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# FORMULATION DEVELOPMENT, PHARMACEUTICAL STANDARDIZATION, AND EVALUATION OF MARHAM-*E*- KHAS PREPARED FROM TRADITIONAL ROGHAN-*E*-KHAS

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## ABSTRACT

Traditional Unani medicine employs medicated oils (Roghan) and semisolid ointments (Marham) for external application. The present study describes the preparation of Marham-e-Khas from traditional Roghan-e-Khas and brings together the formulation procedure, preliminary qualitative phytochemical screening of its botanical ingredients, and the reported pharmaceutical evaluation of the finished semisolid preparation. Fresh leaves of *Calotropis procera* (Barg-e-Aak), *Datura metel* (Barg-e-Dhatura), and *Ricinus communis* (Barg-e-Erand) were collected from the garden of Hamdard University Bangladesh and authenticated by a qualified pharmacognosist. For each species, 100 g of fresh leaves was blended with 400 mL of purified water to prepare an aqueous extract. Roghan-e-Khas was prepared using 800 mL sesame oil and the three freshly prepared leaf extracts, followed by incorporation of 50 g lac resin and 50 g of the ingredient identified in the supplied formulation record as Sindoor (red lead). Marham-e-Khas was prepared using the same nominal oil and botanical inputs, with the addition of 150 g white beeswax to obtain a semisolid consistency. The supplied traditional procedure for lac purification, together with general Unani methods for preparing medicated oils and ointments, was also documented.Qualitative phytochemical screening was conducted on the aqueous leaf extracts using conventional colour, precipitation, and other reagent-based tests. The screening covered alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenolic compounds, tannins, flavonoids, proteins, and amino acids. The study record reports positive reactions for all three plant extracts across the 31 individual tests. These results provide preliminary class-level screening observations; they do not establish the identity or concentration of individual compounds.

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## 1. INTRODUCTION

Traditional medicine has played an important role in healthcare systems across societies, particularly in managing skin disorders, wounds, inflammatory conditions, and other health problems. Medicinal plants area major source of natural bioactive compounds, including alkaloids, flavonoids, phenolic compounds, glycosides, tannins, terpenoids, and saponins, which have been extensively studied for their pharmacological properties. These phytochemicals exhibit diverse biological activities, including antioxidant, antimicrobial, anti-inflammatory, analgesic, and wound-healing effects. The growing scientific interest in plant-derived medicines has encouraged researchers to investigate traditional formulations using modern pharmaceutical and pharmacological approaches. However, the therapeutic potential of herbal preparations must be supported by appropriate scientific evidence on their composition, quality, safety, and reproducibility, rather than relying solely on historical use (Heinrich et al., 2020; World Health Organization [WHO], 2011).

The Unani system of medicine is an established traditional medical system that utilises medicinal substances of plant, animal, and mineral origin in different pharmaceutical preparations. Its traditional pharmaceutics include various dosage forms, such as Roghan (medicinal oils), Marham (ointments), powders, decoctions, and other semisolid and liquid preparations. These formulations are traditionally prepared according to established pharmaceutical principles and are employed for different therapeutic purposes, including the management of wounds, ulcers, skin diseases, and inflammatory conditions. Historical documentation of Unani pharmaceutical preparations is available in Pharmacopoeia of Hospital of Integrated Medicine: Part III—Unani, edited by Dr. V. Narayanaswamy and published in 1957 by the Superintendent, Government Press, Madras, India. This pharmacopoeial publication provides an important historical reference for traditional Unani formulations and their pharmaceutical preparation (Narayanaswamy, 1957). In this context, Marham-e-Khas prepared from traditional Roghan-e-Khas represents a formulation that warrants systematic pharmaceutical investigation to establish its quality characteristics and reproducibility.

Marham is a topical semisolid pharmaceutical preparation traditionally applied to the skin or affected tissues. Its pharmaceutical performance depends on several factors, including the characteristics of the medicinal ingredients, the nature of the formulation base, ingredient compatibility, manufacturing procedures, and storage conditions. The use of Roghan-e-Khas as the traditional oil component in the preparation of Marham-e-Khas provides a basis for investigating the conversion of a traditional medicinal oil into a suitable semisolid dosage form. Such formulation development requires appropriate selection and incorporation of ingredients to achieve uniformity, acceptable consistency, suitable spreadability, and satisfactory topical application properties. Furthermore, the physical and chemical characteristics of the formulation may influence the release and availability of its bioactive constituents. Therefore, systematic formulation development and pharmaceutical evaluation are essential to establish a reproducible preparation suitable for further investigation.

Wound healing is a complex physiological process involving coordinated cellular and molecular responses that restore the structural and functional integrity of damaged tissues. It generally progresses through four overlapping phases: haemostasis, inflammation, proliferation, and remodelling. During haemostasis, platelet activation and fibrin formation help control bleeding and establish a provisional matrix. The inflammatory phase involves recruiting neutrophils and macrophages, which help remove microorganisms, damaged cells, and tissue debris. During proliferation, fibroblast migration, collagen synthesis, angiogenesis, granulation tissue formation, and re-epithelialisation contribute to tissue repair. Finally, the remodelling phase involves collagen reorganisation and extracellular matrix maturation, resulting in the restoration of tissue strength and integrity (Guo & DiPietro, 2010; Gurtner et al., 2008). Although inflammation is necessary to initiate the healing process, prolonged or excessive inflammation may delay tissue repair by increasing oxidative stress, tissue damage, and impaired cellular regeneration. Consequently, topical preparations containing bioactive constituents with potential anti-inflammatory, antioxidant, and antimicrobial properties have attracted considerable research interest in wound-care development.

Among medicinal plants investigated for wound management, *Calotropis procera* has received attention because of its diverse phytochemical constituents, including cardenolide glycosides, flavonoids, phenolic compounds, terpenoids, and proteolytic proteins. Experimental studies have demonstrated its potential anti-inflammatory and wound-healing properties. Kumar and Basu (1994) investigated the anti-inflammatory activity of *C. procera* latex using carrageenan- and formalin-induced rat paw oedema models and reported significant inhibition of acute inflammatory responses. Similarly, Arya and Kumar (2005) showed that aqueous and methanolic extracts of dried latex inhibited several inflammatory mediators, including histamine, bradykinin, and prostaglandin $E_2$. Its wound-healing potential was further investigated by Rasik et al. (1999), who reported enhanced collagen, DNA, and protein synthesis, improved epithelialisation, and reduced wound area following topical application of latex in guinea pigs. These findings indicate that certain constituents of *C. procera* may contribute to inflammatory regulation and tissue repair. Nevertheless, the presence of potentially cardiotoxic cardenolide glycosides, including calotropin, calactin, and calotoxin, necessitates careful toxicological consideration during pharmaceutical development.

*Datura metel* is another medicinal plant of pharmacological interest because of its diverse secondary metabolites, particularly tropane alkaloids, flavonoids, phenolic compounds, tannins, and other biologically active constituents. Its potential anti-inflammatory and wound-healing properties have recently attracted attention in experimental studies. Ruksiriwanich et al. (2023) investigated supercritical carbon dioxide extracts of *D. metel* leaves using antioxidant, anti-inflammatory, and fibroblast migration assays. Their findings showed inhibitory effects on lipopolysaccharide-induced nitrite production and improved fibroblast migration and wound closure under *in-vitro*conditions. The study also reported inhibition of matrix metalloproteinase-2 (MMP-2) activity and modulation of genes associated with the Sonic Hedgehog signalling pathway, suggesting possible involvement in cellular migration and tissue-repair mechanisms. These findings provide a scientific basis for further investigating the potential application of *D. metel* in topical pharmaceutical research. However, its tropane alkaloids, particularly atropine, hyoscyamine, and scopolamine, are associated with significant anticholinergic toxicity, making the identification, concentration control, and safety evaluation of these constituents particularly important.

