

OPTIMISATION OF *AVICENNIA MARINA* HEARTWOOD EXTRACT NANOEMULSION USING BOX-BEHNKEN DESIGN: FORMULATION CHARACTERISTICS AND ANTIFUNGAL EVALUATION

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ABSTRACT

Objective: This study aimed to develop a practically suitable *Avicennia marina* heartwood extract nanoemulsion and evaluate its physicochemical characteristics, stability, and anti-dandruff activity.

Methods: The heartwood extract was obtained by maceration using 96% ethanol, followed by phytochemical screening. Nanoemulsion formulations were developed using Response Surface Methodology with a box-behnken design to investigate the effects of virgin coconut oil (VCO), Tween 80, and sorbitol on viscosity and particle size. The extract concentration was fixed at 0.5%, and one blank formulation was used as a control. Physicochemical evaluation included viscosity, particle size, pH, homogeneity, and stability testing at room temperature. Anti-dandruff activity was assessed against *Pityrosporum ovale* using the disk diffusion method on potato dextrose agar.

Results: The viscosity model was statistically significant ($p < 0.05$), with Tween 80 and its interaction with VCO identified as dominant factors influencing viscosity. Although the particle-size model did not reach statistical significance, several formulation variables showed meaningful effects on droplet size. A formulation consisting of 1.13% VCO, 15.96% Tween 80, and 2.18% sorbitol produced a nanoemulsion with a viscosity of 1.12 mPa-s and an experimentally confirmed minimal particle size of 51.42 nm within the studied Design space. The selected formulation remained physically stable during storage and exhibited strong anti-dandruff activity, with an inhibition zone diameter of 14.63 mm against *P. ovale*.

Conclusion: This study demonstrates that a practically stable nanoemulsion of *A. marina* heartwood extract can be achieved through formulation-guided optimisation, yielding favourable physicochemical properties and effective anti-dandruff activity, thereby supporting its potential application in topical anti-dandruff formulations.

Keywords: *Avicennia marina*, Nanoemulsion, Box-behnken design, Anti-dandruff activity, Formulation optimization

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INTRODUCTION

Dandruff is a common scalp disorder characterised by excessive flaking, pruritus, and inflammatory responses, which may adversely affect scalp health and quality of life. One of the primary factors contributing to dandruff is the overgrowth of scalp-associated microorganisms, particularly *Pityrosporum ovale*, a lipophilic fungus that exists typically as part of the scalp microflora but can proliferate excessively under conditions of increased sebum production and altered scalp homeostasis. The management of dandruff, therefore, requires strategies that control fungal proliferation while maintaining scalp compatibility [1-3].

Mangrove plants have attracted increasing attention as sources of bioactive compounds with antifungal and anti-inflammatory potential. Among them, *Avicennia marina* (Forssk.) Vierh, a mangrove species widely distributed in coastal regions, has been reported to possess diverse pharmacological activities. The heartwood of *A. marina* contains secondary metabolites, such as flavonoids and terpenoids, which exhibit antifungal and antihistaminic effects. In addition, extracts of *A. marina* have demonstrated notable antioxidant activity, suggesting a potential role in mitigating scalp inflammation associated with oxidative stress. These properties indicate that *A. marina* heartwood extract may serve as a promising natural active ingredient for anti-dandruff formulations [4-7].

To enhance the delivery and performance of plant-derived bioactive compounds, nanoemulsion-based systems have been widely explored in pharmaceutical and cosmetic applications. Nanoemulsions are transparent or translucent dispersions with droplet sizes typically ranging from 20 to 500 nm, characterised by high kinetic stability and large interfacial surface area. Such systems

can improve the solubility of poorly water-soluble compounds, facilitate penetration into the skin, and enhance bioavailability [8, 9]. Moreover, nanoemulsions are generally regarded as non-toxic, capable of stabilising sensitive active compounds, and suitable for scalable production, making them attractive carriers for topical delivery of herbal extracts. Previous studies have shown that nanoemulsion-based extract formulations exhibit faster, more efficient penetration than conventional extract preparations [10, 11].

The development of an effective nanoemulsion formulation requires systematic optimisation of its constituent components, as formulation variables may interact and collectively influence critical quality attributes. Response surface methodology using Box-Behnken Design is a statistical optimisation approach that enables the evaluation of main effects and interaction effects among formulation variables with a relatively small number of experimental runs. This design provides an efficient and reliable means of understanding factor-response relationships and is considered superior to conventional one-factor-at-a-time approaches that fail to capture interaction effects [12-14].

Therefore, this study aimed to optimise the formulation of an *Avicennia marina* heartwood extract nanoemulsion using a Box-Behnken Design, evaluating the effects of oil phase, surfactant, and cosurfactant concentrations on physicochemical characteristics and anti-dandruff activity.

MATERIALS AND METHODS

Research materials

This study employed an experimental design to evaluate the effects of formulation variables, namely the oil phase, surfactant, and cosurfactant, on the physicochemical characteristics (viscosity and

particle size) and anti-dandruff activity of a nanoemulsion containing *Avicennia marina* (Forssk.) Vierh. Heartwood extract. The study was conducted at the Phytochemical Laboratory, Cosmeceutical Laboratory, and Physical Pharmacy Laboratory of the Faculty of Pharmacy, Universitas Sumatera Utara. The research process involved maceration extraction of *A. marina* heartwood using 96% ethanol, followed by phytochemical screening and characterisation of the crude extract. Nanoemulsion formulations were developed using a Box-Behnken Design with varying component concentrations, employing 0.5% extract as the active phase and a blank formulation as a control. Evaluations included viscosity, particle size, and physical stability under room temperature conditions, while anti-dandruff activity was assessed against *Pityrosporum ovale* using the disk diffusion method on Potato Dextrose Agar (PDA).

