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Characterization and Safety of *Nothopanaxscutellarium*Ethyl Acetate Fraction Gel Ethosome

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Abstract : *Nothopanax scutellarium* is one of plant that has several biological activities such as obstetrics, kidney disease, inflammatory disease, and promotion of hair growth. Ethosomes are the ethanolic phospholipid vesicles which are used mainly for transdermal drug delivery of drugs. Ethosomes have higher penetration rate through skin. It contains soft vesicle, composed of hydroalcoholic or hydroglycolic phospholipid in which concentration of alcohol is relatively high. Ethosomes are a novel carrier for enhanced skin delivery. The aims of this research were to formulate and obtain ethosome which formed by thin layer hydration method and to know the safety of gel ethosome which contain ethyl acetate fraction. Safety test measured by scoring and categorizing within chorioallantoins membran. (HET CAM). The formulation of ethosome that using 20% ethanol and the ratio of phosphatidylcholine and ethyl acetate fraction were 1:2. The characterization from the ethosome suspension indicated that the entrapment efficiency value of 52.79% with particle size distribution value (Z-Average) 110.5 nm, polydispersity index (PdI) 0.393 and zeta potential value -11.0 mV. The result of safety test showed that 0.5% and 1% gel ethosome contain ethyl acetate fraction of scutellarium leaves have mild irritation effect.
Keywords : *Nothopanax scutellarium* Merr, ethosome, characterization, HET CAM, safety.

Introduction

Nothopanax scutellarium Merr. which locally known as Mangkokan leaves, is one of plant that have several advantage such as obstetrics, kidney disease, inflammatory disease, and promotion of hair growth[1] Mangkokan leaves contain flavonoid such as quercetin, kaempferol, myricetin, luteolin dan apigenin.[2] Based on previous research, ethyl acetate fraction of mangkokan leaves formulated in the form of hair tonic can improve hair growth better than the positive control (Minoxidil) with a concentration of 0.5% and 1%, and in the study also showed that the ethyl acetate fraction leaves mangkokan are mild irritation of the membrane korio alantois with a score of 4.54[3].

Ethosome penetration enhancers system is a type of vesicles (vesicular enhancer) are widely studied in recent years. Ethosome carriers that are non-invasive delivery that enable bioactive compound to reach the deep skin layers or the systemic circulation. They are composed mainly of phospolipids, high concentration of ethanol and water. Ethosome were able to enhance permiation and activity ammonium glycyrrhizinate and

apigenin[4,5]. Some studies reported that ethosome penetrate the skin and allow enhanced delivery of various compounds to the deep skin or to the systemic circulation [6,7]. Based on those reasons, so in this study will formulate a gel formulation of ethyl acetate fraction ethosome mangkokan leaves which are expected to have the effect more effective and safer for the development of research in the field of cosmeceutical.

Materials and Methods

Materials

Mangkokan leaves was purchased from Balai Penelitian Tanaman Obat dan Aromatik (Balittro), chloroform, ethyl acetate, n-hexane, ethanol, soybeanphosphatidylcholine (Phospholipon 90G, Lipoid GmbH, Germany), phosfat buffer pH 5, dichloromethane, propylene glycol,quercetine, Carbopol 940, triethanolamine, sodium metabisulphite, methylparaben, propylpraben, Leghorn egg, Sodium chloride, Sodium Lauryl Sulphate.

Method

Extraction and fractionation

The dried mangkokan leaves powder were macerated using chloroform and ethyl acetate, respectively. The sample was macerated using chloroform for 24 hours, and then the residue was macerated again using ethanol for 48 hours. The extracts solution was evaporated to give crude ethanolic extracts. The extract was partitioned subsequently with hexane and ethyl acetate. The organic solvent was evaporated using rotary vacuumevaporatorin temperature 40-50^{o}Cto give ethyl acetate fraction.

