# The Utilization of *Toona sinensis* (A. Juss) M. Roem Leaf Extract in the Form of Polymeric Nanoparticles for Anti-Aging Purposes

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**Abstract:** *Toona sinensis* (A. Juss) M. Roem leaves are hold potential as a natural source of antioxidants. Natural antioxidants work synergistically to stabilize *Reactive Oxygen Species* (ROS) during the photoaging process. *T. sinensis* leaves contains phytochemical compounds, such as phenolics and flavonoids as major compounds in inhibiting elastase enzymes. To facilitate the delivery of Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) as anti-aging agents, a nanotechnology-based delivery system in the form of polymeric nanoparticles was developed using the ionic gelation method. This study compared the inhibitory abilities of Surian Leaves Extract (SLE), n-Hexane Fraction Surian Leaves (nHFSL), Ethyl Acetate Fraction Surian Leaves (EAFSL), and Water Fraction Surian Leaves (WFSL) on elastase enzymes and characterized the properties of the obtained SLE and EAFSL nanoparticles. The results indicated that both SLE and EAFSL exhibited higher inhibition percentages against elastase enzymes compared to WFSL and nHFSL. The Relative Fluorescence Unit (RFU) value is related to activity of elastase enzyme, where higher RFU value, higher the activity of elastase enzyme and vice versa. If RFU value decreases, inhibitory power of extract against elastase enzyme increases, due to IC50 value for EDS of 12.351 ± 0.092 and IC50 value for FEADS of 15.5865 ± 0.546 in addition to total flavonoid content of Surian Leaf Extract (EDS) of 33.19 mg / g and total phenolic content of FEADS of 365 mg / g which can reduce the ability of elastase to break down fluorescent substrates, so that resulting fluorescence signal becomes lower and vice versa. Based on the inhibition percentage against elastase enzymes, SLE nanoparticles were developed using the ionic gelation method with chitosan (polycation) and alginate (1%:1%) as polymers, and NaTPP (polyanion) as a cross-linker. This approach suggested that SLE and EAFSL nanoparticles can enhance anti-aging activity with reducing the required dosage.

**Keywords:** *Toona sinensis*, anti-aging, elastase enzyme, nanoparticles.

## 1. Introduction

*Toona sinensis* (A. Juss) M. Roem is a plant that holds potential as a natural antioxidant source and is extensively utilized in traditional medicine for treating various conditions, such as care for skin [1,2]. Among the bioactive compounds present in it, we can find gallic acid, methyl gallate, kaempferol, quercetin, rutin, quercitrin, palmitic acid, and linoleic acid [3,4,5]. Notably, quercitrin and quercetin compounds, abundant in Surian leaves, exhibit antioxidant, anti-inflammatory, and antiallergic activities [6,7,8]. The separation of bioactive compounds from herbal plants often involves extraction, ethanol extract of surian leaves (*T. sinensis*) produced is used as an antioxidant, where the resulting extract still contains many bioactive compounds [9,10,11,12]. With technological advancements, techniques for isolating chemical compounds such as fractionation have emerged, facilitating the isolation of active compounds from natural extracts into their pure forms. The ethanol extract and active fraction resulting from this separation can be seen at the level of pharmacological activity, solubility, stability and bioavailability in the body [13]. Previous research stated that to increase the pharmacological activity of extracts and active fractions of herbal plants, developments in the delivery system can be carried out [14].

The antioxidant activity of quercetin and quercitrin found in *T. sinensis* is very strong, exhibit robust antioxidant activity. This potent antioxidant activity functions synergistically to stabilize *Reactive Oxygen Species* (ROS) during the process of photoaging [15, 16]. ROS is responsible for telomere shortening, autophagy, and stem cell fatigue, consequently triggering the aging process [17]. Aging is characterized by skin damage due to the impact of free radicals on biomolecules such as lipids, proteins (enzymes), or nucleic acids, leading to the emergence of fine lines and wrinkles on the face [18]. The wrinkle appearance and the decline in elasticity can be attributed to the progressive thinning of the dermis layer [19]. Dermal thinning (dermal atrophy) is a condition characterized by a reduction in the amount of *Extra Cellular Matrix* (ECM), particularly elastin and collagen, within the dermal layer. The production of elastin and collagen diminishes, leading to an overall increase in degradation as the skin ages. This results in structural alterations in elastin, collagen, and other ECM proteins [13]. ECM constitutes the outermost layer of the skin and is composed of fibroblasts and proteins (collagen and elastin) [20].

For individuals aged over 20, their skin experiences a 1% decline in elastin and collagen levels annually, underscoring the pivotal role of ECM in preserving skin elasticity [20]. The degeneration of ECM is associated with heightened activity of specific enzymes implicated in skin aging, including hyaluronidase, elastase, and collagenase. In vitro, the activity of elastase enzymes can be hindered through the utilization of the neutrophil elastase inhibitor screening kit. The inhibitory mechanism occurs via interaction with the active $Zn^{2+}$ site, which either cleaves the active enzyme (elastase) or binds it into an inactive complex form. The $Zn^{2+}$ site contributes to antioxidant and anti-aging processes by inhibiting *Reactive Oxygen Species* (ROS) as a co-factor that quenches free radicals in the cytoplasm [21]. To increase the pharmacological activity of the ethanol extract of surian leaves and ethyl acetate fraction of surian leaves as antiaging, a delivery system can be developed in the form of nanoparticles with natural polymers [15].

Nanoparticles with natural polymers such as chitosan, alginate, and pectin originate from natural sources and have received significant attention in the cosmetics industry. Chitosan is used in the cosmetics industry as an efficient and stable carrier, helping to increase the solubility and bioavailability of active substances [17, 22]. Alginate is used for controlled-release cosmetic formulations. Pectin is often used in the preparation of hydrophilic matrices for orodispersible delivery. The use of these natural polymers in the cosmetics industry reflects a drive towards the development of safer and more effective cosmetic delivery systems as well as an increased focus on environmentally friendly and biodegradable ingredients [18].

Although there are many nanoparticle studies using natural polymers that look at increasing the activity of various herbal plants, to date there has been no report that comprehensively investigates the effect of using natural polymers (chitosan and alginate) in ethanol extract of surian leaves and ethyl acetate fraction of surian leaves nanoparticles in increasing antiaging activity. In addition, to the best of our knowledge, there have been no studies that have looked at the activity of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) in inhibiting the elastase enzyme. Here, we extensively studied the effect or influence of natural polymers in the form of SLENP and EAFSLNP in increasing antiaging activity compared to the use of Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) alone. So this research can prove that the cosmetic delivery system in the form of nanoparticles with natural polymers (chitosan and alginate) from SLE and EAFSL is able to increase antiaging activity compared to using pure SLE and EAFSL. It is hoped that in the future SLENP and EAFSLNP will be developed into antiaging nanohydrogel preparations which will be followed by hemolytic testing to see their toxicity.

## 2. Method

### *2.1. Collection of plant materials*

Fresh leaves of surian (*Toona sinensis* (A. Juss) M. Roem) were collected in the month of October, 2022 from Rantau Suli Village, East Jangkat, Merangin Regency, Jambi Province, Indonesia and identified by a taxonomist (Dr. Silva Abraham) from the Directorate of Scientific Collection Management of the National Research and Innovation Agency in Central Jakarta, where voucher specimen (No. B-4601/II.6.2/DI.05.07/12/2022) was deposited. The fresh leaves were washed thoroughly to remove dirt and soil, then dried and stored at room temperature. These leaves were grinded and then kept in closed container and stored at room temperature until they will be used for the next process. Information about the plant, the location and date of collection were stated in the Directorate of Scientific Collection Management of the National Research and Innovation Agency in Central Jakarta. In this experimental research that authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

*2.2. Extraction and Fractionation of Surian Leaves*

Before extraction, simplicia was prepared. The surian leaves that have been taken are put into a holding container. The process begins with wet sorting with the aim of separating leaves from dirt such as soil, stems and stems that have been damaged. then washed with clean running water to remove impurities and then weighed 11 kg. Then the wet sorted leaves are chopped into the smallest pieces so that extraction process is faster because leaf surface area is large, then dried in an oven at 50ºC. Drying should not exceed a temperature of >70ºC, because it destroys the antioxidant compounds contained in surian leaves [11].

The extraction of crude Surian leaf material was performed using the maceration method with 70% ethanol as the solvent. The extraction process unfolded as follows: Approximately 5.98 kg of crude Surian leaf material was weighed and placed into a macerator. 70% ethanol was added until complete submersion of the crude Surian leaf material was achieved, in a ratio of 1 part crude Surian leaf powder (5.98 kg) to 10 parts 70% ethanol solvent (60 liters). The mixture was left undisturbed for 24 hours. The resulting liquid extract, totaling 45 liters of macerate, was collected. The macerator was then replenished with an equal quantity of 70% ethanol. The solvent used is ethanol because ethanol is a safe and non-toxic solvent [2]. This solvent can dissolve phytochemical compounds more optimally [3]. Ethanol solvent was chosen because ethanol is a solvent that is polar in its use, meaning that solvent can filter or extract compounds that are polar, semipolar and non polar, not toxic, able to mix with water and requires less heat for concentration, so that during the concentration process from liquid extract to thick extract, the time required is relatively less [8]. Ethanol is a solvent that can extract flavonoids, saponins, tannins, anthraquinones, terpenoids and alkaloids [23]. The extraction process occurred for 3 × 24 hours. The liquid extract obtained was concentrated using a rotary evaporator at a temperature of 50°C. Further concentration was achieved using a water bath at 50°C until a dense Surian leaf extract was obtained [24].

Fractionation of the Surian leaf extract was executed through the *Liquid-iquid Extraction* (LLE) method, utilizing three solvents differing in polarity: n-hexane, ethyl acetate, and water. The fractionation process was executed as follows: Approximately 50 g of the Surian leaf extract was weighed and ground with 400 mL of n-hexane in a mortar. The resultant filtrate was collected in a separatory funnel until the n-hexane solvent was fully employed. An equivalent amount of water was introduced into the separatory funnel, followed by 15 minutes of agitation, intermittently releasing air every 5 minutes. The mixture in the separatory funnel was left undisturbed for 24 hours, allowing complete separation of the two solvents. The n-hexane fraction was isolated from the water fraction. This separation process was reiterated until a nearly colorless n-hexane fraction was obtained. The same separatory funnel was employed for the ethyl acetate fraction, wherein an equal amount of ethyl acetate was added alongside water. Shaking and separation ensued as before. The n-hexane, ethyl acetate, and water fractions were concentrated using a rotary evaporator at 50°C. The subsequent concentration involved a water bath at 50°C, culminating in the acquisition of dense n-hexane, ethyl acetate, and water fractions of Surian leaves [25].

