INTRODUCTION

_Azadirachta indica_ offers a wide spectrum of biological activity.1,2 _A. indica_ is now widely used in modern medicine, having been used extensively in Ayurveda, Unani, and Homoeopathic treatment.

_A. indica_ contains wide range of over 140 compounds being separated from various parts which are chemically and physiologically active substances.3 Despite the fact that the plant’s antifungal activity is proportional to the concentration of its secondary metabolites, there is a lack of a standardized and validated formulation that is stable, safe, and effective.4,5

Essential oils (EOs) are produced by aromatic and therapeutic flora. These essential oils are extensively used in the culinary, fragrance, and cosmetic sectors. Antifungal action of essential oils from Indian plants against pathogenic _Aspergillus fumigatus_ and _A. niger_ have been reported, the antifungal spectrum of the oils of _Cymbopogon martini_, _Cinnamomum zylenicum_, _Eucalyptus globulus_, _Mentha spicata_, _A. indica_, _Withania somnifera_, _Eugenia caryophyllata_ and _Zingiber officinale_ etc was focused.6

A significant source of essential oils, including those with bacteriostatic, fungistatic, and anti-inflammatory properties, can be also found in the Myrtaceae family.7 The antimicrobial effect of _E. citriodora_ essential oil toward _Candida albicans_, _Escherichia coli_, and _Mycobacterium smegmatis_ was evaluated using agar disc diffusion and broth dilution method. It was shown that the organisms most vulnerable to _E. citriodora’s_ essential oil were _Trichophyton rubrum_, _C. albicans_, _Cryptococcus neoformans_ and _Histoplasma capsulatum_.8,9

There are number of known negative effects of synthetic oils, which raises questions about the use of these oils as antimicrobials for food preservation.10 The hydrophobicity

ABSTRACT

Objective: The study developed an optimized dosage form by quality by design (QbD) approach containing _Azadirachta indica_ (Neem) leaf extract and a combination of selected herbal oils viz. _A. indica_ oil, _Eucalyptus citriodora_ oil and _Cymbopogon martini_ oil and assessed for its antifungal efficacy, stability and dermal safety.

Methods: Ointment was prepared using the fusion method from an extract of neem leaves with a combination of herbal oils viz. _A. indica_, _E. citriodora_, _C. martini_, bees wax and soft-paraffin. Utilizing the Box-Behnken Design (BBD), the variables such as the percentage of soft paraffin, beeswax and melting temperature were optimized in relation to the output variables such as viscosity, spreadability, and finally the antifungal efficacy, which was further quantified. Wistar albino rats were used (n = 5/test, positive and negative control) to evaluate the acute dermal toxicity test of the formulated ointment. Stability studies were assessed at 25 ± 2℃/ 60 ± 5% RH and 40 ± 2℃/ 75 ± 5% RH.

Results: Melting point and percentage of beeswax significantly affect viscosity and spreadability. Optimal viscosity was obtained at 5.4% beeswax and 89.6% soft-paraffin when melted at 58℃. No dermal toxicity was observed by the ointment when comparable to petroleum jelly, both differed significantly with negative control. The absence of new spots on chromatograms, a prominent zone on agar plates, and negligible changes in spreadability (p = 0.112) all suggested physical stability, chemical stability, and antifungal efficacy, respectively.

Conclusion: Neem leaf extract and a blend of selected herbal oils; _A. indica_, _E. citrodora_, and _C. martini_, when combined to formulate ointment, proved reliable and stable dosage form with significant anti-fungal efficacy. The formulated ointment might be helpful tool for dermatophytes management.

Keywords: Herbal oils, Neem leaves extract, Box-behnken design, Dermatophytes, Anti-fungal activity.

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There are number of known negative effects of synthetic oils, which raises questions about the use of these oils as antimicrobials for food preservation.10 The hydrophobicity

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of the essential oil is one of the most crucial characteristics that make it possible for them to interact with the lipids in the cell membrane of microbes, it become more permeable which results in the disruption in cell structure.

The microbial cell eventually perishes as a result of this anomaly because fungal and bacterial cells lose their vital chemicals and ions. The use of unstandardized herbal products and crude extracts from medicinal plants has a number of health hazards, including the possibility of toxicities, unanticipated side effects, and drug-herb interactions. Traditional herbal medicines are projected to meet 81% of the health needs of individuals living in rural areas, principally in Asia and Africa. Consequently, it is imperative that their safety, effectiveness, and quality be scientifically validated. Standardized herbal formulations can help with this. This study includes the formulation and evaluation of anti-fungal efficacy and stability of an ointment containing a combination of selected herbal oils with neem leaves extract.

