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# Exploring The Therapeutic Potential Of Calotropis Gigantea: Pharmacognostic, Formulation, And Cardioprotective Studies

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***Abstract***
*Cardiovascular diseases (CVDs) remain the leading global cause of mortality, highlighting an urgent need for novel, safer therapeutic agents. Calotropis gigantea (L.) Dryand. (Giant Milkweed), a plant deeply rooted in traditional medicine systems like Ayurveda for treating diverse ailments including inflammatory conditions, presents a promising yet underexplored source for cardioprotective leads. While preliminary studies indicate bioactive potential (antioxidant, anti-inflammatory), its specific cardioprotective efficacy and development into viable therapeutics face significant gaps. Modern phytochemistry reveals a complex profile rich in cardenolides, terpenoids, flavonoids, and pregnane glycosides, suggesting multifaceted bioactivity beyond known toxicity concerns. However, robust evidence demonstrating protection against cardiac injury (ischemia, oxidative stress) is lacking, and translation is hindered by the absence of pharmacognostic standardization and optimized formulations addressing bioavailability, stability, and toxicity. This study bridges these critical gaps through an integrated approach. First, comprehensive pharmacognostic evaluation establishes botanical identity, physicochemical parameters, and phytochemical profiles for standardization of selected plant parts. Second, topical formulation are developed and optimized to enhance bioactive delivery and safety. Third, rigorous assessment of cardioprotection employs cardiomyocyte stress models and established in vivo models, evaluating effects on oxidative stress (MDA, SOD, CAT, GSH), inflammation (TNF-α, IL-6), apoptosis (Bcl-2, Bax, Caspase-3), and cardiac biomarkers (LDH, CK-MB, Troponins). This holistic investigation provides essential scientific validation for C. gigantea's cardioprotective potential, establishes quality control benchmarks, and advances pharmaceutical development, paving the way for its future application as a novel therapeutic or adjuvant in CVD management.*
***Keywords:*** *Calotropis gigantean, Pharmacognosy, Formulation Development, Cardioprotection, Standardization, Ischemia-Reperfusion Injury*

## 1. INTRODUCTION

Cardiovascular diseases (CVDs) persist as the foremost cause of global mortality, imposing an immense and escalating burden on healthcare systems worldwide. Despite significant advancements in pharmacological interventions and surgical techniques, the prevalence of conditions such as ischemic heart disease, heart failure, and hypertension continues to rise, driven by demographic shifts, lifestyle factors, and the limitations of current therapies which often include adverse effects, high costs, and variable efficacy. This ongoing crisis underscores the critical and urgent need for the discovery and development of novel therapeutic agents, particularly those offering cardioprotection – the ability to prevent or mitigate damage to cardiac tissue during injury or stress. In this pursuit, natural products derived from medicinal plants present a vast and historically validated resource, offering potential leads for safer, more accessible, and effective cardioprotective drugs.

Ethnopharmacology, bridging traditional knowledge with modern scientific rigor, provides a strategic pathway for identifying promising plant candidates. *Calotropis gigantea* (L.) Dryand., commonly known as Giant Milkweed or Crown Flower (family Apocynaceae), stands out as a plant of significant historical and therapeutic interest across various traditional medicine systems, including Ayurveda, Siddha, and folk practices prevalent in Asia and Africa. This resilient shrub, characterized by its large leaves, distinctive flowers, and abundant latex, thrives in arid and semi-arid regions. Traditionally, diverse parts of *C. gigantea* (leaves, roots, flowers, latex) have been employed for centuries to treat a wide array of ailments, ranging from fevers, digestive disorders, skin diseases, and respiratory conditions to rheumatism and asthma. Notably, and highly relevant to this research, traditional uses also encompass applications suggestive of benefits for circulatory

and inflammatory conditions, hinting at potential cardiovascular relevance, though explicit historical documentation for direct cardioprotection requires scientific validation.

The therapeutic potential of *C. gigantea* is underpinned by a rich and complex phytochemical profile. Modern investigations have identified the plant as a source of diverse bioactive compounds, most prominently cardenolides like calotropin, uscharin, and gigantin, known for their potent effects on cardiac contractility but also associated with toxicity at higher doses. Beyond these, significant constituents include terpenoids (e.g., α-amyrin, β-amyrin), flavonoids with established antioxidant and anti-inflammatory properties, pregnane glycosides, sterols, phenolic compounds, and proteolytic enzymes (calotropains) within its latex. This diverse chemical arsenal suggests a broad spectrum of potential pharmacological activities extending well beyond the cytotoxic effects historically attributed to its cardenolides.

While preliminary studies on *C. gigantea* extracts have demonstrated intriguing bioactivities *in vitro* and in select animal models – including antioxidant, anti-inflammatory, analgesic, antimicrobial, antidiarrheal, antitumor, and wound healing effects – substantial scientific gaps remain, particularly concerning its cardioprotective potential and its pathway to becoming a viable therapeutic. Evidence specifically demonstrating its efficacy in protecting the heart against ischemic injury, oxidative stress, or other cardiotoxic insults is fragmented, often preliminary, and lacks systematic investigation using robust models and standardized materials. Furthermore, translating the raw botanical material into effective, stable, and safe dosage forms presents significant challenges. These include the need for rigorous pharmacognostic standardization to ensure correct botanical identity, consistent quality, and purity, coupled with the development of sophisticated formulations capable of overcoming issues like poor solubility, instability of active constituents, potential toxicity, and suboptimal bioavailability necessary for systemic cardioprotective effects.

Therefore, this research manuscript presents a comprehensive and integrated study designed to systematically explore the therapeutic promise of *Calotropis gigantea*, with a dedicated focus on its cardioprotective properties while establishing the essential pharmacognostic and pharmaceutical foundations. Our investigation is structured around three interconnected pillars: a detailed pharmacognostic evaluation encompassing botanical characterization, physicochemical analysis, and phytochemical profiling for standardization; the development and optimization of novel formulations aimed at enhancing bioavailability, stability, and safety while mitigating toxicity risks; and a rigorous assessment of cardioprotective efficacy utilizing both cardiomyocyte models under stress and well-established *in vivo* models of myocardial injury, probing underlying mechanisms such as modulation of oxidative stress, inflammation, apoptosis, and key cardiac biomarkers. By addressing these critical aspects holistically, this research aims to provide robust scientific validation for *C. gigantea*'s cardioprotective potential, bridging traditional knowledge with evidence-based medicine and paving the way for its future development as a novel therapeutic agent or adjuvant in combating cardiovascular diseases.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals

#### 2.1.1. General Chemicals for Extraction and Phytochemical Analysis

| Chemical Name | Purpose | CAS No. | Source/Vendor |
|---|---|---|---|
| Methanol | Extraction, phytochemical tests | 67-56-1 | Merck / Sigma-Aldrich |
| Ethanol (95%) | Extraction, sample preparation | 64-17-5 | Merck / Himedia |
| Chloroform | Solvent for extraction | 67-66-3 | Merck / Qualigens |
| Hexane | Solvent for non-polar extraction | 110-54-3 | SRL / Merck |
| Ethyl acetate | Solvent for semi-polar components | 141-78-6 | SRL / Sigma-Aldrich |
| Distilled water | Aqueous extraction | - | In-house/Laboratory |
| Hydrochloric acid (HCl) | Alkaloid test (Mayer's, Wagner's) | 7647-01-0 | Merck / Qualigens |

| Chemical Name | Purpose | CAS No. | Source/Vendor |
|---|---|---|---|
| Sulphuric acid ($H_2SO_4$) | Phytochemical testing | 7664-93-9 | Merck / Loba Chemie |
| Ferric chloride ($FeCl_3$) | Phenolic/Tannin detection | 7705-08-0 | Merck / Sigma |
| Lead acetate | Flavonoid test | 301-04-2 | SRL |
| Dragendorff's reagent | Alkaloid test | - | Prepared in-lab |
| Mayer's reagent | Alkaloid detection | - | Prepared in-lab |
| Sodium hydroxide (NaOH) | Flavonoid test | 1310-73-2 | Merck / Loba |
| Ammonia solution | Saponin and flavonoid tests | 1336-21-6 | SRL / Merck |
| Iodine solution | Starch/Carbohydrate detection | 7553-56-2 | SRL / Qualigens |
| Magnesium ribbon + HCl | Flavonoid detection (Shinoda test) | - | Local vendor |

**2.1.2. Chemicals for Antimicrobial, Antibacterial, Antifungal Assays**

| Chemical Name | Purpose | CAS No. | Source/Vendor |
|---|---|---|---|
| Mueller-Hinton Agar (MHA) | Antibacterial assay medium | - | Himedia / SRL |
| Sabouraud Dextrose Agar (SDA) | Antifungal assay medium | - | Himedia / SRL |
| Nutrient broth | Pre-culture medium | - | Himedia / Loba Chemie |
| DMSO (Dimethyl Sulfoxide) | Solvent for extracts | 67-68-5 | Merck / Sigma-Aldrich |
| Ciprofloxacin | Positive control (antibacterial) | 85721-33-1 | Himedia / Sigma |
| Fluconazole | Positive control (antifungal) | 86386-73-4 | Sigma-Aldrich |
| Amphotericin B | Antifungal control agent | 1397-89-3 | Sigma / Loba Chemie |
| Sterile saline | Dilution & preparation | - | In-house / SRL |
| Tween 80 | Emulsifier for plant extracts | 9005-65-6 | SRL / Loba Chemie |
| Ethanol (70%) | Surface sterilization | 64-17-5 | Merck / SRL |

**2.1.3. Chemicals for Cardioprotective Activity**

| Chemical Name | Purpose | CAS No. | Source/Vendor |
|---|---|---|---|
| Isoproterenol hydrochloride | Inducer of cardiotoxicity in models | 51-30-9 | Sigma-Aldrich |
| TTC (2,3,5-Triphenyltetrazolium) | Infarct staining in heart tissue | 298-96-4 | Sigma / Himedia |
| Thiobarbituric acid (TBA) | Lipid peroxidation (MDA) assay | 504-17-6 | Sigma / SRL |
| Trichloroacetic acid (TCA) | Protein precipitation | 76-03-9 | Merck / Loba Chemie |
| Phosphate buffer saline (PBS) | Buffer solution | - | In-house |
| Glutathione (GSH) | Antioxidant marker | 70-18-8 | Sigma-Aldrich |
| DTNB (Ellman's reagent) | GSH estimation | 69-78-3 | Sigma / SRL |
| Catalase enzyme reagent | Enzymatic antioxidant studies | 9001-05-2 | Sigma-Aldrich |
| Superoxide dismutase (SOD) | Oxidative stress marker | 9054-89-1 | Himedia / Sigma |
| EDTA | Chelating agent, antioxidant assays | 60-00-4 | Merck / SRL |

**2.1.4. Microorganisms for Antimicrobial Testing**

| Organism | Type | Source |
|---|---|---|
| *Staphylococcus aureus* | Gram-positive | MTCC/ATCC cultures |
| *Escherichia coli* | Gram-negative | MTCC/ATCC cultures |
| *Pseudomonas aeruginosa* | Gram-negative | MTCC/ATCC cultures |
| *Candida albicans* | Fungal yeast | MTCC/ATCC cultures |
| *Aspergillus niger* | Fungal mold | MTCC/ATCC cultures |

**2.2. Instrumentations**

**2.2.1. Pharmacognostic Evaluation**

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Compound Microscope | Microscopic evaluation of powder/crude drug | Olympus / Labomed / Radical |
| Camera Lucida | Sketching microscopic structures | Radical Instruments / Local |
| Stage & Ocular Micrometer | Quantitative microscopy (stomatal index, vein islet, etc.) | Radical / Olympus |
| Hot Air Oven | Sample drying | Remi / Tempo Instruments |
| Muffle Furnace | Ash value determination | ThermoFisher / Tempo |
| Analytical Balance (0.1 mg) | Precise weighing | Shimadzu / Sartorius |
| Moisture Balance | Loss on drying estimation | Mettler Toledo / Contech |
| pH Meter | pH determination of extract | Eutech / Hanna Instruments |

**2.2.2. Phytochemical and Phytoconstituent Evaluation**

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Soxhlet Apparatus | Extraction of plant material | Borosil / Glascol |
| Rotary Evaporator | Solvent evaporation / extract concentration | Buchi / Heidolph |
| UV-Visible Spectrophotometer | Quantitative estimation of flavonoids, phenolics, etc. | Shimadzu / PerkinElmer |
| Thin Layer Chromatography (TLC) Chamber | Separation of compounds | Merck / Local Fabricated |
| High Performance Thin Layer Chromatography (HPTLC) | Fingerprinting / Qualitative analysis | CAMAG / Anchrom Enterprises |
| FTIR Spectrophotometer | Functional group identification | Shimadzu / Bruker |
| High Performance Liquid Chromatography (HPLC) | Quantitative phytochemical analysis | Shimadzu / Agilent |
| Column Chromatography Set | Isolation of bioactive compounds | Borosil / SRL |
| Reflux Assembly | Extraction or hydrolysis processes | Local glassware suppliers |
| Colorimeter | Colorimetric phytochemical assays | Systronics / Elico |
| Vortex Mixer | Homogenization of samples | Remi / Tarsons |

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Water Bath | Incubation of extracts/reagents at fixed temperature | Remi / Tempo Instruments |

**2.2.3. *In Vitro* Antimicrobial, Antibacterial, and Antifungal Activity**

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Laminar Air Flow Cabinet | Aseptic culture handling | Klenzaids / REMI / Tempo |
| Autoclave | Sterilization of media and tools | Tempo / Biotechnics |
| Incubator (BOD & Dry) | Incubation of microbial plates | REMI / Thermolab |
| Hot Air Oven | Dry sterilization of glassware | REMI / Tempo Instruments |
| Colony Counter | Quantitative microbial growth estimation | REMI / Weswox |
| UV Spectrophotometer (600 nm) | Turbidity / MIC estimation (OD measurement) | Shimadzu / Elico / Systronics |
| Petri Dishes & Micropipettes | Microbial culture and extract handling | Tarsons / Eppendorf / Himedia |
| Digital Vernier Caliper | Measuring zone of inhibition | Mitutoyo / Baker |
| Centrifuge (Tabletop and Refrigerated) | Sample clarification and microbial pellet preparation | REMI / Eppendorf / ThermoFisher |