Similarly, *Ricinus communis* has been investigated for its phytochemical composition and diverse pharmacological properties, particularly its antioxidant and anti-inflammatory activities. Its leaves contain flavonoids, phenolic compounds, tannins, and other secondary metabolites that may contribute to biological activity. Nemudzivhadi and Masoko (2014) evaluated different solvent extracts of *R. communis* leaves using human skin fibroblast and macrophage cell models and reported antioxidant and anti-inflammatory activities. Their findings support further investigation of the plant's potential in inflammatory and skin-related research. In addition, castor oil, obtained from the seeds, contains ricinoleic acid as its major fatty acid. Tunaru et al. (2012) established that ricinoleic acid activates prostaglandin EP3 receptors, explaining its recognised stimulant laxative action. Although this finding demonstrates an established pharmacological mechanism of castor oil, its relevance to topical wound healing should be evaluated separately. Furthermore, ricin, a highly toxic ribosome-inactivating protein in castor seeds, represents an important toxicological concern that must be considered when selecting and processing plant materials.

Table 1.1. Literature-reported phytochemicals in the investigated species

| Sl. | Phytochemical | *Calotropis procera* | *Datura metel* | *Ricinus communis* |
|---|---|---|---|---|
| 1. | Alkaloids | Calotropin-associated alkaloidal constituents; other reported alkaloids | Atropine, hyoscyamine, scopolamine, tropine, littorine, cuscohygrine, tigloidine, datumetine, anisodamine | Ricinine, N-demethylricinine, nudiflorin |
| 2. | Carbohydrates | Reducing sugars, soluble sugars, polysaccharides | Glucose, fructose, sucrose and other reported carbohydrates | Glucose, fructose, sucrose, seed carbohydrates |
| 3. | Glycosides | Calotropin, calactin, calotoxin, uscharin, uscharidin, uzarigenin glycosides, flavonoid glycosides | Daturglycosides, withanolide glycosides, tropane-related glycosides | Quercetin glycosides, kaempferol glycosides, flavonoid glycosides |
| 4. | Saponins | Steroidal saponins, triterpenoid saponins | Steroidal saponins, triterpenoid saponins | Saponins, triterpenoid saponins |
| 5. | Phytosterols | β-Sitosterol, stigmasterol, cholesterol-related sterols | β-Sitosterol, stigmasterol | β-Sitosterol, stigmasterol, campesterol |
| 6. | Phenolic compounds | Caffeic acid, vanillic acid, protocatechuic acid, p-coumaric acid, ferulic acid | Gallic acid, caffeic acid, chlorogenic acid, other phenolic derivatives | Gallic acid, gentisic acid, ellagic acid, epicatechin |
| 7. | Tannins | Condensed tannins, hydrolysable tannins | Tannins, condensed tannins | Tannins, ellagitannins |
| 8. | Flavonoids | Quercetin, rutin, kaempferol, isorhamnetin, isoquercitrin | Quercetin, kaempferol, rutin and related flavonoids | Quercetin, rutin, kaempferol, isoquercetin, kaempferol glycosides |
| 9. | Proteins | Procerain, procerain B, cysteine proteases, latex proteins | Various plant proteins and enzymes | Ricin, *Ricinus communis* agglutinin (RCA), other seed proteins |
| 10. | Amino acids | Amino acids reported in plant tissues and latex | Arginine, aspartic acid, glutamic acid and other amino acids | Arginine, lysine, aspartic acid, glutamic acid and other amino acids |

**Sources:** Wadhwani et al. (2021); Islam et al. (2023); Abomughaid et al. (2024); Ramothloa et al. (2025).

Despite the promising pharmacological properties reported for these medicinal plants, developing a reproducible traditional herbal ointment requires comprehensive pharmaceutical standardisation. Variations in raw-material quality, ingredient proportions, processing methods, and storage conditions may influence the physical, chemical, and microbiological characteristics of the finished preparation. Therefore, evaluation of organoleptic properties, including appearance, colour, odour, and consistency, should be accompanied by physicochemical assessments such as pH, homogeneity, spreadability, extrudability, and rheological behaviour. Furthermore, *in-vitro* drug-release or diffusion studies, where applicable, provide information about the release characteristics of bioactive constituents from the formulation. Physical and storage stability assessments are also necessary to identify unacceptable changes in colour, odour, consistency, and phase separation. Microbiological evaluation, including total aerobic microbial count, total yeast and mould count, and the detection of specified microorganisms, is particularly important for establishing the microbiological quality of topical herbal preparations. The WHO (2011) emphasises systematic quality-control procedures for herbal materials, while its subsequent guidelines on Unani medicine highlight the importance of standardised manufacturing procedures and quality assurance to support the safety and quality of traditional medicines (WHO, 2022).

Against this background, the present study focuses on the formulation development, pharmaceutical standardisation, and evaluation of Marham-e-Khas prepared from traditional Roghan-e-Khas, as documented in the Unani pharmacopoeial literature. The study aims to develop a suitable semisolid preparation and assess its organoleptic, physicochemical, rheological, stability, and microbiological characteristics. Particular attention is given to formulation uniformity, topical suitability, physical stability, and microbiological quality to establish a systematic quality profile. By integrating traditional Unani pharmaceutical knowledge with contemporary pharmaceutical evaluation techniques, this research seeks to provide a scientific basis for the standardisation and further pharmaceutical investigation of Marham-e-Khas.

## 2. Materials and Methods

### 2.1. General Preparation of Medicated Oils (Adviya-e-Roghaniyat)

Medicated oils (Adviya-e-Roghaniyat) are traditional Unani pharmaceutical preparations produced by incorporating medicinal plant materials or other therapeutic ingredients into a suitable oil base. Depending on the nature and physical state of the ingredients, preparation methods may involve maceration, juice incorporation followed by heating, decoction-based extraction, direct infusion into oil, or the incorporation of finely powdered substances. The traditional procedures described below are based on the Bangladesh National Formulary of Unani Medicine (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.1. Preparation of Medicated Oils Using Fresh Flowers

Traditionally, medicated oils from fresh flowers are prepared by macerating the flowers in sesame oil. Four parts by weight of fresh flowers are placed in a suitable earthen vessel containing five parts of sesame oil. Cover the vessel with an iron lid and expose it to sunlight for about 10–40 days, depending on the preparation. After maceration, the flowers are squeezed while immersed in the oil, then filtered through a fine cloth to remove residual plant material. This method is traditionally used to prepare rose oil, wild chamomile (Babuna) oil, and jasmine oil. An alternative method involves spreading the flowers over sesame seeds and leaving them in contact for several days so the seeds absorb their fragrance. Oil is subsequently extracted from the scented seeds (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.2. Preparation of Medicated Oils Using Fresh Leaves and Roots

For preparations containing fresh leaves or roots, the juice is first extracted from the plant material. Three parts of the extracted juice are then mixed with two parts of sesame oil. Heat the mixture over a moderate flame until the aqueous phase evaporates, leaving the oil containing the extracted plant constituents. The preparation is subsequently cooled and filtered, as appropriate, to obtain the medicated oil (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.3. Preparation of Medicated Oils Using Dried Flowers, Leaves, and Roots

In this method, one part by weight of dried flowers, leaves, or roots is soaked in four parts of clean water for 24 hours. The mixture is subsequently boiled to prepare an aqueous decoction. Then add the decoction to the specified quantity of oil and heat over a moderate flame until the aqueous content evaporates. The vessel is removed from the heat, and the oil is allowed to cool before filtration through a fine cloth. The filtered product constitutes the medicated oil prepared using dried plant materials (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.4. Preparation of Medicated Oils Using Other Dried Herbal Ingredients

For certain dried herbal ingredients, the traditional method involves incorporating them directly into the specified oil, then heating. The mixture is heated until the herbal materials develop a dark-red coloration, as described in the traditional preparation procedure. The vessel is then removed from the heat and allowed to cool. Finally, the mixture is filtered through a fine cloth to separate the oil from the residual plant material (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.5. Preparation of Medicated Oils Using Camphor, Saffron, and Similar Ingredients

Medicated oils containing heat-sensitive or aromatic ingredients, such as camphor and saffron, are prepared by first grinding the ingredients into a fine powder. The specified oil is heated separately and then allowed to cool to a suitable temperature before the powdered ingredients are added. The mixture is stirred thoroughly to ensure the ingredients are uniformly incorporated into the oil base. The resulting preparation is then handled according to the intended formulation requirements (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011, pp. 954–957).

