

Lampiran c4. Bukti korespondensi dengan editor Karil JIB No4 pada usulan pertama

No	Permasalahan	Komentar Tim PAK DIKTI	Klarifikasi
4	<p>Karil no 4</p> <p>Judul Artikel: The anti-inflammatory activity of <i>p</i>-methoxycinnamic acid (PMCA) in the nanostructured lipid carrier (NLC) system using combinations of solid lipid; beeswax-oleum cacao and liquid lipid; virgin coconut oil (VCO)</p> <p>(Penulis ke 1 dari 4 penulis) dan sebagai Corresponding Author</p> <p>Research Journal of Pharmacy and Technology (RJPT), Vol. 12, Issue 8, 2019, Hal: 3619-3625</p> <p>ISSN: 0974-360X(Online), 0974-3618(Print)</p> <p>Doi: 10.5958/0974-360X.2019.00617.6</p> <p>Impact Factor: SJR 2018: 0,19; Q3: Coverage: 2011-ongoing H index: 14 Similarity Index (Turnitin): 6%</p>	<p>Tidak ada masalah. Dapat diterima sebagai syarat khusus</p>	<p>Tambahan Kronologi dan bukti korespondensi dengan editor jurnal terlampir</p>

Kronologi korespondensi dengan editor jurnal untuk Karil JIB No 4

No	Tanggal	Keterangan
1	21 Februari 2019 (email 1: author)	Pengiriman artikel dan <i>copyright transfer agreement</i> dan melalui web jurnal; <i>Manuscript Submission Successful</i>
2	22 Februari 2019 (email 2: editor)	Pemberitahuan status artikel RJPT-7415 akan segera diinformasikan
3	6 April 2019 (email 3: editor)	Pemberitahuan <i>Accepted</i> dan biaya proses USD 118
4	8 April 2019 (email 4: author)	Pembayaran biaya proses
5	22 Juli 2019 (email 5: editor)	Pengiriman galley proof oleh editor
6	24 Juli 2019 (email 6: author)	Pengiriman lis koreksi galley proof ada bukti
7	26 Juli 2019 (email 7: editor)	Pemberitahuan revisi galley proof sdh dilakukan
8	14 Agustus 2019 (email 8: editor)	Pemberitahuan artikel telah terbit pada <i>Research J. Pharm. and Tech. 12(8): August 2019</i>

Paper Submission

Dari: [tristiana_erawati \(era_ffua@yahoo.co.id\)](mailto:tristiana_erawati_era_ffua@yahoo.co.id)

Kepada: editor.rjpt@gmail.com

Tanggal: **Kamis, 21 Februari 2019** 22.30 GMT+7

Dear Editor of the Research Journal of Pharmacy and Technology (RJPT)

I hereby submit my manuscript entitled " **The anti-inflammatory activity of *p*-methoxycinnamic acid (PMCA) in the nanostructured lipid carrier (NLC) system using combinations of solid lipid; beeswax-oleum cacao and liquid lipid; virgin coconut oil (VCO)**" intended to publish in your esteemed journal as Original Article.

Please find the document in the attachment.

Thank you and best regards,

Dr. Tristiana Erawati M., MSi, Apt.
Department of Pharmaceutics
Faculty of Pharmacy, Airlangga University
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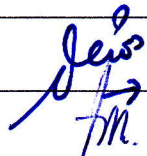
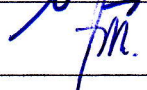

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Surabaya, 20 Februari 2019



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1	Dewi Melani Hariyadi	Universitas Airlangga/ Co-Author	20/2 2019 /2	
2	Noorma Rosita	Universitas Airlangga/ Co-Author	20/2 2019	
3	Tutiek Purwanti	Universitas Airlangga/ Co-Author	20/2 2019	

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Dari: Research Journal of Pharmacy and Technology Monika Daharwal (editor.rjpt@gmail.com)

Kepada: era_ffua@yahoo.co.id

Tanggal: **Jumat, 22 Februari 2019** 12.52 GMT+7

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Kepada: era_ffua@yahoo.co.id

Tanggal: Sabtu, 6 April 2019 17.23 GMT+7

Ref. No - RJPT-7415

Date - 06/04/2019

To,
Tristiana Erawati
Department of Pharmaceutics
Faculty of Pharmacy, Airlangga University,
Surabaya, Indonesia
tristiana-e-m@ff.unair.ac.id

Subject - Acceptance Letter-RJPT

Dear Author,

With reference to your article titled **"The anti-inflammatory activity of p-methoxycinnamic acid (PMCA) in the nanostructured lipid carrier (NLC) system using combinations of solid lipid; beeswax-oleum cacao and liquid lipid; virgin coconut oil (VCO)"** Author by Tristiana Erawati^{1*}, Dewi Melani Hariyadi¹, Noorma Rosita¹, Tutiek Purwanti¹. We wish to bring to your kind notice the following

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(Dr. Mrs. Monika S. Daharwal)

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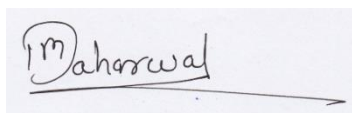
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Re: Confirmation request regarding the publication of the manuscript

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Kepada: era_ffua@yahoo.co.id

Tanggal: **Senin, 22 Juli 2019** 13.21 GMT+7

Dear Author,

Regards

The submitted manuscript is ready for publication in Research Journal of Pharmacy and Technology (RJPT) Vol. 12 Issue 8, August 2019.

The gallery proof of the paper is attaching herewith for any necessary correction if required, please convey by E. Mail as in the attached format before **July 27, 2019**.

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Kepada: editor.rjpt@gmail.com

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Dear Dr. Monika Daharwal

Thank you for your very quick response.

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To make it easier to find words that need to be corrected, I also attach articles that have given pink highlights.

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Best Regards,

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Article Number (original Submitted manuscript No)	RJPT- 7415 / 21-02-2019
Only Corresponding authors name	Tristiana Erawati
I would like to recheck the corrections: corrections to be done within week very carefully	Yes No

List of corrections

Page number	Column (Left / Right)	Paragraph number from top/ Name of Paragraph	Line number from top of paragraph	Delete this text (Error)	Replace deleted text with (correction)
1	Center	1/Abstract	1	... caco cacao ...
1	Center	1/Abstract	14	... NLC FI (60 : 40) NLC
1	Center	1/Abstract	14	... same equivalent ...
3	Left	Oclusivity test:	4	... vials were and vials and ...
3	Right	RESULTS: 1. The pH Value and Viscosity	3 and 4	... cacao with VCO liquid lipid,	... cacao and VCO as liquid lipid,
5	Left	1	1	Table 6.	Table 5.

5	Right	Figure 4	3 and 4	... and with PMCA (NE) nanoemulsion..	..and with nanoemulsion –PMCA (NE)..
5	Right	Figure 5	4	PMCA nanoemulsion	nanoemulsion-PMCA
6	Right	2	15	... NLC-PMCA F I (60 : 40) NLC-PMCA ...
6	Right	2	16	... provide the same anti-inflammatory activities as produce anti-inflammatory activity equivalent to ...
6	Right	CONCLUSIONS	13	... had better had equivalent ...
6	Right	CONCLUSIONS	14	... than as ...
6	Right	CONCLUSIONS	16	... ear skin mice ear skin of mice ...