Prapation of ethosome

Ethosome was prepared according to the thin film hydration methode[10]. Soya Phospatydylcholine and ethyl acetate fraction (2:1) were dissolved in dichloromethane in a 50 ml round bottomed flask. The mixture was evaporated in a rotary vacuum evaporator at 50-150 rpm, 40^{o}C for 40 min. The film then degasses using nitrogen and stored in the refrigerator for 24 hours. The film was hydrated with 20% ethanol, 15% propylene glycol and add with buffer phosfat pH 5 solution using rotary vacuum evaporator at 39°C with a speed of 100 rpm and with the aid of glass beads to be easily peeled off. and stored in the refrigerator (4-8 °C) until further use. Etosom suspension that has been stored for 24 hours then reduced in size by usingultrasonication[8].

Characterization of ethosome[9,10,11]

Particle shape visualization

Characterization of the ethosomewas performed using particle visualization. Particle shape visualized using a TEM(TransmissionElectronMicroscopy).One drop of suspension ethosome dropped on carbon-coated copper gridand allowed to be absorbed. The non-absorbed suspension wasremoved byblottingpaper.Then dried at room temperature. The ethosome morphology was analyzed using TEM(Transmission ElectronMicroscopy).

Particle size distribution, zeta potentialand polydispersity index (PdI)

The particle size distribution of ethosomeand zeta potential were determined byDynamic Light Scatteringmethod (Malvern Zetasizer). Distilled water solution is inserted into the fluid tank as a baseline,then the sample is introduced into the fluid tank dropwise to a concentration sufficient. After that will be measurable particle size ethosome globule and also zeta potential.

Entrapment efficiency of ethosom

To obtain the fraction ethosome, the unentrapped part was removed using ultracentrifuge50 000 rpm at 4°C for 60 min[12] . The sediment of the ethosome was kept after removal of the supernatant. TLC Densitometry was performed to evaluate the drug absorption from the ethosomes. The entrapment efficiency (EE) was calculated according to the following formula

$$EE = DE/(DE + DS) \times 100$$

Where DE: the drug content measured from the parvules; DS: the drug content measured from supernatant.

Preparation of ethosomal gel

Ethosomal gel was prepared with two kinds of different concentrations 0.5% and 1%. The amount of Carbopol (0.5%) was slowly added to aquadest and kept for 24h. Triethanolamine was added to it drop wise. Sodium metabisulfite was dissolved into aquademineralisata. Then, methyl paraben and propyl paraben were dissolved in propylene glycol. The mixture was added to the carbopol mixture and homogenized with continuous stirring 1000 rpm. After the gel base formed, then the ethosome suspension was added until homogenous.

Gel evaluation[13,14]

a. Homogeneity

Gels were tested for homogeniety by visual characteristic container. They were tested for their appearance and presence of any aggregates.

b. pH measurement

The pH of the ethosomal gel was determined by using digital pH meter

c. Viscosity study

The viscosity of different formulation was measured using Brookfield viscometer. The reading was taken at 20 rpm using spindle no.1

d. Particle Size Distribution

The gel(1 g) was dissolved in distilled water until homogeneous and then taken one drop of solution was then dispersed in 10 mL of water. After that, 1 mL of the dispersion was incorporated into the cuvette to examine particle size distribution using the Particle Size Analyzer (PSA).

Stability test[15]

Stability was determined by physical appearance content uniformity and release study at 4^{o}C, 40^{o}C, and 25^{o}C at different time intervals 0-2 months.

Cycling test

Gels were stored at 4°C for 24 hours and then was removed and placed at 40°C for 24 hours. This treatment was one cycle. The experiment was repeated for 6 cycles. The organoleptic characteristic and pH were evaluated before and after the test

Safety Test

The test was performed according to hen's egg test – chorioallantoic membrane (HET-CAM) test method with slight modification[15], using fertile 50-60 g white leghorn chicken eggs (7 days).The eggs were incubated at 38.3 °C and 58 % relative humidity when incubating in a still-air incubator or at 37.8 ± 0.3ºC and 58 ± 2% relative humidity. Partially of eggshell was removed. The inner membrane was moistened with 0.9% NaCl.Test solutions(0.3 mL) was added directly onto the CAM surface. Sodium lauryl sulfate (1%) was used as positive control, as negative control was 0.9% NaCl. The reactions on the CAM was observed ina period of 5 minutes. The appearance of: haemorrhage (Bleedings), vascular lysis (Blood vessel disintegration), coagulation (protein denaturation intra- and extra vascular) was monitored and recorded in seconds. The time for each reaction was calculatedin irritation score (IS)[14] based on the below formula.