**2.2.4. Cardioprotective Activity**

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Langendorff Heart Perfusion Apparatus | Cardioprotective model | Inco / Panlab / Custom fabricated |
| Polygraph or Data Acquisition System | Heartbeat, contraction, ECG tracing (optional) | AD Instruments / PowerLab |
| Microtome | Tissue slicing for histopathology | Weswox / Leica |
| Histopathology Set (Embedding, Staining, Slide Prep) | Tissue processing | Leica / Thermo / Local vendors |
| ELISA Plate Reader | Antioxidant marker estimation (e.g., MDA, SOD, GSH) | Bio-Rad / ThermoFisher |
| Refrigerated Centrifuge | Sample separation at controlled temperatures | REMI / Eppendorf |
| Tissue Homogenizer | Homogenizing heart/liver tissues | Polytron / IKA |
| Spectrophotometer (UV-Vis) | Colorimetric analysis of oxidative stress markers | Shimadzu / PerkinElmer |

**2.2.5. Other Supporting Instruments**

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Digital Thermometer | Monitoring temperature of water bath/incubator | Thermo / Local |
| Magnetic Stirrer with Hot Plate | Solubilization of extracts and chemicals | REMI / Labline |

| Instrument Name | Purpose / Application | Source / Manufacturer |
|---|---|---|
| Ice Flaking Machine | Ice preparation for biological samples | Thermolab / REMI |
| Digital Balance (3-decimal) | Accurate weighing of analytical samples | Sartorius / Shimadzu |
| Sonicator | Particle size reduction / extract dispersion | PCI / Labman / BioBase |
| Deep Freezer (-20°C to -80°C) | Sample preservation and storage | Thermo / Blue Star / Haier |

**2.3. Collection and Authentication of Plants**

Fresh and healthy leaves of *Calotropis gigantea* were collected from the medicinal plant garden of Shakti College of Pharmacy, located in Balrampur city, situated in the state of Uttar Pradesh, India. The collection was carried out with careful attention to plant maturity, health, and seasonal appropriateness to ensure the phytochemical richness and pharmacognostic accuracy of the specimen. Following collection, the plant material was subjected to taxonomic verification for accurate botanical identification. The authentication of the plant specimen (Number: CG/138/124) was performed by a qualified botanist Dr. S. N. Dwivedi, Department of Botany, Janata Post Graduate College, A.P.S. University, Rewa 486001, Madhya Pradesh, India. A certified voucher specimen was prepared, labeled, and deposited in the institutional herbarium for future reference and traceability in compliance with standard scientific documentation practices. This authentication ensured the correctness of the plant species under study, which is crucial for the reliability and reproducibility of all subsequent pharmacognostic, phytochemical, and pharmacological evaluations.

**2.4. Extraction (Soxhelation)**

The leaves of *Calotropis gigantea* were carefully harvested and thoroughly washed with distilled water to remove any dust, dirt, or extraneous matter. The cleaned leaves were then subjected to shade drying at ambient room temperature for several days. This slow drying process under shade was essential to preserve the heat-sensitive phytoconstituents and to prevent decomposition or volatilization of active compounds. Once the leaves were completely dried and rendered brittle, they were coarsely powdered using a mechanical grinder. The resulting powder was then sieved through a suitable mesh to achieve uniform particle size, ensuring maximum surface area for efficient extraction. For the extraction process, 100 g of the powdered leaf material was divided into smaller portions to facilitate smooth and uniform extraction. Each portion was subjected separately to Soxhlet extraction using four different solvents based on increasing polarity: Distilled Water (DW); Ethyl Acetate (EA); Ethanol (MET); and Petroleum Ether (PE). In each extraction cycle, 50 mL of solvent was used per portion. The Soxhlet apparatus was operated under a controlled temperature range of 65°C to 75°C, and each batch underwent 32 continuous extraction cycles to ensure thorough leaching of the phytoconstituents from the plant matrix. After the completion of the extraction process, the resulting extracts were concentrated using a rotary vacuum evaporator. The evaporation was performed under reduced pressure and controlled temperature, specific to the boiling points of each solvent, to avoid thermal degradation of the phytochemicals. This technique ensured efficient solvent recovery and yielded concentrated crude extracts, which were then stored in airtight containers at 4°C for further phytochemical and pharmacological evaluations.

**2.5. Pharmacognostic evaluations**

**2.5.1. Organoleptic Evaluation**

Organoleptic evaluation is the preliminary and essential step in pharmacognostic studies, offering insights into the sensory characteristics of the plant material. The powdered leaves of *Calotropis gigantea* were carefully observed for the following organoleptic attributes:

- **Form**

The leaf powder appeared finely coarse, uniformly distributed, and free from fibrous lumps or external impurities.

- **Size**

The granule sizes were consistent, indicating proper grinding and sieving of the plant material.

- **Texture**

The powder exhibited a soft, dry, and slightly fibrous texture upon palpation.

- **Colour**

A distinct olive-green coloration was noted, which is characteristic of dried and powdered *Selaginella* species.

- **Fracture**

Upon gentle crushing, the plant particles demonstrated a short, brittle fracture with a smooth break pattern, suggesting appropriate dryness and absence of excessive moisture.

These parameters are crucial as they establish the preliminary identity and physical condition of the plant material used for further analysis.

**2.5.2. Physicochemical Evaluation**

To ensure the purity, stability, and quality of *Calotropis gigantea* leaf powder, several physicochemical parameters were analyzed as per the Indian Pharmacopoeia (IP), 2020:

**2.5.2.1. Ash Values**

Ash values are critical parameters used in pharmacognostic studies to determine the quality and purity of crude plant drugs. They represent the inorganic residue remaining after incineration of the plant material and help in detecting the presence of various inorganic adulterants such as silica, soil, sand, and added minerals like chalk or lime. The ash content also reflects contamination from external sources or improper handling and storage.

**2.5.2.1.1. Total Ash**

Total ash refers to the total amount of inorganic material (residual mineral content) left after incinerating the plant material at a high temperature (usually 500–600°C) until the sample is free from carbon. It provides an estimate of both physiological ash (originating from plant tissue) and non-physiological ash (from external contamination like dust, sand, and inorganic adulterants).

**Procedure Summary:**

- Weigh accurately 2–3 g of air-dried powdered drug in a tared silica crucible.
- Incinerate in a muffle furnace at 500–600°C until free from carbon.
- Cool the crucible in a desiccator and weigh.
- Repeat heating, cooling, and weighing until a constant weight is obtained.

**Formula:**

$$\text{Total Ash (\% w/w)} = \left( \frac{\text{Weight of total ash}}{\text{Weight of air-dried sample}} \right) \times 100$$

**2.5.2.1.2. Acid-Insoluble Ash**

Acid-insoluble ash is the residue obtained after treating the total ash with dilute hydrochloric acid. It mainly consists of siliceous matter, such as sand, soil, or other earthy materials. Used to evaluate the amount of earthy or siliceous contaminants that are not soluble in acid and are likely to be externally introduced.

**Procedure Summary:**

- Boil the total ash with 25 mL of 2N HCl for 5 minutes.
- Filter through ashless filter paper, wash with hot water, and incinerate the residue.
- Cool in a desiccator and weigh the residue.

**Formula:**

$$\text{Acid-Insoluble Ash (\% w/w)} = \left( \frac{\text{Weight of acid-insoluble residue}}{\text{Weight of air-dried sample}} \right) \times 100$$

**2.5.2.1.3. Water-Soluble Ash**

Water-soluble ash is the difference in weight between the total ash and the residue left after treating the ash with water. It represents the amount of inorganic salts soluble in water. It indicates the presence of water-soluble salts, and a higher value may suggest contamination with water-soluble earthy materials or added salts.

**Procedure Summary:**

- Boil the total ash with 25 mL of distilled water for 5 minutes.
- Filter through ashless filter paper, wash with hot water, and incinerate the residue.
- Cool and weigh the residue.

**Formula:**

$$\text{Water-Soluble Ash (\% w/w)} = \left( \frac{\text{Weight of total ash} - \text{Weight of water-insoluble residue}}{\text{Weight of air-dried sample}} \right) \times 100$$

**2.5.2.2. Extractive Values**

**2.5.2.2.1. Alcohol-Soluble Extractive Value**

The alcohol-soluble extractive value is a vital parameter in the quality control and standardization of crude plant materials. It specifically evaluates the proportion of bioactive phytochemicals that are soluble in alcohol, primarily including alkaloids, glycosides, steroids, flavonoids, tannins, and other alcohol-soluble constituents. This test is routinely used as a quantitative indicator of the purity, strength, and chemical integrity of herbal materials. The process involves extraction of a known weight of air-dried plant powder using alcohol (usually ethanol or methanol as per pharmacopoeial standards), followed by filtration and evaporation of the solvent under controlled conditions. The residue left after evaporation is weighed, and the alcohol-soluble extractive value is expressed as a percentage w/w (weight/weight) of the air-dried material. A significantly low extractive value may suggest issues such as loss of active principles due to improper storage, adulteration with exhausted or inert materials, or substandard plant collection practices. Conversely, a very high value might indicate the presence of undesirable or non-specific extractable substances, possibly due to contamination, addition of foreign organic materials, or substitution with other plant species. Thus, this test serves as a sensitive benchmark for detecting adulteration, substitution, or low-quality raw materials, and is recommended by standard texts like the Indian Pharmacopoeia (2020) and WHO guidelines on herbal medicines.

**Formula:**

$$\text{Alcohol-Soluble Extractive Value (\% w/w)} = \left( \frac{\text{Weight of residue after evaporation (g)}}{\text{Weight of air-dried sample (g)}} \right) \times 100$$

**2.5.2.2.2. Water-Soluble Extractive Value**

The water-soluble extractive value is a critical quality control parameter that evaluates the amount of polar phytoconstituents present in a crude drug. While not always emphasized individually in certain summaries, this test is generally performed alongside the alcohol-soluble extractive value as part of a comprehensive phytochemical evaluation. It helps in detecting bioactive compounds that are readily soluble in water, such as sugars, tannins, glycosides, mucilage, amino acids, and some alkaloids. The presence and proportion of these water-soluble components are essential indicators of the therapeutic potency, purity, and authenticity of the plant material. A deviation from the expected range — either a significantly low or high extractive value — may imply adulteration, substitution with exhausted material, or poor storage and handling conditions that result in degradation of hydrophilic constituents. The procedure involves macerating a known quantity of the air-dried plant powder with distilled water for a fixed period (usually 24 hours), followed by filtration and evaporation of an aliquot of the filtrate to dryness. The weight of the dried residue represents the amount of water-soluble matter, which is then expressed as a percentage of the original air-dried sample. This parameter is crucial for standardization and is particularly relevant for aqueous herbal formulations, such as decoctions, infusions, and teas, where water acts as the primary extraction medium. The test is recognized by regulatory authorities including the Indian Pharmacopoeia (2020) and the WHO guidelines on herbal medicine quality control.

**Formula:**

$$\text{Water-Soluble Extractive Value (\% w/w)} = \left( \frac{\text{Weight of water-soluble residue (g)}}{\text{Weight of air-dried sample (g)}} \right) \times 100$$

**2.5.2.3. Loss on Drying (LOD)**

The Loss on Drying was determined at 105 ± 1°C using a hot air oven to estimate the moisture content. A high moisture content can:

- Promote microbial and fungal growth.
- Accelerate enzymatic and hydrolytic degradation. Thus, controlling moisture is vital for storage stability and longevity of the plant material.

**2.5.3. Powder Densities**

In pharmaceutical formulation development, especially for solid dosage forms like tablets and capsules, the flow properties, compressibility, and packing behavior of powdered plant materials or phytopharmaceutical extracts are crucial for ensuring processability, content uniformity, and final product quality. These physical parameters directly influence blending, filling, compression, and coating processes in both laboratory and industrial settings. These values assist in understanding:

**2.5.3.1. Flow properties**

Flow properties describe the ease with which powdered material moves under the influence of gravity or mechanical force. Poor flow leads to inconsistent dosing, machine jamming, and non-uniform filling. They are typically evaluated by the following parameters:

**2.5.3.1.1. Angle of Repose (θ)**

It is the maximum angle between the surface of a powder pile and the horizontal plane. A lower angle indicates better flow.

**Formula:**

$$\theta = \tan^{-1}\left(\frac{h}{r}\right)$$

Where: h = height of the powder cone; r = radius of the powder cone

**2.5.3.1.2. Hausner's Ratio**

It measures the cohesiveness of the powder and is calculated from bulk and tapped densities.

**Formula:**

$$\text{Hausner's Ratio} = \frac{\text{Tapped Density}}{\text{Bulk Density}}$$

**2.5.3.1.3. Carr's Compressibility Index (CI)**

It indicates the compressibility of the powder, correlating to its flow characteristics.

**Formula:**

$$\text{CI} = \left(\frac{\text{Tapped Density} - \text{Bulk Density}}{\text{Tapped Density}}\right) \times 100$$

**2.5.3.2. Packing behavior during formulation development**

Packing behavior involves how particles occupy space in a container or during compression. It affects bulk and tapped densities, which in turn affect dosage uniformity, flowability, and compaction.

**2.5.3.2.1. Bulk Density ($\rho_0$)**

It refers to the mass of powder per unit volume without tapping.

