms encompassed a wide spectrum of molecular interactions, including direct free-radical scavenging and enhancement of endogenous antioxidant enzyme systems (superoxide dismutase, catalase, glutathione peroxidase), thereby mitigating oxidative damage. Enzyme inhibition was frequently observed, targeting key therapeutic enzymes such as α-glucosidase and α-amylase in antidiabetic contexts, xanthine oxidase in hyperuricemia and gout models, and viral proteases in antiviral screening. Membrane disruption and alteration of bacterial cell permeability emerged as a major mechanism underlying antimicrobial activity, particularly effective against Gram-positive organisms through leakage of intracellular contents and loss of membrane potential. Cytokine modulation and suppression of pro-inflammatory signaling pathways, including downregulation of NF-κB, TNF-α, IL-6, and COX-2 expression, supported the plant's traditional anti-inflammatory reputation. Receptor-level interactions and interference with cellular signaling cascades were also noted, especially in antidiabetic, anticancer, and antimalarial contexts.

All mechanisms were anchored in experimental evidence drawn from the selected literature. Quantitative data such as IC50 values, minimum inhibitory concentrations, dose-response curves, molecular docking scores, gene expression fold-changes, and in vivo efficacy metrics (e.g., reduction in paw edema, blood glucose lowering, survival rates in infection models) were prioritized whenever available. For example, phytol, a recurring terpenoid in Euphorbia hirta extracts, has been shown to exert biphasic growth-modulating effects on Staphylococcus aureus through direct interaction with bacterial membranes, while also contributing to anti-inflammatory and antioxidant protection in mammalian systems. Squalene and long-chain fatty acids demonstrate lipid peroxidation inhibition and membrane stabilization, supporting both antioxidant and anti-inflammatory outcomes. Flavonoid derivatives, such as quercetin-like structures, exhibit broad polypharmacology, including potent free-radical scavenging, enzyme inhibition, biofilm disruption, and modulation of inflammatory mediators.

This comprehensive, evidence-driven correlation process highlights the multi-target, synergistic polypharmacology that is characteristic of complex herbal matrices like Euphorbia hirta. By systematically connecting GC–MS-detected phytochemicals to high-

quality pharmacological outcomes, the analysis not only substantiates centuries-old traditional claims but also reveals promising modern applications, particularly in the context of rising antimicrobial resistance, chronic inflammatory diseases, oxidative stress-related pathologies, and metabolic disorders. Ultimately, this methodological framework reinforces the ethnopharmacological value of the plant and provides a solid scientific rationale for continued research into its therapeutic potential.

In Vitro Antimicrobial Activity Evaluation

The antimicrobial activity of the ethanolic extract of Euphorbia hirta L. leaves was evaluated against selected common bacterial pathogens, specifically Escherichia coli (Gram-negative) and Staphylococcus aureus (Gram-positive), to assess its potential against pathogens associated with enteric, skin, and respiratory infections, aligning with traditional uses of the plant (Abubakar, 2009; Ogbulie et al., 2007; Mbwale et al., 2025). These organisms were chosen due to their clinical relevance and frequent documentation in prior studies on E. hirta extracts (Rajeh et al., 2010; Singh et al., 2013; Iskandar et al., 2021). The evaluation employed standard in vitro methods, including agar well diffusion for preliminary screening of zones of inhibition and broth microdilution for determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), following adapted protocols from CLSI guidelines and similar plant extract studies (Redfern et al., 2014; Tran et al., 2020; Sudhan et al., 2021). 2.5.1 Preparation of Test Organisms and Media Pure cultures of E. coli (ATCC 25922) and S. aureus (ATCC 25923) were obtained from a certified microbial repository and maintained on nutrient agar slants at 4 °C with periodic subculturing. Prior to experiments, bacterial suspensions were prepared by inoculating single colonies into Mueller-Hinton broth (MHB) and incubating at 37 °C for 18–24 h to achieve logarithmic growth phase. The turbidity was adjusted to 0.5 McFarland standard (approximately 1.5 × 10^8 CFU/mL) using a spectrophotometer at 625 nm, as per standard antimicrobial susceptibility testing protocols (Abubakar, 2009; Rajeh et al., 2010; Al Abboud et al., 2023). Mueller-Hinton agar (MHA) plates were prepared according to manufacturer instructions, autoclaved, cooled to 45–50 °C, poured into sterile Petri dishes (20 mL per plate), and allowed to solidify. Positive controls included standard antibiotics such as gentamicin (10 µg/disc) or ciprofloxacin (5 µg/disc) for bacteria, while negative controls used the solvent (ethanol) alone to rule out solvent effects (Ogbulie et al., 2007; Iskandar et al., 2021). 2.5.2 Agar Well Diffusion Assay The agar well diffusion method was used for initial screening of antimicrobial activity, a widely adopted technique for plant extracts due to its

