Anti-inflammatory Effect and Physical Stability Test of Nanoemulgel Containing Marjoram (Origanum majorana L.) Essential Oil

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Abstract. The ability of marjoram essential oil (MEO) to reduce pro-inflammatory agents, C-reactive protein (CRP), is gaining attention these days. The nanoemulgel development is needed to increase permeation of the active components. Furthermore, proving the anti-inflammatory effectiveness and stability of MEO nanoemulgel is the key to the success of the formulation. This study aimed to formulate and perform anti-inflammatory and physical stability studies on a nanoemulgel formulation containing MEO. MEO (Darjeeling CAS 8015-01-8) nanoemulgel was prepared. The edema volume formation with carrageenan is a method used for anti-inflammatory test. The stability studies used are accelerated methods and cycling tests. MEO nanoemulgel shows anti-inflammatory activity, where 2% MEO nanoemulgel produces a better anti-inflammatory effect compared to 1% MEO because it has a greater reduction in edema volume and CRP levels. Independent T-test results show that there are no significant differences in all physical preparation parameters, before and after stability test. In tests, the nanoemulgel formulation prepared in this study was found to be safe and did not cause erythema or edema when applied to the skin.

Keywords: Marjoram, Nanoemulgel, Antiinflamatory.

1 Introduction

Inflammation is a defensive reaction process by which the body attempts to neutralize and eradicate harmful substances when infection, tissue damage, or other disorders occur. This is how the immune system starts the healing process by identifying and eliminating dangerous or alien stimuli [1]. Diseases involving inflammatory processes in the body in Indonesia have quite high incidence rates, with a national prevalence of asthma 2.4%, acute respiratory infections 9.3%, pneumonia 4%, joint diseases 7.3%

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[2]. Early inflammatory arthritis has an annual incidence of 115 to 271 per 100,000 individuals, while undifferentiated inflammatory arthritis (UA) has an incidence of 41 to 149 per 100,000 persons. Thirteen to fifty-four percent of individuals with undifferentiated inflammatory arthritis go on to develop rheumatoid arthritis (RA), whereas twenty-one to eighty-seven percent of them never progress beyond this stage.

It is known that there is a relationship between oxidative stress and chronic inflammation in rheumatism [3]. Reactive oxygen species (ROS) are considered media- tors of tissue damage in arthritis patients. Changes in the balance of oxidants and anti- oxidants can cause tissue damage. Therefore, the simultaneous administration of anti- oxidants is also important in inflammatory arthritis, besides topical non-steroidal anti-inflammatory drugs.

Marjoram essential oil (MEO) has recently received attention for its ability to reduce the inflammatory substance C-reactive protein (CRP). Marjoram essential oil (MEO) contains terpene compounds such as sabinene, p-cymene, and thymol, and high-content components include carvacrol and terpinen-4-ol. Studies have shown that MEO can act as an anti-inflammatory agent by reducing proinflammatory substances such as TNF-α, IL-1β, IL-6, and IL-10. In another study, carvacrol, one of the largest components of MEO, was able to reduce the pro-inflammatory mediator C-reactive protein (CRP), and there was no effect on kidney damage.

A dermal pharmacokinetics study showed that terpinene-4-ol and carvacrol could only diffuse less than 10% even though both have a molecular weight of less than 500 daltons, indicating that they still have the potential to be delivered through the skin [4], [5]. The nanoemulgel development is needed to increase permeation of the active components. This research is follow-up research, where in previous research it was found that marjoram nanoemulgel with a combination of polysorbate 80 surfactant and polyethylene glycol 400 9,958:6,042 could produce nanoemulgel with good characteristics. Furthermore, proving the anti-inflammatory effectiveness and stability of MEO nanoemulgel was carried out in this research.

2 Material and Method

2.1 Material

The materials used in this research were marjoram essential oil (Darjeeling, Mar-joram Oil Egypt® CAS #8015-01-8), polysorbate (polysorbate) 80 (Petronas), polyeth-ylene-glycol (PEG) 400 (Petronas), aquadest (Multi Kimia Raya Nusantara), DMDM-Hidantoin (Clariant), Trietanolamin (Multi Kimia Raya Nusantara), Carbophol 940 (Dunia Kimia Jaya), Xanthine (Merck), Karagenan (Sigma), Voltaren® Emulgel.