After producing Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves EAFSL), we continued testing non-specific characteristics of Simplicia Surian Leaves (SSL), SLE and EAFSL, namely determining the pH by weighing 0.5 g of each SSL, SLE and EAFSL dissolving it in 50 mL of distilled water in a glass beaker, then measure the pH with a pH meter which has previously been calibrated with a standard buffer solution (pH 4 and pH 7). Measurements are carried out on newly created and saved SSL, SLE and EAFSL [11]. Not only was non-specific characterization carried out but also qualitative phytochemical screening was carried out using the Thin Layer Chromatography (TLC) test [11].

2.3 *Identification of phytochemicals by LCMS/MS in SLE and EAFSL*

Identifying secondary metabolites in SLE and EAFSL were conducted using LCMS/MS. The LCMS/MS was carried out in the Advanced Characterization Laboratory, National Research and Innovation Agency (BRIN), Cibinong, Indonesia. The sample was filtered through a 0.2 μm PTFE membrane. The analysis using LCMS UHPLC Vanquish Tandem Q Exactive Plus Orbitrap HRMS

ThermoScientific, Accucore C18 column, 100 x 2.1 mm, 1.5 m (ThermoScientific), flow rate 0, 2 mL/min. The eluent was water + 0.1% formic acid (A) and acetonitrile + 0.1 % formic acid (B), gradient 0-1 min (5% B), 1-25 min (5-95% B), 25-28 minutes (95%B), 28-30 minutes (5%B). The column temperature was 3$^{o}$C with an injection volume of 2.0 µL. The analysis used a molecular weight interval of 100-1500 m/z and positive ionization mode. The database used for the qualitative identification of the chemical constituents in the fraction was Compound Discoverer 3.2. The quantitative analysis was calculated from the peak area of identified compounds.

*2.4. Determination of Elastase Enzyme Activity*

The assessment of Elastase enzyme activity was carried out using the *Neutrophil Elastase Inhibitor Screening Kit Method*. The process commenced with the preparation of solutions as detailed in the following section [26].

*a. Test Compounds*

Surian Leaves Extract (SLE), n-Hexane Fraction Surian Leaves (nHFSL), Ethyl Acetate Fraction Surian Leaves (EAFSL), and Water Fraction Surian Leaves (WFSL) were prepared at concentrations of 0.6 mg/mL, 1 mg/mL, 1.4 mg/mL, and 1.8 mg/mL, respectively, utilizing DMSO as the solvent. Each test compound was diluted fourfold to attain the desired final assay concentrations using an assay buffer. Then, 25 microliters of each diluted test compound were added to distinct wells of a 96-well plate [26].

*b. Inhibitor Control, Enzyme Control, Background Control*

Inhibitor control stock 1:25 was diluted with assay buffer. Subsequently, 25 µl of the diluted inhibitor control were added to separate wells of the plate. To establish separation in a well of the plate, 25 µl of assay buffer were added. It is noteworthy that enzyme control should be included in every test run. To create separation in a well of the plate, 75 µl of assay buffer were added [26].

*c. Preparation of Enzyme Solution*

For each well (excluding the background control well), a 50 µl solution of neutrophil elastase was prepared according to the following table.

| Reagent | Volume |
|---|---|
| Assay Buffer | 48 µl |
| Neutrophil Elastase Stock Solution | 2 µl |

Subsequently, 50 µl of the diluted neutrophil elastase solution were added to each well labeled as test compounds, inhibitor control, and enzyme control. Importantly, this solution was not added to the background control well. Thorough mixing was carried out followed by an incubation period of 5 min at 37°C. Throughout the incubation, the plate was shielded from light. The total volume for all wells in this step, encompassing test compounds (s), inhibitor control, enzyme control, and background control, amounted to 75 µl [26].

*d. Reaction Mixture*

Reagents were prepared as required for the intended number of tests. 25 µl of the reaction mixture were prepared for each well, following the details in the table below.

| Reagent | Working Reagent |
|---|---|
| Assay Buffer | 23 µl |
| Substrate | 2 µl |

The reaction mixture, totaling 25 µl, was introduced into every reaction well, encompassing the test compound, inhibitor control, enzyme control, and background control. Thorough mixing was conducted, followed by an immediate measurement of the plate [26].

*e. Measurement and Calculation*

Fluorescence (RFU) was measured at λEx = 400 nm / λ Em = 505 nm within a microplate. Readings occurred under kinetic conditions for 30 min at 37°C. The plate was covered to shield it from light during incubation. Fluorescence readings were taken every certain minute (0, 10, 20, and 30 min). After the absorbance values were obtained, calculations were performed to determine the percentage of elastase enzyme activity against the test compound and the percentage of elastase enzyme inhibition against the test compound, using the following formulas:

$$\%Activity = \frac{\Delta\ RFU\ Test\ Compound}{\Delta\ RFU\ Enzyme\ Control} \times 100\%$$

$$\%\ Inhibition = 1 - \frac{\Delta\ RFU\ Test\ Compound}{\Delta\ RFU\ Enzyme\ Control} \times 100\%$$

Subsequently, a profile graph depicting NE activity at various test compound concentrations and a graph illustrating the correlation between the percentage of activity values and the percentage of inhibition values of the test compound were generated [26].

*2.4. Preparation of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP)*

To create and obtain stable nanoparticles, one of the best methods is the ionic gelation method using a positive natural polymer and a negatively charged crosslinking agent [27, 57]. In this study, Sodium Tri Poly Phosphate (NaTPP) was used as an anionic crosslinking agent and chitosan as a cationic polymer. Chitosan, amounting to 200 mg, was weighed and dissolved in 200 ml of acetic acid (chitosan was incrementally added to the 2% acetic acid solution, undergoing stirring with a magnetic stirrer until complete dissolution), and pH adjustment to pH 4 was carried out. The use of 2% acetic acid as a chitosan solvent is very necessary, where chitosan is very easily soluble in acetic acid solvent. In this case, so that the pH is not too acidic, NaOH is added little by little to keep the pH at pH 4, because chitosan is stable at this pH. 50 mg of alginate was weighed, dissolved in 50 ml of distilled water, homogenized through stirring, and the pH was attuned to pH 6.5. NaTPP, quantifying 28 mg, was weighed, combined with 40 ml of distilled water, stirred to homogeneity, and pH was adjusted to pH 3.6 [28, 30].

The dissolved ethanol extract of Surian leaves was combined with chitosan (mass 1). The micropump apparatus, consisting of 2 magnetic stirrers, was prepared. On the left side, there was a solution of SLE/EAFSL added chitosan (mass 1), and on the right side, a solution of NaTPP (mass 2) was present. The micropump principle involved the gradual transfer of mass 1 drop by drop into mass 2 until all of mass 1 was transferred to mass 2. After the complete transfer of mass 1, the micropump was deactivated. Mass 3 (extract + chitosan + NaTPP) was transferred to another magnetic stirrer, and then sodium alginate was added drop by drop at a speed of 1000 RPM for 1 hour [29, 30].

Nanoparticles were crafted through the employment of the ionic gelation technique, involving chitosan which is a polycation polymer which has a positive charge from the amine group ($-NH^{3+}$) and alginate polymers with a concentration of 1% each, namely 1 gram weight of chitosan and alginate dissolved in 100 ml of a suitable solvent, alongside an Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) concentration of 20 mg/20 ml = 1 mg/ml = 1000 ppm was fashioned by blending 20 ml of ethanol with the quantified Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) (homogenization ensued), and employing the cross-linker NaTPP (Sodium Tripoly Phosphate) which is a polyanion which has a negatively charged phosphate group ($PO4^{3-}$).

This process occurs through an electrostatic mechanism, where the polyphosphate ion from NaTPP interacts with the positively charged amino group in chitosan and produces a nano-sized gel structure. The use of Alginate as an anionic polymer can produce ionic gelation with divalent cations such as calcium (Ca2+) which will eventually produce a strong three-dimensional network [27,28]. To control the size and stability of the resulting nanoparticles, parameters such as the chitosan-NaTPP ratio, stirring speed, and pH are optimized. The formed nanoparticles are characterized physicochemically starting from particle size, PDI, zeta potential, Adsorption Efficiency [27, 28, 29].

### *2.5. Characterization of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP)*

The characterization of nanoparticles was performed to ensure the quality of the produced nanoparticles. Indicators of nanoparticle quality included particle size, Poly Dispersity Index (PDI), zeta potential value, and resulting Encapsulation Efficiency (EE) [30].

*a. Determination of Particle Size, Poly Dispersity Index (PDI), and Zeta Potential*

Particle size and PDI index were determined using a Particle Size Analyzer (PSA) utilizing the Dynamic Light Scattering (DLS) method. Meanwhile, zeta potential was assessed using a Zetasizer Zen3600, employing a 10-fold dilution of the sample in an aqueous medium at room temperature [31].

*b. Encapsulation Efficiency (EE)*

20 mg/20 ml of NPES underwent centrifugation at 12000 rpm for 45 minutes at 40°C. The concentration of free drug (in the supernatant, 1.0 ml) was determined by quantifying quercetin content from the supernatant using a UV-Vis spectrophotometer at the pre-determined maximum wavelength (426 nm) [26].

$$\%\ EE = \frac{(Quercetin\ content\ -\ ZA\ content\ in\ the\ supernatant)}{Total\ ZA\ content} \times 100\%$$

c. Nanoparticle Morphology

The examination of nanoparticle morphology was conducted using negative staining Transmission Electron Microscopy (TEM). In summary, a drop of the sample, diluted with water to approximately 0.05 mg/mL, was placed on a Formvar copper mesh 200. It was allowed to adsorb, and the excess was removed using filter paper. A drop of uranyl acetate 2% solution (w/v) was added and allowed to contact the sample for 5 minutes. Excess water was removed, and the sample was air-dried before vesicles were observed using TEM operating at 200 KV.

### *2.6. Data analysis*

The experimental results included three replications, and the data were expressed as mean ± standard deviation (SD). The data were analyzed by an ANOVA ($p < 0.05$) using SPSS, and $p < 0.05$ was considered to be statistically significant.

