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Physical Stability and Antibacterial activity of Black Cumin Oil (*Nigella sativa* L.) Nanoemulsion Gel

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Abstract: Black cumin oil is a herbal product that hasthe potential to treat certain ailments. One of its benefits is its antibacterial activity. The purpose of this research was to formulate this oil into nano emulsion gels and then test the physical stability and antibacterial activity. Black cumin oil was formulated into nanoemulsion gels in various concentrations of 5%, 7%, and 9%. Physical stability tests were conducted by recording effects of storage at room temperatures ($25^0C \pm 2^0C$), high temperatures ($40^0C \pm 2^0C$), and low temperatures ($4^0C \pm 2^0C$), a centrifugation test and a cycling test. Antibacterial activity assay usedthe disc method and the results were statistically analyzed using ANOVA to see if there were any significant differences among inhibiting zones. The results showed that in yelloworange- and translucent nano emulsion gels, no phase separation occurs, and they have a globule size of below 1 micrometer. Nanoemulsion gels are stable at room temperatures ($25^0C \pm 2^0C$) and low temperatures ($4^0C \pm 2^0C$). Nanoemulsion gels have a smaller inhibiting zonethan the black cumin oil ($P < 0.01$). In addition, there were significant differences between the inhibiting zone of negativeblanks and the inhibiting zone of preparations containing black cumin oil ($P < 0.01$).
Keywords: black cumin oil, antibacterial,inhibiting zone, nanoemulsion gel, *Nigella sativa* L., physical stability

Introduction

Nowadays, herbal products are used as an alternative to synthetic chemical drugs. One such product is black cumin (*Nigella sativa* L.). The content of black cumin oil has been investigated and found to have beneficial antibacterial pharmacological effects[1]. Based on this, black cumin oil is used as the oil phase and active substance in this study and is formulated into nanoemulsion gel (nanoemulgel) dosage form.

Nanoemulsionconsists of nanosized globules of liquid dispersed in another liquid. Nanoemulsion is in the form of a liquid, such as water, lotion, or gel[2]. Emulsion gel (emulgel) has advantages over emulsion preparation and gel preparation[3].

Nanoemulsion gel (nanoemulgel) dosage form was chosen because this preparation is suitable for topical use, and its small globule size is expected to penetrate the skin better thus increasing antibacterial effectiveness. Furthermore, dosage forms are also becoming more stable and convenient to use by consumers.

Skin is the outermost tissue of the human body which means people are very aware of and very sensitive to the appearance of their skin[4]. Skin serves as the body's defense against environmental hazards[5]. Therefore, the skin is the part of the body which is most often exposed to or comes into contact with chemical and biological substances, and mechanical impact. An example is that the skin harbors bacteria [6].

In fact, there are skin microorganisms called normal flora[7].. Normal flora is not harmful to humans under normal conditions. However, normal flora can cause infections in other parts of the body, such as during surgery, or whenever the immune system is weakened.Furthermore, the microorganisms that live on the skin can cause problems, such as *Staphylococcus aureus*in infected hospital patients. These conditions accentuate the need for antibacterials notwithstanding the emergence of antibacterial resistant bacteria. Taking this into account, it is necessary to find other effective antibacterialstages in this study from developing nanoemulsion gel (nanoemulgel) formula using various concentrations of black cumin and to test physical stability and in vitro antibacterial activity of black cumin oil prepared innanoemulsion gel dosage form.

This study aims to develop and evaluate the physical stabilityand antibacterial activity ofnanoemulsion gel containing black cumin oil with different concentrations.

Materials and Methods

Materials

Homogenizer (CKL Multimix®, USA), pH-meter tipe 510 (Eutech Instrument, Singapore), Brookfield viscometer (Brookfield Engineering Laboratories, USA), *particle size analyzer zetasizer* ver. 6.20 (Malvern, Germany), centrifugator (Kubota 5100, Japan), tensiometer Du Nuoy (Cole Parmer Surface Tensiomat 21, USA), picnometer (OTTX), electrical heating (Ika®, Germany & Corning, USA), autoclave (Hiroyama, Japan), incubator (Memmert, Germany), Laminar Air Flow (ESCO, USA), micro pipet (Socorex®, Switzerland), Whatman™ filter paper no.41, paper disc, black cumin oil (obtained from PT. Prima Agritech Nusantara, Indonesia), Tween 80 (Sigma Aldrich,USA), Carbomer 940, propylene glycol, Sigma Aldrich, USA), ethanol (96%) (Merck, Germany), sodium hydroxide (Merck, Germany), distilled water, Nutrient agar media (Difco™, USA), Mueller-Hinton medium (Oxoid, UK), Sodium Chloride (Merck, Germany), and culture of *Staphylococcus aureus*(Laboratory of Microbiology, Faculty of Pharmacy, University of Indonesia)

Methods

1. Characteristics of Black Cumin Oil

Organoleptic characterization including the color and odor, pH measurement, surface tension, interfacial tension and oil density was taken.

