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Original Research Article

Moringa oleifera Seed Oil Nanoemulsion Using Tween 80 and Polyethylene Glycol 400: Oil Characterization and Formula Optimization

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ABSTRACT

Moringa oleifera is a useful plant known for its antialopecia properties. Nanoemulsions have been proven effective as a carrier for the delivery of lipophilic compounds in *Moringa oleifera* seed oil to increase the permeation of active ingredients. This study aimed to characterize the physical and chemical properties of *Moringa oleifera* seed oil and identify its composition using GCMS, optimize the formulation of a nanoemulsion containing *Moringa oleifera* seed oil, using Tween 80 as a surfactant and PEG 400 as a co-surfactant. The optimal formulation was determined using Design-Expert through the simplex lattice design approach. A total of 14 formulations were tested for particle size response, polydispersity index (PDI), and zeta potential. The results showed that *Moringa oleifera* seed oil contained 12 fatty acids, with oleic acid as the most abundant (71.52%), followed by palmitic (8.05%), stearic (5.99%), behenic (5.31%), and arachidic acid (2.97%).The acid value, peroxide, density, saponification, refractive index, and moisture content of the oil were 0.46 mg KOH/g oil, 6.28 meq O_2/kg oil, 0.915 grams/cm^3, 253.5 mg KOH/g oil, as well as 1.4668 and 0.12% w/w, respectively. The optimal formulation obtained consisted of 6.401% *Moringa oleifera* seed oil, 36% Tween 80, and 18.599% PEG 400. Evaluation of the optimal formula showed particle size response value was 237.06 nm, PDI 0.467, zeta potential -20.89 mV, transmittance value 97.7%, spherical shape in observations using TEM. Nanoemulsion containing high quality of *Moringa oleifera* seed oil was able to be formulated, which may serve as a promising dosage form for hair growth treatments.

Keywords: Simplex lattice design, *Moringa oleifera* seed oil, Tween 80, PEG 400, Nanoemulsion

Introduction

Hair plays a crucial role in human identity and aesthetics. Several hair-related disorders, are alopecia, discoloration, and hirsutism.[1] Among these, alopecia, particularly androgenic alopecia (AGA), is a common dermatological disorder that significantly affects mental well-being and overall quality of life. An epidemiological survey conducted in China reported an AGA prevalence of 21.3% in men and 6.0% in women.[2] Due to its high prevalence, alopecia has become a subject of extensive research. Some treatments used for alopecia, such as minoxidil and finasteride, have unpredictable side effects,[3] including dizziness, allergic dermatitis, and cardiovascular disease.[4] As a result, there is a growing need for natural alternatives in the search for new anti-alopecia agents. One such potential treatment is *Moringa oleifera* seed oil.

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Moringa oleifera Lamk. is a plant originating from the sub-Himalayan region of North India. This plant is widely distributed in tropical and subtropical areas at altitudes of approximately 2000 m.[5] All plant parts such as pods, seed, bark, leaves, and flowers can be consumed and used by humans.[6] Seed contains high-quality fatty xacids such as oleic acid (> 70%), with a large amount of oil (up to 40%).[7] Additionally, there is phytosterol content such as stigmasterol and β sitosterol.[8] Korassa *et al*. (2022) found that the phytosterol compounds and fatty acids in *Moringa oleifera* seed oil have potential as anti alopecia agents.[4] The lauric and linoleic acid in the oil are hair nutrients and can accelerate growth by inhibiting the enzyme 5 α-reductase.[4] However, one of the main limitations of *Moringa oleifera* seed oil is its poor applicability and practicality in topical use. As an oily liquid, it has the potential to cause dandruff. Therefore, a delivery system and dosage form are required to enhance user comfort, improve the penetration of active ingredients through the scalp and stratum corneum barrier, facilitate accumulation in hair follicles, and ensure physical stability. Furthermore, the use of *Moringa oleifera* seed oil in preparations requires the consideration of several factors, such as stability, benefits, and safety.

Nanotechnology is a controlled delivery widely utilized in pharmaceuticals, cosmetics, and food industries to optimize the dispersion of active ingredients.[9] Nanoparticle preparations such as solid lipids, nanogels, polymers, liposomes, and nanoemulsion have been shown to prevent external degradation of herbal medicines and increase their bioavailability.[10] Among these, nanoemulsion is a promising carrier for the delivery of lipophilic compounds, improving the permeation of active ingredients. This method provides a transparent appearance and exhibits greater stability against creaming and flocculation compared to conventional emulsion preparations.

