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Formulation and Assessment of Herbal Hydrogel and Nanoemulsion for Dandruff Management

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ABSTRACT

Dandruff, which is characterized by excessive flaking, irritation, and microbial development, is common on the scalp. Using nanoemulsion (NE) and hydrogel formulations, this study examined the effectiveness of *Rosmarinus officinalis* (RE) leaf extract and *Trigonella foenum-graecum* (fenugreek, FE) seed extract in managing dandruff. Preformulation tests involving solubility, antioxidant activity, thin-layer chromatography (TLC), UV spectroscopy, FTIR, antimicrobial testing, and phytochemical screening were used to establish the extracts' medicinal potential and confirm the existence of bioactive components. In addition to continuous drug release, the hydrogel formulation demonstrated appropriate physicochemical characteristics, including the right pH, viscosity, spreadability, drug content, and antifungal and antioxidant activity. According to stability tests, the hydrogel worked better at lower temperatures. A 2^3 factorial design was used for optimization in order to get over the constraints of nanoemulsion stability. At room temperature, the modified nanoemulsion showed improved drug diffusion, small particle size, sufficient zeta potential, acceptable polydispersity index, and good physicochemical and microbiological stability. Both formulations demonstrated strong antifungal activity and therapeutic promise, however the nanoemulsion demonstrated better stability and efficacy. These results suggest that topical delivery solutions based on herbs, such as hydrogel and polyherbal nanoemulsion formulations, could be useful natural substitutes for managing dandruff..

Keywords: *Rosmarinus officinalis*, *Trigonella foenum-graecum*, Tea tree oil, hydrogel formulation**,** nanoemulsion, antifungal activity, stability study and dandruff treatment.

INTRODUCTION

The scalp's excessive scaling, itching, and irritation are frequently accompanied by dandruff, which is primarily caused by fungal colonization and aberrant keratinization. This condition is rare. Dandruff affects a large population worldwide and is primarily linked to Malassezia species, increased sebum production, and individual susceptibility [1,2,3]. Although traditional anti-dandruff medications like azole antifungals and keratolytic agents are effective, they may result in adverse effects such as scalp dryness or irritation, frequent use of topical treatments, which has led many experts to explore safer alternatives [4]. Polyherbal formulations are becoming more popular because of their potent therapeutic benefits and reduced incidence of side-effects. Antifungal, antioxidant, and anti-inflammatory properties have been demonstrated in herbal extracts such as Trigonella foenum-graecum and Rosmarinus officinalis, which are potential candidates for treating dandruff. The use of herbal extracts in advanced topical drug delivery systems can enhance therapeutic performance [5,6,7]. The ease of application, spreadability, and extended drug release make Hydrogel systems a preferred option for topical administration [8,9]. Nanoemulsion systems enhance drug penetration, stability, and bioavailability by virtue of their nanoscale droplet size and large surface area [10,11]. Its current work was undertaken to develop and test new formulations of polyherbal hydrogel and nanoemulsion solutions for treating dandruff effectively, while also measuring their physicochemical traits, overall stability, and antifungal activity.

Relevant conflicts of interest/financial disclosures: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

MATERIALS AND METHODS

Materials

Extracts of Rosmarinus officinalis (leaf) and Trigonella foenum-graecum (seed) were purchased from Vogofy Oil Pvt. Ltd. Kalyan (Mumbai). While other chemicals like ethanol from M/S Gogia and Company, tween 80, citric acid, PEG 400, glycerol, and triethanol amine were purchased from Molychem and Carbopol 974 P NF from Lubrizol, tea tree oil was purchased from Sahyadri Essential Oil.

Preformulation studies

The purpose of preformulation studies is to describe the physicochemical characteristics of medicinal ingredients and how they interact with different formulation ingredients.

Organoleptic Evaluation

Rosemary and fenugreek extracts were evaluated for various organoleptic characterization such as color and odor.

Solubility study

The solubility of both the extracts in various solvent was determined. The solubility was quantified as per the standard procedure. In the present work, solubility of extract was determined in solvents like water, methanol, ethanol, DMSO and PEG-400.

Phytochemical analysis [12,13]

The extracts of fenugreek seeds and rosemary leaves were tested for the presence of several phytoconstituents. Standard qualitative chemical methods were used for phytochemical screening in order to identify secondary metabolites, including phenols, flavonoids, tannins, alkaloids, saponins, and glycosides. These investigations, which followed procedures outlined in previously published literature, confirmed the extract's rich phytochemical profile, particularly its noteworthy flavonoid and phenolic content, which bolsters its antioxidant and antimicrobial properties.

Thin layer chromatography [14,15]

Precoated silica gel G plates were used as the stationary phase in the TLC of both extracts. Different combinations of solvents were used to optimize the mobile phase. Following development, the presence of polyphenols and flavonoids was verified by calculating the Rf values of the isolated spots and comparing them with existing literature.

Determination of λ max and calibration curve

The extracts and TTO were combined to make a standard stock solution of 1000 µg/ml by carefully measuring 25 mg of each sample and dissolving it in 25 ml of ethanol. The stock solutions were further diluted as necessary using the same solvent. A double beam UV-Visible spectrophotometer (Shimadzu, UV-1800) 10 mm quartz cuvettes was used to scan each diluted solution independently in the 200–800 nm region. The wavelength of maximum absorption (λ max) was determined using the appropriate UV absorption spectra. Standard calibration curves were created in ethanol for quantitative analysis. While RE and FE solutions were prepared at 200–1000 µg/ml, TTO solutions were made at 50–300 µg/ml. Absorbance was measured at their respective maximum absorption wavelengths. Similar was done using combination of phosphate buffer 4.75 with PEG 400 for RE (30-80 µg/ml) and FE (100-600 µg/ml) concentration.

FTIR analysis

The KBr pellet technique was used to record the FTIR spectra of the extracts and TTO. Samples were crushed into thin pellets, combined with dry potassium bromide, and scanned between 4000 and 400 cm^{-1}. To determine which distinctive functional groups were present in the samples, the spectra were examined.

Evaluation activity of extracts

Total phenolic content [16]

Gallic acid was used as the standard in the Folin-Ciocalteu technique to calculate the total phenolic content (TPC) of both extracts. Extract solutions (10 ppm) and gallic acid solutions (5–30 ppm) were made.

Folin-Ciocalteu reagent and 7.5% sodium carbonate were combined with each sample and standard, and distilled water was used to adjust the volume. A blank was made in the same way without the sample, and the mixes were incubated in the dark for half an hour. A UV-visible spectrophotometer was used to measure absorbance at 725 nm, and the gallic acid calibration curve was used to compute TPC.

Antioxidant activity of extracts by DPPH method [16]

The antioxidant capability of both extracts was assessed by measuring their ability to scavenge DPPH radicals. Free radical scavenging is demonstrated by the yellow hydrazine that is produced when the purple DPPH radical combines with antioxidants that donate hydrogen. 10 mg of extracts were dissolved in 10 ml of ethanol (1000 µg/ml) to create extract solutions, which were then further diluted. Diluted samples were combined with 3 ml ethanol and 1 ml of 0.1 mM DPPH solution for analysis, and they were then allowed to sit at room temperature for half an hour in the dark. A UV-Visible spectrophotometer was used to detect absorbance at 517 nm, using ethanol as a blank. Reduced absorbance demonstrated the extracts' efficient neutralization of free radicals and validated antioxidant activity.

Antimicrobial study of the extracts and TTO by Agar well diffusion method [17,18]

Antimicrobial activity of the extracts was evaluated using the agar well diffusion method against Staphylococcus aureus. Nutrient agar medium was prepared, sterilized by autoclaving at 121°C for 15 minutes, poured into petri plates, and allowed to solidify. The bacterial culture was swabbed onto agar plates. Wells were made using a sterile cork borer, and different concentrations of extracts and tea tree oil were added. Plates were incubated at 37°C for 24 hours, and zones of inhibition were measured to assess antimicrobial activity.

Formulation and Evaluation of Hydrogel

For full hydration, required quantity of carbopol 974 was soaked in 50 mL of distilled water for the entire night. For even dispersion, 20 mL of distilled water was then added and agitated for two hours using a magnetic stirrer. Glycerin and polyethylene glycol 400 were added gradually while being constantly stirred. The required quantity of FE and RE were weighed, the total volume was set at 100 ml. Triethanolamine was added to the gel to change its pH, and citric acid was utilized as a preservative [19]

Table 1: Hydrogel formula

Ingredient	Amount
Rosemary leaf extract	1%
Fenugreek seed extract	1%
Carbopol 974 P NF	1.5%
Glycerol	3%
PEG- 400	4%
Citric acid	0.1%
Tri ethanolamine	Qs
Water	100 ml

Physical Evaluation: Color, homogeneity, and the presence of grittiness were among the organoleptic characteristics assessed for the formulated hydrogel.