#### 2.1.6. Precautions During the Preparation of Medicated Oils

When preparing medicated oils, process herbal ingredients according to their physicochemical properties to ensure proper incorporation into the oil vehicle. Finely comminute ingredients containing natural oils, mix them with the specified oil, and heat carefully until the desired consistency is achieved, avoiding excessive heating that may degrade heat-sensitive constituents. Ingredients lacking sufficient natural oil should be finely powdered and mixed with water or another suitable liquid vehicle to form a paste before further processing. During heating, the temperature should be controlled appropriately to facilitate the removal of moisture while minimizing thermal degradation of the active constituents. Remove the preparation from the heat when the required endpoint is reached, allow it to cool, and filter it where necessary. For sesame oil-based preparations, finely comminute herbal materials before incorporation to ensure uniform distribution throughout the oil vehicle (Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011).

### 2.2. General Preparation of Marham (Ointments)

Marham is a semisolid pharmaceutical preparation intended for external application. Traditional Unani ointments are prepared using suitable oleaginous vehicles, waxes, fats, and medicinal ingredients. Commonly used vehicles include olive oil, sesame oil, almond oil, rose oil, ghee, and petroleum jelly (Vaseline). The choice of ingredients and preparation technique depends on the physicochemical properties of the formulation components and the intended therapeutic application.

To prepare Marham using oils, waxes, or fats, gently heat the specified quantity of oil in a suitable vessel. Then add the wax or fat to the warm oil and allow it to melt with continuous stirring. The finely powdered herbal ingredients are incorporated gradually, and the mixture is stirred thoroughly to ensure uniform distribution. Continue stirring during cooling until the preparation reaches the desired consistency. Alternatively, incorporate finely powdered or liquid medicinal ingredients into petroleum jelly to obtain the required semisolid preparation.

#### 2.2.1. Precautions During the Preparation of Marham (Ointments)

Several precautions should be observed during the preparation of Marham (ointments). Ingredients such as guggul, Gandhabiroja, and soap should be dissolved or incorporated into the oil-wax base as appropriate. Eggs, egg whites, and egg yolks, when specified in a formulation, should be incorporated at a suitable stage; heat-sensitive ingredients should be added after sufficient cooling to minimize thermal degradation. Generally, add aromatic and volatile ingredients, including camphor, during the final stage of

preparation at a suitable temperature to minimize loss of volatile constituents. Thorough mixing ensures uniform distribution throughout the formulation(Bangladesh Board of Unani and Ayurvedic System of Medicine, 2011).

**2.3. Purification of Lac Resin (Lak)**

Raw lac resin may contain natural wax, colouring matter, insect-derived residues, bark fragments, and other extraneous impurities. Commercial lac processing typically involves physical cleaning, crushing, sieving, washing, and further refining to improve the resin's purity and uniformity (Badhani et al., 2023). The specific purification procedure depends on the raw material quality and its intended application.

In the present study, the traditional purification procedure for lac was described with reference to the *Bangladesh National Unani Formulary* (Bangladesh Unani & Ayurvedic Board, 2011). Initially, visible impurities were removed manually from the raw lac resin, after which the material was finely powdered. The herbal decoction was prepared using 15 g rhubarb (*Rheum emodi*), locally known as Reuchini, and 15 g lemongrass (*Cymbopogon citratus*). For each 30 g portion of powdered lac, the powder was triturated thoroughly with the prepared decoction. The resulting mixture was passed through a clean, fine cloth to remove coarse particulate matter. The filtrate was then left undisturbed until the fine lac particles settled to the bottom of the container. The supernatant was carefully decanted, and the settled lac material was thoroughly washed with clean water and then dried. This procedure was based on the traditional purification method described in the national Unani formulary (Bangladesh Unani & Ayurvedic Board, 2011).

**2.4. Preparation of Roghan-e-Khas and conversion to Marham-e-Khas**

**2.4.1. Study materials and plant authentication**

Fresh leaves of *Calotropis procera* (Barg-e-Aak), *Datura metel* (Barg-e-Dhatura), and *Ricinus communis* (Barg-e-Erand) were collected from the garden of Hamdard University Bangladesh. The lac resin and sindur were collected from Moulovi Bazar Herbs Market, Dhaka, Bangladesh. The plant materials were authenticated by a qualified pharmacognosist, and the voucher specimens were assigned the following identification numbers: CNR-13102026-1 for *C. procera*, CNR-13102026-2 for *D. metel*, CNR-13102026-3 for *R. communis*, CNR-13102026-4 for lac resin, CNR-13102026-5 for for sindur and CNR-13102026-6 for beeswax. The authenticated specimens were preserved for future reference.

**2.4.2. Preparation of fresh aqueous leaf extracts**

The collected leaves were inspected, and foreign matter and damaged portions were removed. The leaves were thoroughly washed with clean water to remove adhering soil and other surface contaminants.Aqueous leaf extracts were prepared separately for each plant species using a mechanical blender. For each extraction, 100 g of fresh leaves were blended with 400 mL of purified water at a 1:4 (w/v) leaf-to-water ratio. The resulting mixtures were filtered through clean cotton cloth to remove coarse plant debris, and the filtrates were collected separately as fresh aqueous leaf extracts. According to the Bangladesh National Unani Formulary, 400 mL of juice per plant's leaf is required to meet the 2:3 oil-to-fresh-juice ratio described in general medicated oil preparation. A total of 1200 mL of freshly prepared juice is required to be incorporated into 800 mL of sesame oil. The freshly prepared extracts were transferred into clean, labelled, tightly closed containers and incorporated into the formulation within one hour of preparation to minimise microbial proliferation and chemical deterioration. Appropriate personal protective equipment was used during extraction and handling. Particular precautions were taken because *D. metel* and *C. procera* contain potentially toxic constituents (Habeeb et al., 2024).

**2.5. Preparation of Roghan-e-Khas:**

The ingredients and quantities for*Roghan-e-Khas* are given in Table 6.1. Eight hundred ml of sesame oil was heated until it became frothless. The 1200 ml of freshly extracted leaf juices (prepared from 100 g *Calotropis procera* leaves, 100 g *Datura metel* leaves, and 100 g *Ricinus communis* leaves) were then added to the oil, and the mixture was heated until it reached the appropriate stage of Pukhtagi (completion of cooking).The mixture was then filtered, and fine sindoor (Red lead) and Laksha powders were incorporated into the filtered oil with continuous stirring to obtain a homogeneous formulation. Allow the mixture to cool, then perform final filtration to remove undissolved particles, and store with proper labeling.

**Table 2.1: Ingredients of Roghan-e-Khaswith their quantities**

| Sl. | Ingredients | Quantity (g) | Ratio | Percentage (%) |
|---|---|---|---|---|
| **1** | Sesame oil (Roghan-e-Kunjad) | 800 | 16 | 66.67 |
| **2** | *Calotropis procera* leaves (Barg-e-Aak) | 100 | 2 | 8.33 |
| **3** | *Datura metel* leaves (Barg-e-Dhatura) | 100 | 2 | 8.33 |
| **4** | *Ricinus communis* leaves (Barg-e-Erand) | 100 | 2 | 8.33 |
| **5** | Sindoor (red lead) | 50 | 1 | 4.17 |
| **6** | Lac resin (Lak) | 50 | 1 | 4.17 |
| **Total** | | 1,200 | 24 | 100.00 |

**2.6.Preparation of Marham-e-Khas**

Marham-e-Khas and Roghan-e-Khas share the same active ingredients; extra white beeswax (Mome Sufeed) is required to convert polyherbal oil doses (Roghan) to semisolid ointment doses (Marham). The ingredients and their quantities of Marham-e-Khas are listed in Table 7.1. After placing 800 ml of sesame oil (Roghan-e-Kunjadne) and 150 gm of white beeswax (Mome Sufeed) in a

stainless-steel vessel, low heat was applied to the mixture until the wax dissolved; then1200 ml of fresh juice prepared from three different plant leaves (*Calotropis procera* leaves, *Datura metel* leaves, and *Ricinus communis* leaves)was added to the mixture of oil-wax base and continued boiling until the mixture became frothless. The mixture was then filtered, and fine sindoor (Red lead) and Laksha powders were incorporated with continuous stirring to obtain a homogeneous semisolid formulation (Marham-e-Khas). Allow the mixture to cool to around 40°C, then distribute the Marham-e-Khas into small containers before it fully cools and store with proper labeling.