Nanoemulsion also increases the surface area and penetration of active substances.[11] In this context, nanoemulsion has been identified as a potential carrier for delivering lipophilic compounds in *Moringa oleifera* seed oil to increase the permeation of active ingredients. This preparation consists of oil and water phases, which are stabilized by the presence of surfactant and cosurfactant as important components.[12]
Surfactants lower interfacial tension by reducing the repulsive force of immiscible liquid.[13] Meanwhile, cosurfactants prevent aggregation and increase pH stability.[14] A study by Tirmiara *et al*. (2019) revealed that the nanoemulsion *Moringa oleifera* seed oil, formulated with Tween 80 and sorbitol as surfactans, resulted in a particle size of 52.25 nm.[15] However, there is no research on the use of Tween 80 and PEG 400 in *Moringa oleifera* seed oil nanoemulsion preparations, so researchers want to do this research. Tween 80 is a non-ionic surfactant with an HLB value of 15, forming an oil-in-water type emulsion.[16] A higher concentration of Tween 80 leads to increased clarity, higher transmittance values, and smaller particle sizes.[13] Polyethylene glycol 400 (PEG 400) is used as cosurfactant to improve the stability of nanoemulsion particularly in pharmaceutical, cosmetic.[17,18] Structurally, PEG 400 is a mid chain hydrocarbon that can be placed between the gaps of the nanoemulsion system by the formation of hydrogen chains so that it can be placed between the gaps nanoemulsion system with the formation of hydrogen chains so as to maximize the emulsification process in the preparation of nanoemulsions.[17]
In this study, the analysis and characterization of *Moringa oleifera* seed oil aim to evaluate its quality and composition after extraction. Existing research on the hair growth activity of *Moringa oleifera* seed oil remains general and has not yet explored its formulation using nanoparticle technology as a hair growth agent. This study aimed to optimize the preparation of nanoemulsion with the concentration of *Moringa oleifera* seed oil, Tween 80, and PEG 400. This was followed by the analysis of the characteristics of *Moringa oleifera* seed oil nanoemulsion. Response characteristic parameters were determined using expert design including particle size, polydispersity index (PDI), zeta potential. The nanometer-scale particle size of nanoemulsions is expected to enhance penetration and facilitate the accumulation of targeted drugs in the dermal layer of the skin.[10] This aims to optimize the topical application of the formulation. An appropriate combination of oil, Tween 80, and PEG 400 is anticipated to further support this objective.

Materials and Methods

Materials
The materials used in this study included *Moringa oleifera* seed oil, Tween 80, PEG 400 (Brataco Indonesia), and distilled water. The instruments used in this study include Particle Size Analyzer (HORIBA SZ-100), Zeta Nanosizer (Zetasizer), Hot Plate Magnetic Stirrer (Thermo Scientific, China), Sonicator (Elma Transsonic 570), and a Transmission Electron Microscope (TEM) (JEOL/EO JEM-1400 version 1.0).

Collection and Identification of Moringa oleifera seed
Moringa oleifera seed was obtained from the Blora region, Central Java, Indonesia in May 2024. *Moringa oleifera* seed was botanically identified in Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Extraction of the oil
The oil was extracted using a cold pressing system (capacity: 2–6 L/h) without the application of heat. Six point one kg of *Moringa oleifera* seed was weighed, the seed skin was peeled and placed into a cold press machine. The extraction process consisted of grinding *Moringa oleifera* seeds and pressing them with a conical screw at a gradually increasing pressure of 250 kg/N. The oil was then expelled through a perforated tube. The oil obtained was stored in a tightly closed container, and the yield value was calculated.[19]

Characterization of physical and chemical properties of Moringa oleifera seed oil
The determination of physical and chemical properties of *Moringa oleifera* seed oil were characterized based on organoleptic, acidnumber, peroxide number, density, saponification number, refractive index, and water content. The determination of density and refractive index followed the AOAC standard methods. Density was measured using a phycnometer, while the refractive index was determined using an Abbe Refractometer.[20]

Analysis of Fatty Acid Composition using GCMS
The oil was hydrolyzed, derivatized, and then analyzed using a GC-MS equipped with an autoinjector. A total of 5-20 mg of *Moringa oleifera* seed oil was dissolved in 1 mL of 20% of boron trifluoride in methanol. The mixture was heated in an ultrasonic bath at 60°C for 20 minutes, and then allowed to cool. Subsequently, 2 mL of n-hexane was added, and the mixture was vortexed, and centrifuged at 4000 rpm for 10 minutes. The upper layer (n- hexane) was collected into a collection vial and injected into the GC-MS. The analysis was performed using a DB-Fast FAME capillary column (30 m × 250 µm × 0.25 µm) on an Agilent GCMS system (8890 GC System, 5977B GC/MSD, 7693A Autosampler).
The GC oven temperature was maintained at 60°C, programmed to 200°C at a rate of 2°C/min, and held constant at 250°C for 48 minutes. High-purity helium was used as the carrier gas at a constant flow rate of 1 mL/min. The split ratio and flow rate were set at 20: l and 20 ml/min, respectively. The injection volume of the oil mixture was 1 µL. The ion source temperature was set to 200°C and while the interface was set to 230°C. The electron impact (EI) ionization mode used an ionization energy of 70 Ev column linear velocity of 36 cm/s, with mass ranging from m/z 30 to 700.[19]

Optimization of Moringa oleifera seed oil nanoemulsion
The formula optimization process was conducted using the simplex lattice design in Design Expert version 13. The response variables considered for optimization included particle size, PDI, and zeta potential. The initial step taken was to adjust the model to the response. Furthermore, the optimal formula was determined by observing the value closest to the desired value. All materials were weighed as shown in Table 1. The mixture was homogenized using a magnetic stirrer for 30 minutes at a temperature of 25°C at a speed of 800 rpm and sonicated for 10 minutes (one cycle). The mixing process was carried out in two cycles.

