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Research Article

Nanostructured Lipid Carriers with the Eichhornia crassipes Extract as a Receptor, ErB-2 Inhibitor in Breast Cancer: An in Silico Study

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ABSTRACT

In 2020, Indonesia had a total of 65.858 new breast cancer cases and the mortality rate reached 17 per 100,000 population in 2021. Human Epidermal Growth Factor Receptor 2 or Receptor, ErB-2 is a protein that is often used as a target for developing breast cancer drugs. Nowadays, an alternative drug from natural compounds has been developed to overcome the dangerous effect of the chemical drugs, for example by using Eichhornia extract. Due to its low solubility in water and bioavailability, it can be modified by a nanostructured lipid carrier as a sender. Therefore, this study aimed to determine the bioactive compounds in Eichhornia extract that can potentially act as Receptor, ErB-2 inhibitors when encapsulated in nanostructured lipid carriers. However, a nanostructured lipid carrier was constructed by encapsulating Eichhornia extract in vitro. According to the results of molecular docking simulations, luteolin, 2-hydroxy-8-(4 hydroxyphenyl)-phenalene-1-one, and 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one have the highest bond energies and exceed the bond energy of the native ligand. The characterization test showed that the manufacturer's nanostructured lipid carriers had an average size of 471.3 nm, a PDI of 0.507, a pH of 6.20, and a viscosity of 0.896 mPa·S. This nanostructured lipid carrier prototype has been successfully synthesized and nearly met the nanodrug characteristics. This present study could give a new insight in developing drug delivery that alleviate the harmful side effects of cancer chemotherapy.

Keywords: Eichhornia; Nanostructures; Lipids; Carrier; Receptor; ErB-2; Breast cancer; Molecular Docking Simulations

Introduction

Breast cancer is a type of cancer that occurs in breast tissue, caused by continuous abnormal cell division. Breast cancer is one of the most prevalent types of cancer found in Indonesia, with an average of 42.1 per 100,000 population and a mortality rate of 17 per 100,000 population in 2021 [1]. Additionally, according to the Ministry of Health in 2021, morbidity of breast cancer in Indonesia is known to be 42.1 per 100,000 [2].

In most breast cancer cases, a protein called human epidermal growth factor receptor 2 or Receptor, ErB-2 is overexpressed. Receptor, ErB-2 is a protein that is often used as a target for breast cancer drug development. Under normal circumstances, Receptor, ErB-2 plays a major role in the growth and differentiation of breast cells. However, various research shows that the development of breast cancer cells are associated with the gene amplification and overexpression of Receptor, ErB-2 [3]. The Receptor, ErB-2 protein is often used as a positive indicator of breast cancer. Therefore, this protein is often used as a therapeutic target to treat breast cancer. Various research has been conducted to develop a drug that can inhibit the Receptor, ErB-2 protein. One example of a chemical drug that inhibits the Receptor, ErB-2

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protein, is lapatinib [4], [5]. However, chemical drugs can often have side effects, leading to much research examining the bioactive components of plants as potential anticancer agents since they are less toxic than synthetic drugs are [6].

Eichhornia, also known as Eichhornia crassipes, is an aquatic plant that is classified as a water weed. This plant has a very high level of spread, so it is a potential threat to the Indonesian aquatic environment [7]. Eichhornia extract contains various bioactive compounds that can have anti-inflammatory, antimicrobial, and anticancer effects. However, this compound has several shortcomings in drug delivery because of its limited solubility and stability in water and low bioavailability [8].

Nanoparticles (NPs) have potential as drug delivery agents that can increase the efficiency of drug delivery to target cells. NPs can be defined as materials that have a size of 1 to 1000 nanometers. These nanoparticles have shown promising results in drug delivery because they can provide protection to the active ingredient, increase bioavailability, increase intracellular penetration and deliver drugs to targeted tissues or organs through surface modification of the carrier [9].

One of the types of nanoparticles in nanostructured lipid carriers. The nanostructured lipid carrier system is also widely used in the pharmaceutical industry because it can deliver hydrophilic and lipophilic drugs directly to target cells and is able to control drug release [10]. The main components of nanostructured lipid carriers are solid lipids, liquid lipids and surfactants. One type of lipid used in manufacturing nanostructured lipid particles is stearin and olein because they are food grade, are included in the category generally recognized as safe (GRAS), and have good compatibility [11].

