Original Article

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In Vitro Analysis of Anti-Inflammatory Activity of Lyotropic Liquid Crystal Nanoparticles from *Echinacea purpurea* Flower Extract

Priscilla Sherly Ayu Santianingtyas, Muhammad Dzakwan*, Fransiska Leviana

Department of S1 Pharmacy, Faculty of Pharmacy, University Setia Budi

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*Corresponding Author:
Muhammad Dzakwan
Email:
mdzakwan9@gmail.com

ABSTRACT

Inflammation serves as the body's defense mechanism in response to tissue damage caused by various factors. Echinacea contains flavonoids such as quercetin, kaempferol, and diosmetin, which exhibit significant anti-inflammatory properties by inhibiting inflammatory enzymes and reducing the production of proinflammatory mediators. This study aims to evaluate the anti-inflammatory effects of echinacea flower extract formulated as Lyotropic Liquid Crystal Nanoparticles (LLCN) and compare its efficacy with viscous echinacea flower extract and diclofenac sodium. Echinacea flower extract was produced using 70% ethanol solvent via ultrasonication. LLCNs were synthesized using a top-down approach and characterized based on particle size, zeta potential, stability, and absorption efficiency. Anti-inflammatory activity was assessed in vitro using 0.2% Bovine Serum Albumin (BSA) solution in Tris Buffer Saline (TBS), with diclofenac sodium as a positive control. The results showed that echinacea flower extract can be converted into LLCN with particle sizes ranging from 10 to 1000 nm. The LLCN formulation of echinacea flower extract showed stronger anti-inflammatory activity compared to the condensed extract, with activity recorded at 54,660 ppm, IC50 for LLCN at 21,823 ppm, and IC50 for diclofenac sodium at 19,984 ppm. Thus, echinacea flower extract LLCN has potential as a natural anti-inflammatory agent, which shows higher efficacy compared to its condensed extract form. This indicates that LLCN technology not only improves the bioavailability, but also the effectiveness of the extract in inhibiting inflammation, thus LLCN has the potential to be applied more widely in the development of natural drugs for anti-inflammatory therapy.

Keywords : *Echinacea purpurea*, Lyotropic Liquid Crystal Nanoparticles, Anti-inflammation, Ultrasonication, Nanotechnology

INTRODUCTION

Inflammation is the body's natural response as a protective mechanism that occurs as a localized response to damage to body cells and tissues. It is caused by various factors such as infections, chemicals, extreme temperatures and physical stress. Symptoms of inflammation in the tissue include a sensation of heat, discoloration to redness, swelling, and pain, which can result in structural changes in the tissue and disrupt its normal function (T. Mulyani, 2023).

Inflammation can be managed with non-steroidal anti-inflammatory drugs (NSAIDs) like diclofenac sodium. Nonetheless, prolonged use of these medications can lead to side effects including nausea, vomiting, gastritis, skin redness, and headaches (Domini et al., 2019). To overcome the side effects that may arise from the use of NSAID drugs,

there are alternative options that can be considered, such as the use of traditional medicines made from natural ingredients that are proven to help reduce inflammation (Mulyani et al., 2017).

Indonesia boasts a rich biodiversity, encompassing numerous plants with medicinal properties. One such plant recognized for its anti-inflammatory effects is *Echinacea purpurea* L., commonly known as echinacea. This purple coneflower, indigenous to North America and part of the Asteraceae family, has gained widespread popularity as an herbal remedy in many countries. It is primarily used for its immunomodulatory, anti-inflammatory, antioxidant, and antiviral benefits, particularly for the prevention and treatment of upper respiratory tract infections (Kumar and Ramaiah, 2011). Echinacea contains various chemical compounds such as flavonoids, alkamides, polysaccharides, lipoproteins, betaine, polyacetylene, saponins, as well as a number of phenolic compounds such as echinacoside, caffeic acid derivatives, and chicoric acid (Coelho et al., 2020).

In vivo studies of polysaccharide extracts from echinase showed decreased levels of several cytokines in mice infected with influenza A WSN virus and affected the course of influenza infection in mice by altering cytokine responses (Fusco et al., 2010). Clinical trials of echinacea extract-based creams and gels showed significant improvement in corneometer hydration index as well as decreased wrinkles after one month of use (Yotsawimonwat et al., 2010). Echinacea extract tested for antioxidants in vitro had the most significant anti-collagenase results with a value of 78.5 ± 0.0% (Chaiyana et al., 2021). Research on standardized hydroalcoholic extracts of the aerial parts of ekinase (SEP) showed that ekinase is a functional immunomodulator with antioxidant effects in vitro and in vivo capable of increasing phagocytosis activity, cytokine release, immune organ index, lymphocyte proliferation, and natural killer cell activity and phagocytosis index at certain doses (Sudeep et al., 2023).

A study aimed at evaluating the in vivo anti-inflammatory activity in carrageenan-induced paw edema in rats by oral administration revealed that a 100 mg/kg dose of echinacea extract could reduce COX-2 expression (Raso et al., 2010). Another study on the in vitro anti-inflammatory activity of echinacea alcohol extract found that it significantly inhibited NO production by lipopolysaccharide (LPS) at lower concentrations of 5-25 μg/ml, but not at higher concentrations (50-200 μg/ml) (Z. Zhai et al., 2009). In addition, echinacea extract (chicoric acid - CA) cultured with peripheral blood mononuclear cells (PBMC) showed the ability to inhibit LPS-induced inflammatory responses in an in vitro assay (Xue et al., 2021).

The application of nanotechnology has made significant contributions in various scientific disciplines and technological fields. A major focus in the development of nanotechnology is the fabrication of nanoparticles for diverse applications, including drug delivery. Lyotropic liquid crystals (LLCs) are important examples of nanoparticle systems with distinctive and attractive features, which serve as drug delivery systems composed of amphiphilic molecules (lipids and polar surfactants) and solvents. Liquid crystals are divided into two main categories: thermotropic and lyotropic. Lyotropic liquid crystals are mainly used in the development of drug delivery systems due to their ability to display different levels of liquid crystallinity with changes in concentration (Chavda et al., 2022).

LLCNs are composed of molecules that have amphiphilic properties and are formed through interaction with solvents, which generally occur in cubic, hexagonal, and lamellar mesophase forms (Mo et al., 2017). LLCN has advantages, namely that it can increase the bioavailability, stability, and durability of plant bioactive compounds (Nieri et al., 2022). In addition, LLCNs enable "on-demand" controlled release of drugs through manipulation of their internal phases, which can be adapted to different administration routes, thereby improving therapeutic effectiveness. With the ability to integrate responses to stimuli, LLCNs can potentially be used in the efficient delivery of hydrophobic and hydrophilic molecules and prolong therapeutic effects, making them a superior delivery system in various medical applications (Leu et al., 2023).

Based on the description above, researchers are interested in developing further research related to the anti-inflammatory activity of echinacea flower extract tested in the LLCN system. This study aims to characterize LLCN through the measurement of particle size, zeta potential, and sorption efficiency, as well as testing anti-inflammatory activity in vitro using the protein denaturation method.

RESEARCH METHOD

Tools

The tools used in this research are analytical scales (Fujitsu), volume pipettes (Pyrex), measuring cups (Pyrex), oven (Memmert), incubator (Memmert), glass funnel (Pyrex), beaker glass (Pyrex), micropipette (Soccorex) to take samples and materials with microliter scale needs, test tubes (Pyrex), drop pipettes (Pyrex), centrifuge (Hettich) to

separate solid and liquid mixture, Erlenmeyer tube (Pyrex) to mix the components of the solution, thermometer (GEA), magnetic stirrer (Texcare), stirring rod (Pyrex), blender (Cosmos), flannel cloth, spectrophotometer (Indotech), water bath (WNB), pH meter (Eutech), and Eppendorf tube (Onemed).

Materials

The materials used in this study consisted of echinacea flowers, 70% ethanol, Bovine Serum Albumin (BSA) (Sigma Aldrich), diclofenac sodium, Phytantriol, Poloxamer 407, distilled water, glacial acetic acid, Tris Base, NaCl, quercetin, gallic acid, and Folin-Ciocalteu reagent.