2. Formulation and production of Nanoemulsion Gel

Nanoemulsion gel formula can be seen in table 1.

Table 1: Composition of ingredients in nano emulsion gel.

Material	***Concentration (% b/b)***			
	Blank Negative	**Formula 1**	**Formula 2**	***Formula 3***
Black Cumin Oil	**-**	5	7	*9*
Tween 80	40	40	40	*40*
Propylene glycol	5	5	5	*5*
Alcohol (96%)	15	15	15	*15*
Distilled water	15	10	8	*6*
Gel Base (carbomer 940 and NaOH)	*25*	*25*	*25*	*25*

Distilled water and Tween 80 were heated in separate containers until the temperature reached 35^{0}C, and then they were mixed and homogenized using a homogenizer with a speed of 3000 rpm. First of all black cumin oil was added and then a mixture of propylene glycol and alcohol (96%) was gradually added and homogenized using a homogenizer with a speed of 3000 rpm. After Nanoemulsion developed it rested for 24 hours. In a separate container Carbomer 940 was dispersed in distilled water, then NaOH solution was added little by little, and then homogenized using a homogenizer with a speed of 2000 rpm to form a gel base. The gel baserested for 24 hours. After that, the gel base was stirred using the homogenizer, then the nanoemulsion was gradually added into the gel base and homogenized using a homogenizer with a speed of 3000 rpm. Finally,the nanoemulsionwas added and the homogenizer speed was increased to 3500 rpm.

3. Evaluation of Nanoemulsion Gel Dosage Form

Organoleptic

Observations on any changes of colour, odor, clarity, and phase separation were made.

Globule Size Distribution

Globule size distribution was measured using zetasizer particle size analyzer ver. 6.20 (Malvern).

The pH value

The pH value of 1% nanoemulsion gel was measured using a pH meter.

Viscosity and Rheological

Rheological and viscosity of nanoemulsion was measured using the Brookfield viscometer at room temperature ($25^0C \pm 2^0C$). Viscosity measurements used one spindle speed and experiments were conducted three times, meanwhile the rheological spindle speed was regulated from low speed to high speed, and from high speed to low speed gradually.

Stability Test

These tests included the cycling test, 8 week storage at temperatures of $4^0C \pm 2^0C$, $25^0C \pm 2^0C$, and $40^0C \pm 2^0C$, and the centrifugation test (speed of 3800 rpm for 5 hours).

Antibacterial Activity Assay

This assay used the disc method. Disc diameters were 5 mm and 12 mm. Testing was conducted on black cumin oil and nanoemulsion gel. Each disc contained black cumin oil or nano emulsion gel which contained black cumin oil at 5 µl, 10 µl. or 20µl. The disc was placed on the surface of the test media and incubated at 37^0C for 24 hours. Inhibition zone diameters were measured using calipers and the results were recorded. The experiment was conducted three times. The data obtained were analyzed statistically using ANOVA.

Table 2: Dosage of formula 1, formula 2, formula 3, and negativeblank on each paper disc.

Dosage Form	Black Cumin Oil Content	Dosage Weight (g)
Formula 1	5 µl	0.0918
	10 µl	0.1836
	20 µl	0.3672
Formula 2	5 µl	0.0656
	10 µl	0.1312
	20 µl	0.2623
Formula 3	5 µl	0.0510
	10 µl	0.1020
	20 µl	0.2040
Negative blank	-	0.0918
	-	0.1836
	-	0.3672

Results and Discussion

1. Characterization of Black Cumin Oil

Table 3: Characterization of black cumin oil

Organoleptic		pH	Surface tension (dyne/cm)	Interfacial tension of oil and distilled water (dyne/cm)	Density (g/ml)
Color	Odor				
chocolate orange (Pantone 174 c)	specific	6.03 6.04 6.02	38.1337	11.8844	0.9181