The high prevalence of cancer in Indonesia, coupled with the various side effects and side effects of anticancer drugs, highlights the need for further drug development innovation. Therefore, this study aimed to determine the bioactive compounds in Eichhornia extract that can potentially act as Receptor, ErB-2 inhibitors for encapsulation into nanostructured lipid carriers, which are generated from stearic acid and red palm oil. The resulting nanostructured lipid carrier could potentially be used as an alternative breast cancer therapeutic that helps alleviate the harmful side effects of the current synthetic drug.

Materials and Methods

This present study used an experimental method, including molecular docking and laboratory analysis. The docking included screening of secondary metabolite compound in Eichhornia extract and molecular docking of the compounds to the ErB-2 protein. Meanwhile, the laboratory analysis involved preparation of Eichhornia extract, preparation of nanostructured lipid carriers, and characterization (organoleptic, pH, particle size, and viscosity of the NLC).

Screening of Secondary Metabolite Compounds in Eichhornia Extract

Collecting secondary metabolite compounds from the literature

Secondary metabolite compounds were collected based on the literature regarding Eichhornia extract (*Eichhornia crassipes*). These compounds are entered into a table along with CID and SMILES codes.

Testing the drug likeness of secondary metabolites using SwissADME

The SMILES code of each compound was entered into the SwissADME site search field. The “Run” button is pressed to display the test results. The logP value, molecular weight, number of hydrogen bond donors and number of hydrogen bond acceptors are recorded. The number of violations of the Lipinski, Ghose, Veber, Egan, and Muegge rules were recorded. Compounds that violate the Lipinski, Ghose, Veber, Egan, and Muegge rules are eliminated.

Toxicity test of secondary metabolites using Protox-II

The SMILES code was entered on the search field of the “Tox prediction” page. The "estrogen receptor ligand binding domain (ER-LBD)” option was selected for analysis. The "Start-Tox Prediction” button was pressed to display the toxicity test results. Compounds with a toxicity value less than 4 were eliminated.

Molecular Docking of Secondary Metabolite Compounds to the ErB-2 Protein

The Receptor, ErB-2 protein crystal structure was obtained from the Protein Data Bank (PDB) with the code 3PP0, and the secondary metabolite compounds were obtained from PubChem. The native ligand 03Q was extracted from the protein

and docked against the Receptor, ErB-2 protein using AutoDock Vina. The bioactive compounds of the Eichhornia extract were then docked with the Receptor, ErB-2 protein using AutoDock Vina with the same grid box coordinates. The highest binding energy of each bioactive compound is recorded. The ligand conformations with the highest binding energies were visualized using BIOVIA Discovery Studio to construct a 2D diagram of the interacting amino acids. Preparation of nanostructured lipid carriers encapsulating Eichhornia extract extract

Preparation of Nanostructured Lipid Carriers Encapsulating Eichhornia Extract

Eichhornia extraction

Eichhornia leaves were cut into small pieces. The resulting pieces of Eichhornia leaves were ground using a blender until they became powder. The Eichhornia powder was subsequently filtered through a sieve until only the fine powder remained. The powder was dissolved in 96% ethanol and stirred using a shaker. The resulting Eichhornia extract was evaporated using a heater until only the concentrated extract remained. Then, as much as 20 mg of the crude extract was weighed using an analytical balance and dissolved in a mixture of 96% ethanol and distilled water. After that, the mixture was left until the ethanol evaporated.