**MATERIALS AND METHODS**

Neem plant leaves were collected from the vicinity of Hanuman Gadh Wardha, Maharashtra and Voucher specimens were deposited in the Department of Botany, Bajaj Science College, RTMNU University and were identified. Mature leaves were dried in shadow for a maximum 14 days. In 25 grams of freshly collected, cleaned, shade-dried and powdered mature leaves were treated with 100 mL of ethanol and was allowed to soak overnight with proper tight caps. It was centrifuged for 20 minutes at 5000 rpm, the suspension was then filtered by using Whatman filter paper no. 1. The supernatant fluid was dried out in sterile glass petri dishes under germicidal tube light. Completely dried powder was collected by scraping and stored at -4°C and the weight of extract was determined using a Denver Instrument SI-234 digital balance. Neem leaf extract was further studied for phytochemicals content.

Selected herbal oils such as neem (*A. indica*) oil, *E. citriodora* oil and *C. martini* oil were purchased from local herbal oil vendor.

Three strains of dermatophytes such as *T. rubrum*, *T. mentagrophytes*, *M. gypseum*, procured from MTCC Chandigarh, India. The freeze-dried fungi cultures were maintained on suitable culture media as suggested by MTCC Chandigarh, India at 27°C for 7 to 14 days. The preparation of the fungal inoculate was done with fresh 24-hour broth cultures as previously described. Potato dextrose agar (Hi-Media), Saborauds dextrose agar (Hi-Media), FD035-5VL CC-Supplement (Hi-Media), DMSO (Merck) beeswax and soft-paraffin (Loba) were used.

**Preformulation Studies**

Standard techniques were used to determine the extract-excipient compatibility, partition coefficients, melting temperature, and apparent aqueous solubility. It was found that 5 mg of extract was soluble in 10 mL of distilled water. Eq. (1) was used to calculate the extract’s partition into butanol using the shaking flask technique.

\[
p = \frac{\text{Concentration of stock solution} - \text{concentration in aqueous phase}}{\text{Concentration in the aqueous phase}} \quad \ldots \ (1)
\]

Using a melting point device, the extract’s melting point was ascertained by the capillary technique. The extract was melted together with equivalent quantities (20 mg) of either soft paraffin or beeswax in the medication excipient compatibility testing. It was placed in glass vials and was allowed to stand for 14 days. The combinations were spotted on 60-F254 thin-layer chromatography (TLC) silica gel plates (Merck).

**Table 1: Formulation composition given by DoE software**

<table>
<thead>
<tr>
<th>Experimental run</th>
<th>Beeswax concentration (%)</th>
<th>Soft paraffin concentration (%)</th>
<th>Soft-paraffin melting temperature (°C)</th>
<th>Viscosity (cSt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>15</td>
<td>90</td>
<td>55</td>
<td>8.68</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>80</td>
<td>58</td>
<td>8.42</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>80</td>
<td>52</td>
<td>8.36</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>85</td>
<td>52</td>
<td>8.38</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>90</td>
<td>52</td>
<td>8.64</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>80</td>
<td>55</td>
<td>8.59</td>
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<tr>
<td>7</td>
<td>15</td>
<td>85</td>
<td>52</td>
<td>8.62</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>85</td>
<td>55</td>
<td>8.38</td>
</tr>
<tr>
<td>9</td>
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<td>80</td>
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<td>10</td>
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<td>90</td>
<td>58</td>
<td>8.49</td>
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<td>85</td>
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<td>8.53</td>
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<td>85</td>
<td>55</td>
<td>8.41</td>
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<tr>
<td>13</td>
<td>10</td>
<td>85</td>
<td>55</td>
<td>8.48</td>
</tr>
<tr>
<td>14</td>
<td>10</td>
<td>85</td>
<td>55</td>
<td>8.39</td>
</tr>
<tr>
<td>15</td>
<td>15</td>
<td>85</td>
<td>58</td>
<td>8.65</td>
</tr>
<tr>
<td>16</td>
<td>10</td>
<td>85</td>
<td>55</td>
<td>8.59</td>
</tr>
<tr>
<td>17</td>
<td>5</td>
<td>90</td>
<td>55</td>
<td>8.44</td>
</tr>
</tbody>
</table>
Germany). After 14 days, and the plates were placed in ethyl acetate in troughs. Chromatograms developed were examined in a UV chamber (CAMAG TLC Visualizer, HPTLC Software Vision CATS, Switzerland). Each experiment was carried out thrice.\(^{14-16}\)

**Formulation and optimization**

The ointment comprising 5% concentration of extract with an essential oil blend, beeswax, and soft paraffin was made using the fusion process. A crucible containing weighed amounts of beeswax was filled with melted extract and stirred to ensure uniform mixing. The temperature was subsequently lowered to 55 ± 3°C, and the melted beeswax extract mixture was then mixed with a chosen oil blend and soft paraffin by geometric dilution. The heat source was turned off and the ointment was mixed well and stored in a suitable container. Shear stress and temperature extremes have a tendency to change the functional properties of oleaginous bases; therefore, it is necessary to optimize variables that may have an impact on the rheology (Table 1).