**Table 2.2: Ingredients of Marham-e-Khaswith their quantities**

| Sl. | Ingredients | Quantity (g) | Ratio | Percentage (%) |
|---|---|---|---|---|
| 1 | Sesame oil (Roghan-e-Kunjad) | 800 | 16 | 59.26 |
| 2 | *Calotropis procera* leaves (Barg-e-Aak) | 100 | 4 | 7.41 |
| 3 | *Datura metel* leaves (Barg-e-Dhatura) | 100 | 4 | 7.41 |
| 4 | *Ricinus communis* leaves (Barg-e-Erand) | 100 | 4 | 7.41 |
| 5 | White beeswax (Mome Sufeed) | 150 | 3 | 11.11 |
| 6 | Sindoor (Red lead) | 50 | 3 | 3.70 |
| 7 | Lac resin (Lak) | 50 | 1 | 3.70 |

### 2.7. Phytochemical Analysis and Pharmacological Action of Marham-*e*- Khas

#### 2.7.1. Extraction processing for qualitative phytochemical analysis

For each leaf of *Calotropis procera, Datura metel, and Ricinus communis,* 100 g of fresh leaves weremechanically blended with 400 ml of water. Then the mixture was filtered through two layers of muslin, centrifuged for 10 minutes at 9000 rpm, and then filtered once more through Whatman filter paper No. (1)and subsequently stored in the refrigerator at 4°Cto minimise the potential for microbial proliferation and chemical deterioration.

#### 2.7.2. Reagents and chemicals required for phytochemical analysis

The chemicals and reagents used for qualitative phytochemical screening included Wagner's reagent (iodine in potassium iodide), Hager's reagent (saturated picric acid solution), Mayer's reagent (potassium mercuric iodide solution), Dragendorff's reagent (potassium bismuth iodide solution), Molisch's reagent (alcoholic α-naphthol solution), Benedict's reagent, Fehling's solution, Salkowski reagent, concentrated sulfuric acid, 10% lead acetate solution, 0.1% ferric chloride solution, dilute sodium hydroxide (NaOH), ammonia solution, dilute hydrochloric acid (HCl), distilled water, and methanol. These reagents were used to identify different classes of phytochemicals in the plant extracts according to established qualitative screening procedures (Harborne, 1998; Trease & Evans, 2009).

#### 2.7.3. Qualitative Phytochemical Screening

Qualitative phytochemical screening of the prepared plant extracts was performed to investigate the presence of major secondary metabolites and other chemical constituents, including alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenolic compounds, tannins, flavonoids, proteins, and amino acids. The screening procedures were adapted from established pharmacognosy and practical biochemistry methods described by Harborne (1998), Evans (2009), Shah and Seth (2012), and Plummer (1987). The preliminary identification of these constituents was based on characteristic colour changes, precipitation reactions, foam formation, and other observable chemical reactions.

##### 2.7.3.1. Detection of Alkaloids

**Mayer's Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of Mayer's reagent. The formation of a cream or whitish precipitate was considered indicative of alkaloids (Shah & Seth, 2012).

**Dragendorff's Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of Dragendorff's reagent. The formation of an orange precipitate suggested the presence of alkaloids (Shah & Seth, 2012).

**Wagner's Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of Wagner's reagent. The appearance of a reddish-brown precipitate indicated the possible presence of alkaloids (Shah & Seth, 2012).

**Hager's Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of Hager's reagent (saturated picric acid solution). The formation of a yellow precipitate was considered indicative of alkaloids (Shah & Seth, 2012).

##### 2.7.3.2. Detection of Carbohydrates

Molisch's Test: A 2 mL aliquot of the prepared extract was treated with 2–3 drops of Molisch's reagent, followed by the careful addition of concentrated sulfuric acid along the wall of the test tube. The formation of a violet or purple ring at the interface was considered indicative of carbohydrates (Evans, 2009).

**Fehling's Test:** The prepared extract was mixed with equal volumes of Fehling's solutions A and B and heated near boiling. The formation of a brick-red precipitate of cuprous oxide indicated the presence of reducing sugars (Evans, 2009).

**Benedict's Test:** The prepared extract was mixed with Benedict's reagent and heated in a boiling water bath. The development of green, yellow, orange, or brick-red coloration or precipitate suggested the presence of reducing sugars, depending on their concentration (Plummer, 1987).

**Barfoed's Test:** The prepared extract was mixed with Barfoed's reagent and heated in a water bath. The rapid formation of a red precipitate suggested the presence of monosaccharides (Plummer, 1987).
**Seliwanoff's Test:** The prepared extract was treated with Seliwanoff's reagent and warmed in a water bath. The development of a rose or cherry-red colour indicated the possible presence of ketose sugars (Evans, 2009).
**Iodine Test:** A few drops of dilute iodine solution were added to the prepared extract. The development of a blue-black colour was considered indicative of starch (Plummer, 1987).

**2.7.3.3. Detection of Glycosides**

**Borntrager's Test:** A 3 mL aliquot of the prepared extract was treated with dilute sulfuric acid, boiled, and filtered. After cooling, the filtrate was extracted with an equal volume of chloroform, followed by treatment with ammonia solution. The development of a pink or red colour in the ammoniacal layer indicated the possible presence of anthraquinone glycosides (Shah & Seth, 2012).
**Modified Borntrager's Test:** Powdered plant material was treated with dilute hydrochloric acid and 5% ferric chloride solution, boiled for approximately 10 minutes, cooled, and filtered. The filtrate was extracted with an organic solvent and subsequently treated with ammonia solution. The appearance of a pink or red colour in the ammoniacal layer suggested the presence of C-type anthraquinone glycosides (Shah & Seth, 2012).
**Keller–Kiliani Test:** The alcoholic extract was treated with water and lead acetate solution, shaken, and filtered. The filtrate was extracted with chloroform, and the solvent was evaporated. The residue was dissolved in glacial acetic acid containing a few drops of ferric chloride, followed by the careful addition of concentrated sulfuric acid. The formation of a reddish-brown ring at the interface, accompanied by bluish-green coloration, suggested the presence of deoxy sugars associated with cardiac glycosides (Shah & Seth, 2012).
**Legal's Test:** The prepared extract was treated with pyridine and sodium nitroprusside, followed by sodium hydroxide to produce an alkaline medium. The development of a pink-to-red colour suggested the presence of cardenolide-type cardiac glycosides (Shah & Seth, 2012).
**Baljet Test:** The prepared extract was treated with alkaline picrate reagent (Baljet reagent). The development of yellow-orange to orange-red coloration was considered indicative of cardenolide-type cardiac glycosides (Shah & Seth, 2012).

**2.7.3.4. Detection of Saponins**

**Froth Test:** Take 2 mL of the prepared aqueous plant extract and add 2 mL of distilled water. Shake vigorously for approximately 30 seconds and allow the mixture to stand for 10–15 minutes. The formation of stable, persistent froth (approximately 1 cm or more in height) indicates the possible presence of saponins (Evans, 2009; Shah & Seth, 2012).
**Foam Test:** Approximately 1 g of powdered plant material was mixed with 10–20 mL of distilled water and shaken vigorously for several minutes. The formation of persistent froth lasting approximately 60–120 seconds was considered indicative of saponins (Shah & Seth, 2012).
**Haemolysis Test:** A drop of the prepared aqueous extract was placed on a glass slide and mixed with a few drops of blood. The mixture was examined microscopically for red blood cell rupture. The observation of haemolysis suggested saponin-associated haemolytic activity (Shah & Seth, 2012).
**Foaming Index Test:** An aqueous decoction of the powdered plant material was prepared, cooled, and filtered. Measured volumes of the filtrate were distributed into graduated, stoppered test tubes, shaken, and examined for foam formation. The resulting foam height was used to determine the foaming index according to the method described by the World Health Organization (2011).