RESEARCH ARTICLE

The Anti-inflammatory Activity of *p*-methoxycinnamic acid (PMCA) in the Nanostructured lipid carrier (NLC) system using combinations of solid lipid, beeswax-oleum cacao and liquid lipid, Virgin Coconut oil (VCO)

Tristiana Erawati*, Dewi Melani Hariyadi, Noorma Rosita, Tutiek Purwanti

Department of Pharmaceutics Faculty of Pharmacy, Airlangga University, Surabaya, Indonesia

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ABSTRACT:

Objective: The nanostructured lipid carrier (NLC) system used the combination of beeswax-oleum cacao and virgin coconut oil (VCO) in this study, it was the development of nanoemulsion (NE) and solid lipid nanoparticle (SLN) delivery systems, which are used as nano-carriers of *p*-methoxycinnamic acid (PMCA). PMCA a secunder metabolite of champheria galanga has antiinflammatory effect which is difficult to dissolve in water. Several combinations of solid lipids (beeswax-oleum cacao) and liquid lipids (VCO) were prepared, namely F I (60:40); F II (70:30); and F III (80:20) in the NLC system. **Material and Method:** The NLC-PMCA system was prepared by the high shear homogenization method, then characterized and subjected to anti-inflammatory activity tests compared with nanoemulsion and solid lipid nanoparticle (SLN). **The results of the research:** The increasing concentration of VCO in the NLC-PMCA system resulted in lower viscosity, and greater entrapment efficiency, namely in the NLC-PMCA system with a combination of beeswax-oleum cacao and VCO of 60:40. NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 (F I) has particle size (236.00 ± 17.15 nm) smaller than SLN-PMCA (665.60 ± 72.59 nm) but bigger than NE-PMCA (29.27 ± 1.85 nm). Occlucivity of F I NLC-PMCA (41.50 ± 4.00 %) higher than SLN-PMCA (33.52 ± 3.65 %) and NE-PMCA (33.52 ± 3.65 %). PMCA in NLC, SLN and NE has the same anti-inflammatory activity based on the observations of the PMN cells number in ear skin mice with edema. **Conclusion:** The NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 was the best formula.

KEYWORDS: anti-inflammatory activity, beeswax, nanostructured lipid carrier (NLC), oleum cacao, *p*-methoxycinnamic acid (PMCA), virgin coconut oil (VCO).

INTRODUCTION:

In a previous study *p*-methoxycinnamic acid (PMCA), which is difficult to dissolve in water, was formulated in the nanoemulsion [1]. It was known the first colloidal system based on nanotechnology that successfully carries active ingredients through the skin barrier for topical use is the nanoemulsion system [2,3]. The PMCA nanoemulsion system using a combination of surfactants (Tween 80 and Span 80), ethanol 96% co-surfactant, and several plant oil phases, namely VCO, corn oil, and soybean oil [3].

From the study, it was determined that PMCA nanoemulsion that uses VCO as an oil phase produces the smallest droplet size. This is because VCO contains fatty acids with C atomic chains, which are shorter than the fatty acid content in corn oil and soybean oil. The smaller size of nanoemulsion droplets results in the greater rate of release of drug ingredients from the carrier system and the rate of penetration of drug ingredients into the skin, so the resulting effectiveness is also higher. However, being in storage for more than 2 months, PMCA nanoemulsion preparations tend to coalescence, so the size of the nanoemulsion droplets grows larger [4]. Than the PMCA formulated in Solid Lipid Nanostructure (SLN) systems, the liquid lipid is replaced with solid lipid using cetylalcohol and Tween 80 as surfactant can inhibit release and extend their anti-inflammatory effects [5] However, the use of solid lipids

that are too ordered on the storage the active ingredients tends to be pushed out, so a combination of solid lipids and liquid lipids is needed to overcome this. The improvement of the SLN system is the NLC system, which consists of a certain amount of solid lipid and liquid lipid matrices. NLC remains in its solid form by controlling the levels of liquid lipids added to the formulation, so the controlled drug release properties for NLC can be achieved [6]. Liquid lipids in NCL can reduce crystalline formation in solid lipids thus increasing system stability on storage [7,8]. Various liquid oils and solid lipids can be used as NLC components, among others for example combination of olive oil and palmitic acid (1.8:4.8) as NLC system to deliver ubiquinone (Q10) as an antiaging [9].

In this research the NCL system as PMCA carrier used VCO as liquid lipid combined with combinations of oleum cacao and beeswax as solid lipid in various ratios and Tween 80 and Span 80 as stabilizer. Furthermore, in the NLC-PMCA system formed characterization and in vivo effectiveness tests are carried out.

MATERIALS AND METHODS:

Materials:

The materials used in the study which unless otherwise stated have pharmaceutical grade purity are p-methoxycinnamic acid (Sigma Aldrich), Tween 80 (Sigma Aldrich), Span 80 (Sigma Aldrich), VCO (UD. Cocos Coconut), beeswax (PT. Kurniajaya Muktisentosa), oleum cacao (Coffee and Cacao Research Center, Jember-Indonesia), Propylene glycol (PT. Brataco), Sodium benzoate (PT. Brataco), Ethanol 96% p.a (E Merck), Na acetate p.a. (E Merck), and Acetic acid p.a. (E Merck)

Sample preparation:

NLC-PMCA system with different ratios of oleum cacao-beeswax as solid lipids and VCO as liquid lipids, namely F I (60:40), F II (70:30), and FIII (80:20) and SLN-PMCA as shown in Table 1, were prepared using high shear hot homogenization method by Ultra-Turrax High Shear Homogenizer IKA T-25. The NLC-APMS system is made by melting beeswax at 70 °C. After beeswax melts, add oleum cacao to beeswax and let the oleum cacao melt. The APMS which has been

Table 1 Material Composition in NLC-PMCA, SLN-PMCA and NE-PMCA

Name Materials	Formula Concentration (% b/v)				
	F I (60:40)	F II (70:30)	F III (80:20)	SLN	NE
PMCA	0.20	0.20	0.20	0.20	0.20
Oleum Cacao	2.97	3.46	3.96	4.95	-
Beeswax	0.99	1.16	1.32	1.65	-
VCO	2.64	1.98	1.32	-	2.66
Tween 80	5.74	5.84	5.94	4.39	18.66
Span 80	14.76	14.66	14.56	16.11	1.92
Propylene glycol	3.50	3.50	3.50	3.50	3.50
Sodium benzoate	0.10	0.10	0.10	0.10	0.10
Buffer Acetate pH 4.2 ± 0.2	Ad 100	Ad 100	Ad 100	Ad 100	Ad 100

dissolved in VCO added into the mixture of beeswax-oleum cacao. And Tween 80 and Span 80 heated at 70 °C mixed with the lipid phase. At the same time sodium benzoate, propylenglycol and phosphate buffer pH 4.2 ± 0.02 were heated at 70°C as water phase. Then the water phase is slowly dispersed into the lipid phase which is stirred with Ultra-Thurax High Shear Homogenizer at a speed of 5000 rpm until all phases of water are exhausted then continued by adding stirring speed to 16,000 rpm for 2 minutes. The next process is the cooling process, which is carried out with a speed decrease at 500 rpm stirred using a magnetic stirrer to reach room temperature (25°C). For NE-PMCA preparation, all components were mix by magnetic stirrer at room temperature, 500 rpm, 5 minutes, than increase mixing speed to 1000 rpm, 10 minutes.

Sample characterization:

Characterization of the sample includes: 1) the pH value inspection using pH-meter, 2) viscosity measurement

using a cone and plate viscometer, 3) particle size measurement and polydispersity index using Delsa™ Nano Submicron Particle Size and Zeta Potential Dynamic Light Scattering, 4) and the melting temperature using Differential Scanning Calorimeter (DSC),

Entrapment efficiency (%EE):

Measurements of percent entrapment efficiency (EE) were carried out by diluting 100 mg NLC with acetate buffer pH 4.20 ± 0.2 then centrifuged 2500 rpm for ±45 minutes and filtered using filter paper. The supernatant was pipetted 2.0 ml and added acetate buffer pH 4.20± 0.2 to 10.0 ml in the measuring flask. The solution was filtered again using Whatman Millipore filter paper 0.22 µm. Then the solution was measured at the maximum wavelength of the PMCA which is then compared with the absorption of standard solutions. The data obtained is the concentration of PMCA in the NLC water phase. The calculation of % EE (percentage of entrapment

efficiency) used the following equation (1).

$$EE (\%) = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100\% \quad (1)$$

Notes:

$W_{\text{initial drug}}$: The amount of active ingredients used

$W_{\text{free drug}}$: The amount of free active ingredients that are in the water phase

Furthermore, the average, SD, and CV calculation were calculated from the efficiency of PMCA entrapment in the NLC system [7].

