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Research Article

Topical Gel Containing *Wrightia tinctoria* Extract: Optimization by Central Composite Design, Characterization and Bio-activity Assessment

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ABSTRACT

The study aimed to develop an herbal anti-*Candida* topical gel using ethanolic extract of leaves from the arid zone plant *Wrightia tinctoria*, also known as Indrajau. This plant from the Apocynaceae family has been investigated for a wide range of medicinal uses, including pain relief, antifungal effects, inflammation reduction, parasite expulsion, ulcer treatment, dysentery remedy, diabetes management, cancer therapy, fever reduction, and wound healing. These effects are due to the presence of multiple bioactive compounds found in different parts of the plant. This research focused on formulating a pharmaceutically stable herbal gel containing *W. tinctoria* extract by applying a central composite design for optimization and evaluating its phytoconstituents, physical and chemical properties, and various quality control parameters including appearance, pH, spreadability, consistency, homogeneity, viscosity, drug release, stability, skin irritation study, and *ex-vivo* anti-*Candida* activity in rats. The herbal gel formulation was found to have a pH value of 6.3 ± 0.1 , indicating that it is unlikely to cause skin irritation. The prepared herbal gel formulation's spreadability value was discovered to be 22.17 ± 1.5 gm.cm/sec, signifying good spreadability. The extrudability of 89.53%, demonstrated excellent squeezability. During dermal irritation studies, no signs of redness or swelling were observed in the treated animals, indicating the formulation's safety. The major skin irritation index (irritability score) was zero, confirming its suitability for topical application. HPLC analysis confirmed the presence of indirubin, a bis-indole alkaloid, as a major active chemical constituent. The prepared herbal formulation also exhibited acceptable stability as per ICH guidelines. Furthermore, when the gel formulations were applied topically to rats infected with *Candida albicans*, notable wound-healing activities were noted. These tests collectively ensured that the formulated gel was stable, safe, effective, and suitable for use.

INTRODUCTION

Most often, humans coexist peacefully with the microorganisms in their surroundings, and only when pathogen levels become exceptionally high may an infection arise.^[1] Over the past twenty years, there has been a notable rise in mycosis, which can vary from systemic infections that are superficial to potentially fatal, particularly impacting immunocompromised individuals.^[2] Fungal infections, affecting over 1 billion people, are inherently contagious and carry a high mortality

rate, estimated at around 1.5 million deaths.^[3-5] These infections are increasingly becoming a concern within hospital settings, emerging as significant challenges for healthcare institutions.^[6]

The term "candidiasis" refers to infections brought on by fungi in the *Candida* genus, manifesting in mucosal (such as oral, esophageal, and vaginal candidiasis), cutaneous, or systemic forms (including systemic candidiasis and bloodstream infections). The global burden of candidiasis is staggering, with over 1 billion cases of

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superficial cutaneous and mucosal candidiasis annually, along with an estimated 130 million cases, respectively. Approximately 750,000 people are afflicted with invasive candidiasis annually, and mortality rates from 40 to 55% are reported. Those with immunodeficiency, exposure to broad-spectrum antibiotics, advanced age, cancer chemotherapy, organ transplantation, and extended hospitalizations in intensive care units are the most common predisposing factors for this illness. Across numerous nations, *C. albicans*, a normal human microflora component, is accountable for over 90% and over 40% of occurrences of mucosal and invasive candidiasis, respectively.^[7] *Candida* infections notably affect skin tissues, leading to inflammation and itching, a condition known as cutaneous candidiasis. Beyond the skin, *Candida* can also cause infections in nails, the mouth, and vaginal tissues. Traditionally, synthetic antifungal agents like nystatin, miconazole, fluconazole, itraconazole, ketoconazole, clotrimazole, 5-flucytosine, amphotericin B, and echinocandins have been employed to treat fungal infections.^[9,10] However, these medications often come with side effects such as diarrhea, elevated serum alkaline phosphatase levels, leukopenia, thrombocytopenia, transient but occasionally lethal pancytopenia, and increased serum transaminase levels. Prolonged use of antifungal agents has also contributed to the emergence of drug-resistant microbes.^[11]

Antifungal resistance poses a significant and escalating threat.^[12] There is an urgent need for safe and effective substitutes for antifungal medications due to the recent growth in resistance to these drugs and their side effects. These alternatives can be used to treat or prevent *Candida* infections.^[13]

In light of these challenges, herbal drugs present a promising avenue for potential treatments.^[14,15] Indian medicinal plants are known for their diverse pharmacological activities, attributed to the presence of various phytochemicals. Extensive literature surveys have identified numerous plants with antifungal properties across the plant kingdom.^[17] Drugs derived from plant sources continue to be major remedies in many countries, particularly in Africa and Asia, due to their widespread availability and relatively fewer side effects compared to synthetic drugs.^[18] However, one of the primary challenges in utilizing herbal drugs lies in the development of appropriate formulations. Many pharmacological dosage forms, such as liquid solutions, sprays, solid powders, gels, creams, and ointments, are available for treating skin-related conditions. Among them, gels are distinguished by being a network of cross-linked polymers that have swelled in a liquid medium. The interaction between the solid-state polymer and the liquid component has a significant impact on the properties of gels. In comparison to other topical preparations and oral administration techniques, gel formulations have a number of benefits for topical drug

delivery, such as simple application, extended contact time, and low side effects. This formulation provides high penetration efficiency, is less greasy, and is readily extracted from the skin. Moreover, gel formulations offer superior application properties and stability compared to ointments and creams. Therefore, they serve as a suitable delivery system for herbal drugs, particularly in treating skin-related disorders.^[19]

Wrightia tinctoria, a remarkable deciduous tree, features a smooth bark and can reach heights of up to 10 meters. Its leaves, arranged oppositely, are lanceolate and typically measure between 8 to 14 cm in length.^[20] This species is widely distributed across various habitats, including roadsides, yards, gardens, parks, and human settlements, thriving in the warmer regions of India.