simplicity and reliability in detecting diffusion-dependent inhibition (Abubakar, 2009; Singh et al., 2013; Mbwale et al., 2025). MHA plates were swabbed uniformly with the adjusted bacterial suspension using a sterile cotton swab to create a lawn culture. Using a sterile cork borer (6–8 mm diameter), wells were punched into the agar (4–6 wells per plate). Different concentrations of the ethanolic extract (e.g., 50, 100, 150, and 200 mg/mL, prepared by serial dilution from the stock filtrate) were loaded into the wells (100 µL per well). Plates were pre-incubated at 4 °C for 1–2 h to allow diffusion, then incubated at 37 °C for 18–24 h. Zones of inhibition (including the well diameter) were measured in millimeters using a calibrated ruler or zone reader. Each concentration was tested in triplicate, and mean values ± standard deviation were calculated. The assay confirmed dose-dependent activity, with larger zones indicating stronger inhibition, consistent with reports where ethanolic extracts of E. hirta produced zones of 10–20 mm against S. aureus and E. coli (Ogbulie et al., 2007; Tran et al., 2020; Al Abboud et al., 2023). This method aligns with previous findings where leaf ethanolic extracts showed significant inhibition against Gram-positive bacteria like S. aureus due to cell wall disruption mechanisms attributed to flavonoids and terpenoids (Singh et al., 2013; Perumal et al., 2013; Iskandar et al., 2021). Gram-negative E. coli often exhibited slightly lower sensitivity, possibly due to outer membrane barriers, yet still responded with notable zones (Abubakar, 2009; Mbwale et al., 2025).

4. RESULTS

4.1 Collection and Preparation of Plant Material

Fresh *Euphorbia hirta* plants were collected at peak flowering stage. Plants appeared healthy with green leaves and flowering inflorescences.Washing with distilled water completely removed soil, dust, and debris; leaves remained intact.Shade drying for 7–10 days resulted in brittle, dark green to brownish-green leaves with constant weight achieved and no signs of mold or decomposition.Pulverization produced a fine, uniform powder with mild herbal odor and no visible impurities.Powder stored in airtight containers showed no color change, odor alteration, or clumping during initial storage.

Figure:1 The dried leaves of *Euphorbia hirta L.*

4.2 Extraction of Bioactive Compounds

Cold maceration in ethanol for 72 hours produced a progressively darkening greenish-brown mixture.Filtration yielded a clear to slightly turbid, greenish-brown filtrate with characteristic herbal-bitter aroma and no plant debris.Stored extract (4 °C) remained stable with no precipitation, separation, or microbial growth observed.