Oclusivity test:

The oclusivity test was carried out by in vitro method. Amount of 5 ml water was put into each vial (10 ml) with the same diameter. Previously, the empty and dry vials were and the vial that filled with water were weighed. The vial that filled with water were capped using a membrane filter (cellulose filter, with 0.45 mm pore, Whatman no. 4), and then tie with a rope and make sure there is no gap between water and membrane. Then put 10 drops of Isopropyl myristate to saturate the membrane and leave it overnight. Sample (0.05 grams) was applied evenly to the membrane then observed for a week. After a week, the water that has been lost were calculate using the following equation (2) [10]:

$$F = \frac{B}{A} \times 100\% \quad (2)$$

Notes:

A = Amount of water lost in control

B = Amount of water lost in the sample

Anti-inflammation effectiveness test:

The effectiveness test of PMCA in the NLC system uses mice as experimental animals. Measuring the ability to reduce the thickness of the ear skin of mice with edema and the number of polymorpho nuclear (PMN) cells, there were 5 animal treatment groups namely; positive control group, negative control group, group with NLC-PMCA treatment, group with SLN-PMCA treatment, and group with NE-PMCA treatment. PMCA effectiveness test was carried out on the skin of mice ears which had been induced inflammation by croton oil (2.5% in acetone) for as much as 10 μ l using micropipette. One hour after being dripped with test samples. Then, 9 hours after treatment, the histological preparations of mice ear were made after the mice were sacrificed and then stained with Hemato-Eosin (HE). The histological preparations were then observed under a microscope. Measurements were made on (edema) skin thickness and the amount of inflammatory cell infiltration of polymorpho nuclear (PMN) or

neutrophils, namely the main inflammatory cells in acute inflammation [4]. Percentage reduction in edema thickness (PTE) was calculated based on the equation(3):

$$\%PTE = \frac{T(K+) - T(S)}{T(K+) - T(K-)} \times 100\% \quad (3)$$

Notes:

% PTE = percent of skin thickness decrease

T (K +) = thickness of skin that had edema (positive control)

T (S) = thickness skin that had edema after sample was applied

T (K-) = thickness of healthy skin (negative control)

The calculation of PMN cells on histological preparations of mice without treatment (K-), after induction of croton oil (K+), after treatment with NLC-PMCA (NLC), after treatment with SLN-PMCA (SLN), and after treatment with nanoemulsion PMCA (NE) were observed using an Olympus CX21 microscope at 400x magnification.

RESULTS:

1. The pH Value and Viscosity:

The measurement results of the pH value and viscosity of the NLC-PMCA system with various ratios of solid lipid combination of beeswax-oleum cacao with VCO liquid lipid, namely FI (60:40); F II (70:30); F III (80:20); SLN-PMCA and NE-PMCA by pH-meter and the cone and plate viscometer can be seen in Table 2. Based on the data pH in Table 2 all of the formula has pH around 4, and the results of the viscosity statistical test using one-way ANOVA with 95% confidence level, obtained results a significance value of 0.002 smaller than 0.05 and the results of the Tukey's HSD test, it can be concluded that NE-PMCA has the lowest viscosity, and for the NLC-PMCA the F I (60:40) has the lowest viscosity.

2. Particle Size and Particle Size Distribution:

Particle size measurement results of the NLC-PMCA system with various ratios of beeswax-oleum cacao as solid lipid with VCO as liquid lipid, namely: FI (60:40); F II (70:30); F III (80:20), SLN-PMCA and NE-PMCA using the Delsa™ Nano Submicron Particle Size and Zeta Potential Dynamic Light Scattering can be seen in Table 3. The results of particle size examination were then subjected to a one-way ANOVA statistical test with 95% confidence degree and Tukey's HSD test. A significance value of 0.239 was obtained, which was more than 0.05. This means that there was no differences in particle size for all NLC-PMCA formulas. And the particle size distribution or polydispersity index (PI) measurement results of all systems were below 0.3, its mean the particle size each formula homogen.

Table 2: pH value and viscosity of NLC-PMCA System with Various ratios of Beeswax-Oleum Cacao as Solid Lipid with VCO as Liquid Lipid, Namely: F I (60:40); F II (70:30); and F III (80:20); SLN and NE

Formula	pH value		Viscosity (cps)	
	Average	SD	Average	SD
F I (60:40)	4.47	0.18	126.90	17.34
F II (70:30)	4.51	0.21	163.10	01.81
F III (80:20)	4.36	0.01	189.83	10.96
SLN	4.48	0.07	425.57	6.43
NE	4.01	0.06	6.13	0.08

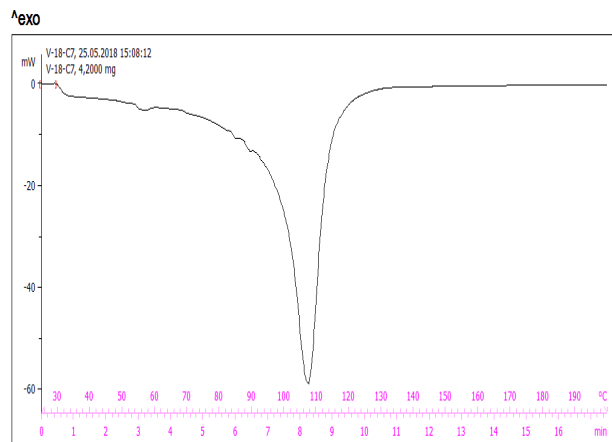
Table 3: Particle/Droplet Sizes and Polydispersity Index (PI) of NLC-PMCA System with Various Ratios of Beeswax-Oleum Cacao as Solid Lipid with VCO as Liquid Lipid, Namely: F I (60:40); F II (70:30); and F III (80:20), NE-APMS and SLN-APMS

Formula	Particle size (nm)		Polidispersity Index (PI)	
	Average	SD	Average	SD
F I (60:40)	236.00	17.15	0.242	0.031
F II (70:30)	241.01	13.72	0.228	0.001
F III (80:20)	255.08	06.10	0.237	0.046
SLN	665.60	72.59	0.461	0.060
NE	29.27	1.85	0.435	0.020

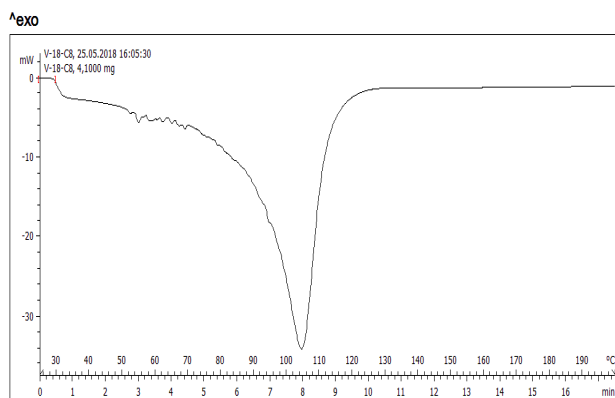
3. Melting Point and Recrystallization Index:

The measurement of the melting point and recrystallization index was carried out on the NLC-PMCA system with various ratios of beeswax-oleum cacao as solid lipid with VCO as liquid lipid, namely: F I (60:40); F II (70:30); and F III (80:20) using Differential Scanning Calorimeter (DSC) compared to oleum cacao, beeswax, and PMCA. The results of the thermogram melting point of the formulas can be seen in Figures 1, 2 and 3, while the melting point parameters of each formula can be seen in Table 4. From the peaks that appear in the thermogram of each sample, the recrystallization index was calculated based on the following equation (4):

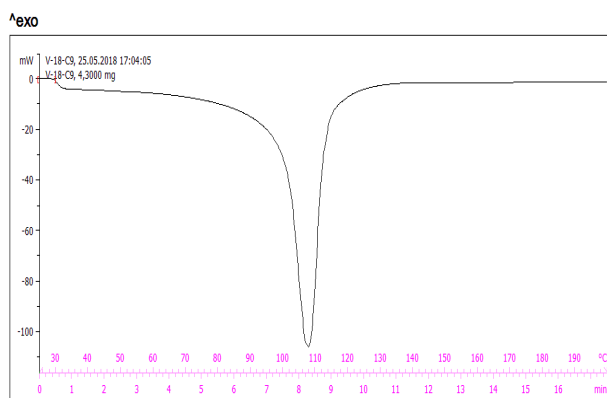
$$\% RI = \frac{\Delta H_{NLC}}{(\Delta H_{Lipid} \times konsentrasi\ lipid)} \times 100\% \dots\dots(4)$$