The leaves of *W. tinctoria* are rich in diverse phytochemical components, including alkaloids, glycosides, flavonoids, tannins, terpenoids and saponins. These compounds contribute to the plant's extensive pharmacological profile, which encompasses analgesic, antifungal, antipyretic, antidiabetic, anticancer, anthelmintic, antiulcer, antidiysenteric, and wound healing activities. Studies have demonstrated the efficacy of extracts derived from plants from similar geographical area in the management of skin conditions and fungi.^[21,22] This emphasizes the plant's potential as a useful source of medicinal compounds for a range of related illnesses.

However, there is a dearth of substantial scientific literature substantiating the medicinal benefits of *W. tinctoria* in treating skin disorders. Moreover, systematic investigations aimed at developing an effective delivery method for *W. tinctoria* to address *Candida*-induced skin infections have been lacking. Consequently, the goal of the current investigation is to assess anti-*Candida* properties of *W. tinctoria* extracts through animal experiments. Acknowledging that the availability of an appropriate formulation is a crucial determinant of practical utility, we have also formulated and evaluated an *in-vivo* topical gel containing *W. tinctoria* extract for its potential anti-*Candida* activity.

In pharmaceutical formulation development, the implementation of the formulation by design approach for the optimization of the relationship between dependent and independent variables by using statistical tools, optimizing the process by using mathematical equations and predicting the response by using the point prediction method in response surface methodology has been widely practiced. Quality by Design practices by implementing statistical optimization designs are widely used for diverse purposes like formulation development^[23-30], total quality management,^[31-34] yield enhancement of phytoconstituents,^[35-36] etc., in the author's lab. The goal of the current study is to optimize the independent variables that were chosen, such as the quantity of PEG



400 and Carbopol 934 used to make the gel formulation and to ascertain the impact of the independent variables on the dependent variables, such as the gel's viscosity and spreadability. The prepared formulation may offer a new vista in finding suitable alternatives to existing therapies for various benefits.

MATERIALS AND METHODS

Materials

Chemicals

Carbopol 934, polyethylene glycol 400, propylparaben, methylparaben and triethanolamine were obtained from Lab Fine, Mumbai.

Microbial strain

The investigation utilized *Candida albicans* as the fungal strain, specifically the reference strain MTCC 9215, obtained from the MTCC, Chandigarh, India.

Plant material

The Botanical Survey of India, Jodhpur, verified the *W. tinctoria* leaves, which were taken in June 2022 at the Central Arid Zone Research Institute campus in Jodhpur, Rajasthan.

Animals

Under registration number PBRI/IAEC/16-01-23/009, the Institutional Animal Ethics Committee accepted the experimental protocol.

Wistar rats (150 ± 20 g) were housed together in groups of two (six rats per group) within clean polypropylene enclosures, with males and females kept separated and maintained under standard environmental conditions. A one-week acclimatization period to the laboratory environment was provided to the animals. The rats were deprived of food for 3 to 4 hours prior to treatment but had unrestricted access to drinking water and standard pelleted food.

Extraction

After carefully cleaning them in distilled water, the leaves of *W. tinctoria* were dried under shade at room temperature [27–42°C]. Then, using an electric blender, blend into a fine powder and put in a container for storage. The extract was prepared via soxhlet extraction at a temperature range of 60 to 80°C. The powdered drug was subjected to extraction with ethanol for a duration of 72 hours. After extraction, the solvent was vacuum-concentrated, and any leftover solvent was removed from the extract by drying it at 45°C.^[37,38] To increase extraction efficiency, the single-variable-at-a-time method was used to pre-optimize the drug-to-solvent ratio and extraction time.

Preliminary phytochemical analysis of ethanolic extract

Based on coloring and precipitation reactions, a qualitative

screening of the leaf extract was carried out to identify the presence of key chemical ingredients.^[38] The investigated constituents included alkaloids, glycosides, tannins, reducing sugars, phlobatannins, flavonoids, saponins, terpenoids, anthraquinone, and cardiac glycosides.

HPLC analysis of leaf extract

A properly diluted extract liquid in ethanol was subsequently loaded onto an Agilent TC-C18 HPLC column (5.0 µm, 4.6 × 250 mm). A mobile phase of methanol and water (75:25, v/v) at a flow rate of 1-mL/min was used to optimize the HPLC system. The column temperature was kept at 25°C, and 290 nm was the wavelength at which peaks were identified. In particular, the bis-indole alkaloid indirubin was detected at 290 nm in contrast to the standard.^[42] The procedure was previously verified in accordance with ICH Q2R1 standards.

