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Hair Serum Nanoemulsion loaded Clove Essential Oil Formulation for Androgenetic Alopecia: Characterization and Hair Growth Activity

Wayan Cintya Ganes Budastra[1,2], Teti Mariam Riandari[2], Laras Novitasari[2,5], Retno Murwanti[3] and Ronny Martien[4*]

1. Department of Pharmacy, Faculty of Medicine and Health Sciences, University of Mataram, Mataram, 83115, Indonesia.
2. Master of Pharmacy Science, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara 55281, Yogyakarta, Indonesia
3. Department of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara 55281, Yogyakarta, Indonesia
4. Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara 55281, Yogyakarta, Indonesia
5. Researcher, PT. Ronature Teknologi Indonesia, Yogyakarta, 55188, Indonesia.

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*Corresponding author
Ronny Martien

Email:
ronnymartien@ugm.ac.id

ABSTRACT

Androgenetic alopecia (AA) is hair loss caused by genetic factor and androgen hormone. Clove essential oil (CEO) contain eugenol which has anti-androgen activity and can be used to treat AA. Formulation CEO into hair serum nanoemulsion overcome the CEO from several disadvantages. The aims of this study were preparation, characterization, and evaluation hair growth activity of hair serum nanoemulsion loaded CEO. The chemical components of CEO were analysed by gas chromatography. The optimum formula was obtained using the D-Optimal Design method and was further characterized for droplet size, size distribution, pH, viscosity, thermodynamic stability, and storage stability. In vivo of skin irritation and hair growth activity were evaluated. The result showed that the GC-MS analysis of the CEO consisted of eight components, which the main component was eugenol (60.72%). The optimum formula consisted of CEO (10.63%), cremophor RH 40 (67.5%), and propylene glycol (21.87%). Formula has the droplet size of 31.26±0.155 nm, the PDI value of 0.24±0.008, the pH of 5.55±0.01, the viscosity of 2386.18±62.77 cP, physically stable, and did not show the skin irritation. The length and weight hair of the treatment group was 2.28±0.12 cm and 2.28±0.12 g, respectively. Clove oil nanoemulsion hair serum is effective in promoting hair growth and may serve as a promising treatment product for alopecia areata.

Keywords: Clove oil, Hair serum, Nanoemulsion, Hair growth, Androgenetic Alopecia

INTRODUCTION

Androgenetic alopecia (AA) is the most common type of hair loss and characterized by pattern baldness. AA is disorder associated with genetic factor and the role of androgen hormone activity (Tanaka et al., 2018). AA has a genetic predisposition caused by an excessive response to androgen, especially testosterone hormone (Lolli et al., 2017). Testosterone circulate in the scalp and reaches the scalp through capillaries. Testosterone was converted to dihydrotestosterone (DHT) by enzyme 5 alpha-reductase (5αR). DHT hormone has fivefold greater affinity with androgen receptor (Kabir & Goh, 2013; Kidangazhiathmana & Santhosh, 2022).

In AA condition, hair follicles differentiation was miniaturized and hair growth cycle was changed. Hair follicle miniaturization caused by terminal hair become vellus hair, leading to hair thinning. The hair growth cycle of head hair is divided into anagen phase (growth phase), catagen phase (transition phase), and telogen phase (resting phase). In normal condition, ratio of anagen phase to telogen phase is 12:1, but in AA

ratio was decreased to 5:1. Shortening of anagen phase duration caused by decreased number and size of dermal papilla cells and lead to hair follicle miniaturization or hair production was decreased. The hair shafts became short and thin (Kabir & Goh, 2013).

Certain medication has been used to treat AA, including systemic and topical drugs. Minoxidil and finasteride are the most common drugs for AA therapy. Minoxidil is vasodilator and was used for first line in AA therapy and finasteride is 5 alpha-reductase enzyme inhibitors, also known as DHT blockers. Long-term use of both drugs causes several side effects such as dermatitis, depression, sexual dysfunction including gynecomastia, libido, erectile dysfunction, and ejaculation disorders (Rai et al., 2018).