Preparation of nanostructured lipid carriers

The oil phase was prepared by heating stearic acid and red palm oil at a ratio of 7:3 at 65°C on a hot plate magnetic stirrer using a water bath. Red palm oil was mixed with stearic acid and then stirred at 600 rpm and 65°C for 10 minutes. The liquid phase was made by dissolving Tween 80 in distilled water. The liquid phase was heated above the hot plate magnetic stirrer until the temperature reached 65°C. The liquid phase was slowly added to the oil phase via dropwise addition while stirring using a stirrer at a speed of 600 rpm and a temperature of 65°C. The Eichhornia extract solution was subsequently mixed with the liquid and oil phase mixture while stirring using a stirrer at a speed of 600 rpm and a temperature of 65°C. Ten milliliters of the mixture was removed using a pipette and transferred inside a conical bottom test tube. Then, homogenization was carried out using an Ultra Turrax IKA T10 at a speed of 10 000 rpm for 6 minutes [12]. A transparent yellow liquid was removed from the bottom conical bottom test tube using a pipette, while the foam layer above was discarded. The homogenization and filtration process was repeated until a sufficient number of samples was obtained.

Characterization of the NLC sample

Organoleptic test

The NLC organoleptic test was carried out visually, using the senses of sight and smell, to observe the physical appearance of the sample. Organoleptic examination was carried out by observing the color, odor, consistency and homogeneity of the NLC.

pH test

pH was measured by taking 10 ml of the NLC sample and then using a calibrated pH meter to measure the pH.

Particle size and viscosity tests

Particle size and viscosity measurements were carried out by sending a 10 ml sample to the ILRC Laboratory, University of Indonesia. Particle size and viscosity tests were carried out using a Horiba-Sz 100z Particle Size Analyzer by applying dynamic light scattering (DLS).

Results and Discussion

Secondary metabolite compound screening

Screening or filtration of secondary metabolite compounds was carried out by testing the drug likeness of the compound based on the Lipinski rule of five, Ghose, Veber, Egan, and Muegge rules, as well as testing the toxicity of each compound. Compounds that violated these rules or have a toxicity level less than 4 were eliminated. This process produced 9 compounds, including luteolin, quercetin, chrysoeriol, gossypetin, kaempferol, apigenin, azaleatin, 2-hydroxy-8-(4-hydroxyphenyl)-phenalene-1-one, and 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalene-1-one. These compounds were subsequently docked as ligands against the Receptor, ErB-2 protein (Table 1).

Table 1. Results of Screening for Five Secondary Metabolites

Ligand Name	LogP <5	MW <500	Hd <5	Ha <10
Azaleatin	4	316.26 g/mol	4	4
Quercetin	3	300.26 g/mol	3	3
Chrysoeriol	6	318.24 g/mol	6	6
Gossypetin	4	286.24 g/mol	4	4
Kaempferol	3	330.29 g/mol	3	3
Apigenin	3	270.24 g/mol	3	3
Luteolin	4	286.24 g/mol	4	4
2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one	6	318.24 g/mol	6	6
2-hydroxy-8-(3,4-dihydroxy-phenyl)-phenalen-1-one	4	286.24 g/mol	4	4

Results of Docking Ligands for the ERB-2 Protein

Molecular preparation and molecular docking were carried out using the AutoDock tool. The size of the grid box used for molecular docking was 40 × 40 × 40, with X, Y, and Z coordinates (17.70, 7.46, and 26.66, respectively). Based on the docking results, the 3 compounds that have the highest bond energies are the phenolic compound 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalene-1-one, with a bond energy of -11.4 kJ/mol; the 2-hydroxy-8-(4-hydroxyphenyl)-phenalene-1-one, with a bond energy of -10.9 kJ/mol; and the flavone compound luteolin, with a bond energy of -9.9 kJ/mol. When the ligand has a bond energy that is much greater than the bond energy of the reference ligand 3Q0, the energy is only -6.4 kJ/mol. The stronger the bond between the ligand and the amino acid is, the more negative the bond energy. These 3 compounds exhibited greater efficacy as protein inhibitors than did the other ligands (Table 2).

Table 2. Gibbs Energy Yield of Molecular Docking

Ligand Name	Classification	Bond Energy (kJ/mol)
03Q	Comparative ligand	-6.4
Azaleatin	Flavones	-9.5
Quercetin	Flavones	-9.4
Chrysoeriol	Flavones	-9.5
Gossypetin	Flavonols	-9.2
Kaempferol	Flavonols	-8.8
Apigenin	Flavones	-9.5
Luteolin	Flavones	-9.9

2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one	Phenolic	-10.9
2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one	Phenolic	-11.4

.