Evaluation of Moringa oleifera seed oil nanoemulsion preparation
Organoleptic test
The test was carried out by visually observing the color, odor, and consistency of the formulations. Subsequently, observations were carried out visually.[14]

Particle size, PDI, and zeta potential
The particle size, PDI, and zeta potential of nanoemulsion were analyzed using Malvern Particle Size Analyzer (PSA) at 25°C with a scattering angle of 90°.[21] The zeta potential was measured after the samples were dissolved in purified water. The zeta potential indicates the electrical charge on the surface of the nanoparticles, suggesting that the colloidal carrier system remains physically stable.[22]

Percentage transmittance
The transmittance percentage value was measured using a UV-Vis spectrophotometer at a wavelength of 650 nm.[23]

Morphology of Moringa oleifera seed oil nanoemulsion
The morphological characteristics of nanoemulsion containing *Moringa oleifera* seed oil were perfomed using a Transmission Electron Microscopy (TEM). Initially, a drop of the sample was placed onto a carbon film copper grid. This was followed by adding a drop of uranyl acetate to enhance the contrast of the sample before analysis. [13]

Statistical analysis

Data analysis was performed using Design Expert version 13 to determine the effect of *Moringa oleifera* seed oil, Tween 80, and PEG 400 on nanoemulsion evaluation response. Statistical analysis was performed using ANOVA, where significance was determined at a p-value ≤ 0.05.[25] Subsequently, the optimum nanoemulsion formula was verified through t-test using IBM SPSS Statistics 23 software.

Table 1: Upper and lower limit values of *Moringa oleifera* seed oil nanoemulsion in Design Expert

Ingredient	Under limit (%)	Upper limit (%)
Moringa oleifera oil	5	9
Tween 80	36	40
PEG 400	16	20

Results and Discussion

Extraction of Moringa oleifera seed oil

In the current study, mature *Moringa oleifera* seeds were used, characterized by their black seed coat**.** The separation between the skin and seed was performed manually. The oil was obtained through cold pressing, a method that applies mechanical pressure to break the cell walls, facilitating oil release.[26] Our extraction produced yellowish oil that was transparent and odorless, with yield of 14.73%. The peeled seed were heated at a temperature of 70 ºC for 15 minutes. This process was carried out to unite and collect oil granules, allowing easy flow from seed flesh and reduced affinity for efficient extraction. Heating was also performed to increase oil dilution, evaporate water to a certain extent, deactivate enzymes, and coagulate proteins, thereby enhancing further separation.[26]

Physicochemical properties of Moringa oleifera seed oil

The physicochemical properties of *Moringa oleifera* seed oil obtained by cold press are shown in Table 2. These values provided information regarding the oxidation status and possible impurities in Moringa oleifera seed oil.[27] The acid value of *Moringa oleifera* oil obtained was 0.46 mg KOH/g, which serves as a crucial indicator of oil rancidity and stability. The acid value is utilized to determine the degradation level of the triglyceride content of the oil through the action of lipase.[28] The high content of fatty acids suggests that many fatty acids have been released from glycerol, where glycerol can help fatty acids form triglycerides. High free fatty acids also affect flavors of oils.[29] The high saponification value was shown by the presence of high molecular weight triacylglycerols, which are beneficial for soap production.[30] The results indicate that the oil has a saponification value that is useful for industrial soap production. Ozcan *et al* (2019) reported the physicochemical properties of *Moringa oleifera* seed oil from Saudi Arabia by cold pressing, reported an acid number of 1.91 mg KOH/g, a peroxide number of 7.2 meq O_2/kg, and a saponification value of 181.3 mg KOH/g. In comparison, the *Moringa oleifera* seed oil obtained in this study exhibited low peroxide values, indicating a low free fatty acid content.[31] The oil has a peroxide value of <10 meq/kg, which can be used to determine the storage stability of the oil as well as to guard against oil rancidity caused by primary oxidation factors such as hydroperoxides, oxygen, temperature, and light. It can be concluded that *Moringa oleifera* seed oil obtained by cold press extraction technique is stable and rancid-free due to its very low hydroperoxidant content.[31] This showed that the characteristics of *Moringa oleifera* seed oil obtained were identical to all previous research.[19] Moisture content value obtained was 0.12%, which could be influenced by factors such as climate conditions, growing places, and the processing methods.[26] According to Anwar, *et al* (2007) the moisture content of Pakistan *Moringa oleifera* seeds is 5.70%.[32] The difference in moisture content between this study and previous findings can be attributed to regional variations in climate, soil conditions, and seed processing techniques. The results also showed that the density of *Moringa oleifera* seed oil was 0.915 g/cm3. According to Barakat (2016), the specific gravity of oil was affected by the molecular weight and components of unsaturation of fatty acid in oil.[29] The refractive index obtained in this study was 1.4668, which was used to determine the purity of oil.[26] The refractive index of a substance is defined as the ratio between the sine of the incident angle and the sine of the angle of the refracted light passing through the medium. Refraction is caused by the interaction between electrostatic forces and electromagnetic forces of the atoms in the liquid molecules. Refractive index testing can be used to determine the purity of an oil and can quickly detecting hydrogenation. The longer the carbon chain and the more double bonds, the greater the refractive index. The refractive index is also affected by factors such as free fatty acid content, oxidation process, and temperature.[33]