Determination of pH: A calibrated digital pH meter was used to measure the hydrogel's pH at room temperature. After dissolving 1 g of gel in 100 mL of

distilled water and letting it sit for two hours, the pH of the mixture was measured [19]

Spreadability: To measure spreadability, a wooden block and pulley device was used. Two glass slides were sandwiched with around 2 g of gel, which was crushed for one minute using a 100 g weight. Spreadability was determined using the formula S = M × L / T, and the amount of time needed for slide separation was noted [19]Where, M-Weight tide to upper slide l- Length moved on the glass slide T-time taken (in sec) taken to separate the slide from each other

Viscosity: A Brookfield viscometer was used to assess rheological behavior. A 50 g sample was put in a beaker and given five minutes to equilibrate. Using spindle 7 at 10 rpm, viscosity was measured and computed using viscometer factors [20]

Drug content: After dissolving 100 mg of hydrogel in 10 mL of ethanol, the mixture was sonicated for two hours. Using ethanol as a blank, the solution was filtered, suitably diluted, and examined at the proper wavelengths using a UV spectrophotometer [21]

Invitro drug release: The in-vitro diffusion study of the prepared gel was carried out using a Franz diffusion cell apparatus. In the Franz diffusion cell, 1 gm of hydrogel was uniformly spread over a cellophane membrane, which was previously soaked in PEG-400 and phosphate buffer pH 4.75 for 24 hours and then sandwiched between the donor and receptor compartments. A mixture of PEG-400 and phosphate buffer (pH 4.75) in a 1:10 ratio was used as the receptor medium. The temperature was maintained at 37 ± 0.5°C. The entire assembly was placed on a magnetic stirrer, and the receptor compartment was continuously stirred using a magnetic bead at 100 rpm. At time intervals of 0, 5, 15, 20, 25, 30, 60, 90, 120, 150 and 180 minutes, 2 mL samples were withdrawn and immediately replaced with 2mL of the same fresh receptor medium. The percent drug release in the receptor fluid was determined spectrophotometrically at respective wavelengths of both RE and FE against the receptor medium as blank [22,23,24]

Antioxidant activity by DPPH method: To create a 1000 ppm solution, 25 mg of gel was dissolved in 25 ml of ethanol. Dilutions of this stock solution were made in increments of 200, 400, 600, 800, and 1000 parts per million. To create the samples, from each of these solution 1 ml was pipetted out, 3 ml of ethanol were added, and then 1 ml of 0.1 mM DPPH solution was added. After that, they were left in a dark room for half an hour at room temperature. The absorbance at 517 nm was measured using ethanol as a blank. When test samples at different concentrations were present, it was found that the absorbance dropped [16,25,26,27]

Antifungal activity: To assess the antifungal potential of the formulated hydrogel, the samples were outsourced to the microbiology laboratory, where it was tested against Candida albicans fungal strain using standard microbiological methods and compared with marketed formulation. Measurements were made of the zones of inhibition surrounding the wells.

Drug excipient interference study: Formulated hydrogel and placebo gel was diluted with phosphate buffer pH 4.75 and PEG-400 in combination and scanned the above solutions using UV spectrophotometer [28]

Stability study: Stability study of hydrogel formulation was subjected to stability study as per ICH guidelines for one month.

Optimization, formulation and evaluation of Nanoemulsion

Using a 2^3 factorial design, the nanoemulsion formulation including FE and RE was optimized using Design Expert software (Version 12). While reducing the number of experimental runs, this method allowed for the simultaneous study of several formulation variables and their interactions. Particle size, zeta potential, and polydispersity index were chosen as responses, whereas Tween 80, tea tree oil, and mixing speed were chosen as independent factors. To create response surface plots and polynomial equations, eight experimental runs were created and statistically examined [29,30]

Preparation Method

Using a 2^3 factorial design, eight nanoemulsion formulations were created by adjusting the amount of tea tree oil, Tween 80 concentration, and mixing speed. To create a coarse emulsion, distilled water was added gradually after Tween 80 and tea tree oil had been combined and swirled at predetermined rates. After adding herbal extracts, the volume was adjusted to 100 ml. For 15 minutes, the mixture was probe-sonicated. DLS (Dynamic light scattering) was used to examine the particle size, zeta potential, and PDI; the optimum formulation was chosen for additional research [31]

Thermodynamic stability: The nanoemulsion's stability was investigated using centrifugation and freeze-thaw testing. Three 12-hour cycles at 4°C and then 12 hours at 25°C were used in the freeze-thaw test to identify instability, and formulations were visually inspected. By centrifuging the optimized nanoemulsion at 2000 rpm for 30 minutes and visually observing any changes, the centrifugation test evaluated metastable situations including phase separation, drug precipitation, or creaming [32]

pH: A digital pH meter was used to measure the pH at room temperature by submerging the probe in the mixture.

Viscosity: Using a Brookfield viscometer, the viscosity was calculated by equilibrating 50 mL of the sample for 5 minutes, measuring it with spindle 3 at 10 rpm, and multiplying the dial reading by the viscometer catalog factor to get the viscosity in centipoise.

Dye solubility, dilution and filter paper test: A small amount of formulation was mixed with methylene blue to examine dispersion in order to verify dye solubility [33] By diluting 1 mL of nanoemulsion with 10 mL of water to check for phase inversion, phase behavior was evaluated. A drop was placed on paper for 30 minutes as part of the filter paper test to look for the creation of an oily ring.

Drug content: After dissolving 1 mL of the formulation in 10 mL of ethanol, it was sonicated for 30 minutes at room temperature. A UV spectrophotometer was used to measure the drug concentration at the appropriate wavelength after the solution was filtered and 1 mL of the filtrate was diluted with 10 mL of ethanol [33]

Invitro drug release: A cellophane dialysis membrane was used for in-vitro drug release experiments. After adding 1 mL of NE formulation, the membrane was sliced and sealed at both ends to create a dialysis bag. This was suspended to improve the solubility of the active ingredients in a beaker filled with phosphate buffer (pH 4.75). To guarantee continuous agitation, the setup was kept at 37 ± 0.5°C on a magnetic stirrer. 2 mL aliquots were taken out and promptly replenished with new buffer at predefined intervals (0, 5, 15, 20, 25, 30, 60, 90 and 120 minutes) in order to preserve sink conditions. Using buffer as a blank, the proportion of drug release into the receptor media was measured spectrophotometrically at the precise wavelengths that corresponded to the extracts of fenugreek and rosemary [30]

Antioxidant activity: To create a 1000 ppm solution, 25 ml of formulation was dissolved in 25 ml of ethanol. Dilutions of this stock solution were made in increments of 200, 400, 600, 800, and 1000 parts per million. To create the samples, from each of these solution 1 ml was pipetted out, 3 ml of ethanol were added, and then 1 ml of 0.1 mM DPPH solution was added. After that, they were left in a dark room for half an hour at room temperature. The absorbance at 517 nm was measured using ethanol as a blank. When test samples at different concentrations were present, it was found that the absorbance dropped [16,25,26,27]

Antifungal activity: To assess the antifungal potential of the formulated NE, the samples were outsourced to the microbiology laboratory, where it was tested against Candida albicans fungal strain using conventional microbiological techniques. The wells surrounding zones of inhibition were measured.

Drug excipient Interference study: Formulated NE and placebo NE was diluted with phosphate buffer pH 4.75 and PEG-400 in combination and scanned the above solutions using UV spectrophotometer [28]

Stability study: Both the optimized formulations were subjected to stability as per ICH guidelines for 1 month [33]

RESULT AND DISCUSSION

Solubility study

Table 2: Solubility of extracts

Solvent	Rosemary leaf extract	Fenugreek seed extract
Ethanol	Soluble	Soluble
Methanol	Soluble	Soluble
PEG-400	Sparingly soluble	Soluble
DMSO	Soluble	Soluble
Water	Soluble	Soluble

Phytochemical Evaluation

Both the extracts showed presence of all phytochemical tests except RE showed absence of alkaloids as shown in table 3.