The second metabolite compounds, luteolin, 2-hydroxy-8-(4hydroxyphenyl)-phenalene-1-one, and 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalene,

The -1-one has the highest binding energy. This is due to the strong bond between the reference ligand and the protein. The stronger a bond is, the greater the energy released; hence, a more negative binding energy is produced. Therefore, these three ligands have the most negative binding energies, which indicates that they are strongly related to the protein.

In comparison, a study revealed that the ligand ZINC43069427 and ZINC95918662 was found to have a binding energy of -11.0 and -8.50 kcal/mol which was relatively lower than that of 2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one [13]. This means, through the docking test of ligand conducted in this research, *Eichhornia crassipes* contained an active compound which was highly more effective with a binding energy of -11.4 kcal/mol. A greater binding energy indicates a greater efficacy as protein inhibitors.

Visualization of the Molecular Docking Results

Visualization of the docking results was performed using the application Discovery Studio (Figure 1-4). According to the docking results between 2-hydroxy-8-(4-hydroxyphenyl)-phenalene-1-one and the ERB-2 protein, there are 3 hydrogen bonds, namely, with the amino acids Arg849, Leu796, and Gly729. Moreover, the ligand 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalene-1-one also has 3 hydrogen bonds with Thr862, Asp863, and Leu726. Luteolin has 4 hydrogen bonds with the amino acids Arg849, Asn850, and Thr862. The comparison ligand 3Q0 has the most hydrogen bonds, namely, 6 bonds with the amino acids Gln799, Met801, Thr862, Asp863, Asn850, and Gly729 (Table 3).

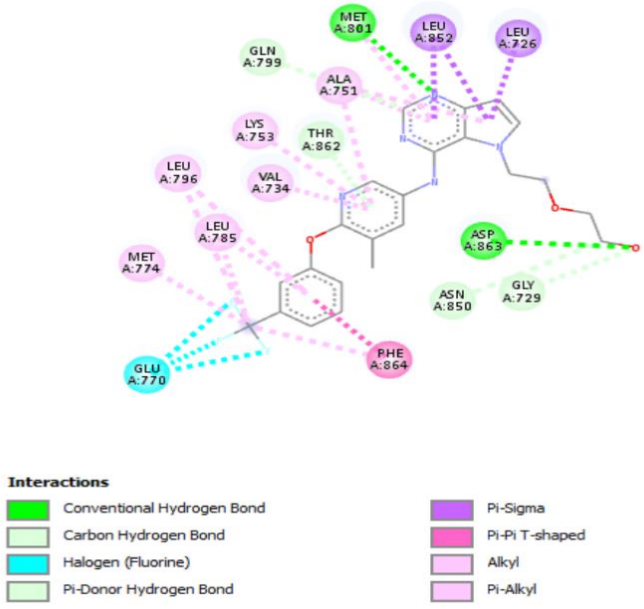

Figure 1. Molecular Docking Simulations Visualization of 3Q0 (Native Ligand)

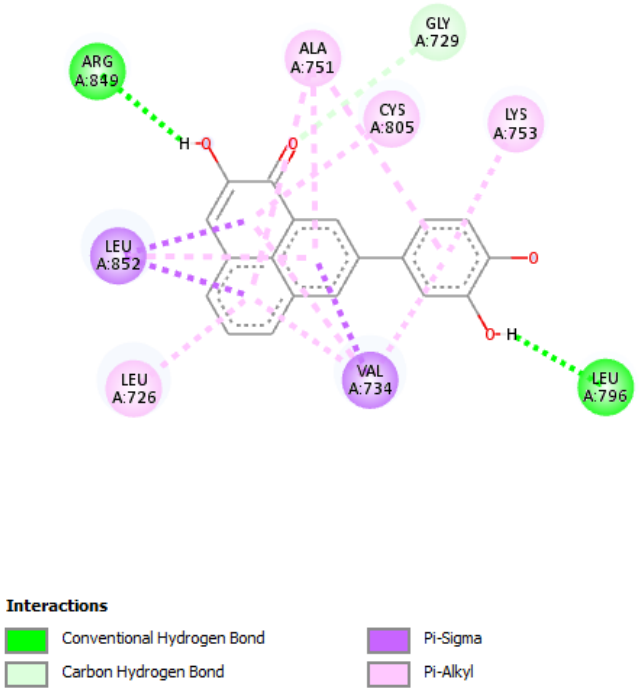

Figure 2. Molecular Docking Simulations Visualization of 2-Hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one

