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Research Article

Preparation and Characterization of Marigolds (*Cosmos caudatus* L.) Leaf Extract-Loaded Nanoemulsion: Physicochemical Properties and *In-Vitro* Release Activities of Nanoemulsion System Using Virgin Coconut Oil (VCO)

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ARTICLE DETAILS

ABSTRACT

Marigolds (*Cosmos caudatus* L.) leaves widely consumed as a vegetable and empirically been used as a medicine for various diseases. In this study, nanoemulsion was prepared using leaf extract of *C. caudatus* with a concentration of 5%, 10%, 15% and using Virgin Coconut Oil (VCO) as an oil phase. The purpose of this study is to know the characteristics of the formulation and to determine the effect of extract concentration on the release rate of the active compound of quercetin of *C. caudatus* leaf extract on the preparation.*C. caudatus* extract was obtained by the ultrasonic method with 96% methanol solvent. Nanoemulsion extracts were made by a low energy method. All formulations were evaluated for the preparation characteristics which included organoleptic test, pH test, particle size, emulsion type, entrapment efficiency, and stability, and quercetin release test of *C. caudatus* leaf extract using Franz cell diffusion method with cellophane membrane.The result of the characteristic evaluation of the research shows that formulation 1, 2, and 3 give a good result that is in accordance with the standard specification of each specified. The quercetin release flux of each nanoemulsion preparation formulation was 13,374 ± 0.216 μg / cm2 / min, 9,617 ± 0.404 μg / cm2 / min and 8,635 ± 0.021 μg / cm2 / min respectively. This result shows that the higher of extract concentration, the longer the release time.

INTRODUCTION

Plants that have many benefits as medicine and are still being developed, one of them is Marigolds (*Cosmos caudatus* L.). *C. caudatus* leaves are traditionally used as appetite enhancing drugs, gastric, bone-boosting, and insect repellents [1]. *C. caudatus* leaf has been proven to contain various types of compounds, one of which flavonol compounds from the group of flavonoid polyphenol compounds is quercetin. The calculation of flavonol content on a leaf of Marigold using standard curve based on wet base (per 100 g fresh samples), ie 51.28 mg quercetin, based on dry base (per 100 g dried samples) of 413.57 mg quercetin [2]. Bioactivity Quercetin is very broad, such as antioxidant, antibacterial, antiedema, antifungal, anti-inflammatory, antitumor, antiviral and others.

Quercetin has the disadvantage of its physicochemical properties ie its low solubility in water, causing limitations in the absorption process and affecting its bioavailability in the body [3]. Innovation in topical preparations is that transdermal drug delivery system is now in great demand by the public due to its ease of use. One of the advancement in manufacture of transdermal preparations is the manufacture of nanoemulsion systems. Transdermal delivery routes have many advantages over oral administration, which are more comfortable for the patient and can easily be stopped in case of undesirable side effects [4].

Nanoemulsion is a drug delivery system consisting of aqueous and oil phases stabilized by a combination of surfactants and cosurfactants [5]. Nanoemulsion has several advantages such as to improve the solubility and bioavailability of the drug as well as thermodynamically stable [6]. Nanoemulsion is formed when the dispersion results are clear

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with no phase separation. The recommended particle size of the Self Nano Emulsifying formulation is 10-200 nm [7]. The small size of nanoparticles causes the extracts soluble and has a high adsorption efficiency in the intestine [8].

The oil that can dissolve the lipophilic active ingredients is an important component in nanoemulsion formulation. In this research, the nanoemulsion system was formulated by leaf extract of *C. caudatus* as active ingredient and VCO as an oil phase. The formulation of the nanoemulsion system was made by variation of extract concentration by 5%, 10%, and 15%, so it can be determined the effect of variation of extract concentration in producing nanoemulsion system product which has the characteristic of chemical physics accordingly and its effect on the release rate of quercetin of *C. caudatus* leaf extract using Franz diffusion cell method.

MATERIALS AND METHODS
Materials
The ingredients used in this study were simplicia of Marigold (*Cosmos caudatus* L.) leaf, methanol solvent pa, 96% methanol (obtained from PT Bratachem, Indonesia), Virgin Coconut Oil (VCO) (PT Harba Bagus). Tween 80 (Sigma Aldrich), span 80 (Sigma Aldrich), propylene glycol (Sigma Aldrich) and oleic acid (PT Brataco, Indonesia), standard quercetin (Sigma Aldrich), phosphate buffer solution pH 6 ± 0.05 and pH 7.4 ± 0.05.