Table 2: Physicochemical properties of *Moringa oleifera* seed oil

Properties	Result
Organoleptic	Oil, Yellow, odourless
Acid value (mg KOH/g)	0.46 ± 0.001
Peroxide value (meq O2/kg)	6.28 ± 0.008
Density (g/cm^3; 25 °C)	0.915 ± 0.002
Saponification value (mg/KOH/g)	253.5 ± 1.460
Refractive index	1.4668 ± 0.0003
Moisture content (% b/b)	0.12 ± 0.005

Fatty acids analysis of Moringa oleifera seed oil using GCMS

The GC-MS analysis result are presented in Figure 1 and Table 3. Based on the results, 37 compounds were identified, including 12 fatty acids. The highest fatty acid was oleic acid, accounting for 71.52% of the total oil, followed by palmitic acid (8.05%), stearic acid (5.99%), behenic acid (5.31%), and arachidic acid (2.97%). These results indicate that the fatty acid composition of *Moringa oleifera* seed oil from Blora is consistent with findings reported in previous studies.[19] Variations in fatty acid composition can be attributed to differences in agroclimatic conditions and *Moringa oleifera* seed varieties. The monounsaturated fatty acid content of *Moringa oleifera* seed oil is similar to that of olive oil.[34] In general, the characteristics and fatty acid content of *Moringa oleifera* seed oil obtained by cold pressing are approximately the same when compared to the literature. The slight differences obtained may be due to differences in plant species, climatic factors, and the cultivation locations.[8]

This oil falls into the high oleic oil category, with high-value potential similar to others.[35] Oils with high oleic content are particularly valuable due to their superior stability and high nutritional value.[36] With a high-quality fatty acid composition, particularly oleic acid (>70%), *Moringa oleifera* seed oil exhibits significant resistance to oxidative degradation.[5] The oleic acid content in *Moringa oleifera* seed oil has been proven to reduce hair loss and promote hair growth.[4] A method that can be used to formulate oil actives is nanoemulsion. This preparation is suitable for efficient penetration of active substances into the skin. The active ingredient will easily penetrate the skin due to the large surface area of the emulsion system. In addition, this preparation is easy to use and does not irritate the skin.[37]

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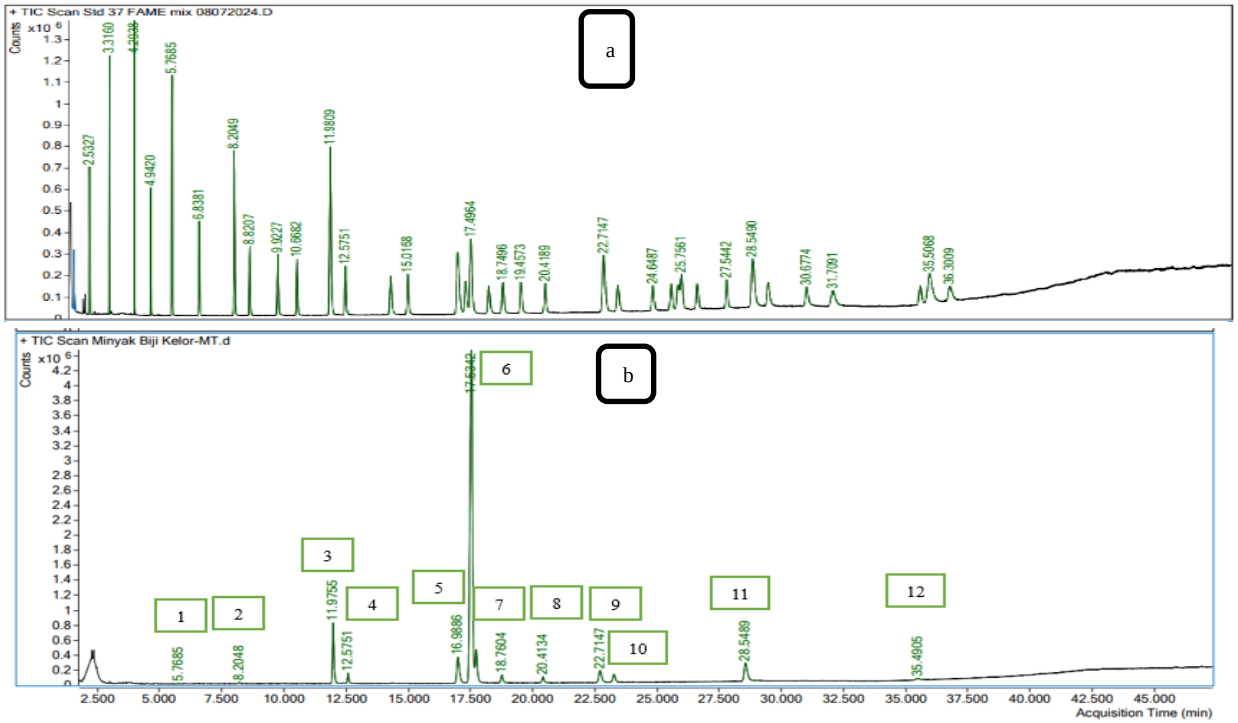


Figure 1: GCMS chromatogram results, (a) FAME standard; (b) *Moringa oleifera* seed oil sample. Note : *Laurate*(Peak 1), *Myristoleate* (peak 2), *Palmitate* (peak 3), *Palmitoleate*(peak 4), *Stearate*(peak 5), *oleate* (peak 6), *Linoleate*(peak 7), *Arachidate*(peak 8,9,10), *Behenate*(peak 11), *Lignocerate*(peak 12).