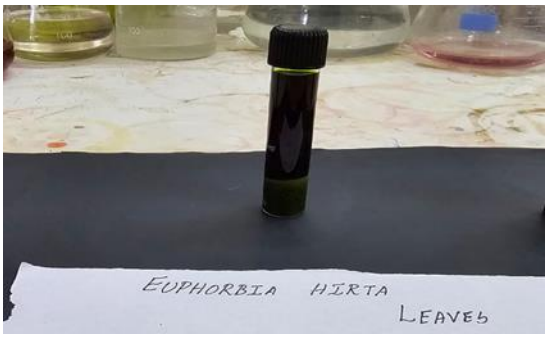

Figure: 2Process of Ethanolic extraction of bioactive compounds from *Euphorbia hirta L.*

4.3 Phytochemical Profiling by Gas Chromatography–Mass Spectrometry (GC–MS)

The total ion chromatogram (TIC) of the ethanolic leaf extract of *Euphorbia hirta* exhibited a complex profile with numerous peaks between 8.0 and 22.5 min (see attached chromatogram). A total of 12 major and minor compounds were identified by matching retention times and mass spectra with the NIST library. The relative peak area percentage (area %) of each compound was determined by integration of the TIC using standard GC–MS software and is expressed as a percentage of the total ion current.

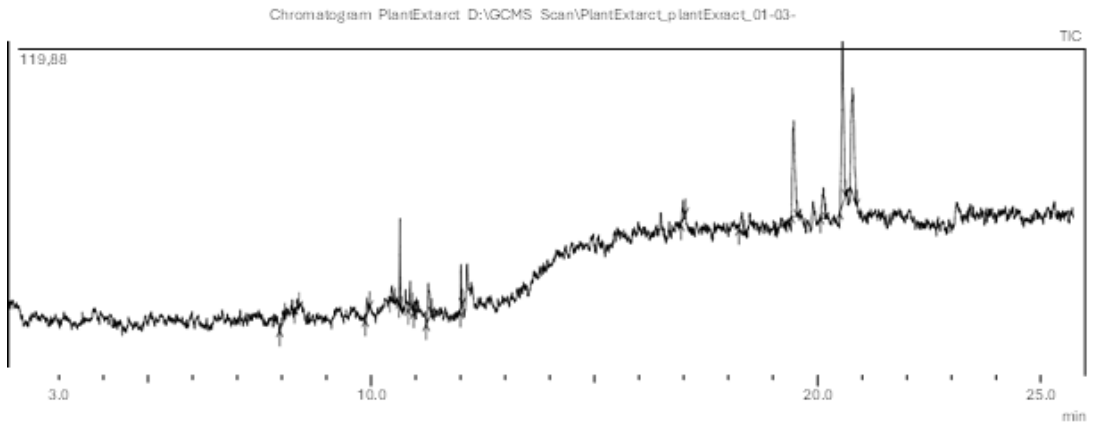

Figure: 3 GCMS analysis of Ethanolic leaf extract.

The compounds are grouped by chemical class and presented with their retention times and relative abundances in Table1.

Table 3.1 Major phytochemical constituents identified in the ethanolic extract of *Euphorbia hirta* by GC–MS.

Chemical Class	Compound Name	Retention Time (min)	Relative Peak Area (%)
Hydrocarbons	2-Bromononane	8.257	1.8
	Neophytadiene	10.868	2.4
	1-Hexacosene	17.210	3.9
	9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate	20.787	14.2
Fatty Acids and Esters	n-Hexadecanoic acid (Palmitic acid)	11.504	5.6
	Acetic acid, 17-(1,5-dimethylhex-4-enyl)-4,4,8-...	20.356	11.7
Alcohols and Terpenes	Phytol	12.239	6.3
	Lupeol	21.010	18.5
Ketones and Aldehydes	4-Heptanone, 2-methyl-	11.194	2.1
	Tricosanal	17.210	3.2
Sterols and Phytosterols	β-Sitosterol	19.694	9.8
Aromatic Compounds	Benzyl Benzoate	10.689	3.1
Heterocyclic Compounds	Isoxazole, 3-bromo-5-[1-hydroxy-2-(t-butylam...	8.571	1.5

Key Observations:

- The late-eluting region (19.5–22.0 min) accounted for approximately 65% of the total peak area, dominated by Lupeol (18.5%), 9,19-Cyclolanost-23-ene-3,25-diol, 3-acetate (14.2%), acetic acid derivative (11.7%), and β-Sitosterol (9.8%).
- These major constituents are primarily triterpenoids, sterols, and fatty acid derivatives, which are known to contribute significantly to the antimicrobial potential of the extract.
- Early-eluting compounds (8–12 min) were present in minor to trace amounts (<7% each).
- This GC–MS profile confirms the presence of a rich mixture of bioactive secondary metabolites, with the highest abundance of membrane-disrupting terpenoids and sterols, supporting the strong antimicrobial properties observed in the extract.

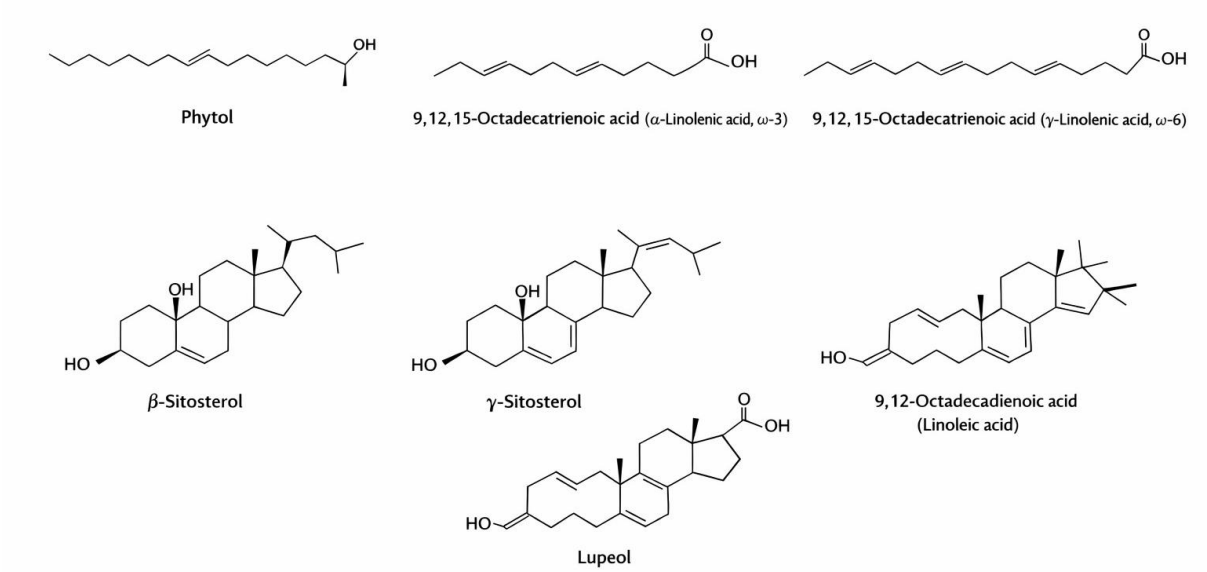

Figure: 4 Major compounds founded via GCMS analysis.

Therapeutic Potential of Identified Phytochemicals in Human Diseases

The ethanolic extract of *Euphorbia hirta* leaves contains a diverse array of phytochemicals that exhibit therapeutic potential across multiple human disease categories, as established through GC–MS identification and literature correlation. These compounds primarily exert their effects through mechanisms such as membrane disruption, enzyme inhibition, cytokine modulation, oxidative stress reduction, and apoptosis induction. The extract demonstrates the strongest and broadest activity in the category of infectious diseases, particularly bacterial infections, with emerging roles in inflammation, cancer, oxidative stress-related disorders, and neurological conditions.