The tools used in this research were gas chromatography - mass spectrometry (GC-MS) Shimadzu GCMS-2010 Plus, spectrophotometer Uv-vis Shimadzu 1280, par-ticle size analyzer Malvern MAL1275495, viscosimeter Brookfield, pH meter Trans instrument HP 9000, plethysmometer, Glory CRP Test Kit (GD-CRP100, 24895).

2.2 Method

Identification of MEO compound using GC-MS (Gas Chromatography- Mass Spectrometry). The study of essential oil compounds was carried out using a gas chromatography-mass spectrometer (Shimadzu 2010 Plus).

Preparation of MEO Nanoemulsion. Three main ingredients are needed to make an emulsion: an oil phase, an aque- ous phase, and a surfactant. The process involved mixing 1% and 2% MEO in a glass vial followed by 5 minutes of mixing. Subsequently, 16% Smix (polysorbate 80 and PEG 400) was mixed with oil phase MEO for 5 min, soni- cated for 10 min, and then incubated in a water bath at 40 °C for 5 min. This process was repeated for three cycles. 100% distilled water was added as the aqueous phase. To evaluate the transparency of nanoemulsions, spectrophotometry was used to measure the transmittance at a wavelength of 650 nm with dis- tilled water as a reference [6].

Preparation of MEO Nanoemulgel. The MEO nanoemulsion was then dispersed in a gel base with the composition: carbophol 940® 1%, triethanolamine 0.1%, DMDM hydantoin (1,3-dimethylol-5,5-dimethyl-hydantoin) 0.03%. Nanoemulgel preparations with drug loading of 1% MEO and 2% MEO were prepared in five replicates, respectively. Carbopol 940® was ground with triethanolamine until a gel mass was formed, and then DMDM-hydantoin was added. 50 mL of the prepared nanoemulsion was added to the gel base and gently stirred at low speed until homogeneous.

Physical characterization of MEO Nanoemulgel.

pH Test. The pH of the gel was measured with a pH meter and the value was recorded after calibration.

Viscosity Test. Viscosity was measured by placing the sample in a Brookfield viscometer with an immersion spindle set at a speed of 50 rpm.

Adhesion Test. For adhesion testing, 500 mg of the formulation was placed on two microscope slides and a load of 1.0 kg was applied for 5 min. The time required for the slides to peel off was recorded.

Spreadability Test. Spreadability was assessed by placing 0.5 grams of gel on a glass cylinder for 1 minute and measuring the distribution on all four sides using a ruler. Every 50-gram weight was added to the load until a constant weight was reached.

Anti-inflammation In Vivo Testing. This test already has an Ethical approval from Health Research Ethics Committee with certificate number 533/YP-

NA/KEPK/STIFAR/EC/VIII/2023, Stifar Yayasan Pharmasi Semarang, Indonesia. A total of 25 male white rats (Rattus norvegicus) were randomly selected and divided into four groups. Each group consists of five 3-month-old male white mice weighing 200–300 grams. Before testing, rats were allowed to acclimate for 7 days and fasted for 18 hours while provided with drinking water. On the day of the experiment, the right hind paw of the rats was coated according to the following treatment groups:

Negative control group : 200 mg nanoemulgel base
Positive control group : 200 mg Voltaren® Emulgel
Normal control group : Nothing was given
Test group 1 : 200 mg 1% of MEO nanoemulgel
Test group 2 : 200 mg 2% of MEO nanoemulgel

After treatment, 0.1 ml of carrageenan solution was injected into the right hind paw of all groups except the normal control group. The volume of the right hind of rat was measured at 0 and 30 min after carrageenan (Vt) injection. At the 30th minute, 1 ml of blood from the tip of the rat's tail was also taken for CRP measurement.

This study used the latex agglutination method to measure C-reactive protein (CRP). The idea behind latex agglutination CRP testing is to use antibodies to bind to particles in order to identify antigens in serum samples. The serum sample being analyzed is mixed with a suspension of latex particles coated with anti-human CRP antibodies to conduct the test. Visible aggregates are a sign that CRP levels have risen to levels that are clinically significant.