Determination and Procurement Of Simplisia

The first step in conducting this study was the procurement of simplisia, which was obtained from UPF Pelayanan Kesehatan Tradisional RSUP Dr. Sardjito Tawangmangu. Simplisia in the form of powder with a degree of fineness of mesh number 60. Determination of plants is carried out in the UPF Pelayanan Kesehatan Tradisional RSUP Dr. Sardjito Tawangmangu.

Identification of Simplisia

Identification of powdered simplisia is carried out by conducting microscopic testing. Microscopic identification of powdered simplisia by comparing according to the American Herbal Pharmacopoei (AHP) monograph.

Drying Shrinkage of Powder

Determination of powder drying shrinkage was carried out using a moisture balance tool. Drying shrinkage is done by weighing 2 grams on a plate that has been marked with a temperature of 105°C. Then flattened and waited until the tool sounded (Putri, 2023). % Drying shrinkage :

$$\frac{(initial\ weight - final\ weight)}{initial\ weight} \text{ x } 100\%$$

Extract Preparation

Extract preparation was carried out by ultrasonication method with 70% ethanol solvent. The echinase powder was extracted using 70% ethanol with a ratio of 1:10 (b/v). The weighed echinacea powder was put into a glass bottle, then added with 70% ethanol and homogenized. The extraction process was carried out using ultrasonic assistance using a probe sonicator with a fixed frequency at 35 kHz. The extraction parameters were set with an ultrasonic time of 20 minutes, ultrasonic power of 300W, and ultrasonic temperature maintained at 75°C (Petkova et al., 2017).

After the extraction process, the filtrate was filtered and concentrated using a rotary vacuum evaporator until a thick extract was obtained. Then, the yield of equine extract was calculated. The yield of echinacea extract was calculated using the formula:

$$\text{Yield} = \frac{weight\ of\ extract}{sample\ weight} \text{x } 100\%$$

Determination of extract water content

Determination of extract water content using gravimetric method and modified from Kemenkes, (2017). About 5 grams of extract is put into a container that has been carefully weighed. The weighing process was carried out after drying at 105°C for 5 hours. Drying continued and repeated weighing was carried out every 1 hour until the difference between two consecutive weighings did not exceed 0.25% or 0.0005 grams.

Phytochemical screening and KLT

Phytochemical screening of the extracts was modified from Depkes, (1987 and 1989).

1. Flavonoid Test

Performed by using 2 tests, namely qualitative tests and chromatogram patterns (KLT). The qualitative test was carried out by dissolving 10 mg of extract in 1 ml to 2 ml of methanol (50%) P with the help of heating. To the solution was added magnesium metal P and 4 drops to 5 drops of concentrated hydrochloric acid P. The presence of flavonoid aglycone was indicated by the occurrence of red or orange color (Cyanidin Reaction or Shibata Reaction).

In testing the chromatogram pattern using the Thin Layer Chromatography Technique (KLT) testing method. The first step is to dissolve the ethanol extract of echinacea with distilled water, which is then referred to as the sample. Eluent or mobile phase that has been specifically determined is prepared and filled into the chamber. Next, the extract was photographed on a KLT plate that had been inserted into a chamber containing TAA mobile phase (Toluen: Acetone: Formic acid = 6: 6: 1).

As a comparison, standard quercetin was bottled as standard (Sari et al., 2022). After that, cyroborate was sprayed as a specific reagent to detect flavonoids (Arsul et al., 2022).

2. Alkaloid Test

A 10 mg sample of the extract was dissolved in 1.5 ml of 2% hydrochloric acid. This solution was then divided into three equal portions in separate test tubes. The first test tube served as a control. In the second test tube, 2 to 3 drops of Dragendorff's reagent

were added. The third test tube received 2 to 3 drops of Mayer's reagent or another precipitating agent. The presence of alkaloids in the sample is indicated by turbidity or a brownish-orange precipitate with Dragendorff's reagent and a yellowish-white precipitate with Mayer's reagent.

3. Steroid and Triterpenoid Tests

A 10 mg extract was dissolved in 0.5 ml of acetic anhydride, followed by 0.5 ml of chloroform. This mixture was transferred to a dry test tube. Subsequently, 1 to 2 ml of sulfuric acid was added dropwise along the tube wall using a pipette (Liebermann-Burchard reaction). A color transition from red to green or purple between the two solution layers indicates the presence of steroids or triterpenoids.

4. Tannin Test

A 10 mg extract was diluted with 2 ml of water, and then 3 drops of iron (III) chloride solution were added. A color change to blue-black suggests the presence of gallotannins in the extract, while a shift to blackish-green indicates catechol tannins.

5. Saponin Test

A 10 mg extract was placed in a test tube with 10 ml of hot water. After cooling, the mixture was vigorously shaken for 10 seconds. The presence of saponins is confirmed if a stable froth forms, lasting at least 10 minutes with a height between 1 cm and 10 cm. The froth should not dissipate upon the addition of 1 drop of 2N hydrochloric acid.

Determination of total flavonoid content

1. Test Solution Of Echinacea Extract

About 0.2 grams of echinacea extract was weighed precisely and put into an Erlenmeyer flask. Then, 25 mL of ethanol P was added and stirred for 30 minutes with a magnetic stirrer. The solution was then filtered into a 25 mL volumetric flask, and ethanol P was added through the filter until it reached the specified measurement mark (Dellima et al., 2023).

2. Comparison Solution

Approximately 50 mg of quercetin was weighed carefully and put into a 50 mL volumetric flask. After that, dissolve the quercetin and add ethanol P until it reaches the predetermined mark. Furthermore, a series of dilutions of the comparison solution with levels of 20, 30, 40, 50, and 60 ppm were made (Dellima et al., 2023).

3. Procedure for Determining Total Flavonoid Content.

A total of 1 mL of test solution and comparison solution series with the same volume were put into separate containers. Then, 1 mL of 2% $AlCl_3$, 1 mL of 0.1 M sodium acetate and 2.0 mL distilled water were added to each container. The solution mixture was shaken and allowed to stand for 30 minutes at room temperature. Absorption measurements were taken at the wavelength of maximum absorption. For blank measurements, carried out without the addition of active substances. The next step is to make a standard curve and calculate the content of the test solution (Dellima et al., 2023).

Determination of total phenol content

1. Test solution of Echinacea Extract

Approximately 0.1g of equine extract was carefully weighed and put into an erlenmeyer flask. Then, 25 mL of methanol P was added and stirred for 30 min using a magnetic stirrer. After that, the solution was filtered into a 25 mL measuring flask, and continued with the addition of methanol P through the filter until it reached the specified mark (Khadijah et al., 2021).

2. Comparison Solution

Approximately 10 mg of gallic acid is weighed carefully and put into a 25 mL measuring flask. Then, dissolve gallic acid using methanol P and supplemented with methanol P until it reaches the specified mark. Next, make a series of dilutions of the comparison solution with levels of 30, 50, 70, 90, 110 ppm respectively (Khadijah et al., 2021).

3. Procedure for Determination Of Total Phenol Content

In a suitable container, 1 mL of test solution and each series of comparison solution were added, followed by 5 mL of distilled water and 1 mL of Folin-Ciocalteu reagent diluted 1:10, then shaken. The mixture was allowed to stand for 8 minutes, then 3 mL of 10% Na_2CO_3 was added. The solution was then allowed to stand for 1 hour at room temperature. The absorbance was measured with a UV-Vis spectrophotometer at the maximum wavelength. For blank measurements, no active substance was added. The next step is to make a standard curve and calculate the concentration of the test solution (Khadijah et al., 2021).

LLCN Formula

Formula modified from Spicer et al., (2005).