2. Formulation and production of Nanoemulsion Gel

Formulation and production of nanoemulsion gel in this study is the result of the optimization process from adding a cosurfactant. A cosurfactant was needed to assistthe surfactant in lowering the interfacial tension and improve the solubilization of non-polar groups [8.] The amount of each material/ingredient in each formula was the same, except for the black cumin oil and distilled water, because it was intended to reduce variations in the antibacterial activity assay. The concentrations of black cumin oil used were 5%, 7%, and 9%. Higher amounts of oil concentrationmade it difficultto make nanoemulsion gel because of instability in the system.

The surfactant used was a non-ionic surfactant, ie. Tween 80, because it is relatively non-toxic and does not irritate anionic or cationic surfactants[9]. Propylene glycol and alcohol 96% (class of short chain alcohol) was used as the cosurfactant because the small molecules can quickly form between oil and water. Alcohol 96% was also used as a penetration enhancer. Carbopol940is a synthetic polymer used asa gelling agent. In this study no preservativessuch as methyl paraben or propyl paraben were used, so the test resultswere not biased on antibacterial activity of black cumin oil with other preservatives.

3. Evaluation of Nanoemulsion Gel

The results of the evaluation of nano emulsion at week one can be seen in table 4.

Table 4: Evaluation of nanoemulsion gel in first week

Dosage form	Colour	Odor	Clarity	Average size of globules	pH	viscosity	rheology
Formula 1	yellow orange +	specific	Translucent	71,67 nm	6,77	12650 cP	pseudoplastic
Formula 2	yellow orange ++	specific	Translucent	131,2 nm	6,65	4900 cP	pseudoplastic
Formula 3	yellow orange +++	specific	translucent	832,7 nm	6,56	3600 cP	pseudoplastic

Description : Yellow orange + = Pantone PMS 1375 c
Yellow orange ++ = Pantone PMS 144 c
Yellow orange +++ = Pantone PMS 138 c
Specific = mix odor like Tween 80 and odor of black cumin oil

Organoleptic

Formulas 1, 2, and 3 have different intensities of yellow-orangecolour. The greater concentration of black cumin oil used in a formula, the orange colournanoemulsion gel dosage form. Formulas 1,2, and 3 are viscous, translucent, homogeneous, and have no phase separation. This indicates that the concentration of surfactant (Tween 80) and cosurfactant (propylene glycol and alcohol 96%) used was appropriate. When applied to the skin, formulas easily dispersed and left a slightly sticky feel. This is because of the high concentration of Tween 80.

pH

In this study Nanoemulsion gels were developedwith the same percentage of ingredients, except black cumin oil and distilled water. Therefore, the pH of black cumin oil (pH 6,03) is the effect of pH nanoemulsion gels. The higher the concentration of black cumin oil, the lower is the pH of nanoemulsion gel. Formula 3, which has 9% of black cumin oil concentration, has more acidic pH than formula 2, which has 7%of black cumin oil concentration, and formula 1, which only has 5%of black cumin oil concentration. The pH value of the three formulas is still close enough to skin pH (near pH 6,50) so as to minimize the chance of irritation.

Globule Size Distribution

The mean diameter of the globule size decreased after 8 weeks storage. It could be due to the irregular long axis molecules that can lead some of the bonded solvent molecules to escape, causing an effective decrease in concentration and decrease in size of the dispersed molecules and lower viscosity[10]. In addition, the high concentration of surfactant used can lead to a lot of micelle formation. Micelles around oil globules only allow a small amount of monochromatic light to penetratethe oil globules therefore there is less light diffraction. The less light that is diffracted, the smaller is the size of the globule read on the particle size analyzer (PSA).