Lab: METTLER STAR® SW 11.00
Figure 1: Thermogram of melting temperature of NLC-PMCA formula I (60:40)



Lab: METTLER STAR® SW 11.00
Figure 2: Thermogram of melting temperature of NLC-PMCA formula II (70:30)



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Figure 3: Thermogram of melting temperature of NLC-PMCA formula III (80:20)

Table 4: Melting Points of PMCA, Oleum Cacao, Beeswax, and NLC-PMCA

Materials	Melting point (° C)	ΔH (J/g)	RI (%)
PMCA	173.00	-110.27	-
Beeswax	59.99	-300.46	-
Oleum Cacao	33.65	-115.17	-
Formula I (60:40)	56.85	-5.37	0.326
	105.52	-698.97	
Formula II (70:30)	55.01	-1.43	0.074
	103.44	-722.52	
Formula III (80:20)	104.13	1421.84	-

4. Entrapment Efficiency:

The results of the determination of PMCA recovery in the NLC, SLN system with different ratios of solid lipids and liquid lipids can be seen in Table 5, it was known that the percentage of recovery of all formulas were > 94%, and the results of the calculation of the % CV value of active materials showed that the % CV value of all formulas were less than 6%, so it can be said that all NLC-PMCA formulas were homogeneous.

The determination of the PMCA in NLC and SLN system entrapment efficiency, the results can be seen in

Table 6. Meanwhile, the results of the one-way ANOVA statistical test with 95% confidence degree followed by the Tukey's HSD test with a significance value of 0.020 <0.05. Thus, it can conclude that F I (60:40) had a higher entrapment efficiency than F II (70:30) and F III (80:20).

5. Occlusivity test:

The result of F1 NLC-PMCA (41.50 ± 4.00 %), SLN-PMCA (33.52 ± 3.65 %) and NE-PMCA (20.30 ± 2.39 %) occlusivites after one week obsevation known the F1 NLC-PMCA system has higher occlusivity.

6. Anti-inflammation effectiveness test:

To determine the PMCA activity in the NLC system, a test was conducted on the reduction in skin thickness that induced edema in the ear skin tissue of the mice, as well as the observation of the number of PMN cells. The NLC-PMCA formula tested for activity was F 1 (60:40) because it had the highest entrapment efficiency. The NLC-PMCA activity test was compared with nanoemulsion system (NE-PMCA) and solid lipid nanoparticle system (SLN-PMCA).

a. Decrease of Skin Thickness at The Ears Mice with Edema:

The results of the PMCA activity test in the NLC system on the thickness reduction of the ear mice skin with edema can be seen in Table 6 and Figure 4. The results of a one-way ANOVA statistical test with 95% confidence degree and Tukey's HSD test on the reduction in the thickness of the skin with edema in the ear skin tissue of mice observed in 5 visual fields with a magnification of 100x, which found a significance value of 0.623 > from 0.05. Skin thickness of mouse ears after exposure with NLC-PMCA, SLN-PMCA and NE-PMCA were not significantly different from the negative control and significantly different from the positive controls. So it can be concluded that NLC-PMCA can provide the same anti-inflammatory activities as SLN-PMCA and NE-PMCA.

b. Number of PMN Cells:

The results of the calculation of the number of PMN cells contained in histology preparations in 5 visual fields with magnifications of 400x can be seen in Table 6 and Figure5. It was known that NLC-PMCA can reduce the number of PMN cells in the ear mice skin with edema better than SLN-PMCA and nanoemulsi-PMCA, but still far compared to the negative control.

Table 5: Recovery Percentage and Entrapment Efficiency of PMCA in NLC and SLN

Formula	Recovery (%)			Entrapment efficiency (%)	
	Average	SD	CV (%)	Average	SD
F I (60:40)	94.37	0.175	0.19	43.37	1.10
F II (70:30)	94.70	0	0	33.99	1.76
F III (80:20)	94.39	1.09	1.16	27.80	8.02
SLN	93.76	3.72	3.95	34.46	2.85

Table 6: The Ear Mice Skin Thickness after 9 Hours Treatment and The Number of PMN Cells in Histological Preparations of Mice Ear Skin Treated with NLC-PMCA, SLN-PMCA, and NE-PMCA Compared to Positive Control and Negative Control. Observation Using Olympus CX21 Microscope, With 100x and 400x Magnifications

Treatment	Skin thickness per 5 field of view average ± SD (µm)	Number of PMN Cells per 5 field of view average ± SD (%)
Positive control	3354.4 ± 364.2	166.6 ± 23.3
Negative control	1390.6 ± 408.6	5.6 ± 0.7
NLC PMCA	1834.9± 355.4	85.6 ± 25.1
SLN PMCA	2177.5 ± 346.4	93.3 ± 17.2
NE PMCA	1862.0 ± 136.9	115.3 ± 43.5

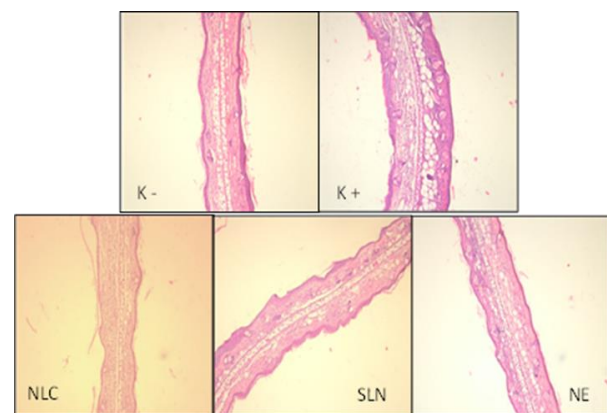


Figure 4: Histology of ear skin thickness of mice in negative control (K-), positive control (K+), with NLC-PMCA (NLC) treatment, with SLN-PMCA (SLN) treatment, and with PMCA (NE) nanoemulsion treatment. Observed using Olympus CX21 microscope with 100x magnification

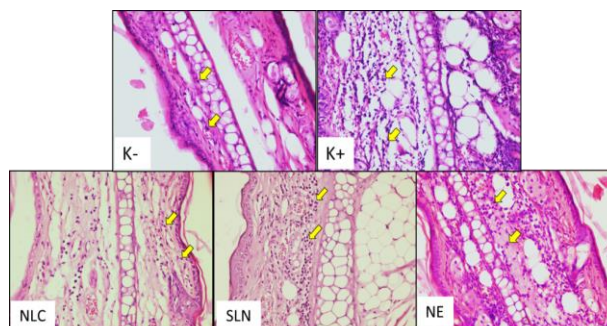


Figure 5: PMN cells in the ears of mice without treatment (K-), after induced by croton oil (K+), after treatment with NLC-PMCA (NLC), after treatment with SLN-PMCA (SLN), and after treatment with PMCA nanoemulsion (NE) Observed with Olympus CX21 microscope at 400x magnification

DISCUSSION:

In this NLC-PMCA study VCO was used as liquid lipid because it was determined that PMCA nanoemulsion that uses VCO as an oil phase produces the smallest droplet size. This is caused by VCO contains fatty acids with C atomic chains which are shorter than the fatty acid content in corn oil and soybean oil. The smaller size of nanoemulsion droplets results in the greater release rate of active ingredients from the carrier system and the penetration rate of active ingredients into the skin, so the resulting effectiveness is also higher [4]. As stabilizers, Tween 80 and Span 80 were used, which are nonionic surfactants, safer than other types of surfactants in the event of skin irritation [11,12]. NLC remains in its solid form by controlling the levels of liquid lipids added to the formulation, its can controlled drug release properties for NLC can be achieved.⁶ The choice of constituent materials and ratios between solid lipids and liquid lipids are important factors in the NLC formulation. To decreased orderness of the solid lipid in this research also used combination of beeswax and oleum cacao.

The data pH value in Table 2 it is known that all NLC-PMCA systems that formed had pH values that are slightly below the pH range of the skin at 4.5-6.5 so it does not causes irritation. From the viscosity data, it is known that F I (60:40) has the lowest viscosity, F I has the highest VCO content, which is the highest liquid lipid. An increase in liquid lipid consetration decreases the viscosity of NLC-APMS, but has no effect on particle size and particle size distribution or polydispersity index (PI). All formula have particle size < 500 nm, it was known a good NLC system has particle size up to 500 nm [7]. And then in Table 3, the results of particle size distribution or polydispersity index (PI) of NLC-PMCA systems were below 0.5 indicated the absence of particle aggregation, and the presence of a homogeneous particle size distribution.[13,14].