Table 1. LLCN Formula Of Equine Extract

No.	Material	Weight (g)
1	Echinacea extract	0,02
2	Phytantriol	10
3	Etanol	5
4	Water	1,8
5	Poloxamer 407	1
6	Water	82,18
7	Total	100

Preparation of LLCN

The liquid crystal nanoparticle system (LLCN) is developed using the top-down approach. Phytantriol is heated to 60°C while being stirred with a magnetic stirrer. Phytantriol, ethanol, and water are added to the echinacea extract and stirred at 150 rpm using a stirring rod to create the oil phase. For the water phase, poloxamer 407 is accurately weighed and dissolved in water by stirring with a stirring rod. The water phase is subsequently combined with the oil phase and homogenized using a sonicator for 5 minutes (Ramadhan et al., 2022).

LLCN Characterization

1. Particle Size Test

Particle size was measured using a Particle Size Analyzer (PSA) with the Dynamic Light Scattering (DLS) method. A sample of 1 mL was put into the cuvette, which was then put into the holder, and the analysis was performed with the appropriate instrument (Zulfa et al., 2019).

2. Zeta Potential Test

Droplet zeta potential was measured using a Zetasizer. The test was carried out by diluting the preparation in sufficient water. After that, the sample is put into a cuvette and measurements are taken with a Zetasizer (Zulfa et al., 2019).

3. Entrapment Efficiency

Determination of the entrapment efficiency was carried out using UV-Vis spectrophotometry, where the LLCN solution of ekinase extract with a dilution of 1:1000 was measured for absorbance in the wavelength range between 200 to 500 nm. After that, the suspension was centrifuged at 20,000 rpm for 20 minutes.

The supernatant was taken as much as 0.1 mL, then the absorption at the maximum wavelength was measured. The percentage value of LLCN absorbed equine extract can be calculated:

$$\% \text{ EE} = \frac{(QT-QS)}{QT} \text{ x } 100\%$$

EE is the Entrapment Efficiency, Qt is the amount of echinacea extract LLCN added and Qs is the amount of echinacea extract LLCN detected in the supernatant (not adsorbed) (Akib et al., 2022).

4. Stability

Physical stability is the length of time for a drug to maintain the physical properties of a product at some time of storage period. Samples were stored for 30 days at controlled room temperature (30°C) and cold temperature (4°C) then particle size and zeta potential were measured. The final results can be evaluated by comparing the particle size before and after testing (Sun et al., 2020).

Anti-inflammatory activity testing of echinacea extract and LLCN of echinacea extract in vitro

1. Preparation of Tris Buffer Saline (TBS) stock solution

A total of 4.35 g NaCl was dissolved in 200 ml distilled water. Then, 605 mg Tris Base was added and the solution was diluted with water to a volume of 400 ml. The pH of the solution was adjusted by adding glacial acetic acid to reach pH 6.3, and then further diluted with water to a final volume of 500 ml (Farida et al., 2018).

2. Preparation of 0.2% BSA Solution in TBS

A total of 0.2 g of Bovine Serum Albumin (BSA) was put into a 100 mL volumetric flask. Then, the flask was filled with TBS solution until it reached a volume of 100 mL (Farida et al., 2018).

3. Preparation of Lowry Reagent

To prepare Lowry reagents using Lowry A and Lowry B, begin by making Lowry A by dissolving 2 grams of sodium carbonate in 100 ml of 0.1 N sodium hydroxide. For Lowry B, dissolve 5 ml of a 1% CuSO4·H2O solution and mix it with 5 ml of a 1% sodium (or potassium) tartrate solution. Lowry C is prepared by adding 2 ml of Lowry B reagent to 100 ml of Lowry A reagent and mixing until homogeneous. Lowry D reagent is prepared by

diluting Folin-Ciocalteau reagent with distilled water in a 1:1 ratio (Nadea et al., 2023).

4. Wavelength Determination

The maximum wavelength of the standard was determined through scanning in the wavelength range of 200-700 nm using 0.2% BSA, Lowry reagent, and distilled water as a blank (Apriliyani et al., 2018).

5. Determination of Operating Time

The operating time of the standard solution was determined by taking measurements at a wavelength of 657 nm during the time range of 0 to 45 minutes, with a time interval of every 1 minute (Suharyanto, 2021).

6.Preparation of Diclofenac Sodium Solution As Positive Control

Dissolve 25.0 mg of diclofenac sodium in a 25 mL volumetric flask with distilled water, and make up to the 25 mL mark to obtain a stock solution with a concentration of 1000 ppm. Subsequent dilutions are then prepared to concentrations of 15, 20, 25, 30, 35, and 40 ppm (Farida et al., 2018).

7. Preparation of Echinacea Extract Stock Solution

Dissolve 1 gram of echinacea extract in a 100 mL volumetric flask with distilled water, and make up to the 100 mL mark to obtain a stock solution with a concentration of 10,000 ppm. Further dilutions are then prepared to concentrations of 10, 20, 30, 40, 50, and 60 ppm (Farida et al., 2018).

Preparation of Extract Stock Solution In The Form of LLCN

Dissolve 1 gram of LLCN in a 100 mL volumetric flask with distilled water, and make up to the 100 mL mark to obtain a stock solution with a concentration of 10,000 ppm. Subsequent dilutions are then prepared to concentrations of 10, 20, 30, 40, 50, and 60 ppm.

Preparation of Negative Control Stock Solution

A 0.2% BSA solution in TBS was added with 1 mL of distilled water into a volumetric flask until it reached a volume of 10 mL (Farida et al., 2018).

Preparation of Blank Control Solution

The blank control solution is distilled water (Farida et al., 2018).

Anti-Inflammatory Activity Testing

1 ml of each solution was taken, then 5 ml of Lowry reagent C was added and shaken until homogeneous. Then, 0.5 ml of Lowry reagent D was added, followed by the addition of 0.2% BSA in TBS to reach the final volume. The solution was incubated for 27 minutes at an ambient temperature of ±25°C. After incubation, the solution was heated in a water bath at approximately ±72°C for 5 minutes. Next, the solution was cooled for 25 minutes at room temperature, and the absorbance was measured using a UV-Vis spectrophotometer at a wavelength of 657 nm (Farida et al., 2018).

Statistical Analysis

Data obtained from the anti-inflammatory activity testing of echinacea flower extract and LLCN were analyzed using the One-Way ANOVA statistical test with SPSS version 23.0 software. This test was chosen to evaluate significant differences between various samples, including the positive control (diclofenac sodium), echinacea condensed extract, and echinacea extract LLCN.

The ANOVA test was used because it is able to compare the means of more than two groups of data to determine whether there are statistically significant differences.

RESULTS AND DISCUSSION

Determination and Procurement of Simplisia

The Echinacea simplisia used in this study was sourced from the Functional Service Unit (UPF) for Traditional Health Services at Dr. Sardjito Hospital, Tawangmangu. Determination was performed by examining the plant's morphology and comparing it to existing literature. Based on the determination results, numbered TL.02.04/D.XI.5/16536.445/2023 from UPF Pelayanan Kesehatan Tradisional RSUP Dr. Sardjito Tawangmangu, it was confirmed that the simplisia used in this research is Echinacea flower (*Echinacea purpurea* L.).

The determination results indicated that the sample used is Echinacea, specifically the flower simplisia, belonging to the Asteraceae family, species *Echinacea purpurea* (L.) Moench, with the synonym *Brauneria purpurea* (L.) Britton. These results confirm the authenticity and accuracy of the simplisia used as the raw material in the study, ensuring that the research is conducted with appropriate and standard-compliant materials.

Identification of Simplisia

The Echinacea simplisia obtained from the UPF Traditional Health Services at Dr. Sardjito Hospital, Tawangmangu, in powder form, underwent microscopic identification. Using a microscope, the powder was examined and identified. The observations revealed that the lower epidermis of the leaves contains cells with wavy anticlinal walls and anomocytic stomata.

Additionally, the pollen grains exhibited a tricolporate structure with spiny exine. In the epidermis of the ray florets, the cells also had wavy

anticlinal walls, anomocytic stomata, and bright areas indicating the presence of secretory channels underneath. These observations confirmed that the analyzed powder is Echinacea powder, aligning with the references from the American Herbal Pharmacopoeia Botanical Pharmacognosy Microscopic Characterization of Botanical Medicines (2011).

