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Formulation And Evaluation Of Anti - Leprotic Ointment By Using *Cascabela Thevetia*

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ABSTRACT

Leprosy known as Hansen's disease is caused by infection of susceptible individuals with acid-fast bacilli (AFB) of the Mycobacterium leprae complex (M. leprae). These rod-shaped AFB were first described by Gerhard Hansen in 1873.The mechanism of transmission of M. leprae has been somewhat obscure historically due to variability in host susceptibility to infection and a very long potential incubation period. Skin contact and vertical transmission are also possible infection mechanisms. *Thevetia Peruviana* belongs to family Apocynaceae and commonly called as a yellow Olender or Pilli Kaner. *Cascabela thevetia* known as an ornamental plant and planted as large flowering shrub or tree standards in garden, parks, road side and on road dividers. All parts of this plant are highly toxic including leaves, bark, fruits, and flowers. The roots and leaves of *Cascabela thevetia* are used in vata, pitta, cough, bronchitis, renal, and skin diseases. Due to Phytoconstituents it shows various activities anti-fungal, anti-bacterial, anti-oxidant, anthelmintics, anti-spermatogenic, anti-inflammatory, anti-urolithic and infertility.

Many therapies in the form of photo therapy, corticosteroids, immuno-modulators, Ultraviolet rays, lasers, hypo-pigmentation, and disguise have been used to treat Vitiligo since centuries. Medicinal plants play a very important role in the development of alternative drugs without the adverse effects of synthetic drugs. Plants and natural products form the basis of both modern and traditional medicines and are currently widely used in the production of commercially produced drugs. Scientific and reliable reports indicated that about 25% of prescribed medicines worldwide are taken from herbs.

Keywords: Leprosy, hypopigmentation, M. leprae, *Cascabela thevetia*, Apocynaceae, melanogenesis, vitiligo.

INTRODUCTION TO LEPROSY

Leprosy known as Hansen's disease is caused by infection of susceptible individuals with acid-fast bacilli (AFB) of the Mycobacterium leprae complex (M. leprae and M. lepromatosis). These rod-shaped AFB were first described by Gerhard Hansen in 1873.M. leprae are slow growing organisms that replicate preferentially in macrophages, endothelial cells, and Schwann cells. They are obligate intracellular organisms and do not grow in artificial media cultures. Their ideal growth occurs between 27-33oC. M. lepromatosis is more recently described as an etiologic agent, though its clinical course may be indistinguishable from infection caused by M. leprae.Leprosy is ubiquitous in tropical countries, particularly underdeveloped and developing countries. In 1990, the World Health Organization (WHO) proposed the global goal of eliminating leprosy by the end of the 20thcentury. Despite the commitment of governments, researchers, and healthcare workers worldwide, disease control has not yet been achieved.