$$\frac{(301 - t\ haemorrhage)x\ 5 + (301 - t\ lysis)x\ 7 + (301 - t\ coagulation)x\ 9}{300}$$

When determining the threshold the degree of severity of each reaction after treatment time has to be recorded according to the following scheme:

Table 1. Scoring scale for the HET-CAM test

core on HET CAM	Category of irritation
0 – 0.9	Nonirritant or practically no irritation
1 – 4.9	Weak or slight irritation
5 -8.9 or 5 – 9.9	Moderate irritation
9 – 21 or 10-21	Strong or severe irritation

Results and Discussion

Extraction of mangkokan leaves is used a cold maceration. This method was chosen because heating can make the levels of flavonoids reduced. The heating process can lead to lower levels of flavonoids total of 15-78%[17].Remacerationwas conducted using chloroform to eliminate non polar compound such as chlorophyll, lipids, and terpenoids[18]. While ethyl acetate has the ability to extract polar compounds such as flavonoids-*O*-glycoside The result of the extraction, is blackish green color and a distinctive odor mangkokan leaves and for the ethyl acetate extract yiled 5.77%.

Fractionation is a separation method that uses two kinds ofsolvents that are not intermingled or who have high levels of polaritydifferent[18]. From the previous research, fractination using ethyl acetate of mangkokan leaves has higher concentration of flavonoid than using extraction methode[3].

Characterization of ethosome

Particle shape visualization

Figure 1 result of visualization of ethosome under transmission electron microscopy. The results of the test showed that ethosome has spherical vesicles, regular with uni lamellar structure which has one layer.

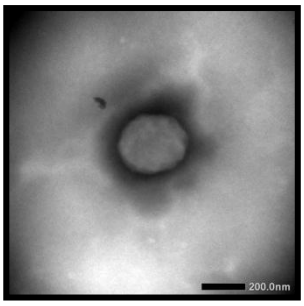

Figure 1. Visualization of ethosomes by Transmission Electron Microscopy (x20000)

Particle size distribution, zeta potential, andpolydispersity index

The particle size and zeta potential will affect the value of the indicator of the quality and appearance ethosome. The use of lipids as a carrier and drug usually makes the particle size distribution of a few nanometers to hundreds of small nanometer[19]. Higher particle size and surface chargezeta potential will improve the stability of the suspension ethosome.The size is getting smaller can be obtained from the ultrasonication. Particles with a round shape will be easier to measure.The particle size by measuring its diameter as it has the same dimensions or with measured D volume. For particles having a shape that is not spherical measured particle size can use the calculation of the Z-average.

Zeta potential is directly related to the distance between the particles in a dispersion system. The more positive or more negative zeta potential, the more happening among particles repel force so that the distance between the particles in the dispersion system will fall further. The farther the distance between the particles

decreases the tendency of the particles to aggregate or defloculation. The value of the zeta potential is good in suspension with a value of more positivethan + 30 mV or more negative than -30 mV[20].

Polydispersity Index (PDI) is the degree of heterogeneity of acollection of particles[17]. PDI value is 0-1, which if PDI <0.5, the system is monodisperse but if the system is polidisperse have PDI values> 0.5. The smaller the value of PDI (close to zero)the more homogeneous particle size distribution[18]. From the data was obtained that ethosome from mangkokan leaves has particle size 110.5 nm, with PDI 0.393 and zeta potential -114 (see Fig 2).