Loss on Drying of Powder

Loss on drying is a non-specific parameter aimed at determining the amount of compounds lost during the drying process. The measurement involves drying the sample at 105°C until a constant weight is achieved, with the results expressed as a percentage (Kemenkes, 2017). The use of a temperature of 105°C in the drying process is considered effective because at this temperature, the bound water in the sample can evaporate without causing thermal decomposition or chemical changes to the essential components of the sample.

This temperature is sufficiently high to evaporate water and other compounds with a lower boiling point than water, ensuring that only water is lost during the drying process, not other compounds that may have a higher boiling point (Andrian et al., 2018). The determination of the loss on drying for the Echinacea powder was conducted using a moisture balance, with three replications. The percentage results of the loss on drying test for the powder are presented in Table 2.

Table 2. Results of the Percentage of Loss on Drying Test for Powder

Replication	Weight (gram)	Result (%)
1	2	8,6
2	2	8,6
3	2	8,5
Average±SD		8,567 ± 0,058

Based on Table 2, the average loss on drying of Echinacea flower powder after three replications is 8.567% with a standard deviation of 0.058. A good criterion for loss on drying is less than 10%, as the percentage of loss on drying also reflects the amount of compounds that evaporate during the process. This is important for maintaining the quality and stability of the dried powder, as well as ensuring that important bioactive compounds remain in the powder (Maryam et al., 2020).

Extraction Process

The ethanol extract of Echinacea flowers in this study was prepared using the ultrasonication method with 70% ethanol as the solvent. Ultrasonication was chosen for its ability to break down plant cell walls, allowing more bioactive compounds to be released into the solvent. This process uses ultrasonic waves (>20,000 Hz) to create cavitation bubbles in the solution. When these bubbles burst, small turbulent flows and high pressures are generated, breaking the plant cell walls and releasing the bioactive materials.

This technology is more efficient than traditional methods as it yields higher extraction with less solvent and time usage, and minimizes heat-induced damage (Verret et al., 2023). Compared to maceration, ultrasonication offers several significant advantages. Ultrasonication is faster in the extraction process, often requiring only a few minutes to an hour to complete, while maceration can take up to several days to achieve the desired extraction results. Additionally, ultrasonication uses less solvent due to its high efficiency in the extraction process, whereas maceration requires a larger amount of solvent to extract bioactive compounds from the plant material (Verret et al., 2023). Ultrasonication is also more effective in releasing bioactive compounds, especially from hard plant cell structures, because of the cavitation generated by ultrasonic waves, whereas maceration is less effective in releasing bioactive compounds trapped in hard cell walls and requires more agitation or longer time (Usman et al., 2023).

In this study, 70% ethanol was chosen as the solvent because it is effective in extracting various bioactive compounds in Echinacea, such as flavonoids, alkamides, and phenolics (including caffeic acid and its derivatives). Ethanol is also known as a safe, non-toxic, and environmentally friendly solvent, which is important for pharmaceutical and food applications (Plaskova and Mlcek, 2023).

The use of 70% ethanol is not only effective but also safe, as regulated by the International

Council for Harmonisation (ICH), which states that ethanol is a Class 3 solvent, considered to be of low toxicological risk to human health (ICH, 2021). The yield results of the ethanol extract of Echinacea flowers are presented in Table 3.

Based on Table 3, 500 grams of Echinacea flower powder were ultrasonicated, yielding 186 grams of thick extract with a recovery rate of 37.2%. Yield calculation is essential for comparing the amount of extract obtained from a specific weight of raw material to the original simplisia weight. It also helps determine the quantity of bioactive compounds extracted. Expressed as a percentage (%), a higher yield reflects a more efficient extraction process (Maryam et al., 2020). Various studies comparing extraction methods, including ultrasonication, have shown that this technique often produces higher yields than traditional methods such as maceration or percolation. For example, research by Shen et al., (2023) highlighted that ultrasonication improves extraction efficiency, yielding significantly more extract. The 37.2% recovery observed in this study underscores the effectiveness of ultrasonication in maximizing extract yield.

Table 3. Yield Results of Ethanol Extract of Echinacea Flowers

Weight of powder (gram)	Weight of extract (gram)	Yield (%)
500	186	37,2

Determination of Moisture Content in Ethanol Extract

The moisture content of the ethanol extract of Echinacea flowers is determined using the gravimetric method. Gravimetric analysis consists of methods in analytical chemistry that determine the amount of an analyte based on its mass. In measuring moisture content, this method involves weighing the sample before and after the drying process.

Determining moisture content is crucial for understanding the amount of water present in the material (Depkes, 2000). High moisture content can cause instability in pharmaceutical preparations by increasing the risk of bacterial exposure, which threatens product safety. Excessive moisture content can also lead to the chemical decomposition of active pharmaceutical ingredients, thereby reducing the effectiveness of the drug. Controlling moisture content in pharmaceutical preparations is essential to ensure that the resulting product is stable and of high quality (Voight, 1994). The results of the moisture content determination for the ethanol extract of Echinacea flowers are shown in Table 4.

Tabel 4. Moisture Content Determination Results of Ethanol Extract of Echinacea Flowers

Replication	Initial weight (gram)	Moisture content (%)
1	5	20,079
2	5	19,766
3	5	19,790
Average±SD		19,878 ± 0,174

According to Table 4, the average moisture content of the ethanol extract of Echinacea flowers, based on three replications, is 19.878% with a standard deviation of ± 0.174. This moisture content is higher than that reported in other studies, which indicate a moisture content of 12% (Petrova et al., 2023). The variation in moisture content can be attributed to factors such as the extraction method and the type of solvent used. According to Anggraini and Kusuma (2020), if a material has a moisture content greater than 10%, it can affect the quality of the material, accelerate mold growth, and lead to the hydrolysis of its chemical constituents. Therefore, it is important to consider effective moisture reduction methods to improve the quality and stability of the extract.

Phytochemical Screening and Thin Layer Chromatography (TLC)

Phytochemical screening of the ethanol extract of Echinacea flowers was performed using qualitative methods with test tubes. The objective of this screening is to identify the presence of secondary metabolite compounds in the Echinacea flower sample.

This process involves testing for chemical constituents such as flavonoids, alkaloids, steroids, triterpenoids, tannins, and saponins by using various reagents to determine the chemical composition of the Echinacea extract. The results of the phytochemical screening for the ethanol extract of Echinacea flowers are presented in Table 5.

Table 5. Results of Phytochemical Screening Of Ethanol Extract Of Echinacea Flowers

Chemical content		Literature Reference (Depkes, 1987, 1989)	Result	Description
Flavanoid		The presence of flavonoid aglycones is indicated by the occurrence of a red or orange color	Red in color	+
Alkaloid	R. Mayer	There is a yellowish white precipitate	No precipitate	-
	R. Dragendorf	There is turbidity or brownish orange precipitate	No precipitate	-
Steroid dan triterpenoid		Color change from red to green or purple between the two layers of solution	No purple ring	-
Tanin		Blackish green color	Blackish green in color	+
Saponin		There is stable froth	There is froth	+

Note : (+) Presence of compound, (-) Absence of compound

The phytochemical screening results reveal that the ethanol extract of Echinacea flowers contains flavonoids, tannins, and saponins. These compounds have significant potential for providing pharmacological effects, including notable anti-inflammatory activity (Ngoua-Meye-Misso et al., 2018). Flavonoids are polyphenolic compounds commonly found in plants and exhibit strong anti-inflammatory properties. Research has shown that flavonoids can inhibit regulatory enzymes or transcription factors that are crucial for controlling mediators involved in inflammation, such as prostaglandins and pro-inflammatory cytokines (Aarland et al., 2017). For instance, the flavonoids present in Echinacea, particularly quercetin, kaempferol, and diosmetin, have demonstrated anti-inflammatory effects by inhibiting the activity of cyclooxygenase (COX) and lipoxygenase (LOX) enzymes, which play key roles in the inflammatory process (Abinaya & Anuradha, 2018).