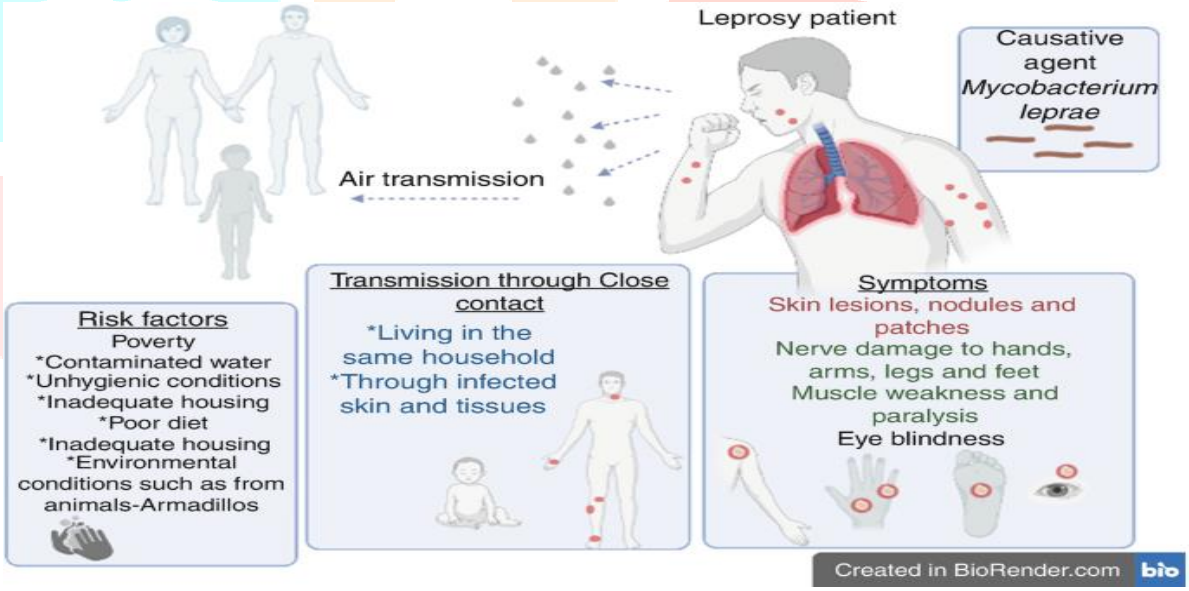

Fig. 1. Mechanism of disease transmission and infection

M. leprae infection induces host cell metabolic reprogramming. Formation of lipid droplets and accumulation of oxidised phospholipids, cholesterol and fatty acids that results in "foamy cells" is a characteristic of infected cells such as that observed in the infected skin lesions, macrophages and Schwann cells. Foamy cell formation is also a characteristic of the related *M. tuberculosis* infection. In addition to up-regulation of lipid metabolism genes in infected host cells, a decrease in mitochondrial ATP formation with a concomitant rise in glycolytic activity has been reported.

1. Disease classification and Pathophysiology

According to the WHO, different forms of leprosy are classified based on the symptoms such as the presence of bacilli in the skin smears and visible lesions. Infection is classified as "paucibacillary" with 1–5 skin patches and no apparent bacteria in skin smears. Individuals with more than five skin patches and visible bacteria in the skin smears are classified as "multibacillary".

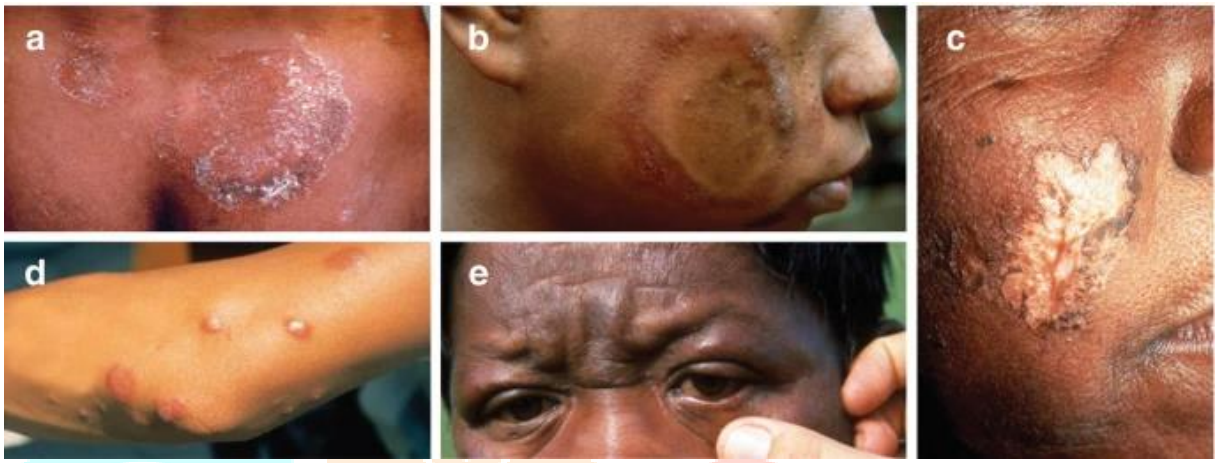

Fig. 1.1 Classification of different forms of leprosy

(**a**) Tuberculoid (TT) leprosy: this shows a leprosy lesion on the lower back that has healed spontaneously and remains only a thin scar with a complete loss of sensation.

(**b**) Borderline tuberculoid (BT) leprosy: this shows an annular lesion on the cheek of a boy with BT.

(**c**) Borderline borderline (BB): this shows a scarred lesion on the right cheek.

(**d**) Borderline lepromatous (BL) leprosy: this shows a few raised and erythematous lesions on the arm of a patient.