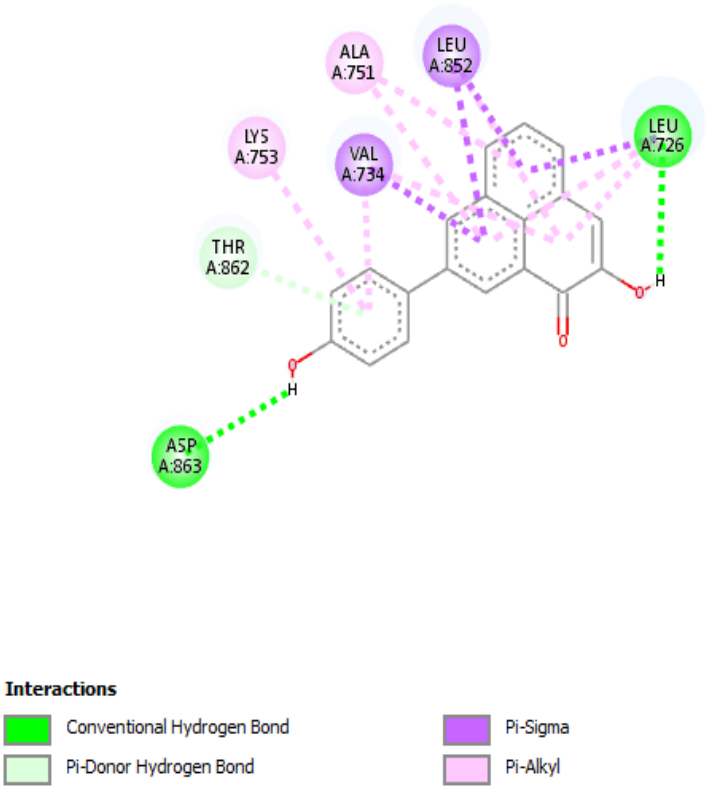

Figure 3. *Molecular Docking Simulations Visualization of 2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one*

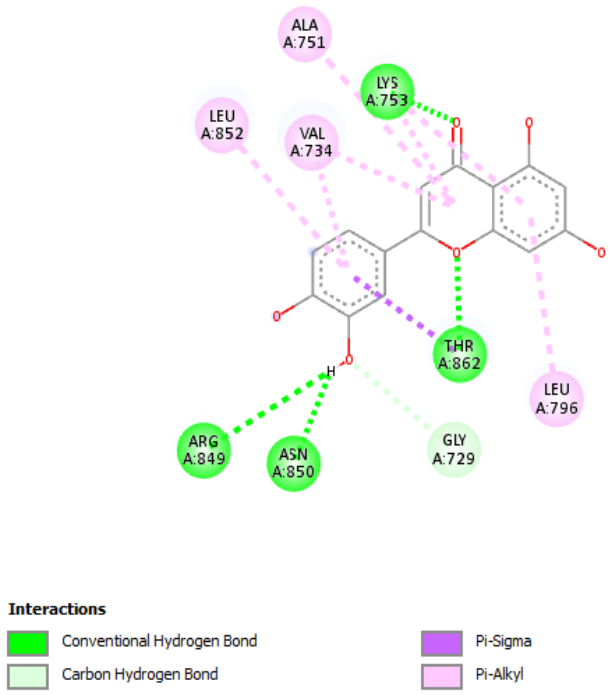

Figure 4. Molecular Docking Simulations Visualization Of Luteolin

Table 3. Comparison of Amino Acid Bonds Between 3 Ligands With The Highest Bond Energy

Amino acids	2-Hydroxy-8-(4 hydroxyphenyl)-phenalen-1-one	2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one	Luteolin
Cys805	V	-	-
Lys753	V	V	V
Val734	V	V	V
Leu796	V	-	V
Leu726	V	V	-
Leu852	V	V	V
Arg849	V	-	V
Ala751	V	V	V
Gly729	V	-	V
Thr862	-	V	V
Asn850	-	-	V
Asp863	-	V	-