EXPERIMENTAL
Extraction of Marigold (Cosmos caudatusL.) Leaves
250 g of simplicial powder was extracted using an ultrasonic extraction method with 96% methanol solvent with a ratio of 1:20 (weight: volume) for 20 minutes [8]. After filtered and obtained macerate, then concentrated by using a rotary evaporator until obtained extract concentrated. The concentrated extract of *C. caudatus* leaves was then filtrated using a Whatman filter paper with a size of 0.8 micros with a ratio of 1:10. This filtering is used to uniform the particle size in the extract.

The concentrated extracts were then subjected to a methanol-free test by dissolving a small amount of extract with H_2SO_4 in a test tube and then adding CH_3COOH and cotton-covered, then heated to boiling. Next is the identification of ester odor on cotton, if the extract does not contain methanol then it does not smell the ester [9].

Phytochemical Test of Flavonoid Compounds C. caudatus Leaf Extract
The content of flavonoids was determined based on the work methods of [10]. C. caudatus leaf extract of 1 g was dissolved in 10 mL measuring flask with methanol solvent. From the mother liquor, 1 ml was dissolved 10 mL in a measuring flask. A total of 0.5 mL of the extract was mixed with 2 mL of distilled water and 0.15 mL of 5% NaNO2 and then allowed to stand for 6 minutes. The solution was added with 0.15 mL AlCl3 10% and restored for 6 minutes. The solution was reacted with 2 mL of 4% NaOH and diluted with aquadest up to a total volume of 5 mL and allowed to stand for 15 min. The change of color to pink to red indicates that the extract contains flavonoid compounds [11].

Determination of Total Content of Quercetin Compound in C. caudatus Leaf Extract Filtrate
One gram of extract dissolved in a 10 mL measuring flask with methanol solvent to the boundary mark, then filtered using 0.8 μm whatman paper. The result of filtration process for F1 was taken 1.5 mL, F2 taken 3 mL and F3 taken 4.5 mL, dissolved 10 times in measuring flask. Measured absorbance of the solution at a maximum wavelength of quercetin with UV-Vis Spectrophotometer [12].

Formulation Design
Nanoemulsion system formulation made as much as 30 mL, each formulation is done with 3 times replication.

Table 1: Design of Formulation Nanoemulsiom

Material Name	Function	Concentration (%)		
		F I	**F II**	**F III**
*C.caudatus*Leaf Extract	Active ingredients	5	10	15
VCO	Oil phase	5	5	5
Tween 80	Surfactant	46,19	46,19	46,19
Span 80	Surfactant	3,82	3,82	3,82
Propylene glycol	co-surfactant	5	5	5
Oleic acid	*Enhancer*	1	1	1
Phosphate buffer pH 6 ad		100	100	1000

Manufacture of Nanoemulsion System C. caudatus Leaf Extract
The nanoemulsion system of the Marigolds extract is made by the low energy method of spontaneous emulsification formation. The filtrate of C. caudatus leaf extract was dissolved

in span 80, then mixed with VCO and then added oleic acid (oil phase) then homogenized. Tween 80 is mixed with propylene glycol and phosphate buffer pH 6 as a water phase. The oil phase is introduced into the water phase by slowly mixing using a magnetic stirrer and then stirring until homogeneous at 1000 rpm at 37ºC for 90 minutes. Stirred until nanoemulsion system (clear view) [12].

Evaluation of Preparations
Organoleptic Test
The method used by observing visually the colors and shapes and using the sense of smell to find the smell of dosage. This test is done by distributing questionnaires to 10 respondents to know that this nanoemulsion really acceptable in society.

pH Test
A total of 1 gram of the preparation was dissolved in 10 ml of aquadest then measured using a calibrated pH meter. The pH of the preparation was adjusted to a pH of 4.5-6.5 [12].

Examination of Emulsion Type
The nanoemulsion type examination is performed by sprinkling a water-soluble dye, ie methylene blue on the surface of the preparation on top of the object glass and observed under an optical microscope [13].