(**e**) Lepromatous leprosy: this leprosy patient has marked loss of the eyebrows and eyelashes and thickening of the facial skin.

2. Diagnosis

The diagnosis of leprosy is based on clinical findings along with results of a SSS or skin biopsy. Importantly, a recent review highlighted that failure to consider leprosy in the differential diagnosis for patients is often the greatest impediment to making the diagnosis.Leprosy can be cured with timely diagnosis and MDT treatment. Untreated, it can cause progressive and permanent damage to the skin, nerves, limbs and eyes. According to the WHO, a case of leprosy was identified as hypopigmented or reddish skin lesions, thickening of peripheral nerves and loss of sensation and skin-smear positive for acid fast bacilli. Skin lesions as the dermatological condition are the indicators in 90% of leprosy cases.

Table 1.Differential diagnosis of leprosy subtypes:

Leprosy subtype	Differential diagnosis consideration
Tuberculoid; Indeterminate	Pityriasis alba; post-inflammatory hypopigmentation; vitiligo; segmental vitiligo; tinea versicolor; nummular eczema.
Borderline tuberculoid	Granuloma annulare; interstitial granulomatous dermatitis; disseminated granuloma annulare; sarcoidosis; erythema annulare centrifugum; tinea corporis
Mid-borderline and borderline lepromatous	Necrobiosis lipoidica; granuloma annulare; necrobiotic xanthogranuloma; gummatous syphilis; Sweet's syndrome; lichen planus; ichthyosis vulgaris; parapsoriasis; mycosis fungoides; mastocytosis; morphea

2.1 Types of leprosy diagnosis

1. **Clinical Findings** - Clinical evaluation is the first step in the diagnosis of leprosy and is generally sufficient in most cases.
2. **Slit-Skin Smear Test** -slit skin smear test has a specificity of 100% and sensitivity of 50% [57–59]. A smear from the nasal mucosa, ear lobe, forehead, chin, extensor surfaces of the forearms, knee, cooler parts of the body, and/or skin lesions was the preferred site for sample collection. After collection, Fite staining or modified Ziehl–Neelsen staining was used to examine acid-fast bacilli (AFB) and calculate the Ridley logarithmic scale or bacterial index (BI) score [23, 60].
3. **Skin Biopsy and Histopathologic Examination** - A biopsy was obtained from the leading margins of the most recent and active skin lesions with the entire thickness of the dermis, at least a portion of the subcutaneous fat lesion, and stained according to the Fite-Faraco method.Biopsy specimens may be further analyzed for granuloma fraction, bacterial index of granuloma (BIG) for grading AFB in tissues, and histopathological index. BIG is a method used to detect AFB bacilli in a given tissue volume. The accuracy of skin biopsy examination depends on the appropriate selection of the location for biopsy.
4. **Polymerase Chain Reaction (PCR)** –this is used to support the suspicion of a clinical diagnosis of leprosy. PCR is highly sensitive in patients with a positive BI, however in those with negative BI it can be much more variable. Over the past 30 years, PCR methods have been developed to amplify various gene targets in M. leprae. PCR techniques have been used

to detect possible environmental sources for the dissemination of M. leprae as well as the aerosol route of infection by means of nasal carriage. The summary sensitivity of the PCR test was 75.3% (95% CI 67.9–81.5), and the specificity was 94.5% (91.4–96.5). Quantitative polymerase chain reaction (qPCR) is at least 20 times more sensitive than microscopic detection and has become increasingly important for early diagnosis and difficult-to-diagnose cases.

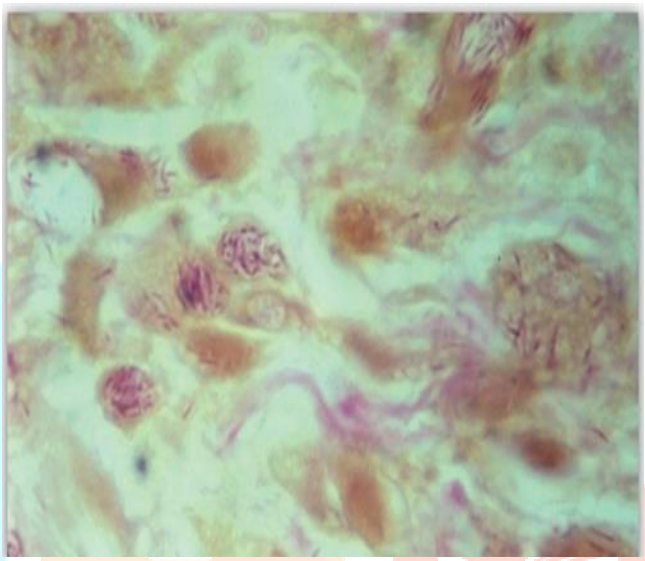

Fig. 2.1. A positive slit skin smear from a patient with multibacillary leprosy. Acid fast bacilli (AFB) are visible. Groups of organisms (globi) are present