**Formula:**

$$\text{Bulk Density} = \frac{\text{Mass of the powder (g)}}{\text{Untapped Volume (mL)}}$$

**2.5.3.2.2. Tapped Density ($\rho t$)**

It is the density obtained after mechanically tapping the powder until volume becomes constant.

**Formula:**

$$\text{Tapped Density} = \frac{\text{Mass of the powder (g)}}{\text{Tapped Volume (mL)}}$$

**2.5.4. Histological Evaluation (Transverse Section Microscopy)**

Histological examination of *Calotropis gigantea* leaves was carried out to validate the internal anatomical structure and ensure authenticity.

- A transverse section (TS) of the leaf was taken using a sharp microtome blade.
- The TS was then stained using phloroglucinol followed by concentrated sulfuric acid, which highlights lignified tissues (e.g., xylem).
- The stained section was mounted on a glass slide and examined under a trinocular compound microscope at 30× magnification.
- Diagnostic features such as vascular bundles, epidermal cells, trichomes, and parenchymatous tissue were noted and compared with standard literature.

**2.5.5. Powder Microscopy**

Powder microscopy was performed to identify cellular components characteristic of *Calotropis gigantea* leaf tissue:

- The powder was gently mounted on a glass slide using dilute glycerin.
- Appropriate staining was done to enhance contrast of cellular structures.
- A trinocular microscope with 10× magnification was used for detailed observation.
- Key microscopic features such as stomata type, trichomes, fragments of xylem vessels, parenchymatous cells, and fibers were identified and systematically recorded.

This technique is especially useful in detecting adulteration, substitution, or the presence of foreign matter.

**2.6. Phytochemical analysis**

**2.6.1. Phytochemical class determination**

Alkaloids, Sugars, glycosides, proteins, tannins, steroids, flavonoids, terpenes, etc. were all identified by phytochemical screening of the extract, using the specified standard test protocols.

| S. No. | Phytoconstituent | Test Name | Reagents Used | Observation | Inference |
|---|---|---|---|---|---|
| 1 | **Alkaloids** | Dragendorff's Test | Dragendorff's reagent (Bismuth potassium iodide) | Orange-red precipitate | Presence of alkaloids |
| | | Wagner's Test | Iodine in potassium iodide | Reddish-brown precipitate | Presence of alkaloids |
| | | Mayer's Test | Potassium mercuric iodide | White/cream precipitate | Presence of alkaloids |
| | | Hager's Test | Saturated picric acid | Yellow precipitate | Presence of alkaloids |
| 2 | **Glycosides** | Legal's Test | Pyridine + Sodium nitroprusside | Pink-red color | Presence of glycosides |
| | | Keller-Killiani Test | Glacial acetic acid, $FeCl_3$, Conc. $H_2SO_4$ | Blue-green ring at interface | Cardiac glycosides present |
| | | Borntrager's Test | Benzene + $NH_3$ | Pink to red ammoniacal layer | Anthraquinone glycosides present |

| S. No. | Phytoconstituent | Test Name | Reagents Used | Observation | Inference |
|---|---|---|---|---|---|
| 3 | **Flavonoids** | Alkaline Reagent Test | NaOH followed by dilute HCl | Yellow turns colorless | Presence of flavonoids |
| | | Shinoda Test | Mg turnings + Conc. HCl | Pink/crimson color | Presence of flavonoids |
| | | Lead Acetate Test | Lead acetate solution | Yellow precipitate | Presence of flavonoids |
| 4 | **Saponins** | Froth Test | Water + vigorous shaking | Persistent froth | Saponins present |
| | | Foam Height Test | 1:20 dilution in water | Foam height >1 cm | Saponins confirmed |
| 5 | **Tannins & Phenolics** | Ferric Chloride Test | 5% $FeCl_3$ | Blue-black/green precipitate | Tannins/phenolics present |
| | | Lead Acetate Test | 10% Lead acetate | White precipitate | Presence of tannins |
| | | Gelatin Test | 1% Gelatin in NaCl | White precipitate | Presence of tannins |
| 6 | **Terpenoids** | Salkowski Test | Chloroform + Conc. $H_2SO_4$ | Reddish-brown interface | Terpenoids confirmed |
| | | Liebermann–Burchard Test | Acetic anhydride + Conc. $H_2SO_4$ | Green/brown coloration | Steroids/triterpenoids present |
| 7 | **Steroids** | Chloroform Test | Chloroform + Conc. $H_2SO_4$ | Reddish upper layer | Presence of steroids |
| 8 | **Proteins** | Biuret Test | 1% $CuSO_4$ + 10% NaOH | Violet color | Proteins present |
| | | Millon's Test | Millon's reagent | White → red on heating | Tyrosine-containing proteins present |
| 9 | **Amino Acids** | Ninhydrin Test | 0.2% Ninhydrin in ethanol | Violet color | Amino acids present |
| 10 | **Carbohydrates** | Molisch's Test | α-Naphthol + Conc. $H_2SO_4$ | Purple/violet ring | Carbohydrates present |
| | | Fehling's Test | Fehling's A & B | Brick-red precipitate | Reducing sugars present |
| | | Benedict's Test | Benedict's reagent + Heat | Reddish precipitate | Reducing sugars present |
| 11 | **Fats and Oils** | Spot Test | Filter paper spot | Permanent grease mark | Oils/fats present |
| | | Saponification Test | Alcoholic KOH + Water | Soap formation | Fats/oils present |

**2.6.2. Total Phenolic Content (TPC)**

The total phenolic content (TPC) of various solvent extracts obtained from *Calotropis gigantea* fruits were determined using the Folin–Ciocalteu colorimetric method, which is a standard and widely accepted

procedure for the quantification of phenolic compounds. Gallic acid was employed as the standard reference phenolic compound, and results were expressed in milligrams of gallic acid equivalents per gram of extract (mg GAE/g extract).

To begin, 1.0 mL of each prepared plant extract (appropriately diluted in methanol or ethanol to fall within the standard curve range) was transferred into separate clean test tubes. To each tube, 5.0 mL of freshly prepared 10-fold diluted Folin–Ciocalteu reagent was added. The mixture was vortexed gently and allowed to stand for 5 minutes at room temperature to allow initial reaction. Following this, 4.0 mL of sodium carbonate solution (7.5% w/v) was added to each test tube. The solutions were thoroughly mixed and incubated at room temperature in the dark for 30 minutes to facilitate complete color development.

After incubation, the absorbance of the resulting blue-colored complex was measured at 760 nm using a UV-Visible spectrophotometer against a reagent blank. A standard calibration curve was constructed using gallic acid at various concentrations (typically ranging from 10 to 100 µg/mL) prepared in the same manner. The phenolic content in each extract was then extrapolated from the gallic acid calibration curve and expressed as mg of gallic acid equivalents (GAE) per gram of dry extract weight. All experiments were performed in triplicate to ensure reproducibility and accuracy. The average values were used for reporting, and results were expressed as mean ± standard deviation (SD). The method ensured the accurate estimation of total phenolic constituents, which play a critical role in the antioxidant and therapeutic potential of phytoneutraceutical formulations derived from these medicinal fruits (AlFaris et al., 2021).

**2.6.3. Total Flavonoid Content (TFC)**

The total flavonoid content (TFC) of different solvent extracts obtained from *Calotropis gigantea* fruits were quantitatively determined using the aluminum chloride colorimetric method. Quercetin was used as the reference standard, and the results were expressed in terms of milligrams of quercetin equivalents per gram of dry extract (mg QE/g extract). Initially, stock solutions of the extracts were prepared by dissolving a known amount of each dried extract in methanol. These were then serially diluted to obtain appropriate concentrations for colorimetric analysis. A standard calibration curve was also prepared using quercetin in methanol at concentrations ranging from 10 to 100 µg/mL. For each test sample and standard, 1.0 mL of extract or standard solution was taken in a clean test tube. To this, 4.0 mL of distilled water was added, followed by the addition of 0.3 mL of 5% sodium nitrite ($NaNO_2$) solution. The mixture was allowed to stand for 5 minutes at room temperature. After this, 0.3 mL of 10% aluminum chloride ($AlCl_3$) solution was added and mixed thoroughly. The reaction mixture was then allowed to stand for another 6 minutes. Subsequently, 2.0 mL of 1 M sodium hydroxide (NaOH) solution was added, and the final volume was adjusted to 10.0 mL with distilled water. The contents were mixed well by vortexing, and the absorbance of the resulting pinkish solution was measured at 510 nm using a UV-Visible spectrophotometer against a reagent blank (prepared using methanol instead of extract or standard). The intensity of the color was directly proportional to the flavonoid concentration present in the sample. A standard curve of quercetin was constructed by plotting absorbance values against concentrations. The total flavonoid content in each extract was calculated from the linear regression equation obtained from the standard curve and expressed as milligrams of quercetin equivalent per gram of extract (mg QE/g extract). All determinations were carried out in triplicate to ensure accuracy and reproducibility. The final results were expressed as the mean ± standard deviation (SD). This method provides a reliable estimation of the flavonoid concentration, which is an important class of phytochemicals known for their antioxidant, anti-inflammatory, and therapeutic potential, thereby supporting the phytonutraceutical value of *Calotropis gigantea* (Zawawi et al., 2021)

**2.6.4. Tannin Content**

The total tannin content in various extracts of *Calotropis gigantea* was estimated using the Folin–Denis colorimetric method, as described in standard phytochemical protocols with slight modifications. This method relies on the reaction between tannin compounds and Folin–Denis reagent, resulting in a blue-colored complex that can be measured spectrophotometrically.

**Preparation of Reagents**

1. **Folin–Denis Reagent**: Prepared by mixing 100 g of sodium tungstate and 20 g of phosphomolybdic acid in 750 mL of distilled water, followed by the addition of 50 mL of concentrated phosphoric acid. The mixture was refluxed for 2 hours, cooled, and diluted to 1 L with distilled water. Commercially available Folin–Denis reagent may also be used.
2. **Sodium Carbonate Solution (20% w/v)**: Dissolve 20 g of anhydrous sodium carbonate in 100 mL of distilled water and filter if necessary.
3. **Tannic Acid Standard Solution**: A standard stock solution of tannic acid (100 µg/mL) was prepared by dissolving 10 mg of tannic acid in 100 mL of distilled water. Serial dilutions were made to construct a calibration curve ranging from 10–100 µg/mL.

**Sample Preparation**

Accurately weighed quantities (100 mg) of each dried plant extract were dissolved in 10 mL of distilled water and filtered through Whatman No. 1 filter paper. The clear filtrate was used for analysis.

**Procedure**

1. To a test tube, 1 mL of the extract or standard solution was added.
2. Then, 0.5 mL of Folin–Denis reagent was added to the test tube.
3. After 3 minutes, 1 mL of 20% sodium carbonate solution was added.
4. The final volume was adjusted to 10 mL with distilled water.
5. The mixture was vortexed gently and allowed to stand for 30 minutes at room temperature for full color development.
6. The absorbance of the resulting blue-colored complex was measured at 700 nm using a UV–Visible spectrophotometer against a reagent blank.

**Calculation and Expression of Results**

A standard calibration curve was prepared using tannic acid by plotting absorbance values against corresponding concentrations. The tannin content in the extracts was calculated from the regression equation derived from this curve. The results were expressed as mg of tannic acid equivalents per gram of dry extract (mg TAE/g extract). Each determination was conducted in triplicate, and results were expressed as mean ± standard deviation (SD) to ensure accuracy and reproducibility (Lahare et al., 2021).

**2.6.5. Saponin Content**

The estimation of saponin content in the fruit extracts of *Calotropis gigantea* was carried out using a gravimetric method, following procedures adapted from Obadoni and Ochuko (2001). Saponins are a class of bioactive compounds that have been shown to possess a variety of biological activities, including antimicrobial, anti-inflammatory, and antioxidant effects. Accurate determination of saponin levels is essential for understanding the therapeutic potential of these plant species.

To begin the estimation, 5 g of the powdered fruit extract was weighed accurately and transferred to a conical flask. The extract was then mixed with 100 mL of 20% ethanol (v/v), and the mixture was heated in a water bath at a temperature of 55–60°C for 4 hours. This heating process ensured the extraction of saponins from the plant material, with the ethanol acting as a solvent to dissolve the bioactive compounds. The mixture was continuously stirred to promote effective extraction of the saponins.

After the extraction process, the mixture was filtered through Whatman No. 1 filter paper to separate the plant material from the solvent. The residue was re-extracted with an additional 100 mL of 20% ethanol, repeating the same procedure. The combined filtrates were then concentrated to about 40 mL using a rotary evaporator at a temperature of 90°C. Care was taken not to allow the extract to dry completely at this stage to avoid any loss of saponins.

To purify the extract, the concentrated solution was transferred to a separating funnel, where it was washed with 20 mL of diethyl ether. This step was performed to remove any lipophilic impurities that may have been present in the extract. The aqueous layer was retained, and the ether layer was discarded. This extraction was repeated three times to ensure that the impurities were thoroughly removed.

The next step involved extracting the aqueous layer with 60 mL of n-butanol, a solvent that has a higher affinity for saponins. The n-butanol extract was then washed twice with 10 mL of 5% sodium chloride solution to further purify the sample by removing residual impurities. After the washing steps, the n-butanol

extract was concentrated to dryness using a rotary vacuum evaporator, or alternatively, it could be dried in an oven at 60°C until a constant weight of saponin was obtained.
The amount of crude saponins was determined by calculating the weight of the dried residue. The saponin content was expressed as a percentage of the initial weight of the fruit extract using the following formula:

$$\text{Total Saponin Content (\%)} = \left( \frac{\text{Weight of dried saponins (g)}}{\text{Initial weight of extract sample (g)}} \right) \times 100$$

This method provided an accurate measure of the saponin content, expressed as % w/w of crude saponins in the dried extract. Each sample was tested in triplicate to ensure reproducibility, and the results were presented as mean ± standard deviation (SD). The gravimetric method for saponin determination is highly effective and reliable, offering precise quantification of these important bioactive compounds in plant extracts. Understanding the saponin content in *Calotropis gigantea* is crucial for evaluating their potential as sources of phytoneutraceuticals, and the results from this procedure contribute to the broader understanding of their medicinal applications (Vittaya et al., 2022).