Based on the results of the melting point examination with DSC in Figures 1, 2, 3 and Table 4 it can be seen that the F III has a different thermogram form than F I and F II. In F III there is only one peak, this can be caused by the decreasing amount of liquid lipids the possibility of molecular interaction between oleum cacao and beeswax would occur, so there was endothermic peak of each solid lipid (oleum cacao and beeswax) an appeared. In F I and F II, there was a shift in endothermic (oleum cacao and beeswax) peaks (56.85 °C and 55.01°C) and a decrease in enthalpy between single solid lipids with NLC-PMCA (5.87 J/g and 1.43 J/g). This can be due to differences in composition between single solid lipids (oleum cacao and beeswax) in the formulas. In the calculation of the recrystallization index for F I and F II, a decrease in the value of RI

(0.326 and 0.074%) was obtained. This shows a decrease in the order of the crystal lattice. From these results, it can be concluded that the addition of PMCA and VCO can affect endothermic conditions (melting point and enthalpy).

The entrapment efficiency of F I higer than F II and F III its caused by the use of a combination of solid lipids and liquid lipids in NLC system can reduce crystallinity in the crystalline matrix and form irregular matrix, thus creating a space where drug molecules can be entrapped in the system [15,16]. Higher content of liquid lipid in NLC-PMCA also occured the less ordered matrices, supported by the recrystallization index data. The lower of recrystallization index of an ingredient occered the less ordered the crystal lattice is, the crystal space can accommodate larger drugs, so the resulting entrapment efficiency would be greater.[17,18]. The addition of liquid lipids also increased stability caused the crystallization process to be inhibited, so drug expulsion during storage can be minimized [17,19]. NLC-PMCA can provide the same anti-inflammatory activities as SLN-PMCA and NE-PMCA

To seek improvement in the delivery system so as to increase the effectiveness of the PMCA as an anti-inflammatory, the rate of release and penetration of the system needs to be determined. An active material can have the desired effect if it can be released from the system, and then penetrate into the skin to the site of action. Easier the active material detaches from the system, the faster penetration will be and the faster effect will be too.

CONCLUSIONS:

Based on the results of the characterization of the NLC PMCA system with ratios of beeswax-oleum cacao solid lipid and VCO lipid of 60:40, 70:30, and 80:20 which included organoleptic, pH, viscosity, particle size, particle size distribution, melting point, entrapment efficiency, and activity tests, in this research it can be concluded that: The increase of the amount of VCO in the NLC-PMCA system results in smaller particle size, lower viscosity, and greater entrapment efficiency, namely in the combination of beeswax-oleum cacao solid lipid and VCO liquid lipid of 60:40. NLC-PMCA with a combination of beeswax-oleum cacao solid lipid and VCO liquid lipid of 60:40 had better anti-inflammatory activity than SLN-PMCA and nanoemulsi-PMCA, based on the observations of the PMN cells number in ear skin mice with edema. The NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 was the best formula.

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ETHICAL CLEARANCE:

All animal experiments were conducted with the permission from Animal Care and Use Committee (ACUC) of Veterinary Faculty, Airlangga University, Surabaya-Indonesia. (Reference number; 2.KE.051.01, 2018).

CONFLICT OF INTEREST:

No conflict of interest is associated with this work.

AUTHOR CONTRIBUTION:

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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RESEARCH ARTICLE

The Anti-inflammatory Activity of *p*-methoxycinnamic acid (PMCA) in the Nanostructured lipid carrier (NLC) system using combinations of solid lipid, beeswax-oleum cacao and liquid lipid, Virgin Coconut oil (VCO)

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ABSTRACT:

Objective: The nanostructured lipid carrier (NLC) system used the combination of beeswax-oleum cacao and virgin coconut oil (VCO) in this study, it was the development of nanoemulsion (NE) and solid lipid nanoparticle (SLN) delivery systems, which are used as nano-carriers of *p*-methoxycinnamic acid (PMCA). PMCA a secunder metabolite of champheria galanga has antiinflammatory effect which is difficult to dissolve in water. Several combinations of solid lipids (beeswax-oleum cacao) and liquid lipids (VCO) were prepared, namely F I (60:40); F II (70:30); and F III (80:20) in the NLC system. **Material and Method:** The NLC-PMCA system was prepared by the high shear homogenization method, then characterized and subjected to anti-inflammatory activity tests compared with nanoemulsion and solid lipid nanoparticle (SLN). **The results of the research:** The increasing concentration of VCO in the NLC-PMCA system resulted in lower viscosity, and greater entrapment efficiency, namely in the NLC-PMCA system with a combination of beeswax-oleum cacao and VCO of 60:40. NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 (F I) has particle size (236.00 ± 17.15 nm) smaller than SLN-PMCA (665.60 ± 72.59 nm) but bigger than NE-PMCA (29.27 ± 1.85 nm). Occlucivity of F I NLC-PMCA (41.50 ± 4.00 %) higher than SLN-PMCA (33.52 ± 3.65 %) and NE-PMCA (33.52 ± 3.65 %). PMCA in F I (60 : 40) NLC, SLN and NE has the equivalent anti-inflammatory activity based on the observations of the PMN cells number in ear skin mice with edema. **Conclusion:** The NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 was the best formula.

KEYWORDS: anti-inflammatory activity, beeswax, nanostructured lipid carrier (NLC), oleum cacao, *p*-methoxycinnamic acid (PMCA), virgin coconut oil (VCO).

INTRODUCTION:

In a previous study *p*-methoxycinnamic acid (PMCA), which is difficult to dissolve in water, was formulated in the nanoemulsion [1]. It was known the first colloidal system based on nanotechnology that successfully carries active ingredients through the skin barrier for topical use is the nanoemulsion system [2,3]. The PMCA nanoemulsion system using a combination of surfactants (Tween 80 and Span 80), ethanol 96% co-surfactant, and several plant oil phases, namely VCO, corn oil, and soybean oil [3].

From the study, it was determined that PMCA nanoemulsion that uses VCO as an oil phase produces the smallest droplet size. This is because VCO contains fatty acids with C atomic chains, which are shorter than the fatty acid content in corn oil and soybean oil. The smaller size of nanoemulsion droplets results in the greater rate of release of drug ingredients from the carrier system and the rate of penetration of drug ingredients into the skin, so the resulting effectiveness is also higher. However, being in storage for more than 2 months, PMCA nanoemulsion preparations tend to coalescence, so the size of the nanoemulsion droplets grows larger [4]. Than the PMCA formulated in Solid Lipid Nanostructure (SLN) systems, the liquid lipid is replaced with solid lipid using cetylalcohol and Tween 80 as surfactant can inhibit release and extend their anti-

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inflammatory effects [5] However, the use of solid lipids that are too ordered on the storage the active ingredients tends to be pushed out, so a combination of solid lipids and liquid lipids is needed to overcome this. The improvement of the SLN system is the NLC system, which consists of a certain amount of solid lipid and liquid lipid matrices. NLC remains in its solid form by controlling the levels of liquid lipids added to the formulation, so the controlled drug release properties for NLC can be achieved [6]. Liquid lipids in NCL can reduce crystalline formation in solid lipids thus increasing system stability on storage [7,8]. Various liquid oils and solid lipids can be used as NLC components, among others for example combination of olive oil and palmitic acid (1.8:4.8) as NLC system to deliver ubiquinone (Q10) as an antiaging [9].

In this research the NCL system as PMCA carrier used VCO as liquid lipid combined with combinations of oleum cacao and beeswax as solid lipid in various ratios and Tween 80 and Span 80 as stabilizer. Furthermore, in the NLC-PMCA system formed characterization and in vivo effectiveness tests are carried out.