Table 3: Phytochemical screening of extracts

Test	Rosemary leaf extract	Fenugreek seed extract
	Result	
Tannins	+	+
Alkaloids	-	+
Saponins	+	+
Flavonoids	+	+
Glycosides	+	+
Steroids	+	+
Phenols	+	+
Carbohydrates	+	+
Proteins	+	+

Thin Layer Chromatography

To determine the phytoconstituents contained in both extracts, TLC analysis was carried out. Toluene was the mobile phase that was employed. The ratio of ethyl acetate to formic acid (3.5: 1.5: 0.5) for RE Under UV illumination, the TLC plate and methanol: water (2.5: 1.5: 1) for FE were visible. Each spot's Rf value was computed for both extracts and compared to values reported in the literature. Rosmarinic acid and quercetin were found to be present in the RE and FE based on Rf comparison [14,15]

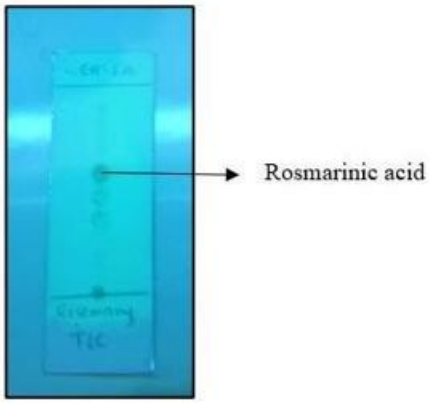


Figure 1: TLC of RE

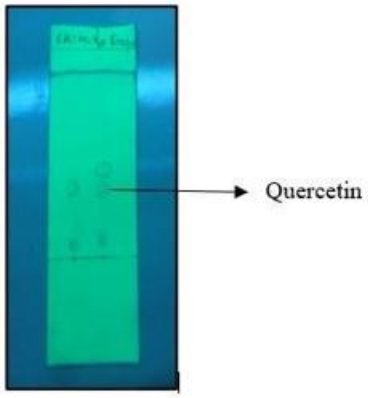


Figure 2: TLC of FE

Spectral characterization by UV spectroscopy

Spectrum and calibration curve of RE

The spectrum of RE in ethanol is shown in [Figure 3] after researching the drug's UV spectra. The highest absorbance (λ max) was reported at 282 nm in ethanol. The straight-line equation obtained was y = 0.0009x + 0.3032, where y is absorbance and x is concentration. The standard calibration curve shows a straight line with a correlation coefficient of 0.9954, indicating the medication follows Beer's law in the concentration range of 200-1000 µg/ml Figure 4].

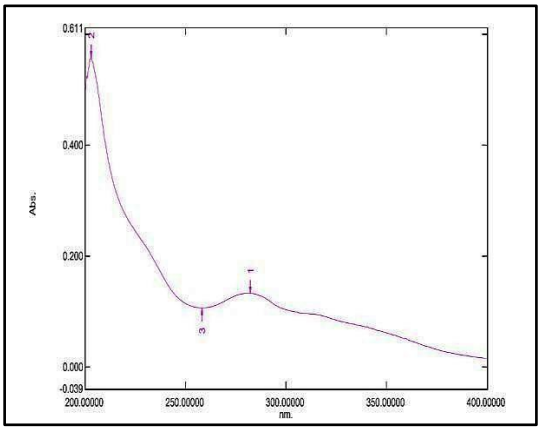


Figure3: Spectra of RE

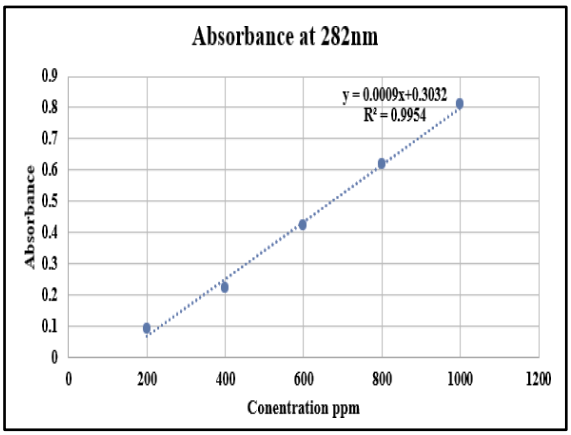


Figure 4: Calibration curve of RE

Spectrum and calibration curve of FE

After investigating the drug's UV spectra, [Figure 5] depicts the spectrum of FE in ethanol. In ethanol, the highest absorbance (λ max) was observed at 254 nm. The straight-line equation was derived as y = 0.001x + 0.0281, where y represents absorbance and x represents concentration. The standard calibration curve shows a straight line with a correlation coefficient of 0.9901. This implies that the drug follows Beer's law in the concentration range of 200-1000 µg/ml [Figure 6].

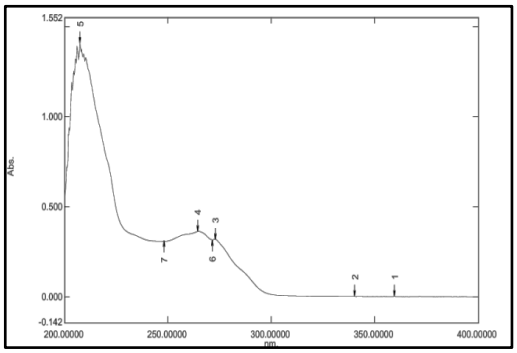


Figure 5: Spectra of FE

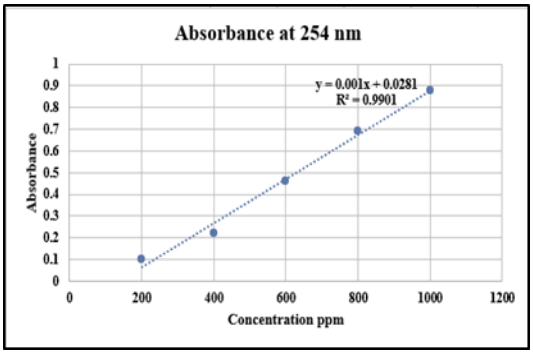


Figure 6: Calibration curve of FE

Spectrum and calibration curve of TTO

Figure 7 depicts the spectrum of tea tree oil in ethanol based on the drug's UV spectra. In ethanol, the highest absorbance (λ max) was measured at 264 nm.The straight-line equation obtained was y = 0.0034x + 0.0539, where y is absorbance and x is concentration. The standard calibration curve shows a straight line with a correlation coefficient of 0.9869, indicating the medication follows Beer's law in the concentration range of 50-300 µg/ml [Figure 8].

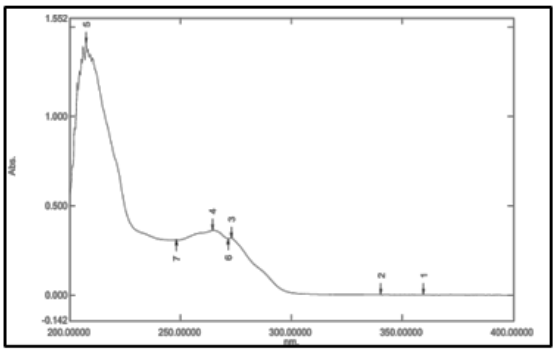


Figure 7: Spectra of TTO

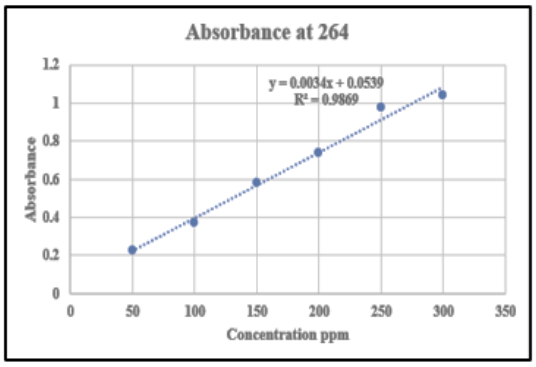


Figure 8: Calibration curve of TTO

Fourier Transform Infrared spectroscopy studies (FTIR)

The FTIR spectra of RE exhibits distinct bands at following wavelengths

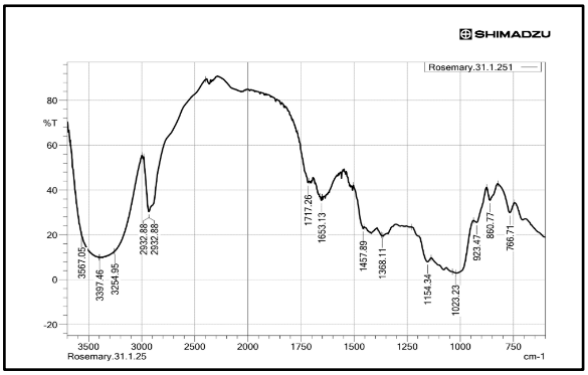


Figure 9: FTIR of RE

Functional group	Observed frequency
Hydroxyl(-OH) stretching	3567.05, 3397.46 cm^{-1}
Carboxylic acid(-COOH) stretching	1717.26 cm^{-1}
Carbonyl (-C=O) stretching	1653.13 cm^{-1}
Ether (C-O-C) stretching	1154.34, 1023.23 cm^{-1}