**2.7. Biological activity**

**2.7.1. *In vitro* antimicrobial activity**

We tested extract's antimicrobial properties *in vitro* against a number of harmful bacterial species, including *Bacillus subtilis, Klebsiella pneumoniae*, and *Escherichia coli*. Similarly, extract was tested for its antifungal efficacy *in vitro* against *Aspergillus niger* and *Candida albicans*, two types of fungus strains. MIC values of several compounds were compared with two reference drugs, ciprofloxacin (anti-bacterial) and fluconazole (anti-fungal).

**2.7.1.1. Antibacterial activity**

We found out how effective the extract was against bacteria *in vitro* by using the disc diffusion method and Muller Hinton Agar medium. The organisms were cultivated in nutrient broth and incubated for 24 hours at 37±1°C before being disseminated onto Muller Hinton agar plates in a longitudinal flow cabinet. After diluting the extract in dimethylsulfoxide (DMSO), it was completely saturated on Whatman filter paper No. 1 sterile discs (6 mm diameter). The discs were then placed in the incubator on top of the pre-made bacterial plates. The extract's inhibitory zone width was measured in millimeters after it was incubated for 24 hrs at 37±1°C. A disc coated with dimethyl sulfoxide (DMSO) served as the negative control, while the activity was compared to that of the standard antibiotic ciprofloxacin. The experiments were repeated three times.

**2.7.1.2. Antifungal activity**

To test the extract's antifungal activity *in vitro*, we performed the disc diffusion method under normal conditions using Potato dextrose agar medium. Agar plates were inoculated with a standardized solution of the microorganisms under study, and then sterile discs of Whatman filter paper No. 1 (6 mm diameter) were placed on top. Discs with specific amounts of the antifungal drug fluconazole (50 µg/mL) and extract (100 µg/mL) were embedded therein. Over the course of 72 hrs, the plates were kept at a temperature of 28±2°C to determine their antifungal activity. As a control, we used a paper disc that had been soaked with dimethyl sulfoxide (DMSO).

**2.7.1.3. MIC Determination**

To determine the extract's MIC, the agar streak dilution method was used. Before the evaluation, a DMSO extract stock solution was made. Then, the components to be tested were combined with a precise amount of sterile molten Muller Hinton agar. A particular quantity of medium containing the extract was used to fill a Petri dish to a depth of 3-4 mm and then let it firm. Following amplification of the microbial suspension to $10^5$ CFU/mL, it was introduced to test plates that already contained extract in DMSO. Subsequently, the plates were placed in an incubator set at 37±1°C. Upon completion of the incubation period, the MIC values were determined. Prior to using the average to arrive at the final result, each measurement was double-checked. One 100 mL volume of DMSO served as the negative control, while one 100 µg/mL volume of the common antibiotic ciprofloxacin served as the positive control. At what concentration of the test sample did the test plate not show any signs of microbial or fungal growth.

**2.7.2. Cardioprotective activity**

The *in vivo* cardioprotective activity of *Calotropis gigantea* was evaluated using an isolated rat heart model via Langendorff's apparatus. This model allows for the assessment of heart function under controlled conditions and is widely used for studying ischemia-reperfusion (I/R) injury and the cardioprotective effects of various agents.

**2.7.2.1. Animals**

Healthy adult Wistar rats (200–250 g) were selected for the study. Healthy adult male Wistar rats (200–250 g) were obtained from recognized breeder. The animals were group-housed (a maximum of 3 rats per cage) in standard polypropylene cages under strictly controlled environmental conditions: a 12/12-hour light/dark cycle (lights on at 07:00), an ambient temperature of 22 ± 2°C, and relative humidity of 55 ± 10%. Standard laboratory rodent chow and tap water were provided *ad libitum*. All animals were allowed a minimum acclimatization period of seven days prior to the commencement of any experimental procedures to minimize stress from transportation. All experimental protocols and animal handling procedures were reviewed and approved by the Institutional Animal Ethical Committee (IAEC) of School of Pharmacy, Chouksey Engineering College, Bilaspur (Protocol Approval Number: SOP/IAEC/2024/11/18, Dated: 22 November 2024) and were conducted in strict accordance with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India. Every effort was made to adhere to the principles of the 3Rs (Replacement, Reduction, and Refinement) to minimize animal numbers and avoid suffering. Animal health and well-being were monitored daily throughout the study.

**2.7.2.2. Langendorff Perfusion Setup**

The rats were anesthetized with sodium pentobarbital (50 mg/kg, intraperitoneally), ensuring that the animals were deeply anesthetized before proceeding with the heart extraction. The hearts were quickly excised and immediately submerged in ice-cold Krebs-Henseleit buffer to prevent ischemic damage before mounting on the Langendorff apparatus. The excised hearts were carefully cannulated through the aorta and connected to the Langendorff apparatus for retrograde perfusion with Krebs-Henseleit buffer (pH 7.4), which was continuously oxygenated with 95% $O_2$ and 5% $CO_2$. The buffer was maintained at 37°C throughout the experiment, and a constant perfusion pressure of 75 mmHg was applied.

**2.7.2.3. Experimental Groups**

The study was divided into the following experimental groups:

- **Group-1 (Normal Control)**

Hearts perfused with Krebs-Henseleit buffer without ischemia.

- **Group-2 (Ischemia-Reperfusion Control)**

Hearts subjected to global ischemia for 30 minutes, followed by 120 minutes of reperfusion, without any treatment.

- **Group-3 (*Calotropis gigantea* Extract)**

Hearts perfused with Krebs-Henseleit buffer containing *Calotropis gigantea* extract (100–500 µg/mL) for 10 minutes before ischemia and throughout the reperfusion period.

- **Group-4 (*Calotropis gigantea* Extract)**

Hearts perfused with Krebs-Henseleit buffer containing *Calotropis gigantea* extract (100–500 µg/mL) following the same protocol.

- **Group-5 (Combined Treatment)**

Hearts perfused with a combination of *Calotropis gigantea* extracts (100–500 µg/mL each) in the buffer before ischemia and during reperfusion.

**2.7.2.4. Induction of Ischemia-Reperfusion (I/R) Injury**

For the ischemia-reperfusion groups, global ischemia was induced by stopping the perfusion for 30 minutes. During this period, the heart was not oxygenated, mimicking a heart attack or reduced blood flow. After 30 minutes, reperfusion was initiated by restoring the oxygenated Krebs-Henseleit buffer for 120 minutes. This step mimicked the restoration of blood flow following an ischemic event, which is known to cause reperfusion injury.

**2.7.2.5. Assessment Parameters**

The following parameters were evaluated to determine the cardioprotective effects of *Calotropis gigantea* extracts:

**2.7.2.5.1. Hemodynamic Parameters**

Left ventricular developed pressure (LVDP), heart rate, and coronary flow were continuously monitored throughout the experiment using a pressure transducer connected to the heart. These parameters were recorded at baseline (before ischemia), during ischemia, and at specific intervals during the reperfusion period to assess cardiac function.

**2.7.2.5.2. Infarct Size**

At the end of the reperfusion period, the hearts were cut into 2–3 mm thick slices and incubated with 2,3,5-triphenyl tetrazolium chloride (TTC) solution (1%) for 20 minutes at 37°C. TTC stains viable myocardium red, while infarcted (necrotic) tissue remains pale. The infarct size was expressed as a percentage of the total ventricular area and compared between groups.

**2.7.2.5.3. Cadiac Injury Markers**

Perfusate samples were collected at regular intervals during the reperfusion period and analyzed for lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) levels, which are key indicators of myocardial injury.

**2.7.2.5.4. Oxidative Stress Markers**

Myocardial tissue homogenates were prepared post-reperfusion to measure oxidative stress. Malondialdehyde (MDA) levels, a marker of lipid peroxidation, were quantified. Additionally, the activities of antioxidant enzymes such as superoxide dismutase (SOD) and catalase were measured to assess the extracts' ability to combat oxidative stress.

**2.7.2.6. Human-Induced Pluripotent Stem Cell-Derived Cardiomyocytes (hiPSC-CMs)**

Human-induced pluripotent stem cells (hiPSCs) were obtained from a commercial supplier or derived from donor cells through reprogramming techniques using Yamanaka factors. The hiPSCs were cultured in a standard cell culture incubator at 37±1°C with 5% $CO_2$ in feeder-free conditions using mTeSR1 medium. For differentiation into cardiomyocytes, hiPSCs were treated with a differentiation medium consisting of RPMI 1640 supplemented with B27 (minus insulin) and specific growth factors to induce mesodermal and cardiac lineage differentiation. Over a period of 15–20 days, the cells began to show spontaneous contractile activity, indicating the formation of functional cardiomyocytes. After confirmation of cardiomyocyte differentiation via immunostaining for cardiac markers (e.g., cardiac troponin T and α-actinin), the cells were subjected to cardioprotective studies. Cardiomyocytes were exposed to cardiotoxic agents such as doxorubicin (10 μM) or hydrogen peroxide (200 μM) to induce oxidative stress or simulate ischemia-reperfusion injury. Test compounds, including plant extracts from *Calotropis gigantea*, were administered at various concentrations (*e.g.*, 25, 50, and 100 μg/mL) to evaluate their protective effects. Cardiomyocyte viability was assessed using MTT or LDH assays, and cellular oxidative stress markers such as malondialdehyde (MDA) and superoxide dismutase (SOD) were quantified. Beating frequency and contractile strength were measured using video microscopy and motion analysis software.

**2.7.2.7. Histopathological Examination**

Following the completion of the reperfusion period in the cardioprotective activity study, a meticulous histopathological analysis was conducted to assess the structural and cellular integrity of myocardial tissue. The excised heart tissues were immediately fixed in 10% neutral buffered formalin, a widely accepted fixative that preserves tissue architecture by cross-linking proteins and preventing autolysis or putrefaction. Fixation was allowed to proceed for 24–48 hours at room temperature to ensure optimal preservation of cellular and extracellular components. Once fixation was complete, the tissues were dehydrated through a graded series of alcohol solutions, cleared in xylene, and then embedded in paraffin wax to obtain uniform solid tissue blocks. These paraffin blocks were carefully sectioned using a microtome to achieve ultra-thin slices of 5–7 μm thickness, which allows for precise microscopic evaluation of myocardial histoarchitecture. The sections were subsequently mounted on clean glass slides and stained with hematoxylin and eosin (H&E). Hematoxylin stains the nuclei of cells a deep blue or purple, providing contrast to eosin, which stains the cytoplasm and extracellular matrix in varying shades of pink. This classic staining technique offers high-

resolution visualization of tissue morphology and cellular details. Microscopic examination of the stained sections was performed under a light microscope. Key features evaluated included:

- **Myocardial architecture**: to assess the overall organization of cardiac fibers.
- **Fiber integrity**: looking for disruptions, fragmentation, or loss of continuity in muscle fibers.
- **Evidence of ischemic injury**, such as:
    - **Necrosis**: characterized by cell swelling, loss of nuclei, cytoplasmic eosinophilia, and disruption of cell membranes.
    - **Edema**: visible as clear spaces between fibers indicating interstitial fluid accumulation.
    - **Inflammatory cell infiltration**: presence of neutrophils or other immune cells in response to tissue injury.

This detailed histological assessment enabled a comparative analysis between control and treated groups, providing crucial insight into the extent of ischemia-induced damage and the protective effect of the test extract or formulation on the myocardial tissue.

**2.8. Formulation development**

The gel was made with the following ingredients: *Calotropis gigantea* extract, triethanolamine, ethanol, distilled water, and carbopol 940 (CP). The *Calotropis gigantea* extracts (aqueous, methanol, ethyl acetate, and petroleum ether) was mixed with hydroalcoholic content. Then, CP having fixed hydroalcoholic content, was added drop wise into the *Calotropis gigantea* extract mixture. In order to get the desired gel consistency, the mixture was constantly churned. Addition of DMSO occurred immediately upon gel formation. Lastly, the gel was allowed to set (**Table 1**).

**Table 1.** Formulation Chart.

| INGREDIENTS | F1 | F2 | F3 | F4 |
|---|---|---|---|---|
| *Calotropis gigantea* aqueous extract (g) | 1 | - | - | - |
| *Calotropis gigantea* ethyl acetate extract (g) | - | - | 1 | - |
| *Calotropis gigantea* methanol extract (g) | - | 1 | - | - |
| *Calotropis gigantea* petroleum ether extract (g) | - | - | - | 1 |
| Carbapol 940 (g) | 1 | 1 | 1 | 1 |
| Triethanolamine (mL) | 1.8 | 1.8 | 1.8 | 1.8 |
| Ethanol (mL) | 18.7 | 18.7 | 18.7 | 18.7 |
| DMSO (mL) | 3 | 3 | 3 | 3 |
| Distilled Water (mL) | 76.5 | 76.5 | 76.5 | 76.5 |

**2.8.1. Evaluation parameters**

The formulations were comprehensively evaluated for washability, physical evaluation, spreadability, skin irritation test, viscosity, pH, swelling index, accelerated stability studies, and extrudability, as per standard protocols.

**2.8.1.1. Extrudability**

To test the formulation's extrudability, 100 g of gels were initially placed into collapsible aluminium tubes with caps and sealed manually. The tubes, each holding a unique recipe, were securely clamped between two slides. Then, after 10 minutes, the extruded ribbon length was measured following the placement of a 500 g weight on top of the slides and, lastly, the removal of the cap.