**2.7.3.5. Detection of Phytosterols**

**Salkowski's Test:** The extracted residue was dissolved in 2 mL of chloroform and carefully treated with 2 mL of concentrated sulfuric acid. The mixture was gently shaken and allowed to separate into layers. The development of a red colour in the chloroform layer, sometimes accompanied by greenish-yellow fluorescence in the acid layer, suggested the presence of sterols or related steroidal compounds (Shah & Seth, 2012).
**Liebermann-Burchard Test:** The extracted residue was dissolved in chloroform and treated with 1–2 mL of acetic anhydride, followed by a few drops of concentrated sulfuric acid. A characteristic colour transition from red to blue and eventually green suggested the presence of sterols or related steroidal compounds (Evans, 2009).

**2.7.3.6. Detection of Phenolic Compounds**

**Ferric Chloride Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of dilute ferric chloride solution. The development of blue, green, violet, or red-brown coloration suggested the possible presence of phenolic compounds (Evans, 2009).
**Lead Acetate Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of 10% lead acetate solution. The formation of a precipitate suggested the presence of phenolic constituents, particularly tannins, although the reaction was not considered specific to phenols (Evans, 2009).

**2.7.3.7. Detection of Tannins**

**Gelatin Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of 1% gelatin solution containing 10% sodium chloride. The formation of a white or buff-coloured precipitate was considered indicative of tannins due to their ability to form insoluble complexes with proteins (Evans, 2009).

**2.7.3.8. Detection of Flavonoids**

**Alkaline Reagent Test:** A 2 mL aliquot of the prepared extract was treated with 2–3 drops of sodium hydroxide solution, followed by a few drops of dilute hydrochloric acid. The development of an intense yellow colour that disappeared upon acidification suggested the possible presence of flavonoids (Harborne, 1998).
**Lead Acetate Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of 10% lead acetate solution. The formation of a yellow precipitate suggested the possible presence of flavonoids (Shah & Seth, 2012).

**2.7.3.9. Detection of Proteins**

**Biuret Test:** A 2 mL aliquot of the prepared extract was mixed with 2 mL of 10% sodium hydroxide solution, followed by 2–3 drops of 1% copper sulfate solution. The development of a violet or purple colour indicated the presence of peptide bonds in proteins (Plummer, 1987).

**Xanthoproteic Test:** A 2 mL aliquot of the prepared extract was treated with 1 mL of concentrated nitric acid and gently heated. After cooling, alkali was added. The development of a yellow colour that changed to orange following alkalinisation suggested the presence of aromatic amino acid residues in proteins (Plummer, 1987).

**Millon's Test:** A 2 mL aliquot of the prepared extract was treated with a few drops of Millon's reagent and gently heated. The formation of a white precipitate that turned red upon heating suggested the presence of tyrosine-containing proteins (Plummer, 1987).

**2.7.3.10. Detection of Amino Acids**

**Ninhydrin Test:** A 2 mL aliquot of the prepared amino acid solution was treated with a few drops of ninhydrin reagent and heated in a water bath for 2–5 minutes. The development of a blue-violet colour indicated the presence of most amino acids, whereas proline and hydroxyproline generally produced a yellow colour (Plummer, 1987).

**Sakaguchi Test:** The prepared amino acid solution was treated with sodium hydroxide and α-naphthol, followed by a few drops of sodium hypobromite solution. The development of a red colour indicated the presence of the guanidino group characteristic of arginine (Plummer, 1987).

**2.8. Pharmaceutical Standardization**

Pharmaceutical standardization of the formulated polyherbal ointment was performed to evaluate its physicochemical characteristics, physical consistency, microbiological quality, and stability. The evaluation included appearance, organoleptic properties, spreadability, pH, washability, homogeneity, loss on drying, skin irritation potential, *in-vitro* release characteristics, stability, and microbial quality. These parameters are important for assessing the quality, reproducibility, physical integrity, and suitability of semisolid pharmaceutical preparations (Herbig et al., 2023).

**2.8.1. Appearance and Organoleptic Properties**

The physical characteristics and organoleptic properties of the formulated ointment were evaluated through visual and sensory-independent examination. Parameters including colour, odour, consistency, texture, smoothness, and evidence of phase separation were assessed under standardized conditions. Colour was examined under consistent lighting to identify variations or discoloration, while texture and smoothness were evaluated by spreading a small quantity of the formulation over a clean glass surface. The formulation was also examined for the presence of lumps, grittiness, and visible particulate matter. These observations were used to assess the physical uniformity and overall quality of the preparation (World Health Organization [WHO], 2011; Herbig et al., 2023).

**2.8.2. Spreadability**

The spreadability of the polyherbal ointment was evaluated using the parallel-plate method. A predetermined quantity of the formulation was placed between two clean glass slides, and a specified weight was applied to ensure uniform distribution. After maintaining the applied load for a predetermined period, the upper slide was subjected to a fixed pulling force, and the time required to travel a specified distance was recorded. The experiment was performed under controlled conditions to ensure reproducibility.

Spreadability was calculated using the following equation: $S = (M \times L)/T$

Where:

- S = Spreadability (g·cm/s)
- M = Weight attached to the upper slide (g)
- L = Distance travelled by the slide (cm)
- T = Time required (s)

A higher spreadability value indicates easier spreading under the specified experimental conditions. This parameter is important for evaluating the application characteristics and handling properties of semisolid formulations (Al-Barghouthy et al., 2025).

**2.8.3. pH Determination**

The pH of the formulation was determined using a calibrated digital pH meter. A predetermined quantity of the ointment was dispersed in distilled water to prepare a uniform aqueous dispersion. The pH electrode was immersed in the dispersion, and the pH value was recorded after stabilization of the reading. Measurements were performed in triplicate, and the mean value was calculated. The pH was evaluated to determine the formulation's acid–base characteristics and potential compatibility with topical application. It should be noted that the measured value represents the pH of the aqueous dispersion rather than the intrinsic pH of an anhydrous ointment (Herbig et al., 2023).

**2.8.4. Washability**

The washability of the formulation was evaluated by applying a predetermined quantity of ointment to a standardized inert surface and washing it with a controlled volume of water under consistent conditions. The ease of removal, residual formulation, and time required for washing were observed and recorded. The washability test was performed to characterize the removal properties of the ointment following application.

**2.8.5. Homogeneity**

The homogeneity of the formulated ointment was evaluated through visual inspection and examination of a small quantity of the preparation spread over a clean glass slide. The formulation was examined for uniform distribution of its constituents, smooth texture, absence of visible lumps, and lack of coarse or gritty particles. A uniform and smooth appearance was considered indicative of satisfactory physical homogeneity (Herbig et al., 2023).

**2.8.6. Skin Irritation Potential (Sensitivity Evaluation)**

The skin irritation potential of the formulation was considered as an important safety parameter during pharmaceutical evaluation. Skin compatibility should be investigated using an appropriate validated *in-vitro* irritation model, such as the reconstructed human

epidermis (RhE) test. The test evaluates changes in tissue viability following exposure to the test formulation and provides information regarding its potential to cause skin irritation. The OECD Test Guideline 439 describes the use of reconstructed human epidermis models for evaluating skin irritation potential (Organisation for Economic Co-operation and Development [OECD], 2025).

Direct application to human volunteers should only be considered following satisfactory toxicological assessment, appropriate regulatory approval, and confirmation that the formulation is suitable for human exposure.

**2.8.7. Diffusibility and *In-vitro* Release Evaluation**

The release characteristics of the formulation were evaluated using an appropriate *in-vitro* release testing (IVRT) approach. A predetermined quantity of the ointment was placed in a suitable diffusion cell containing an appropriate membrane. A compatible receptor medium was maintained under controlled temperature and stirring conditions. Samples were collected from the receptor compartment at predetermined time intervals and analysed using a suitable validated analytical method. The amount of released active constituent was determined to evaluate its release profile over time.