Table 3: Fatty acid compounds of *Moringa oleifera* seed oil from GCMS analysis

Peak	tR minutes		Area		% Relative Fatty acid		FAME	
	Standard	*Moringa oleifera* seed oil	Standart	*Moringa oleifera* seed oil	Standart	*Moringa oleifera* seed oil	compound name	Standart
1	1.9331		401565.7		0.97	0		C4
2	2.5327		756983.4		1.83	0	*Caproate*	C6
3	3.316		1117629		2.7	0	*Caprylate*	C8
4	4.2938		1464595		3.54	0	*Caprate*	C10
5	4.942		765736.3		1.85	0		C11
6	5.7685	5.7685	1758716	18282.3	4.25	0.04	*Laurate*	C12
7	6.8381		906860.3		2.19	0		C13
8	8.2049		2025140	61204.9	4.89	0.14	*Myristate*	C14
9	8.8207	8.2049	859363.2		2.07	0	*Myristoleate*	C14:1
10	9.9227		1004700		2.43	0		C15
11	10.6682		894107		2.16	0		C15:1
12	11.9809	11.9755	3412276	3545357	8.24	8.05	*Palmitate*	C16
13	12.5751	12.5751	902465.3	562653	2.18	1.28	*Palmitoleate*	C16:1
14	14.3524		1003786		2.42	0	*Margarate*	C17
15	15.0168		895742.1		2.16	0		C17:1
16	16.9778	16.9886	2148697	2640036	5.19	5.99	*Stearate*	C18
17	17.2857		952284.8		2.3	0	*eladiate*	C18:1 (n9)
18	17.4964	17.5342	2300275	3.2E+07	5.55	71.52	*oleate*	C18:1 (n9)
19	18.204		766174		1.85	0	*Linolelaidate*	C18:2 (n6)
20	18.7496	18.7496	848129.5	627630	2.05	1.42	*Linoleate*	C18:2 (n6)
21	19.4573		789900.4		1.91	0		C18:3 (n6)
22	20.4189	20.4134	752851.8	451855	1.82	1.03		C18:3 (n3)
23	22.7147	22.7147	2097494	1307879	5.06	2.97	*Arachidate*	C20

24	23.2712	23.2712	826683.7	724887	2	1.65		C20:1 (n9)
25	24.6487		694866.5		1.68	0		C20:2 (n6)
26	25.3617		678773.8		1.64	0		C20:3 (n6)
27	25.6264		700517.3		1.69	0		C21
28	25.7561		1070832		2.59	0	*Arachidonate*	C20:4 (n6)
29	26.3881		720133.1		1.74	0		C20:3 (n3)
30	27.5442		717156.5		1.73	0	*EPA*	C20:5 (n3)
31	28.549	28.549	1955556	2339764	4.72	5.31	*Behenate*	C22
32	29.1702		761721.9		1.84	0	*Erucate*	C22:1 (n9)
33	30.6774		694237.9		1.68	0		C22:2 (n6)
34	31.7091		837697.8		2.02	0		C23
35	35.1286		569785.4		1.38	0	*DHA*	C22:6 (n3)
36	35.5068	35.4905	1676772	269402	4.05	0.61	*Lignocerate*	C24
37	36.3009		691063.3		1.67	0	*Nervorate*	C24:1 (n9)

Optimization of Moringa oleifera seed oil nanoemulsion

Nanoemulsion preparation consisted of 14 run formulas with different concentrations of *Moringa oleifera* seed oil, Tween 80, and PEG 400, (Table 4). The result of 14 runs showed that all *Moringa oleifera* seed oil nanoemulsion formulations showed a slightly thick consistency and a clear yellowish color. However, runs 1,2,4,10,12 showed turbidity while the remaining appeared transparent (see Figure 2). The response variables in this study included particle size, PDI value, and zeta potential. Based on the results of the evaluation test, some nanoemulsions exhibited a clear yellow (transparent) appearance, and some formulations developed turbidity. The particle size across the 14 runs ranged from 13.01 to 442.3 nm. The parameter was significantly influenced by the concentration of surfactant, as higher surfactant levels were associated with smaller particle sizes.[38]

Figure 2: Run Formula of *Moringa oleifera* seed oil nanoemulsion

The PDI value, which significantly influences characeteristics of nanoemulsion stability, across runs 1 to 14 ranged from 0.2233 to 0.6043.[39] The range of PDI values is usually between 0-1. PDI serves as a measure of system uniformity, a low PDI value indicates a more uniform particle distribution in a monodisperse system.[41] When the value is close to zero, the particle distribution is more homogeneous. Meanwhile, values above 0.5 indicate increasing heterogeneity.[42] Higher heterogeneity can lead to increased particle agglomeration due to collisions between particles.[43] PDI is calculated as the ratio of the standard deviation to the mean droplet size44. In this study, the zeta potential value obtained was between -31.02 to -15.08. Zeta potential is used to determine the surface charge of nanoemulsion droplets.[45] The results of this evaluation show homogeneity, and size distribution of the nanoemulsion preparation.[14] The physical characteristics of *Moringa oleifera* seed oil nanoemulsion are presented in Table 4.