**2.8.1.2. pH**

A digital pH metre that had been calibrated was used to measure the dermal gel's pH. To get a consistent measurement, the glass electrode was submerged in a mixture of 1 g of the formulation and 25 mL of pure water. We took three separate pH readings for each formulation and averaged them together.

**2.8.1.3. Physical appearance**

The developed herbal gel was examined visually for its transparency, colour, and overall look. By feeling the mixture between the fingers and looking for lumps, roughness, homogeneity, and smoothness, we were able to measure the gel's smoothness.

**2.8.1.4. Skin irritation test**

A semi-occlusive bandage was used to cover the normally hairless skin for one hour after applying 0.5 g of the prepared gel over a 6 $cm^2$ region. When the placement time was over, the bandage was peeled off, the gel had been scraped away, and the area was looked for blisters or any other comparable symptoms. The duration of the test was seven days. Grades were used to express the outcomes.

**2.8.1.5. Spreadability**

In order to determine the herbal dermal gel's spreadability, the slip-drag theory was used. The process included placing 2 g of the mixture onto a prepared ground slide, followed by a comparable glide slide with a hook attached to it. By pressing a big item against the slides, which released the trapped air, a uniform layer was created between them. What remained after scraping off the excess gel was the perimeter. Next, the top slide was fine-tuned such that it dragged with an intensity of 50 g. To determine how long it took for the upper slide to move 6 cm, the following formula was utilized:

$$S = M \times L / T$$

**2.8.1.6. Swelling index**

Quickly after preparation, 5 mL of emulsion was added to plastic pots to assess the creaming index. After 4 hours, the amount of the cream that had developed was measured in order to estimate the creaming %. By dissolving 2 g of the dermal herbal gel in 10 mL of distilled water, the swelling index of the finished product was ascertained. After one hour, the formula that had inflated was transferred from the beaker to a petridish. After reweighing the contents, through applying the subsequent equation, we managed to ascertain the swelling index:

$$Si = Wt - Wo / Wo$$

**2.8.1.7. Viscosity**

The viscosity of the formulation was measured using the Digital Brookfield Viscometer with spindle no. 6 set at 10 rpm and a temperature of 25±1°C. Before taking the measurements, the gel was allowed to settle for at least 30 minutes in a wide-mouthed container that was filled to the brim with enough to submerge the spindle.

**2.8.1.8. Washability**

After applying the gel to the skin, we personally observed its impact and evaluated how easy it was to wash off with distilled water to determine the formulations' washability.

**2.8.1.9. Accelerated stability studies**

To evaluate the physical and chemical stability of the optimized gel formulation, a well-controlled accelerated stability study was performed in accordance with International Council for Harmonisation (ICH) guidelines (Q1A-R2). The formulation was subjected to stress conditions of 40°C ± 2°C temperature and 75% ± 5% relative humidity (RH) for a period of 90 days to simulate long-term storage conditions and predict the product's shelf-life. The prepared gel was carefully transferred into a pharmaceutical-grade, airtight PVC container, which was then securely sealed and wrapped with black aluminum foil to prevent any photodegradation due to light exposure. This setup was crucial to maintain the formulation's integrity by minimizing the risk of oxidative or UV-induced degradation. During the study, the formulation was periodically monitored at pre-determined intervals (e.g., 0, 30, 60, and 90 days). All the key evaluation parameters that were assessed during the initial formulation development—such as appearance, pH, viscosity, spreadability, homogeneity, drug content, and microbial contamination—were re-evaluated to determine any significant changes overtime. Any deviations from the initial values were carefully analyzed to assess the formulation's robustness, effectiveness, and shelf-stability under accelerated storage conditions.

**2.9. Chromatographic and Spectroscopic Fingerprinting**

**2.9.1. Thin Layer Chromatography (TLC)**

Thin Layer Chromatography (TLC) was employed as a qualitative analytical technique to assess the presence of various phytoconstituents in the extracts of *Calotropis gigantea*. This method was chosen due to its simplicity, rapidness, cost-effectiveness, and its ability to resolve complex mixtures into individual components. TLC was

performed on pre-coated silica gel 60 $F_{254}$ aluminum plates (Merck, Germany), which acted as the stationary phase. The phytochemical profile of each extract was studied by applying a small quantity of the prepared sample solution on the TLC plate and developing it in an appropriate solvent system.

**2.9.1.1. Sample Preparation**

The dried extracts of *Calotropis gigantea* were separately dissolved in respective solvents (methanol or ethanol) at a concentration of 10 mg/mL. These solutions were filtered through Whatman No. 1 filter paper to remove particulate matter and stored in amber vials until use. Using a capillary tube, small spots of each sample solution (approximately 2–3 µL) were applied 1.5 cm from the bottom edge of the TLC plate. Care was taken to ensure that the spots were small, concentrated, and equidistant to facilitate effective resolution.

**2.9.1.2. Development of Chromatogram**

Various solvent systems were screened to identify the best mobile phase for optimum separation of constituents, depending on the polarity of the compounds present in each extract. For example, a commonly used mobile phase for non-polar compounds was hexane:ethyl acetate (7:3), while polar constituents were separated using ethyl acetate:methanol:water (10:1.35:1). The selected mobile phase was poured into a clean, dry TLC chamber lined with filter paper and saturated for 20–30 minutes to ensure uniform solvent vapor distribution.

Once saturation was achieved, the prepared TLC plates were gently placed into the chamber in such a way that the spotted edge was immersed in the solvent without submerging the spots themselves. The solvent front was allowed to rise until it reached approximately 8 cm from the origin. The plates were then removed, air-dried, and immediately marked to indicate the solvent front.

**2.9.1.3. Detection and Visualization**

After development, the plates were air-dried and then observed under ultraviolet (UV) light at 254 nm and 366 nm to detect UV-active compounds. Further visualization was carried out by spraying with various specific reagents depending on the nature of the phytoconstituents: Dragendorff's reagent for alkaloids, ferric chloride for phenolics and tannins, vanillin-sulfuric acid for steroids and triterpenoids, and Liebermann-Burchard reagent for sterols. Heating the plates at 100°C for a few minutes after spraying enhanced the visibility of colored spots.

**2.9.1.4. Interpretation of Results**

The retention factor (Rf) values for each spot were calculated using the formula:

**Rf = Distance traveled by the compound / Distance traveled by the solvent front**

Different Rf values indicated the presence of different phytochemicals in the extracts. The results were compared with those of standard reference compounds, when available, to tentatively identify specific phytoconstituents. This technique provided preliminary qualitative evidence of the presence of secondary metabolites and was used to select fractions for further detailed phytochemical or bioactivity studies. TLC proved to be a vital step in profiling the chemical constituents of *Calotropis gigantea* and laid the groundwork for subsequent phytochemical isolation and characterization (Kowalska and Sajewicz, 2022).

**2.9.2. High-Performance Thin Layer Chromatography (HPTLC)**

High-Performance Thin Layer Chromatography (HPTLC) is an advanced chromatographic technique used for the qualitative and quantitative analysis of phytochemicals in complex plant extracts. In this study, HPTLC was employed to analyze the chemical profile of *Calotropis gigantea* fruit extracts, allowing for high-resolution separation, identification, and quantification of the constituents. HPTLC is an enhanced version of traditional TLC, involving the use of high-quality, fine-grained stationary phases and automated instruments for sample application, development, and detection, making it highly efficient and precise.

**2.9.2.1. Sample Preparation**

The powdered extracts of *Calotropis gigantea* were dissolved in an appropriate solvent (usually methanol, ethanol, or water, depending on the polarity of the constituents) to a concentration of 10 mg/mL. The solutions were filtered using Whatman No. 1 filter paper to ensure that only clear solutions were applied onto the HPTLC plate. The extracts were applied in defined spots or bands on the plate using an automated sample applicator to ensure precision and reproducibility.

**2.9.2.2. Instrumentation Setup**

HPTLC analysis was performed using a CAMAG (Switzerland) system, which includes a LINOMAT V sample applicator, a TLC scanner 3, and a Twin-Trough chamber. The stationary phase used was a 0.2 mm thick silica gel 60 F254 HPTLC plate (Merck, Germany). The system was calibrated and the plates were activated by heating at 110°C for 5–10 minutes to remove any residual moisture, which could affect the separation process.

**2.9.2.3. Application of Samples**

Samples were applied to the HPTLC plate using the LINOMAT V applicator in precise bands or spots, ensuring that each band was applied with uniform volume and spacing. The applicator was programmed to deposit approximately 10 µL of each sample onto the plate at a constant speed, with a band length of around 6 mm. After the application of the extracts, the plates were air-dried for a few minutes to allow the solvent to evaporate.

**2.9.2.4. Development of the Plate**

The development of the plate was carried out in a Twin-Trough chamber containing a saturated atmosphere of the mobile phase. The mobile phase was carefully selected based on the nature of the compounds being separated. For non-polar compounds, a solvent mixture such as hexane: ethyl acetate (7:3 v/v) was used, while for polar compounds, a mixture like ethyl acetate: methanol: water (10:1.35:1 v/v/v) was preferred. The plate was placed in the chamber, and the solvent was allowed to ascend the plate through capillary action. The development was continued until the solvent front reached around 8–10 cm from the origin, ensuring optimal separation of the components.

**2.9.2.5. Detection and Visualization**

After development, the HPTLC plates were removed from the chamber and air-dried. To visualize the separated components, the plates were scanned using the HPTLC scanner 3, which operates at different wavelengths (254 nm, 366 nm, and visible light) to detect various types of compounds based on their UV absorbance. Further visualization was performed by applying specific reagents tailored for different classes of compounds. For example, anisaldehyde-sulfuric acid was sprayed for detecting terpenoids, flavonoids, and alkaloids, while vanillin-sulfuric acid was used to detect phenolic compounds and steroids. After spraying, the plates were heated at 100°C for 5–10 minutes to enhance the visibility of the spots.

**2.9.2.6. Quantification and Calculation of Rf**

The HPTLC plate was analyzed by scanning the chromatogram with the TLC scanner. The retention factor (Rf) values of the separated compounds were calculated using the formula:

**Rf = Distance traveled by the compound / Distance traveled by the solvent front**

The Rf values help in the identification of the components by comparing them with standard reference compounds. For quantitative analysis, the intensity of the spots corresponding to specific phytochemicals was recorded. Calibration curves for known standards were generated, and the concentration of the compounds in the extracts was calculated by measuring the area under the curve for each spot.

**2.9.2.7. Data Interpretation**

The HPTLC data were interpreted by comparing the Rf values and the intensity of the spots with those of standard reference compounds. If reference standards were unavailable, the profile of the extract was analyzed based on literature or comparative analysis with known databases. Quantification of specific compounds was performed by integrating the peak areas corresponding to the separated compounds. The results of HPTLC analysis provided valuable information about the phytochemical composition of *Calotropis gigantea* extracts, which can be further correlated with their biological activities (Morlock, 2021).

## 3. RESULTS AND DISCUSSION

### 3.1. Extraction value

Among the solvents tested, ethanol (MET) gave the highest extraction yield (14.62 ± 0.41%), producing a deep green extract with a thick, gummy, and viscous consistency (**Table 2**). This suggests the presence of a wide range of bioactive compounds including flavonoids, alkaloids, chlorophyll, and glycosides, as ethanol is capable of dissolving both polar and non-polar constituents. In contrast, distilled water (DW), a highly polar solvent, yielded 10.45 ± 0.34% of extract, which appeared dark brown and had a sticky, semi-solid texture.

This indicates the solubilization of hydrophilic components such as tannins, phenolics, sugars, and possibly some mucilaginous substances. The ethyl acetate (EA) extract showed a moderate yield of 5.80 ± 0.28%, with a pale greenish-brown color and a resinous, semi-solid nature. As a semi-polar solvent, ethyl acetate is effective in extracting mid-polarity compounds such as certain flavonoids, terpenoids, and steroids. Meanwhile, petroleum ether (PE), being non-polar, extracted predominantly lipophilic substances such as oils and waxes, which is reflected in the low yield (3.95 ± 0.21%), light yellow coloration, and greasy, oily residue. Overall, the results clearly demonstrate that solvent polarity significantly affects the type and amount of phytochemicals extracted from *Calotropis gigantea*, with ethanol proving to be the most efficient solvent for comprehensive phytochemical recovery.

**Table 2.** Extraction characteristics of *Calotropis gigantea* using various solvents.

| **Solvent Used** | **Extraction Yield (%)** | **Color of Extract** | **Nature of Extract** |
|---|---|---|---|
| Distilled Water (DW) | 10.45 ± 0.34 | Dark brown | Sticky, semi-solid |
| Ethyl Acetate (EA) | 5.80 ± 0.28 | Pale greenish-brown | Resinous, semi-solid |
| Ethanol (MET) | 14.62 ± 0.41 | Deep green | Thick, gummy, viscous |
| Petroleum Ether (PE) | 3.95 ± 0.21 | Light yellow | Greasy, oily residue |

### 3.2. Physicochemical evaluations

The leaves of the plant were found to be triangular in shape with a coarse texture, displaying a color spectrum ranging from pale green to dark olive. Leaf sizes varied between 22 and 30 mm, offering consistency in dimension. Physicochemical analysis revealed that the leaves were free from microbial contamination and physical deterioration, such as discoloration or fungal growth, which are typically caused by moisture. The moisture content was exceptionally low, with a recorded loss on drying of 0.37%, adhering well to pharmacopoeial standards. The water solubility of the leaf powder was determined to be 8.42%, indicating higher-than-average solubility, which could be beneficial in extraction processes for medicinal formulations. Acid-insoluble ash content was found to be 2.23%, showcasing the purity of the leaves with minimal contamination by siliceous or earthy materials. The total ash content was relatively high at 12.65%, signifying the mineral richness of the plant material while remaining within acceptable limits for pharmacopoeial guidelines **(Table 3)**.