Determination of anti-Candida activity of prepared extracts

The antifungal activity of the produced extract against *C. albicans* was evaluated. The fungal strain was incubated for seven days at 28°C after being subcultured on potato dextrose agar (PDA) medium. Using the disk diffusion method, the extract's antifungal activity was assessed. Fluconazole (5 µg/mL) and various doses of the extract (25, 50, and 100 µg/mL) were utilized to assess the extract's anti-*Candida* properties. After the samples were placed on the agar plates, they were left to incubate for three days at room temperature. The effectiveness of the extract against *C. albicans* was assessed by measuring the inhibition zones in millimeters.^[38]

Optimization Studies for Herbal Gel Preparation

Central composite design based experiments

To maximize the anti-*Candida* action of the herbal gel containing *W. tinctoria* leaf extract, the formulation by design method was used. The response surface methodology's central composite design was employed to optimize the gel's viscosity and spreadability. The amount of Carbopol 934 and PEG 400, were chosen as independent factors that had an impact on the dependent variables, which were the gel's viscosity and spreadability. The selected critical variables were optimized by using Design Expert software. The CCD model generated 13 experimental runs for optimization of the prepared gel containing *W. tinctoria* leaves extract. In total of 13 experimental runs, five were central experimental runs and four were the axial point runs. The selected model given four alpha values runs going beyond the lower and upper limit of the selected factors.

The quadratic polynomial equation and the factor coefficient values, which are determined by ANOVA and computed in equation 1, show how the independent variables affect the dependent variables.

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_1\beta_2X_1X_2 + \beta_1X_1^2 + \beta_2X_2^2 \quad (\text{Eq...1})$$

Whereas the Y represents the response variable and the β_0 represents the intercept in the equation and X1 and X2 are the individual impacts of both selected variables where X1 is the amount of carbopol 934 and the X2 is the amount of the PEG 400 and X1X2 is the combined effect of both variables on the dependent variable, i.e., viscosity of gel and its spreadability. In the quadratic polynomial equation, the impact of the individual square of the independent variables on the selected dependent variables was also studied.

Preparation of herbal topical gel formulation

The formulated extract was chosen for the preparation of the plant extract based topical gel preparation. To begin, 20 mL of filtered water was mixed with a selected quantity, as per the experimental recipe of the gelling agent, Carbopol 934, and left to swell for half an hour before stirring to formulate a gel. One gram of the extract was placed in a different container together with the appropriate amount of polyethylene glycol 400, following ten minutes of sonication, per CCD guidelines. Next, 0.5 mL of propyl 4-hydroxy benzoate and 0.05 mL of methyl hydroxyl benzoate were added to 5 mL of distilled water. Finally, the thoroughly combined materials were continuously stirred into the Carbopol 934 gel. Subsequently, 1.5 mL of triethanolamine was added slowly, drop by drop, to adjust the pH to 6.8 to 7 and to attain the desired gel consistency. For optimization purposes, viscosity and spreadability were determined of all formulations as outlined below.

Model validation

The herbal topical gel was prepared under specific conditions suggested by the CCD model of response surface methodology where the amount of Carbopol 934 (1.5 gm) and the amount of PEG 400 (5 mL) while remaining other excipients are constant. The experimental values of the selected responses, i.e., viscosity of the gel and its spreadability, were validated by comparing with the predicated values given by the mathematical model. To better comprehend the relationship between particular variables and answers, contour plots were created. The point prediction function was employed to ascertain the ideal concentrations of particular variables. The prepared optimized formulation was further analyzed for the other quality parameters of the herbal topical gel and was also discussed in the subsequent section of the research paper.

Evaluation of Formulated Herbal Topical Gel

Physical appearance

The formulated gel was visually examined for its color and the presence of any gritty particles.³⁹

Measurement of pH

Using a digital pH meter, the pH of the optimized herbal gel formulation was measured at 25°C. To guarantee total coverage, the pH meter's glass electrode was completely submerged in the gel system. After combining one gram of the prepared gel sample with distilled water to create a homogeneous solution, the pH was assessed.^[39]

Consistency

The prepared herbal gel formulation's consistency was measured by dropping a cone that was attached to a holding rod from a fixed distance of 10 cm so that it fell in the center of the glass cup that held the gel. From the gel's surface to the tip of the cone inside the gel, the cone's penetration was measured. Ten seconds later, the cone's distance traveled was recorded.^[39]

Homogeneity

Once the gel had set in the container, the prepared herbal gel formulation was visually inspected to ensure homogeneity.^[39]

Spreadability

The spreadability of the prepared herbal gel formulation was assessed using a locally designed device that included a wooden block with a pulley at one end. A rectangular ground plate was covered with two grams of herbal gel. After then, the herbal gel was positioned between this plate and a second plate that had the same dimensions as the stationary ground plate. To create a consistent layer of herbal gel between the two plates and release any trapped air, a 1-kg weight was placed on top of each of the plates for 5 minutes. Then, using a piece of rope fastened to the hook, it was pulled 100 g. The top plate's time (measured in seconds) to traverse a distance of 5 cm was recorded; the faster two slides separate, the more spreadable the material.

The spreadability was calculated using the formula below, i.e.,

$$S = M \times L / T^{[39]}$$

Where M stands for the weight fastened to the upper slide, L stands for the glass slide's dimension and T is the amount of time required for the slides to separate.