Stability Test. For stability testing, the cycling test method is used. The preparation was stored at 4±2oC for 24 hours, then stored at 40±2oC for the next 24 hours. This treatment is 1 cycle of treatment and testing is continued for up to 6 cycles [7].

Dermal Acute Irritation Test of MEO Nanoemulgel Preparations. A healthy male New Zealand rabbit is shaved such that the back hair measures 2.5 × 2.5 cm2, and 0.5 grammes of the nanoemulgel mixture is applied. At0,6, and 24 hours, the reaction to the degree of erythema and edoema was evaluated. The assessment of skin irritation was done visually. The following scale was used to evaluate skin irritation: 0 denotes no skin irritation, 1 mild skin irritation, 2 certain skin irritation, 3 moderate skin irritation, and 4 scarring [7].

3 Result and Discussion

3.1 GC-MS (Gas Chromatography-Mass Spectrometry) Analysis

The components of bioactive compounds in marjoram essential oil (MEO) have been reported to have a variety of potential activities. The two largest components of MEO were trans-sabinene hydrate (retention time 18.135 min, mol and peak area 15.54%) and terpinene-4-ol (retention time : 20.317 min and peak area 26,70%) (Fig. 1). The

other compounds are alpha pinene, beta pinene, sabinene, cis sabinene, and gamma terpinene. This result is almost the same as previous research which also studied the identification of MEO components [8], [9].

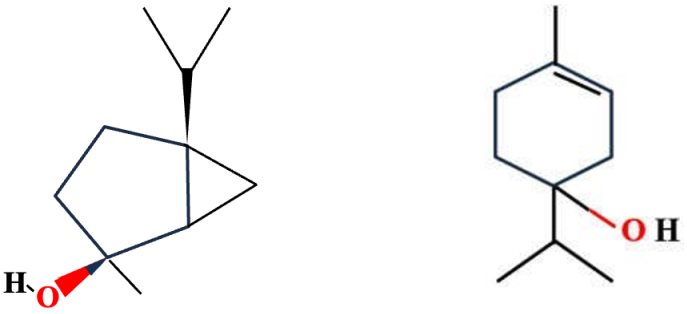

Fig. 1. Chemical structure of two largest compounds on MEO (a) trans-sabinene hydrate (b) terpinene-4-ol

3.2 Physical characterization of MEO Nanoemulgel and Its Stability Test

The pH values of MEO nanoemulgel preparations with 1% and 2% content were still in the range of 4.5–6.5 before and after the stability test. The pH value of the pre- pared nanoemulgel formulation corresponds to that of the skin. The pH value of the prepared formulation is optimal for topical application without causing skin irritation [10]. Viscosity is an important factor in drug delivery through the skin. The viscosity of topical formulations directly affects drug release, distribution, stability, and ease of use throughout the body [11]. The therapeutic efficacy of topical preparations depends on their spreadability. The ability of a topical application to spread over the entire skin surface is called spreadability. The lower the viscosity, the higher the spreadability of preparation. Optimal spreadability allows topical formulations to come out of the container more easily, even under low shear stress. In this research, it can be seen that the greater the MEO loading, the viscosity will decrease, followed by a decrease in the adhesive strength of the preparation, but both still meet the criteria for semi-solid adhesive strength, namely more than one second [12].

Table 1. Physical characteristics of MEO nanoemulgel before and after stability test

Physical characteristic Test	Before cycling test	After cycling test	Before cycling test	After cycling test
pH	5.16 ± 0.16	5.15 ± 0.14	5.28 ± 0.29	5.02 ± 0.21
Viscosity	5.63 x 10^5 cps	5.13 x 10^5 cps	5.29 x 10^5 cps	5.21 x 10^5 cps
Spreadability	3.8 ± 0.27 cm	4.1 ± 0.07 cm	4.2 ± 0.24 cm	4.1 ± 0.16 cm
Adhesion Power	1.55 ± 0.32 s	1.65 ± 0.25 s	1.40 ± 0.23 s	1.32 ± 0.16 s

The stability test (using the cycling test method) showed that the 2% MEO nanoemulgel did not experience significant changes in the test values for the physical characteristics of the gel, this was indicated by a p value > 0.05 from different test method, independent samples t-test, which means there wasno difference before and after the stability test.