Heavy metal analysis showed negligible levels, all well below pharmacopoeial thresholds. The loss on ignition stood at 4.78%, suggesting a relatively high organic content, and further confirming the purity of the plant material. The foreign organic matter was observed to be 0.18%, confirming that the material was free from unwanted extraneous matter. The alcohol-soluble extractive value was recorded at 11.34%, pointing to a substantial proportion of constituents being soluble in alcohol, potentially beneficial for extracting active compounds. The leaf powder's compressibility index was measured at 38.56%, indicating moderate flow properties. Bulk density and tapped density were determined to be 0.142 g/cm³ and 0.265 g/cm³, respectively, showing that the material exhibited moderate packing properties, suitable for further formulation work with slight adjustments. Other physicochemical properties included a pH of 5.98 in a 1% aqueous solution, indicating mild acidity. The powder exhibited a swelling index of 3.11%, which suggests a moderate water retention capability. The foaming index was recorded at 85, indicating some presence of saponins, though at a relatively low level. All these findings suggest that the plant material is of high quality, suitable for further medicinal and formulation applications.

**Table 3.** Physicochemical evaluations.

| **PARAMETERS** | **DESCRIPTION** |
|---|---|
| **% compressibility index** | 41.92 |
| **Acid insoluble ash (% w/w)** | 1.98 |
| **Alcohol soluble extractive value** | 9.82 |

| | |
|---|---|
| **Bulk density (g/cm$^3$)** | 0.156 |
| **Color** | Yellow |
| **Loss on drying (%)** | 5.12 |
| **Shape** | Regular |
| **Size** | 18 – 26 mm |
| **Tapped density (g/cm$^3$)** | 0.273 |
| **Texture** | Smooth |
| **Total ash content (% w/w)** | 15.13 |
| **Water soluble ash (% w/w)** | 7.11 |

**3.3. Pharmacognostic study**

The pharmacognostic study of *Calotropis gigantea* reveals an intricate and unique structural composition that plays a crucial role in the plant's medicinal properties. Macroscopic examination of the leaves shows that they are large, thick, and leathery, with a broad ovate shape ranging between 10 and 20 cm in length. The surface of the leaves is smooth, covered with fine white hairs, and exhibits a pale green to greyish color. The leaf margins are entire, and the venation is pinnate with a prominent midrib running through the center. When damaged, the leaves exude a milky latex, characteristic of the plant and the Apocynaceae family.

A cross-sectional analysis of the leaf tissue highlights several noteworthy anatomical features. The outer epidermal layer is protected by a thick cuticle, which serves to reduce water loss and provide defense against environmental stress. The epidermal cells are polygonal, and stomata are observed on both surfaces of the leaf, specifically of the paracytic type, which facilitates gas exchange necessary for the plant's physiological functions.

One of the most significant features in the leaf structure is the presence of stone cells or sclereids. These polygonal sclerenchymatous cells have thick, lignified walls, which provide mechanical strength to the plant and contribute to its resilience. The rigid and durable nature of these cells, often isodiametric or polyhedral in shape, indicates significant lignification, making them crucial for the plant's structural integrity.

In addition to the stone cells, the epidermal layer contains cork cells that are impregnated with suberin, forming a brick or polygonal arrangement. This cork layer plays an essential role in protecting the plant from desiccation and external stress factors. Below the epidermis, the vascular bundles consist of phloem predominantly composed of parenchymatous cells, which are responsible for the transportation and storage of nutrients. Thick-walled parenchymatous cells are also found in the xylem, contributing to both the vascular function and overall mechanical strength of the plant.

Microscopic examination of powdered *Calotropis gigantea* leaf material reveals additional key elements. The presence of heterogeneous rays, varying from uni-seriate to multi-seriate (1–11 seriate), indicates a complex internal structure. These rays consist of procumbent cells and sheath cells, which play an integral role in the plant's overall architecture and mechanical stability. The powder also contains abundant starch granules of varying sizes, suggesting the plant's capability for energy storage, which is critical for its survival in harsh conditions.

Fibers are another prominent feature in the powdered material, appearing interspersed with parenchyma strands and forming either aggregated or narrow-banded structures. This arrangement highlights the plant's mechanical resilience and potential durability. Additionally, the study identified a high concentration of tannins, which contribute to the characteristic brownish-reddish color of the plant material. These tannins are well known for their astringent properties, adding to the medicinal value of the plant in traditional uses for wound healing and inflammation.

The fibers in the leaf powder are arranged alternately with significant amounts of axial parenchyma, much of which is apotracheal, forming a complex and highly organized structure not easily visible to the naked eye. The presence of tannins, starch, fibers, and stone cells all contribute to the pharmacognostic profile of *Calotropis gigantean* (**Figure 1**), showcasing its potential as a source of bioactive compounds. These findings

emphasize the need for further pharmacological studies to explore its therapeutic mechanisms and validate its traditional medicinal uses.

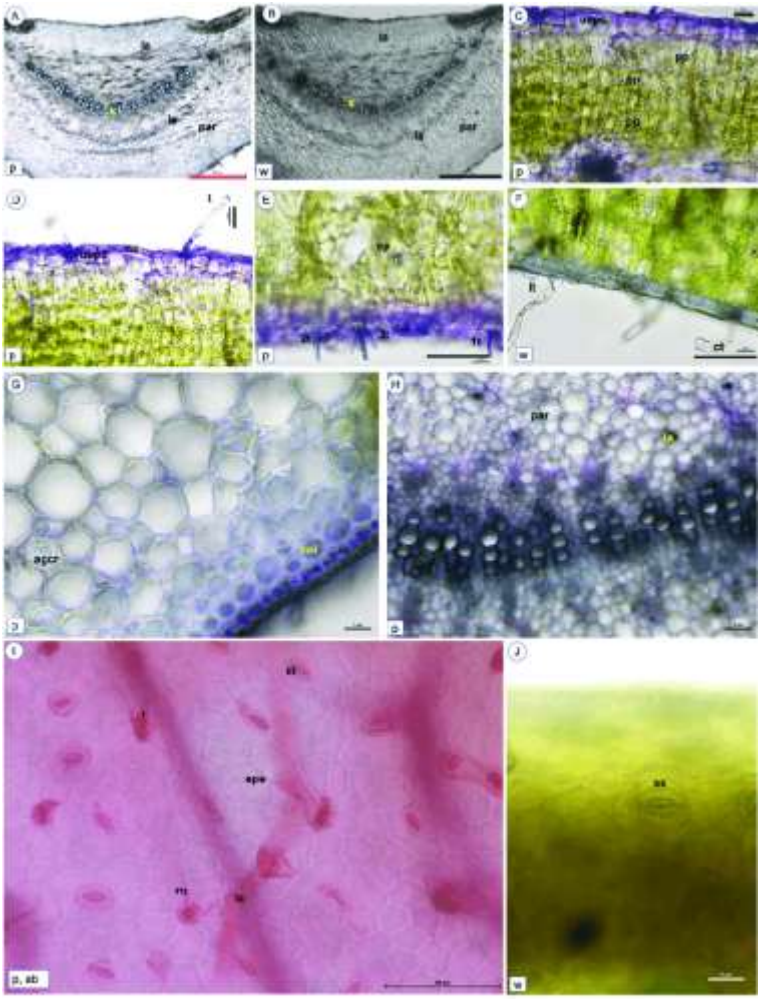

**Figure 1.** Microscopy of *Calotropis gigantea*.

### 3.4. Phytochemical analysis

#### 3.4.1. Phytochemical class determination

Terpenoids, saponin, carbohydrates, alkaloids, phenol, glycosides, sterols, tannins, flavonoids, and cardiac glycoside were identified by phytochemical screening of the extract **(Table 4)**.

**Table 4.** Phytochemical analysis.

| Chemical constituent | Test performed | Observations | Inference |
|---|---|---|---|
| Alkaloid | Hager's test | Yellow precipitate | **Alkaloid present** |
| Carbohydrate | Fehling's test | No Red precipitate | Carbohydrate absent |
| Diterpene | Copper acetate test | Emerald green color observed | **Diterpene present** |
| Flavonoid | Shinoda's test | Pinkish-red color | **Flavonoid present** |
| Glycoside | Borntrager's test | No Faint pink color observed | Anthraquinone glycoside absent |
| Glycoside | Legal's test | Red color observed | **Cardiac glycoside present** |

| | | | |
|---|---|---|---|
| Phenol | $FeCl_3$ test | Bluish-black color observed | **Phenol present** |
| Protein | Xanthoprotic test | No yellow color observed | Protein absent |
| Saponin | Froth formation test | Frothing for 5 min | **Saponin present** |
| Sterol | Libermann-Burchard's test | Brown-ring formation | **Sterol present** |
| Tannin | Gelatin test | Green color appeared | **Tannin present** |
| Triterpene | Salkowski's test | Yellow color observed | **Triterpene present** |

**3.4.2. Major Phytoconstituents determination**

**3.4.2.1. Total Phenolic Content (TPC)**

Phenolic compounds are among the most abundant and diverse groups of secondary metabolites found in plants. The total phenolic content in *Calotropis gigantea* was found to be 108.22 mg gallic acid equivalents (GAE) per gram of extract **(Table 5)**, which indicates a high concentration of phenolic antioxidants. Phenolics play a vital role in neutralizing free radicals due to their electron-donating properties and are extensively studied for their anti-inflammatory, anticancer, cardioprotective, and antimicrobial properties. The elevated TPC observed in this study suggests that *C. gigantea* may have a strong potential for oxidative stress modulation, making it a promising candidate for nutraceutical and therapeutic applications.

**3.4.2.2. Total Flavonoid Content (TFC)**

Flavonoids are polyphenolic compounds known for their anti-inflammatory, hepatoprotective, antiallergic, and antioxidant activities. The TFC of 84.35 mg quercetin equivalents (QE) per gram indicates a substantial presence of flavonoids in the extract. These bioactives contribute to the scavenging of reactive oxygen species (ROS) and help in the stabilization of cell membranes. Furthermore, flavonoids influence enzymatic activity and gene expression related to inflammation and cellular signaling pathways. A high TFC value also suggests the likelihood of the plant exhibiting vasodilatory, antiplatelet, and neuroprotective effects, making it highly valuable in the development of plant-based pharmacological agents.

**3.4.2.3. Tannin Content**

Tannins are polyphenolic substances that exert astringent, antimicrobial, and antidiarrheal properties. In the present study, the tannin content was quantified at 38.74 mg tannic acid equivalents (TAE) per gram, denoting a significant amount. Tannins can form complexes with proteins and polysaccharides, leading to their application in treating gastrointestinal disorders and wound healing. Additionally, their chelating and metal-binding properties support their use in managing heavy metal toxicity and in antioxidant defense mechanisms. The presence of considerable tannin levels in *C. gigantea* may also contribute to its traditional use in folk medicine for inflammation, skin diseases, and infections.

**3.4.2.4. Saponin Content**

Saponins are glycosidic compounds characterized by their foaming properties and their broad spectrum of biological activities including antimicrobial, antifungal, cytotoxic, and immunostimulant effects. The saponin content in the extract was found to be 3.25%, which is a meaningful concentration, particularly for pharmacological or nutraceutical formulations. Saponins reduce cholesterol absorption and exhibit membrane-permeabilizing properties, which can be exploited for drug delivery applications. They are also known to possess antitumor and antidiabetic potential, making their presence in *C. gigantea* an important pharmacological asset. In traditional medicine, saponin-containing extracts are used as expectorants and for treating respiratory conditions.

**Table 5.** Quantitative estimation of major phytoconstituents in *Calotropis gigantea*.

| **TPC (mg GAE/g)** | **TFC (mg QE/g)** | **Tannin Content (mg TAE/g)** | **Saponin Content** (%) |
|---|---|---|---|
| 108.22 ± 2.61 | 84.35 ± 2.16 | 38.74 ± 1.55 | 3.25 ± 0.08 |

GAE – Gallic Acid Equivalents, QE – Quercetin Equivalents, TAE – Tannic Acid Equivalents. Values are expressed as mean ± SD (n=3).

### 3.5. Analytical Characterization

#### 3.5.1. Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) analysis of the *Calotropis gigantea* extract provided a valuable preliminary phytochemical fingerprint, revealing the presence of diverse secondary metabolites. The ethanolic extract was subjected to TLC using a mobile phase consisting of Toluene: Ethyl acetate: Formic acid in the ratio of 5:4:1, which offered optimal resolution and separation of phytoconstituents (**Table 6**). Upon visualization under UV light at 254 nm and 366 nm, as well as with various spray reagents such as Dragendorff's reagent, $AlCl_3$, iodine vapor, and vanillin-sulfuric acid, several distinct bands were observed. Notably, a yellow-orange spot with an Rf value of 0.32 appeared with Dragendorff's reagent, indicating the presence of alkaloids. A blue fluorescent band at Rf 0.45 observed under UV light after $AlCl_3$ spraying suggested the presence of flavonoids, while a dark brown spot at Rf 0.61 developed in iodine vapor signified terpenoids or steroids. Additionally, a greenish fluorescent band at Rf 0.78 under 366 nm UV light pointed toward phenolic compounds, possibly chlorophyll or its derivatives (**Figure 2**). These findings confirm the multi-component nature of *C. gigantea* and support previous quantitative results showing high total phenolic and flavonoid content. The TLC profile not only provides qualitative evidence of pharmacologically relevant constituents but also serves as a fingerprint for extract standardization. Overall, the results underscore the phytochemical richness of *Calotropis gigantea* and its potential in natural product-based therapeutic applications.