Extrudability

The amount of gel that extruded from the tip while a continuous load was applied to the pan was used to measure extrudability. Standard-capped collapsible aluminum tubes were filled with the gel mixture, and the ends were crimped shut to seal. The tube's weight was noted. The tube was clamped after being positioned between two glass slides. After covering the slides with 500 g, the cap was taken off. Weighing and collecting the extruded gel's quantity. The following calculation was used to determine the percentage of gel extrusion and



graded excellent, good and fair at >90%, >80%, >70% extrudability, respectively.^[39]

Extrudability = $\frac{\text{Amount of gel extruded from the tube}}{\text{Total volume of gel that is inside the tube}} \times 100$

In-vivo skin irritation study

Six animals were split into two groups for the study: Group I and group II. One day prior to the study, the back skin area of each animal was shaved to create a 5 by 2 cm area for application. Each rat's back skin was evenly covered with one square centimeter of the gel formulation (1 g). The investigation spanned four days. Upon completion of the study, the animals were checked for any signs of skin irritation, such as swelling or redness. Scoring was conducted according to the parameters utilized by Draize-scoring,^[37] score of 2 or lower was considered to exhibit no irritation.

Evaluation of anti-Candida activity in animals

Six rats were split into two groups at random: Group I was treated with 2 mg/kg of hydrocortisone Group II was given a prepared herbal gel treatment (1 g) Group-III was given a marketed gel preparation (1 g), containing clotrimazole 1% w/w, For three days in a row, experimental rats (Groups II and III) received hydrocortisone at a dose of 2 mg/kg body weight to suppress their immune systems.

Creation of the external wound

Using sterile surgical blades, an external cut with a skin depth round of about 1-cm radius was made on the animal's posterior mid-dorsal side 24 hours after the last hydrocortisone treatment.

Bio-inoculation

Fresh *C. albicans* spores were collected in saline, and a suspension containing 104 CFU/mL count was made. This suspension (0.5 mL) was applied topically onto the wound as a single dose to induce infection.^[18]

Treatment with prepared herbal gel formulation

After wound creation developed gel/marketed preparation was applied on the wound at regular intervals of twice a day for 8 days.^[18]

Test to recover the pathogen

On 9th day, the material recovered from the dry wound using a sterile swab (treated and controlled wound) was

stained with gram-positive and LPCB staining methods and observed under a microscope.

Analysis of blood parameters

After the animals were killed on the tenth day, blood was drawn and stored in a glass vial with EDTA, an anticoagulant, so that the hematological parameters could be examined. Using a routine procedure, the total and differential count of WBC was measured in groups of rats that were treated and infected.^[41]

Statistical analysis

Tukey's multiple comparison was used after a one-way analysis of variance (ANOVA) for all statistical analyses. When $p < 0.05$, statistically significant differences were considered to exist. The values were shown as mean \pm standard deviation for each.^[41]

RESULTS AND DISCUSSION

Extraction and Phytochemical Screening Preparation

After experimenting with various time intervals, the optimal yield percentage of the extracted *W. tinctoria* leaves was determined to be 15.62%. Results are shown in Table 1. The results of the phytochemical screening revealed the presence of alkaloids as a major active constituent. Scientific research has documented in the literature that this plant's primary active component is indirubin alkaloid and that this extract has antifungal properties The parameters for validated analytical method are given as Table 2.^[42]

Determination of Anti-Candida Activity

The extract showed strong dose-dependent anti-*Candida* activity with a maximum inhibition zone of 8.3 mm at 25 $\mu\text{g/mL}$.^[44]

Optimization Study

Analysis of effect of independent variable on viscosity of gel

The CCD model study the impact of both independent variables, i.e., the amount of Carbopol 934 and the amount of PEG 400 on the dependent variable viscosity and spreadability of the herbal gel (Table 3). The CCD model suggested the quadratic polynomial model for the analysis of the factors. The ANOVA analysis suggested that the model was highly statistical significance with

Table 1: Optimization of extract of leaves of *W. tinctoria*

S. No.	Extraction method	Weight of powdered drug	Extracting solvent	Extraction time (hrs)	Yield (% w/w)
1	Soxhlet extraction	100 g	Ethanol (300 mL)	24	6.9 \pm 1.03
				48	11.02 \pm 1.4
				72	15.62 \pm 0.5

Table 2: Validation parameters as per ICH-Q₂R₁ guidelines.

S. No.	Parameter	Value
1	Specificity	Specific
2	Linearity	0.999
3	Accuracy (%)	98.78 ± 0.1443
4	Limit of detection (µg /mL)	3.32
5	Limit of quantification (µg /mL)	7.53
6	Retention time (min.)	8.78
7	Robustness	Robust

Table 3: Range (s) of factors taken for CCD based optimization.

Factor	Name	Low level	Centre level	High level
1	Carbopol 934	1.25	1.50	1.75
2	PEG 400	3.00	5.00	7.00

p-value less than 0.05 and a lack of fit value of 3.92. The correlation coefficient (R^2) value of 0.9921 for the model was high. The predicted R^2 of 0.9548 aligns reasonably well with the adjusted R^2 of 0.9864, with a difference of less than 0.2, indicating robustness in the model's predictive capabilities. The coefficient values of the intercept and the effect of the independent variable on responses are defined in equation 2.

$$Y = 6552 + 453.61A + 333.34B + 136.25AB - 252.81A^2 - 44.31B^2 \quad (\text{Eq...2})$$