Table 4: Results of FTIR (RE)

The FTIR spectra of FE exhibits distinct bands at following wavelengths

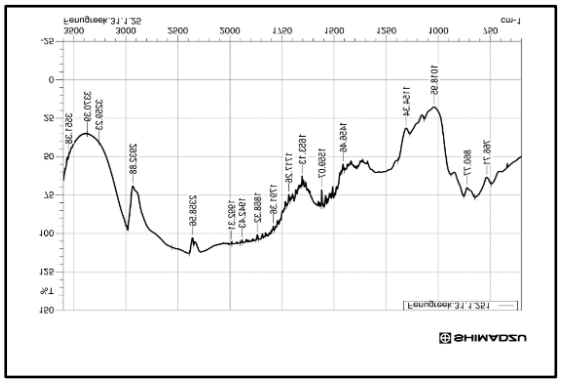


Figure 10: FTIR of FE

Functional group	Observed frequency
Hydroxyl(-OH) stretching	3551.38, 3370.39, 3259.23 cm^{-1}
Carbonyl(-C=O) stretching	1717.26 cm^{-1}
Aromatic C=C stretch and C=O conjugation	1653.13, 1559.07 cm^{-1}

Table 5: Results of FTIR (FE)

The FTIR spectra of exhibits distinct bands at following wavelengths.

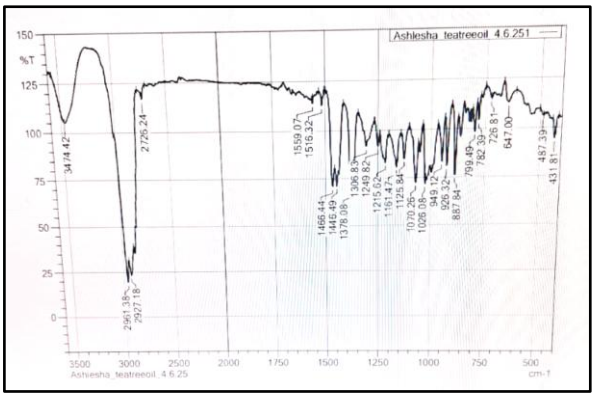


Figure 11: FTIR of TTO

Functional group	**Observed frequency**
Hydroxyl(-OH) stretching	3474.42 cm^{-1}
Alkanes & Alkenes C-H stretch	2927.18, 2961.38 cm^{-1}
Aromatics C=C stretch	1516.32 cm^{-1}
Ether C-O-C Stretching	1456.46, 1559.07 cm^{-1}
Alkenes =C-H bend	887.84 cm^{-1}

Table 6: Results of FTIR (TTO

Evaluation activity of extracts

Total phenolic content

The Folin–Ciocalteu colorimetric method, which is based on the reduction of the reagent by phenolic compounds under alkaline circumstances, was used to assess the total phenolic content. This method produced a blue complex that was detected at 765 nm. Results were reported as milligrams of gallic acid equivalents per gram of extract after the extracts were reacted with the reagent and sodium carbonate

Table 7: Observation of standard gallic acid

Concentration (ppm)	**Absorbance**
5	0.156
10	0.312
15	0.452
20	0.589

25	0.699
	0.812
Rosemary	0.489
Fenugreek	0.371

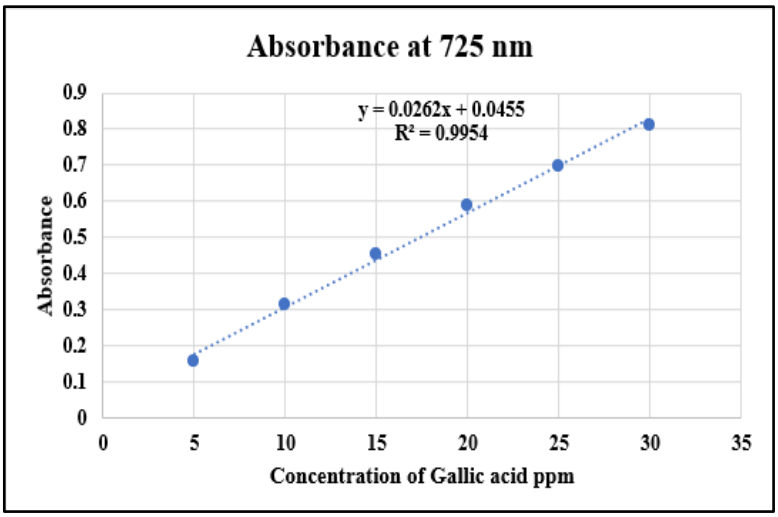


Figure 12: Calibration curve of gallic acid

Antioxidant activity of extracts by DPPH method

The DPPH radical scavenging experiment was used to assess the extracts' antioxidant capacity. When DPPH is lowered by antioxidants, it loses its intense purple hue and turns colorless. The scavenging activity of both extracts was concentration-dependent. Table 8 provides detailed results, while Figure 13 and 14 display comparisons with the standard. IC 50 value for RE was found to be 503.111 µg/ml, FE 614.454 µg/ml and for standard ascorbic acid it was found to be 373.293 µg/ml. Both the extracts have moderate antioxidant activity as compared to ascorbic acid.

$$\text{Percent Inhibition} = \frac{\text{Abs of control - Abs of test}}{\text{Abs of control}} \times 100$$

Table 8: Percent inhibition for standard and extracts

Concentration	Ascorbic acid	Rosemary leaf extract	Fenugreek seed extract
200	28.45%	36.90%	34.12%
400	53.19%	41.79%	42.11%
600	64.02%	59.57%	49.15%
800	75.58%	67.50%	55.12%
1000	81.24%	87.50%	73.8%

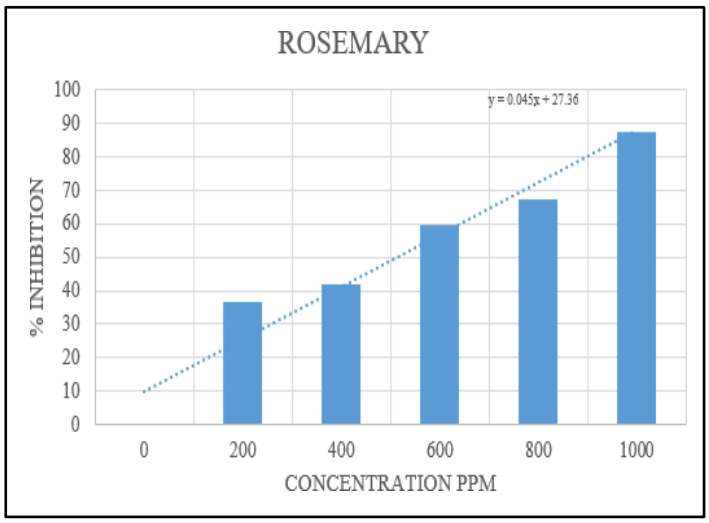


Figure 13: Antioxidant activity of RE

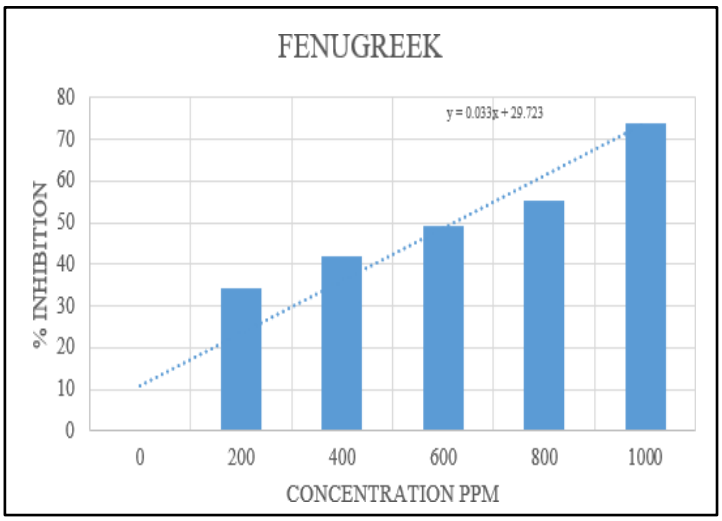


Figure 14: Antioxidant activity of FE

Antimicrobial activity

When tested against Staphylococcus aureus, zone of inhibition shown antibacterial activity. At 1.5% RE, the maximum ZOI was 10.6 mm, but at 1–1.5% FE, it was 5.6 mm. At 1%, the RE and FE combination displayed 11 mm. At 3%, TTO displayed 10.6 mm, which when combined with 1% RE and FE was used.