1. INTRODUCTION

Synonyms - Nerium oleander.

Biological source – It consists of dried leaves and seeds of oleander of Nerium indicum Linn.

Family- Apocynaceae.

Parts use - leaves and seeds.

Chemical constituents - Polysaccharides, Cardenolides, glycosides and Triter Penoids

Geographical source - It is mainly found in the United States, India, West indies, also in native to the Mediterranean Region including northern Africa, southern Europe.

Use - Heart Conditions, Asthma Epilepsy, Cancer, painful menstrual periods, leprosy, malaria, and ringworm infection.

Fig.1.1. *Cascabela thevetia* flower and plant

Thevetia Peruviana belongs to family Apocynaceae and commonly called as a yellow Olender or Pilli Kaner. *Cascabela thevetia* known as an ornamental plant and planted as large flowering shrub or tree It can grow in wide range of polluted soil and dump site. The use of plant-based natural products in the treatment and prevention of diseases and health enhancement has led to the significant attention of the scientific community and the public nowadays. Medicinal plants play a very important role in the development of alternative drugs without the adverse effects of synthetic drugs. Scientific and reliable reports indicated that about 25% of prescribed medicines worldwide are taken from herbs.

Cascabela thevetia is a multipurpose small evergreen plant widely distributed in the tropical and subtropical regions of India. It belongs to the family Apocynaceae and is commonly known as yellow oleander. It is also locally known as "Kanar or Bitti" in Nepal and India It is mostly found in Asia, Central America, and tropical Africa. It is an evergreen tropical shrub or small flowering tree that bears yellow flowers and deep green or black color fruits. All parts of this plant are highly toxic including leaves, bark, fruits, and flowers. The seeds are used as purgative and also seed oil is used to treat jaundice and infections and as insecticides. The roots of *Cascabela thevetia* are applied to tumors. The roots and leaves of *Cascabela thevetia* are used in vata, pitta, cough, bronchitis, renal, and skin diseases. Due to Phyto - constituents it shows various activities anti-fungal, anti-bacterial, anti-oxidant, anthelmintics, anti-spermatogenic, anti-inflammatory, anti – urolithic and infertility.

The term hypo-pigmentation shows lower pigmentation, which means considerably lower melanin than normal skin. The word depigmentation should not be confused, indicating the complete absence of melanin due to the significant melanocyte loss. The differences between hypo-pigmented disorders and depigmentation disorders are clinically complicated. Hypo-pigmentation is one of the conditions under Vitiligo, where Unani formulations are one of the most effective alternatives to other medicines systems.

After 30 years of extensive clinical trials at NRIUMSD, CCRUM Dept., AYUSH GOI, New Delhi. It's reported that formulations of Unani have performed well in hypo-pigmentation. While the results of the clinical trials were highly satisfactory, well-designed mechanistic studies are limited, and the mechanism of these potential therapies urgently needs to be explored.

Many therapies in the form of photo therapy, corticosteroids, immuno-modulators, Ultraviolet rays, lasers, hypo-pigmentation, and disguise have been used to treat Vitiligo since centuries. Few scientific evidence has shown that *Cascabela thevetia* plants have built in ingredients in Leprosy care.

MATERIAL AND METHOD:

Firstly, the collected leaves, flowers, and latex were cleaned with water and arranged properly. The leaves and flowers were left for shade drying for two weeks. The drying process was accomplished in the laboratory at room temperature with natural airflow. The room was monitored from time to time. After complete drying, the dried leaves and flowers were hand crushed to reduce their size and further used for the extraction process.