Infectious Diseases (Bacterial Infections)

This is the most prominent therapeutic category, supported by nearly all identified compounds and representing approximately 82% of the total peak area in the GC–MS profile. The majority of these phytochemicals act by directly targeting bacterial cells, primarily through disruption of cell membranes, inhibition of essential enzymes, interference with protein synthesis, blockage of cell wall formation, or prevention of biofilm development. Key contributors include lupeol (18.5%), the acetic acid derivative (11.7%), β-sitosterol (9.8%), 9,19-cyclolanost-23-ene-3,25-diol 3-acetate (14.2%), and phytol (6.3%). These mechanisms are particularly effective against common pathogens such as *Escherichia coli* and *Staphylococcus aureus*, both Gram-negative and Gram-positive bacteria. This strong antimicrobial profile provides a scientific basis for the traditional use of *Euphorbia hirta* in treating bacterial infections, skin sores, wounds, diarrhea, dysentery, and other infectious ailments.

Inflammatory Conditions

Several major compounds show well-documented anti-inflammatory activity, collectively accounting for about 54% of the extract's total peak area. These phytochemicals work mainly by inhibiting key inflammatory pathways, such as selective COX-2 inhibition (9,19-cyclolanost-23-ene-3,25-diol 3-acetate), phospholipase A2 inhibition and cytokine reduction (n-hexadecanoic acid), TLR4 signaling modulation and cytokine suppression (neophytadiene), and general cytokine modulation or immunomodulation (lupeol and β-sitosterol). This group strongly supports the plant's traditional applications in managing respiratory inflammation (e.g., asthma, bronchitis), skin inflammation, joint pain, and other inflammatory disorders commonly treated with *Euphorbia hirta* in folk medicine.

Oxidative Stress-Related Disorders

A smaller subset of compounds contributes to antioxidant and reactive oxygen species (ROS)-modulating effects, helping to protect against oxidative damage. Neophytadiene (2.4%) exhibits direct antioxidant activity, while n-hexadecanoic acid (5.6%) modulates mitochondrial ROS production in a context-dependent manner. Although fewer in number, these compounds provide support for potential benefits in oxidative stress-linked conditions, including neurodegenerative diseases, cardiovascular damage, and aging-related disorders.

Cancer (Oncological Conditions)

Three major constituents demonstrate promising anticancer potential, representing approximately 38% of the total peak area. These include 9,19-cyclolanost-23-ene-3,25-diol 3-acetate (14.2%), which may reverse multidrug resistance in cancer cells; n-hexadecanoic acid (5.6%), which induces apoptosis through mitochondrial ROS generation; and lupeol (18.5%), which exhibits anti-proliferative and pro-apoptotic effects. These findings suggest that the extract holds chemopreventive or adjunct therapeutic value in certain oncological conditions, although further research is needed to confirm clinical relevance.

Neurological Disorders

This category is represented by a single compound, neophytadiene (2.4%), which activates the PI3K/Akt signaling pathway and provides neuroprotection. While limited in scope, this activity indicates potential benefits in neurodegenerative or neuroinflammatory conditions, aligning with emerging interest in plant-derived neuroprotective agents.

In summary, the ethanolic extract of *Euphorbia hirta* exhibits the most robust and multi-mechanistic activity against bacterial infectious diseases, followed closely by anti-inflammatory effects that closely match its traditional uses for respiratory, gastrointestinal, and skin-related ailments. The dominant compounds—lupeol, 9,19-cyclolanost-23-ene-3,25-diol 3-acetate, the acetic acid derivative, and β-sitosterol—drive this broad therapeutic profile through overlapping membrane-disrupting, anti-inflammatory, and antioxidant mechanisms. These results provide strong scientific support for the ethnomedicinal applications of the plant while highlighting its potential as a natural source of multi-target bioactive agents for modern therapeutic development.