In the above, the polynomial equation defines the effect of the independent variable amount of Carbopol 934 and the amount of PEG 400 on the viscosity of the herbal gel. The individual effect of both independent variables shows a positive correlation with the viscosity of the gel. A similar effect of both variables has been seen in the experimental run 1 and 6 in Table 4 which showed the positive correlation between Carbopol 934 and PEG 400 with viscosity. On the other hand, the viscosity of herbal gel exhibited a comparable positive association with the combined effect of both variables. When preparing gel, the concentration of the gelling ingredient is essential because it determines the gel's viscosity and consistency. Too low a concentration may result in a solution with minimal consistency, while too high a concentration can lead to excessively viscous gels that distribute unevenly and are difficult to handle. Through testing various gel formers, it was found that gels containing cellulose sodium salt and extract from *W. tinctoria* leaves exhibited phase separation and were thus not suitable. After formulating gels with different concentrations of Carbomer 934, it was observed that gels containing low concentrations of Carbomer 934 liquefied within 6 hours, indicating inadequate gelling. Increasing the carbomer concentration improved gelling slightly, but liquefaction still occurred after a day. However,

gels containing an optimum concentration of Carbopol 934 formed consistent and sleek gels that remained stable without liquefaction during storage. At further higher concentrations, the gel became excessively dense and adhesive, making distribution challenging. Carbopol 934 is widely used in herbal topical gels due to its ability to provide the desired rheological properties and formulation quality.^[45-49] Kusuma and colleagues conducted a similar investigation to examine the effect of carbopol on gel viscosity incrementally up to ideal values.^[50] In another study, topical gel of curcumin was developed using Carbopol 934 as the critical factor for the viscosity of the gel. The results showed that the gel's viscosity increased as the quantity of carbopol increased.^[51]

Statistical model analysis for spreadability

In ANOVA analysis for the second response variable (spreadability), was identified the impact of the change in the amount of Carbopol 934 and PEG 400. The model was significant, having R^2 value of 0.9984. The predicted R^2 (0.9909) and adjusted R^2 (0.9973) values with a difference of less than 0.2 showed model robustness and significance. The model analysis by ANOVA reflects that the selected model should be significant, having *p*-value less than 0.05 and having a lack of fit value (F-value) is 4.0. Because of the noise, the low F-value indicates that the lack of fit is not significant, with just a 10.69% chance of occurring. The impacts of the selected variable on the response variable are defined by the polynomial equation 3 given below.

$$Y = 22.164 + 2.44A + 2.21B + 1.73AB - 0.68A^2 + 0.24B^2 \quad (\text{Eq...3})$$

In given ANOVA coefficient-based polynomial equation, the quantitative relation between selected factors on the spreadability of the gel is given. Both selected factors have a similar positive correlation with spreadability, which was also confirmed from experimental runs 7 and 10 of Table 4. Also the combined effect of both factors shows a positive correlation with the spreadability of the prepared optimized herbal gel of extract of leaves of *W. tinctoria*. In a similar study on formulating turmeric essential oil-loaded nanoemulgel using CCD model, Carbopol exhibited a positive correlation with the spreadability of the prepared nanoemulgel.^[52]

Contour plots

Fig. 1 graphically demonstrates the impact of both variables on the selected response in 2D and 3D format. Notably, the plots clearly demonstrated how the quantities of PEG 400 and Carbopol 934 increased the viscosity and spreadability. The contour plot shapes further underscore the significant interactions between the amount of Carbopol 934 and the PEG 400 on the viscosity and spreadability of the prepared herbal topical gel containing extract of *W. tinctoria* leaves.



Herbal Gel for Anti-candida Activity

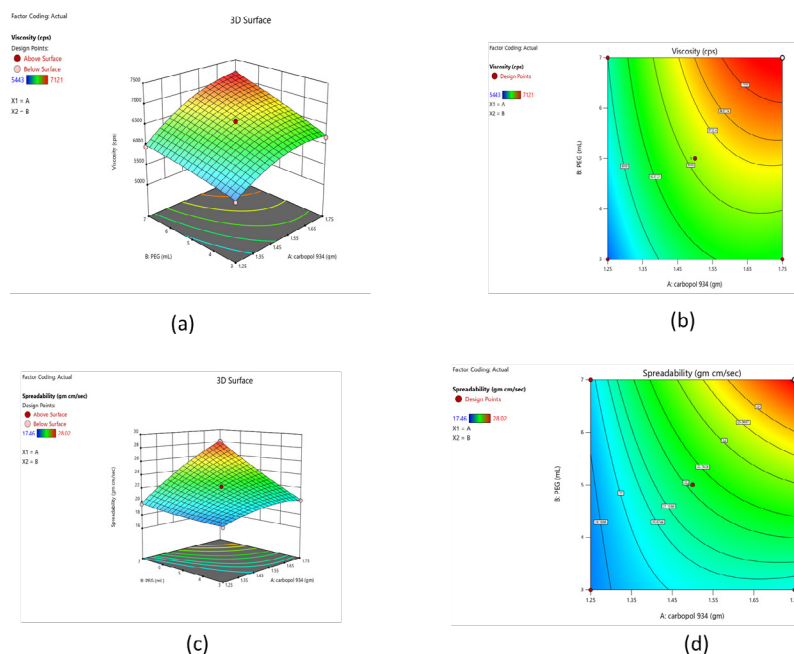


Fig. 1: Graphical representation of 2D and 3D contour plots of response variables. A) 3D contour plot of viscosity; b) 2D contour plot of viscosity; c) 3D response surface contour plot for spreadability; d) 2D contour plot for spreadability

Table 4: Runs of CCD model on the herbal topical gel of extract of *W. tinctoria*

