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Formulation of Nanostructured Lipid Carrier Gel From Mulberry Root Extract (*Morus alba* L.) as Whitening Agent using Zebrafish Modelling

Formulasi Gel *Nanostructured Lipid Carriers* Ekstrak Akar Murbei (*Morus alba* L.) sebagai Pencerah Kulit pada Model Zebrafish

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Abstract: Mulberry is a plant that has been shown to be effective as a whitening agent. The objective of this research was to evaluate the efficacy of mulberry root extract (MRE) and its nanostructured lipid carrier (NLC) extracts in terms of their ability to lighten skin tone by using zebrafish as a model. NLC were formulated into gel preparations. The whitening effect of MRE and NLC was evaluated, and the melanin content of the extracts was found to be the result of the evaluation. Based on the results of morphological observations, a decrease in melanin levels was seen in the eye. In the mulberry root extract, the melanin level decreased with an increase in the concentration of the mulberry root extract. In the NLC of mulberry root extract, a decrease in melanin levels was obtained at an increased NLC concentration of mulberry root extract. It can be concluded that mulberry root NLC has the ability to act as a whitening agent, which is distinguished by the reduction of melanin content.

Keywords: Mulberry root extract, nanostructured lipid carrier, whitening agent, zebrafish.

Abstrak : Akar murbei (*Morus alba* L.) merupakan salah satu tanaman yang memiliki khasiat sebagai pencerah kulit. Penelitian ini bertujuan untuk menentukan aktivitas pencerah kulit secara in vivo menggunakan pemodelan dengan zebrafish pada ekstrak dan NLC ekstrak akar murbei yang diformulasikan menjadi sediaan gel. Pengujian inhibisi tirosinase dilakukan pada ekstrak dan gel NLC ekstrak akar murbei, aktivitas pencerah kulit dilihat dari kadar melanin. Hasil yang diperoleh memperlihatkan penurunan level melanin seiring dengan peningkatan konsentrasi ekstrak akar murbei. Demikian pula terjadi penurunan level melanin pada peningkatan konsentrasi NLC. Disimpulkan, gel NLC ekstrak akar murbei memiliki aktivitas sebagai pencerah kulit ditandai dengan kadar melanin yang menurun.

Kata Kunci: Ekstrak akar murbei, gel, *nanostructured lipid carriers*, pencerah kulit, zebrafish.

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INTRODUCTION

SKIN is the outer barrier of the body, which is impermeable and has a function as a protector from various exposures in the environment, such as UV rays, pollution, dust, and bacteria. UV rays will induce the formation of melanin pigments, which are stimulated by tyrosinase, and they will increase melanin synthesis in the skin and cause hyperpigmentation[1]. Both the hydroxylation of tyrosine to dihydroxy-phenylalanine (L-DOPA) and the oxidation of L-DOPA to DOPA quinone require the catalytic activity of tyrosinase[2].

One of the ways to inhibit melanin formation is to inhibit tyrosinase[3]. Compounds derived from plants inhibit tyrosinase activity and are used for skin whitening preparations, such as ellagic acid, oxyresveratrol, chlorophorin, and norartocarpanone[4]. The content in mulberry plants that has activity as an inhibitor of the tyrosinase enzyme is oxyresveratrol, where the highest oxyresveratrol content is found in the roots[5]. To inhibit the pigmentation process, oxyresveratrol will be formulated in topical dosage forms rather than oral, because the active substances can interact longer with the skin. The nanoparticles commonly used for the manufacture of cosmetic preparations are nanostructured lipid carriers (NLC)[6]. The application of nanoparticles in cosmetic preparations is to increase drug delivery and decrease side effects[6].

The zebrafish (*Danio rerio*) is a species of dwarf tropical fish native to India and South Asia[7]. These animals also have the same melanophore gene in zebrafish as the melanocyte gene in mammals[8]. Some of the advantages of using zebrafish include adaptation mechanisms to the environment, fairly varied instincts, circadian patterns, and various other adaptation mechanisms. The melanin pigment in the zebrafish is present on its surface, making it easier to observe the pigmentation process[9]. For the purpose of inhibiting the pigmentation process, oxyresveratrol is formulated in topical dosage forms, such as cosmetics, rather than oral, because the active substance can interact with facial skin for longer.

Nowadays, the cosmetic industry uses many developments in nanoparticle technology, one of them is NLC. The application of nanoparticles in cosmetic preparations is intended to provide more targeted delivery of active cosmetic ingredients due to their small particle size and to reduce side effects[3]. The study aimed to determine the whitening effect using zebrafish modelling on extracts and NLC extracts of *Morus alba* L. To determine the effectiveness of the mulberry root plant as a skin lightener, an in vivo activity test was carried out using modelling with zebrafish. Activity tests were carried out on samples of 96% ethanol extract of mulberry roots and NLC containing mulberry root extract. Testing of the NLC of mulberry root extract was carried out to determine whether there was an influence on the skin-lightening effect of making nanoparticle preparations.