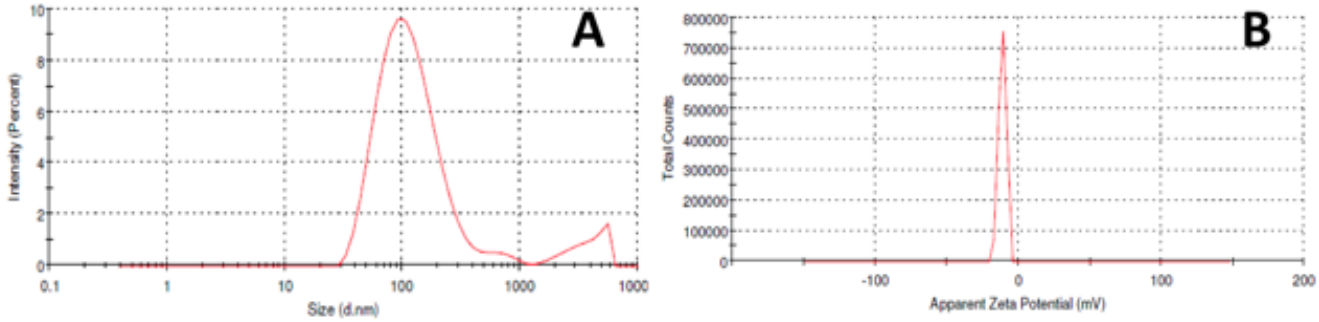

Figure 2. Particle size (A) and zeta potential of ethosome mangkokan leave (B)

Entrapment efficiency

Ethosome suspension was centrifugated with 50 000 rpm for 1 hour to separate the suspension into supernatant and sediment. Test of entrapment efficiency ofethosome in this study conducted direct method by measuring the concentrations of quercetin as a standard for fraction of mangkokan leaves, that is bound to nanovesicle.Percentage entrapment of quercetin was calculated by comparing the concentrations ofquercetin measured in sediment ethosome with an average concentration ofquercetin in suspension intact ethosomeand then calculated using TLC-densitometry. From the test was obtained that the percentage for entrapment efficiency of ethosome from mangkokan leaves was 52.79%

Gel evaluation

From the evaluation were obtained that gel ethosome mangkokan leaves have a homogen appearance (Fig 3) with pH 5.61 (0.5%) and 6.4 (1%). Thus means that gel ethosome suitable for skin which has pH balance 4.5 – 6.5. The viscosity of this gel ethosome was 980 cps considering the mangkokan leaves was used for hair growth dan applied in the scalp. From the measurement of particle size was obtained that the gel ethosome from mangkokan leaves has size 240.5 nm with PdI 0.493 and zeta potential -73.8 (Fig 4).

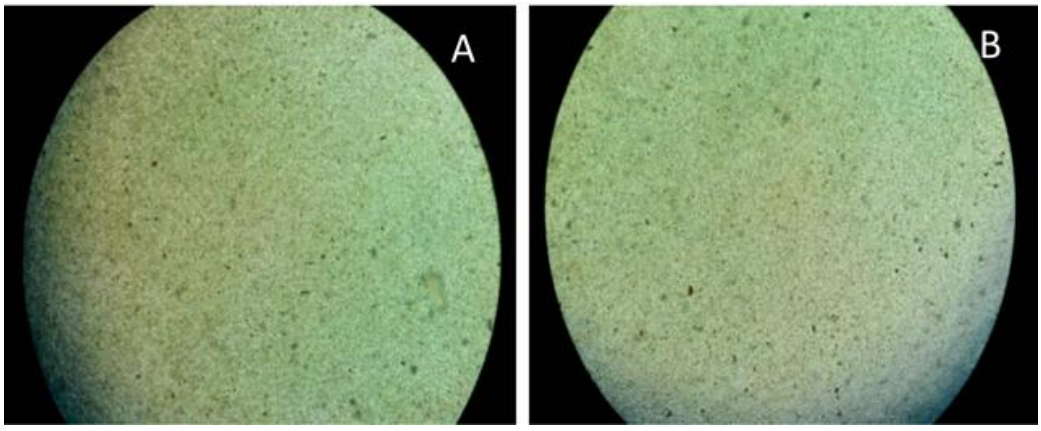

Figure 3. Homogeneity of gel: A. 0.5%, and B 1% (Magnificant 10x)