Clove (*Syzygium aromaticum L.*) is spice plant native to Indonesia and the one of aromatic plants which have the distinctive aroma. Empirically, aromatic oil obtained from clove leaves was used to stimulate hair growth by applied on the head scalp regularly in the morning and evening. Essential oil was produced from cloves contain eugenol as the main component. Eugenol has the anti-androgen properties by the inhibiting of androgen hormone activity. Clove essential oil has promising potential herbal medicine to treat AA and safe for long-term. However, eugenol is volatile, not heat resistant, easy oxidized, and lead to chemical structure of compound was changed. In addition, eugenol cause skin irritation (Abadi et al., 2020; Ogawa et al., 2010; Rahmi et al., 2021; Xue et al., 2019).

Nanoemulsion as an advanced technology of drug delivery system has been applied to enhance stability and bioavailability of essential oil as therapeutic agent (Nirmala et al., 2020; Yakoubi et al., 2021). Nanoemulsion is a class of emulsions, typically has droplet sizes between 20 and 200 nm, thermodynamically stable, and provide stability against creaming, sedimentation, flocculation and coalescence (Moradi & Barati, 2019; Shaker et al., 2019). Research conducted by Nirmala et al. (2020) and Agnish et al. (2022) showed that the encapsulation of essential oils into a nanoemulsion delivery system has been proven effective in protecting the volatile components of essential oils from various forms of damage of degradation while also enhancing the stability and bioavailability of these components. Accordingly, the present study aims to prepare and characterize the clove essential oil as a hair serum nanoemulsion in hair growth nutraceutical preparations. Hair growth activity was evaluated to for its potency by *in vivo* studies.

MATERIALS AND METHODS

Materials

Clove essential oil (*S. aromaticum L.*) Cremophor® RH 40 were purchased from Lansida. Butylated Hydroxytoluene (BHT) was purchased from Sigma-Aldrich. Propylene glycol, glycerine, and distilled water were purchased from PT. Brataco. Minoxidil 2% (Regrou ®) was obtained from PT. Surya Dermato Medica Laboratories.

Animals

Male New Zealand White rabbits of approximately age between 3-4 months and weight 2-3 kg obtained from Terminal Kelinci, Bantul, Yogyakarta, Indonesia. All rabbits were maintained with controlled condition and acclimatized for 7 days before treatment. Food and drink standard provided ad libitum. The experimental protocol is approved by Ethical Clearance Committee, Faculty of Veterinary Medicine, Gadjah Mada University (No. 082/EC-FKH/Eks./2022).

Identification the chemical compound

The sample injection volume was 1 µL with a concentration of 1% in methanol. Identification using GC-MS instrument. The condition of instrument was followed: oven temperature 280ºC, injector and detector temperature were 250 °C ,cross-linked 5% methyl phenyl silox, capillary column (30 m × 0.25 mm, film thickness 0.25 m). Flow rate of helium as carrier gas was 1 mL/min.

Formulation and optimization of the hair serum nanoemulsion loaded clove essential oil (HSN-CEO)

Formula optimization was conducted with D-Optimal design using Design-Expert software. The composition of components nanoemulsion including CEO, cremophor RH 40 (surfactant) and propylene glycol (co-surfactant) was used as independent variable and the responses were droplet size and polydispersity index (PDI). The optimization conditions for HSN-CEO were predicted by assessing the priority values of each response using Design-Expert software. The validation of these optimal conditions was conducted by comparing experimental results with the predicted values provided by the software. Statistical analysis was performed using a one-sample t-test with a 95% confidence interval ($p>0.05$). Additionally, the analysis was carried out using Design-Expert software, considering both the

95% confidence interval (CI) and the 95% prediction interval (PI).