Measurement of Particle Size
Nanoemulsion particle size was measured using Particle Size Analysis (PSA) Zetasizer "Malvern", Particle Size Analyzer nanoemulsionparticle size range 5-200 nm [7].

Entrapment Efficiency
A total of 1 gram of preparation is added with methanol solvent up to 10 ml. Then centrifuged at 2500 rpm for 45 minutes. The amount of unabsorbed quercetin will be dispersed in methanol in the supernatant. The supernatant of centrifugation result is determined using UV-Vis spectrophotometer [12]. Interpretation of the results of the trapability test, ie the trapability produced between 50-100% for the active ingredients of natural material extract. The interpretation of the percentage of sorption results is calculated using the following formulation:

$$\%EE = \frac{TD - FD}{TD} \times 100\%$$

Information;
EE = Entrapment efficiency percentage
TD = Total compounds contained in the formulation
FD = Number of compounds in the water phase (not absorbed)

Test of Preparation Stability
The nanoemulsion preparations were each stored at low temperature (4± º20C), room temperature (28±20ºC), high temperature (40±º20C) for 6 weeks, then organoleptic observation (discoloration, odor, phase separation, clarity) and measurement of pH by observation every 2 weeks [12].

Quercetin Release Test from the Preparation
The membrane used in the quercetin release test is a cellophane membrane that has been cut according to the disk size of the Franzdiffusion cell and has been soaked with aquades for one night (± 12 hours). Franz diffusion cells are one of the tools to test drug permeation through the skin in vitro, a vertical system permeation. The receptor compartment is filled with phosphate buffer pH 7.4 about 16 ml which are maintained at about 37 ± 0.5ºC and stirred with magnetic stirring at 250 rpm. Then the cellophane membrane is placed between the donor compartment and the receptor compartment with the dermal side directly related to the receptor medium. Samples weighed as much as ± 1 gram were then applied to the surface of the cellophane membrane. Then samples from receptor compartments at minute 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, 300, 330 and 360 were ± 2 mL using syringe. Each sampling is replaced with a phosphate buffer solution of pH 7.4 ± 0.05 with the same amount and temperature. Samples were measured uptake at a maximum wavelength of quercetin with UV-Vis spectrophotometer [12].

Release test result data which is the curve of the relationship between the cumulative quantity of quercetin active compound released from the nanoemulsion system of Marigolds extract (µg / cm^2) over time (min), calculated from the obtained level each time (µg / mL) which has been correlated using the Wurster formulation [14].

RESULTS AND DISCUSSION
Ultrasonic Extraction Result of Marigolds (Cosmos caudatusL.) leaves
The results of C. caudatus leaf extract were positively free from methanol, evidenced by the absence of odorous odors. That is because there

is no H atom of methanol attached by OH atoms of acetic acid, this reaction is influenced by H_2SO_4 which is a strong acid and serves as a catalyst so as not to cause an odor reaction from the ester.

Table 2: The results of Ultrasonic Extraction

Solvent	Extract Color	The weight of concentrated extract (g)	Rendemen (b/b)(%)
Methanol 96%	greenish brown	28.40	11.36

Data are expressed as mean±SD, n=3

Phytochemical Test of Flavonoid Compounds C. caudatus Extract

The results obtained on the methanol extract of C. caudatus leaf color change from brown to red. The principle of the AlCl3 colorimetric method is the formation of a complex between AlCl3 and keto groups in C-4 atoms and also with hydroxy groups on C-3 or C-5 atoms crossed from flavones and flavonols (Markham, 1988). This test proves that a positive C. caudatus leaf extract contains flavonoid compounds.

Determination of Total Content of Quercetin Compound of C. caudatusLeaf Extract Filtrate

Quercetin levels for each formulation were measured by measuring the absorbance of quercetin at a wavelength of 371.6 nm. After measurement using UV, the result of absorbance of leaf extract of C. Caudatus, the average absorbance result of F1 = 0204, F2 = 0.378, and F3 = 0.545, so that the average concentration for F1, F2, F3 is 1.27 ppm, 2.66 ppm, and 4.00 ppm. From these concentrations, it is known that the level of quercetin in caudatus leaf extract in F1 is 1.27%, F2 is 2.66% and F3 is 4,00%.