Chemicals and reagents used included distilled water; DPPH (Sigma-Aldrich, St. Louis, MO, USA); 96% ethanol; *A. marina* heartwood; butylated hydroxytoluene (BHT); analytical-grade ethanol and vitamin C (Merck, Darmstadt, Germany); buffer solutions (pH 4 and pH 7); Potato Dextrose Agar (PDA); propylene glycol; sorbitol; Tween 80; and virgin coconut oil.

The main instruments and software used were an NDJ-8S viscometer, a particle size analyser (Fritsch Analysette 22 NanoTec), Design-Expert® software v13, a rotary evaporator (Heidolph), homogenisers (IKA® RW20 digital and SCILOGEX), a UV-Vis spectrophotometer (UV PROBE 1800), a pH meter (Milwaukee Mi151), an oven (Deo-24), an analytical balance (Fujitsu), and a water bath (Bone).

Heartwood samples of *A. marina* were collected from the Pangkalan Susu coastal area, North Sumatra Province, Indonesia. Botanical identification was confirmed, and a voucher specimen was deposited at Herbarium Medanense (voucher No. 2522/MEDA/2024). Fresh heartwood was cleaned to remove adhering impurities, weighed to determine wet weight, dried in a drying cabinet until constant weight, reweighed to obtain dry weight, ground into powder, and stored in sealed containers to prevent moisture absorption and contamination. All procedures involving human participants in this study were conducted in accordance with ethical standards and approved by the Health Research Ethics Committee of Universitas Sumatera Utara (Ethical approval No. 284/KEPK/USU/2025).

Characterisation, phytochemical screening, and extraction

Characterisation of *Avicennia marina* heartwood simplisia was conducted to ensure compliance with crude drug quality standards prior to formulation. The characterisation comprised macroscopic and microscopic examinations, determination of moisture content, total ash content, acid-insoluble ash content, and water-soluble and ethanol-soluble extractive values. In addition, phytochemical

screening was performed to qualitatively identify major secondary metabolites, including alkaloids, flavonoids, saponins, tannins, glycosides, and steroids/terpenoids present in the heartwood [15, 16].

For extraction, 300 g of *A. marina* heartwood powder was macerated with 96% ethanol at a 1:5 (w/v) ratio in a closed container for 3 days with occasional stirring. The maceration process was repeated three times to ensure exhaustive extraction. The combined filtrates were subsequently concentrated using a rotary vacuum evaporator at 50 °C until complete solvent removal, yielding a concentrated crude extract for further formulation [15, 16].

Antioxidant activity

Antioxidant activity was evaluated using the IC₅₀ (inhibitory concentration 50) value, which represents the concentration required to scavenge 50% of free radicals. The obtained data were analysed using linear regression, with sample concentration plotted on the X-axis and percentage of free radical scavenging activity on the Y-axis [17–23].

Single-factor evaluation

Preliminary optimisation was conducted to select appropriate surfactant and cosurfactant combinations based on the nanoemulsion's particle size and distribution. Several combinations, including Tween-sorbitol, Tween-PEG, and Tween-Span 80, were evaluated to determine the most effective pair for producing the smallest particle size. This screening step was conducted to support the proper selection of surfactants/cosurfactants and their practical concentrations, as nanoemulsion systems often require high surfactant levels and therefore demand judicious selection of surfactant and cosurfactant composition to achieve desirable attributes. Based on the screening outcomes and formulation feasibility (i. e., ability to form a homogeneous dispersion without phase separation), the factor ranges for the subsequent Box-Behnken Design were set to VCO 1–9%, Tween 80 6–16%, and sorbitol 1–3%, to capture the concentration effects within a practically acceptable design space [24, 25].

Formula design using box-behnken design

Optimisation was performed using Response Surface Methodology (RSM) with a Box-Behnken Design. Three independent variables were investigated: virgin coconut oil (X_1), Tween 80 (X_2), and sorbitol (X_3). The responses optimised were the nanoemulsion's viscosity (Y_1) and particle size (Y_2) (table 1). The applied concentration ranges were constrained by preliminary formulation observations, particularly phase separation and instability at intermediate oil concentrations, which necessitated the use of a practical, non-symmetric factor range. Consequently, the Design space reflects formulation feasibility rather than idealised statistical symmetry [26–28].

Table 1: Variation of variables in the box-behnken design

Symbol	Independent variable	Actual level code		
		-1	0	+1
X1	VCO (%)	1	2	9
X2	Tween 80 (%)	6	9	16
X3	Sorbitol (%)	1	2	3

Preparation of *Avicennia marina* nanoemulsion

The nanoemulsion formulation process began by dissolving concentrated *A. marina* heartwood extract and butylated hydroxytoluene into virgin coconut oil. The mixture was homogenised 259 rpm for 10 min at room temperature (25±2 °C). Subsequently, distilled water and propylene glycol were added gradually in small portions under continuous stirring until a uniform nanoemulsion was obtained, and the mixture was further stirred for an additional 10 min to ensure complete homogenisation. The final batch size was adjusted to a constant total volume of 100 ml for all formulations [29].