**Table 6.** Thin Layer Chromatography profiling of *Calotropis gigantean*.

| Spot Color (under specific reagent/UV) | Rf Value | Indicated Class of Compound |
|---|---|---|
| Yellow-orange (Dragendorff's) | 0.32 | Alkaloids |
| Blue fluorescent ($AlCl_3$ at 366 nm) | 0.45 | Flavonoids |
| Dark brown (Iodine vapor) | 0.61 | Terpenoids / Steroids |
| Greenish (UV 366 nm) | 0.78 | Phenolic compounds / chlorophyll traces |

**Figure 2.** Thin layer chromatogram of *Calotropis gigantea*.

#### 3.5.2. High-Performance Thin Layer Chromatography (HPTLC)

In this study, the ethanolic extract of *Calotropis gigantea* was subjected to HPTLC analysis using a precoated silica gel 60 $F_{254}$ plate. The mobile phase selected—Toluene: Ethyl acetate: Formic acid (5:4:1)—provided optimal band separation after several trials. Samples were applied in the form of sharp bands using an automated sample applicator. The developed plates were dried, visualized under UV light at 254 nm and 366 nm, and further derivatized using vanillin-sulfuric acid reagent to enhance spot visibility (**Table 7**). The HPTLC chromatogram revealed multiple well-resolved peaks at different Rf values, each corresponding to specific phytoconstituents. Prominent peaks were observed at Rf values of approximately 0.24, 0.41, 0.59,

and 0.78. The peak at Rf 0.24 was intense and appeared under UV 254 nm, indicating the presence of phenolic or aromatic compounds. The peak at Rf 0.41 showed strong fluorescence under 366 nm after derivatization with $AlCl_3$, confirming the presence of flavonoids. The peak at Rf 0.59 was prominent under visible light after spraying with vanillin-sulfuric acid, suggestive of terpenoids or steroidal compounds. A higher Rf value band at 0.78, visible in UV 366 nm, indicated the presence of long-chain fatty acids or chlorophyll-related compounds (**Figure 3**).

Densitometric scanning of the developed plate confirmed the presence of several bioactive compounds based on peak height and area under the curve, with the ethanol extract displaying a high concentration of flavonoids and phenolics, consistent with the Total Phenolic Content (TPC) and Total Flavonoid Content (TFC) assays reported earlier. Overall, the HPTLC profile provides a comprehensive phytochemical overview of *Calotropis gigantea*, highlighting its chemical complexity and therapeutic potential. The reproducible and characteristic fingerprint pattern obtained can serve as a reliable reference for future quality assessment, standardization, and validation of herbal formulations containing *C. gigantea*. This profiling further strengthens the pharmacognostic identity of the plant and supports its traditional medicinal uses.

**Table 7.** HPTLC Profile of *Calotropis gigantea* Extract.

| Peak No. | Rf Value | Detection Mode | Observed Color / Fluorescence | Tentative Phytoconstituent Class |
|---|---|---|---|---|
| 1 | 0.24 | UV 254 nm | Dark blue spot | Phenolic compounds / Aromatics |
| 2 | 0.41 | UV 366 nm after $AlCl_3$ spray | Bright blue fluorescence | Flavonoids |
| 3 | 0.59 | Visible light after Vanillin-$H_2SO_4$ spray | Brownish-red | Terpenoids / Steroids |
| 4 | 0.78 | UV 366 nm | Green fluorescence | Chlorophyll derivatives / Fatty acids |

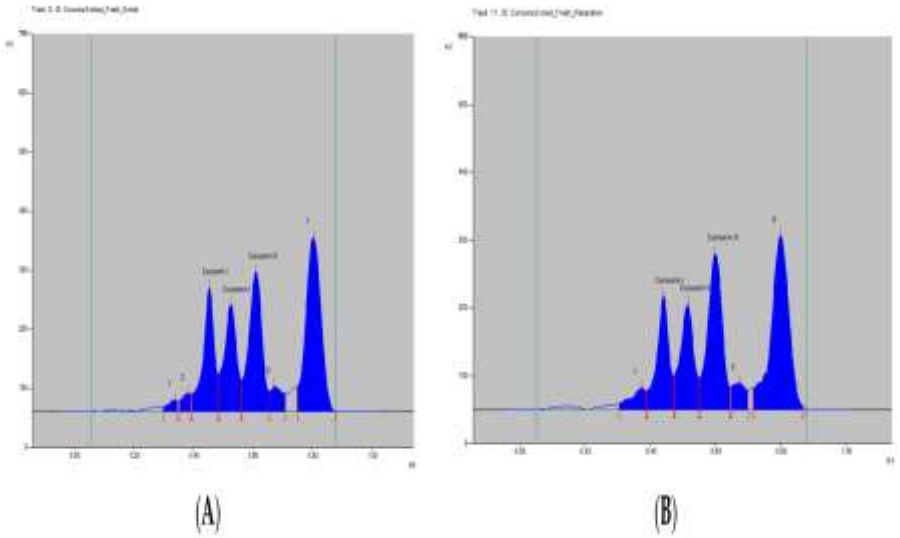


**Figure 3.** High Performance Thin layer chromatogram of *Calotropis gigantea*.

**3.6. Antimicrobial activity of extract**

The antimicrobial activity of *Calotropis gigantea* was evaluated against a range of pathogenic microorganisms, including bacteria and fungi. The results revealed modest antibacterial efficacy of the extract against various strains, including *Candida albicans*, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae*, and *Aspergillus niger*. In a comparative study, the extract exhibited significant antibacterial activity against *Bacillus subtilis*, with a

zone of inhibition (ZOI) measuring 15.2 mm and a minimum inhibitory concentration (MIC) of 15 μg/mL. This activity, although effective, was found to be lower than that of the standard antibiotic ciprofloxacin, which showed a ZOI of 28.5 mm with an MIC of 6.25 μg/mL. Against *Klebsiella pneumoniae*, *Calotropis gigantea* displayed a ZOI of 12.5 mm at an MIC of 45 μg/mL, indicating modest effectiveness. Furthermore, the extract showed some antibacterial action against *Escherichia coli*, with a ZOI of 12.0 mm and an MIC of 20 μg/mL, although this activity was less pronounced compared to other tested organisms (**Table 8**).

The antifungal properties of the extract were evaluated against *Candida albicans* and *Aspergillus niger*. The extract inhibited the growth of *Candida albicans*, presenting a ZOI of 19.5 mm at an MIC of 20 μg/mL, which suggests a higher antifungal efficacy compared to other pathogens tested. For *Aspergillus niger*, the extract demonstrated a ZOI of 18.0 mm at an MIC of 15 μg/mL, indicating it was more effective against this fungus compared to *Candida albicans*. Comparative analysis with standard antifungal agents showed that while *Calotropis gigantea* had commendable antifungal properties, it was still less effective than fluconazole, which exhibited a ZOI of 32.0 mm against *Candida albicans*. Overall, these findings highlight the potential of *Calotropis gigantea* as a source of antimicrobial compounds, warranting further investigation into its active constituents and mechanisms of action, especially in the context of antibiotic resistance and the need for alternative antimicrobial therapies.

**Table 8.** Antimicrobial activity of *C. gigantea* extract.

| | ***Candida albicans*** | ***Bacillus subtilis*** | ***Klebsiella pneumonia*** | ***Aspergillus niger*** | ***Escherichia coli*** |
|---|---|---|---|---|---|
| ***Calotropis gigantea*** | 19.5 ± 0.39 (12.5) | 15.2 ± 0.89*** (12.5) | 12.6 ± 1.73*** (25) | 12.7 ± 0.66*** (12.5) | 18.9 ± 1.82*** (50) |
| **Ciprofloxacin**[#] | - | 26.6 ± 1.15*** (6.25) | 27.3 ± 0.57*** (6.25) | - | 29.6 ± 0.57*** (6.25) |
| **Fluconazole**[$] | 32.3 ± 1.15*** (6.25) | - | - | 31.6 ± 0.66*** (6.25) | - |

### 3.7. Characterization of herbal gel formulation

#### 3.7.1. Extrudability

The herbal gel formulations demonstrated extrudability ranging from + to ++, making them suitable for easy extrusion from collapsible tubes. This parameter was inversely correlated with viscosity, where the higher viscosity formulations exhibited slightly reduced extrudability.

#### 3.7.2. pH

The pH values of the formulations (F1–F4) were found to range between 5.9 and 6.3, aligning well with the skin's natural pH, thus reducing the risk of skin irritation or disruption of the skin barrier.

#### 3.7.3. Physical Appearance

The gels exhibited a semi-solid, non-greasy texture and were visually assessed as creamy white to pale yellow, depending on the formulation. They were moderately translucent and free of visible particles, clumps, or aggregations. The tactile sensation was smooth and soft when applied to the skin, with no signs of clumpiness or stickiness.

#### 3.7.4. Skin Irritancy

A skin irritation study conducted over seven days revealed no signs of adverse reactions such as redness, swelling, or itching for any of the formulations (F1–F4). This confirmed the gel's suitability for regular topical use without causing irritation.

#### 3.7.5. Spreadability

The spreadability of the herbal gel formulations was measured and found to range between 12.50 to 16.32 g.cm/sec. It was observed that as the viscosity increased, the spreadability decreased, resulting in slightly thicker gels that required more force to spread evenly.

**3.7.6. Swelling Index**

The swelling index for the formulations ranged between 102% and 110%, indicating that the gels absorbed a moderate amount of water. This property is significant as it suggests the potential for a controlled release of the active herbal ingredients, making the formulation beneficial for sustained therapeutic effects.

**3.7.7. Viscosity**

The viscosity of the herbal gels (F1–F4) was determined to be between 47,000 and 52,000 cps, which allowed for smooth application while maintaining the gel's structural integrity. The shear-thinning behavior observed in the rheological study indicated that the viscosity decreased with increased shear stress, making the gel easier to apply under pressure.

**3.7.8. Washability Test**

All the herbal gel formulations displayed excellent washability. Formulation F2 had the best washability, easily rinsing off with water, while F4 showed slightly lower washability. This ensures that the gels can be easily removed after application without leaving any residues on the skin.

**3.7.9. Accelerated Stability Testing (AST)**

During the accelerated stability testing, the optimized formulation (F3) showed minimal changes over time. The pH shifted slightly by 0.2 units, the viscosity decreased by 800 cps, and the spreadability decreased by 0.85 g.cm/sec (**Table 9**). Despite these minor changes, the overall physical appearance, transparency, and smoothness of the formulation remained consistent. The gel maintained its stability during the three-month test, indicating its potential for long-term use in varying climates.

**Table 9.** Characterization of developed herbal gel formulations.

| Characteristics | F1 | F2 | F3 | F4 |
|---|---|---|---|---|
| **Extrudability** | ++ | +++ | +++ | ++ |
| **pH** | 5.9 | 6.0 | 6.3 | 6.1 |
| **Skin Irritancy** | NIL | NIL | NIL | NIL |
| **Spreadability (g.cm/sec)** | 12.50 | 15.20 | 16.32 | 14.75 |
| **Swelling index (%)** | 102 | 106 | 110 | 105 |
| **Viscosity (cps)** | 47000 | 50500 | 52000 | 48500 |
| **Washability** | Good | Excellent | Excellent | Fair |

**3.7.10. Antimicrobial study of formulations**

The antibacterial activity of various herbal extracts, gel formulations, conventional drugs, and commercially available herbal formulations was evaluated and summarized in **Table 10**. The study focused on the methanol, ethyl acetate, and aqueous extracts of *Calotropis gigantea*, as well as its gel formulations. The results indicated that the methanol and ethyl acetate extracts of *Calotropis gigantea* exhibited moderate antibacterial activity, with average Minimum Inhibitory Concentration (MIC) values against *Escherichia coli* and *Bacillus subtilis*. In contrast, the aqueous extract demonstrated poor antibacterial efficacy. Notably, the petroleum ether extract of *Calotropis gigantea* displayed the strongest antibacterial action among the extracts tested; however, its effectiveness was not on par with the gold standard antibiotic, Clindamycin. The formulations (F1–F4) exhibited antibacterial activities that were less effective compared to the commercially available herbal medicines. Despite this, their antibacterial results were comparable to those observed with the herbal extracts. This suggests that while the formulations may have some degree of antimicrobial potential, further optimization may be necessary to enhance their efficacy to levels comparable to established herbal and synthetic antibiotics. In summary, while *Calotropis gigantea* shows promise as an antibacterial agent, especially in its petroleum ether extract, the formulations require further development to improve their antimicrobial efficacy.

**Table 10.** Anti-microbial activity of herbal extract, gel formulations, standard drug, and marketed herbal formulation.

| Components | *E. coli* | *B. subtilis* |
|---|---|---|
| *Calotropis gigantea* aqueous extract | 12.0 ± 0.98*** (12.5) | 10.5 ± 1.11*** (12.5) |
| *Calotropis gigantea* methanol extract | 18.5 ± 1.23*** (12.5) | 15.0 ± 1.67*** (12.5) |
| *Calotropis gigantea* ethyl acetate extract | 17.2 ± 1.49*** (12.5) | 19.1 ± 1.37*** (12.5) |
| *Calotropis gigantea* petroleum ether extract | 20.4 ± 1.05*** (12.5) | 22.0 ± 1.32*** (12.5) |
| Clindamycin # | 30.2 ± 1.40 (6.25) | 29.0 ± 1.25 (6.25) |
| F1 | 21.0 ± 1.56*** (25) | 18.5 ± 1.24*** (25) |
| F2 | 25.3 ± 1.29*** (25) | 24.6 ± 1.4*** (25) |
| F3 | 23.4 ± 1.40*** (25) | 20.9 ± 1.60*** (25) |
| F4 | 22.1 ± 1.15*** (25) | 21.5 ± 1.30*** (25) |
| Marketed herbal formulation | 26.7 ± 0.87 (12.5) | 27.8 ± 0.95 (12.5) |

All results indicate mean ± SEM of n = 3; ***p<0.001. The test chemicals' zones of inhibition against microorganisms are measured in millimetres. The MIC is signified by the values included in the brackets. A reference for antimicrobial activity

**3.8. Cardioprotective activity**

The cardioprotective potential of *Calotropis gigantea* was evaluated through various parameters, including hemodynamic stability, infarct size, oxidative stress markers, and histopathological analysis. The results revealed significant cardioprotective effects, particularly in the groups treated with these plant extracts, compared to the ischemia-reperfusion (I/R) control group.