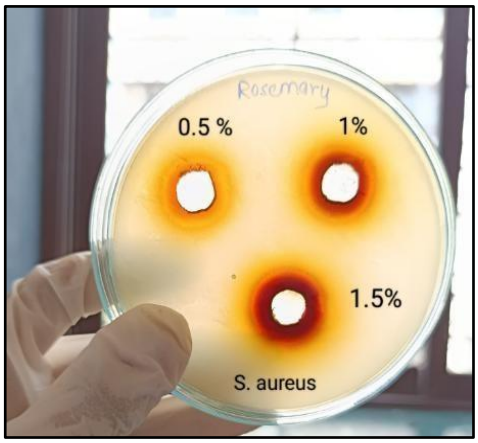


Figure 15: Antimicrobial activity of RE

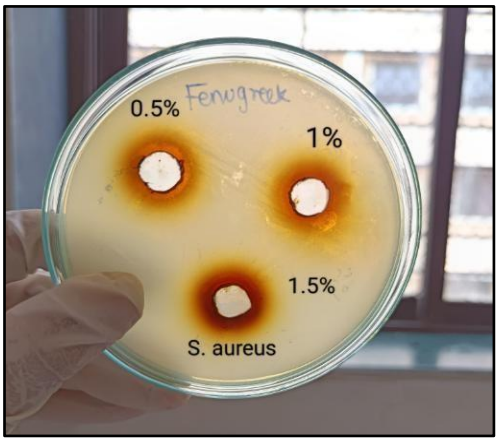


Figure 16: Antimicrobial activity of FE

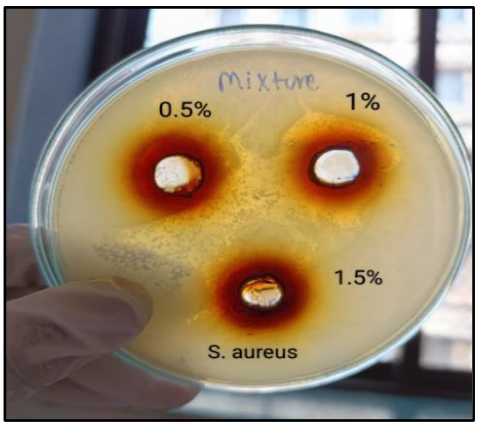


Figure 17: Antimicrobial activity combined extracts

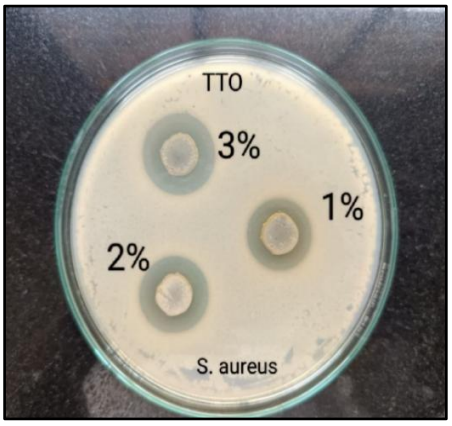


Figure 16: Antimicrobial activity of TTO

Table 9: ZOI of extracts

Concentration	Zone of Inhibition (mm)		
	Rosemary extract	Fenugreek extract	Combined extract
0.5%	8.0	5.0	8.3
1%	9.3	5.6	11
1.5%	10.6	5.6	10.6

Evaluation of Hydrogel

Physical evaluation

The hydrogel exhibited a brown colour, homogeneous formulation without grittiness with a characteristic odor, indicating acceptable organoleptic properties.



Figure 17: Hydrogel formulation



Figure 18: Grittiness

pH determination

The pH of the prepared formulation was found to be 6.82, which is within the acceptable

range for topical applications.

Determination of Viscosity

The viscosity of the formulation was found to be 10,000 Cps, as measured using a Brookfield

viscometer. This high moderate viscosity indicates a stable, semi-solid consistency suitable for topical scalp application, ensuring prolonged retention on the scalp.



Figure 19: Viscosity measurement by Brookfield viscometer

Spreadability

The spreadability of the formulation was found to be 17 gm.cm/sec, indicating good spreading ability under minimal applied force. This suggests the formulation can be easily applied over the scalp affected area, enhancing patient compliance.

Drug Content

The drug content of the formulation was found to be 84.88% for RE and 78.9% for FE, indicating efficient incorporation of the active ingredients into the hydrogel matrix.

In-vitro drug release

The in vitro drug release profile of the formulated topical hydrogel containing RE and FE was evaluated which is used for the treatment of dandruff. The study was performed using a Franz diffusion cell with a synthetic cellophane semipermeable membrane to simulate skin permeation conditions.



Figure 20: In-vitro release study of hydrogel

Table 10: In-vitro release study of hydrogel

Time(min)	% Drug Release extract	% Drug Release Fenugreek extract
0	3.44	1.64
5	6.75	4.72
15	14.05	16.51
20	19.07	24.87
25	28.52	31.81
30	34.31	49.67
60	55.62	53.47
90	66.92	61.97
120	74.45	65.25
150	81.47	71.42
180	87.88	75.60

Rosemary extract (RE) and fenugreek extract (FE) exhibited **87.88%** and **75.60%** drug release respectively within 180 minutes, indicating efficient and sustained release profiles for both herbal components.

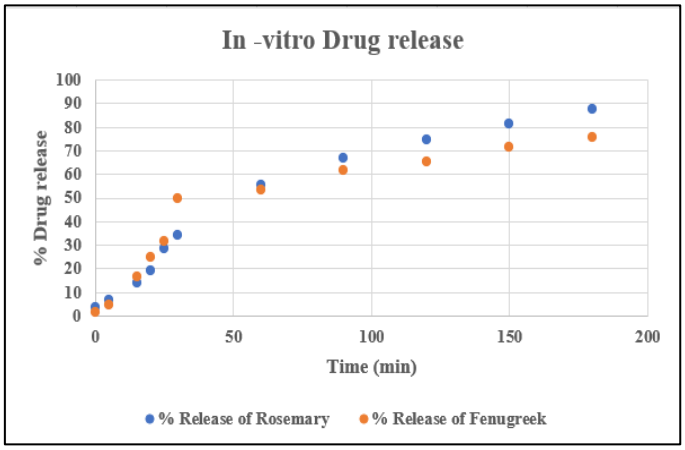


Figure 21: Graph of In-vitro drug release study of hydrogel

Antioxidant activity

In the present investigation, DPPH, a persistent free radical, interacts with antioxidants to change color, and the antioxidant activity is directly correlated with the absorbance reduction. Figure 22 displays the hydrogel antioxidant activity as determined by the DPPH experiment. IC 50 value for hydrogel was found to be 570.631 µg/ml

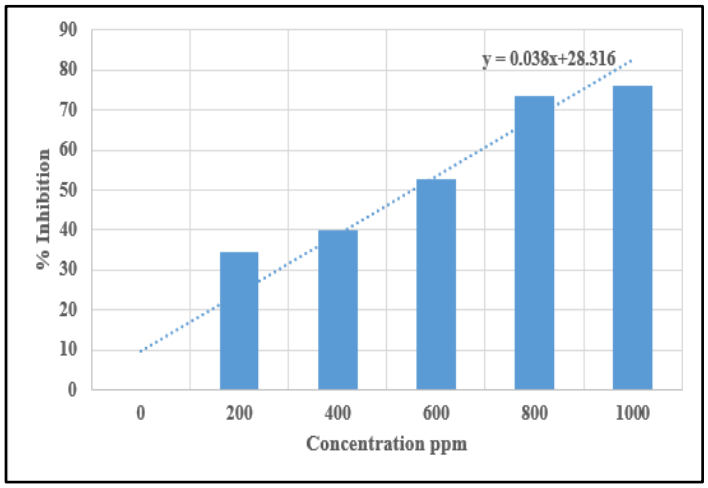


Figure 22: Antioxidant activity of hydrogel

Antifungal activity

Hydrogel's antifungal efficacy against Candida albicans was demonstrated via agar well diffusion. The inhibition zone of the prepared hydrogel was 17.09 mm, whereas the blank gel showed none. The commercial formulation measured 22.02 mm. The findings support hydrogel's strong antifungal properties, indicating that it could be a viable substitute for current treatments.