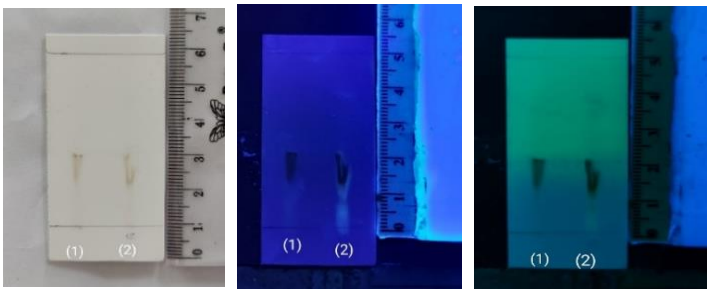
In addition to flavonoids, tannins, and saponins in the ethanol extract of Echinacea flowers also contribute significantly to its anti-inflammatory activity. Tannins, another group of polyphenolic compounds present in the extract, exhibit anti-inflammatory properties primarily by inhibiting inflammatory enzymes and reducing capillary permeability, which in turn decreases edema and other inflammatory responses (Yang et al., 2018). Saponins, complex glycoside compounds that can form foam in aqueous solutions, also possess various biological effects, including anti-inflammatory properties.

The anti-inflammatory mechanism of saponins involves the inhibition of histamine release from mast cells and the modulation of pro-inflammatory cytokine production and other inflammatory mediators (Rajesh et al., 2019). Additionally, saponins enhance cell permeability, allowing other active compounds to more easily enter cells and exert their therapeutic effects (Sundari et al., 2022). The combined presence of these three active compounds (flavonoids, tannins, and saponins) in the ethanol extract of Echinacea flowers underpins its potential for strong anti-inflammatory effects. Flavonoids inhibit key enzymes in the inflammatory pathway, tannins reduce capillary permeability and local inflammatory responses, and saponins modulate cytokine production and inflammatory mediators (Poudel et al., 2019).

Flavonoid testing was also carried out using Thin Layer Chromatography (TLC), with a silica gel GF 254 plate as the stationary phase and a mobile phase consisting of toluene, acetone, and formic acid (Sari et al., 2022). In this test, flavonoid compounds were analyzed using quercetin as the reference standard. The TLC results, illustrated in Figure 1 below, revealed that the Rf value of the quercetin

standard was 0.4 under visible light, UV 254 nm, and UV 366 nm. The sample displayed an Rf value of 0.4 under the same conditions, and an additional Rf value of 0.24 under UV 254 nm.

These results indicate that the compounds in the ethanol extract of Echinacea show similarities in spot shape, color, and Rf value to the quercetin standard. When the Rf value of a compound matches that of the standard, it suggests the presence of similar or identical compounds. Conversely, if the Rf value differs, it is likely that the compound is different.



(a) (b) (c)

Figure 1. Identification of Flavonoids From Ethanol Extract Of Echinacea Using TLC Method

Description: (a) Under visible light, (b) Under UV 366 nm light, (c) Under UV 254 nm light, (1) Quercetin standard, (2) Sample

Determination of Total Flavonoid Content

The total flavonoid content is measured using the colorimetric method with a UV-Vis spectrophotometer. In this procedure, $AlCl_3$ and sodium acetate serve as reagents. The interaction between $AlCl_3$ and flavonoid compounds results in the formation of a complex involving hydroxyl groups paired with ketones or adjacent hydroxyl groups. CH_3COONa also functions to identify the 7-hydroxyl group.

The ethanol extract of Echinacea flowers was then analyzed to determine its flavonoid content using a UV-Vis spectrophotometer with a quercetin standard solution. Quercetin was chosen as the standard because it is one of the most widely distributed flavonoid compounds. Quercetin, a flavonoid from the flavonol group, has a keto group at C-4 and hydroxyl groups at neighboring C-3 or C-5 atoms from flavones and flavonols. Flavonoid compounds are major polyphenol components that have been extensively studied as one of the most important anti-inflammatory agents in many medicinal plants (Rahman et al., 2022).

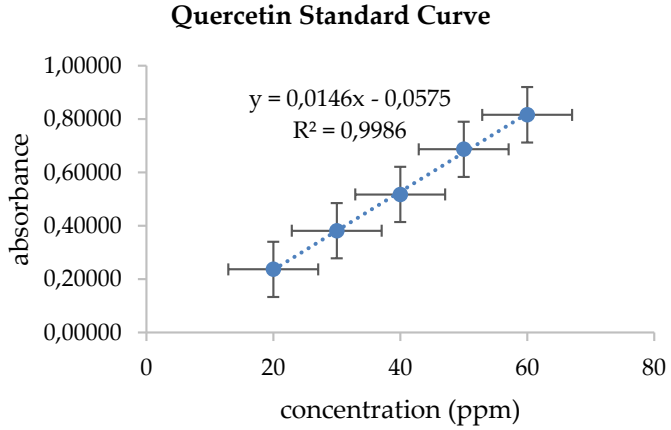


Figure 2. Standard Curve of Quercetin

The determination of the maximum wavelength revealed the highest absorbance at 431 nm. The total flavonoid content was analyzed by generating a linear regression equation from the standard curve, as shown in Figure 2. The resulting linear regression equation was Y = 0.0146X - 0.0575. In the assessment of total flavonoid content, the average concentration obtained was 10.268 mgQE/g. The unit mgQE/g denotes milligrams of Quercetin Equivalent per gram, indicating the amount of quercetin equivalent to the flavonoid content present in one gram of extract (Ramonah, 2023). This means that each gram of extract contains the equivalent of 10.268 milligrams of quercetin. The results of the total flavonoid content determination in the ethanol extract of Echinacea flowers are displayed in Table 6.

Table 6. Results of Total Flavonoid Content Determination in Ethanol Extract of Echinacea Flowers

Replication	Absorbance	Content (mg QE/g)
1	0,227	10,256
2	0,228	10,292
3	0,227	10,256
Average±SD		10,268 ± 0,021

Another study reported that the total flavonoid content in Echinacea flower extract is 10.85 mgQE/g (Georgieva et al., 2014). This comparison shows that the total flavonoid content measured in this study (10.268 mgQE/g) closely approximates the results obtained in the other study. The high total flavonoid content indicates the potential of Echinacea flower extract as an effective antioxidant and anti-inflammatory agent. Flavonoids such as quercetin found in Echinacea flower extract play a significant role in its biological activity.

Quercetin is known to have anti-inflammatory effects by inhibiting the enzymes cyclooxygenase and lipoxygenase, which are responsible for producing inflammatory mediators such as prostaglandins and leukotrienes. Quercetin can also inhibit the release of histamine and pro-inflammatory cytokines, thus reducing the inflammatory response (Maleki et al., 2019). The high flavonoid content in Echinacea flower extract plays a major role in its anti-inflammatory activity, which has the potential to be further developed as a raw material in natural-based anti-inflammatory drug formulations.

Determination of Total Phenol Content

The determination of total phenol content in Echinacea flower extract was conducted using the Folin-Ciocalteu method. The Folin-Ciocalteu reagent contains a complex of phosphomolybdate / phosphotungstate acids. The principle of the Folin-Ciocalteu method involves the oxidation of phenolic hydroxyl groups and the reduction of phosphomolybdate under basic conditions by the aromatic nucleus of phenolic compounds to form a molybdenum-tungsten complex.

The reaction occurs under basic conditions, necessitating the use of sodium carbonate to create a basic environment. Sodium carbonate converts phenolic compounds into phenolate ions due to proton dissociation. The phenolic-hydroxyl groups react with the Folin-Ciocalteu reagent to form a blue phosphomolybdate-phosphotungstate complex. The blue color intensity increases with the concentration of phenolic compounds formed. The higher the phenol concentration, the more phenolic compounds will reduce heteropoly acid, resulting in a darker blue color (Ramonah, 2023).