Organoleptic Test

The organoleptic test was performed on 10 respondents and obtained the result that F1, F2, and F3 have a different color. The color obtained from F1 is yellow; F2 is brownish yellow and brown on F3. The aroma produced from F1, F2, and F3 is a characteristic odor, there is no difference between the three formulations. The texture of the dosage obtained by the result is homogeneous in the form of a clear liquid with a slightly thick texture. Differences in dosage color due to different concentration of extract on each formulation.

pH Test

pH test results of the three formulations have met the criteria that can be tolerated pH to not irritate the skin ie 4.5-6.5 [15]. The results of pH measurements tend to be acidic because quercetin is acidic. The pH values of F1, F2, and F3 did not differ significantly and even tended to be similar, indicating that the variation of extract concentration did not affect the pH of the preparation. In the nanoemulsion system of C. caudatus leaf extract using phosphate buffer pH 6 which maintains the pH.

Table 3: The results of pH

Formulation	pH ± SD
F1	4.733 ± 0.058
F2	4.800 ± 0.100
F3	4.800 ± 0.060

Data are expressed as mean±SD, n=3

Emulsion Type

The results obtained after observation of the type of emulsion to each of the formulations are the dissolved methylene blue dye in the preparation. Based on the observation using optical microscope under 400x magnification, it was found that the particles in the dosage diffuse evenly throughout the water, then the emulsion type of nanoemulsion preparation of *C.caudatus* leaf extract is O/W.

Particle Size

The results show that the three tested formulations have met the particle size criteria for nanoemulsion preparations of 5-200 nm [7]. The particle size results differed, the particle size of the preparation increased from F1 <F2 <F3. This indicates that the variation of extract concentration affects the size of the dosage particles. The greater concentration of extracts added in the nanoemulsion system will increase the particle size of the dosage, possibly due to the overly fast stirring process during the manufacture of the nanoemulsion system.

Table 4: The results of Ultrasonic Extraction

Formulation	Particle Size± SD
F1	10.99 ± 0.015
F2	13.43 ± 0.160
F3	13.95 ± 0.116

Data are expressed as mean±SD, n=3

Entrapment Efficiency
Based on the calculation result of trap efficiency, it is known that the increase of the concentration of the leaves of Marigolds extract as the active ingredient in F3 shows the increasing of entrapment efficiency. The amount of entrapment efficiency is caused by the amount of active compound in the preparation, the higher the active compound the greater the entrapment efficiency. The result of low Entrapment efficiency can be caused by less stirring time because the formation of ionic gelation one of them is influenced by the length of stirring time.

Table 5: The results of Entrapment Efficiency

Formulation	Entrapment Efficiency (%)
F1	99.00 ± 0.004
F2	99.02 ± 0.008
F3	99.25 ± 0.006

Data are expressed as mean±SD, n=3

Physical Stability of the Preparation: Organoleptic Observation
The result of observation of the three dosage formulationtions during storage for 6 weeks at high temperature (40±2oC), the nanoemulsion preparation remained stable, no phase separation and phase inversion, but the color of the dosage became increasingly bright, the aroma of the preparations unchanged and the texture of the dosage more dilute (liquid). Storage at room temperature (28 ± 20C) showed that the nanomaterials remained stable and showed no significant physical changes, the three nanoemulsion formulationtions remained clear, homogeneous, color, aroma, and texture of the preparations were unchanged from before. While storage at low temperatures (4±20C), the third preparation color tends to fade (decrease), the aroma of the preparations also decreases, and the texture becomes thicker than before but the three preparations do not freeze and do not undergo deposition.

The results show that storage at different temperatures may affect the organoleptic preparation, the nanoemulsion preparations of the Marigolds leaf extract, tending to be stable at high temperature storage and room temperature.

Observation of pH Stability
The result of initial pH measurement during storage at high temperature (40±2oC) with a measurement every 2 weeks resulted that the pH of the preparation of each formulation decreased. Measurements at room temperature (28±o20C), obtained the initial pH of the preparation decreased pH value until the 4th week, but at 6th week increased. Different results were shown at pH measurements at low temperature (4±20oC), initial pH of the preparation and for 6 weeks of storage increased.

Based on the measurements, it was found that the pH of the nanoemulsion preparation on the first day of preparation up to the 6thweek after storage had changed the pH value but still met the physiological skin pH range of 4.5-6.5 [15]. The decrease in pH value during storage may be due to the oxidation process which can increase the acidity, increase the pH in the preparation due to the release of VCO in the nanoemulsion system that increases the basicity.