3.3 Anti-inflammation In Vivo Testing

Measuring the edoema volume in test rats and the levels of C-reactive protein (CRP) in test animals following carrageenan induction were the two methods used to perform the anti-inflammatory activity test (Fig. 2). Carrageenan induction will cause the release of histamine and serotonin within the first 90 minutes after induction. This is what causes swelling in feet od rats due to carrageenan [13].

Procalcitonin (PCT), C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), and counts of neutrophils and white blood cells are the primary markers of inflammation. Six hours following acute inflammation, the liver produces CRP, an acute phase protein. The CRP test is a very sensitive and specific marker ofinflammation [14]. In previous research, marjoram could reduce CRP levels. In addi- tion, marjoram also has a mechanism to reduce the secretion of pro-inflammatory cy- tokines and gene expression in ox-LDL activated macrophages [8].

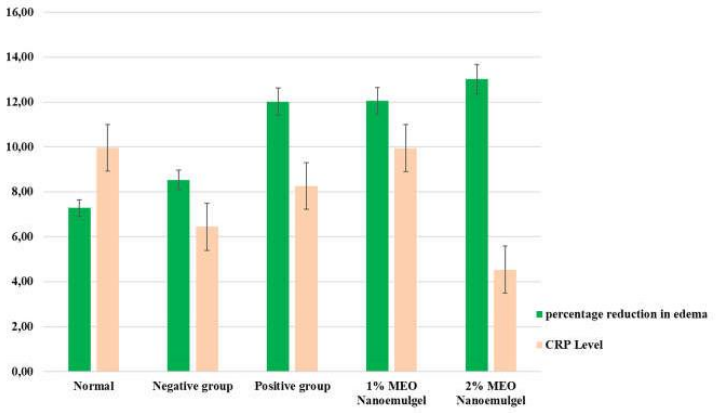

Fig. 2. Percentage reduction in edema and CRP levels 30 minutesafter carrageenan induction

The results showed that 2% MEO nanoemulgel has greater anti-inflammatory power than 1% and also positive control. Statistical tests using One-way ANOVA showed that there was no difference in the percentage value of edema reduction in all groups (p value>0.05). However, in percentage reduction in edema of 1% MEO Nanoemulgel was 12.05$\pm$1.77% and CRP levels of 99.40$\pm$9.81ppm). While 2% MEO nanoemulgel has a decrease in levels of 13.02+3.74%.

The statistical test results of CRP values showed that the CRP level of the normal group and the MEO 2% nanoemulgel group showed a significant difference ($p<0.05$).

2% MEO Nanoemulgel had CRP levels of45.4±46.81ppm at 30 minutes (50% lower than the normal group). Essential oils are also known as enhancers in topical preparations, therefore nanoemulgel with higher levels allows it to have higher activity [15].

3.4 Dermal Acute Irritation Test of MEO Nanoemulgel Preparations

Skin irritation potential studies were performed on all prepared MEO nanoemulgel (Table 2). The irritation test is carried out to determine the irritation effect of the gel preparation after use on the skin, so that the level can be determined safety of the gel preparation before being sold to the public.

Table 2. Skin irritation studies: erythema and edema

Preparations	erythema score, hour to-			edema score, hour to-		
	0	**6**	**24**	**0**	**6**	**24**
1% MEO Nanoemulgel	0	0	0	0	0	0
2% MEO Nanoemulgel	0	0	0	0	0	0

This irritation test is carried out to prevent side effects on the skin. The results showed that the preparation did not cause any reaction symptoms (either erythema or edema) up to 24 hours of use. Therefore, this study found that the MEO nanoemulsion gel was non-irritating and could be used for topical application [16].

4 Conclusion

MEO nanoemulgel exhibits anti-inflammatory activity; however, due to a larger reduction in edoema volume and CRP levels, MEO nanoemulgel with 2% loading has a stronger anti-inflammatory effect than MEO 1%. There were no appreciable variations in any of the physical preparation parameters between before and after the stability test, according to the independent T test results.

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