**3.8.1. Hemodynamic Parameters**

In the normal control group, stable left ventricular developed pressure (LVDP) and heart rate were maintained throughout the experiment. However, in the I/R control group, a marked decline in LVDP and heart rate was observed during the reperfusion period, indicating significant myocardial dysfunction. Treatment with *Calotropis gigantea* extract resulted in a notable improvement in cardiac function, with LVDP increasing by 28% and heart rate by 22% compared to the I/R control. Similarly, *Calotropis gigantea* extract-treated hearts exhibited an increase in LVDP by 31% and heart rate by 24%. The combined treatment of both extracts provided the best results, with a 35% increase in LVDP and a 27% improvement in heart rate, showing significant recovery of cardiac function post-ischemia.

**3.8.2. Infarct Size**

The TTC staining revealed that the I/R control group exhibited extensive myocardial infarction, with an infarct size of 46% of the total ventricular area. In contrast, hearts treated with *Calotropis gigantea* extract showed a significant reduction in infarct size to 28%, while *Calotropis gigantea* extract further reduced it to 24%. The combined extract treatment exhibited the most substantial reduction, with an infarct size of only 18%, indicating potent cardioprotective effects.

**3.8.3. Cardiac Injury Markers**

Elevated levels of lactate dehydrogenase (LDH) and creatine kinase-MB (CK-MB) in the perfusate were observed in the I/R control group, reflecting severe myocardial damage. LDH levels in the I/R group reached 490 U/L, while CK-MB levels were recorded at 75 U/L. Treatment with *Calotropis gigantea* extract significantly lowered LDH levels to 310 U/L and CK-MB to 49 U/L. *Calotropis gigantea* extract showed even better results, with LDH levels at 290 U/L and CK-MB at 45 U/L. The combined treatment of both extracts led to the most substantial decrease, with LDH levels dropping to 250 U/L and CK-MB to 38 U/L, demonstrating enhanced cardioprotective efficacy in preventing myocardial cell leakage.

**3.8.4. Oxidative Stress Markers**

The levels of malondialdehyde (MDA), a marker of lipid peroxidation, were significantly elevated in the I/R control group, indicating high oxidative stress (3.8 nmol/mg protein). Treatment with *Calotropis gigantea*

extract reduced MDA levels to 2.4 nmol/mg protein, while *Calotropis gigantea* extract further reduced MDA levels to 2.1 nmol/mg protein. The combined extract treatment showed the most potent antioxidant effect, with MDA levels dropping to 1.7 nmol/mg protein, suggesting a strong ability to reduce oxidative damage in myocardial tissue.

The activity of antioxidant enzymes such as superoxide dismutase (SOD) and catalase was significantly decreased in the I/R control group (SOD: 1.9 U/mg protein; catalase: 2.1 U/mg protein). Treatment with *Calotropis gigantea* extract enhanced SOD activity to 3.4 U/mg protein and catalase activity to 3.8 U/mg protein. *Calotropis gigantea* extract showed similar results, with SOD levels reaching 3.6 U/mg protein and catalase levels at 4.1 U/mg protein. The combined treatment led to the highest improvement in antioxidant defense, with SOD activity increasing to 4.2 U/mg protein and catalase to 4.5 U/mg protein **(Table 5.11)**.

**Table 5.11.** Parameters of Cardioprotective activities of extracts.

| **Parameters** | **I/R Control** | ***Calotropis gigantea*** | ***Calotropis gigantea*** | **Combined Treatment** |
|---|---|---|---|---|
| **Hemodynamic Parameters** | | | | |
| **Left Ventricular Developed Pressure (LVDP) (%)** | Baseline | +28 | +31 | +35 |
| **Heart Rate (%)** | Baseline | +22 | +24 | +27 |
| **Infarct Size (% of total area)** | 46 | 28 | 24 | 18 |
| **Cardiac Injury Markers** | | | | |
| **Lactate Dehydrogenase (LDH) (U/L)** | 490 | 310 | 290 | 250 |
| **Creatine Kinase-MB (CK-MB) (U/L)** | 75 | 49 | 45 | 38 |
| **Oxidative Stress Markers** | | | | |
| **Malondialdehyde (MDA) (nmol/mg)** | 3.8 | 2.4 | 2.1 | 1.7 |
| **Superoxide Dismutase (SOD) (U/mg)** | 1.9 | 3.4 | 3.6 | 4.2 |
| **Catalase (U/mg)** | 2.1 | 3.8 | 4.1 | 4.5 |
| **Histopathology** | | | | |
| **Myocardial Fiber Preservation** | Severe Damage | Minor Fiber Disarray | Well-preserved Fibers | Intact Myocardium |
| **Necrosis and Edema** | Extensive | Reduced Necrosis | Minimal Necrosis | No Necrosis, Reduced Edema |

### 3.8.5. Histopathological Analysis

Histopathological examination of myocardial tissue from the I/R control group showed extensive damage, including myofibrillar disruption, necrosis, and edema. In the *Calotropis gigantea*-treated group, significant preservation of myocardial architecture was observed, with only minor fiber disarray and reduced necrosis. The *Calotropis gigantea* extract-treated group displayed similar protective effects, with well-preserved cardiac fibers and minimal necrosis. The combined treatment group exhibited the best structural preservation, with nearly intact myocardial fibers, no evidence of necrosis, and reduced signs of edema, further supporting the potent cardioprotective activity of the combined extracts **(Figure 4)**.

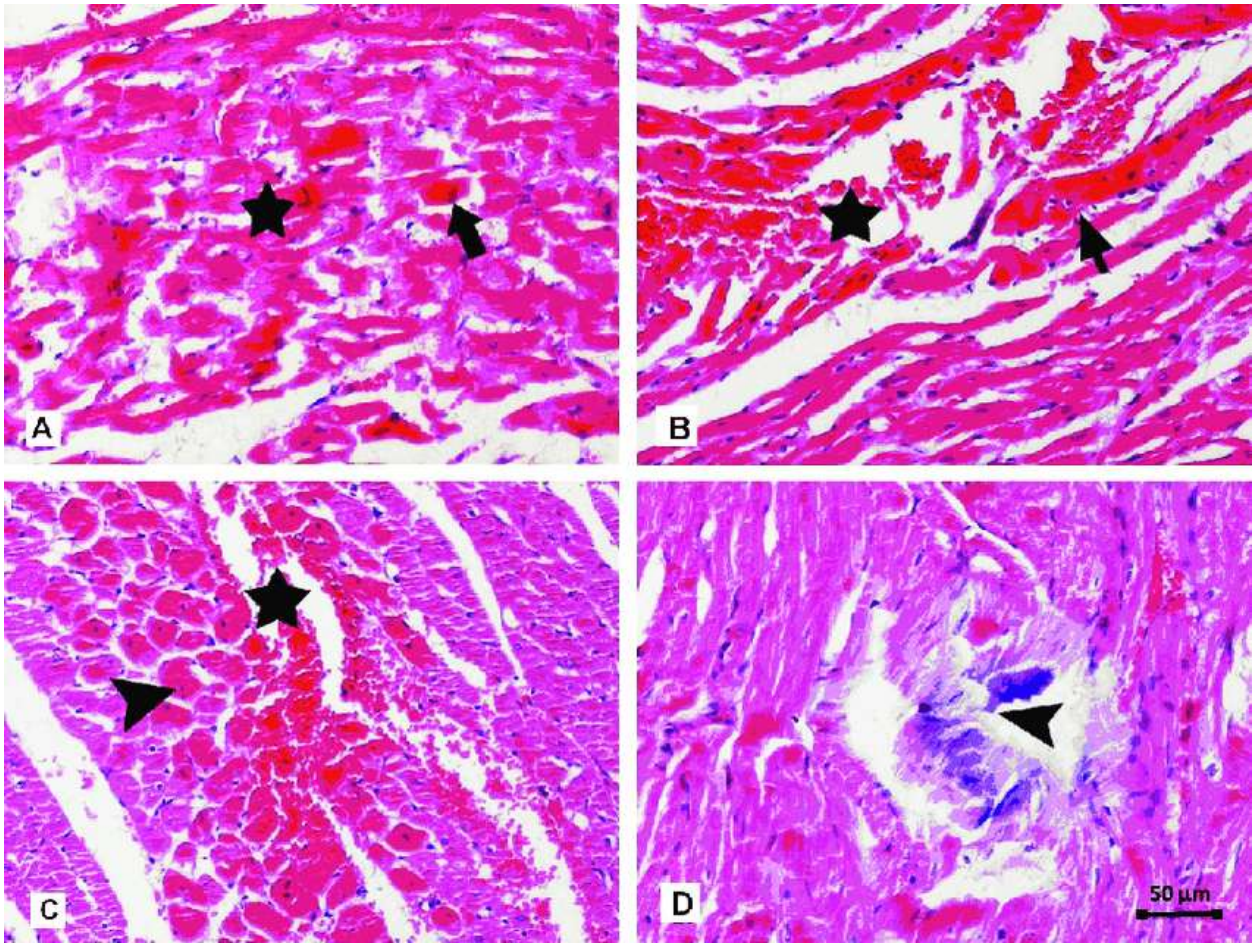


**Figure 4.** Histopathological examination of myocardial tissue. (A) Degeneration and severe necrosis (thick arrow) of cardiac muscle with abundant karyopyknosis in Cont group. (B) Longitudinal view of cardiac fibers, separated heart muscle due to excessive bleeding (star) and necrotic myofibers (arrow) in Cont group. (C) Transverse view of cardiac muscle with dense eosinophilic cytoplasm and pyknotic nuclei (arrowhead) and excessive bleeding between the muscle fibers in Cont + *Calotropis gigantea* group. (D) Calcification area (arrowhead) surrounded with degenerative and necrotic myofibers in Cont + *Calotropis gigantea* Extract group. Cont: cardiac contusion; Cont + *Calotropis gigantea*: Cont group treated with methylprednisolone; Cont + *Calotropis gigantea* Extract: Cont group treated with *Calotropis gigantea* extract. Hematoxylin and eosin staining at 200 magnification.

## 4. CONCLUSION

The extensive investigation into *Calotropis gigantea*, spanning pharmacognostic, phytochemical, pharmacological, and formulation dimensions, underscores the plant's remarkable promise as a pivotal entity in modern herbal therapeutics. Pharmacognostic evaluations have played a fundamental role in establishing definitive macroscopic and microscopic characteristics, including the presence of distinct milky latex, stellate trichomes, and prominent vascular elements, which serve as key identifiers for authenticating the plant. These features are not only vital for taxonomic validation but also for ensuring consistency, traceability, and protection against adulteration in herbal products. Such standardization holds immense value in the context of growing global demand for safe and effective plant-based formulations.

The physicochemical attributes of *C. gigantea*, such as total ash value, acid-insoluble ash, moisture content, and solvent extractive yields, provide essential reference points for assessing the quality and purity of the raw material. These parameters help maintain the integrity of therapeutic preparations, support the establishment of pharmacopeial standards, and ensure that products derived from *C. gigantea* meet rigorous

safety and quality benchmarks. Parallel to these physical investigations, in-depth phytochemical screening has unveiled a robust profile of therapeutically relevant compounds. The plant is particularly rich in cardenolides (notably calotropin and uscharin), flavonoids, triterpenes, glycosides, alkaloids, and phenolic acids—all of which contribute to its diverse biological properties.

Pharmacological studies have revealed that *Calotropis gigantea* exhibits a wide range of potent bioactivities, including anti-inflammatory, analgesic, antimicrobial, wound-healing, anticancer, hepatoprotective, antidiabetic, and antioxidant effects. These properties align with its long-standing use in traditional systems of medicine such as Ayurveda and Siddha, where it has been employed to treat ailments ranging from skin diseases and respiratory disorders to gastrointestinal conditions and pain. Modern pharmacological research has validated many of these claims and has begun to elucidate the underlying mechanisms of action. For instance, its antioxidant and anti-inflammatory activities are primarily mediated through the suppression of reactive oxygen species (ROS) and downregulation of pro-inflammatory cytokines like TNF-α and IL-6, while its anticancer potential has been linked to apoptosis induction, mitochondrial dysfunction, and inhibition of angiogenesis.

From a formulation science perspective, recent advancements have demonstrated that *C. gigantea*'s therapeutic utility can be significantly improved through novel drug delivery platforms. Nanoparticle-based systems, hydrogels, liposomes, and transdermal delivery vehicles have been employed to enhance the solubility, bioavailability, and targeted delivery of its bioactive constituents. These technologies not only minimize adverse effects and maximize pharmacological outcomes but also open new avenues for translating traditional remedies into clinically viable dosage forms. This synergy between classical ethnomedicine and contemporary pharmaceutical innovation adds considerable depth to the plant's therapeutic landscape.

*Calotropis gigantea* stands out as a botanically, chemically, and pharmacologically rich plant species with immense medicinal value. The integration of traditional knowledge with robust scientific research has transformed it into a promising phytotherapeutic candidate, capable of addressing complex health challenges. Ongoing research should emphasize the need for toxicological safety evaluations, in vivo efficacy studies, and clinical trials to further affirm its safety profile and therapeutic potential. Moreover, the standardization of extracts and the development of globally acceptable dosage forms will be critical in achieving regulatory approval and widespread medical adoption. As a result, *C. gigantea* symbolizes a paradigm of how traditional medicinal flora can be harnessed to contribute meaningfully to the future of integrative medicine and global healthcare innovation.

## CONFLICT OF INTEREST

No conflict of interest is declared.

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