## 3. Results and Discussion

We are currently in a new era of developing bioactive compounds from herbal plants, which play a pivotal role in skin care, aiding in enhancing skin appearance and delaying premature aging [26]. With technological advancements, bioactive compounds derived from *T. sinensis* herbal plants, particularly for anti-aging purposes, have been isolated using several methods, such as maceration extraction and liquid-liquid fractionation [8]. The Surian Leaves Extract (SLE) yield was 33.278%, while the Ethyl Acetate Fraction Surian Leaves (EAFSL) yield was 0.221%. The EAFSL yield produced is small because there are many losses during the fractionation process. From the research results of Simplicia Surian Leaves (SLS), SLE and EAFSL that have been

produced have an acidic pH, namely in the pH range 4-6, this can be proven by the distinctive aroma of surian leaves which smells very sour. SLS has a pH of 5.176 ± 0.0378, SLE has a pH of 4.463 ± 0.5951, EAFSL has a pH of 6.153 ± 0.2757. The acidity level of an SLE and EAFSL also influences the content of the active compounds [11]. The availability of bioactive compounds from these herbal sources holds the promise of being further optimized through nanotechnology-based delivery systems. This advancement is expected to lead to the creation of superior anti-aging cosmetic products that excel in terms of safety and the targeted benefits for users [31,32].

Based on the results of Thin Layer Chromatography (TLC) identification, the stationary phase used was F 254 silica gel plates and the mobile phase or eluent chloroform and methanol were used in a ratio of 9:1. The stain was viewed under UV light at λ 254 nm and λ 366 nm with the marker (control) used being Quercetin which has antioxidant activity which works synergistically towards the ability to stabilize the role of Reactive Oxygen Species (ROS) in the photoaging process (antiaging activity). The identification results from TLC Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) produced Rf values of 0.4 (Quercetin), Rf 0.5 (flavonoids), Rf 0.3 (phenolic). This shows that SLE and EAFSL contain quercetin because they have the same Rf as the quercetin marker.

### *3.1. Identification of phytochemicals by LCMS/MS in SLE and EAFSL*

The LCMS/MS analysis of Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) showed the presence of several identified major compounds. The percentage of all peak areas was calculated, then the identified peak area higher than 1% was considered the major compound in SLE and EAFSL.

Gallic acid was the highest compound percentage in SLE. Quercetin was the highest compound percentage in EAFSL. Flavonoids are identified abundantly in SLE and EAFSL. The major compounds in the SLE and EAFSL were phenolics and flavonoids. The major compounds in SLE and EAFSL based on LCMS/MS analysis such is Table 1 below,

Table 1. The major compounds in Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL)

| Sample | Molecular Formula | Compound Name |
|---|---|---|
| Surian Leaves Extract (SLE) | $C_7H_6O_5$ | Gallic acid (1) |
| | $C_{14}H_6O_8$ | Ellagic acid (2) |
| | $C_{15}H_{10}O_7$ | Quercetin (3) |
| | $C_{21}H_{20}O_{11}$ | Quercitrin (4) |
| | $C_{15}H_{10}O_5$ | Apigenin (5) |
| Ethyl Acetate Fraction Surian Leaves (EAFSL) | $C_{15}H_{10}O_7$ | Quercetin (1) |
| | $C_{15}H_{10}O_6$ | Kaempferol (2) |
| | $C_7H_6O_5$ | Gallic acid (3) |
| | $C_{21}H_{20}O_{11}$ | Quercitrin (4) |
| | $C_{14}H_6O_8$ | Ellagic acid (5) |

Several scientific studies have been conducted to assess the anti-aging effects of bioactive compounds from natural sources [25]. The results of the in vitro determination of anti-elastase enzyme activity on Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) using the neutrophil elastase inhibitor screening kit method indicated that *T. sinensis* contains secondary metabolites (flavonoids and phenolics) that exhibited excellent elastase inhibition against the photoaging process, surpassing other fractions. Based on the research findings, the total flavonoid content in SLE was 33.19%, and the total phenolic content was 153.10%. Hence, its influence is highly significant in combating skin aging as an antioxidant, skin brightener, and sunblock agent. SLE and EAFSL can facilitate skin rejuvenation, resulting in a more

youthful, healthier, radiant appearance, and anti-aging effects. The development of SLE and EAFSL nanoparticles could yield natural skincare products that are rapidly absorbed by the skin's surface layers [33,34,35].

### *3.2. Determination of Elastase Enzyme Activity*

#### *3.2.1. Elastase Enzyme Activity of Surian Leaves Extract (SLE)*

The inhibition effect on the elastase enzyme was evaluated in vitro using the *Neutrophil Elastase Inhibitor Screening Kit Method*. The *Neutrophil Elastase Inhibitor Screening Kit Method* aims to see potential of inhibitors in inhibiting activity of the elastase enzyme in vitro. This process begins with preparation of an elastase enzyme solution which is mixed with test compound as an inhibitor. The activity of elastase enzyme is observed through changes in fluorescence that occur on specific elastase substrates. This is an important first step in the development of drugs aimed at treating conditions associated with excessive elastase enzyme activity, such as premature aging [20]. The results are presented in Table 2, Figures 1, and Figure 2. SLE demonstrated dose-dependent elastase inhibition when incubated for 15 minutes before substrate addition. Changes in absorbance were measured at 405 nm every 0, 10, 20, and 30 min, resulting in the % inhibition and % activity values presented in the table below.

**Table 2.** % Activity and % Inhibition of Surian Leaves Extract (SLE)

| No. | Sample | Fluorescence SLE ±SD | Fluorescence Enzyme Control ±SD | Activity (%)±SD | Inhibition (%) ±SD | RSD (precison) |
|---|---|---|---|---|---|---|
| 1. | SLE 1800 µg/mL | 0.08±0.01 | 0.11±0.01 | 75.86±2.58 | 24.14±2.58 | 0.10 |
| 2. | SLE 1400 µg/mL | 0.08±0.01 | 0.11±0.01 | 69.82±4.31 | 30.18±4.31 | 0.14 |
| 3. | SLE 1000 µg/mL | 0.09±0.01 | 0.11±0.01 | 80.17±3.45 | 19.83±3.45 | 0.17 |
| 4. | SLE 600 µg/mL | 0.09±0.01 | 0.11±0.01 | 83.42±2.61 | 16.58±2.61 | 0.15 |

In the table above, it can be seen that increasing the concentration of Surian Leaf Extract (EDS) does not affect the elastase inhibition activity. EDS with a concentration of 1400 µg/mL has the highest % inhibition of 30.18% compared to EDS with a concentration of 1800 µg/mL which only has a % inhibition of 24.14%. This is due to the saturation point, where all active sites of the enzyme have been bound to the elastase inhibitor, so that increasing the concentration no longer has a significant impact on elastase inhibition [20], as well as the comparison between time and % elastase inhibition as seen in the graph below:

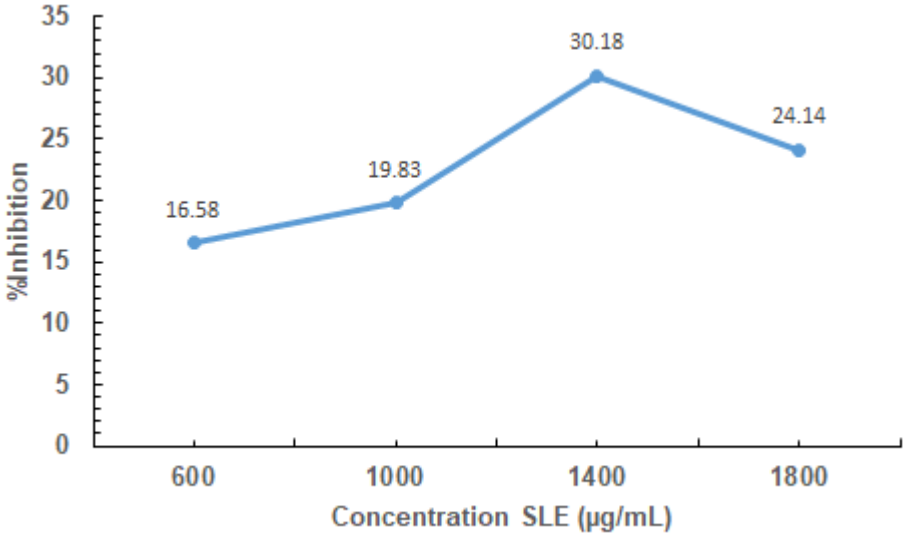



**Figure 1.** Relationship between time and % value inhibition elastase of Surian Leaves Extract (SLE)

The graph above shows that increasing the concentration of Surian Leaf Extract (SLE) does not affect the inhibitory power of SLE on the elastase enzyme. SLE with a concentration of 1400 µg/mL has the highest inhibitory power of 30.18% compared to SLE with a concentration of 1800 µg/mL which only has an inhibitory power of 24.14%. This is due to the saturation point, where all active sites of the enzyme have been bound to the inhibitor, so that increasing the concentration no longer has a significant impact on inhibiting the elastase enzyme [36].

The determination of anti-elastase activity was conducted to assess Surian Leaves Extract (SLE) capability to degrade elastase. Concerning the process of skin aging, elastase inhibitors hold the potential for addressing skin elasticity loss or wrinkle formation [26,37]. Elastase enzyme is a serine protease with the ability to degrade elastin and hydrolyze nearly all *Extra Cellular Matrix* (ECM) proteins within the connective tissue, including collagen, elastin, and fibronectin. Inhibiting elastase activity can serve as a target to preserve elastin proteins, counter *Reactive Oxygen Species* (ROS), mitigate photoaging effects, and prevent ECM structural damage [26,38].