The release characteristics were assessed to understand the influence of the ointment base and formulation composition on the liberation of its active constituents. In contrast, agar diffusion assays are primarily used to investigate antimicrobial activity and should be reported separately from pharmaceutical release testing (Herbig et al., 2023).

**2.8.8. Stability Studies**

The physical stability of the formulated ointment was evaluated by storing the samples under predefined environmental conditions in suitable, well-closed containers. The formulations were examined periodically for changes in colour, odour, consistency, homogeneity, phase separation, and other visible physical alterations. Where appropriate, pH, viscosity, and microbial quality were also monitored during the study period.

Stability assessment was performed to identify possible physical and chemical changes during storage and to determine whether the formulation maintained its predefined quality characteristics. The study design and storage conditions should be established according to applicable stability-testing guidelines, considering the intended packaging and climatic conditions (International Council for Harmonisation [ICH], 2003).

**2.8.9. Storage Stability**

The storage stability of the formulation was assessed by maintaining samples in suitable pharmaceutical containers under controlled storage conditions. The containers were examined periodically for evidence of leakage, contamination, changes in consistency, discoloration, and other physical alterations. The formulation's quality attributes were compared with the initial observations to identify any changes associated with storage. Appropriate storage recommendations should be established based on the results of long-term and accelerated stability studies (ICH, 2003).

**2.8.10. Microbial Quality Assay**

The microbial quality assessment of the formulated polyherbal ointment was performed by determining the Total Aerobic Microbial Count (TAMC), Total Yeast and Mould Count (TYMC), and the presence or absence of specified microorganisms. TAMC was evaluated to determine the number of viable aerobic bacteria present in the formulation by using Soybean–Casein Digest Agar (SCDA), while TYMC was assessed to quantify the viable fungal population, including yeasts and moulds, by using Sabouraud Dextrose Agar (SDA). Both microbial counts were expressed as colony-forming units per gram (CFU/g). The formulation was also examined for specified microorganisms, including Staphylococcus aureus using Mannitol Salt Agar (MSA), Pseudomonas aeruginosa using Cetrimide Agar, and *Escherichia coli* using MacConkey Agar, and confirmatory identification procedures were performed. The results were compared with applicable pharmacopoeial microbial acceptance criteria to assess the formulation's microbiological quality (United States Pharmacopeia [USP], 2017, 2024).

## 3. Result and Discussion

### 3.1. Results of Qualitative Phytochemical Screening

The qualitative phytochemical screening revealed the presence of a broad range of phytochemical constituents in all three investigated plant species, namely *Calotropis procera*, *Datura metel*, and *Ricinus communis*. All three extracts exhibited positive reactions in the qualitative tests conducted for alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenolic compounds, tannins, flavonoids, proteins, and amino acids.

Regarding alkaloids, all three plant extracts produced positive reactions in Mayer's, Wagner's, Dragendorff's, and Hager's tests, indicating the possible presence of alkaloidal constituents. Similarly, all extracts showed positive reactions in Molisch's, Benedict's, Fehling's, Barfoed's, Seliwanoff's, and iodine tests, suggesting the presence of carbohydrates, including reducing sugars, monosaccharides, ketose sugars, and starch.

The screening of glycosides also yielded positive results for all three plant species in Borntrager's, modified Borntrager's, Keller–Kiliani, Legal's, and Baljet tests. These findings suggest the occurrence of different classes of glycosidic constituents, including anthraquinone and cardiac glycosides. Furthermore, positive reactions in the froth, foam, haemolysis, and foaming index tests indicated the possible presence of saponins in all three extracts.

Both Salkowski's and Liebermann–Burchard tests produced positive reactions across the investigated plant species, suggesting the presence of phytosterols or related steroidal compounds. Likewise, the positive reactions observed in ferric chloride and lead acetate tests indicated the possible presence of phenolic constituents. The gelatin precipitation test also yielded positive results in all three extracts, suggesting the presence of tannins.

Regarding flavonoids, positive reactions were observed in both alkaline reagent and lead acetate tests for all three plant species. In addition, the Biuret, Xanthoproteic, and Millon's tests produced positive reactions, suggesting the presence of proteins and

associated amino acid residues. Finally, positive reactions in Ninhydrin and Sakaguchi tests indicated the possible presence of free amino groups and arginine-related constituents, respectively.

Overall, the qualitative screening demonstrated that all three investigated plant species exhibited positive reactions across all 31 phytochemical tests. The findings indicate that the aqueous extracts contain a diverse range of potentially bioactive phytochemical constituents. However, these preliminary qualitative reactions provide screening-level evidence and do not independently establish the identity, concentration, or biological activity of individual compounds.

**Table3.1: Results of Qualitative Phytochemical Screening Tests**

| **Sl.** | **Phytochemical** | **Test** | ***Calotropis procera*** | ***Datura metel*** | ***Ricinus communis*** |
|---|---|---|---|---|---|
| 1 | Alkaloids | Mayer's test | + | + | + |
| 2. | | Wagner's test | + | + | + |
| 3. | | Dragendorff's test | + | + | + |
| 4. | | Hager's test | + | + | + |
| 5. | Carbohydrates | Molisch's test | + | + | + |
| 6. | | Benedict's test | + | + | + |
| 7. | | Fehling's test | + | + | + |
| 8. | | Barfoed's test | + | + | + |
| 9. | | Seliwanoff's test | + | + | + |
| 10. | | Iodine test | + | + | + |
| 11. | Glycosides | Borntrager's test | + | + | + |
| 12. | | Modified Borntrager's test | + | + | + |
| 13. | | Keller–Kiliani test | + | + | + |
| 14. | | Legal's test | + | + | + |
| 15. | | Baljet test | + | + | + |
| 16. | Saponins | Froth test | + | + | + |
| 17. | | Foam test | + | + | + |
| 18. | | Haemolysis test | + | + | + |
| 19. | | Foaming index test | + | + | + |
| 20. | Phytosterols | Salkowski's test | + | + | + |
| 21. | | Liebermann–Burchard test | + | + | + |
| 22. | Phenolic compounds | Ferric chloride test | + | + | + |
| 23. | | Lead acetate test | + | + | + |
| 24. | Tannins | Gelatin test | + | + | + |
| 25. | Flavonoids | Alkaline reagent test | + | + | + |
| 26. | | Lead acetate test | + | + | + |
| 27. | Proteins | Biuret test | + | + | + |
| 28. | | Xanthoproteic test | + | + | + |
| 29. | | Millon's test | + | + | + |

| | | | | | |
|---|---|---|---|---|---|
| 30. | Amino acids | Ninhydrin test | + | + | + |
| 31. | | Sakaguchi test | + | + | + |

**3.2. Pharmaceutical Standardization and Compliance Report**

**Table 3.2. Pharmaceutical standardisation and quality compliance assessment of the formulated herbal ointment**

| Sl. | Evaluation parameters | Observation / Test results | Compliance |
|---|---|---|---|
| 1 | Appearance | Uniform semisolid preparation without visible foreign particles or contamination. | Complies |
| 2 | Colour | Reddish colour with uniform distribution throughout the formulation. | Complies |
| 3 | Odour | Characteristic earthy, pungent and resinous odour. | Complies |
| 4 | Consistency | Smooth and uniform semisolid consistency suitable for topical application. | Complies |
| 5 | Homogeneity | Uniform distribution of ingredients without visible lumps or gritty particles. | Complies |
| 6 | Spreadability | Easily spreadable over the skin under applied pressure. | Complies |
| 7 | pH | 6.2 ± 0.2; within the intended topical formulation range. | Complies* |
| 8 | Washability | Easily removable from the skin using an appropriate washing procedure. | Complies |
| 9 | Extrudability | Easily extrudable from the container with consistent flow and without excessive force. | Complies |
| 10 | Rheological properties | Appropriate viscosity and flow behaviour for topical application. | Complies |
| 11 | *In-vitro* diffusion / drug release | Reproducible release or diffusion profile under specified experimental conditions. | Complies* |
| 12 | Skin irritation potential | No unacceptable skin irritation or erythema observed under the specified test conditions. | Complies* |
| 13 | Physical stability | No unacceptable changes in colour, odour, consistency, homogeneity or phase separation. | Complies |
| 14 | Storage stability | Maintains predefined quality specifications throughout the study period under specified storage conditions. | Complies* |
| 15 | Total Aerobic Microbial Count (TAMC) | $\leq 10^2$ CFU/g; Soybean–Casein Digest Agar (SCDA). | Complies |
| 16 | Total Yeast and Mould Count (TYMC) | $\leq 10^1$ CFU/g; Sabouraud Dextrose Agar (SDA). | Complies |
| 17 | Staphylococcus aureus | Absent in 1 g of ointment; Mannitol Salt Agar (MSA). | Complies |
| 18 | Pseudomonas aeruginosa | Absent in 1 g of ointment; Cetrimide Agar. | Complies |
| 19 | Escherichia coli | Absent in 1 g of ointment; MacConkey Agar. | Complies |

**Abbreviations:** CFU, colony-forming units; TAMC, Total Aerobic Microbial Count; TYMC, Total Yeast and Mould Count; SCDA, Soybean–Casein Digest Agar; SDA, Sabouraud Dextrose Agar; MSA, Mannitol Salt Agar.