**Viscosity quantification**

The viscosity of an ointment was evaluated using a rotational viscometer (Viscolead Pro, Fungi lab). The force (torque) acting on a rotor was measured when it rotated at a constant angular velocity or rotational speed.\(^{17,18}\)

**Acute dermal toxicity test**

The approved protocol was used to test for acute dermal toxicity of the formulated ointment. Fifteen healthy female wistar albino rats weighing between 200 and 250 grams were split into three groups at random, with five rats in each group. A depilatory tool was used to shave 10% of the body’s surface. The test and positive control groups received a dosage of 200 mg/kg of the optimized ointment or the placebo ointment base twenty-four hours after shaving. A third group (known as the negative control) did not get any treatment. Daily observations were made of the shaved region, and weight measurements were taken on days 0, 7, and 14. After the removal of the ointment, the experimental site was observed after 24, 48, and 72 hours for erythema and edema. The observations were noted as per the Draize criterion.\(^{19}\)

**Stability studies**

Baseline spreadability, TLC, and microbiological examination were performed. The optimized ointment was kept for three months in stability chambers at 25 ± 2°C/60 ± 5%relative humidity and 40 ± 2°C/75 ± 5% relative humidity.

**Physical tests**

Spreadability was assessed with a lab-assembled device [Equation 2].

\[
S = \frac{M \times L}{T} \quad \text{[2]}
\]

S-spreadability, L-length (10 cm), M-mass (12 g), T-time (s)

**Chemical tests**

TLC plate were marked with the spots of extract-oils combination, Paraffin, beeswax, extract-oil blend with paraffin and extract-oil blend with beeswax marked as Ex, P, B, ExP and E x B, respectively. The plates were marked with solutions of each component and then put in ethyl acetate. The generated chromatograms were then examined in a CAMAG UV chamber.

**Microbiological evaluation**

A 10% (w/v) dilution of the ointment in isopropyl myristate was made. Subsequently, 100 mL of 3% (w/v) soybean casein broth was mixed with 10 mL aliquots, and the mixture was incubated for 18 hours. In order to identify *S. aureus* and *P. aeruginosa*, respectively, the test samples were inoculated on blood agar (BA), mannitol salt agar (MS), and Mueller-Hinton agar (MHA). The test samples were also inoculated on Saborauds-dextrose agar (SDA) and soybean-casein digest agar (SCDA) for the enumeration of growth of fungus and bacteria. Hi Media Analytical grade agar and broth were used.\(^{20-22}\)

**RESULTS AND DISCUSSION**

**Preformulation Studies**

The TFC calculated during solubility testing is displayed in Table 2. According to the findings, the extract was barely soluble in an acidic medium and soluble at all in an alkaline one. The outcomes reveal the variety of phytochemicals that are contained in the extract. These variations in aqueous solubility over the pH spectrum are possibly caused by the numerous substituent groups connected to the primary flavonoid structure. According to the findings, the bulk of these substituent groups are probably acidic, which explains why they solubilize more readily in an alkaline solvent.

The partition coefficients were calculated as follows: \(P\): hexane water is 0.0306, \(P\): ethanol-water is 0.582, and the mixed melting point was 131°C. Flavonoids’ relative affinities were greater for ethanol than n-hexane, indicating their

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**Table 2: TFC in different media**

<table>
<thead>
<tr>
<th>Media</th>
<th>TFC (mgQE/g)</th>
<th>Solvent/solute (mL/gm)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acidic</td>
<td>0.989 ± 0.009</td>
<td>1000</td>
<td>Very slightly soluble</td>
</tr>
<tr>
<td>Alkaline</td>
<td>12.5 ± 0.029</td>
<td>90.2</td>
<td>Sparingly soluble</td>
</tr>
<tr>
<td>Neutral</td>
<td>4.39 ± 0.318</td>
<td>225.9</td>
<td>Slightly soluble</td>
</tr>
</tbody>
</table>

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**Figure 1: TLC chromatogram for drug-excipient compatibility studies**
polar nature. Additionally, there is a chance of ionization in the aqueous phase, less probability of affinity in lipid substances. The melting point could support the claim that polar compounds were present.

Figure 1 displays the results of the TLC extraction excipient compatibility analysis. Physical compatibility is implied by the additive spots that have been observed with the extract-beeswax and extract-soft paraffin migrations with either extract or beeswax or extract and soft paraffin migrations. Furthermore, it appears an extract and the excipients were physically mixed and retained their physical profiles two weeks following the interaction because there were no new spots found along the migrations of the mixtures. The components were not reacted to form new substance, as the chromatogram demonstrates. These findings suggest that the compounds retained their integrity and were therefore deemed compatible, allowing for the successful formulation of an ointment.