The mechanism of elastase enzyme inhibition can occur through interaction with the active $Zn^{2+}$ site, which either cleaves the active enzyme (elastase) or binds it into an inactive complex form. The $Zn^{2+}$ site participates in antioxidant and anti-aging processes to inhibit reactive oxygen species (ROS), which act as cofactors that eliminate free radicals in the cytoplasm [20]. If the enzyme has reached saturation point then all active sites of the enzyme have been bound to the elastase inhibitor and there is no further increase in the reaction rate even if the concentration is increased. The RFU value is still stable and EDS can still be tested if there is a decrease in the RFU value but EDS is still able to have elastase inhibition activity even under maximum working conditions [39], this can be seen in the NE (Neutrophil Elastase) Activity profile image below:

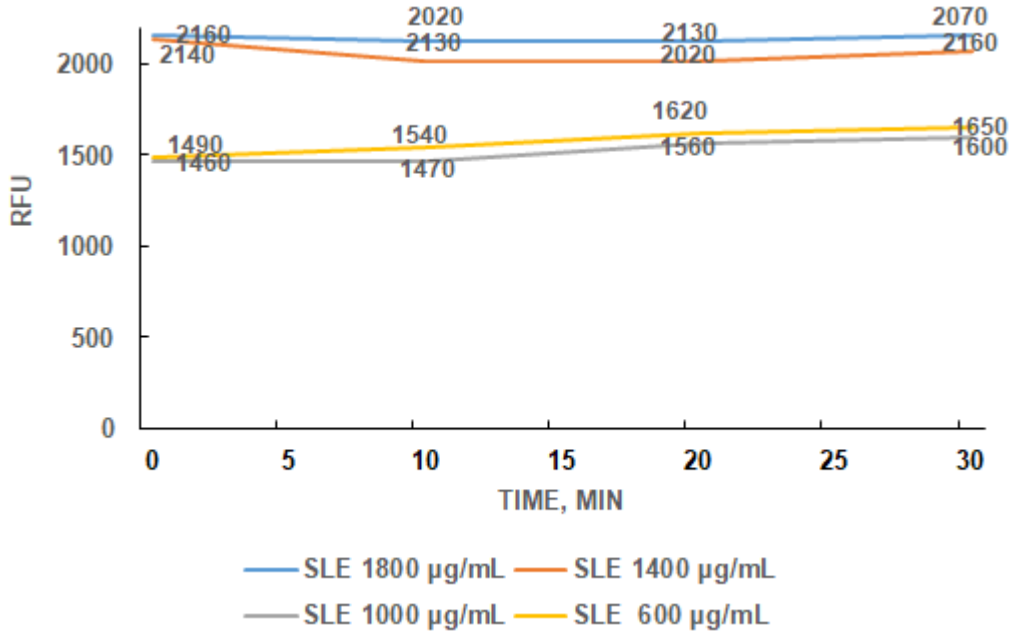



**Figure 2.** NE (Neutrophil Elastase) Activity Profile with Various Surian Leaves Extract (SLE)

RFU (*Relative Fluorescence Unit*) is a measurement unit used in analyses involving fluorescence detection. Fluorescence is detected using charged devices, where labeled fragments, separated in capillaries through electrophoresis, are energized by laser light and pass through a detection window. The "RFU peak" is the relative maximum point along the analyzed data graph. The height of an RFU can range from 0 to several thousand [39]. The Relative Fluorescence Unit (RFU) value is related to the activity of the elastase enzyme, where the higher the RFU value, the higher the activity of the elastase enzyme and vice versa. If the RFU value decreases, the inhibitory power of the extract against the elastase enzyme increases, because the presence of active inhibitory compounds in the Surian Leaf Extract (EDS) reduces the ability of elastase to break down

fluorescent substrates, so that the resulting fluorescence signal becomes lower and vice versa the RFU value increases because the inhibitory power of the extract against the elastase enzyme decreases, so that more fluorescent substrates are broken down and produce stronger and more fluorescence signals [39].

*3.2.2. Elastase Enzyme Activity of Ethyl Acetate Fraction Surian Leaves (EAFSL)*

The inhibition effect on the elastase enzyme was evaluated in vitro using the neutrophil elastase inhibitor screening kit method. The results are presented in Table 3, Figures 3, and Figure 4. The Ethyl Acetate Fraction Surian Leaves (EAFSL) demonstrated dose-dependent elastase inhibition when incubated for 15 min before substrate addition. The concentration/dose of EAFSL given in study directly affects length of incubation time required to observe elastase enzyme response. The higher dose of EAFSL, the shorter the incubation time usually requires because enzyme inhibitory response can occur more quickly. In addition, length of measurement time after incubation is also important, because it provides information about the stability of elastase enzyme inhibition of compound being tested over time. Changes in absorbance were measured at 405 nm every 0, 10, 20, and 30 min, resulting in the % inhibition and % activity values presented in the table below.

**Table 3.** % Activity and % Inhibition of Ethyl Acetate Fraction Surian Leaves (EAFSL)

| No. | Sample | Fluorescence EAFSL±SD | Fluorescence Enzyme Control±SD | Activity(%)±SD | Inhibition(%) ±SD | RSD (precision) |
|---|---|---|---|---|---|---|
| 1. | EAFSL 1800 µg/mL | 0.09±0.00 | 0.11±0.01 | 77.58±3.45 | 22.42±3.45 | 0.15 |
| 2. | EAFSL 1400 µg/mL | 0.10±0,01 | 0.11±0.01 | 83.62±4.31 | 16.38±4.31 | 0.26 |
| 3. | EAFSL 1000 µg/mL | 0.10±0.00 | 0.11±0.01 | 84.48±2.59 | 15.52±2.59 | 0.16 |
| 4. | EAFSL 600 µg/mL | 0.09±0.00 | 0.11±0.01 | 81.03±0.86 | 18.97±0.86 | 0.04 |

In the table above, it can be seen that the higher the concentration of FEADS, the higher the % inhibition of elastase, but this is not always significant with a FEADS concentration of 600 µg/mL with a FEADS concentration of 1400 µg/mL and 1000 µg/mL, where FEADS with a concentration of 600 µg/mL has the highest inhibition of 18.97% compared to FEADS concentration of 1400 µg/mL only has an inhibition of 16.38% and FEADS concentration of 1000 µg/mL only has an inhibition of 15.52 mg/mL, this is because the solubility of the active compound at a concentration of 1400 µg/mL is less soluble, causing precipitation and reducing the number of molecules to interact with the enzyme. Finally, it causes the inhibition of elastase to be smaller compared to FEADS, the small concentration has perfect and homogeneous solubility and produces a large number of molecules in inhibiting the elastase enzyme [39]. This can be seen clearly in the image below:

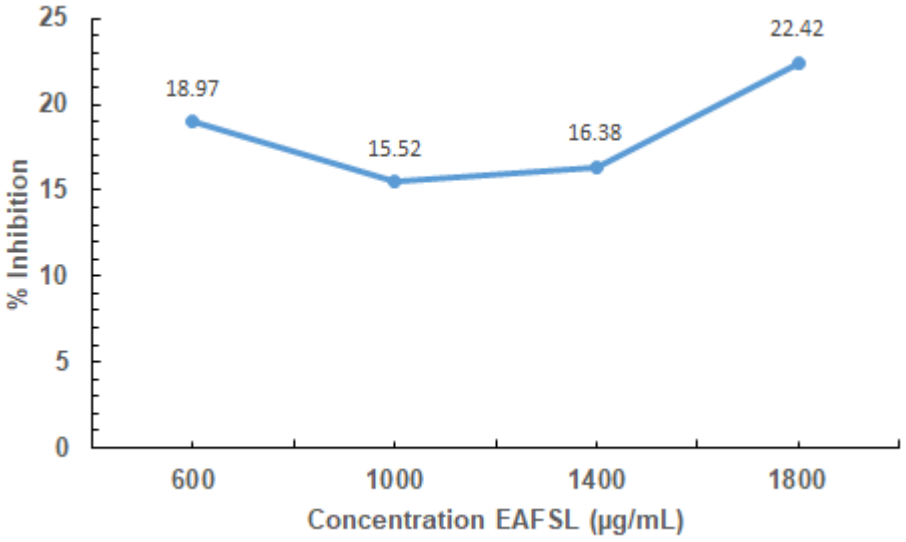



**Figure 3.** Relationship between Time and % Value Inhibition Elastase of Ethyl Acetate Fraction Surian Leaves (EAFSL)

In the graph above, it can be seen that increasing the concentration of Ethyl Acetate Fraction Surian Leaves (EAFSL) increases the inhibition of the elastase enzyme, but this is not always significant with an EAFSL concentration of 600 µg/mL with an EAFSL concentration of 1400 µg/mL and 1000 µg/mL, where EAFSL with a concentration of 600 µg/mL has the highest inhibition of 18.97% compared to EAFSL concentration of 1400 µg/mL only has an inhibition of 16.38% and EAFSL concentration of 1000 µg/mL only has an inhibition of 15.52 mg/ml, this is because the solubility of the active compound at high concentrations is less soluble, causing precipitation and reducing the number of molecules to interact with the enzyme. Finally, it causes its inhibition of the elastase enzyme to be smaller compared to EAFSL with a small concentration that has perfect and homogeneous solubility and produces a large number of molecules in inhibiting the elastase enzyme [39].

The graph illustrates that Ethyl Acetate Fraction Surian Leaves (EAFSL) exhibits the same elastase inhibition activity as Surian Leaves Extract (SLE). Thus, it can be inferred that EAFSL contains bioactive compounds capable of stabilizing *Reactive Oxygen Species* (ROS) during the photoaging process [15]. Based on the research findings, the phytochemical compounds present in EAFSL, with anti-aging activity, belong to the flavonoid and phenolic groups, as determined by *Thin Layer Chromatography* (TLC) with $R_f$ values of 0.607 (flavonoid) and 0.391 (phenolic) [11].

Elastase is a protease enzyme that reduces elastin levels by breaking peptide bonds. Consequently, inhibiting elastase activity within the dermis layer can help maintain skin elasticity. Measurement of elastase enzyme inhibition involves tracking the kinetic conversion of N-Succinyl-(Ala)-3-Nitroanilide to p-nitroaniline (substrate) using a UV-Vis spectroscopy at a 405 nm wavelength, utilizing *Neutrophil Elastase* (NE) as the enzyme. Research results indicated that EAFSL acts as an elastase enzyme inhibitor, contributing to the preservation of skin elasticity [40, 41]. This underscores the potential of both SLE and EAFSL for developing natural-based cosmetic nanoparticle formulations containing active compounds.

Flavonoid compounds extracted and fractionated from *T. sinensis* consist of the pure compound quercetin [10]. Quercetin exhibits robust antioxidant activity and inhibits elastase, enabling the restoration of elasticity after mice were exposed to elastase treatments once a week for four weeks, followed by administering 0.5 mg of quercetin for 10 days [42]. Quercetin is the most effective inhibitor of elastase release [41]. This antioxidant activity synergistically works to stabilize *Reactive Oxygen Species* (ROS) during the photoaging process. The quercetin content in Ethyl Acetate Fraction Surian Leaves (EAFSL) varies with the concentration of EAFSL used in testing, whereby higher concentrations result in increased quercetin levels. This correlation between EAFSL concentration and quercetin content aligns with the elevation of EAFSL concentration,

leading to an increase in the *Relative Fluorescence Unit* (RFU) value. This is observed in the NE (neutrophil elastase) activity profile depicted below :