Based on the comparison of amino acid bonds between 2-hydroxy-8-(4-hydroxyphenyl)-phenalene-1-one, 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalene-1-one, and luteolin, there are 4 identical amino acid bonds between the three ligands, namely, Lys753, Val734, Leu852, and Ala751. To inhibit the ERB-2 protein, the ligand must bind to the amino acids Lys724, Lys736, and Cys805 [14]. Based on these results, only the compound 2-hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one forms one pi-alkyl bond with the amino acid Cys805. The amino acid Lys753 is also a crucial

amino acid in the ATP binding site. The reference ligands 03Q, 2-hydroxy-8-(4-hydroxyphenyl)-phenalen-1-one, and 2-hydroxy-8-(3,4-dihydroxy-phenyl)-phenalene

The -1-one residue forms a pi-alkyl bond with the amino acid Lys753. However, luteolin also forms a conventional hydrogen bond with the amino acid Lys753. Binding to the ATP binding site results in the inhibition of ERB-2 (Table 4).

Table 4. Comparison of the Types of Bonds Formed With the Interacting Amino Acids

Ligand	Interacting Amino Acid			
	Conventional hydrogen bond	**Carbon hydrogen bond**	**Pi-Alkyl**	**Pi-Sigma**
Reference ligand	Met801, Asp863	Gln799, Thr862	Val734, Lys753, Ala751, Leu796, Leu785, Met744	Leu852, Leu726
2-Hydroxy-8-(4-hydroxyphenyl)-phenalene-1-one	Arg849, Leu796	Gly729	Ala751, Cys805, Lys753	Leu852, Val734
2-Hydroxy-8-(3,4-dihydroxyphenyl)-phenalene-1-one	Asp863, Leu726	Thr862	Lys753, Ala751	Leu852, Val734
Luteolin	Lys753, Thr862, Arg849, Asn850	Gly729	Leu796, Ala751, Val734, Leu852	Thr862

Organoleptic test results

The organoleptic test results showed that the nanostructured lipid carrier, which was made from stearin and olein at ratios of 7:3 and 2.5% (w/w) Tween 80 surfactant, had a liquid and homogeneous consistency. The NLC results did not show any signs of separation or agglutination, thus indicating that the NLC was homogeneous. The NLC sample had a yellow and slightly cloudy-like color and was a colloidal liquid (Figure 5). This yellow color was obtained from a mixture of red palm oil, which was red–orange, and stearic acid, which was white. The color of the NLC products was yellow when Eichhornia extract was added. The absence of the dark green color shows that the Eichhornia extract was encapsulated in the NLC. The NLC results also revealed a distinctive aroma due to the use of the raw materials, namely, stearic acid and red palm oil (RPO).

Figure 5. *Nanostructured Lipid Carrier Sample After Processed in Ultra-turrax High Shear Homogenizer (Ultra-Turrax IKA T10)*

pH test results

The results of the pH test using a pH meter showed that the NLC had a pH of 6.20 ± 0.05. The pH value of nanostructured lipid carrier that is considered as safe for the human body was in the range of 5-7. Thus, the NLC could be classified as a safe NLC (Figure 6) considering its neutral pH [15].

Figure 6*. Testing the pH of NLCs using a pH meter*

Viscosity Test Results

A viscosity test was carried out to test the viscosity of the formulation nanostructured lipid carrier. The viscosity test results show that the average viscosity of the NLC after 3 repetitions is 0.896 mPa·S. A moderate viscosity is required to allow for easy dispersion and administration while also providing good stability [16].