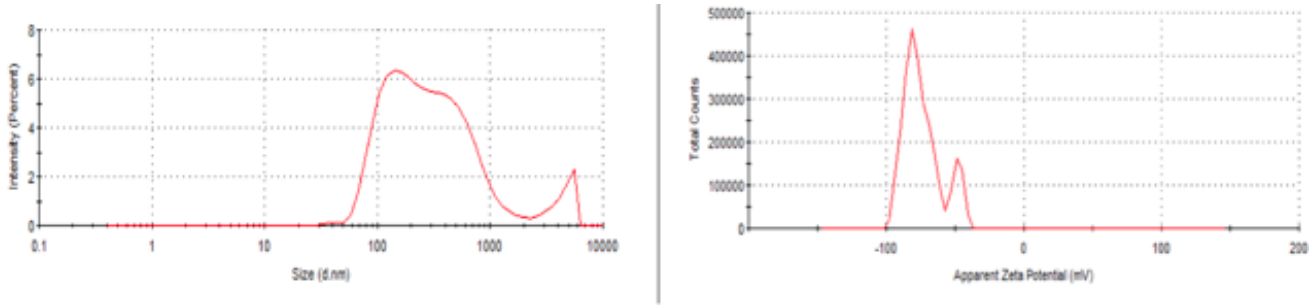

Figure 4. Particle size and zeta potensial of ethosomal gel

Physical stability test

Figure 5 showed stability test result of the thosomal gel stored at low, room and high temperature for 2 months. The observation of the organoleptic each storage samples at low temperature, room temperature, and high temperature for 8 weeks at intervals of every two weeks. There was a decrease in the pH of the gel formulation. It is caused due to an oxidation reaction by the interaction of carbon dioxide contained in the air with water (H_2O) contained in a gel form carbonic acid which is acidic so it can increase the acidity. From the results of organoleptic examination at a temperature of 40 $^{\circ}$Cgel filled in the container experienced a reduction in time, this is because the water on the basis of the gel is reduced.

Cycling testaims to determine the stability of a preparation with extreme changes in temperature conditions. This test is performed on stocks stored at low temperature (4°C ± 2°C) for 24 hours, then stored at high temperature (40°C ± 2°C) for 24 hours. The move is called a cycle. This test is performed for 6 cycles to simulate the temperature changes each day to determine the stability of the gel. After 12 days of either gel or gel transethosome non-transethosome does not change the smell, color and does not occur syneresis. That is to say that both the gel formulation is stable.