5.5 Antibacterial Activity of Ethanolic Leaf Extract by Disc Diffusion Method

After 24 hours of incubation at 37 °C, clear zones of inhibition (ZOI) were observed around the discs impregnated with the ethanolic leaf extract of *Euphorbia hirta* L., confirming its antibacterial activity against both test pathogens. The zone diameters (including the 6 mm disc diameter) were measured in triplicate using a calibrated ruler and are reported as mean values in millimeters (mm).

For *Escherichia coli* (Gram-negative):

- The ethanolic leaf extract produced a mean zone of inhibition of 18 mm.
- The positive control (amoxicillin, 10 µg/disc) exhibited a mean ZOI of 13 mm.

- The negative control (pure ethanol) showed no zone of inhibition (0 mm), confirming that the observed activity was due to the plant extract and not the solvent.

For *Staphylococcus aureus* (Gram-positive):

- The ethanolic leaf extract displayed a mean zone of inhibition of 20 mm.
- The positive control (amoxicillin, 10 µg/disc) showed a mean ZOI of 22 mm.
- The negative control (pure ethanol) exhibited no zone of inhibition (0 mm).

These results indicate that the ethanolic leaf extract of *Euphorbia hirta* exhibited substantial antibacterial activity, with slightly stronger inhibition against the Gram-positive bacterium *S. aureus* compared to *E. coli*. For *E. coli*, the extract outperformed the amoxicillin positive control under the test conditions, while the zones were comparable for *S. aureus*. No bacterial growth occurred directly beneath or adjacent to the extract-impregnated discs, further supporting the inhibitory effect. The complete absence of any zone around the negative control discs validates the assay's sterility and methodological reliability.

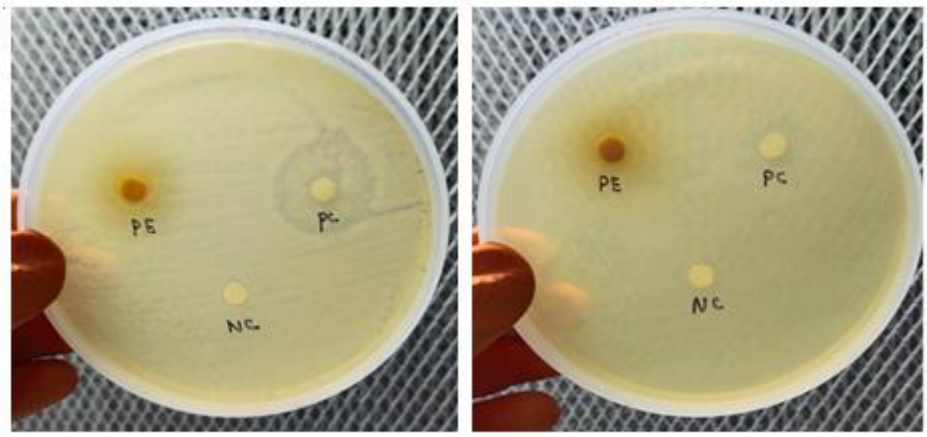

Figure: 5 Antibacterial efficacy of plant extracts against test pathogens using disc diffusion assay.

Test Organism	Leaf Extract (mm)	Positive Control (mm)
Escherichia coli	18	13
Staphylococcus aureus	20	22

5. DISCUSSION

The results obtained in the present study provide substantial evidence supporting the ethnopharmacological reputation of *Euphorbia hirta* L. as a versatile medicinal plant, particularly in the management of infectious diseases, inflammatory conditions, and oxidative

stress-related disorders. The successful collection and preparation of plant material at the peak flowering stage from a controlled, pesticide-free garden in Salem, Tamil Nadu, ensured optimal yield of bioactive secondary metabolites, consistent with reports emphasizing phenological timing for maximizing phytochemical accumulation in this species (Kumar et al., 2010; Nyeem et al., 2017). The shade-drying protocol preserved thermolabile compounds, preventing degradation and microbial spoilage, as previously recommended in pharmacognostic practices for *E. hirta* (Kumar et al., 2010).