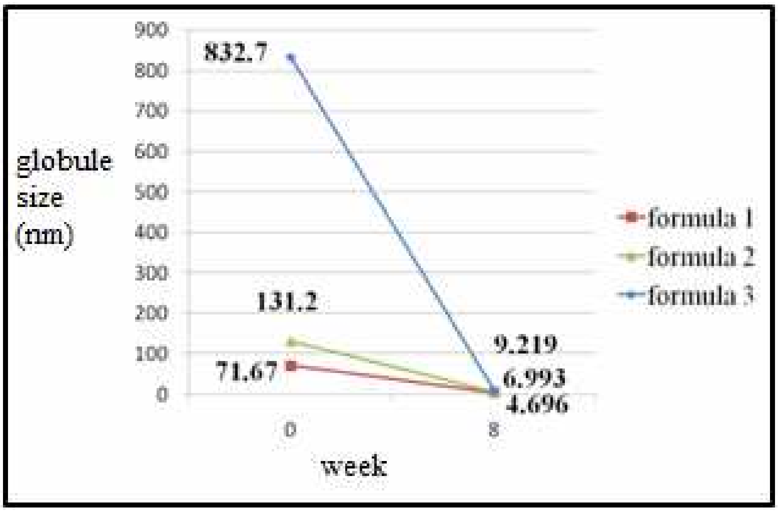

Figure 1: Graph of average globule diameter changes in nanoemulsion gel

Viscosity and Rheology

Viscosity of the three formulas was measured using spindle no. 3. In this study it was found that the viscosity valuein formula 1 is greater than in formula 2 and formula 3. The addition of the same carbomer gel base in all formulas causes a decreased the number of carboxylate group are ionized at formula which contain more black cumin oil (formula that is more acidic). This is may be caused by the repulsive force between the carboxylate group so the development of carbomer structure is less than usual. The viscosity of a substance that indicates the pseudoplastic flow cannot be expressed with a single value because there is no linear part of the curve

Rheological measurements of formula 1 used spindle no. 3 and 4, while formulas 2 and 3 used spindle no. 2 and 3. Diagrams show thepseudoplastic flow properties. There is an increasing shear rate in pseudoplastic flow resulting froma decrease in viscosity. The rheogram curved due to shearing work on molecules of long chain materials, such as Tween 80 Viscosity of all nanaoemulsion gels decreased after 8 weeks of storage. This could have been caused by a lack of watertight storage packaging resulting in the gel absorbing water from the environment thereby increasing the volume of water in the formula.

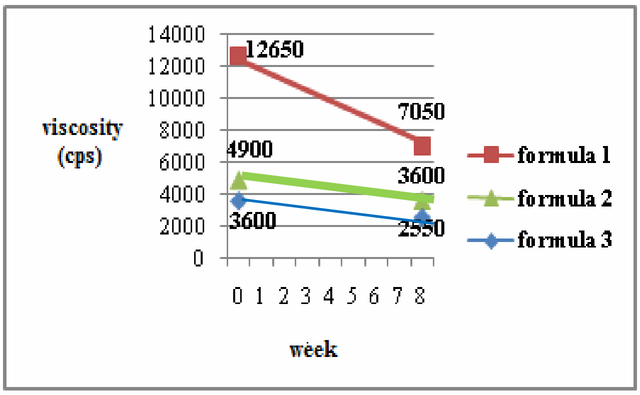

Figure 2: Graph of average viscocity changes in nanoemulsion gel

Stability Test

Physical stability test results showednanoemulsion gel is stable at room temperatures ($25^0C \pm 2^0C$) and low temperatures ($4^0C \pm 2^0C$) for 8 weeks. The level of acidity (pH) of all nanoemulsion gels during storage did not show any significant statistical difference (P > 0,05). The stability suggests that the selection and concentration of surfactant and cosurfactantwas appropriate. Black cumin oil is an active ingredient in this formula, which is efficacious as an antibacterial, it also has antioxidant and antifungal activity, thereby reducing contamination by microorganism oxidation of air catalysts with or without light[11]. After8 weeks at higher storage temperatures ($40^0C \pm 2^0C$) instability occurred, except in formula 1. The level of acidity (pH) of all nanoemulsion gels during storage showeda significant statistical difference ($P < 0,05$). This could be caused by exposingcarbomerat more than normal temperatures (room temperature) which can reduce stability. In addition to that, Tween 80 and carbomerare sensitive to oxidation[12]. In the centrifugation test instability occurred, except in formula 1, while in the cycling test formula 3 showed instability.