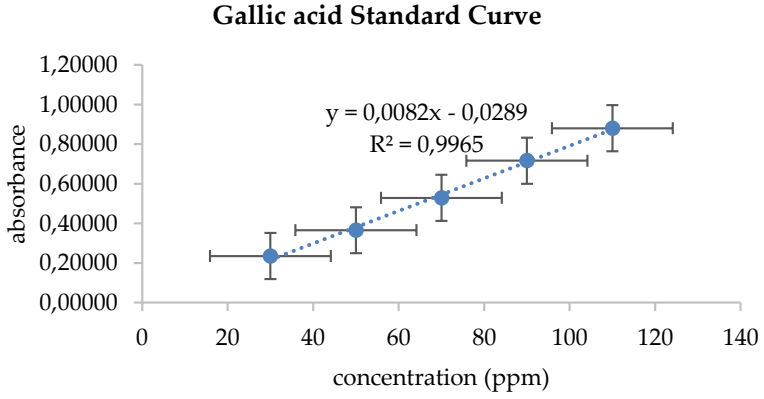


Figure 3. Standard Curve of Gallic Acid

The determination of the maximum wavelength indicated the highest absorbance at 653 nm. The total phenolic content was assessed by developing a linear regression equation for the standard curve, as shown in Figure 3. The resulting linear regression equation was Y = 0.0082X - 0.0289.

The unit mg GAE/g stands for milligrams of Gallic Acid Equivalent per gram, which represents the total phenolic concentration in the sample, expressed as milligrams of gallic acid equivalent per gram of extract (Dumitru & Gănescu, 2022). The determination of total phenolic content revealed that the average total phenolic concentration was 66.531 mg GAE/g, indicating that each gram of extract contains phenols equivalent to 66.531 milligrams of gallic acid. The results of the total phenolic content determination in the ethanol extract of Echinacea flowers are presented in Table 7.

Table 7. Results of Total Phenol Content Determination in Ethanol Extract of Echinacea Flowers

Replication	Absorbance	Content (mgGAE/g)
1	0,572	66,709
2	0,568	66,175
3	0,572	66,709
Average±SD		66,531 ± 0,308

Another study reported that the main phenolic compound contained in Echinacea flower extract is cichoric acid. The concentration of cichoric acid in Echinacea flower extract varies between 63.66 to 70.31 mg/g (Chen et al., 2015). This result shows that the total phenol concentration measured in this study (66.531 mgGAE/g) falls within the range comparable to the cichoric acid concentration reported by previous studies.

Research by Kumar and Ramaiah (2011) mentioned that cichoric acid has significant biological activities, including antioxidant and anti-inflammatory properties. The cichoric acid content in Echinacea contributes significantly to its therapeutic properties. The combination of various phenolic compounds in the Echinacea flower extract plays an important role in its anti-inflammatory activity, which has the potential to be further developed as a raw material in natural-based anti-inflammatory drug formulations.

LLCN Preparation Results

The preparation of Lyotropic Liquid Crystals Nanoparticles (LLCN) in this study was carried out using the top-down method. The top-down method was chosen because it allows better control over particle size and distribution, and can minimize the degradation of active ingredients during the manufacturing process (J. Zhai et al., 2019).

In this procedure, the oil phase consisting of Echinacea extract, Phytantriol, ethanol, and water was mixed homogeneously, while the aqueous phase containing Poloxamer 407 and water was prepared separately. The two phases were then combined and homogenized before being sonicated for 5 minutes. The sonication process aims to reduce particle size and assist in the formation of a stable lyotropic liquid crystal structure (Chountoulesi et al., 2018). Echinacea extract was used due to its strong anti-inflammatory activity, thanks to its active compounds such as flavonoids and alkylamides (Vieira et al., 2023).

Phytantriol was used as a cubic phase former that helps improve the stability and bioavailability of the active compounds. Phytantriol is an amphiphilic lipid capable of forming unique liquid crystal structures, such as cubic and hexagonal phases (Astolfi et al., 2019). This cubic phase consists of two interpenetrating aqueous nanochannels that do not touch, allowing for the protection of active compounds from degradation and controlled release, thereby enhancing therapeutic effectiveness (Chong et al., 2015). Ethanol was used as an effective solvent in the extraction and nanoparticle formation process. Ethanol not only dissolves bioactive compounds well but is also considered safe and environmentally friendly (Dong et al., 2006). Water was used as the dispersion medium to form micelles and liquid crystal structures (Nguyen et al., 2010). Poloxamer 407 functions as a stabilizer and surfactant in the LLCN formulation. Poloxamer helps stabilize the nanoparticles by reducing surface tension and preventing particle aggregation, thus enhancing the physical stability of the LLCN system (Hartnett et al., 2014).

Characterization of LLCN

1.Particle Size, Polydispersity Index (PI), Zeta Potential Test Results

The particle size and polydispersity index (PI) of Lyotropic Liquid Crystals Nanoparticles (LLCN) of Echinacea flower extract were tested using a Particle Size Analyzer (PSA). This instrument operates using the Dynamic Light Scattering (DLS)

method, which is highly effective for measuring particle size distribution in the nanometer to micrometer range (Filippov et al., 2023). This measurement is crucial to ensure that the formed nanoparticles have an appropriate size for pharmaceutical applications, particularly to ensure enhanced bioavailability and stability of bioactive compounds (Mehdizadeh et al., 2022). The polydispersity index (PDI) indicates particle size homogeneity; a low PDI value signifies a narrow and more uniform particle size distribution (Dai et al., 2022).

In this study, the LLCN formulation of Echinacea extract was evaluated at two different temperatures: controlled room temperature and cold temperature, with storage periods of up to 30 days. This testing is crucial to assess the physical stability of the LLCN formulation of Echinacea extract under varying storage conditions. The results of the particle size and polydispersity index (PI) tests for the LLCN formulation of Echinacea extract before and after 30 days of storage are shown in Table 8.

Table 8. Particle Size, Polydispersity Index (PI), and Zeta Potential of LLCN of Echinacea Extract Before and After 30 Days of Storage

No.		Controlled room temperature (30°C) 1 Day	Controlled room temperature (30°C) 30 Days	Cold temperature (4°C) 1 Day	Cold temperature (4°C) 30 Days	Requirements	Remark
1	Particle Size (nm)	263,100 ± 3,835	229,367 ± 0,451	303,333 ± 17,368	611,100 ± 21,161	10-1000nm	Meets requirements
2	Polydispersity Index (PI)	0,217 ± 0,011	0,367 ± 0,029	0,459 ± 0,026	0,492 ± 0,041	0,01 to 0,5-0,7	Meets requirements
3	Zeta Potential (mV)	-22,737 ± 1,348	-19,623 ± 1,157	-23,737 ± 1,831	-22,860 ± 0,745	> +30 mV or < -30 mV	Meets requirements

In this test, the particle size distribution results show that the LLCN of Echinacea extract has an average particle size within the optimal range, which is 10-1000 nm (Gorantla et al., 2022). Smaller particles have a larger surface area, allowing for increased interaction with biological targets and enhanced anti-inflammatory efficacy (Mehdizadeh et al., 2022). From the measurements, it can be seen that the particle size at controlled room temperature (30°C) decreased after 30 days of storage. Conversely, at cold temperature (4°C), the particle size significantly increased after 30 days of storage. The increase in particle size at cold temperature (4°C) indicates the possibility of particle aggregation during storage at low temperatures, causing the particles to combine into larger particles (Jakubek et al., 2023).

The polydispersity index indicates the distribution of nanoparticles in the formulation. In this study, the polydispersity index ranges from 0.01 to 0.5-0.7 for monodisperse particles. Monodisperse nanoparticles can enhance the stability of the nanoparticle system as they exhibit homogeneous size, shape, and weight. A higher polydispersity index means more particles are aggregated, or in other words, the formulation is becoming less stable (Rabima & Sari, 2019). The polydispersity index test results before and after the storage period show a narrow particle size distribution, with the polydispersity index values within the range of 0.01 to 0.5-0.7. A low polydispersity index indicates that the formed particles have a narrow size distribution, which indicates stability and uniformity of the particles in the formulation (Maslizan et al., 2022).