Run	Factor 1 Amount of Carbopol 934 (gm)	Factor 2 Amount of PEG 400 (mL)	Viscosity (cps)	Spreadability (gm.cm/sec)
1	1.25	3	5567	18.66
2	1.5	7.82843	6987	25.93
3	1.5	2.17157	6034	19.66
4	1.5	5	6539	22.15
5	1.5	5	6576	22.2
6	1.85355	5	6744	24.36
7	1.75	7	7121	28.02
8	1.75	3	6189	20.11
9	1.5	5	6601	22.29
10	1.14645	5	5443	17.46
11	1.5	5	6497	22.01
12	1.5	5	6547	22.17
13	1.25	7	5954	19.63

Table 5: Comparison between predicted and experimental values

Analysis	Predicted value	Observed value	%Bias
Viscosity	6552	6547	0.076%
Spreadability	22.164	22.17	0.027%

Model validation

The optimized conditions, as indicated by the point prediction feature of Design Expert software, improved the viscosity and spreadability of the prepared herbal gel formulation containing *W. tinctoria* leaf extract. Specifically, the formulation displayed better viscosity and spreadability while maintaining constant levels of other excipients. These conditions included the amount of carbopol 934 (1.5 gm) and PEG 400 (5 mL). The model-suggested values of the selected factors are listed in Table 5. Under specific conditions, the predicted optimized viscosity was 6552 cps and the spreadability was 22.16 gm cm/sec in comparison with experimental values. Taking the amount of the selected factors showed the viscosity of gel was 6547 and its spreadability was 22.17 gm cm/sec, which is closer to the predicted value by the CCD model (Table 5). The model suggested the optimization success, having the R^2 value of 0.9921 for viscosity and R^2 0.9984 for the spreadability of the prepared herbal gel. The high value of R^2 suggested the model's robustness and effectiveness in predicting and achieving optimal results in herbal gel formulation. This is further corroborated by the fact that the herbal gel's viscosity and spreadability only differ by 0.076 and 0.026%, respectively.

Evaluation of Topical Herbal Gel Formulation

Physical appearance

It was discovered that the herbal gel composition was homogeneous and devoid of grit, making it simple to apply to the skin.

Table 6: Stability study of prepared herbal gel formulation

Parameters	Time (in days)						
	0	30	60	90	120	150	180
Color	No change						
Odour	No change						
Homogeneity	Homogenous						
pH	6.30	6.27	6.22	6.15	6.11	6.10	6.08
Viscosity (cps)	6545	6543	6535	6528	6520	6510	6490
%Drug content	72.3	69.5	66.93	62.43	58.8	55.47	53.1

Measurement of pH

The prepared herbal gel formulation's pH value was discovered to be 6.3 ± 0.1 , meaning that skin irritation is unlikely to occur.

Consistency

The gel's consistency indicates its ability to be uniformly and precisely discharged when the tube is squeezed. The distance covered by a falling cone has an inverse relationship with consistency. It was discovered that the prepared herbal gel formulation had a consistency value of 6.7 mm. A sufficient consistency is necessary to extrude the gel from the tub since high consistency gels may not extrude from the tube while low viscosity gels may flow swiftly.^[39]

Homogeneity

After the prepared herbal gel formulation was set in the container, it was visually inspected to ensure homogeneity. Homogeneity was found in the formulation.

Spreadability

The spreadability is an important parameter as it shows the behavior of prepared herbal gel while squeezed from the tube. The developed herbal gel formulation's spreadability values were found to be 22.17 ± 1.5 gm.cm/sec, which is considered satisfactory.

Extrudability

When it comes to delivering the appropriate amount of gel from a jar or extruding gel from collapsible tubes, gel packing has become increasingly important. The amount of gel that extruded from the tube when a specific load

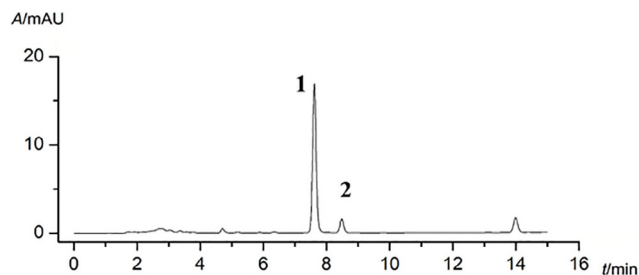




Fig. 2: HPLC chromatogram of *W. tinctoria* at 290 nm showing peaks; Indirubin (2)

Table 7: In-vivo skin irritation investigation of prepared herbal gel formulation

Sample	Zero day	4 th day
Prepared herbal gel formulation reflecting zero score i.e. non-irritant nature		

was applied was used in the current investigation to measure the extrudability of the gel formulation. Better extrudability results from extruding a larger volume of gel. The extrudability value (%) of the prepared herbal gel formulation was found to be 89.53%, reflecting acceptable squeezability.

Rheological properties of the formulated gel

Using Brookfield viscometer (DV-II+Pro), the generated herbal gel formulation's viscosity was measured at 6547 cps at 50 rpm by using spindle number 95, and its torque (%) was 82.7%, indicating appropriate rheological behavior.