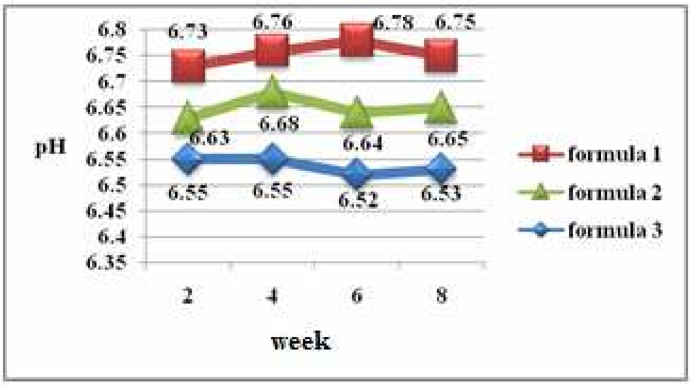

Figure 3: Graph of pH changes during storage (8 weeks) at low temperature ($4^0C \pm 2^0C$

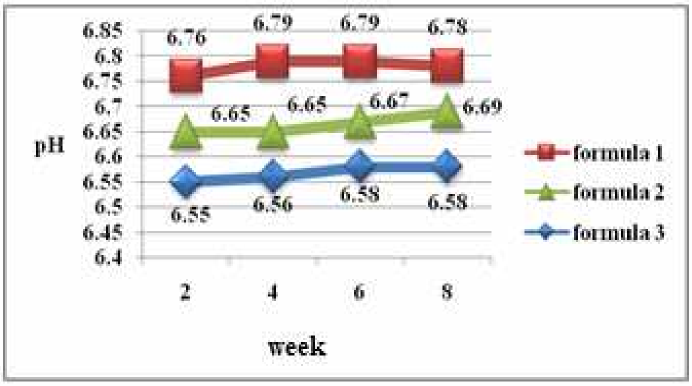

Figure 4: Graph of pH changes during storage (8 weeks) at room temperature ($25^0C \pm 2^0C$)

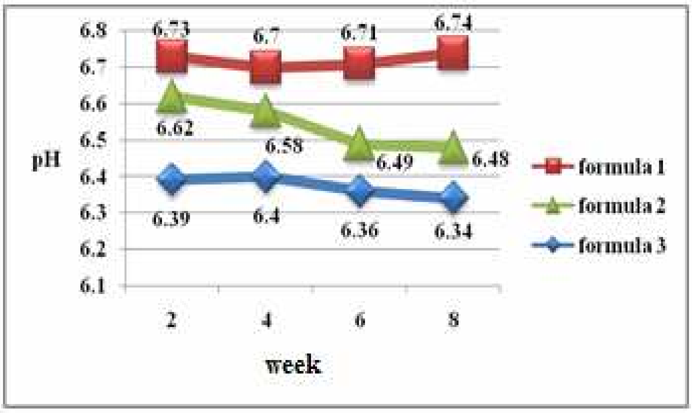

Figure 5: Graph of pH changes during storage (8 weeks) at high temperature ($40^0C \pm 2^0C$)

Antibacterial Activity Assay

The results of antibacterial activity assay were statistically analyzed using ANOVA, then followed by LSD testing if there were significant differences among the inhibiting zones using PASW® Statistics 18 software.

Table 5: The results of antibacterial activity assay

Sample	Inhibiting zones (mm) with paper disc (diameter 6 mm)			Inhibiting zones (mm) with paper disc (diameter 12 mm)		
	Content of black cumin oil			Content of black cumin oil		
	5 µl	**10 µl**	**20 µl**	**5 µl**	**10 µl**	**20 µl**
Black cumin oil	15,25	17,50	26,25	25,00	27,00	34,00
	16,60	18,30	22,00	26,50	28,00	36,00
	14,25	15,85	23,25	25,75	30,00	33,00
Mean	**15,37**	**17,22**	**23,83**	**25,75**	**28,33**	**34,33**
SD	**1,18**	**1,25**	**2,18**	**0,75**	**1,53**	**1,53**
Formula 1	10,50	11,00	11,75	16,75	18,50	20,00
	11,00	12,00	12,25	16,00	17,50	20,25
	10,00	11,25	12,00	16,50	18,30	20,50
Mean	**10,50**	**11,42**	**12,00**	**16,42**	**18,10**	**20,25**
SD	**0,50**	**0,52**	**0,25**	**0,38**	**0,53**	**0,25**
Formula 2	10,75	12,50	13,25	16,25	18,25	20,00
	10,00	10,50	13,50	14,50	18,00	20,50
	11,00	13,00	13,75	16,50	18,50	20,75
Mean	**10,58**	**12,00**	**13,50**	**15,75**	**18,25**	**20,42**
SD	**0,52**	**1,32**	**0,25**	**1,09**	**0,25**	**0,38**
Formula 3	9,00	10,00	13,00	14,00	17,75	20,75
	9,50	13,00	13,25	14,50	16,50	20,30
	10,00	11,00	12,75	14,30	18,00	20,00
Mean	**9,50**	**11,33**	**13,00**	**14,27**	**17,42**	**20,35**
SD	**0,50**	**1,53**	**0,25**	**0,25**	**0,80**	**0,38**