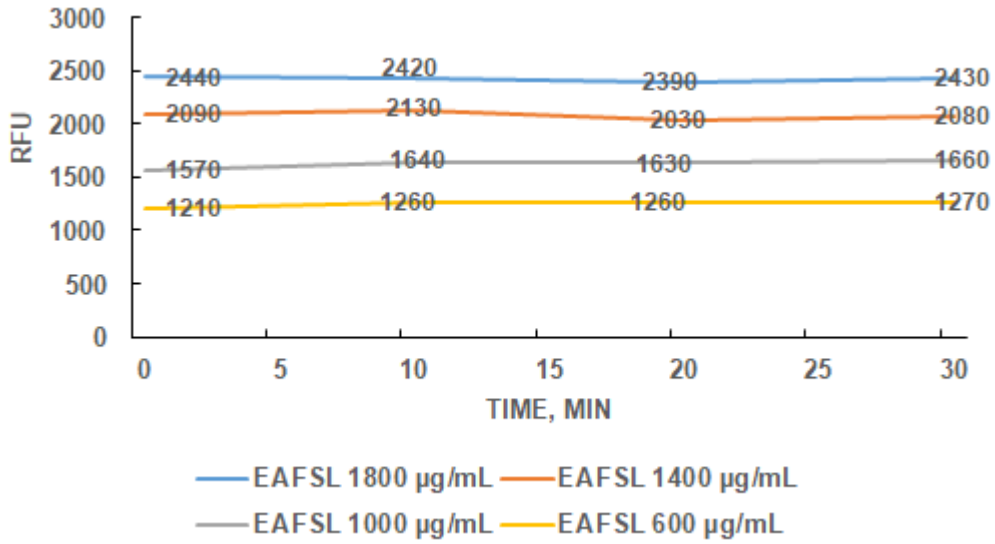



**Figure 4.** NE Activity Profile with Various Ethyl Acetate Fraction Surian Leaves (EAFSL)

*3.2.3. Elastase Enzyme Activity of n-Hexane Fraction Surian Leaves (nHFSL)*

The inhibitory effect on elastase enzyme activity was investigated in vitro using the neutrophil elastase inhibitor screening kit method, and the results are presented in Table 4, Figures 5, and Figure 6. The nHFSL exhibits elastase inhibition in a dose-dependent manner, with a 15 min incubation period before introducing the substrate. Changes in absorbance were measured at 405 nm at intervals of 0, 10, 20, and 30 min, yielding the % inhibition and % activity as outlined in the table below.

**Table 4.** % Activity and % Inhibition of n-Hexane Fraction Surian Leaves (nHFSL)

| No. | Sample | Fluorescence nHFSL±SD | Fluorescence Enzyme Control ±SD | Activity (%)±SD | Inhibition (%)±SD | RSD (precision) |
|---|---|---|---|---|---|---|
| 1. | nHFSL 1800 µg/mL | 0.04±0.01 | 0.11±0.01 | 36.20±4.31 | 63.80±4.31 | 0.06 |
| 2. | nHFSL 1400 µg/mL | 0.11±0.01 | 0.11±0.01 | 95.68±6.89 | 4.32±6.89 | 1.59 |
| 3. | nHFSL 1000 µg/mL | 0.10±0.00 | 0.11±0.01 | 87.06±2.58 | 12.94±2.58 | 0.19 |
| 4. | nHFSL 600 µg/mL | 0.12±0.01 | 0.11±0.01 | 99.13±6.03 | 0.97±6.03 | 6.21 |

From the provided table, it is evident that the elastase inhibition activity of n-Hexane Fraction Surian Leaves (nHFSL) becomes more pronounced with higher concentrations of nHFSL, while the percentage of elastase enzyme activity diminishes with increasing nHFSL concentration. However, the generated data lacks significance; specifically, nHFSL 1400 µg/mL displays a smaller percentage inhibition than nHFSL 1000 µg/mL. Furthermore, it exhibits a wider range of percentage inhibition across concentrations compared to the substantial percentage inhibition results seen in Surian Leaves Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL). Additionally, at a concentration of 600 µg/mL, nHFSL demonstrates minimal inhibitory potency at only 0.97%, unlike SLE and EAFSL, which maintain considerable inhibition ranging from 16.58% to 18.97%. This is because nHFSL does not contain chemical compounds that can provide an antiaging effect, where nHFSL, through chemical screening test results, contains tannins, monoterpenes and quinones which have antibacterial activity [11]. This is graphically depicted below.

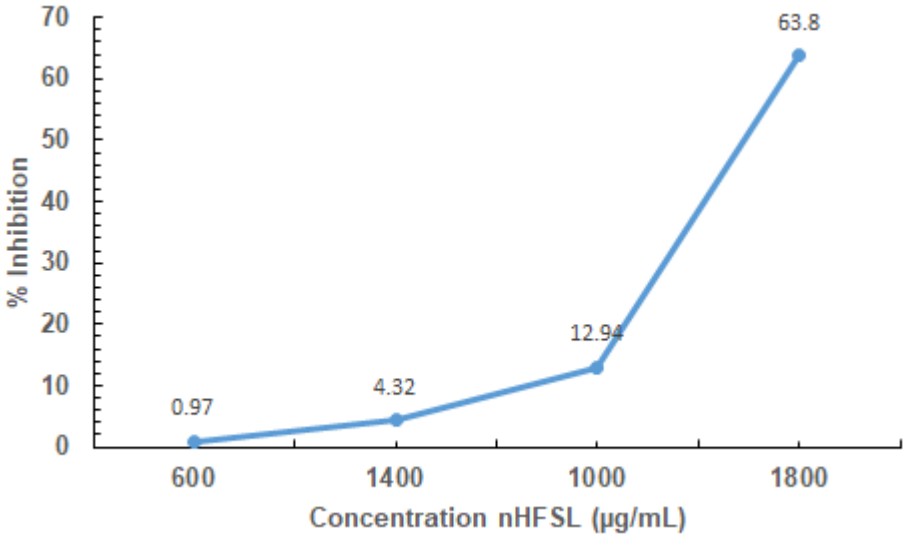



**Figure 5.** Relationship between Time and % Value Inhibition Elastae of n-Hexane Fraction Surian Leaves (nHFSL)

The graph above shows that the higher the concentration of n-Hexane Fraction Surian Leaves (nHFSL), the stronger the inhibition of the elastase enzyme. nHFSL with a concentration of 1800 µg/mL has the highest inhibition of 63.8% compared to nHFSL concentrations of 600 µg/mL; 1400 µg/mL and 1000 µg/mL only have an inhibition of 0.97%; 4.32% and 12.94%. This is because at high concentrations many bioactive compounds are found in nHFSL that can inhibit the elastase enzyme compared to nHFSL with low concentrations [43].

The percentage of elastase inhibition in FnHDS is not significant in value from various concentrations compared to EDS and FEADS, this is because the flavonoid and phenolic content is smaller in FnHDS compared to the total flavonoid and phenolic content which is higher in FEADS. FEADS contains the highest total flavonoid content compared to EDS and FnHDS [44]. The difference in the percentage of inhibition of FnHDS 1800 µg/mL is very far compared to FnHDS 1000 µg/mL, this is because FnHDS 1800 µg/mL has more interactions with the target enzyme so that it inhibits its activity more effectively, because the number of inhibitor molecules available to bind the enzyme increases with higher concentrations and reduces the level of substrate breakdown and vice versa [39].

The outcomes of phytochemical screening demonstrated that both SLE and EAFSL contain flavonoids and polyphenols, whereas nHFSL consists of saponins, steroids, and terpenoids, with only trace amounts of flavonoids and phenolics. According to the research findings, SLE exhibits a total flavonoid content of 33.19 mg/g and a total phenolic content of 153.10 mg/g. These factors contribute to the robust anti-aging activity of SLE in elastase enzyme inhibition. Polyphenols play a pivotal role in restraining elastase activity [46]. Plant-derived polyphenols have been scientifically proven to counteract free radicals, which act as triggers for the skin aging process [45,47, 48]. The *Neutrophil Elastase* (NE) activity of nHFSL at various concentrations is depicted in the figure below.

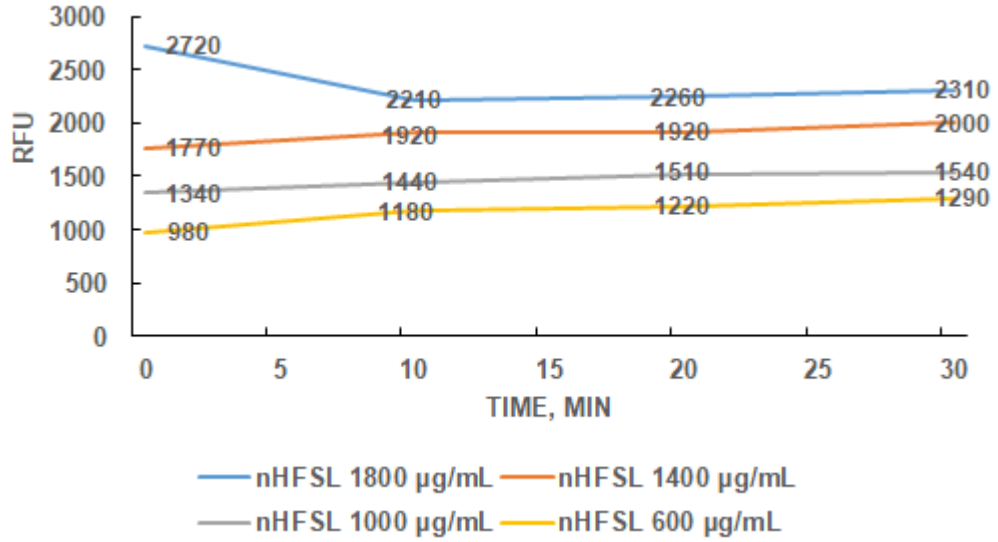



**Figure 6.** NE Activity Profile with Various n-Hexane Fraction Surian Leaves (nHFSL)

In the Figure 6, it can be explained that the Neutrophil Elastase Activity from FnHSL does not show a good graph because there is a very high increase in the RFU value in FnHSL concentration of 1800 µg/mL so that the results seen are not significant. While the NE Activity profile seen in ESL and FEASL has a significant graph, where the increasing concentration of ESL and FEASL will also increase the RFU (Relative Fluorescence Unit) value. In addition, the increase in the RFU value from minute 0 to minute and decreased significantly from minute 10 to minute in FnHSL 1800 µg/mL is because at minute 0, the fluorescent substrate is still available to be degraded by the enzyme, so that the inhibition of the elastase enzyme is getting weaker. While at 10 minutes, most of the substrate has been degraded and produces a lower fluorescence signal, so that elastase inhibition is getting higher [46].*3.2.4. Elastase Enzyme Activity of Water Fraction Surian Leaves (WFSL)*

The inhibition of elastase enzyme activity was studied in vitro using the neutrophil elastase inhibitor screening kit method, and the results are presented in Table 5, Figures 7, and Figure 8. The Water Fraction Surian Leaves (WFSL) exhibits dose-dependent elastase inhibition, with a 15-minute incubation period before introducing the substrate. Changes in absorbance were measured at 405 nm at intervals of 0, 10, 20, and 30 minutes, yielding % inhibition and % activity values as outlined below.