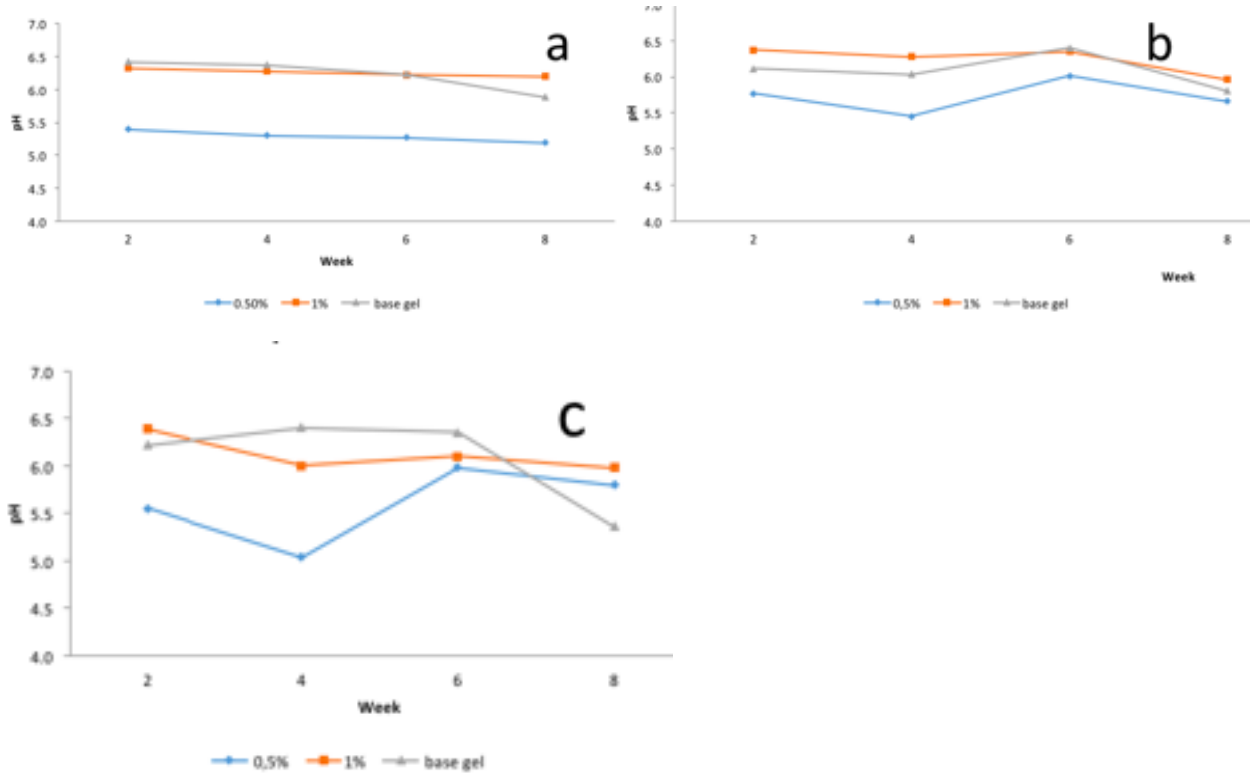

Figure 5. pH of gel after stored for 2 months (a) 4^{o}C, (b) 25^{o}C and (c) 40^{o}C

Hen's egg test – chorioallantoic membrane (HET-CAM)

HET-CAM test results showed that the hemmorrhage occured in 250 sec(F1= 0.5%) and 180 sec (F2 1%) as seen in Figure 6. For 5 minutes observation time does not happen lysis and coagulation reactions for all three groups of test preparation. Based irritation scores obtained, then to the negative controls included in the category are not irritants, the positive control and gel ethosome 0.5 % and 1 % are mild irritation with score 0.85 and 2.01. This irritation wascaused by the material properties of propylene glycol that demonstrated slight irritant effect[21,22].

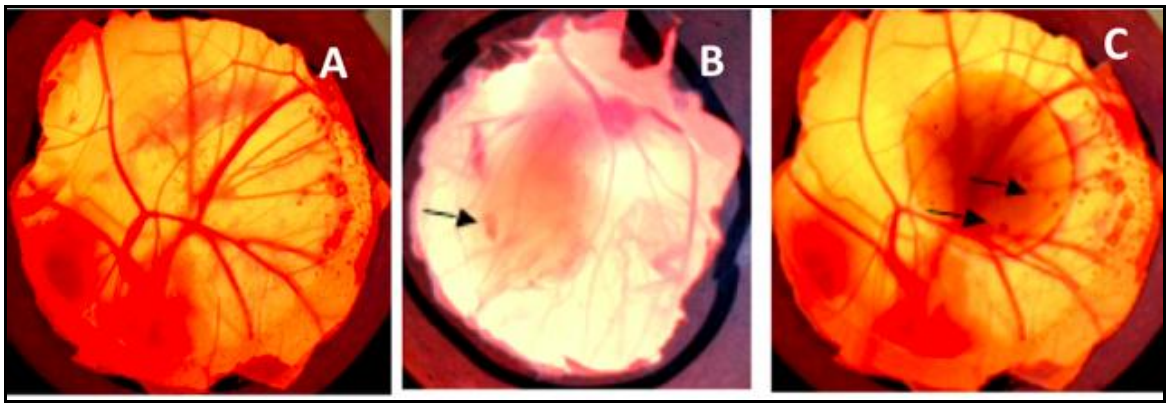

Figure 6. Photograph of HET CAM results; (A) Normal CAM; (B). CAM with 0.5% sample and (C) CAM with 1% sample

Conclusions

From the above results indicated that the gel ethosome of mangkokan leaves has a good character and demonstrated mild irritant effect.

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