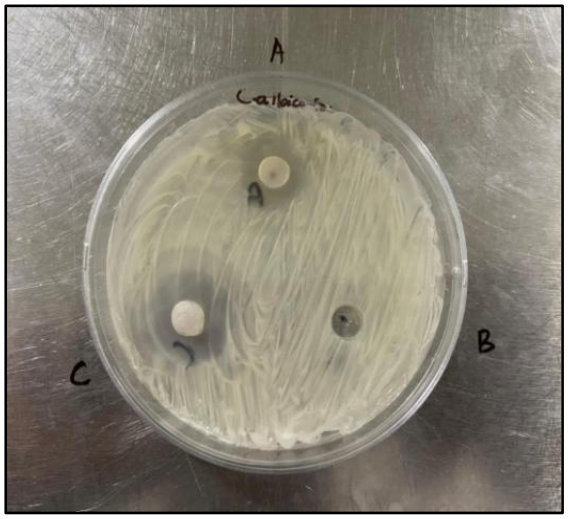


Figure 23: Antifungal activity of hydrogel

Drug excipient Interference study of hydrogel

Formulated gel and placebo gel was diluted with phosphate buffer pH 4.75 and scanned the above solutions using UV spectrophotometer. It was observed that there was no interfering peak with both extracts, indicating no drug-excipient interference.

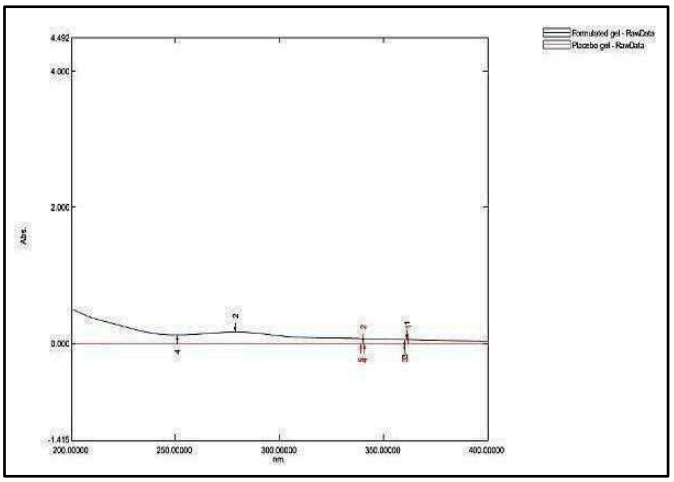


Figure 24: Overlay spectra

OPTIMIZATION OF NANOEMULSION BY USING 2^3 FACTORIAL DESIGN

2^3 Factorial design

Table 11 represents independent variables of the formulation

Independent variables	Levels	
	Low (-)	High (+)
Tween 80	3	5
Tea tree oil	1	3
Mixing speed	500	1000

Dependent variables: Particle size, Zeta potential and Polydispersity index

Table 12 represents the experimental runs (Formulations) given by DOE software and their obtained responses

Run	Factor 1 A: Tween 80	Factor 2 B: Tea tree oil	Factor 3 C: Mixing speed	Response 1 Particle size nm	Response 2 Zeta potential mV	Response 3 PDI
F1	3	1.5	1000	39.5	-41.6	0.469
F2	3	3	500	27.5	-54.2	0.668
F3	5	1.5	500	32.3	-45.6	0.277
F4	3	1.5	500	20.1	-56.6	0.402
F5	5	1.5	1000	18.7	-40.6	0.421
F6	5	3	1000	31.7	-52	0.599
F7	5	3	500	66.4	-45.1	0.539
F8	3	3	1000	51.2	-60.3	0.565

Effect of independent variables on responses

Particle size (R1)

A coded polynomial equation was created to evaluate the impact of the formulation factor on particle size.

Coded equation: (R1: Particle size) 35.93 + 8.27B + 3.50AB − 11.43AC.

Particle size increased with increasing concentration of tea tree oil (B). Particle size decreased as a result of the size-decreasing effect of the interaction between Tween 80 and mixing speed (AC). Particle size rose slightly when Tween 80 and tea tree oil (AB) were increased combined due to their interaction.

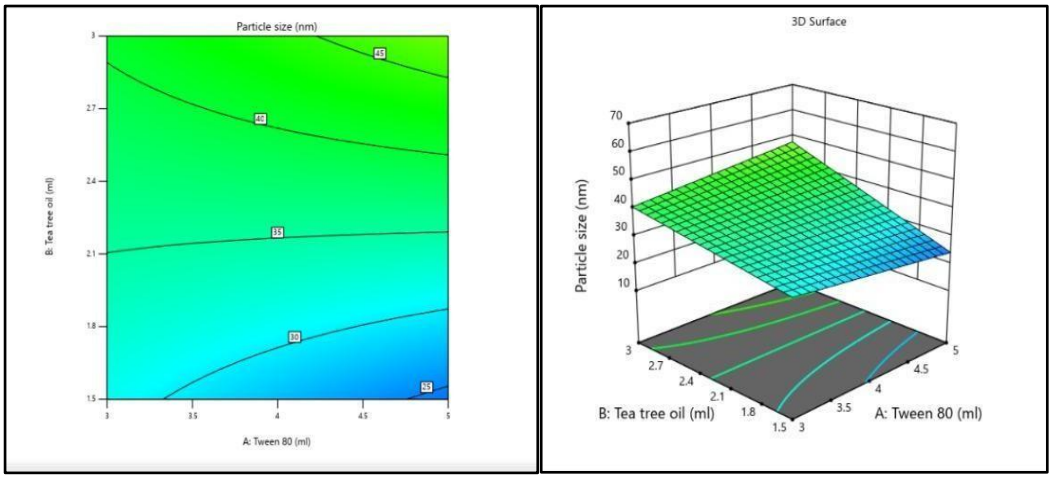


Figure 25: 2D contour and 3D surface plot of particle size

Zeta potential (R2)

A coded polynomial equation was created to evaluate the impact of the formulation factor on zeta potential.

Coded equation: (R2: Zeta potential) -50.12 + 4.30 A - 4.03 B - 4.75 BC

As Tween 80 (A) concentration rose, zeta potential dropped. At greater concentrations, tea tree oil (B) raised zeta potential. Zeta potential was further raised by the combination of tea tree oil and mixing speed (BC) when both were raised simultaneously, demonstrating a favorable combined effect on zeta potential.

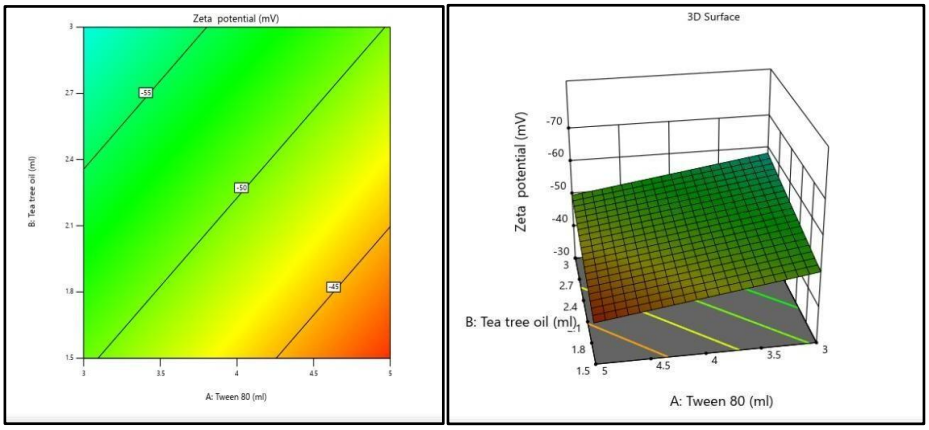


Figure 26: 2D contour and 3D surface plot of zeta potential

Polydispersity index

A coded polynomial equation was created to evaluate the impact of the formulation factor on PDI.

Coded equation: (R3: PDI) 0.4925 + 0.1003 × B

The concentration of tea tree oil (B) raised PDI, indicating that greater oil levels resulted in a less homogeneous emulsion and a wider variety of particle sizes. PDI was not significantly impacted by other variables such as Tween 80 (A), mixing speed (C), or their interactions. More consistent and stable nanoemulsion droplets were obtained with a lower concentration of tea tree oil.