**Table 5.** % Activity and % Inhibition of Water Fraction Surian Leaves (WFSL)

| No. | Sample | Fluorescence EFSL±SD | Fluorescence Enzyme Control±SD | Activity (%)±SD | Inhibition (%)±SD | RSD (precision) |
|---|---|---|---|---|---|---|
| 1. | WFSL 1800 µg/mL | 0.06±0.21 | 0.11±0.01 | 55.17±3.31 | 44.83±3.31 | 0.07 |
| 2. | WFSL 1400 µg/mL | 0.10±0.01 | 0.11±0.01 | 87.06±5.62 | 12.94±5.62 | 0.43 |
| 3. | WFSL 1000 µg/mL | 0.11±0.00 | 0.11±0.01 | 92.24±3.44 | 7.76±3.44 | 0.44 |
| 4. | WFSL 600 µg/mL | 0.11±0.01 | 0.11±0.01 | 96.55±5.17 | 3.45±5.17 | 1.49 |

In the table above, it can be seen that the higher the concentration of Surian Leaf Water Fraction (SLWF), the higher the elastase inhibition. SLWF with a concentration of 1800 µg/mL has the highest % elastase inhibition of 44.83% compared to SLWF concentrations of 600; 1000; 1400 µg/mL only has % elastase inhibition of 3.45%; 7.76% and 12.94%. This is because at high concentrations, many bioactive compounds are found in SLWF that can inhibit the elastase enzyme compared to FADS with low concentrations [48]. This can be seen clearly in the Figure 7:

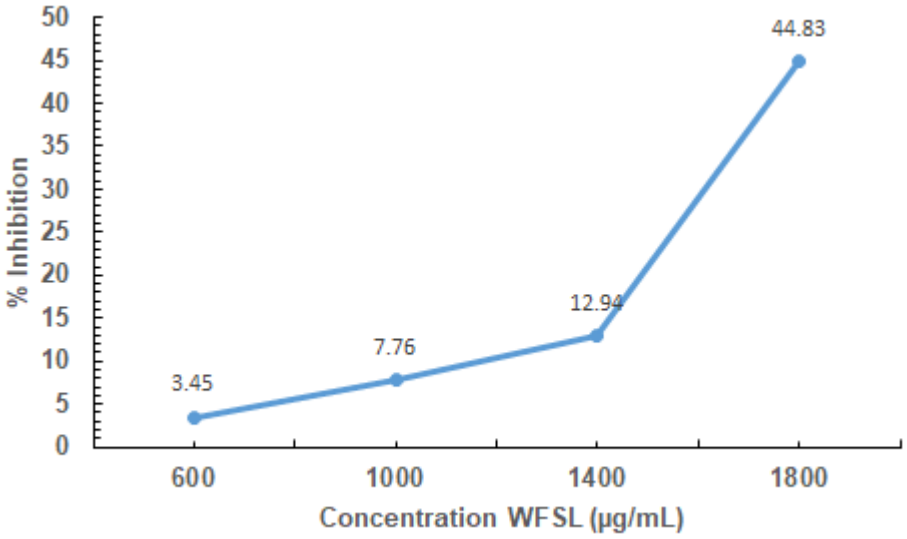



**Figure 7.** Relationship between Time and % Value Inhibition Elastase of Water Fraction Surian Leaves (WFSL)

The graph above shows that the higher the concentration of Water Fraction Surian Leaves (WFSL), the stronger the inhibition of the elastase enzyme. WFSL with a concentration of 1800 µg/mL has the highest inhibition of 44.83% compared to WFSL with a concentration of 600 µg/mL; 1000 µg/mL and 1400 µg/mL only have an inhibition of 3.45%; 7.76% and 12.94%. This is because at high concentrations many bioactive compounds are found in WFSL that can inhibit the elastase enzyme compared to WFSL with low concentrations [49]. The very wide range of % inhibition of elastase concentration of FADS 1800 µg/mL with FADS 1400 µg/mL is caused by FADS 1800 µg/mL having more interactions with the elastase enzyme so that the inhibitory activity is more effective, because the number of inhibitor molecules available to bind the enzyme increases with higher concentrations and reduces the level of substrate breakdown, so the resulting fluorescence signal will decrease, so that the inhibitory power against the elastase enzyme is greater and vice versa [50].

The substantial range of inhibition percentage at each Water Fraction Surian Leaves (WFSL) concentration is attributed to the presence of chemical compounds trapped within the aqueous fraction or water fraction. Based on phytochemical screening tests, WFSL contains a minimal amount of flavonoids and phenolics, resulting in limited inhibition potential against elastase enzymes. This phenomenon leads to WFSL exhibiting only slight anti-aging activity, which accelerates the intracellular elastase-induced degradation of elastin and consequently contributes to skin aging [46,50]. Although elastase inhibition is influenced by phenolic and flavonoid content, the extent to which elastase is inhibited depends on the concentration of these compounds in WFSL. Notably, the concentration of 600 µg/mL WFSL displays a minor inhibitory effect of only 3.45%. The Neutrophil Elastase (NE) activity capability of WFSL can be observed in the figure below.

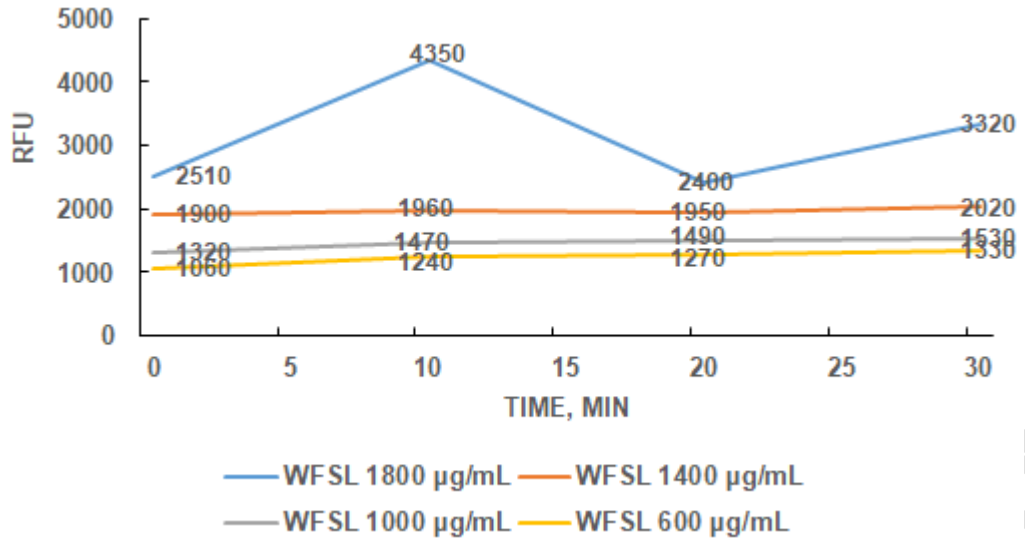



**Figure 8.** NE Activity Profile with Various Water Fraction Surian Leaves (WFSL)

In the Figure 8, it can be explained that the Neutrophil Elastase Activity from WFSL does not show a good graph because there is an increase and decrease in the RFU value which is very high at different times in WFSL concentration of 1800 µg/mL so that the results seen are not significant. At the 0, 10, 20, and 30 minutes, the WFSL RFU value increases and decreases, this is due to the dynamics of the interaction between the active compounds in WFSL with the elastase enzyme and the fluorescent substrate. At the 10th and 30th minutes, the RFU value increases because the elastase enzyme is still active and the fluorescent substrate is degraded, producing a strong fluorescence signal and the inhibition of the water fraction against the elastase enzyme is getting weaker and vice versa [51]. Meanwhile, the Neutrophil Elastase Activity profile seen in SLE and FEASL has a significant graph, where the increasing concentration of SLE and FEASL will increase the RFU (Relative Fluorescence Unit) value. This can be seen clearly in the combined graph at Figure 9.

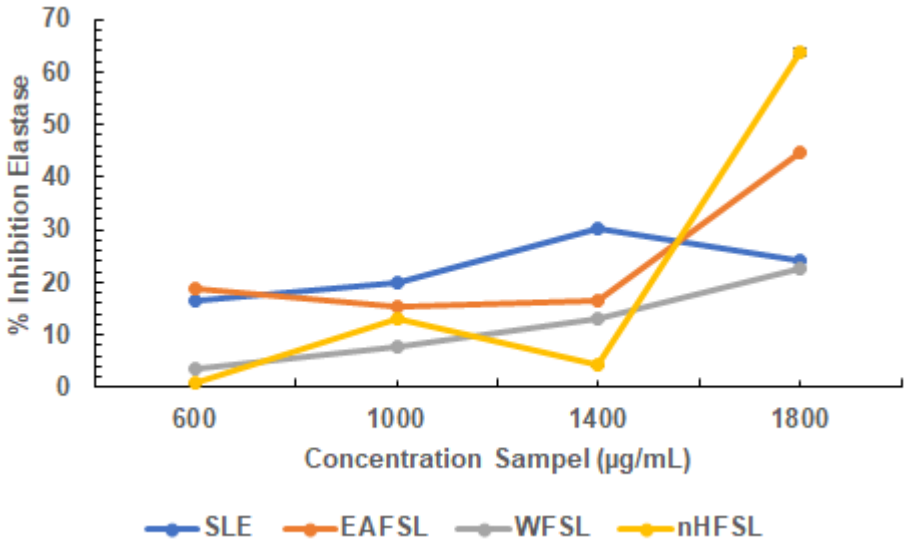



Figure 9. Graph of the relationship between sample concentration and % inhibition elastase

The graph above can be explained that the relationship between the dose or concentration of Surian Leaves Extract (SLE), Water Fraction Surian Leaves (WFSL), Ethyl Acetatte Fraction Surian Leaves (EAFSL) and n-Hexane Fractin Surian Leves (nHFSL) is not linear with the activity

and inhibition of the elastase enzyme. This is due to the saturation effect, where all active enzyme sites have been bound to the inhibitor, so that increasing the concentration does not affect and the presence of antagonistic compounds or competition between compounds in the extract can inhibit the effectiveness of inhibition. Therefore, increasing the concentration of extracts or fractions is not always followed by an increase in inhibition against the elastase enzyme [36,39].

There are other factors that influence such as the limited solubility of active compounds at high concentrations, causing precipitation and reducing the number of molecules to interact with the enzyme. In addition to the above, other factors such as extract toxicity, changes in the chemical structure of active compounds, and enzyme feedback mechanisms can also contribute or cause no increase in inhibitory activity even though the concentration of extracts or fractions is increased [43,49].