Hair serum nanoemulsion loaded CEO was prepared using spontaneous emulsification method by mixing CEO and 1% BHT as oil phase with cremophor RH 40 and propylene glycol. The components were then mixtured for 1 h at 400 rpm and temperature 25°C. The mixture was further emulsified with water and 10% glycerine as aqueous phase.

Stability studies

Stability studies were conducted by storage at room temperature for 30 days. The appearance, droplet size, pH and viscosity were determined at day 30.

Skin irritation studies

The study was conducted using patch-test method. The back of the rabbit was divided into several of area exposed as include HSN-CEO, negative control (serum base), positive control (serum in market) and normal control (not given treatment). Each area was exposed by applying the sample to area of the skin. The sample residue was removed after 4 h of exposure. The responses such as erythema and oedema were observed at 1, 24, 48 and 72 h after the patch to be opened. The skin irritation scores of were determined using calculation of Primary Irritation Index (PII).

Hair growth activity studies

The studies were conducted for 3 weeks by divided the back of the rabbit into several of area exposed as include normal control (not given treatment), negative control (distilled water), positive control (minoxidil 2%), CEO and HSN-CEO. Each area exposed was applied with 1 ml of the sample for twice a day and observed the hair growth by measuring hair length every week. Hair weight was obtained by shaving and weighing the hair at day 21.

RESULTS AND DISCUSSION

Chemical Composition of CEO

The GC-MS analysis identified eight components in CEO (Table I). Eugenol was the predominant component, making up 60.72%, followed by caryophyllene at 27.62%. The concentrations of the other components were below 0.5%. The result was in line with recent study that showed CEO contains eugenol as the major component (Amelia et al., 2017).

Table I. Results of chemical compound content of clove oil based on GC-MS analysis

No	Chemical compund	Retention time (minute)	% area
1	Diaseton alkohol	3.887	2.89
2	Eugenol	15.440	60.72
3	alfa-Copaene	15.753	0.55
4	Kariofilen	16.039	27.62
5	alfa-Humulen	16.233	2.91
6	Eugenil asetat	16.371	4.40
7	Delta-Cadinena	15.574	0.23
8	Kariofilen oksida	16.929	0.69

Formulation and optimization of the HSN-CEO

Preparation of the HSN-CEO was started with the preliminary test to determine the upper and lower limit the components of nanoemulsion. The range concentration of CEO, cremophor RH 40 dan propylene glycol was showed the good quality result is 10.00-12.50% v/v, 54-74.08% v/v, and 14.82-36% v/v, respectively. The result was further used to determine the formula design using D-Optimal design. A total of 14 formula exhibited droplet sizes within the nanoemulsion range of 20-200 nm (Table II). Additionally, the formulations had PDI values below 0.5, indicating a uniform distribution of droplet sizes.

ANOVA analysis results for the droplet size response (Y1) indicate that the proposed model is significant with a p-value of 0.0135 ($p<0.05$) (Table III). The adequate precision value obtained in the analysis test is 6.9517, which indicates that the linear model can be used to adequately explain the droplet size response because the value is >4. Additionally, the analysis test also produced an R2 value of 0.5430, indicating that the components of oil, surfactant, and cosurfactant as independent variables influence the droplet size by 54.3%.The regression equation indicates that the response of droplet size, the concentration of CEO (A), Cremophor RH 40 (B), and Propylene glycol (C) have a synergistic effect in increasing droplet size because the coefficients A, B, and C are positive. The component that has the greatest influence on the increase in droplet size is CEO, because it has the highest coefficient value. The results obtained are in line with previous research which states that droplet size will continue to increase as the oil concentration is further increased (Li et al., 2022).