The measurement results of the polydispersity index before storage show that at controlled room temperature (30°C), the polydispersity index is 0.217 ± 0.011, while at cold temperature (4°C) it is 0.459 ± 0.026. These values indicate that before storage, the particles at controlled room temperature have a more homogeneous size distribution compared to particles at cold temperature. After 30 days of storage, the polydispersity index at room temperature increased to 0.367 ± 0.029, while at cold temperature it increased to 0.492 ± 0.041. The increase in the polydispersity index after storage indicates a decrease in the homogeneity of the particle size distribution, with cold temperature showing a higher increase in the

polydispersity index compared to controlled room temperature.

Small particle size and narrow size distribution are crucial for applications in drug delivery systems, as they can affect drug release and stability of the formulation during storage (Waheed and Aqil, 2021). Good particle size stability can also prevent particle aggregation, which can reduce the pharmacological effectiveness of the extract used (Fan et al., 2020). The results of these measurements conclude that storage at controlled room temperature is more stable in maintaining particle size and size distribution compared to storage at cold temperature, which tends to cause larger particle aggregation and an increase in the polydispersity index.

Zeta potential testing was conducted to determine the stability of the nanoparticle dispersion system (Pochapski et al., 2021). Zeta potential measures the electrical charge present on the surface of suspended particles and is an important indicator of colloid stability (Mukherjee and Okram, 2023). The higher the zeta potential value, the greater the repulsive force between particles, which can prevent agglomeration or clumping of particles (Zulkafli and Wanatasanappan, 2021). Zeta potential values greater than +30 mV or less than -30 mV indicate high particle stability, as these particles tend to remain dispersed and not clump together. With high stability, the particles will remain evenly dispersed in the medium, reducing the risk of aggregation and sedimentation that can affect the performance of the colloidal system (Savchenko and Velichko, 2019). The zeta potential test results can be seen in Table 8.

In this study, zeta potential testing was conducted under two temperature conditions: controlled room temperature (30°C) and cold temperature (4°C), both before storage and after 30 days of storage. The measurement results show that at controlled room temperature (30°C), the zeta potential value before storage was -22.737 mV and after 30 days of storage was -19.623 mV. Meanwhile, at cold temperature (4°C), the zeta potential value before storage was -23.737 mV and after 30 days of storage was -22.860 mV.

The obtained zeta potential values indicate that the nanoparticles remained stable during storage, both at controlled room temperature and cold temperature. The decrease in zeta potential after 30 days of storage under both temperature conditions still falls within a range that indicates sufficient stability, being less than -30 mV (Huo et al., 2019). This good stability suggests that the nanoparticles did not undergo significant aggregation during the storage period, making them reliable for further applications in drug delivery (Kurpiers et al., 2020).

Overall, the zeta potential test results show that Lyotropic Liquid Crystal Nanoparticles (LLCN) from Echinacea extract have good stability during storage at both tested temperature conditions.

2. Entrapment Efficiency Test Results

Entrapment efficiency is a parameter that determines the percentage of active substances absorbed by the Lyotropic Liquid Crystals Nanoparticles (LLCN) of Echinacea extract compared to the total Echinacea extract added. In general, entrapment efficiency in nanoparticles can enhance bioavailability and increase the accumulation of therapeutic agents at the target site (Loo et al., 2020). In this study, the method used involved calculating the amount of drug not trapped in the liquid using centrifugation and UV-Vis spectrophotometry. The principle of this method is to separate the nanoparticles from the free drug solution using centrifugation. After centrifugation, the supernatant containing the free drug is separated, and the drug concentration in the supernatant is measured using a UV-Vis spectrophotometer.

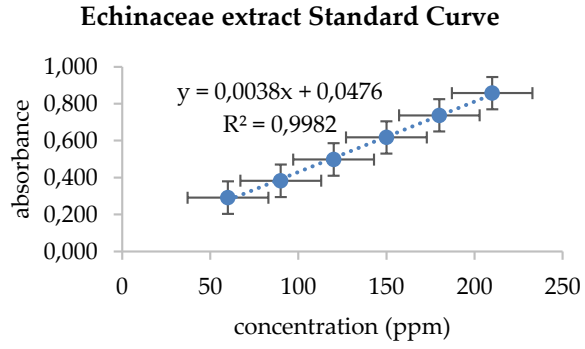


Figure 4. Standard Curve of Echinacea Extract

The analysis of entrapment efficiency was conducted by creating a linear regression equation of the standard curve. The standard curve is shown in Figure 4, and the obtained linear regression

equation is Y = 0.0038X - 0.0476. This linear regression equation was then used to calculate the entrapment efficiency by subtracting the amount of Echinacea extract detected in the supernatant (unencapsulated) from the total amount of Echinacea extract added to the LLCN formulation, and then multiplying by 100%.

Table 9. Entrapment Efficiency of Echinacea Extract LLCN

Replication	EE (%)
1	88,569
2	88,438
3	88,177
Average±SD	88,395 ± 0,200

In this study, entrapment efficiency was tested in three replications, showing an average entrapment efficiency of 88.395%. Effective absorption is achieved when the entrapment efficiency percentage approaches 100%, and a good entrapment efficiency percentage should be greater than 60% (Supraba et al., 2021). The high entrapment efficiency in this test indicates that the method used in the preparation of LLCN from Echinacea extract is quite effective, as evidenced by the high absorption efficiency results.

A nanoparticle system is considered successful if it has high entrapment efficiency because it can reduce matrix components, meaning the drug can exert pharmacological effects at a smaller or more efficient dose (Supraba et al., 2021). The efficiency value obtained being more than 60% and close to 100% indicates that almost all active compounds incorporated into the nanoparticle system can be effectively absorbed, reducing compound loss during the formulation and storage process.

The high entrapment efficiency in nanoparticles is very important because it can increase the bioavailability of bioactive compounds found in plant extracts. High bioavailability means that more active compounds can reach target cells or tissues in the body, there by maximizing the therapeutic potential of the extract. This high absorption efficiency has significant pharmacological implications, as the drug dose required to achieve the desired pharmacological effect can be lower compared to conventional dosage forms, thereby reducing the potential for side effects and treatment costs.

The entrapment efficiency result with an average of 88.395% and a standard deviation of ± 0.200 in this study shows the great potential of LLCN Echinacea extract as an effective drug delivery system for anti-inflammatory and other therapeutic applications.

Results of Anti-Inflammatory Activity Testing

1. Determination of Wavelength

The maximum wavelength (λmax) is the wavelength at which electronic excitation occurs, resulting in the highest absorbance. Measurements at the maximum wavelength are conducted because the change in absorbance per unit concentration is greatest at this wavelength, making the analysis most sensitive (Rahayu et al., 2009).

In this study, the maximum wavelength was measured using a negative control, which is a solution of 0.2% BSA in TBS added with Lowry reagent. Measurements were made using UV-Vis spectrophotometry in the wavelength range of 200-700 nm. The results showed that the maximum wavelength of the negative control solution of 0.2% BSA in TBS was 657 nm with an absorbance of 0.5153.

2. Determination of Operating Time (OT)

The determination of operating time was performed on the negative control solution, which is 0.2% BSA in TBS added with Lowry reagent, at a wavelength of 657 nm for 60 minutes. The purpose of determining the operating time is to find the time required for the absorbance of the negative control solution to stabilize and be ready for reading on UV-Vis spectrophotometry.

By determining the operating time, we can ensure that the measured absorbance values are representative and consistent, thus serving as a basis for subsequent measurements. The results of the operating time determination for the negative control solution showed stable time between 20 - 27 minutes.

Results of Anti-Inflammatory Activity Testing

Diclofenac as a positive control, the condensed extract, and the LLCN Echinacea extract.

In this study, a Bovine Serum Albumin (BSA) solution was used as a protein model to evaluate the anti-inflammatory capability of Echinacea extract formulated in Lyotropic Liquid Crystal Nanoparticles (LLCN) and condensed Echinacea extract. Bovine Serum Albumin (BSA), a globular protein, is extensively utilized in biochemical and pharmaceutical research due to its high structural similarity to human serum albumin, stability, and ease of denaturation (Jahanban-Esfahlan et al., 2019).