- **Comminution of Dried Samples:**
  The completely dried leaves and root bark were comminuted with mortar and pestle and grinder to make small particles.
- **Extraction Procedure:**
  A triple cold maceration extraction procedure was performed with 200 g of leaves and flowers of *C. thevetia* soaked with 1000 mL of methanol and water in different conical flasks. Dried latex was crushed, and 15 grams of latex was also soaked in 200 ml of methanol, and water in different conical flasks with occasional shaking for 72 hours. Each plant sample (Leaf, Latex, and flower) was extracted separately in two different solvents to obtain six different extraction samples. After each extraction liquids were strained and filtered. The methanol and water macerate of *C. thevetia* was concentrated to dry in a rotary evaporator under reduced pressure and controlled temperature (45 °C) for final extracts. Then obtained crude drug was kept in a petri dish, covered with aluminum foil, and preserved in a desiccator for a few days. All the extracts were then refrigerated at 4 °C until use.

Phyto-chemical Screening:

The confirmatory qualitative phyto-chemical screening of plant extracts was performed to identify the main classes of compounds (tannins, saponins, flavonoids, alkaloids, phenols, glycosides, steroids, and terpenoids) present in the extracts following standard protocols.

I. **Detection of alkaloids:** Extract were dissolved individually in dilute hydrochloric acid and solution were clarified by filtration.

a. **Mayer's test:** The extract of *C. thevetia* was treated with Mayer's reagent [potassium mercuric iodide]. Then it forms yellow precipitate which indicates the presence of alkaloids.

b. **Dragondroffs test:** The extract was treated with dragondroff reagent [solution of potassium bismuth iodide]. It forms red precipitate which indicates presence of alkaloids.

II. **Detection of phenols**

a. **Ferric chloride test:** The extract was treated with three drops of ferric chloride solution. It forms bluish green colour which indicates the presence of phenols.

III. **Detection of flavonoids**

a. **Alkaline reagent test:** The extract was treated with few drops of sodium hydroxide solution. Forms yellow intense colour, which becomes colourless on addition of dil.HCl, indicates presence of flavonoids.

b. **Lead acetate test:** the extract was treated with lead acetate solution. It forms yellow colour precipitate indicates presence of flavonoids.

IV. **Detection of terpenoids**

The extract was added to 2 ml of acetic anhydride and conc. Sulphuric acid. it forms blue, green ring which indicates presence of terpenoids.

V. **Detection of saponins**

a. **Forth test:** The extract was diluted with distilled water to 20 ml and this was shaken in graduated cylinder for 15 minutes. It forms 1cm layer of "honey comb" forth indicates the presence of saponins.

VI. **Detection of tannins**

a. **Ferric chloride test:** The extract was dissolved in water. The solution was clarified by filtration; 10% of ferric chloride solution was added to clear filtrate. This was observed for change in colour to bluish black.

b. Lead acetate test: The extract was dissolved in water and 10% of lead acetate solution was added. It forms yellow precipitate which confirms tannins.

VII. Detection of reducing sugars

a. Fehling's test: filtrate was hydrolyzed with dil. HCl, neutralized with alkali and heated with Fehling's A & B solutions. Forms red precipitate which indicates the reducing sugars.

Sr. No.	Secondary metabolites	Aqueous	Chloroform	Methanol
1	Carbohydrates	+++	+++	+++
2	Alkaloids	+++	+	+++
3	Phenols	++	+	+++
4	Flavonoids	-	-	+
5	Terpenoids	+++	++	+++
6	Saponins	+++	+++	+++
7	Tannins	+	++	+++
8	Glycosides	-	-	+
9	Coumarins	++	+	++
10	Acids	+	-	-
11	Proteins	+	-	+
12	Steroids	++	-	+
13	Quinones	+	+	++