Evaluation of nanoemulsion

The nanoemulsion formulations were evaluated through a series of

physicochemical and safety assessments. Organoleptic evaluation was conducted to observe sensory characteristics, including aroma, colour, and consistency, both immediately after preparation and during storage. The emulsion type was determined using methylene blue as an indicator; a uniform distribution of the blue colour indicated an O/W (oil-in-water) emulsion. The pH of the formulations was measured to ensure compatibility with the physiological skin pH, as inappropriate pH levels may induce scalp irritation, itching, or flaking. Homogeneity testing was performed through visual and microscopic observation to confirm uniform dispersion of components and the absence of sedimentation or agglomeration within the preparation. In addition, potential skin irritation was assessed by applying the nanoemulsion directly to the arms of 12 volunteers three times daily for three consecutive days,

both after formulation and at the end of the storage period, with reactions such as redness or itching recorded as indicators of irritation [30, 31].

Characterisation of the nanoemulsion

The nanoemulsion formulations were characterised for viscosity and particle size. Viscosity was measured using an NDJ-8S viscometer by placing 100 ml of the nanoemulsion sample in a sample container and immersing spindle 1 to the marked level prior to measurement; the viscometer speed was adjusted to an appropriate rpm to obtain a stable reading. Measurements were performed in triplicate and reported as mean $\pm$ SD. The viscosity of the nanoemulsion was considered acceptable when it was below 5 mPa.s.

Particle size was measured using a particle size analyser (Fritsch Analysette 22 NanoTec) based on laser light scattering, in which the sample was exposed to laser light and the resulting light scattering was used to calculate particle diameter. For analysis, distilled water was used as the dispersant, and the instrument software dispersant settings were set to refractive index 1.333 and viscosity 0.89 mPa.s. Measurements were conducted in triplicate and reported as mean $\pm$ SD. The nanoemulsion particle size was expected to fall within the 20–500 nm range, consistent with typical nanoemulsion systems [26, 29].

Cycling test of optimal nanoemulsion

The nanoemulsion preparation was stored at 4 °C for 24 h, then transferred to a climatic chamber at 40 $\pm$ 2 °C for 24 h. This process was repeated six times, followed by organoleptic, pH, homogeneity, and phase separation testing [32].

Anti-dandruff activity

The anti-dandruff activity test was conducted by dipping a cotton swab into the fungal culture, then applying it evenly to the surface of the PDA. The paper disc that had been dipped into the nanoemulsion preparation was placed on the medium, then pressed to ensure it adhered perfectly. The plate was incubated at 35 to 37 °C for 1 d, and the inhibition zone for anti-dandruff activity was measured using a digital calliper. The experiment was repeated three times to ensure consistency of results [33, 34].

Optimal formula stability

Stability was tested against the optimal formula, namely, viscosity and particle size. Particle size was measured at weeks 0, 6, and 12, and the samples were stabilised using a particle size analyser. The preparation was stored for 12 w at room temperature [26, 29].

Statistical analysis

Formulation optimisation and response surface analysis were performed using Design-Expert® v13 with a Box–Behnken Design, and ANOVA was used to evaluate the effects of variables and interaction terms on viscosity and particle size, with statistical significance defined at $P < 0.05$ and P -values reported for the overall model and relevant terms. Model adequacy and validation were assessed using lack-of-fit testing, R^2 , adjusted R^2 , predicted R^2 , adequate precision (>4), and coefficient of variation (CV%). At the same time, ANOVA assumptions were checked using residual diagnostics (normal probability plots and residuals versus predicted values), and a Box–Cox transformation was considered when needed. Numerical optimisation was conducted using a desirability function to identify a practically suitable formulation within the investigated design space; responses with non-significant overall models and/or limited predictive performance were interpreted as exploratory for trend assessment and treated as practical

constraints, followed by experimental confirmation of the selected formulation. Anti-dandruff activity (inhibition zone diameters) was analysed using one-way ANOVA, and statistical significance in tables/fig. was indicated using superscripts ($P < 0.05$, $P < 0.01$).

RESULTS AND DISCUSSION

Characterisation, phytochemical profile, and extraction

The processing of *Avicennia marina* (Forssk.) Vierh. Heartwood began with approximately 26 kg of fresh material. After drying, 18 kg of dried simplisia was obtained, which was subsequently ground into powder, yielding a final weight of 12 kg. This reduction in mass reflects the removal of moisture and non-structural components during drying and comminution, which are essential steps to ensure the raw material's stability prior to extraction.

Macroscopic examination was conducted to observe the physical characteristics of the simplisia, including shape, colour, aroma, and taste. As shown in fig. 1, the fresh heartwood of *A. marina* exhibited a circular shape and a greyish-white colour, accompanied by a characteristic bitter taste. These macroscopic features are consistent with previously reported descriptions of *A. marina* heartwood and serve as primary identification parameters for crude drug authentication. Microscopic examination further revealed the presence of complex starch granules, xylem and phloem transport tissues, and structural elements such as rays, fibre lumens, and sclerenchyma, confirming the anatomical integrity of the heartwood material [6, 35, 36].