Release Test
The Fig.1 shows the cumulative quantity of quercetin in the preparation of the nanoemulsion system is increasing with increasing time. In F1, the release of quercetin through the membrane undergoes a waiting period from minute 0 to 150, then in the 180th minute, the quercetin begins to detach in the compartment medium. F2 has a waiting period from 0 to 240 minutes, in the 27th minute quercetin began to detach. F3 has a longer waiting period ie from 0 to 330 minutes, in the 360th minute there is a quercetin concentrated in the receptor compartment medium.
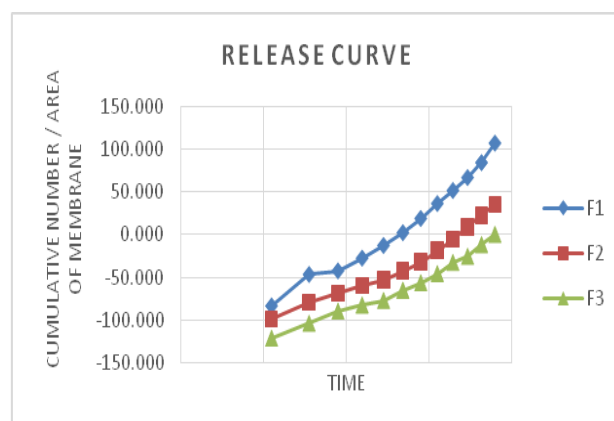

Figure 1: Release Curve

Based on the release result, it can be determined the flux value of each formulation. The flux value is the slope of the regression result between mass transported per unit area against time at steady state condition. The steady state condition is represented by a linear curve, having a relation coefficient value (r) equal to or close to 1. Thus,

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the flux is calculated using a curve having a correlation coefficient (r) close to 1.
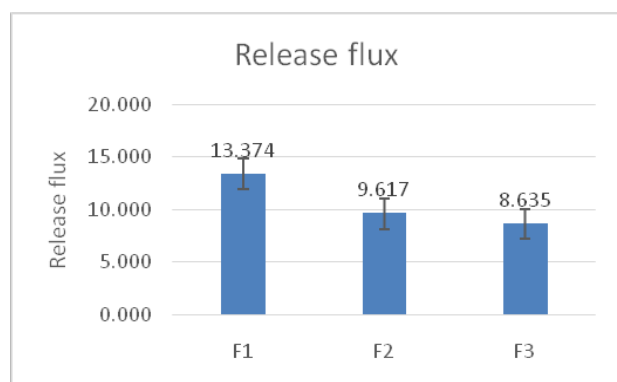
Figure 2: Release Flux

The results of the data analysis show that the inter-formulationtion has a significant difference because the One Way ANOVA test data release test data has a significance of 0.000 <0.05, it indicates that the three formulations have significant differences From the known flux calculation that the flux value FI> F2> F3. This proves that the smaller the concentration of extracts used, the faster the release rate of quercetin from the preparation. From the research conducted, F1 with the least extract content (5%) yields the highest release flux, this is caused by at least quercetin which can be bound by VCO oil so that quercetin is more easily released than F3.

In the nanoemulsion of *C. caudatus* leaf extract, the release of quercetin through the cellophane membrane is influenced by the presence of enhancers which can disrupt the density of the skin structure resulting in increased permeability so that quercetin can penetrate the stratum corneum barrier. However, in the nanoemulsion preparation of Marigolds leaf extract, there is still a lot of quercetin in free form because no purification of the compound before it is mixed as the active ingredient. The amount of flux generated from the release calculation shows a low yield, this is due to a shorter period of time during the discharge process.

CONCLUSION
The nanoemulsion system of the Marigolds leaf extract has clear and homogeneous characteristics, the particle size meets the range of 5-200 nm, the Entrapmentefficiency of 99% - 99.25%, the type of water-oil emulsion (O / W), the stability of the pH value of the nanoemulsion system obtained during storage meets the range

physiological pH of the skin. Different variations in concentration of leaf extract of Marigolds (*Cosmos caudatus* L.) affect the release of active compounds of quercetin in the nanoemulsion system, the higher the concentration of the extract the longer the release time.

CONFLICT OF INTEREST
There are no conflicts of interest.

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