Table II. Formula design and results of characterization from HSN-CEO

Run	Composition (%v/v)			Response	
	Clove oil	Cremophor RH 40	Propylene glycol	Droplet size (nm)	PDI
1	11.65	65.85	22.50	36.19	0.387
2	11.06	66.70	22.24	37.30	0.338
3	12.01	66.12	21.87	34.76	0.302
4	11.61	66.23	22.16	33.79	0.279
5	11.51	66.62	21.87	36.98	0.336
6	12.25	65.63	22.12	40.22	0.400
7	10.99	67.14	21.87	28.55	0.191
8	11.06	66.70	22.24	32.05	0.262
9	10.54	67.50	21.96	32.70	0.295
10	11.23	66.27	22.50	33.75	0.345
11	12.25	65.63	22.12	40.09	0.398
12	10.50	67.00	22.50	36.64	0.336
13	11.06	66.70	22.24	34.97	0.285
14	10.00	67.50	22.50	24.84	0.145

The linear model is also the recommended model based on the analysis of the PDI response value (Table III). The results of the analysis on the linear model have met the requirements of the D-Optimal Design analysis because it obtained a p-value of 0.0088 ($p<0.05$), which is a significantly recommended model. The adequate precision value obtained in the analysis test is 7.1227, which indicates that the linear model can be used to adequately explain the PDI response value because the value is >4. In addition, the analysis test also produced an R2 value of 0.5775, indicating that the components of oil, surfactant, and cosurfactant as independent variables influence the PDI value by 57.75%. The three components of the nanoemulsion (CEO, cremophor RH 40, and propylene glycol) have a direct relationship with the PDI value, and these three components exhibit synergy in increasing the PDI value because coefficients A, B, and C are positive (Table II). The component that has the greatest influence on the increase in PDI value is propylene glycol, because it has the largest coefficient value. Referring to previous studies, it is stated that the increase in PDI value can be caused by the high concentration of co-surfactants (Algahtani et al., 2022).

The concentration of CEO, cremophor RH 40 and propylene glycol in the optimum formula were obtained 10.63%; 67.5%; and 21.87%, respectively. The optimum formula was further measured to determine the size of droplet and PDI value. The measurement result showed that average droplet size was 31.26±0.15 nm (31.20 nm, 31.15 nm, and 31.44 nm). Droplet diameters ranging from 10 to 1000 nm enable their accumulation in hair follicles, facilitating the delivery of therapeutic agents (Tampucci et al., 2022). Based on the result, the droplet size of HSN-CEO was in range capable of penetrating through hair follicle as site of action for AA treatment.

The average PDI value of optimum formula was 0.240±0.008 (Table II). The results of the study showed that HSN-CEO droplets are monodisperse, meaning they have a uniformly distributed droplet size. The smaller of PDI value prevent nanoemulsion from various factors that cause the unstable condition such as creaming, sedimentation, flocculation and coalescence. It maintains the long lasting stability of the nanoemulsion preparation. The droplet size and polydispersity index affected the stability of nanoemulsion (Alhasso et al., 2023).

Stability studies

Visual observations were not showed physical changes after 30 days of storage. The periodic measurement result showed that droplet size and viscosity were increased continuously, whereas pH value was decreased (Table IV). However, the change of droplet size, pH, and viscosity at day 30 was not significantly different compared to day 0 based on the statistical analysis result ($p>0.05$). The stability of the HSN-CEO exhibited a good physical stability for 30 days at room temperature.

Table III. Results of the agreement conditions for statistical parameters and regression equations in the experimental design

Response (Y)	Model	R^2	Adequate precision	Regression equations
Droplet size (Y_1)	Linear*	0.5430	6.95170	Y_1= 39.5182A+27.2497B+35.1919C
PDI (Y_2)	Linear*	0.5775	7.1227	Y_2=0.383534A+0.157984B+0.428392C

Description:
**)= significant recommended (p =0.05); A = concentration of CEO; B = concentration of Cremophor RH 40; C = concentration of Propylene glycol*

Table IV. Results of the stability studies from formula optimum of HSN-CEO in 30 days at temperature room.