Table 4: *Moringa oleifera* seed oil nanoemulsion run formula and their evaluation results

Run	Formulation ingredient (%)				Response parameters		
	Oil	Tween 80	PEG 400	Aquadest	Particle size (nm)	PDI	Zeta potential (mV)
R1	9	36	16	39	442.2	0.2586	-16.61
R2	7	36	18	39	361.6	0.3682	-17.43
R3	5	36	20	39	14.23	0.6043	-24.26
R4	7.67	36.67	16.67	38.99	374.4	0.3073	-15.49
R5	7	38	16	39	207.5	0.5213	-16.79
R6	5.67	36.67	18.67	38.99	26.94	0.6002	-17.29
R7	5	40	16	39	13.01	0.4455	-18.81
R8	5	36	20	39	169.7	0.5929	-29.08
R9	5	38	18	39	13.57	0.5813	-31.02
R10	9	36	16	39	442.3	0.2233	-18.94
R11	5	40	16	39	20.31	0.3161	-15.08
R12	7	38	16	39	160.2	0.5224	-20.06
R13	5.67	38.67	16.67	38.99	15.37	0.5946	-22.55
R14	6.33	37.33	17.33	39.01	16.47	0.5206	-20.28

Verification of the optimal formula of Moringa oleifera seed oil nanoemulsion

The concentration of oil, surfactant, and cosurfactant, as well as their interactions, significantly influenced the characteristics of

nanoemulsion. This influence was represented by coefficients, where a positive value (+) indicated a direct proportional relationship meaning that higher concentrations resulted in greater response values. Conversely, a negative coefficient (-) signified an inverse relationship, where an increase in concentration led to a decrease in the response.[46]

High amounts of surfactant can have toxic effects when used in food or other products.[42] In this study, Tween 80 was used as surfactant due to its low irritation and toxicity, high stability, and small molecules, thereby minimizing the size of nanoemulsion droplets.[47] The cosurfactant, PEG 400 was also used to increase the stability and improve drug absorption.[17] As presented in Table 5, the statistical analysis results revealed that the selected models for response variables were linear for particle size, and were quadratic for PDI, and linear for zeta potential. Additionally, *Moringa oleifera* seed oil, Tween 80, and PEG 400 had a significant effect among all formulas ($p < 0.05$). Figure 3 illustrates the composition of *Moringa oleifera* seed oil, Tween 80, and PEG 400 in modeling. The diagram indicates that the highest response values are represented in the red area, while lower values appear in the yellow area.

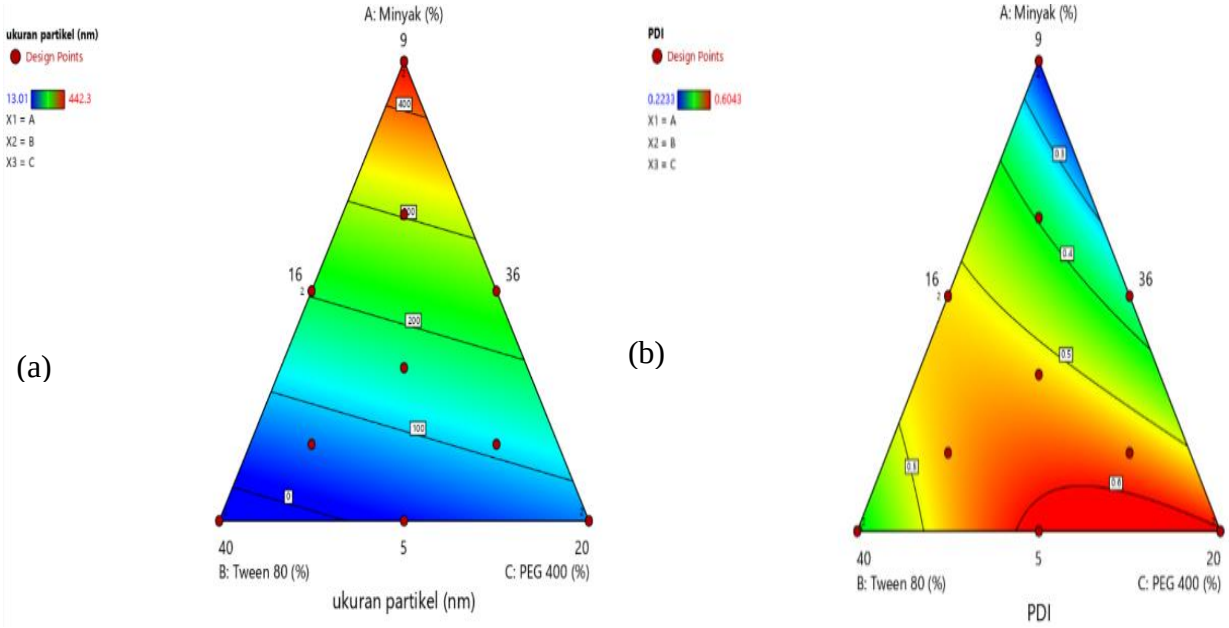
The mixture of *Moringa oleifera* seed oil, Tween 80, and PEG 400 affected particle size. *Moringa oleifera* seed oil and PEG 400 have a positive effect on particle size. Meanwhile, Tween 80 had a negative effect on particle size with a coefficient value of -17.17. As shown in Figure 3a, the blue areas represent smaller nanoemulsion particle sizes, while the color transition from green to yellow and red indicates increasing particle size This suggests that higher concentrations of Tween 80 result in smaller nanoemulsion particles. Previous studies had shown that Tween 80 could produce smaller droplet sizes in emulsion.[48] As a surfactant, Tween 80 consists of both hydrophobic and hydrophilic components, allowing it to bridge the oil and water phases by reducing interfacial tension. This reduction in interfacial tension decreases the free energy required for nanoemulsion formation.[49] The combination of the three materials influenced the PDI value. The quadratic model on PDI showed that *Moringa oleifera* seed oil and Tween had a negative effect, while PEG 400 had a positive effect on the PDI value. As illustrated in Figure 3b, areas with a red color gradient correspond to higher PDI values due to the PEG 400 component, while blue areas indicate lower PDI values associated with *Moringa oleifera* seed oil and Tween 80. This showed that greater amount of *Moringa oleifera* seed oil and Tween can reduce the PDI value of nanoemulsion. Yang *et al* (2023) showed that increasing the concentration of Tween 80 can reduce the droplet size of nanoemulsions. Tween 80 lowers the oil-water interfacial tension, reducing the energy required to break emulsion droplets and thereby decreasing their diameter. However, this also leads to a significant increase in the PDI value of the nanoemulsion. Larger droplet sizes tend to aggregate, resulting in flocculation and phase separation.[50]