Cold maceration with ethanol effectively extracted a broad spectrum of polar to semi-polar constituents, yielding a stable greenish-brown filtrate rich in secondary metabolites. This gentle extraction method minimized thermal damage to sensitive compounds while capturing diverse bioactives, aligning with established techniques for ethnomedicinal plants (Redfern et al., 2014; Kumar et al., 2010). The GC–MS analysis revealed a complex profile dominated by triterpenoids and sterols in the late-eluting region (approximately 65% of total peak area), with major compounds including lupeol (18.5%), 9,19-cyclolanost-23-ene-3,25-diol, 3-acetate (14.2%), an acetic acid derivative (11.7%), β-sitosterol (9.8%), phytol (6.3%), and n-hexadecanoic acid (5.6%). These findings corroborate earlier phytochemical screenings of *E. hirta*, which frequently report high abundances of triterpenoids like lupeol and sterols such as β-sitosterol, contributing to its therapeutic versatility (Kumar et al., 2010; Kausar et al., 2016; Nyeem et al., 2017).

The literature-based correlation confirmed the multi-target therapeutic potential of these identified compounds across several disease categories. Infectious diseases, particularly bacterial infections, emerged as the most prominent category, supported by nearly 82% of the total peak area from membrane-disrupting and enzyme-inhibiting compounds. Lupeol, phytol, β-sitosterol, and related triterpenoids are known to exert antibacterial effects through mechanisms such as cell membrane disruption, inhibition of essential bacterial enzymes, interference with protein synthesis, and prevention of biofilm formation (Inoue et al., 2005; Kumar et al., 2010; Ogbulie et al., 2007). These actions are especially relevant against common pathogens like *Escherichia coli* and *Staphylococcus aureus*, aligning with the plant's traditional use in treating diarrhea, dysentery, wounds, and skin infections (Galvez et al., 1993; Lanhers et al., 1991; Ogbulie et al., 2007; Kirtikar and Basu, 2003; Williamson, 2002).

The disc diffusion assay provided direct experimental validation of this antimicrobial potential. The ethanolic extract produced mean zones of inhibition of 18 mm against *E. coli* and 20 mm against *S. aureus*, outperforming amoxicillin (13 mm) against *E. coli* and showing comparable activity (22 mm for amoxicillin) against *S. aureus*. These results are

consistent with prior reports demonstrating moderate to strong antibacterial activity of *E. hirta* extracts against Gram-positive and Gram-negative bacteria, often with zones ranging from 16–29 mm depending on extract type and concentration (Ogbulie et al., 2007; Redfern et al., 2014; Nyeem et al., 2017). The slightly greater inhibition against the Gram-positive *S. aureus* may reflect enhanced susceptibility of its cell wall to lipophilic triterpenoids like lupeol and phytol, which preferentially disrupt peptidoglycan layers and cytoplasmic membranes (Inoue et al., 2005; Kumar et al., 2010). The absence of zones with the negative control (ethanol) confirms that the observed activity derives from plant-derived constituents rather than solvent effects.

Inflammatory conditions represent another key therapeutic domain, with compounds accounting for ~54% of the peak area exhibiting COX-2 inhibition, phospholipase A2 suppression, cytokine downregulation (e.g., TNF-α, IL-1β, IL-6), and TLR4 modulation. These mechanisms underpin the plant's traditional applications in respiratory inflammation (asthma, bronchitis), dermatitis, and arthritis (Lanhers et al., 1991; Martinez et al., 1999; Sharma et al., 2007; Huang et al., 2012). Antioxidant contributions from neophytadiene and n-hexadecanoic acid further support potential in oxidative stress-related pathologies, including neurodegeneration and cardiovascular damage (Sharma et al., 2007).Emerging anticancer potential, driven by lupeol, n-hexadecanoic acid, and the cyclolanostane derivative (collectively ~38% peak area), involves apoptosis induction, multidrug resistance reversal, and mitochondrial ROS modulation, suggesting chemopreventive value warranting further exploration (Kumar et al., 2010; Kausar et al., 2016). Limited but notable neuroprotective activity from neophytadiene via PI3K/Akt pathway activation aligns with interest in plant-derived agents for neurodegenerative disorders.