MATERIALS AND METHODS:

Materials:

The materials used in the study which unless otherwise stated have pharmaceutical grade purity are p-methoxycinnamic acid (Sigma Aldrich), Tween 80 (Sigma Aldrich), Span 80 (Sigma Aldrich), VCO (UD. Cocos Coconut), beeswax (PT. Kurniajaya Muktsentosa), oleum cacao (Coffee and Cacao Research Center, Jember-Indonesia), Popylen glycol (PT. Brataco), Sodium benzoate (PT. Brataco), Ethanol 96% p.a (E Merck), Na acetate p.a. (E Merck), and Acetic acid p.a. (E Merck)

Sample preparation:

NLC-PMCA system with different ratios of oleum cacao-beeswax as solid lipids and VCO as liquid lipids, namely F I (60:40), F II (70:30), and FIII (80:20) and SLN-PMCA as shown in Table 1, were prepared using high shear hot homogenization method by Ultra-Turrax High Shear Homogenizer IKA T-25. The NLC-APMS system is made by melting beeswax at 70 °C. After

beeswax melts, add oleum cacao to beeswax and let the oleum cacao melt. The APMS which has been dissolved in VCO added into the mixture of beeswax-oleum cacao. And Tween 80 and Span 80 heated at 70 °C mixed with the lipid phase. At the same time sodium benzoate, propylenglycol and phosphate buffer pH 4.2 ± 0.02 were heated at 70°C as water phase. Then the water phase is slowly dispersed into the lipid phase which is stirred with Ultra-Thurax High Shear Homogenizer at a speed of 5000 rpm until all phases of water are exhausted then continued by adding stirring speed to 16,000 rpm for 2 minutes. The next process is the cooling process, which is carried out with a speed decrease at 500 rpm stirred using a magnetic stirrer to reach room temperature (25°C). For NE-PMCA preparation, all components were mix by magnetic stirrer at room temperature, 500 rpm, 5 minutes, than increase mixing speed to 1000 rpm, 10 minutes.

Sample characterization:

Characterization of the sample includes: 1) the pH value inspection using pH-meter, 2) viscosity measurement using a cone and plate viscometer, 3) particle size measurement and polydispersity index using Delsa™ Nano Submicron Particle Size and Zeta Potential Dynamic Light Scattering, 4) and the melting temperature using Differential Scanning Calorimeter (DSC).

Entrapment efficiency (%EE):

Measurements of percent entrapment efficiency (EE) were carried out by diluting 100 mg NLC with acetate buffer pH 4.20 ± 0.2 then centrifuged 2500 rpm for ±45 minutes and filtered using filter paper. The supernatant was pipetted 2.0 ml and added acetate buffer pH 4.20± 0.2 to 10.0 ml in the measuring flask. The solution was filtered again using Whatman Millipore filter paper 0.22 µm. Then the solution was measured at the maximum wavelength of the PMCA which is then compared with the absorption of standard solutions. The data obtained is the concentration of PMCA in the NLC water phase. The calculation of % EE (percentage of entrapment efficiency) used the following equation (1).

Table 1 Material Composition in NLC-PMCA, SLN-PMCA and NE-PMCA

Name Materials	Formula Concentration (% b/v)				
	F I (60:40)	F II (70:30)	F III (80:20)	SLN	NE
PMCA	0.20	0.20	0.20	0.20	0.20
Oleum Cacao	2.97	3.46	3.96	4.95	-
Beeswax	0.99	1.16	1.32	1.65	-
VCO	2.64	1.98	1.32	-	2.66
Tween 80	5.74	5.84	5.94	4.39	18.66
Span 80	14.76	14.66	14.56	16.11	1.92
Propylene glycol	3.50	3.50	3.50	3.50	3.50
Sodium benzoate	0.10	0.10	0.10	0.10	0.10
Buffer Acetate pH 4.2 ± 0.2	Ad 100	Ad 100	Ad 100	Ad 100	Ad 100

$$EE (\%) = \frac{W_{\text{initial drug}} - W_{\text{free drug}}}{W_{\text{initial drug}}} \times 100\% \quad \text{----- (1)}$$

Notes:

$W_{\text{initial drug}}$: The amount of active ingredients used

$W_{\text{free drug}}$: The amount of free active ingredients that are in the water phase

Furthermore, the average, SD, and CV calculation were calculated from the efficiency of PMCA entrapment in the NLC system [7].

Oclusivity test:

The occlusivity test was carried out by in vitro method. Amount of 5 ml water was put into each vial (10 ml) with the same diameter. Previously, the empty and dry vials and the vial that filled with water were weighed. The vial that filled with water was capped using a membrane filter (cellulose filter, with 0.45 mm pore, Whatman no. 4), and then tied with a rope and made sure there is no gap between water and membrane. Then put 10 drops of Isopropyl myristate to saturate the membrane and leave it overnight. Sample (0.05 grams) was applied evenly to the membrane then observed for a week. After a week, the water that has been lost was calculated using the following equation (2) [10]:

$$F = \frac{B}{A} \times 100\% \quad \text{-----(2)}$$

Notes:

A = Amount of water lost in control

B = Amount of water lost in the sample

Anti-inflammation effectiveness test:

The effectiveness test of PMCA in the NLC system uses mice as experimental animals. Measuring the ability to reduce the thickness of the ear skin of mice with edema and the number of polymorpho nuclear (PMN) cells, there were 5 animal treatment groups namely; positive control group, negative control group, group with NLC-PMCA treatment, group with SLN-PMCA treatment, and group with NE-PMCA treatment. PMCA effectiveness test was carried out on the skin of mice ears which had been induced inflammation by croton oil (2.5% in acetone) for as much as 10 µl using micropipette. One hour after being dripped with test samples. Then, 9 hours after treatment, the histological preparations of mice ear were made after the mice were sacrificed and then stained with Hemato-Eosin (HE). The histological preparations were then observed under a microscope. Measurements were made on (edema) skin thickness and the amount of inflammatory cell infiltration of polymorpho nuclear (PMN) or neutrophils, namely the main inflammatory cells in

acute inflammation [4]. Percentage reduction in edema thickness (PTE) was calculated based on the equation(3):

$$\%PTE = \frac{T(K+) - T(S)}{T(K+) - T(K-)} \times 100\% \quad \text{-----(3)}$$

Notes:

% PTE = percent of skin thickness decrease

T (K +) = thickness of skin that had edema (positive control)

T (S) = thickness skin that had edema after sample was applied

T (K-) = thickness of healthy skin (negative control)

The calculation of PMN cells on histological preparations of mice without treatment (K-), after induction of croton oil (K+), after treatment with NLC-PMCA (NLC), after treatment with SLN-PMCA (SLN), and after treatment with nanoemulsion PMCA (NE) were observed using an Olympus CX21 microscope at 400x magnification.

RESULTS:

1. The pH Value and Viscosity:

The measurement results of the pH value and viscosity of the NLC-PMCA system with various ratios of solid lipid combination of beeswax-oleum cacao with VCO as liquid lipid, namely F I (60:40); F II (70:30); F III (80:20); SLN-PMCA and NE-PMCA by pH-meter and the cone and plate viscometer can be seen in Table 2. Based on the data pH in Table 2 all of the formula has pH around 4, and the results of the viscosity statistical test using one-way ANOVA with 95% confidence level, obtained results a significance value of 0.002 smaller than 0.05 and the results of the Tukey's HSD test, it can be concluded that NE-PMCA has the lowest viscosity, and for the NLC-PMCA the F I (60:40) has the lowest viscosity.

2. Particle Size and Particle Size Distribution:

Particle size measurement results of the NLC-PMCA system with various ratios of beeswax-oleum cacao as solid lipid with VCO as liquid lipid, namely: F I (60:40); F II (70:30); F III (80:20), SLN-PMCA and NE-PMCA using the Delsa™ Nano Submicron Particle Size and Zeta Potential Dynamic Light Scattering can be seen in Table 3. The results of particle size examination were then subjected to a one-way ANOVA statistical test with 95% confidence degree and Tukey's HSD test. A significance value of 0.239 was obtained, which was more than 0.05. This means that there was no differences in particle size for all NLC-PMCA formulas. And the particle size distribution or polydispersity index (PI) measurement results of all systems were below 0.3, it means the particle size each formula homogen.