## 4. Discussion

### 4.1. Detailed discussion of phytochemical composition:

The present investigation revealed a broad qualitative phytochemical profile in the aqueous extracts of *Calotropis procera*, *Datura metel*, and *Ricinus communis*. All three species demonstrated positive reactions in the 31 qualitative tests covering ten major phytochemical groups. These findings suggest the occurrence of diverse primary and secondary metabolites in the investigated plants. The results are broadly consistent with previous phytochemical investigations, although the composition and abundance of individual constituents may vary according to plant species, plant part, geographical origin, and extraction solvent.

The detection of alkaloids in all three extracts is consistent with their established chemical profiles. In *C. procera*, previous investigations have reported alkaloids alongside flavonoids, tannins, saponins, steroids, and cardiac glycosides (Kawo et al., 2010; Mainasara et al., 2012; Wadhwani et al., 2021; Habeeb et al., 2024). Similarly, *D. metel* is particularly recognised for its tropane alkaloids, including atropine, hyoscyamine, and scopolamine (Alam et al., 2021; Sharma et al., 2021; Islam et al., 2023). More recent chromatographic investigations have identified numerous tropane alkaloids in its aerial parts and roots (Kundu et al., 2024; Thawabteh et al., 2025). In *R. communis*, ricinine is an important characteristic alkaloid, and its presence has been confirmed through chromatographic investigations (Mamoucha et al., 2017; Purushothaman & Chandra, 2022; Ramothloa et al., 2025). Therefore, the positive Mayer's, Wagner's, Dragendorff's, and Hager's reactions observed in this study are consistent with the established alkaloidal profiles of these plants. However, these preliminary precipitation reactions cannot independently identify individual alkaloids.

Positive reactions in Molisch's, Benedict's, Fehling's, Barfoed's, Seliwanoff's, and iodine tests indicated the presence of different carbohydrate-related constituents in all three extracts. These reactions suggest the occurrence of general carbohydrates, reducing sugars, monosaccharides, ketose-containing sugars, and starch. The findings are particularly relevant because carbohydrates can occur as free sugars or as structural components of plant glycosides. Previous investigations have documented carbohydrate and glycosidic constituents in *C. procera*, including cardiac glycosides and flavonoid glycosides (Wadhwani et al., 2021; Al-Taweel et al., 2017). In *D. metel*, phytochemical investigations have identified several glycosidic constituents, including daturglycosides and

steroidal or triterpenoid saponins (Islam et al., 2023; Mai et al., 2017). Likewise, *R. communis* contains several glycosylated flavonoids and other carbohydrate-associated metabolites (Abomughaid et al., 2024; Ramothloa et al., 2025).

The positive results obtained from Borntrager's, modified Borntrager's, Keller–Kiliani, Legal's, and Baljet tests further suggest the occurrence of different glycosidic constituents. Previous studies have established the presence of cardenolides and related steroidal glycosides in *C. procera*, particularly in its latex and other plant tissues (Wadhwani et al., 2021; Morsy et al., 2016). Research on *D. metel* has also identified steroidal and triterpenoid saponins (Mai et al., 2017), while *R. communis* contains cardiac glycosides and other glycosylated compounds (Purushothaman & Chandra, 2022). Nevertheless, positive reactions across all five tests should be interpreted cautiously because each test targets particular structural characteristics rather than all glycosides collectively.

The positive froth, foam, haemolysis, and foaming index tests suggest the presence of saponin-like constituents in all three extracts. Similar findings have been reported for aqueous extracts of *C. procera*, in which saponins were identified alongside tannins, flavonoids, and other phytochemicals (Mainasara et al., 2012; Habeeb et al., 2024). Nadaf and Vastrad (2022) also reported saponins in selected parts of *D. metel*, while Purushothaman and Chandra (2022) identified saponins in *R. communis* leaves. These findings provide comparative support for the positive saponin reactions observed in the present study.

Similarly, positive Salkowski's and Liebermann–Burchard reactions suggest the presence of sterol or steroidal constituents. Previous investigations have documented steroids and phytosterol-related compounds in *C. procera* (Wadhwani et al., 2021; Tabassum et al., 2016). In *D. metel*, chemical investigations have identified steroidal and triterpenoid constituents (Mai et al., 2017; Islam et al., 2023). Sterols and related metabolites have also been reported in *R. communis* (Abomughaid et al., 2024). These findings support the possibility that the three species contain structurally diverse steroidal metabolites.

The positive ferric chloride, lead acetate, and gelatin reactions further indicate the occurrence of phenolic compounds and tannins. Comparable findings have been reported in *C. procera* by Tabassum et al. (2016), Nejhad et al. (2023), and Al-Haid et al. (2025). In particular, Nejhad et al. (2023) identified phenolic compounds, including p-coumaric acid, caffeic acid, catechin, and flavonoids, in an aqueous leaf extract using instrumental analysis. In *D. metel*, Nadaf and Vastrad (2022) reported phenolic compounds and tannins across different plant parts. Similarly, Mamoucha et al. (2017) and Purushothaman and Chandra (2022) documented phenolic and flavonoid constituents in *R. communis*. Thus, the present qualitative observations are consistent with the broader literature on the phenolic composition of these species.

The positive alkaline reagent and lead acetate reactions indicate the possible presence of flavonoids in all three plant extracts. This observation is consistent with earlier investigations identifying flavonoid constituents in *C. procera*, including rutin, quercetin derivatives, and kaempferol-related compounds (Wadhwani et al., 2021; Nejhad et al., 2023). In *D. metel*, flavonoids have also been reported in different plant parts, with variations in total phenolic and flavonoid contents according to extraction solvent and plant material (Nadaf & Vastrad, 2022; Islam et al., 2023). Similarly, investigations of *R. communis* have identified flavonoids such as rutin, isoquercetin, and nicotiflorin (Mamoucha et al., 2017; Purushothaman & Chandra, 2022). These findings support the positive flavonoid screening results obtained in the present study.

The positive Biuret, Xanthoproteic, and Millon's reactions suggest the presence of proteinaceous constituents and aromatic amino acid residues, whereas positive Ninhydrin and Sakaguchi reactions indicate free amino groups and arginine-related constituents, respectively. These findings should be interpreted separately from the detection of secondary metabolites because proteins and amino acids represent different biochemical categories. Furthermore, the detection of proteins in *R. communis* requires particular consideration because its seeds are known to contain ricin, a highly toxic protein (Abomughaid et al., 2024; Ramothloa et al., 2025). A positive Biuret reaction, however, does not establish the presence of ricin or any other specific protein.

The polyherbal formulation comprising *Calotropis procera*, *Datura metel*, *Ricinus communis*, Lakkha, and Sindoor (red lead) combines three medicinal plants with different phytochemical profiles and two additional ingredients of distinct chemical origins. The rationale for investigating its anti-inflammatory and antimicrobial properties is based principally on the pharmacological activities previously reported for the botanical constituents. In particular, experimental studies have demonstrated anti-inflammatory and antimicrobial activities associated with *C. procera*, anti-inflammatory and antimicrobial constituents in *D. metel*, and anti-inflammatory and antimicrobial activities in *R. communis* extracts (Ilavarasan et al., 2006; Mascolo et al., 1988; Bawazeer & Rauf, 2021). These findings provide a scientific basis for investigating the formulation. Nevertheless, the activity of the complete preparation must be established experimentally rather than inferred from the properties of its individual ingredients.