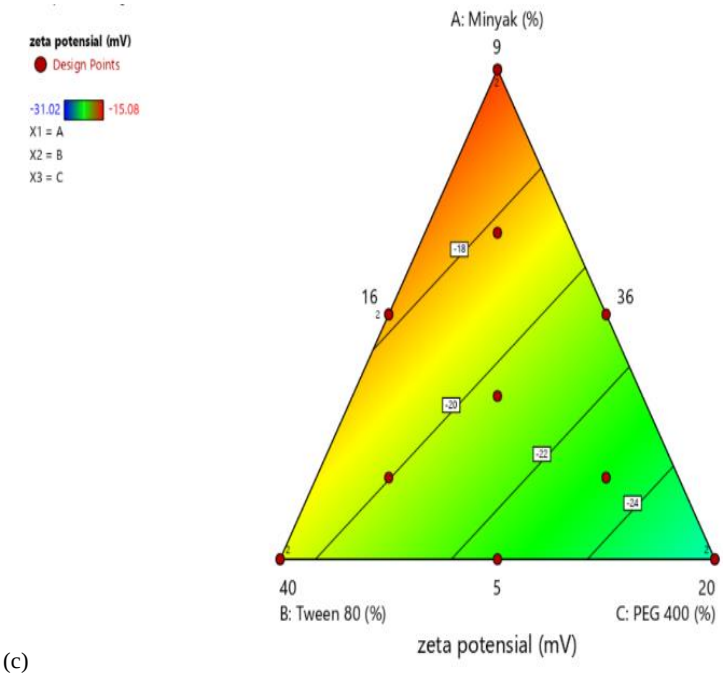
The results of the zeta potential analysis revealed a significant effect. PEG 400 had the greatest impact on reducing the zeta potential, with a coefficient value of -1.57. In contrast, *Moringa oleifera* seed oil and Tween 80 increased the zeta potential value. As illustrated in Figure 3c, PEG 400 is represented in green, indicating a decrease in zeta potential, whereas *Moringa oleifera* seed oil and Tween 80 are shown in yellow and red, signifying higher zeta potential values. Miksusanti et al (2023) reported that a zeta potential value of more than +30 mV or less than -30 mV will produce a relatively stable dosage form.[14] Therefore, the negative value obtained in this study suggests that nanoemulsion had a negative charge and was capable of resting repulsive forces to produce a stable preparation.[22]

Table 5: Results of ANOVA statistical analysis of nanoemulsion characteristics

Characteristic	Mathematics Equation	Mathematics models	Anova model (p value)
Particle size	+103.29 (A) – 17.17 (B) +8.44(C)	linear	<0.0001
PDI	-1.27 (A) - 0.32 (B) +0.52 (C) + 0.05 (A)(B) -0.015 (A)(C) +0.03 (B)(C)	quadratic	0.0007
Zeta potential	+0.89 (A) +0.02 (B) -1.57 (C)	linear	0.0477

Note : A: Oil; B: Tween 80; C: PEG 400





(c)

Figure 3: Counter plot model of *Moringa oleifera* seed oil nanoemulsion response, representing (a) particle size, (b) PDI, (c) zeta potential