+++ strong presence ++ positive + present - absent

Table 2. Phytochemical Screening Test For Cascabela Thevetia Leaf Extract

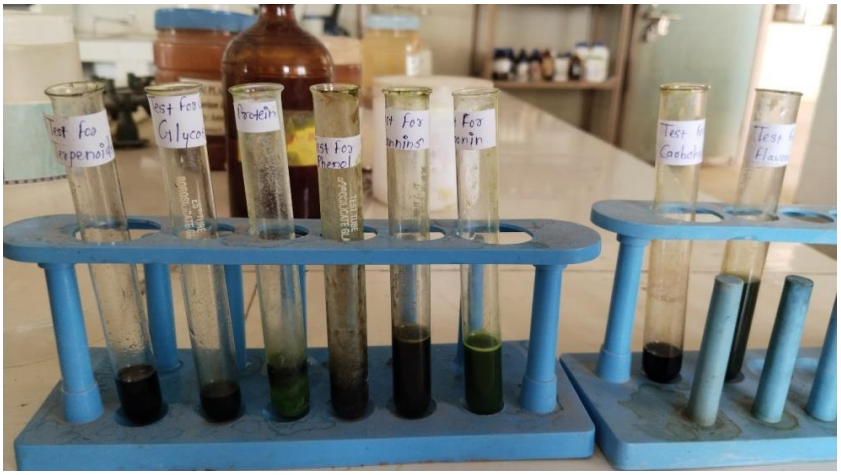

Fig. 2.2. Detection of secondary metabolites by using methanolic extract of *Cascabela thevetia* leaves

2. **Antibacterial activity of the leaf extracts Microorganisms:** Gram positive Streptococcus mutans, Staphylococcus aureus, Staphylococcus epidermidis and Gram-negative bacteria Enterotoxigenic Escherichia coli (ETEC), Enteropathogenic Escherichia coli (EPEC), Klebsiella pneumoniae and Pseudomonas aeruginosa.All bacterial strains from -80 °C were cultureand managed on Muller Hinton Broth at 37 °C.

3. **Assessment of antibacterial activity: -** Pure Dimethyl sulfoxide (DMSO) as solvent was used to prepare the working extracts for assessing the antibacterial activity to the concentration of 50 mg/ml of 0.5 ml. Antibacterial activity of prepared leaf extracts was assessed by disc diffusion method. The cotton swab was used to uniformly spread the overnight bacterial culture into the Muller Hinton Agar (MHA) plate at the surface and permitted to dry. Sterilized paper discs that were prepared by using What man No.1 filter paper (5 mm in diameter) were impregnated with 20 µl of 50 mg/ml leaf extracts and DMSO as a negative control. Kanamycin (30µg/disc) and Erythromycin (15 µg/disc) were applied as the positive control. Antibiotics (Kanamycin and Erythromycin) and impregnated discs with leaf extracts and DMSO were distributed and pressed gently on MHA plates by using sterile forceps and needles. The MHA plate was then incubated at 37°C for 18 hours and after that presence and/or absence of antibacterial activity were observed and measured, if the zone of inhibition was present, by using the transparent scale in diameter (millimeter).

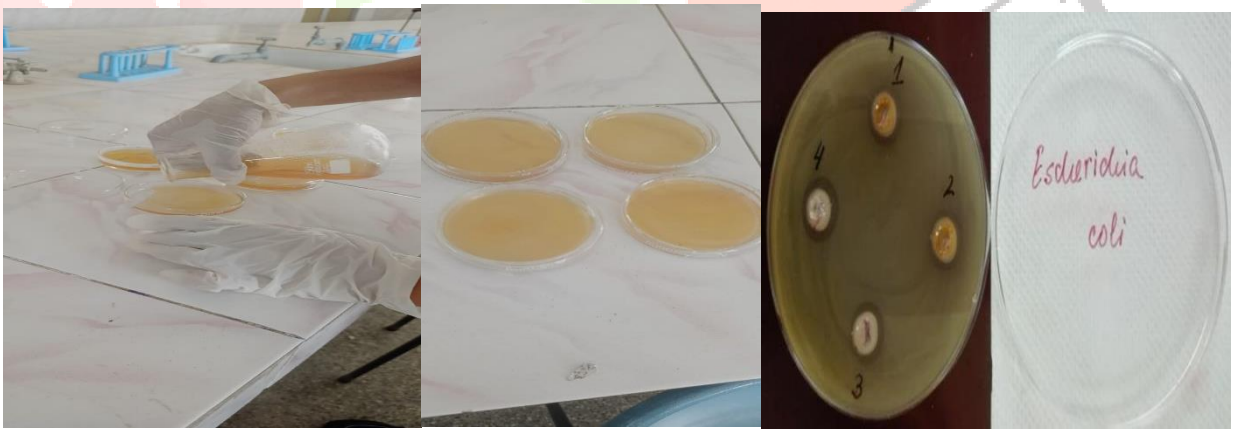

Fig. 2.3. Assessment of anti-bacterial & anti leprotic activity of C. *thevetia* ointment by disc diffusion method.