The results obtained showed that antibacterial activity of black cumin oil was better than nanoemulsion gel dosage form. There was a significant difference ($P < 0,01$) between the inhibiting zone of black cumin oil with nanoemulsion gels. This is could be due to solubilization of black cumin oil in nanoemulsion gel so its activity is reduced. Another factor that may lead to lower antibacterial activity of nanoemulsion gel compared to black cumin oil is that it is not homogenous. This can affect the concentration of black cumin oil in nanoemulsion gel and ultimately have an effect on the inhibiting zone. Formulas which do not add preservatives, such as paraben class, and antioxidants such as BHT, can alsobe the reason why antibacterial activity of nanoemulsion gel is less than black cumin oil. This means that black cumin oil acts as a preservative and antioxidant for nanoemulsion gel so its activity as an antibacterial active ingredientis reduced. In addition, the size of inhibiting zone can be influenced by the rate of diffusion and interaction with media.

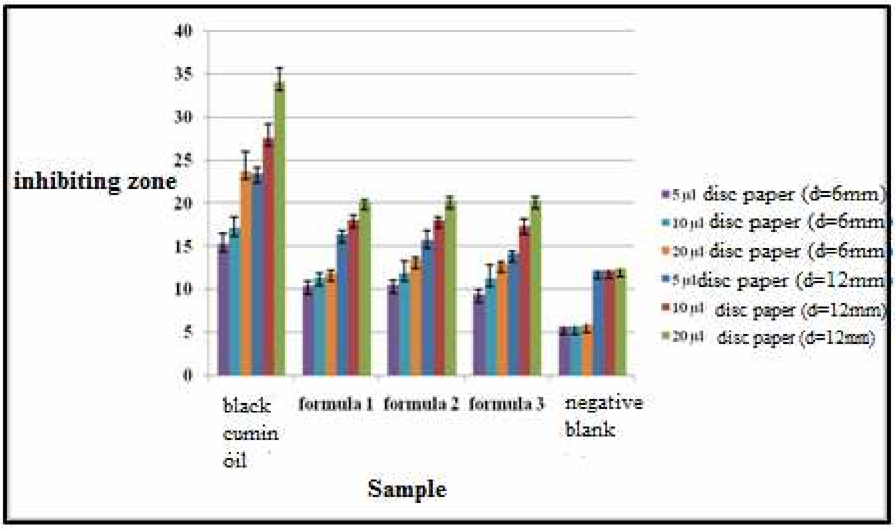

Figure 6: Graph of average inhibiting zone

The higher the concentration of black cumin oil, the greater is the inhibiting zone. In general, there is a significant statistical difference (P < 0,01) between nanoemulsion gel with black cumin oil in concentrations of 5 µl, 10 and 20 µl, and between nanoemulsion gel with black cumin oil in concentrations of 10 µl with 20 µl. Increasing the number of negative blanks showed no significantincrease in the inhibiting zone. The inhibiting zone of nanoemulsion gel showed a significant difference compared to the negative blanks.

Conclusion

Developingnanoemulsion gel in this study was the result of optimization using surfactant Tween 80 with the addition of cosurfactant propylene glycol and ethanol 96%. The nano emulsion gel formed was viscous, translucent, homogeneous with yellow-orange color. The rheology was pseudoplastic and the average globule diameter below 1 µm (1000 nm).

The physical stability test result showednanoemulsion gel was stable at room temperatures (25^0C $\pm$ 2^0C) and low temperatures (4^0C $\pm$ 2^0C) for 8 weeks. Instability occurredat higher temperatures(40^0C $\pm$ 2^0C)for 8 weeks and in the centrifugation test, except in formula 1, whereas it showed instability in the cycling test in formula 3.

The results of in vitro antibacterial activity assay using the disc method showed that black cumin oil hasa greater inhibiting zone than nanoemulsion gels. In addition, there were significant differences (P< 0.01) between the negative blank inhibiting zone with the nanoemulsion gels inhibiting zone.

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