MATERIALS AND METHODS

MATERIAL. Fresh mulberry root (*M. alba* L. root) was purchased from Rumah Sutera, Bogor, West Java, Indonesia. It was harvested in January 2020. The determination of mulberry roots (*M. alba* L.) was carried out at the Herbarium Bogoriense (BO), Research Centre for Biology, Indonesian Institute of Sciences (LIPI), Bogor, West Java, Indonesia, with certificate number B-521/IPH.3/KS/III/2020.

Chemical and Reagents. Ethanol 96%, stearic acid, virgin coconut oil, carbomer 940, tween 80, span 80, triethanolamine, propylene glycol, methyl paraben, propyl parabens, phosphate buffer, methyl cellulose, pure water, and perfume were purchased from Brataco, Indonesia. Kojic acid was purchased from Thornhill, Kanada. Water hyacinth, fish food, and fish growth media (sea salt water) were purchased from Fisharia Farm, Jakarta.

Equipments. Particle Size Analizer and Zetasizer 200 (Malvern, United Kingdom), Transmission Electron Microscopy (JEOL, Tokyo, Japan), Olympus CX 23 microscope (Olympus, Tokyo, Japan), waterbath, Viscometer Brookfield (Middleboro, Massachusetts), Eurostar stirrer (IKA, Germany), Ultra-turrax T25 (IKA, Germany), pH meter Ultra BASIC (Denver Instrument, New York, United States).

METHODS. Extract Preparations. The mulberry root was cleaned of impurities, then chopped and dried in an oven. The dried mulberry root was then mashed and sieved with a sieve number of 4/18 (4760 μm/1000 μm). The extract was made using the sonication method with 96% ethanol, then filtered to obtain the filtrate. The filtrate was then thickened with a rotary evaporator to obtain a thick extract. Then the extracts evaluated included organoleptic, pH, solvent mixtures, and moisture content.

NLC Preparations. NLC was prepared using the solvent evaporation method by mixing solid lipid (stearic acid) with liquid lipid (virgin coconut oil) and using non-ionic surfactants tween 80 and span 80. The formulation of NLC is described in Table 1. The mixing was done with an ultraturrax homogenizer at a speed of 20,000 rpm. The nanoparticles that were obtained were analysed, and some of the parameters that were looked at were particle size, polydispersity index, zeta potential, and particle morphology[10].

Table 1. Formulation of NLC mulberry root extract.

Ingredients	Percentage (%)	Function
Mulberry root extract	0.63	Active ingredients
Stearic acid	0.63	Lipid carrier
Virgin coconut oil	0.63	Lipid carrier
Tween 80 & Span 80	7.00	Non-ionic surfactant
Water	90.50	Solvent

Gel Nanoparticle Preparations. Gel preparations made using carbomer 940 as a gelling agent were developed using water to obtain a gel base. Then mixed the base gel with NLC containing mulberry root extract using a stirrer at a speed of 300 rpm to obtain a homogeneous NLC gel of mulberry root. The gel preparation was then evaluated, including organoleptic, homogeneity, dispersibility, pH, viscosity, and rheology.

Phenotype-Based Evaluation Using Zebrafish. The animals used for research were zebrafish in the embryonic phase. It was obtained from a fish cultivation place in Bogor, West Java, Indonesia. Adult zebrafish (a ratio of 3 males and 2 females) were separated in an aquarium. As soon as the light cycle started, the divider was removed, and zebrafish began to spawn. Embryos were collected and separated from damaged and dead embryos. The embryos were then grouped according to the sample to be tested. The embryos were incubated at 28.5°C for 24 hours. Into the medium container for fish embryos, and the ingredients and samples were dissolved to be tested evenly on the fish growth medium. Testing was carried out up to 48 hours after the embryos hatched. The embryos were dechorionated and mounted in 3% methylcellulose on a depression slide. Melanin formation was observed in the embryo using the Olympus CX 23 Microscope[11].

Estimation of Melanin Content. Photos of five embryos from different treatments were taken at random and subjected to ImageJ analysis. To further investigate the melanin content in zebrafish, we performed the analysis using ImageJ software, a public-domain Java image processing and analysis program. The area in the eye where pigmentation is most intense was selected in the software, and the integrated density was calculated. Integrated density was used to estimate ocular melanin content[11].