4. **Method Of Preparation of Ointment**

In this method the constituents of the base were placed together in a melting pan and allowed to melt together at 70°C. After melting, the ingredients were stirred gently maintaining temperature of 70°C for about 5 minutes and then cooled with continuous stirring. Formulation of ointment done by incorporating 10 g of the semisolid methanolic extract of *cascabela thevetia L*. into the

various bases by triturating in a ceramic mortar with a pestle to obtain 100 g of herbal ointments containing 10 % w/w.

5.1 Formula for Anti-leprotic ointment

Sr.no.	Ingredients	Quantity taken	Role of ingredient
1.	Cascabela thevetia extract	10g	Anti-leprotic, Anti-bacterial
2.	Neem extract	5g	Anti-microbial
3.	catechu	3g	Anti-inflammatory
4.	Psoralea corylifolia	3g	Muscle tonifier
5.	Wool fat	5g	Emollient
6.	Hard paraffin	3g	Emollient
7.	Ceto stearyl alcohol	5g	Emulsifying agent
8.	White soft paraffin	8.5g	Ointment base
9.	Propylene glycol	2g	Humectant

5. Evaluation parameter of anti-leprotic ointment

The evaluations were carried out on the ointment by using the following parameters.

1. Color and odour

Color and odour of ointment, examine by visual examination.

2. Loss on drying

1 g of ointment was placed in the Petri dish and heated in the water bath at 105 °C every 30 min until it gets constant weight**.**

3. PH

The pH of ointment was determined by digital pH meter. 1 g of ointment was dissolved in 50 ml of distilled water and the pH was measured.

4. Diffusion study

The diffusion study was carried out by preparing agar nutrient medium of any concentration. It was poured into Petri dish. A hole bored at the center and ointment was placed in it. The time taken for the ointment to get diffused was noted.

5. Viscosity

The viscosity of the ointment can be determined by using brook field viscometer from the laboratory.

6. Spradability

The formulated anti-leprotic ointment's spreadability was determined by glass slide method.

7. Stability study

The stability studies are carried out for the prepared ointment at temperature of 37oC for 2 months**.**

Sr.no.	Physico-chemical parameter	Observation
1	Color	Dark reddish brown
2	Loss on drying	0.2%
3	PH	6.2
4	Viscosity	1176 cps.
5	Stability	Stable at PH 6.2
6	Odour	Characteristic
7	spreadability	5-6cm
8	Diffusion of ointment	2.2 cm in 1 minute

Fig.2.4 F1,F2,F3 formulation of anti-leprotic ointment by using *cascabela thevetia* extract

RESULT AND DISCUSSION

The preliminary in vitro antimicrobial activity of the Methanolic extract of *Cascabela thevetia L.* presented showed excellent activity against Staphylococcus aureus. The in vitro antimicrobial activity of the Ethanolic extract *Cascabela thevetia* of based herbal ointments. In various case history show that most of the infections are caused by thegram-positive bacteria such as Staphylococcus aureus and Streptococcus pyogenes. Less common cause by the gram-negative bacteria such as Escherichia coli.This observation indicates that the activity due to the presence of large varieties of phytoconstituents present in the extract. Hence, the Both methanolic and ethanolic leaf extracts of *Cascabela Thevetia* generated almost the same mean zone of inhibitory area ranging from 7 to 10 diameter in millimeters (mm). Observed antibacterial activities of the ointment are due to the presence of active constituents of the extract and the activity also possesses as ointment.

CONCLUSION

From the current investigation, it can be concluded that both methanolic and ethanolic *Cascabela thevetia* leaf extracts, specifically ethanolic extracts showed potent DPPH radical scavenging activity, indicating substantial antioxidant activity.Combined methanolic leaf extracts of C. *thevetia* and Azadirachata indica were also revealed to have outstanding antioxidant activity. Further investigation by utilizing other methods of assessing antioxidant activity need to be carried out to confirm the present outcomes. This study indicates that Cascabela Thevetia could be a potent source of biopharmaceutical agents.

From this study the prepared ointment shows the anti- leprotic activity against Acid fast bacilli. This formulated ointment is active against gram positive bacteria.

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