The solution with the highest desirability value was close to 1.0. Based on the results obtained using Design Expert version 13, the desirability value was 0.761, indicating an optimal formulation that met the desired physical characteristics. The optimal formula consisted of 6.401% *Moringa oleifera* seed oil, 36% Tween 80, and 18.599% PEG 400. The measurement results of the optimal formula showed a particle size of 237.06 nm, and PDI value of 0.4672. These values align with the specified criteria, as nanoemulsion particle sizes typically range from 10 to 1000 nm.[51] In general, smaller particle sizes increase surface area, thereby enhancing drug absorption. Additionally, the nanoscale formulation reduces emulsification time.[11] The PDI value indicates that nanoemulsion particles are stable and can reduce the occurrence of precipitation.

In this study, the PDI value of *Moringa oleifera* seed oil nanoemulsion preparation met nanoemulsion the criteria. A smaller PDI value indicates a narrower distribution, leading to a more uniform particle size.[25] The zeta potential value of *Moringa oleifera* seed oil nanoemulsion was found to be -20.89. Additionally, the transmittance percentage of the optimum formula was close to 100%, indicating excellent optical clarity. A higher transmittance percentage is generally associated with smaller nanoemulsion particle sizes.[52] Transmittance values can be used to quantitatively assess nanoemulsion stability, homogeneity, and optical clarity.[53] Compared to the results, the predicted value obtained from the design expert version 13 showed a confidence level of 95%. The complete data on the optimal formula test are presented in Table 6.

Based on the TEM results, utilizing bar scales of 100 nm and 500 nm, the droplets in nanoemulsion form spherical globules (Figure 4). This electron microscope provides high-resolution imaging, enabling the detailed visualization of microstructures and structural transitions within the nanoemulsion.[14] The complete TEM results obtained in this study are shown in Figure 4. Furthermore, based on Table 7, the particle size, PDI, and zetapotential of the optimum formula observation results did not show significant differences from the software-predicted values (p> 0.05). This shows that the results obtained from the test were valid.

Table 6: Results of the optimal formula test of *Moringa oleifera* seed oil nanoemulsion

Response Parameters	Result
Organoleptic	liquid, transparant yellow, odourless
Particle size (nm)	237.06 ± 35.39
PDI	0.467 ± 0.080
Zeta potential (mV)	-20.89 ± 2.961
Transmittance (%)	97.7 ± 0.265

Data represent the mean ±standard deviation (SD and n = 5).

Table 7: Results of one sample T-Test prediction and optimal formula test results

Parameters	Prediction	Result	p value	Significance
Particle size (nm)	200	237.06	0.079	Not significant
PDI	0.416	0.467	0.227	Not significant
Zeta potential (mV)	-22.421	-20.89	0.186	Not significant

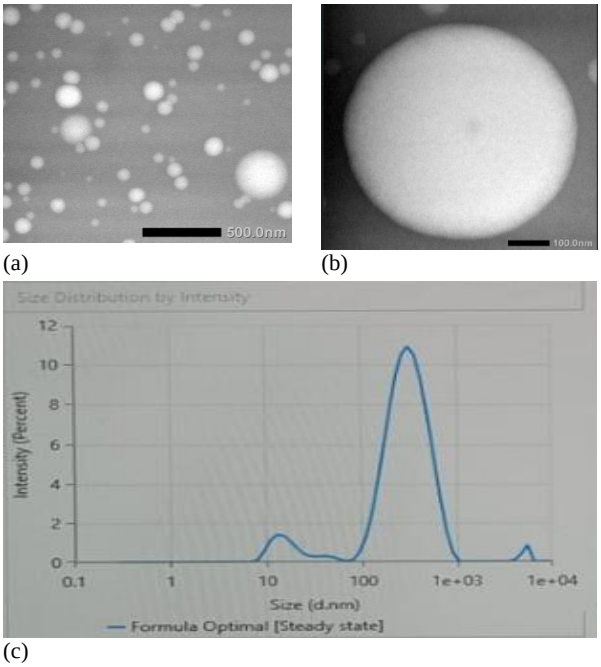


Figure 4: Particle size distribution and morphology of nanoemulsion (a) 10.000 times (Scale bar 500 nm); (b) 40.000 times magnification (Scale bar 100 nm); (c) particle size distribution of nanoemulsion

Conclusion

This study demonstrated that *Moringa oleifera* seed oil contains 12 types of fatty acid, with oleic being the most abundant (71.52%) , followed by palmitic (8.05%), stearic (5.99%), behenic (5.31%), and arachidic (2.97%). The concentration of *Moringa oleifera* seed oil, Tween 80, and PEG 400 significantly affect the characteristics of nanoemulsion such as particle size, PDI value, and zeta potential. The results indicate that the optimal formulation consisted of 6.401% *Moringa oleifera* seed oil, 36% Tween 80, and 18.599% PEG 400. This study further highlights the potential application of *Moringa oleifera* seed oil as a high-quality, oleic acid-rich oil in nanoemulsion preparations, which may serve as a promising dosage form for hair growth treatments.

Conflict of Interest

The authors declare no conflicts of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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