Fig. 1: Result of macroscopic examination of *Avicennia marina* (Forssk.) Vierh. Heartwood

The quality of the simplisia was evaluated through a series of characterisation tests, with the results summarised in table 2. The moisture content of the crude drug was determined to be 7.32%, which complies with the maximum allowable limit of ≤ 10 . Maintaining low moisture content is critical, as elevated moisture levels may promote enzymatic degradation and microbial growth, thereby reducing crude drug stability. The water-soluble extractive content was 4.86%, indicating the presence of polar constituents, while the ethanol-soluble extractive content reached 10.57%, suggesting that the heartwood contains compounds soluble in both polar and semi-polar solvents. These values are within the acceptable ranges specified in national quality standards. In addition, the total ash content was 2.35%, reflecting the overall mineral content, whereas the acid-insoluble ash content was 0.59%, representing inorganic residues such as silica or heavy metals. The low acid-insoluble ash value indicates minimal contamination by extraneous inorganic matter and is consistent with established crude drug quality requirements [15, 16].

Table 2: Results of simplisia characterisation

Parameters	Results
Moisture content	7.32%
Water-soluble extract content	4.86%
Ethanol-soluble extract content	10.57%
Total ash content	2.35%
Acid-insoluble ash content	0.59%

Phytochemical screening was performed to obtain an initial profile of secondary metabolites present in *A. marina* heartwood, and the results are presented in table 3. The screening demonstrated the presence of alkaloids, flavonoids, tannins, saponins, steroids/triterpenoids, and glycosides. Alkaloids were identified using Mayer, Bouchard, and Dragendorff reagents; positive reactions were indicated by the formation of characteristic precipitates in at least 2 of the 3 tests. Tannins were confirmed by the appearance of a

dark blue colouration, while the formation of a reddish-purple colour indicated steroids and triterpenoids. Glycosides were identified by the appearance of a purple ring in the glycone test, and saponins were confirmed by the formation of stable foam with a height of approximately 1.8 cm. The presence of these metabolite classes supports the potential biological activity of the heartwood extract and provides a chemical basis for its subsequent formulation and anti-dandruff activity [15, 16].

Table 3: Results of phytochemical screening of *Avicennia marina* (Forssk.) Vierh. Heartwood

Chemical compound group	Results
Alkaloids	+
Flavonoids	+
Tannins	+
Saponins	+
Steroids/Triterpenoids	+
Glycosides	+

“+” = detected (positive); “-” = not detected (negative).

Extraction of *Avicennia marina* (Forssk.) Vierh. Heartwood was extracted using the maceration method with 96% ethanol as the extraction solvent. A total of 8.400 g of dried heartwood simplisia was used as the raw material, yielding 348.200 g of a thick, dark brown extract with a characteristic plant aroma. Based on these values, the extraction yield was determined to be 4.14%, which is within the typical range reported for heartwood-derived plant materials. The use of ethanol as the solvent is appropriate for the extraction of polar to semi-polar secondary metabolites, as indicated by the phytochemical screening results. Accordingly, the obtained extract was considered suitable for further formulation into a nanoemulsion delivery system.

Antioxidant activity

The IC₅₀ value of the *Avicennia marina* (Forssk.) Vierh heartwood extract, as shown in table 4, has an IC₅₀ of 48.88 µg/ml, indicating a powerful antioxidant activity. According to previous research, antioxidant testing of *Avicennia marina* (Forssk.) Vierh. Leaves yielded

an IC₅₀ value of 5.43 µg/ml. Based on this study, the IC₅₀ value falls within the same category; however, the difference in antioxidant activity is attributed to the use of different solvents in the analysis, as methanol has greater reactivity with DPPH than ethanol, ethyl acetate, or isopropanol. Therefore, the IC₅₀ value obtained may differ from those using methanol as the solvent. However, the differences in antioxidant values are not significantly different, and still fall within the range of very strong antioxidants [17–23].

Single-factor evaluation of surfactant-cosurfactant combinations

The results of the single-factor evaluation examining the influence of surfactant-cosurfactant combinations on nanoemulsion particle size are presented in table 5. Among the combinations tested, Tween 80/sorbitol produced the smallest particle size, measuring 281 nm, whereas other combinations, such as Tween 80–PEG 40, Tween 80–Span 80, and Span 80–PEG 40, resulted in substantially larger particle sizes ranging from 600 to 700 nm.

Table 4: Antioxidant activity of *Avicennia marina* (Forssk.) Vierh. Heartwood

Test solution	IC ₅₀	Antioxidant intensity
<i>Avicennia marina</i> heartwood extract	48.88 µg/ml	Very strong
Vitamin c	4.8 µg/ml	Very strong

Table 5: Single-factor evaluation of surfactant-cosurfactant combinations on nanoemulsion particle size

Surfactant	Cosurfactant	Particle size (nm)
Tween 80	Sorbitol	281 nm
Tween 80	PEG 40	700 nm
Tween 80	Span 80	600 nm
Span 80	PEG 40	700 nm

As shown in table 5, the combination of Tween 80 and sorbitol produced nanoemulsion droplets with particle sizes below 500 nm, meeting the general requirements for topical nanoemulsion preparations. This finding indicates that the Tween 80/sorbitol system provides a more favourable interfacial environment for nanoemulsion formation compared with the other combinations evaluated. The observed effect can be attributed to the complementary physicochemical properties of the two components. Tween 80, with a high hydrophilic-lipophilic balance (HLB) value of approximately 15, is highly hydrophilic and effective in reducing interfacial tension, thereby limiting droplet coalescence during the early stages of nanoemulsion formation. In addition, Tween 80 is widely regarded as non-toxic and suitable for topical applications. Sorbitol, on the other hand, enhances the solubility of the aqueous phase, contributes to an increase in the viscosity of the dispersion medium, and exhibits good

physicochemical stability, which collectively support the formation of smaller and more uniform droplets. The synergistic interaction between Tween 80 and sorbitol, therefore, explains the superior performance of this combination and justifies its selection for subsequent optimisation using a box-behnken design [37–39].