Day	Visual	Average of droplet size (nm)±SD	pH±SD	Viscosity (cP)±SD
0	clear	31.26±0.155	5.55±0.01	2386.18±62.77
8	clear	31.35±0.133	5.54±0.01	2458.70±40.54
15	clear	31.42±0.073	5.51±0.01	2470.02±42.87
22	clear	31.62±0.045	5.47±0.02	2490.09±22.98
30	clear	31.79±0.069*	5.45±0.01*	2494.87±22.01*

Description:
**)= P value > 0.05, not significantly different to Day 0*

The pH value of HSN-CEO was 5.55±0.01. Based on the result, the HSN-CEO preparation was safe for topical use because the pH value was ranging in 5-6. The pH value falls within a range that does not cause skin irritation (Changediya et al., 2019). The viscosity of HSN-CEO was 2386.18±62.77 cP, falling within the typical range for serum formulations, which is 800-3000 cP (Kamishitta et al., 1992). Based on the result, the HSN-CEO had high viscosity and resulting in higher resistance to flow. Glycerine, used as a humectant in this formulation at a concentratuin of 10-20%, contributed to an increase in vicosity (Savic et al., 2021).

Skin irritation

Result of the skin irritation test on male New Zealand white rabbits wasn't showed erythema and oedema responses after exposured four sample. The scores is 0 at 1, 24, 48, and 72 h. The response category was classified as very mild because the primary irritation index are in the range of 0.0-0.4 which indicates very mild or negligible (Badan POM RI, 2022). The CEO contain eugenol as the main compound, which has the potency to cause irritation and dermatitis if exposed to the skin directly. Encapsulated of the CEO in nanoemulsion formulation wasn't showed the dermal toxicity. Formulating essential oils into nano-delivery systemscan help minimize irritation caused by active substances (Ullah et al., 2022). Therefore, the HSN-CEO preparation s safe for topical use.

Hair growth activity

This in vivo hair growth activity studies were used two parameters, the hair length and the hair weight. The hair length was influenced by the duration of the anagen phase. The characteristic of anagen phase is active growth of the hair shaft (Orasan et al., 2016; Wall et al., 2022). Whereas the hair weight was related to hair density. The hair density was impact from duration of the kenogen phase. During kenogen phase, the follicle was empty that occurs after telogen phase and before entering to anagen phase. The increased of the duration kenogen phase due to activation of androgen receptors lead to narrowing of the hair follicles, resulting in thinning of the hair follicles and reducing hair density (Aini, 2017; Katzer et al., 2019; Wall et al., 2022).

The result of testing hair growth activity in male New Zealand white rabbits showed that the HSN-CEO group had the longest of hair length at days 7, 14, and 21, with the value of 0.81±0.07 cm, 1.27±0.08 cm, and 2.28±0.12 cm, respectively. The hair length obtained from the HSN-CEO group was longer than pure CEO group for 3 weeks. The statistical analysis was using to find out the difference of hair length between the HSN-CEO group with pure CEO group (Figure I). The result of the study showed a significant difference ($p<0.005$) in hair length on days 7, 14 and 21.