Table 2: pH value and viscosity of NLC-PMCA System with Various ratios of Beeswax-Oleum Cacao as Solid Lipid with VCO as Liquid Lipid, Namely: F I (60:40); F II (70:30); and F III (80:20); SLN and NE

Formula	pH value		Viscosity (cps)	
	Average	SD	Average	SD
F I (60:40)	4.47	0.18	126.90	17.34
F II (70:30)	4.51	0.21	163.10	01.81
F III (80:20)	4.36	0.01	189.83	10.96
SLN	4.48	0.07	425.57	6.43
NE	4.01	0.06	6.13	0.08

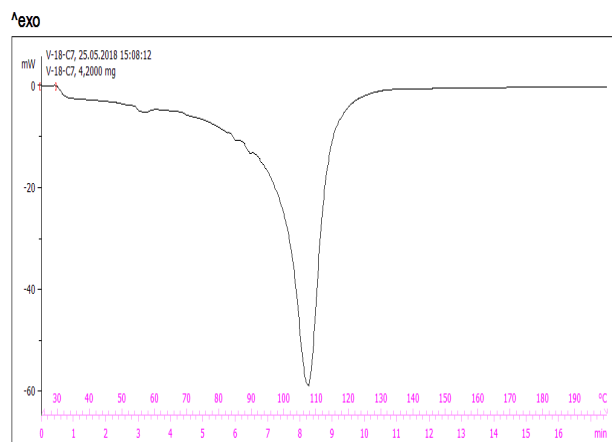
Table 3: Particle/Droplet Sizes and Polydispersity Index (PI) of NLC-PMCA System with Various Ratios of Beeswax-Oleum Cacao as Solid Lipid with VCO as Liquid Lipid, Namely: F I (60:40); F II (70:30); and F III (80:20), NE-APMS and SLN-APMS

Formula	Particle size (nm)		Polidispersity Index (PI)	
	Average	SD	Average	SD
F I (60:40)	236.00	17.15	0.242	0.031
F II (70:30)	241.01	13.72	0.228	0.001
F III (80:20)	255.08	06.10	0.237	0.046
SLN	665.60	72.59	0.461	0.060
NE	29.27	1.85	0.435	0.020

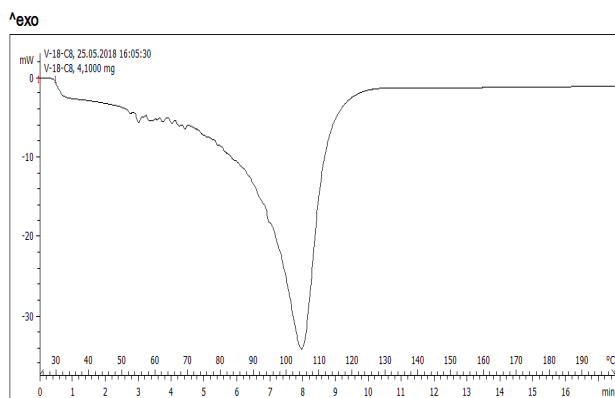
3. Melting Point and Recrystallization Index:

The measurement of the melting point and recrystallization index was carried out on the NLC-PMCA system with various ratios of beeswax-oleum cacao as solid lipid with VCO as liquid lipid, namely: F I (60:40); F II (70:30); and F III (80:20) using Differential Scanning Calorimeter (DSC) compared to oleum cacao, beeswax, and PMCA. The results of the thermogram melting point of the formulas can be seen in Figures 1, 2 and 3, while the melting point parameters of each formula can be seen in Table 4. From the peaks that appear in the thermogram of each sample, the recrystallization index was calculated based on the following equation (4):

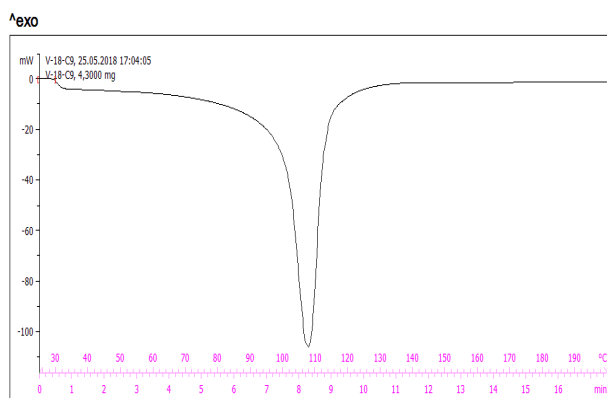
$$\%RI = \frac{\Delta H \text{ NLC}}{\Delta H \text{ Lipid} \times \text{Konsentrasi lipid}} \times 100 \text{ -----(4)}$$



Lab: METTLER STAR® SW 11.00
Figure 1: Thermogram of melting temperature of NLC-PMCA formula I (60:40)



Lab: METTLER STAR® SW 11.00
Figure 2: Thermogram of melting temperature of NLC-PMCA formula II (70:30)



Lab: METTLER STAR® SW 11.00
Figure 3: Thermogram of melting temperature of NLC-PMCA formula III (80:20)

Table 4: Melting Points of PMCA, Oleum Cacao, Beeswax, and NLC-PMCA

Materials	Melting point (° C)	ΔH (J/g)	RI (%)
PMCA	173.00	-110.27	-
Beeswax	59.99	-300.46	-
Oleum Cacao	33.65	-115.17	-
Formula I (60:40)	56.85	-5.37	0.326
	105.52	-698.97	
Formula II (70:30)	55.01	-1.43	0.074
	103.44	-722.52	
Formula III (80:20)	104.13	1421.84	-

4. Entrapment Efficiency:

The results of the determination of PMCA recovery in the NLC, SLN system with different ratios of solid lipids and liquid lipids can be seen in Table 5, it was known that the percentage of recovery of all formulas were > 94%, and the results of the calculation of the % CV value of active materials showed that the % CV value of all formulas were less than 6%, so it can be said that all NLC-PMCA formulas were homogeneous.

The determination of the PMCA in NLC and SLN system entrapment efficiency, the results can be seen in

Table 5. Meanwhile, the results of the one-way ANOVA statistical test with 95% confidence degree followed by the Tukey's HSD test with a significance value of 0.020 <0.05. Thus, it can conclude that F I (60:40) had a higher entrapment efficiency than F II (70:30) and F III (80:20).

5. Occlusivity test:

The result of F1 NLC-PMCA (41.50 ± 4.00 %), SLN-PMCA (33.52 ± 3.65 %) and NE-PMCA (20.30 ± 2.39 %) occlusivites after one week obsevation known the F1 NLC-PMCA system has higher occlusivity.

6. Anti-inflammation effectiveness test:

To determine the PMCA activity in the NLC system, a test was conducted on the reduction in skin thickness that induced edema in the ear skin tissue of the mice, as well as the observation of the number of PMN cells. The NLC-PMCA formula tested for activity was F 1 (60:40) because it had the highest entrapment efficiency. The NLC-PMCA activity test was compared with nanoemulsion system (NE-PMCA) and solid lipid nanoparticle system (SLN-PMCA).

a. Decrease of Skin Thickness at The Ears Mice with Edema:

The results of the PMCA activity test in the NLC system on the thickness reduction of the ear mice skin with edema can be seen in Table 6 and Figure 4. The results of a one-way ANOVA statistical test with 95% confidence degree and Tukey's HSD test on the reduction in the thickness of the skin with edema in the ear skin tissue of mice observed in 5 visual fields with a magnification of 100x, which found a significance value of 0.623 > from 0.05. Skin thickness of mouse ears after exposure with NLC-PMCA, SLN-PMCA and NE-PMCA were not significantly different from the negative control and significantly different from the positive controls. So it can be concluded that NLC-PMCA can provide the same anti-inflammatory activities as SLN-PMCA and NE-PMCA.

b. Number of PMN Cells:

The results of the calculation of the number of PMN cells contained in histology preparations in 5 visual fields with magnifications of 400x can be seen in Table 6 and Figure5. It was known that NLC-PMCA can reduce the number of PMN cells in the ear mice skin with edema better than SLN-PMCA and nanoemulsi-PMCA, but still far compared to the negative control.