Optimisation of nanoemulsion formulation using box-behnken design

The Box-Behnken Design was applied to optimise the nanoemulsion formulation of *Avicennia marina* (Forssk.) Vierh. Heartwood extract by evaluating the combined effects of virgin coconut oil (VCO, X₁), Tween 80 (X₂), and sorbitol (X₃) on viscosity (Y₁) and particle size (Y₂). The factor ranges were determined from preliminary formulation trials to ensure nanoemulsion formation and system stability, while the centre point (5% VCO, 11% Tween 80, and 2%

sorbitol) was replicated to estimate experimental error and assess formulation reproducibility. The experimental Design matrix and corresponding responses are presented in table 6, which summarises 17 experimental runs generated by the Behnken Design.

The viscosity values ranged from 1.14 to 1.52 mPa·s, while particle sizes ranged from 57 nm to 380.66 nm, indicating that variations in formulation components significantly influenced the nanoemulsion's physicochemical characteristics [26–28].

Table 6: Box behnken design results

Run	Factor 1 VCO (%)	Factor 2 tween 80 (%)	Factor 3 sorbitol (%)	Response 1 viscosity (mPa·s)	Response 2 particle size (nm)
1	5	11	2	1.28	280.66
2	5	11	2	1.27	277
3	1	16	2	1.14	57
4	5	11	2	1.26	232.33
5	9	11	3	1.36	353.33
6	5	11	2	1.23	217.33
7	1	11	1	1.14	109.66
8	5	6	3	1.3	282
9	1	11	3	1.14	89.66
10	9	16	2	1.34	299
11	5	6	1	1.31	291.33
12	1	6	2	1.16	137
13	5	16	3	1.19	137
14	5	16	1	1.22	173.33
15	5	11	2	1.23	181.33
16	9	11	1	1.44	371
17	9	6	2	1.52	380.66

The statistical significance of the models was evaluated using analysis of variance (ANOVA), as shown in table 7. For viscosity, the model was statistically significant ($p = 0.0079$), indicating that the selected independent variables adequately explained the response variability. Tween 80 (B) and the interaction between VCO and Tween 80 (AB) exhibited significant effects on viscosity ($p < 0.01$), while the interaction between VCO and sorbitol (AC) also showed a significant contribution ($p < 0.05$). In contrast, VCO (A) and sorbitol (C) did not demonstrate

significant independent effects when considered individually. These results indicate that viscosity is primarily influenced by the surfactant concentration and its interaction with the oil phase rather than by single-factor effects alone. The 2FI model for viscosity, as suggested by Design-Expert, exhibited an adequate signal-to-noise ratio (Adeq Precision > 4) and a low coefficient of variation, supporting its suitability for navigating the formulation Design space, despite the negative predicted R^2 value [26–28].

Table 7: Analysis of variance (ANOVA) models of responses

Response	Source	Sum of squares	df	Mean square	F-value	p-value
Viscosity (Y_1)	Model	0.1411	6	0.0235	5.77	0.0079
	A (VCO)	0.0005	1	0.0005	0.123	0.7331
	B (Tween 80)	0.0708	1	0.0708	17.37	0.0019
	C (Sorbitol)	0.0077	1	0.0077	1.9	0.1981
	AB	0.0696	1	0.0696	17.1	0.002
	AC	0.0231	1	0.0231	5.67	0.0386
	BC	0.006	1	0.006	1.46	0.2544
	Residual	0.0407	10	0.0041	–	–
	Cor Total	0.1818	16	–	–	–
Particle size (Y_2)	Model	1.03E+05	6	17177.43	2.99	0.061
	A (VCO)	6898.31	1	6898.31	1.2	0.2988
	B (Tween 80)	63365.9	1	63365.9	11.04	0.0077
	C (Sorbitol)	13339.53	1	13339.53	2.32	0.1584
	AB	49745.7	1	49745.7	8.66	0.0147
	AC	32530.05	1	32530.05	5.67	0.0386
	BC	3974.49	1	3974.49	0.6921	0.4249
	Residual	57422.54	10	5742.25	–	–
	Cor Total	1.61E+05	16	–	–	–

Information: A = VCO (%), B = Tween 80 (%), C = Sorbitol (%). Significance threshold: $p < 0.05$.

Regarding particle size, the ANOVA results in table 7 indicate that the 2FI model did not reach statistical significance ($p = 0.0610$). However, Tween 80 (B) and the interaction between VCO and Tween 80 (AB) showed significant effects on particle size ($p < 0.05$), while the interaction between VCO and sorbitol (AC) also contributed significantly ($p < 0.05$). These findings suggest that although the overall model did not reach statistical significance, specific formulation variables and their interactions played important roles in controlling droplet size formation within the nanoemulsion system. Given the negative predicted R^2 value, particle size was not interpreted as a fully inferential optimisation

response but was instead treated as a practical formulation constraint within the desirability function and subsequently assessed through experimental confirmation at the selected formulation point [30, 31].