RESULTS AND DISCUSSION

Extract Preparation. The extraction process was carried out by the sonication method at room temperature using 96% ethanol. Obtain 220 g of dried mulberry root, then sonicated using 96% ethanol for as much as 9.0 L. The dried mulberry root was soaked in solvent for 24 hours prior to sonication, allowing the solvent to fully permeate the root and facilitate the breakdown of the root's cell walls and dissolution of its compound content. The macerate was sonicated for 45 minutes before being filtered, concentrated, and finally obtained using a rotary evaporator. The goal of the evaluation of the extract was to ascertain its qualities in order to ascertain its correct formulation and storage conditions. Standards and safe limits for extracts as a quality natural material product must be established for their evaluation[12]. The evaluation of the extract was shown in Table 2.

Table 2. The result of extract evaluation.

Evaluation	Results
Organoleptic	Thick brown extract
Solvent mixture	Soluble in acetone, span 80, propylene glycol, ethanol 96%
pH	4.79
Moisture content	9.92%

Nanostructured Lipid Carrier Preparation. Based on the optimisation results that have been done previously[13], the NLC formula made using a mix of lipids, namely stearic acid and virgin coconut oil, with a ratio of 50:50 will produce a homogeneous and liquid form of NLC. This liquid form of NLC was desirable because it can easily be separated for characterization purposes. The higher the amount of mixed lipid used, the thicker the NLC will be. High surfactant concentrations can reduce particle size. NLC was made using ultra-turrax by keeping the temperature at 40°C so that the melted fat phase did not re-solidify during the mixing process, resulting in a homogeneous mixing of nanoparticles between the oil phase and the water phase. The speed used was 20,000 rpm. The nanoparticle formulation of white mulberry root extract has smaller particle sizes than regular emulsion[14]. The evaluation of NLC was shown in Table 3 and Figure 1.

Gel Nanoparticle Preparations. Selection of a gel dosage form for NLC containing mulberry root extract so that the NLC is physically stable and increases the convenience of applying NLC containing mulberry root extract. The choice of carbomer 940 as a gelling agent is because the carbomer 940 base gel has a more attractive organoleptic appearance and has good viscosity and dispersibility. Gel is a semi-solid preparation with a high water content, and water is a good growth medium for microorganisms, so in gel preparations, preservatives are needed to maintain the stability of the preparation. The preservatives used are methyl paraben and propyl paraben. The Gel NLC evaluation was shown in Table 4.

Table 3. The results of NLC evaluation.

Evaluation	Results
Particle size	216.2 nm
Polidispersity index	0.488
Potential zeta	-35.6 mV

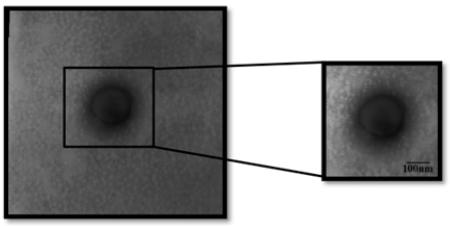

Figure 1. The morphology of NLC using TEM.

Table 4. The results of gel NLC evaluation.

Evaluation	Results
Organoleptic	Semi solid, yellowish white color
Homogeneity	Homogenic
Dispersibility	Qualify
Viscosity	130,532 Ps
Rheology	Thixotropic plastic
pH	5.75

Previous studies have shown that the introduction of a new mulberry extract as a nutraceutical product and the development of a suitable dosage form can increase the quality of the final product[15]. The mulberry leaf extract in a nutraceutical product developed by Surini et al. research has been formulated into hydrogel beads. Hydrogel beads made from mulberry leaf extract have the potential to be used as an intriguing orally administered nutraceutical[15].

Phenotype-Based Evaluation using Zebrafish. Zebrafish is a type of fish that is widely used for modelling in various studies, one of which is used as a model to determine the effectiveness of a substance that has activity as a whitening agent. This is because zebrafish have melanophore genes that are similar to melanocyte genes in mammals. Zebrafish have many different colour pigments. Melanin makes black melanophores, pteridines and carotenoids make yellow or orange xanthophores, and purines make iridophores (silver or gold). During development, most of the zebrafish's coloured tissue comes from its central nerve cells. About 24 hours after fertilisation, melanin is first made in the retinal pigment epithelium. The central nervous system then gives melanophore precursor cells, which can start the process of melanogenesis. As soon as 24 hours after fertilisation, pigment cells start to show up on the top and sides of the head[16].

Based on the results of morphological observations, a decrease in melanin levels was seen in the eye. The eye was chosen because the eye is the location that has the highest amount of melanin. Treated by giving kojic acid, mulberry root extract, and mulberry root extract NLC for two days against the zebrafish, then observed using a microscope. In the positive control with kojic acid, there was a decrease in the amount of melanin that was greatest in the eye area, and in the normal group, there was no decrease in melanin levels, which was indicated by a dark black colour in the eye area. In the mulberry root extract, the melanin level decreased with an increase in the concentration of the mulberry root extract. And in the NLC of mulberry root extract, a decrease in melanin levels was obtained at an increased NLC concentration of mulberry root extract. However, there was an increase in the number of zebrafish that died with an increase in the NLC concentration of mulberry root extract. This is because the NLC given contains tween 80 in increased concentrations[17].