The elastase activity test used in this study requires validation of the analysis method to ensure the reliability and accuracy of the results obtained. The validation of the analysis method used is the precision method. The precision for the % elastase inhibition graph from SLE is 0.14; the precision for the % elastase inhibition graph from FEASL is 0.15; the precision for the % elastase inhibition graph from nHSL is 2.01 and the precision for the % elastase inhibition graph from WFSL is 0.60, all of which precision results do not exceed 2% so that it is essential to ensure that the method is able to measure elastase activity consistently and in accordance with the objectives of the analysis. Therefore, the importance of validation of this precision method must be an integral part of the development of bioactivity tests in drug discovery studies, especially those intended for anti-aging applications. If this precision method validation process does not exist, the data results cannot be scientifically accounted for, thus weakening the credibility of the research results [50].

### *3.3. Characterization of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) before storage*

The results of the characterization of SLENP and EAFSLNP production are presented in the Table 6 below.

**Table 6.** SLENP and EAFSLNP Characterization

| Sample | Particle Size (nm)±SD | Poly Dispersity Index ±SD | Zeta Potential (mV)±SD | Encapsulation Efficiency (%)±SD |
|---|---|---|---|---|
| SLENP 1000 µg/mL | 173.00 ±1.60 | 1.40±0.02 | -22.10±0.20 | 97.31±0.60 |
| EAFSLNP 1000 µg/mL | 186.00±1.10 | 1.95±0.13 | -16.70±1.70 | 98.50±0.20 |
| Control (+) AHANP 1000 µg/mL | 216.10±1.50 | 1.94±0.15 | -23.90±1.80 | 97.78±0.50 |

In this study, the fabrication of Surian Leaves Extract Nanoparticles (SLENP) was carried out using the ionic gelation method, which is the modification process of cross-linking between a polyelectrolyte and its multivalent ion counterpart. The synthesis of these nanoparticles involves the combination of two polymers: chitosan and alginate, each at a concentration of 1%. Chitosan, as a cationic polymer, reacts with multivalent anions to form cross-linked particles [51]. Meanwhile, alginate, derived from brown seaweed, is a natural polymer with significant potential in Indonesia, although its utilization remains limited [52,53]. Chitosan was selected due to its biocompatibility, non-toxicity, and biodegradability.

The cross-linker employed in this study is Sodium Tri Poly Phosphate (NaTPP), known for its multivalent anion properties that facilitate cross-linking with chitosan [53,54]. The cross-linking reaction between chitosan and NaTPP yields more stable nanoparticles with improved membrane penetration characteristics. This is evident in the characterization parameters of Surian Leaves

Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP), encompassing particle size, Poly Dispersity Index (PDI), Zeta potential, and Encapsulation Efficiency (EE). From the research results, SLENP and EAFSLNP before storage provide nanometer sized particles of around 50 - 200 nm with very good % EE (adsorption), but the PDI and zeta potential are not good because the particle size is not uniform or polydispersity and It makes it easier for agglomeration to occur so that the nanoparticles are only stable for 3 months. This can be seen as SLENP and EAFSLNP experiencing sedimentation after 2 months of storage. To overcome this, it is very important to develop natural polymer-based nanoparticles to be developed into nanohydrogel preparations that have the potential to be antiaging.

The characteristics of SLE and FEASL nanoparticles before storage were compared with the positive control of AHA nanoparticles. It turned out that SLE and FEASL nanoparticles had good characteristics such as particle size, PDI, zeta potential and % EE compared to the positive control of Alpha Hydroxy Acid (AHA) nanoparticles.

*3.4. Characterization of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) after two months storage at room temperature*

The results of the characterization of SLENP and EAFSLNP production are presented in the Table 7 below.

**Table 7.** SLENP and EAFSLNP Characterization

| Sample | Particle Size (nm)±SD | Poly Dispersity Index ±SD | Zeta Potential (mV)±SD | Encapsulation Efficiency (%)±SD |
|---|---|---|---|---|
| SLENP 1000 µg/mL | 977.40 ±5,90 | 0.72±0,52 | -17.50±1,83 | 94.06±0,30 |
| EAFSLNP 1000 µg/mL | 958.10±2.00 | 0.78±0.19 | -19.90±4.57 | 93.52±0.12 |
| Control (+) AHANP 1000 µg/mL | 627.40±1.00 | 1.70±0.20 | -10.60±2.27 | 86.32±0.50 |

If we look at the results of characterization of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP), it turns out that Poly Dispersity Index (PDI) and zeta potential do not provide a significant difference, but what can be seen is that the Encapsulation Efficiency (EE) value is getting smaller in percentage and particle size is getting bigger during two months of storage at room temperature. This is influenced by the PDI value. exceeds >0.7 so that sedimentation occurs quickly during two months of storage and easy formation of agglomeration due to small zeta potential value during storage.

In addition, the PDI value > 0.7 indicates significant particle size non-uniformity, which can negatively impact the physical stability and biological performance of the delivery system, especially in anti-aging applications that require high efficiency and safety [55].

The characteristics of SLE and FEASL nanoparticles after storage for three months compared to the positive control of AHA nanoparticles, it turns out that SLE and FEASL nanoparticles have good characteristics such as particle size, PDI, zeta potential and % EE compared to the positive control of Alpha Hydroxy Acid (AHA) nanoparticles.

This was also done by one-way ANOVA statistical analysis for the value of nanoparticle characteristics such as particle size, PDI, zeta potential and % EE. From the results above, the significance <0.05 means there is a significant change between groups before and after storage, where the particle size after storage becomes larger due to the possibility of aggregation during the storage period, but in contrast to the PDI value, it actually decreases to <1.00, which indicates that the particle size distribution becomes more uniform, although the particles generally enlarge. The decrease in zeta potential and entrapment efficiency (%EE) values after storage indicates a decrease in system stability and the ability of the formulation to retain active substances. This indicates that storage significantly affects the physicochemical characteristics of nanoparticles, and stability optimization is needed to maintain the effectiveness of the formulation over a certain period of time [55].

*3.4.1. Surface morphology of nanoparticles, Particle Size and Poly Dispersity Index (PDI)*

Particle size and PDI determination were conducted using a Particle Size Analyzer (PSA) with the Dynamic ight scSattering (DLS) method. The use of the PSA instrument aimed to measure particle size distribution [54]. The measurement principle of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP), relies on light scattering, revealing a particle size of 173.00 nm and 186.00 nm. And after 3 months of storage, particle size increased to 958.10 nm and 977.40 nm. This finding signifies that the particle size of SLENP adheres to the required range for effective drug delivery systems (50-200 nm). This is visually depicted in the Figure 10. The surface morphology of SLENP and EAFSLNP was characterized through Transmission Electron Microscopy (TEM). TEM is an advanced imaging technique utilizing an electron beam, as opposed to light, to examine the ultrastructure of materials at the nanoscale.

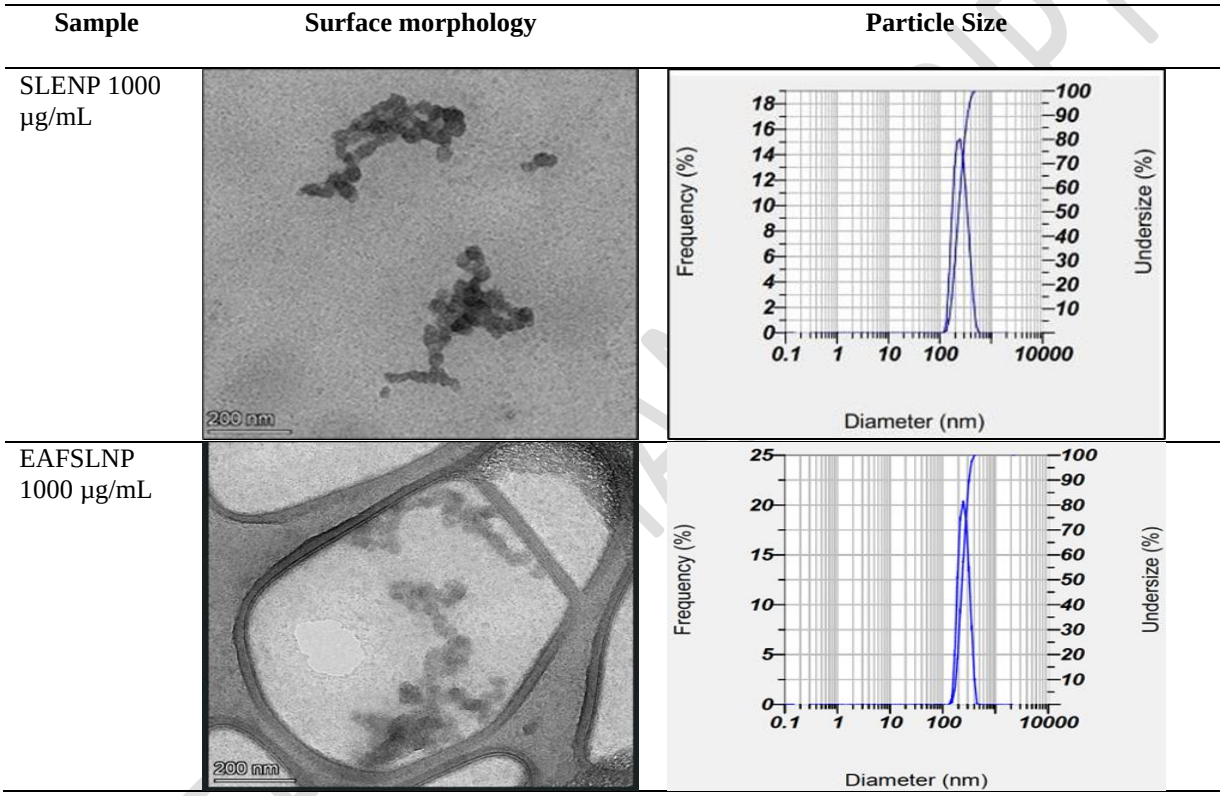



**Figure 10.** Surface morphology of nanoparticles and the distribution curve of particle size of nanoparticles.

The distribution curve of the particle size forms a single bell-shaped peak, indicating that the particle size of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) at a concentration of 1000 µg/mL with chitosan and alginate polymers, both at 1%, exhibits good uniformity. The peaks on the curve illustrate the distribution range of particle sizes. The Poly Dispersity Index (PDI) value provides insight into the breadth or narrowness of the size distribution, with a value < 0.1 indicating a very narrow distribution. Higher PDI indicate increased instability of the nanoparticles. This is because high particle non-uniformity leads to quicker flocculation and coalescence of nanoparticles [55].