HPLC analysis of leaf extract

The HPLC analysis of extract of leaves of *W. tinctoria* has shown the presence of bis-indole alkaloid indirubin as major bioactive compound (Fig. 2). Literature reports have also confirmed that indirubin has significant antifungal property.^[28] However, previous studies clearly demonstrate that agro-ecological zoning,^[53-55] seasonal and geographical variations,^[50] good agricultural/collection practices,^[56] and various other factors during the cultivation and collection of herbal drugs^[57-59] significantly impact the concentrations of active constituents. Hence indirubin was established as main marker compound in this study. The HPLC method described has been thoroughly validated according to the guidelines set forth by the ICH, ensuring its reliability and accuracy. The validation parameters such as precision, accuracy, linearity, and stability are all within acceptable limits as per the ICH Q2R1 guidelines, indicating that the method is robust and suitable for identifying the



Table 8: Status of the fungal hyphae from the wound of the treated and untreated group

Group	Subject	Staining method	
		Gram staining	LPCB staining
I	Group with <i>C. albicans</i> infection but not treated.	+++	+++
II	Group with <i>C. albicans</i> infection treated using herbal gel	+	+
III	Group with <i>C. albicans</i> infection treated using marketed gel	++	++

+++ = Highly infected, ++ = Moderate infected, + = Least infected

Table 9: WBC count in rat blood

Group	Subject	Number of WBCs in blood ($10^3 \mu\text{L}$)	
		Day 1	Day 9
I	<i>C. albicans</i> infected + untreated group	8.06 ± 0.571	8.85 ± 0.492
II	<i>C. albicans</i> infected + treated with herbal gel	$6.2 \pm 0.650^*$	$7.35 \pm 0.376^*$
III	<i>C. albicans</i> infected + treated with marketed gel	$5.95 \pm 0.151\text{ns}^*$	$6.11 \pm 0.278^{**}$

Values are expressed as MEAN \pm SD. One-way ANOVA followed by Tukey's test was applied for comparison between the gel-treated mice groups and the negative control (* $p < 0.05$, ** $p < 0.001$ vs control).

main active constituents of the selected plant (Table 2). Additionally, adherence to ICH guidelines confirms the industrial acceptability of the analytical procedure, which is crucial for ensuring the quality and reliability of the results. It's noteworthy that similar analytical protocols for phyto-constituents and drugs have been established in the author's lab and is widely accepted globally,^[60-64] further affirming the credibility and relevance of the methodology described.

Drug release study of prepared herbal gel formulation

Maximally 84.23% drug in 6 hours was released from the prepared herbal gel formulation, confirming with the expected application time for pharmaceutical gel and was found acceptable.

Accelerated stability study of prepared herbal gel formulation

The results of accelerated stability study of prepared herbal gel formulation were shown in Table 6. The evaluation of the formulated herbal gel's reliability over its shelf life is crucial for ensuring its quality, efficacy, and safety. Following the guidelines set by the International Council for Harmonization (ICH) is a standard approach in this regard. The stability testing conducted at various intervals (0, 1, 2, 3, 4, 5, and 6 months) at 40°C temperature and 75% RH, revealed no observable alterations in key parameters such as color, odor, homogeneity, pH, rheological properties, or total content of the gel formulation. This indicates that the gel maintained its integrity and quality throughout the duration of the study. The manufactured dosage form's stability is conclusively demonstrated by the stability study carried out in accordance with ICH rules, which is crucial for determining the dosage form's shelf life. The

fact that the formulated herbal gel remained stable for up to 6 months (Table 6), as demonstrated by the results, underscores its suitability for practical application and commercialization.

In-vivo skin irritation study

The Draize grading system was used in the skin sensitivity study to evaluate dermal reactions. Observations were collected at different times, including right after patch removal and again 24, 48 and 72 hours later. During the preliminary examination, rats exhibited no skin reactions following any of the three successive exposures, which occurred at intervals of 3 minutes, 1 and 4 hours. In the confirmatory test, the animals treated with the prepared herbal gel formulation showed no signs of redness or swelling. The reaction was consistently rated as "0" at all observation time points. The primary dermal irritation index for the prepared herbal gel was recorded as zero. This suggested that the prepared formulation was safe and appropriate for topical application.

Undoubtedly, a crucial component of preclinical safety evaluation is the skin irritation test used to evaluate the possible irritating effect of pharmaceutical compounds, especially for dermal applications. Such tests help in evaluating the risk of adverse reactions like redness, itching, or pain upon contact with the product. In this study, the skin irritation test was carried out to assess local inflammation, typically indicated by erythema (redness) and edema (swelling), following direct skin exposure to the formulated herbal gel. The absence of erythema or edema (Table 7), as per the Draize scoring system, indicates that the formulated herbal gel did not induce any significant irritation or adverse reaction upon contact with the skin. These results support the formed herbal gel preparation's potential for further development