In this study, the contour shading is used to visualise response gradients across the evaluated formulation space rather than to define a regulatory design space region. The effects of combined formulation variables on viscosity and particle size are illustrated in the contour plots presented in fig. 2. These contour plots were employed to visualise formulation trends and interaction patterns rather than to establish predictive optimisation surfaces. As shown

in fig. 2A–C, an increase in VCO concentration resulted in a corresponding increase in viscosity, while an increase in Tween 80 concentration also increased viscosity, although to a lesser extent. This observation is consistent with previous findings that oil content

strongly influences emulsion viscosity by increasing the dispersed-phase volume fraction and promoting a denser droplet structure. Higher oil concentrations increase resistance to flow, leading to thicker emulsion systems [30, 31].

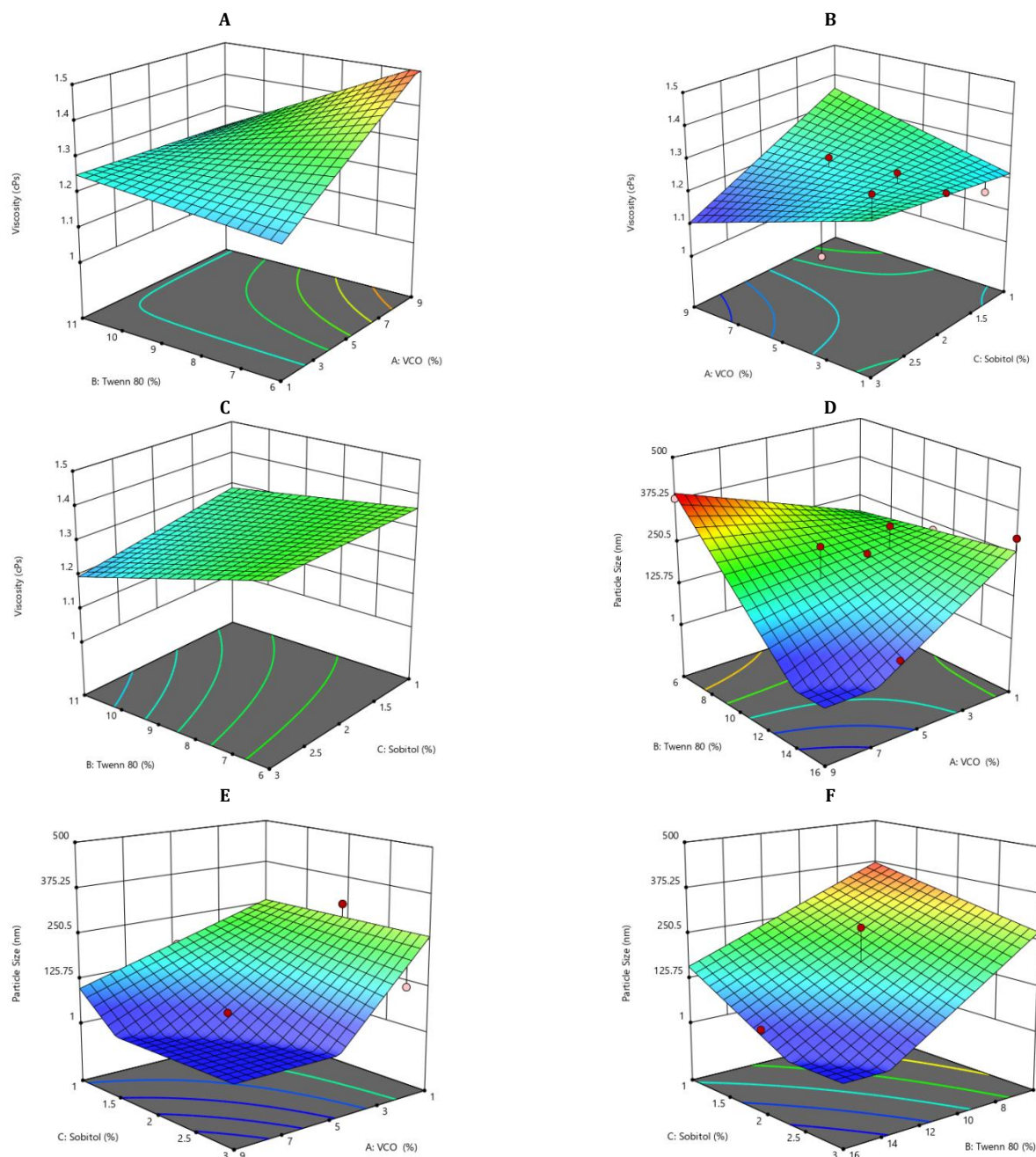


Fig. 2: Contour plots generated using design-expert showing the combined effects of X_1 (VCO, % w/w), X_2 (Tween 80, % w/w), and X_3 (sorbitol, % w/w) on viscosity (A–C; mPa·s) and particle size (D–F; nm) within the investigated factor ranges (VCO 1–9%, Tween 80 6–16%, sorbitol 1–3%)

The contour plots for particle size (fig. 2D–F) show that increasing VCO concentration led to a significant increase in droplet size, whereas increasing Tween 80 concentration led to a reduction in particle size. This behaviour can be attributed to Tween 80's ability to reduce interfacial tension and stabilise newly formed droplets during emulsification. Higher surfactant concentrations enhance surface coverage of oil droplets, thereby preventing coalescence and promoting the formation of smaller particles. Similar relationships between oil concentration, surfactant concentration, viscosity, and particle size in nanoemulsion systems have been reported

previously. Numerical optimisation using a desirability function identified a formulation composed of 1.13% VCO, 15.96% Tween 80, and 2.18% sorbitol as the most practically suitable within the investigated Design space.