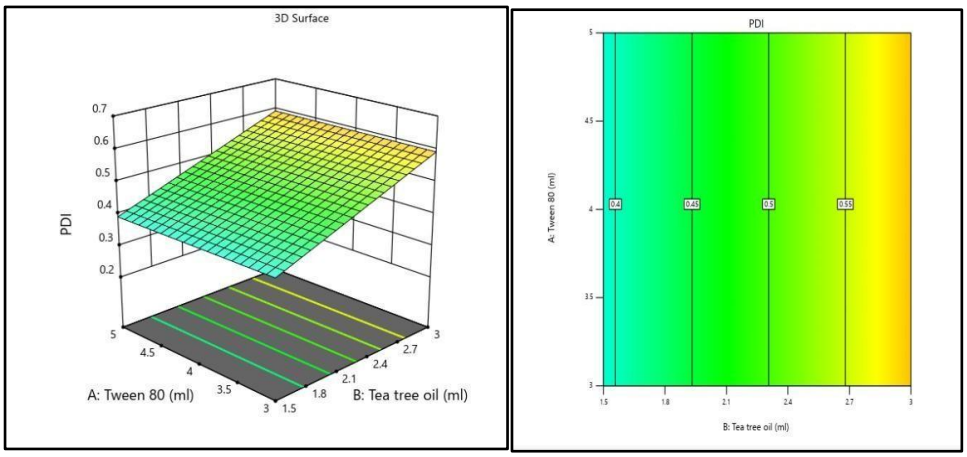


Figure 27: 2D contour and 3D surface plot of PDI

Interpretation of Overlay plot

In order to guarantee product quality, design space analysis demonstrated how formulation and process variables interact. The ideal parameters were determined to be 500 rpm mixing speed, 3 ml tea tree oil, and 5 ml Tween 80 using Design Expert software. With an anticipated particle size of 59.125 nm, a zeta potential of -46.875, and a PDI of 0.593, this combination satisfied the necessary standards for drug delivery and nanoemulsion stability

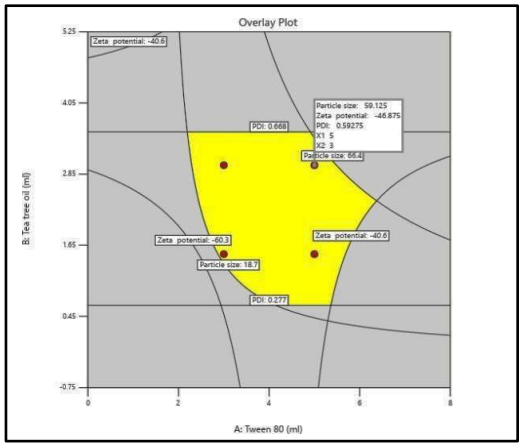


Figure 28: Overlay plot

Characterization of optimized NE formulation



Figure 29: Nanoemulsion formulation

Particle size

The particle size of the optimized NE was found to be 65.2 nm which reflects significant reduction in particle size, aligning with the desired criteria for efficient drug delivery. This result was very close to the predicted value from the optimization model, confirming the accuracy of the optimization process.

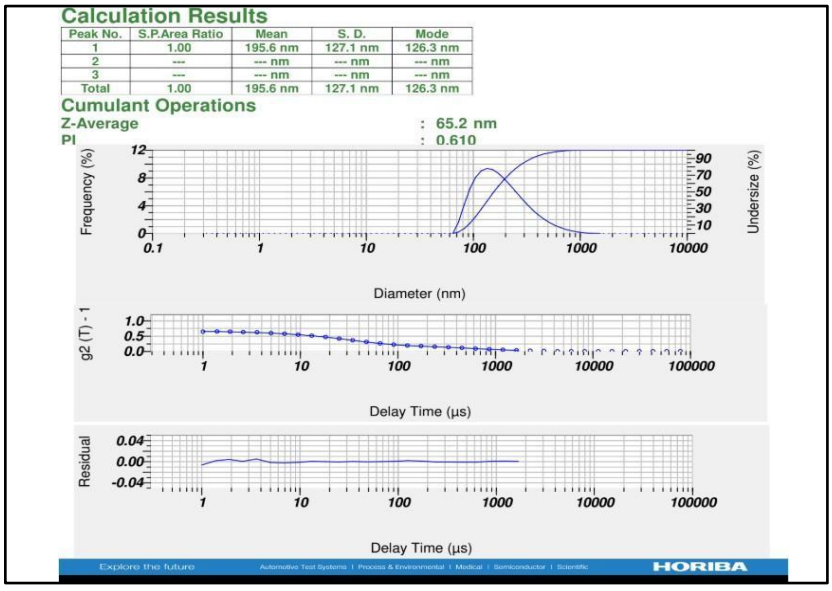


Figure 30: Graph of particle size

Polydispersity Index

The PDI represents the size populations within the given sample. It measures the degree of heterogeneity in particle size. The PDI value less than 1.0 represents a narrow size distribution (high uniformity). The PDI for optimized NE was found to be 0.610 lies within the range indicating consistent size distribution.

Zeta potential

Higher zeta potential values enhance electrostatic repulsion and inhibit particle aggregation, reflecting dispersion stability. Excellent stability is indicated by values of ±30 mV or above. Strong repulsive forces and good colloidal stability within the design space were confirmed by the optimized nanoemulsion −48.2 mV.

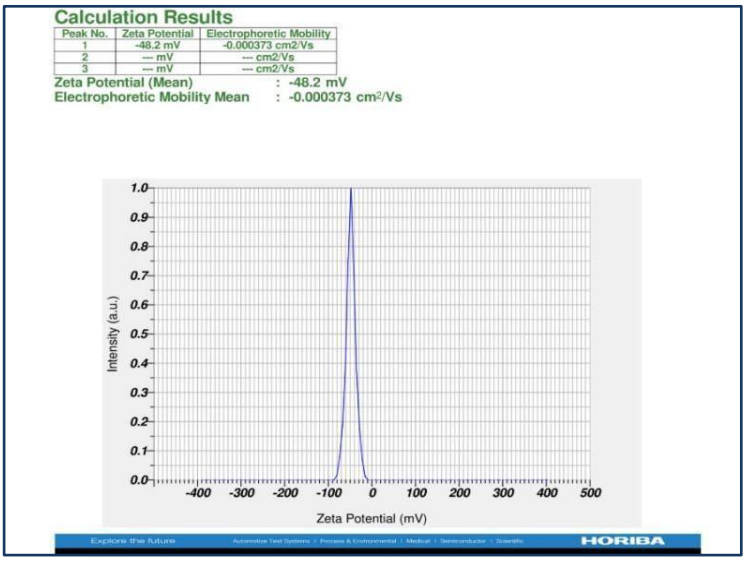


Figure 31: Graph of zeta potential

Thermodynamic stability

Freeze thaw cycle: No phase separation was observed after subjecting the NE at two different temperatures i.e, RT and freezing temp for 3 cycles.

Centrifugation test: No phase separation was observed after subjecting the NE at 2000 rpm for 30 minutes.

pH determination

It was discovered that the NE's pH was 5.53. The slightly acidic pH is suitable for scalp application which maintains skin compatibility.

Viscosity

It was found to be 80 Cps. The low viscosity supports easy spreadability and uniform application on the scalp making it ideal for NE based formulations.

Dye solubility test

The formulation exhibited a uniform dispersion of methylene blue dye throughout the sample. This suggests that the dye, being water-soluble, was well distributed in the continuous phase. Such behaviour confirms the oil-in- water (O/W) nature of the NE formulation.

Dilution test

The formulation remained uniform and stable with no signs of separation. The formulation is stable upon dilution confirming its O/W nature and suitability for aqueous environments.

Filter paper test

Even after 30 minutes after applying a drop of the NE to the surface, no greasy ring was seen. This suggests that the oil phase is evenly distributed throughout the formulation and has high stability. The lack of phase separation attests to the homogeneity and successful emulsification.

Drug content

The drug content of rosemary and fenugreek extracts in NE was found to be 85.55%, 79.9% indicating

efficient encapsulation and uniformity in the formulation.

In-vitro drug release

The in vitro drug release profile of the formulated NE containing RE and FE was evaluated which is used for the treatment of dandruff. The study was performed using a Franz diffusion cell with a synthetic cellophane semipermeable membrane to simulate skin permeation conditions.