The sample absorbance values were obtained from the remaining reaction between denatured BSA and anti-inflammatory compounds. In in vitro anti-inflammatory activity testing, BSA is used to observe how a substance can prevent protein damage caused by heat or chemicals (Anyasor et al., 2019). The addition of Lowry reagent in anti-inflammatory activity testing using BSA and the protein denaturation method aims to measure the concentration of non-denatured protein after treatment with the test compound. Lowry reagent reacts with amino acids in the protein, producing a blue color complex whose intensity is proportional to the amount of protein.

In vitro anti-inflammatory activity is measured by determining the IC50 (inhibition concentration 50) value. The IC50 value is used to measure the effectiveness of the sample in inhibiting protein denaturation, with the IC50 value indicating the concentration at which protein denaturation inhibition reaches 50%. The IC50 value is calculated using the linear regression equation between concentration (X) and inhibition percentage (Y).

The linear regression equation is used because the relationship between inhibitory power and concentration yields a linear equation. From the equation Y = ax + b obtained, the IC50 value is then calculated. In the inhibition test, if the inhibition percentage is more than 20%, the sample is considered to have anti-inflammatory activity (Novika et al., 2021). The formula for calculating the inhibition percentage is as follows. % Inhibition:

$$\frac{Absorbance\ of\ Negative\ Control - Absorbance\ of\ Sample}{Absorbance\ of\ Negative\ Control} x100\%$$

The IC50 value indicates the concentration of the extract needed to inhibit 50% of the total radicals. A sample is considered to have very strong anti-inflammatory activity if its IC50 value is less than 50 ppm, strong if its IC50 value is between 50-100 ppm, moderate if its IC50 value ranges between 100-150 ppm, weak if its IC50 value is between 151-200 ppm, and inactive if its IC50 value exceeds 200 ppm (T. Mulyani, 2023). The results of the anti-inflammatory activity test can be seen in Table 10.

Table 10. Results of Anti-Inflammatory Activity Testing

No.	Sample	IC_{50} (ppm)	Anti-inflammatory Activity
1	Sodium Diclofenac	19,984 ± 0,229	Very strong
2	Echinacea Extract	54,660 ± 0,094	Strong
3	LLCN Echinacea Extract	21,823 ± 0,176	Very strong

The positive control used was sodium diclofenac at a concentration of 1000 ppm, which was then serially diluted to 15, 20, 25, 30, 35, and 40 ppm. According to the results of the anti-inflammatory activity test of Echinacea extract shown in Table 10, the IC50 value of sodium diclofenac is 19.984 ppm, indicating that sodium diclofenac, as a positive control, exhibits very strong anti-inflammatory activity with an IC50 value of less than 50 ppm (T. Mulyani, 2023). This result demonstrates the potent anti-inflammatory activity of sodium diclofenac, making it a standard for evaluating the anti-inflammatory effectiveness of Echinacea extract and LLCN Echinacea extract.

Sodium diclofenac was selected as the positive control because it is one of the most widely used Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) in anti-inflammatory research. NSAIDs function by inhibiting the cyclooxygenase (COX) enzyme, a key mediator in the inflammatory process (Gan, 2010). The use of sodium diclofenac as a positive control provides a strong and reliable comparison for assessing the effectiveness of the compounds or formulations being tested, in this case, Echinacea extract and LLCN Echinacea extract.

The anti-inflammatory activity test of Echinacea extract was conducted by reading the absorbance of the test extract solution that had been added with 0.2% BSA. The stock solution of the extract was prepared by dissolving 1 gram of extract in 10,000 ppm, then serially diluted to 10, 20, 30, 40, 50, and 60 ppm. Based on the results of the anti-

inflammatory activity test in Table 10, the IC50 value of Echinacea extract is 54.660 ppm, indicating that the extract has strong anti-inflammatory activity because it has an IC50 value between 50-100 ppm (T. Mulyani, 2023).

This IC50 value indicates that Echinacea extract does have anti-inflammatory capabilities, although its effectiveness is not as strong as sodium diclofenac. The anti-inflammatory activity of Echinacea extract can be attributed to the bioactive compounds such as flavonoids, alkylamides, and caffeic acid, which are known to have anti-inflammatory properties.

The anti-inflammatory activity test of Lyotropic Liquid Crystal Nanoparticle (LLCN) Echinacea extract was conducted by measuring the absorbance of the LLCN test solution mixed with 0.2% BSA. The LLCN stock solution was prepared using 1 gram of LLCN dissolved to 10,000 ppm, followed by serial dilutions to 10, 20, 30, 40, 50, and 60 ppm. Based on the anti-inflammatory activity test results of LLCN Echinacea extract in Table 10, the IC50 value of 21.823 ppm indicates that LLCN Echinacea extract possesses very strong anti-inflammatory activity, as an IC50 value of less than 50 ppm is considered very potent (T. Mulyani, 2023).

The LLCN Echinacea extract demonstrated a significant increase in anti-inflammatory activity, approaching the effectiveness of diclofenac sodium. The anti-inflammatory activity of LLCN Echinacea extract is evidenced by the IC50 value, which shows the extract's ability to neutralize 50% of total radicals. This categorizes LLCN Echinacea extract as having very strong anti-inflammatory activity, comparable to diclofenac sodium, which is also considered highly potent.

Using LLCN Echinacea extract can reduce the required drug dosage and simultaneously decrease the risk of side effects commonly associated with conventional anti-inflammatory drugs such as diclofenac sodium. LLCN enhances the absorption and effectiveness of active compounds due to its small particle size, resulting in a larger surface area (Mohsin et al., 2016). Furthermore, nanoparticle formulation offers additional benefits, including increased stability and durability of bioactive compounds (Nieri et al., 2022).

Nanotechnology, particularly in the form of LLCN, has proven to enhance the availability of bioactive compounds from plants. This study demonstrates that LLCN Echinacea extract is more effective in reducing inflammation compared to regular Echinacea extract, approaching the effectiveness of diclofenac sodium as a control standard. These results indicate that LLCN technology could be a potential development to enhance the activity of medicinal compounds, especially in anti-inflammatory applications.

The statistical analysis of the data was conducted using normality, homogeneity, and One Way ANOVA tests in SPSS software. The Shapiro-Wilk normality test showed a significance value (sig) greater than 0.05, indicating that the data is normally distributed. The homogeneity test also showed a significance value (sig) greater than 0.05, indicating that the samples are distributed homogeneously. The One Way ANOVA test was performed to evaluate the differences among the samples.

The results indicated a significance value (sig) of 0.000, demonstrating a highly significant difference in IC50 values among the positive control (diclofenac sodium), the concentrated Echinacea extract, and the LLCN Echinacea extract. These results conclude that the treatments applied to the Echinacea extract, both in concentrated form and as LLCN nanoparticles, have a significantly different effect on anti-inflammatory activity. The findings suggest that the LLCN Echinacea extract offers a substantial enhancement in anti-inflammatory activity compared to the concentrated Echinacea extract.

DISCUSSION

This study has several limitations, such as the potential variability in the preparation of LLCN that may affect particle size and absorption efficiency. Small variations in the ultrasonication process may lead to inconsistent results. In addition, the in vitro model used, although effective for initial assessment, does not fully reflect the complexity of biological conditions in the human body. For future research, in vivo tests in animal models and more consistent LLCN preparation methods such as high-pressure homogenization are suggested to improve the accuracy of the results.

CONCLUSIONS

Based on the results and discussion, it can be concluded that Echinacea purpurea flower extract can be formulated into a Lyotropic Liquid Crystal Nanoparticles (LLCN) system that meets the required standards. There is a significant increase in anti-inflammatory activity between concentrated Echinacea flower extract and Echinacea LLCN extract, as evidenced by the lower IC50 value of Echinacea LLCN (21.823 ppm) compared to concentrated extract. This IC50 value was comparable to the positive control, diclofenac

sodium, indicating that the LLCN formulation of Echinacea flower extract significantly enhanced the anti-inflammatory activity.

Recommendations for future research include in vivo tests to confirm the effects of LLCN in animal models and clinical trials to evaluate its safety in humans. Potential clinical applications of these LLCNs include the development of safer and more effective nanoparticle-based anti-inflammatory drugs, with lower risk of side effects compared to long-term NSAID therapy.

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