Experimental confirmation at this composition yielded viscosity and particle size values closely aligned with predicted responses, with deviations below 10%, thereby supporting the practical reliability and reproducibility of the selected nanoemulsion formulation rather than a purely statistical optimum [30, 31]

Based on the desirability function approach, the optimal formulation point was determined and is presented in table 8. The optimal composition consisted of 1.13% VCO, 15.96% Tween 80, and 2.18% sorbitol, resulting in a predicted viscosity of 1.12 mPa·s and a

particle size of 51.42 nm, with a desirability value of 1.00. A desirability value close to 1 indicates a high level of agreement between the predicted optimal solution and the predefined optimisation criteria, reflecting high prediction accuracy [30, 31].

Table 8: Optimal point solutions

VCO (%)	Tween 80 (%)	Sorbitol (%)	Response		Desirability
			Viscosity (mPa·s)	Particle size (nm)	
1.13%	15.96%	2.18%	1.12 mPa·s	51.42 nm	1.00

The viscosity stability of the optimal formulation during storage is presented in table 9. The initial viscosity value of the optimal nanoemulsion was 1.52 mPa·s, which gradually decreased over the 12 w storage period. This reduction in viscosity may be influenced by storage temperature and environmental conditions, which can affect the nanoemulsion system's structural integrity and reduce internal resistance to flow. Despite this decrease, the viscosity values remained within an acceptable range for topical nanoemulsion formulations [26, 29].

Changes in particle size and pH during storage are summarised in table 10. The particle size increased from 57 nm at week 0 to 217.33

nm at week 6 and 371.66 nm at week 12. Although particle size increased over time, the nanoemulsion remained within the nano-size range (<500 nm), indicating that the formulation maintained its nanoemulsion characteristics throughout the storage period. The observed increase in particle size may be associated with gradual droplet aggregation or Ostwald ripening, which can occur during prolonged storage. The formulation pH decreased from 5.69 at week 0 to 5.36 at week 12, indicating a slight increase in acidity during storage. This pH shift may result from the degradation of formulation components, leading to the formation of acidic or basic by-products. Nevertheless, the pH values remained within the acceptable range for topical preparations (4.5–6.5) [26, 29].

Table 9: Viscosity stability results of the optimal formula

Time (weeks)												
0	1	2	3	4	5	6	7	8	9	10	11	12
1.52	1.43	1.36	1.34	1.33	1.3	1.19	1.17	1.08	0.92	0.79	0.66	0.56
1.51	1.45	1.36	1.35	1.3	1.3	1.19	1.15	1.07	0.9	0.79	0.67	0.56
1.51	1.44	1.36	1.35	1.3	1.3	1.19	1.16	1.06	0.98	0.78	0.67	0.59

Table 10: Particle size stability results of the optimal formula and pH stability

Parameter	Week 0	Week 6	Week 12
Particle size (nm)	57	217.33	371.66
pH	5.69	–	5.36

The physical stability of the optimal nanoemulsion was further evaluated using a cycling test, and the results are presented in table 11. No significant changes in colour, aroma, consistency, or phase separation were observed before and after the cycling test,

indicating good physical stability of the formulation. Additionally, pH and viscosity values after the cycling test remained within acceptable limits, supporting the stability of the optimised nanoemulsion under stress storage conditions [32].

Table 11: Results of cycling test of optimal formula

Parameter	Before the cycling test	After the cycling test
Colour	Brown	Brown
Aroma	<i>Avicennia marina</i> aroma	<i>Avicennia marina</i> aroma
Consistency	Thick and transparent	Thick and transparent
Phase separation	Stable system	Stable system
pH	5.51±0.005	5.36±0.00
Viscosity (mPa·s)	1.51±0.005	1.36±0.00

Data are presented as mean±SD (n = 3).

Anti-dandruff activity

Anti-dandruff activity was evaluated using the selected nanoemulsion formulation of *Avicennia marina* (Forssk.) Vierh. heartwood extract against *Pityrosporum ovale*, a fungus associated

with dandruff development. The inhibitory effect of each test solution was determined by measuring the diameter of the inhibition zone using a digital calliper. The results of the anti-dandruff activity test are presented in table 12 and fig. 3.

Table 12: Results of anti-dandruff activity test

Test solution	Inhibitory power
Optimum nanoemulsion	14.63±1.4
Ketokonazole 2%	29.70±0.8
Blank	3.9±0.1

Data are presented as mean±SD (n = 3). Statistical significance was assessed using one-way ANOVA; $p < 0.05$ was considered significant

As shown in table 12, the selected nanoemulsion exhibited notable

inhibitory activity against *P. ovale* (14.63±1.4 mm), which was

substantially higher than the blank formulation (3.9 ± 0.1 mm), while remaining lower than the positive control (ketoconazole 2%). One-way ANOVA showed a significant overall difference among the three groups

($p = 1.37 \times 10^{-7}$). Pairwise comparisons using two-sided Welch's t-tests indicated that the optimum nanoemulsion differed significantly from the blank ($p = 0.0054$) and from ketoconazole 2% ($p = 0.00037$) [33, 34].