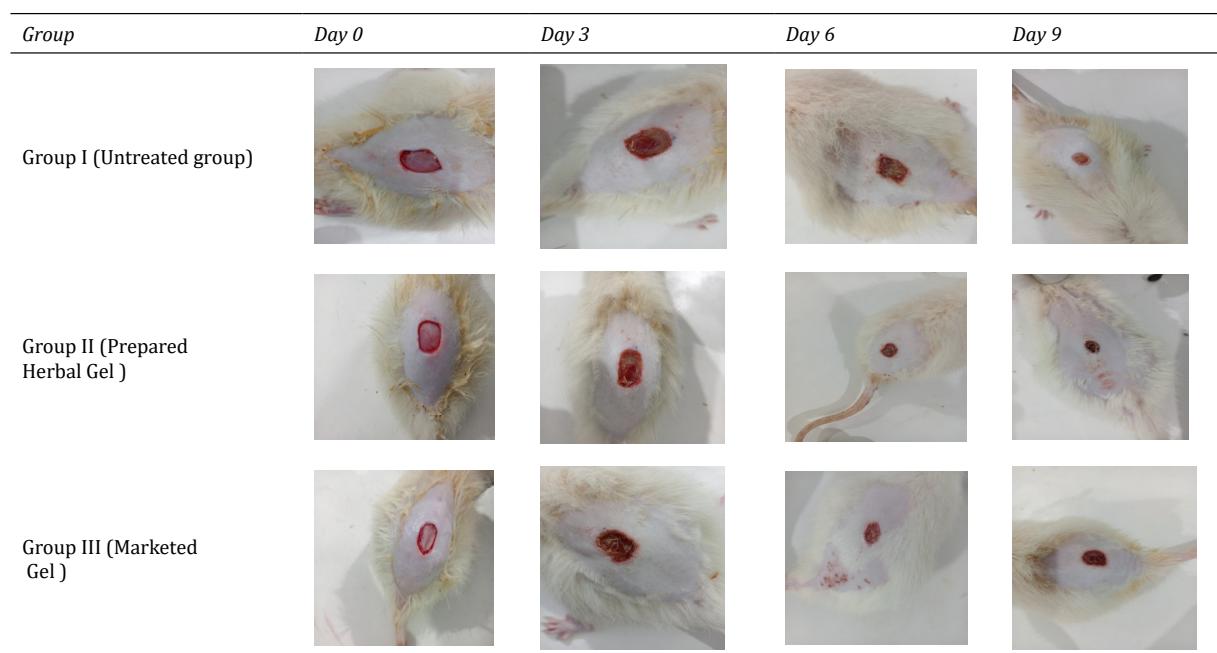


Fig. 3: *In-vivo* wound healing activity of gel formulation: Representative photographs of *C. albicans* infected wound after different treatment for different periods

Group	Group I Hydrocortisone	Group II (Prepared gel)	Group III (Marketed gel)
LPCB staining			
Gram staining			

Fig. 4: LPCB and gram staining of fungal hyphae from wounds

and prospective usage as a pharmaceutical product by indicating that it can be deemed safe and non-irritating for dermal application.

Evaluation of in-vivo anti-Candida activity in animal model by using rats

In this assessment, an 8-day application of a produced herbal gel formulation was used to treat the open wounds of animals infected with *C. albicans*. These treated wounds were showed visible healing sign (Fig. 3). Significant wound healing was seen when the gel formulation was applied topically to *C. albicans* infected rats.

LPCB and gram staining

Staining is crucial for identifying and classifying different types of bacteria, fungi, and other microorganisms. Staining also helps in the diagnosis of infectious diseases by enabling the identification of pathogens in clinical samples. After 24 to 48 hours of incubation, the isolate from rat wounds formed creamy to white yeasty colonies with smooth surfaces on SDA plates.

LPCB staining of C. albicans

LPCB staining revealed round and unilateral budding yeast cells.



Gram staining of *C. albicans*

The fungus cells indicated gram-positive reaction, which is a characteristic feature of taken fungus cell species. On the 9th day, a swab taken from the area of the healing wound revealed the presence of fungal hyphae using both the Grams and LPCB staining techniques (Fig. 4 and Table 8). Swabs taken from the rats' wound areas revealed the presence of *C. albicans*. Groups II and III did not exhibit a substantial fungal infection, but group I, the infected but untreated group, had a heavily *C. albicans* infected lesion.^[65]

Analysis of blood parameters

The elevated WBC count due to *Candida* infection was restored with treatment by applying herbal gel (Table 9). White blood cells participate in the immune process. These cells fight fungal infection and oversee the repair process and also protect the body from damage due to toxins and aid wound healing and tissue repair.^[53] They support your body's defenses against illnesses and infections. Our bodies produce more white blood cells when we are ill in order to combat the bacteria, viruses, or other foreign things that are causing our sickness. This raises the white blood cell count, whereas illnesses like HIV/AIDS and cancer diminish it. Because people with deficiencies in their cell-mediated immune response are more likely to develop superficial but not disseminated candidiasis, clinical observations in previously published have suggested that antibodies are crucial to the host's defense against the disease.^[55]

CONCLUSION

By forming a biofilm, the opportunistic pathogen *Candida albicans* can survive at multiple anatomical sites. The study aimed to develop an antifungal herbal topical gel formulation utilizing the extract of leaves from the desert plant *W. tinctoria*. Results indicated that the formulated gel containing the ethanolic extract of *W. tinctoria* leaves exhibited optimal anti-*Candida* activity. The plant's alkaloids are most likely responsible for this antifungal action. A formulation study was conducted, encompassing physicochemical and phytochemical screening, yielding satisfactory results. The herbal gel formulation demonstrated promising drug content and release profiles. Furthermore, a skin irritation study conducted on rabbits revealed no irritancy, indicating that the formulation was well-tolerated by skin tissues. Stability testing conducted over a six-month period showed no significant changes in the physicochemical properties of the formulation, further confirming its stability. In conclusion, the study highlights the antifungal potential of *W. tinctoria* leaves, particularly in ethanolic extract form. The developed herbal gel formulation presents a promising option for topical antifungal treatment, with favorable characteristics in terms of efficacy, safety, and stability.

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