Particle Size Analysis Test Results

Z-average is the average particle diameter calculated through dynamic light scattering (DLS). From the results of particle size testing, the average Z-average obtained was 471.3 nm. This shows that the sample can be said to be a nanoparticle due to its nanosized (<1000 nm). However, nanoparticles must be smaller than 150 nm to extravasate from the circulation into tumor cells. The blood vessel system in tumors is different from that in ordinary tissue because, in general, the blood vessels in tumor tissue are larger, denser, more permeable and easily leaked. As a result, drugs with high molecular weights and sizes can accumulate in tumor tissue [9]. This shows that the sample particle size is still too large for the material to be used as a drug delivery agent to tumor tissue. This Z-average value, which is still relatively high, could be due to a lack of homogenization and can be solved by increasing the homogenization duration (Table 5).

Another research was conducted by an NLC formulation with a solid lipid to liquid lipid ratio of 6:4 and using a sonicator with an amplitude of 20%-40% for 1.5 to 4.5 minutes. The result shows the formation of a NLC with a particle size that is less than 150 nm. This shows that the solid to liquid lipid ratio as well as the type of machine used significantly affects the particle size of the nanostructured lipid carrier [17].

Table 5. Z-average Value and PDI from the Results of Particle Size Analysis

Repetition	*Z – Average*	*Polydispersity Index* (PDI)
1	254.2 nm	0.679
2	667.1 nm	0.381
3	492.5 nm	0.460
Rate - rate	**471.3 nm**	**0.507**

The polydispersity index (PDI) reflects the level of heterogeneity of a sample based on its size and represents the size distribution of nanoparticles in the sample. The average polydispersity index (PDI) was 0.507. A PDI value less than 0.7 indicates that the sample is well dispersed and is categorized as monodisperse. However, in drug delivery systems using lipid-based nanoparticles, the PDI value must be less than or equal to 0.3. The PDI value of the sample still exceeded the limit, so it was not suitable for use as a drug-based delivery nanolipid system. A high PDI can be caused by uneven homogenization. The homogenization process was carried out using a conical bottom test tube, so some parts were not well dispersed [18].

This research involved a new innovation of NLC through the use of *Eichhornia crassipes.* In the future, obtaining a smaller value of PDI of 0.09 ± 0.10 for even distribution of particles is required [19]. This range of value will ensure drug delivery in the near future. In order to obtain this value, further research is required including finding the optimum solid:liquid lipid ratio and maximum homogenization *Ultra-turrax High Shear Homogenizer.* In addition, coating of NLC shows an increase in encapsulation stability and entrapment efficiency [20]. To ensure encapsulation, an Encapsulation Efiency (EE) test is required [21].

However, there are several limitations involved in this NLC prototype. Due to ethical and resource limitations, the NLC prototype was not able to be tested in vivo. Therefore, the efficiency and effect of the NLC prototype cannot be tested on a real breast cancer cell. Instead, the characteristics of the NLC were only compared to previous research. Additionally, due to time constraints and apparatus limitations, the Eichhornia extract obtained was not pure and still contained other components such as ethanol which might have affected the NLC characteristics.

Conclusion

The compounds Ne-1-one and 2-hydroxy-8-(3,4-dihydroxyphenyl)-phenalen-1-one from Eichhornia extract have great potential as medications for inhibiting the Receptor, ErB-2 protein. These three ligands strongly bind to proteins that exceed the native ligands with binding energies of -9.9 kJ/mol, -10.9 kJ/mol, and -11.4 kJ/mol, respectively. Docking visualization revealed that these compounds can form bonds with the crucial amino acid Lys753, which inhibits the ATP binding site of the Receptor, ErB-2 protein. In addition, 2-hydroxy-8-(4 hydroxyphenyl)-phenalene-1-one also forms a bond with the amino acid Cys805, which is a key amino acid that inhibits the Receptor, ErB-2 protein. Furthermore, these compounds can be generated using nanostructured lipid carriers made from stearic acid and olein with a ratio of 7:3 and 2.5% (w/w) tween 80 as the surfactant. The nanostructured lipid carrier made using this formulation was shown to have a liquid and homogeneous consistency, with no signs of separation or agglutination. The pH test showed a pH of 6.20, which lies within the safe range for the human body. The viscosity test showed that the average viscosity was 0.896 mPa·S, which indicates adequate dispersion. Even though the sample is classified as a nanosized particle, the Z-average and PDI values are still too large for use as drug delivery agents. More attention and precision are needed to ensure that the sample is homogenized more evenly.

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