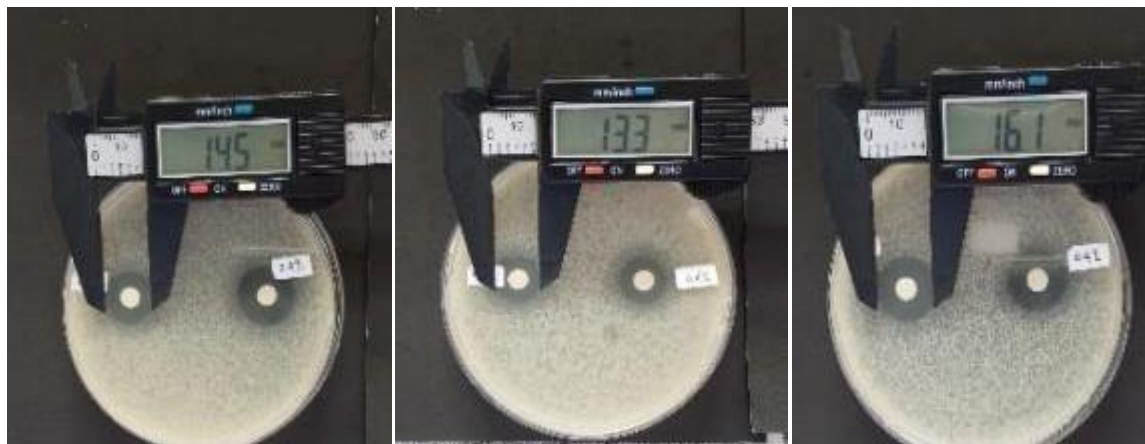


Fig. 3: Inhibition zones of *Pityrosporum ovale*

The selected *A. marina* heartwood extract nanoemulsion produced a measurable inhibition zone against *P. ovale* and performed substantially better than the blank formulation, supporting that the antifungal effect originates from the extract rather than the excipient system. This is consistent with prior reports that plant-derived actives can inhibit *P. ovale* in agar-based assays, supporting the feasibility of botanical anti-dandruff candidates [31, 32]. In addition, nanoemulsion-based delivery systems have been widely reported to improve the dispersion of hydrophobic actives and enhance interfacial contact, thereby contributing to improved apparent antimicrobial performance relative to non-nano dispersions [35–37].

A plausible mechanistic explanation is that the nanoemulsion system provides a large interfacial surface area and facilitates more uniform distribution of lipophilic phytochemicals, thereby increasing contact between active constituents and fungal cell membranes. This can complement the intrinsic antifungal mechanisms of flavonoids, including membrane disruption and interference with cell wall-associated processes, thereby supporting growth inhibition of *P. ovale* [33, 34].

Several limitations should be acknowledged. First, antifungal assessment relied on disk diffusion, which may be influenced by diffusion behaviour of the formulation matrix; complementary assays such as broth microdilution to determine MIC/MFC would strengthen the antifungal evidence. Second, the particle size model did not reach overall statistical significance; therefore, particle size was interpreted as exploratory and treated as a practical constraint followed by experimental confirmation at the selected formulation point. Third, particle size increased during 12 w storage, indicating retention within the nano-range (<500 nm) but not size invariance; further stabilisation strategies (optimising surfactant/co-surfactant balance or adding suitable stabilisers) may improve long-term kinetic stability.

From a translational perspective, the findings support the potential of a topical anti-dandruff nanoemulsion containing *A. marina* heartwood extract. The pH remained within the acceptable topical range, and cycling testing showed no phase separation, suggesting practical stability under stress conditions. However, clinical relevance will require additional safety evaluation (standardised irritation/sensitisation protocols) and efficacy assessment in scalp-relevant models and/or clinical settings.

CONCLUSION

This study demonstrates the novelty and practical value of a Box-Behnken Design-guided approach to develop *Avicennia marina*

(Forssk.) Vierh. heartwood extract nanoemulsion as a topical anti-dandruff candidate. By systematically interrogating the formulation space of the oil phase, surfactant, and cosurfactant, the work establishes a reproducible formulation pathway that yields physicochemically acceptable nanoemulsion characteristics and meaningful antifungal activity against *Pityrosporum ovale* beyond the blank vehicle. Collectively, these findings support the feasibility of using optimisation-driven nanoemulsion delivery to enhance the topical performance of plant-derived actives and provide a practical foundation for further product-oriented development, including broader antifungal profiling, extended stability optimisation, and standardised safety evaluation prior to clinical translation.

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AI-USE DISCLOSURE STATEMENT

The authors used ChatGPT for grammar checking and improving sentence clarity. The authors reviewed and edited the output as needed and take full responsibility for the final content of the manuscript.

AUTHORS CONTRIBUTIONS

All authors significantly contributed to the conception, Design, data acquisition, analysis, and interpretation. They were involved in drafting and revising the article for important intellectual content. All authors agreed to submit the work to the current journal, approved the final version for publication, and agreed to be accountable for all aspects of the research.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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