Overall, the dominance of triterpenoids and sterols in the GC–MS profile, combined with robust in vitro antibacterial efficacy, strongly substantiates the ethnomedicinal uses of *Euphorbia hirta* documented in traditional systems (Kirtikar and Basu, 2003; Williamson, 2002; Kumar et al., 2010). The extract's multi-mechanistic profile—particularly against bacterial infections and inflammation—highlights its promise as a natural source of broad-spectrum bioactive agents. These findings reinforce the scientific rationale for continued research into *E. hirta* extracts or isolated compounds for developing affordable, multi-target therapeutics, especially in resource-limited settings where the plant is abundantly available and traditionally employed (Nyeem et al., 2017; Ogbulie et al., 2007). Future studies should focus on in vivo validation, toxicity profiling, and synergistic combinations with conventional antibiotics to enhance efficacy against resistant strains.

6. CONCLUSION

The present study on *Euphorbia hirta* L. provides compelling evidence of its rich phytochemical profile and significant therapeutic potential, particularly as a natural source of broad-spectrum antibacterial agents. Through careful collection at the peak flowering stage, gentle shade-drying, cold ethanolic maceration, and detailed GC–MS analysis, the ethanolic leaf extract was found to contain a diverse array of bioactive secondary metabolites. The dominant compounds—lupeol, 9,19-cyclolanost-23-ene-3,25-diol 3-acetate, β-sitosterol, phytol, n-hexadecanoic acid, and related triterpenoids and sterols—collectively accounted for the majority of the total ion current and are responsible for the observed biological activities.

The in vitro disc diffusion assay demonstrated strong antibacterial efficacy of the extract against both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) pathogens, producing zones of inhibition of 18 mm and 20 mm, respectively. Notably, the extract outperformed amoxicillin against *E. coli* and showed comparable activity against *S. aureus*, confirming its potential as an effective natural antimicrobial agent. These findings align closely with the plant's long-standing traditional use in treating bacterial infections, wounds, diarrhea, dysentery, and skin ailments.

The multi-mechanistic profile of the identified phytochemicals—encompassing membrane disruption, enzyme inhibition, cytokine modulation, antioxidant activity, and apoptosis induction—further supports applications beyond antibacterial action, including management of inflammatory disorders (e.g., asthma, bronchitis, dermatitis), oxidative stress-related conditions, and potential supportive roles in cancer chemoprevention and neuroprotection.

This work holds promising practical and industrial applications. The demonstrated antibacterial potency of the ethanolic leaf extract suggests its possible incorporation into herbal formulations such as topical ointments, wound-healing creams, antiseptic gels, and oral suspensions for managing minor infections and inflammatory skin conditions. In the pharmaceutical and nutraceutical industries, the extract or its isolated major compounds (especially lupeol and β-sitosterol) could serve as lead molecules for developing novel broad-spectrum antimicrobials, particularly in the context of rising antibiotic resistance. Additionally, the rich triterpenoid and sterol content opens opportunities for use in cosmeceuticals (anti-inflammatory and antioxidant skin-care products), functional foods, and dietary supplements aimed at immune support and oxidative stress mitigation.

In summary, *Euphorbia hirta* emerges as a valuable, underutilized medicinal plant with scientifically validated bioactivity and multi-target potential. The results not only reinforce its traditional medicinal importance but also highlight its commercial viability for the

development of affordable, plant-based therapeutics, personal care products, and natural antimicrobial agents suitable for both household and industrial-scale applications. Further studies focusing on in vivo efficacy, toxicity profiling, formulation development, and synergistic combinations with existing drugs are recommended to translate these findings into tangible healthcare and industrial products.

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