Table 5: Recovery Percentage and Entrapment Efficiency of PMCA in NLC and SLN

Formula	Recovery (%)			Entrapment efficiency (%)	
	Average	SD	CV (%)	Average	SD
F I (60:40)	94.37	0.175	0.19	43.37	1.10
F II (70:30)	94.70	0	0	33.99	1.76
F III (80:20)	94.39	1.09	1.16	27.80	8.02
SLN	93.76	3.72	3.95	34.46	2.85

Table 6: The Ear Mice Skin Thickness after 9 Hours Treatment and The Number of PMN Cells in Histological Preparations of Mice Ear Skin Treated with NLC-PMCA, SLN-PMCA, and NE-PMCA Compared to Positive Control and Negative Control. Observation Using Olympus CX21 Microscope, With 100x and 400x Magnifications

Treatment	Skin thickness per 5 field of view average ± SD (µm)	Number of PMN Cells per 5 field of view average ± SD (%)
Positive control	3354.4 ± 364.2	166.6 ± 23.3
Negative control	1390.6 ± 408.6	5.6 ± 0.7
NLC PMCA	1834.9± 355.4	85.6 ± 25.1
SLN PMCA	2177.5 ± 346.4	93.3 ± 17.2
NE PMCA	1862.0 ± 136.9	115.3 ± 43.5

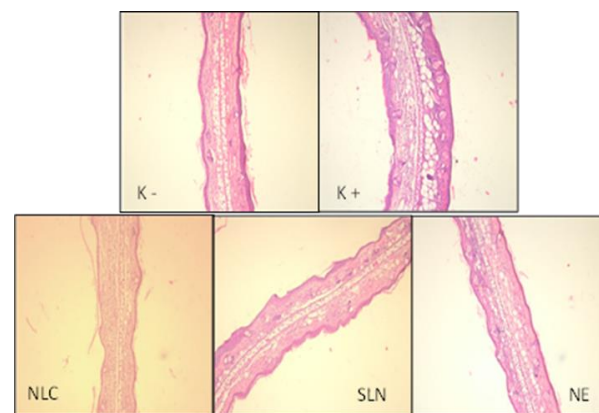


Figure 4: Histology of ear skin thickness of mice in negative control (K-), positive control (K +), with NLC-PMCA (NLC) treatment, with SLN-PMCA (SLN) treatment, ..and with nanoemulsion-PMCA (NE).. treatment. Observed using Olympus CX21 microscope with 100x magnification

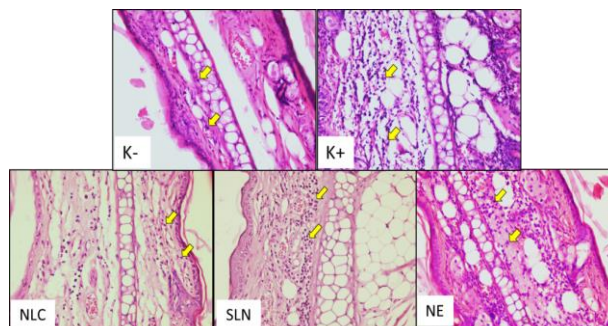


Figure 5: PMN cells in the ears of mice without treatment (K-), after induced by croton oil (K +), after treatment with NLC-PMCA (NLC), after treatment with SLN-PMCA (SLN), and after treatment with nanoemulsion PMCA (NE) Observed with Olympus CX21 microscope at 400x magnification

DISCUSSION:

In this NLC-PMCA study VCO was used as liquid lipid because it was determined that PMCA nanoemulsion that uses VCO as an oil phase produces the smallest droplet size. This is caused by VCO contains fatty acids with C atomic chains which are shorter than the fatty acid content in corn oil and soybean oil. The smaller size of nanoemulsion droplets results in the greater release rate of active ingredients from the carrier system and the penetration rate of active ingredients into the skin, so the resulting effectiveness is also higher [4]. As stabilizers, Tween 80 and Span 80 were used, which are nonionic surfactants, safer than other types of surfactants in the event of skin irritation [11,12]. NLC remains in its solid form by controlling the levels of liquid lipids added to the formulation, its can controlled drug release properties for NLC can be achieved.⁶ The choice of constituent materials and ratios between solid lipids and liquid lipids are important factors in the NLC formulation. To decreased orderness of the solid lipid in this research also used combination of beeswax and oleum cacao.

The data pH value in Table 2 it is known that all NLC-PMCA systems that formed had pH values that are slightly below the pH range of the skin at 4.5-6.5 so it does not causes irritation. From the viscosity data, it is known that F I (60:40) has the lowest viscosity, F I has the highest VCO content, which is the highest liquid lipid. An increase in liquid lipid consetration decreases the viscosity of NLC-APMS, but has no effect on particle size and particle size distribution or polydispersity index (PI). All formula have particle size < 500 nm, it was known a good NLC system has particle size up to 500 nm [7]. And then in Table 3, the results of particle size distribution or polydispersity index (PI) of NLC-PMCA systems were below 0.5 indicated the absence of particle aggregation, and the presence of a homogeneous particle size distribution.[13,14].

Based on the results of the melting point examination with DSC in Figures 1, 2, 3 and Table 4 it can be seen that the F III has a different thermogram form than F I and F II. In F III there is only one peak, this can be caused by the decreasing amount of liquid lipids the possibility of molecular interaction between oleum cacao and beeswax would occur, so there was endothermic peak of each solid lipid (oleum cacao and beeswax) an appeared. In F I and F II, there was a shift in endothermic (oleum cacao and beeswax) peaks (56.85 °C and 55.01°C) and a decrease in enthalpy between single solid lipids with NLC-PMCA (5.87 J/g and 1.43 J/g). This can be due to differences in composition between single solid lipids (oleum cacao and beeswax) in the formulas. In the calculation of the recrystallization index for F I and F II, a decrease in the value of RI

(0.326 and 0.074%) was obtained. This shows a decrease in the order of the crystal lattice. From these results, it can be concluded that the addition of PMCA and VCO can affect endothermic conditions (melting point and enthalpy).

The entrapment efficiency of F I higer than F II and F III its caused by the use of a combination of solid lipids and liquid lipids in NLC system can reduce crystallinity in the crystalline matrix and form irregular matrix, thus creating a space where drug molecules can be entrapped in the system [15,16]. Higher content of liquid lipid in NLC-PMCA also occured the less ordered matrices, supported by the recrystallization index data. The lower of recrystallization index of an ingredient occered the less ordered the crystal lattice is, the crystal space can accommodate larger drugs, so the resulting entrapment efficiency would be greater.[17,18]. The addition of liquid lipids also increased stability caused the crystallization process to be inhibited, so drug expulsion during storage can be minimized [17,19]. NLC-PMCA can produce anti-inflammatory activity equivalent to SLN-PMCA and NE-PMCA

To seek improvement in the delivery system so as to increase the effectiveness of the PMCA as an anti-inflammatory, the rate of release and penetration of the system needs to be determined. An active material can have the desired effect if it can be released from the system, and then penetrate into the skin to the site of action. Easier the active material detaches from the system, the faster penetration will be and the faster effect will be too.

CONCLUSIONS:

Based on the results of the characterization of the NLC PMCA system with ratios of beeswax-oleum cacao solid lipid and VCO lipid of 60:40, 70:30, and 80:20 which included organoleptic, pH, viscosity, particle size, particle size distribution, melting point, entrapment efficiency, and activity tests, in this research it can be concluded that: The increase of the amount of VCO in the NLC-PMCA system results in smaller particle size, lower viscosity, and greater entrapment efficiency, namely in the combination of beeswax-oleum cacao solid lipid and VCO liquid lipid of 60:40. NLC-PMCA with a combination of beeswax-oleum cacao solid lipid and VCO liquid lipid of 60:40 had equivalent anti-inflammatory activity as SLN-PMCA and nanoemulsi-PMCA, based on the observations of the PMN cells number in ear skin of mice with edema. The NLC-PMCA with the combination of beeswax-oleum cacao and VCO of 60:40 was the best formula.

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ETHICAL CLEARANCE:

All animal experiments were conducted with the permission from Animal Care and Use Committee (ACUC) of Veterinary Faculty, Airlangga University, Surabaya-Indonesia. (Reference number; 2.KE.051.01, 2018).

CONFLICT OF INTEREST:

No conflict of interest is associated with this work.

AUTHOR CONTRIBUTION:

We declare that this work was done by the author(s) named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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