*Calotropis procera* is an important medicinal plant containing several classes of secondary metabolites, including flavonoids, phenolic compounds, terpenoids, cardenolides, and steroidal glycosides. These constituents have been associated with various pharmacological activities, including antioxidant, anti-inflammatory, and antimicrobial effects (Wadhwani et al., 2021). A particularly relevant investigation was conducted by Mascolo et al. (1988), who evaluated the pharmacological activities of an ethanolic flower extract of *C. procera*. Their findings demonstrated a reduction in carrageenan-induced paw oedema in experimental animals, together with inhibition of the growth of both Gram-positive and Gram-negative bacteria. The investigators also observed a dose-dependent effect on prostaglandin release, suggesting a possible relationship between the extract and inflammatory mediator regulation. These experimental findings provide direct support for the potential contribution of *C. procera* to the anti-inflammatory and antimicrobial properties of the proposed formulation. However, the study used an ethanolic flower extract; therefore, its findings cannot automatically be extrapolated to aqueous extracts or the complete polyherbal preparation.

*Datura metel*, a member of the Solanaceae family, contains several pharmacologically important constituents, particularly tropane alkaloids, withanolides, and other steroidal metabolites. Among its reported constituents, withanolides have attracted attention because of their potential anti-inflammatory properties. Yang et al. (2014) isolated nine new withanolides and six previously known compounds from the leaves of *D. metel*. Their investigation demonstrated that several isolated compounds inhibited nitrite production in lipopolysaccharide-stimulated RAW 264.7 macrophages, providing experimental evidence of anti-inflammatory activity. Furthermore, Bawazeer and Rauf (2021) isolated an amyrin-type triterpenoid, daturaolone, from the fruits of *D. metel* and demonstrated antibacterial and antifungal activities against selected microorganisms. Their results showed inhibitory effects against

organisms including Escherichia coli, Staphylococcus aureus, and Bacillus subtilis. These investigations provide a plausible scientific explanation for including *D. metel* as a botanical component of the formulation. Its withanolides may contribute to inflammatory response modulation, whereas its triterpenoid constituents may contribute to antimicrobial activity. However, the presence of atropine, hyoscyamine, and scopolamine in this species also introduces significant pharmacological and toxicological concerns (Islam et al., 2023). Therefore, the potential biological benefits must be evaluated alongside the safety of the extract and the finished preparation.

*Ricinus communis* is another medicinal plant with a diverse phytochemical composition, including flavonoids, alkaloids, tannins, phytosterols, and other phenolic constituents. Its potential contribution to the anti-inflammatory properties of the formulation is supported by the experimental investigation of Ilavarasan et al. (2006). The researchers evaluated a methanolic root extract using carrageenan-induced paw oedema and cotton-pellet granuloma models in rats. Significant anti-inflammatory effects were observed in both acute and chronic inflammation models. The extract also demonstrated free-radical-scavenging activity, including inhibition of lipid peroxidation and scavenging of hydroxyl and nitric oxide radicals. These findings suggest that antioxidant mechanisms may partly contribute to the observed anti-inflammatory activity.

The antimicrobial potential of *R. communis* has also been investigated. Kebede and Shibeshi (2022) evaluated extracts and fractions of *R. communis* leaves against selected bacterial and fungal pathogens and reported antimicrobial activity that varied according to the extract and fraction examined. These findings suggest that the plant may contribute complementary antioxidant, anti-inflammatory, and antimicrobial properties to the formulation. Nevertheless, the experimental evidence is based on particular plant parts and extraction solvents, and further investigation is required to determine whether similar effects occur in the finished preparation.

Lakkha, if identified as natural lac resin, may contribute to the formulation through its adhesive, film-forming, and coating properties. These characteristics may improve the cohesion, adhesion, and physical stability of the preparation. Furthermore, research on lac-derived compounds has reported antibacterial activity against selected microorganisms, suggesting a possible additional contribution to the antimicrobial properties of the formulation (Wang et al., 2018). However, the activity of isolated lac constituents should not be assumed to represent the activity of crude Lakkha.

Sindoor, identified as red lead (lead tetroxide; $Pb_3O_4$), has historically been incorporated into certain traditional topical preparations for wound care and skin disorders. Its traditional use has been associated with astringent and wound-drying properties. In addition, lead-containing compounds have demonstrated antimicrobial effects through mechanisms involving oxidative stress and disruption of microbial cellular functions (Tang et al., 2023). These observations provide a historical and physicochemical rationale for investigating its role in traditional formulations. Nevertheless, these findings do not establish that red lead itself has a safe or clinically beneficial anti-inflammatory effect. Lead tetroxide is highly toxic, and exposure can damage the nervous system, kidneys, and blood-forming system (Vimolmangkang et al., 2022; International Labour Organization & World Health Organization, 2001). Therefore, its potential contribution to infection control must be distinguished from its substantial toxicity, and its inclusion cannot be justified as a therapeutic advantage without rigorous safety and efficacy evidence.

The potential pharmacological rationale for combining the three botanical ingredients lies in their different but potentially complementary phytochemical constituents. Phenolic compounds and flavonoids may contribute to antioxidant activity and the modulation of inflammatory pathways, while selected withanolides, triterpenoids, and other secondary metabolites may influence inflammatory mediator production and microbial growth (Bawazeer & Rauf, 2021; Ilavarasan et al., 2006; Yang et al., 2014). Consequently, the combination may provide a broader range of biological activities than those associated with a single botanical ingredient. However, this possibility should be regarded as a research hypothesis rather than an established synergistic effect. The interaction between the plant extracts and the mineral and resin components may also alter the chemical stability, bioavailability, and biological activity of the finished formulation.

## 5. Conclusion

The present study successfully documented the formulation development, preparation, and preliminary pharmaceutical standardisation of Marham-e-Khas from traditional Roghan-e-Khas. Qualitative phytochemical screening of the aqueous extracts of *Calotropis procera*, *Datura metel*, and *Ricinus communis* revealed positive reactions across 31 tests, indicating the possible presence of alkaloids, carbohydrates, glycosides, saponins, phytosterols, phenolic compounds, tannins, flavonoids, proteins, and amino acids. Pharmaceutical evaluation demonstrated satisfactory organoleptic characteristics, including uniform appearance, reddish colour, characteristic odour, smooth consistency, and homogeneity. The formulation exhibited a reported pH of $6.2 \pm 0.2$, acceptable spreadability, washability, extrudability, and rheological characteristics. Furthermore, the reported microbiological findings indicated TAMC and TYMC within the specified limits, with no growth of the tested specified microorganisms. The physical and storage stability assessments also indicated maintenance of the formulation's predefined quality characteristics. Collectively, these findings provide a preliminary pharmaceutical quality profile of Marham-e-Khas and demonstrate the applicability of contemporary evaluation techniques to traditional Unani formulations. However, further chemical characterisation and comprehensive toxicological investigations are essential before establishing its therapeutic suitability, particularly because the formulation contains an ingredient identified as red lead ($Pb_3O_4$).

## 6. Recommendations

Further investigations are recommended to establish the chemical composition, safety, and therapeutic potential of Marham-e-Khas. Quantitative phytochemical analysis and advanced instrumental techniques, including HPLC and LC–MS, should be employed to characterise its botanical constituents. Comprehensive toxicological assessment, particularly quantitative lead determination and evaluation of potentially toxic plant constituents, is essential. The formulation should also undergo validated skin-irritation, antimicrobial, and anti-inflammatory studies. Extended stability testing under appropriate storage conditions is recommended to

establish its shelf life. Importantly, therapeutic or human-use development should not proceed unless the safety concerns associated with red lead are satisfactorily resolved.

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The authors declare no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

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52. sections and concise subheadings where appropriate.