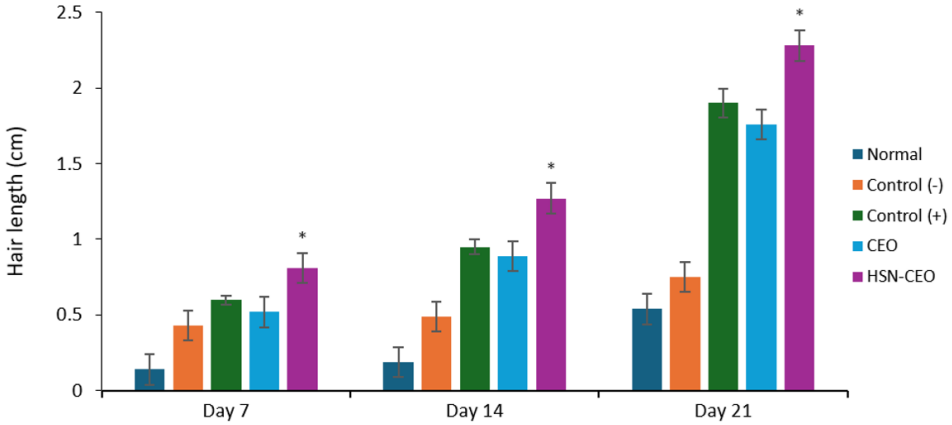

Figure 1. The results of the difference in hair length of mice from various test animal groups on day 7, day 14, and day 21
Description: (*) Different significant (p < 0.05) to CEO

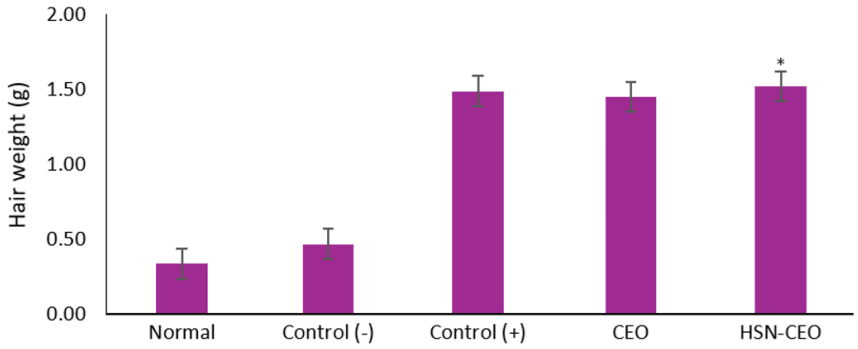

Figure 2. The results of the difference in mouse hair weight from various test animal groups on day 21
Description: (*) Different significant (p < 0.05) to CEO

Based on the observations result of hair growth activity, the hair length of rabbits on the five treatments was rapidly growing after the second week. This study demonstrates that the hair growth cycle reaches the anagen phase after the 14th day. This phase was characterized by the hair is generated and extends growth actively. Hair growth can be stimulated by provide the nutrition or medicine to prolong the duration of anagen phase and resulting to increase the length of the hair shaft (Orasan et al., 2016). In this study, administration of minoxidil 2%, pure CEO, and HSN-CEO has the role to stimulate the hair growth.

Based on the result (Figure 2) the HSN-CEO group had the highest of hair weight with value of 1.52±0.07 g. The hair weight of the HSN-CEO group was greater than the pure CEO group. The statistical analysis was resulted p-value of 0.049 ($p<0.05$), which was showed significant difference. Administration of minoxidil 2%, pure CEO, and HSN-CEO stimulated the growth of hair shaft compared to normal condition. Minoxidil 2% is a vasodilator agent that stimulates hair groth, but its long-term use can cause various serious side effects (Rai et al., 2018). Clove oil is a potential alternative, with its eugenol compund demonstrating a strong effect in stimulating hair growth (Shahtalebi et al., 2016).

The CEO as therapeutic agent containing eugenol compound which has the pharmacological activity by anti-androgen. The pure CEO was used in this study had the larger concentration than the HSN-CEO. Based on the hair growth activity test, development of CEO to nano-sized droplet had been successfully to be more stimulate the hair growth than pure CEO. In addition, the HSN-CEO was showed better efficacy for promote hair growth than synthetic drug. Therefore, the HSN-

CEO preparation could be nutraceutical product for the treatment of baldness caused by androgen hormone activity.

CONCLUSION

Hair serum nanoemulsion loaded clove essential oil was prepared based on selected optimum formula. The formula was characterized for droplet size, size distribution, pH, and viscosity, stability, and skin irritation. HSN-CEO is effective in promoting hair growth and has the potential to be a treatment for androgenetic alopecia. However, further research is needed to analyze consumer preferences for this product.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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