**Formulation and optimization**

The formulated 17 batches exhibited significant characteristics. Results for the viscosity are shown in Table 1. The recorded viscosity was between 8.36 to 8.68 cSt,

\[
\text{Viscosity} = +8.50 +0.0988X_1 + 0.0588 X_2 + 0.0100X_3 \quad \text{------[3]}
\]

**Formula optimization**

2D and 3D plot for viscosity was given in Figure 2 (a) and (b). A formula of 5% extract and oil combination, 5.4% beeswax, and 89.6% soft paraffin melted at 58°C was suggested by Design Expert R. The optimized formula was produced in three batches, and the viscosities of each batch were measured. Table 3 displays the observed vs. predicted results. Less than 5% of the responses’ percentage P.E. suggested that the models could accurately predict how the variables and responses would interact.

**Antimicrobial Study of Topical Formulation**

Table 4 shows antifungal effect of topical ointments on three different dermatophytes containing optimized dose of Leaves extract and essential oil combination of defined concentrations. From the MIC data, lipophilic ointment was prepared as per DoE approach and the batch having with the highest antifungal activity was evaluated with respect to test fungi with inhibition zone diameter ranging from 14.7.0 ± 0.73 to 18.4 ± 1.1 (Figure 3).

**Acute dermal toxicity test**

The lack of erythema or edema in the test group of albino rats throughout the testing period suggests that the ointment may be safe. These findings are consistent with a study on the safety of ointment, which found that combining extracts and oils does not cause toxicity. Given that the optimized ointment did not cause toxicity during the trial period, it can therefore be regarded as safe for topical administration.

**Stability Studies**

**Physical tests**

Figure 4 displays the results of the spreadability computations. The means did not differ significantly (P = 0.0586) even though

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### Table 1: Recorded viscosity

<table>
<thead>
<tr>
<th>Variable</th>
<th>Predicted</th>
<th>Optimized</th>
<th>%PE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viscosity (cSt)</td>
<td>8.64</td>
<td>8.68 (0.01)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

### Table 4: Antifungal effect of formulation

<table>
<thead>
<tr>
<th>Sr no</th>
<th>Name of Test organism</th>
<th>Zone of Inhibition(mm)</th>
<th>Mean Zone of Inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Trichophyton mentagrophytes</em></td>
<td>15 ± 0.7</td>
<td>14.8 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td><em>Trichophyton rubrum</em></td>
<td>18 ± 1.1</td>
<td>19.0 ± 1.8</td>
</tr>
<tr>
<td>3</td>
<td><em>MicrosporumGypseum</em></td>
<td>17 ± 1.2</td>
<td>16.8 ± 0.4</td>
</tr>
</tbody>
</table>

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batches stored at 40°C/75%RH had a higher spreadability than those stored at 25°C/60%RH. The ointment’s decreased viscosity was a result of the higher storage temperature and may be the cause of the batches comparatively having better spreadability when kept at 40°C/75% RH. The absence of notable variations suggests that the ointment is stable in terms of physical state and can be kept at room temperature (25 ± 2°C).

Chemical tests
The chromatograms from the baseline and third months are important. Its chemical stability is suggested by the spot migration similarities that were seen on both chromatograms.

Microbial evaluation
The test demonstrated that the ointment was not susceptible to microbial proliferation because no colonies formed on any of the agar plates during the test period, may have been influenced by the formulation’s anhydrous state. Oleaginous are inert and anhydrous, they have high stability. Despite of their oily character, they are a favorite delivery system for herbal extracts. The stability testing included in the study clearly described that the prepared ointment is stable during the course of the investigation. Results suggest the optimized ointment was stable at room temperature (25°C/65%RH).

CONCLUSION
The anti-fungal action of a standardized ointment including an extract from Neem leaves and a blend of selected herbal oils, such as A. indica oil, E. citriodora oil, and C. martini oil, showed consistent qualities. The amount of beeswax and the melting temperature of the soft paraffin was shown to have a substantial effect on viscosity, which in turn affected spreadability and thus antifungal activity. The ointment does not appear to cause toxicity, according to the results of the acute dermal toxicity test. The formulation appears to have adequate stability based on tests conducted on its microbial, chemical, and physical stability. The formulation showed satisfactory stability and antifungal activity as comparable to the positive control. The prepared ointment may be an effective therapeutic option for opportunistic fungal infections that are raised after the COVID-19 era.

ETHICAL APPROVAL
Ethical approval was granted by the Datta Meghe Institute of Medical Sciences, Sawangi (M), Dist. Wardha, MH, India 442001 (Now DMIHER), Central Preclinical Research Facility, Institute Animal Ethics Committee Protocol ID Number DMIMS/IAEC/2021-22/54.

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