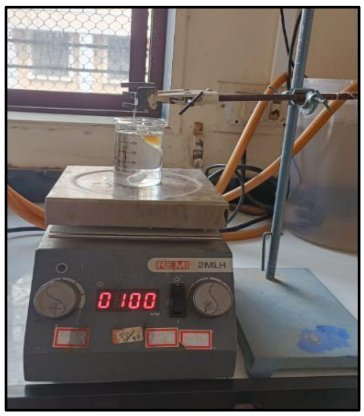


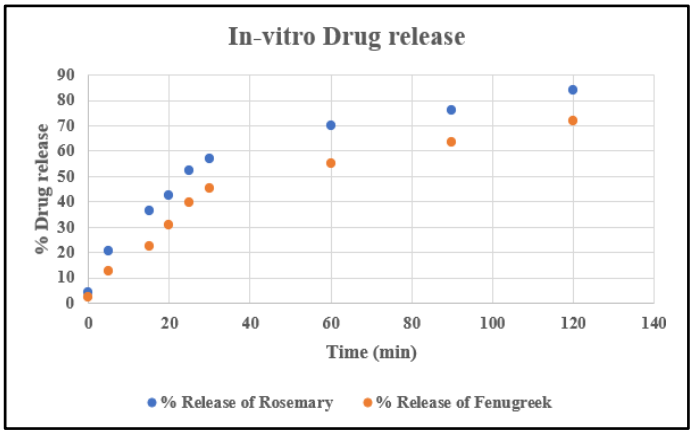
Figure 32: In-vitro drug release study of NE

Table 13: In-vitro drug release of NE

Time(min)	% Drug Release extract	% Drug Release Fenugreek extract
0	4.18	2.14
5	20.33	12.42
15	36.56	22.44
20	42.5	30.74
25	52.51	39.75
30	56.97	45.34
60	70.05	55.21
90	75.95	63.37
120	84.22	71.67

RE and FE exhibited 84.22% and 71.67% drug release respectively within 120 minutes, indicating efficient and sustained release profiles for both herbal components.

Figure 33: Graph of In-vitro drug release of NE



Antioxidant activity

The antioxidant activity of NE was evaluated using the DPPH (2,2-diphenyl-1-picrylhydrazyl) test, which measures an antioxidant's ability to scavenge free radicals. In this experiment, DPPH, a persistent free radical, interacts with antioxidants to change color, and the antioxidant activity is directly correlated with the absorbance reduction. Figure 34 displays the NE antioxidant activity as determined by the DPPH experiment. IC 50 value of NE was found to be 551.75 µg/ml.

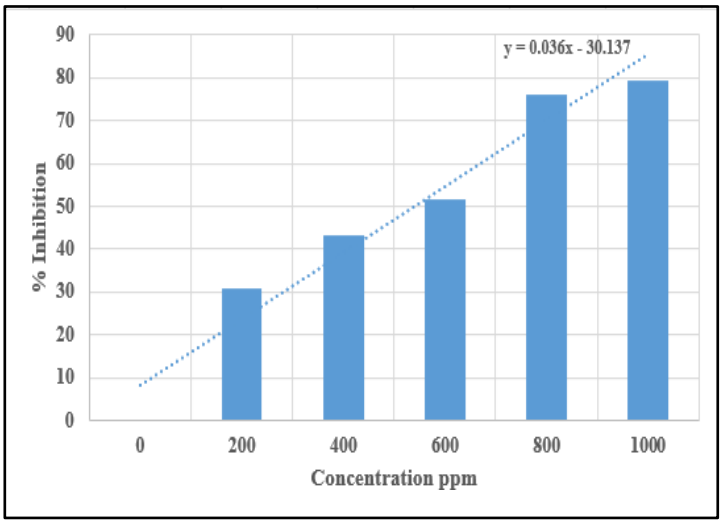


Figure 34: Antioxidant activity of NE

Antifungal activity

The agar well diffusion technique was used to test the NE's antifungal efficacy against Candida albicans. Using conventional procedures, the investigation was conducted at an outside microbiological lab. 1 (formulated NE), 2 (blank), and 3 (marketed formulation) were the three samples that underwent testing as shown in fig. 5.40. Formulated NE showed significant ZOI of 17.51 mm whereas blank showed no ZOI and marketed formulation showed greater ZOI.

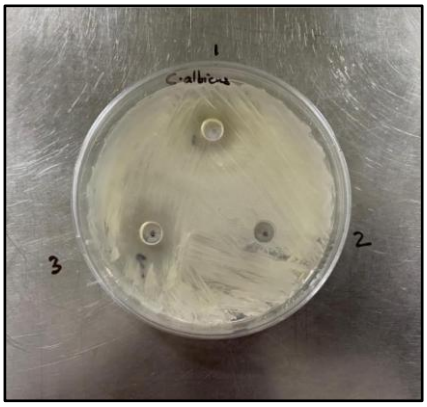


Figure 35: Antifungal activity of NE

Drug excipient Interference study

Formulation and placebo nanoemulsion were diluted with phosphate buffer pH 4.75 and scanned the above solutions using UV spectrophotometer. It was observed that there was no interfering peak with both extracts, indicating no drug-excipient interference.

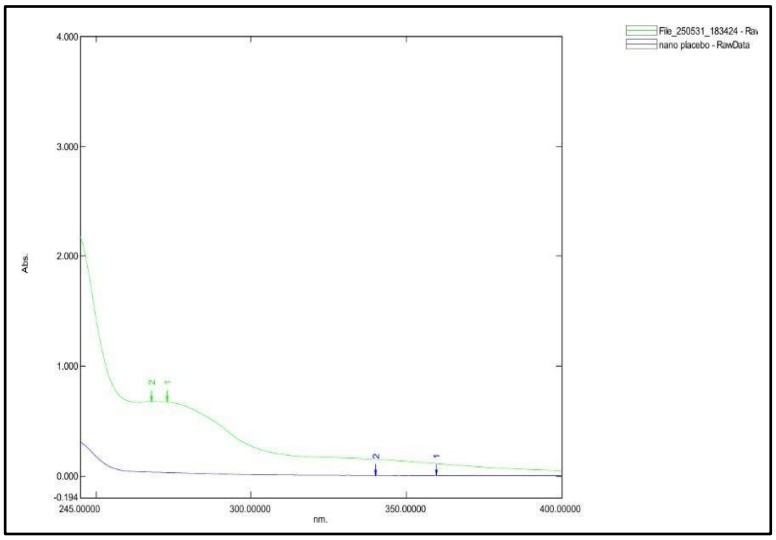


Figure 36: Overlay spectra

Stability study

Stability study was carried out for hydrogel at Refrigerated temperature (5°C± 3°C) and accelerated temperature (40± 2°C;75 ±5% RH) and for NE at Refrigerated temperature (5°C± 3°C), room temperature (25± 2°C;60 ±5% RH) and accelerated temperature (40± 2°C;75 ±5% RH). The results indicated that hydrogel was not stable at RT it showed microbial growth, while it was stable at refrigerated conditions with less variations in their physical characteristics such as pH, viscosity, spreadability and drug content. NE remained stable under all conditions with less variations in their particle size, zeta potential and PDI over the study period of one month.

Table 14: Stability study of Hydrogel

Parameters	Storage condition	0 day	1 month	1 month 10 days
Visual appearance	5°C ± 3°C	No change	No change	No change
	40± 2 °C/ 75% ± 5%	No change	Microbial growth	
Viscosity	5°C ± 3°C	10,000 Cps	9,900 Cps	9,900 Cps
	40± 2 °C/ 75% ± 5%	10,000 Cps	-	-
Spreadability	5°C ± 3°C	17 gm.cm/sec	17.23 gm.cm/sec	17.26 gm.cm/sec
	40± 2 °C/ 75% ± 5%	17 gm.cm/sec	-	-
pH	5°C ± 3°C	6.82	6.89	6.88
	40± 2 °C/ 75% ± 5%	6.82	-	-
Drug content	5°C ± 3°C	84.88%, 78.9%	85.12%, 79.66%	85.38%, 79.12%
	40± 2 °C/ 75% ± 5%	84.88%, 78.9%	-	-

Table 15: Stability study of NE

Parameters	25°C ± 2°C 60% ± 5% RH	40± 2 °C 75% ± 5% RH	5°C ± 3°C	Result
Appearance	Brown	Brown	Brown	Stable
pH	5.53	5.20	5.46	Stable
Particle size	65.2 nm	87.5 nm	46.7 nm	Stable
PDI	0.610	0.624	0.670	Stable
Zeta potential	-48.2 mV	-56.2 mV	-30.9 mV	Stable

CONCLUSION

The herbal hydrogel and nanoemulsion that were formulated showed good antifungal and antioxidant activities in addition to satisfactory stability, which qualified them for patient-friendly scalp treatment. Better stability, sustained release, and higher antibacterial protection were demonstrated by the nanoemulsion, which could contribute to longer-lasting alleviation. Tea tree oil, fenugreek seed, and rosemary leaf extracts work together to provide a safe, all-natural remedy. With consistent usage, this mixture may help enhance scalp health, eliminate dandruff, and regulate microbial growth. Overall, the study indicates that for individuals looking for herbal therapeutic choices, this formulation based on herbal nanoemulsion may be a convenient, safe, and effective substitute for traditional dandruff treatments.

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