Zebrafish is a type of fish that generally lives in rice field water, where rice field water is water that contains lots of oxygen and has an ecosystem suitable for fish growth[18]. The addition of tween 80, which is a compound used as soap, will cause foam to appear when it is added to the fish growth medium and reduce oxygen levels in the water. This made it difficult for the zebrafish to get oxygen, and they eventually died. According to Ting, Yuwen et al. stated that the effective concentration of Tween 80 for experiments with zebrafish modelling was at a concentration of <300 ppm. The histogram for estimating melanin levels using ImageJ software is shown in Figure 2.

Estimation of Melanin Content. To estimate melanin content, the ImageJ analysis application was carried out by measuring the integrated density in the eye area. The eye was the location where pigmentation was most intense. ImageJ analysis can help estimate melanin levels by measuring the integrated density of zebrafish images obtained from a microscope. The image was then selected to determine which part of the eye has the highest amount of melanin because the ocular epithelium in the eye undergoes a melanogenesis process 24 hours after fertilisation[9,19].

Figure 2 showing the relationship between the sample groups with various concentration series of mulberry root extract and it's NLC on the x-axis with % integrated density on the y-axis, results were obtained that were in accordance with the morphological results observed using a microscope. The other methods to determine melanin content have been developed from previous research on a potent, non-cytotoxic, and anti-melanogenic natural compound. It may be developed as a skin-whitening agent with low side effects for cosmetic uses. The melanin content was measured with B16-F10 melanoma cells[20]. The estimation of ocular melanin content was shown in Figure 3.

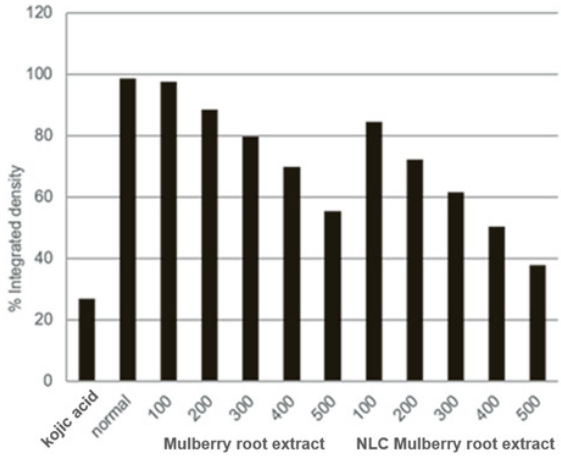

Figure 2. Estimating melanin levels using ImageJ software in each group.

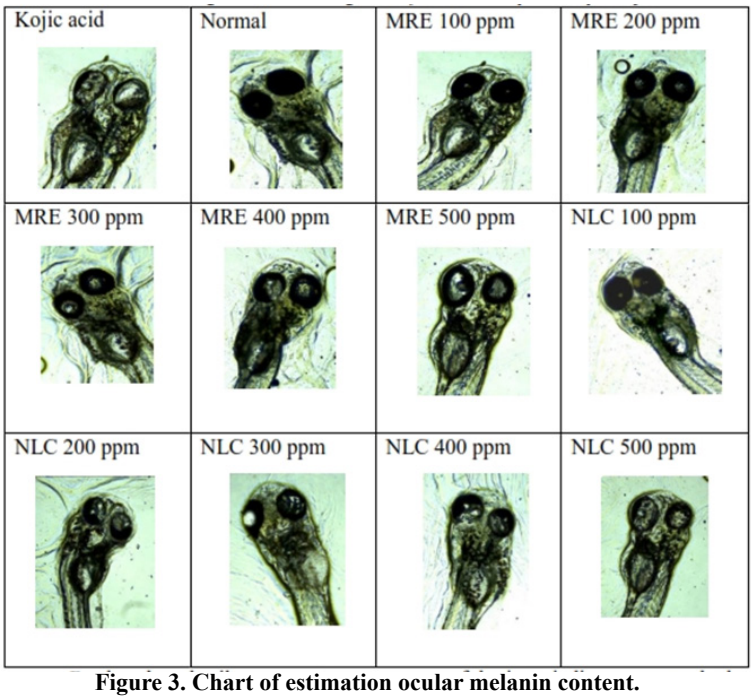

Figure 3. Chart of estimation ocular melanin content.
Note: MRE: Mullbery root extract; NLC: Nanostructured Lipid Carriers

CONCLUSION

Ethanolic extract of mulberry root (*M. alba* L.) and nanostructured lipid carriers containing mulberry root extract with concentration 100-500 ppm has a whitening agent activity on zebrafish modelling.

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