SLENP and EAFSLNP at a concentration of 1 mg/mL using chitosan and alginate polymers, both at 1%, tend to have smaller particle sizes around 173.00 nm and 186.00 nm. This phenomenon is attributed to the formation of nanoparticles via the ionic gelation method, which involves solidifying liquid droplets dispersed in an oil or organic phase. The procedure entails mixing two liquid phases, one containing chitosan and the other containing multivalent anions [56]. With a more uniform size, particles will be stable due to lower size variation. If particle size variation is high, agglomeration can occur more rapidly. A wide particle size distribution leads to lower

nanoparticle stability. A low PDI value indicates that stabilizers prevent particle agglomeration. The recommended range for good Poly Dispersity Index (PDI) values is provided in the Table 8.

**Table 8**. Poly Dispersity Index (PDI)

| PDI | Note |
|---|---|
| < 0.05 | Monodisperse |
| < 0.08 | Nearly monodisperse |
| 0.08 - 0.7 | Most favorable |
| > 0.7 | Very polydisperse |

The results of Poly Dispersity Index (PDI) for Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) at a concentration of 1 mg/mL using chitosan and alginate polymers, both at 1%, yielded a value of 1.40 and 1.95. This value suggests that a PDI greater than 0.70 indicates a wide distribution of particle sizes and the potential for sedimentation. Not only that, the particle size and PDI value during storage increased, where the SLENP and EAFSLNP particle sizes increased to 958.10 nm and 977.40 nm while the PDI value increased to 0.72 s.d 1.70. This indicates significant particle size non-uniformity, which can negatively impact the physical stability and biological performance of the delivery system, especially in anti-aging applications that demand high efficiency and safety. Therefore, thorough formulation optimization is needed to achieve a more homogeneous particle size to improve product stability and efficacy. Unstable nanoparticles are at risk of aggregation or sedimentation, which can reduce the bioavailability of the active ingredient and reduce the efficacy in delaying signs of skin aging [55].

*3.4.2. Zeta Potential*

Zeta potential refers to the electric charge between colloidal particles. The Zetasizer Zen3600 was employed to determine zeta potential, involving a 10-fold sample dilution in an aqueous medium at room temperature. Zeta potential values are used to assess stability concerning temperature, pH, and humidity variations [54]. In this study, a zeta potential value of -22.10 mV and -16,70 nm was obtained. This negative value indicates electric stability, where a higher zeta potential hinders flocculation. A reduction in zeta potential enables particles to attract and aggregate, leading to flocculation. Consequently, a favorable zeta potential value signifies stronger particle repulsion, resulting in a stable dispersion formulation [55,56]. The acceptable range for a good zeta potential value is presented in the Table 9.

**Table 9**. Zeta potential [mV]

| Zeta Potential | Note |
|---|---|
| 0 to ± 5 mV | Flocculation |
| ± 10 mV to ± 30 mV | Less stable (very short stability) |
| ± 30 mV to ± 40 mV | Moderate stability |
| ± 40 mV to ± 60 mV | Good stability |
| > ± 61 mV | Very good stability |

Zeta potential measures the repulsive force between particles. A zeta potential value of ±30 mV provides good stability, while ±60 mV offers excellent stability. Around ±20 mV provides only short-term stability, and a zeta potential value within the range of ±5 mV indicates rapid aggregation. This applies to surfactants with low molecular weight and pure electrical stabilization, but not to stabilizers with high molecular weight, which possess steric stabilization properties [48]. The zeta potential value of SLE dan EAFSL nanoparticles at -22.10 mV and -16,70 nm can provide sufficient stabilization. This can be observed in the zeta potential curve in the Figure 11.

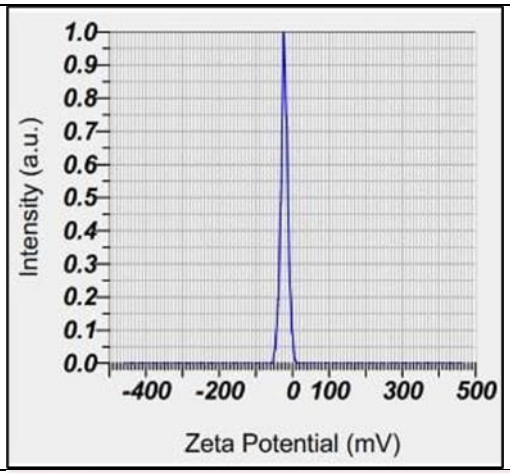
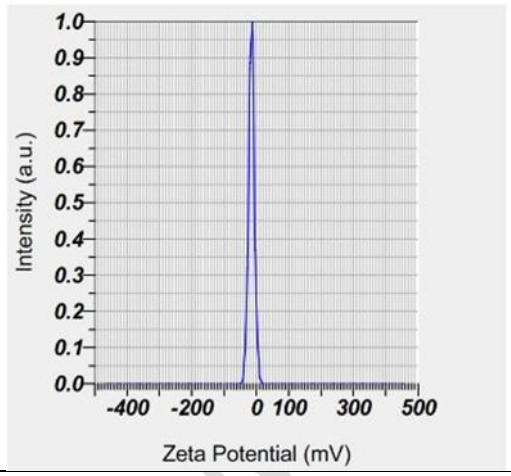
| Sample | Zeta Potential |
|---|---|
| SLENP 1000 µg/mL |  |
| EAFSLNP 1000 µg/mL |  |



**Figure 11.** Zeta Potential Curve of SLENP and EAFSLNP

The zeta potential curve narrows as the intensity increases, indicating that the resulting zeta potential values show the stability of Surian Leaves Extract Nanoparticles (SLENP) during storage. Zeta potential testing was carried out using a voltage of 150 V. This voltage value was derived from the conductivity data of the nanoparticle formulation. Conductivity measures the ability of a solution to conduct electricity. An increase in conductivity value corresponds to an increase in zeta potential value. For conductivity values of < 5 mS/cm, the voltage used for zeta potential measurement is 150 V, while for values of 5-30 mS/cm and > 30 mS/cm, the respective voltages used are 50 V and 10 V [57,58, 59-61].

The zeta potential of Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) is -22.10 mV and -16,70 nm, which indicates a relatively weak zeta potential value of < ± 30 mV. However, the nanoparticles are stabilized by a PVA layer through steric stabilization. SLENP and EAFSLNP carry a negative charge due to ionized carboxyl groups. The presence of cationic polymers like chitosan forms a stable network on the polymer surface. This network shields the surface charge and moves away from the particle surface, resulting in a slightly negative zeta potential [53,54,62-64]. If seen after storage, the zeta potential of SLENP and EAFSLNP decreased, resulting in the nanoparticles becoming unstable again due to precipitation.

*3.4.3. Encapsulation Efficiency (EE)*

The %EE assesses how effectively nanoparticles trap the drug. Factors influencing %EE include the solubility of drug particles in the entrapping polymer, the polymer formulation, Molecular Weight (MW), and interactions with the polymer. Encapsulation involves a process or

technique to coat a core whether it is solid, liquid, gas, or cellular with a specific protective material that reduces damage to the active compound [59, 60, 63,64].

The %EE for Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) nanoparticles is 97.31% and 98.50%. Encapsulation efficiency is a parameter indicating the percentage of successfully coated SLE within the nano-shell [58,59, 61]. This demonstrates that a substantial amount of the active compound in SLE is effectively entrapped within the nano-shell, resulting in a highly successful process for creating nanoparticles to trap the drug. The absorption of SLENP and EAFSLNP during 3 months of storage experienced a reduction in % EE. This can be seen in the research results that the % EE produced after 2 months of storage became 86.32% and 94.06% and had the impact that SLENP and EAFSLNP experienced a decrease in antiaging activity in inhibiting elastase enzyme.

*3.5. Comparison of* SLE *and* EAFSL *Activities on Elastase Enzyme Inhibition with SLENP and EAFSLNP Activities on Elastase Enzyme Inhibition.*

From the results of elastase enzyme activity testing carried out using the *Neutrophil Elastase Inhibitor Screening Kit method*, it was found that Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) had a greater % inhibition of the elastase enzyme compared to pure SLE and EAFSL forms, where the resulting % SLE and EAFSL inhibition was 30.18% and 22.42% while the % inhibition of SLENP and EAFSLNP is 39.00% and 87.30%, this can be seen in Figure 12 below,

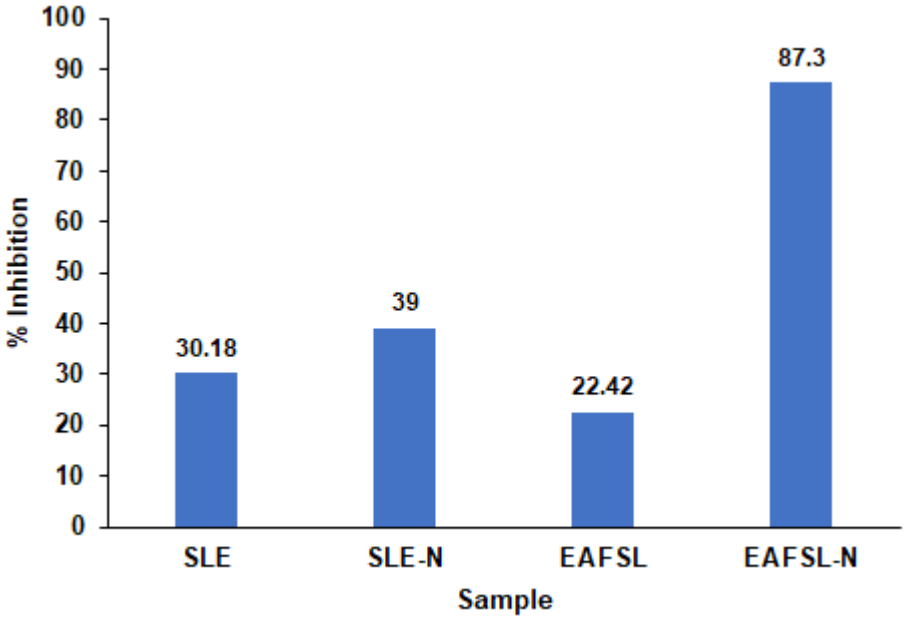



Figure 12. Comparison of % Inhibition on the elastase enzyme of SLE, EAFSL and SLENP, EAFSLNP

This research can prove that cosmetic delivery system in form of nanoparticles with natural polymers (chitosan and alginate) from SLE and EAFSL is able to increase antiaging activity. To improve the characterization of SLENP and EAFSLNP regarding PDI and zeta potential, they can be developed into nanohydrogels so that the SLENP and EAFSLNP can be absorbed in the gel base so as to prevent rapid agglomeration and deposition [62,63]. Based on the graph above, it can be concluded that the presence of a delivery system in the form of nanoparticles can increase the inhibitory power against the elastase enzyme compared to pure surian leaf extract or fraction.

The limitations in the study are about the stability of nanoparticles during storage, although the use of the ionic gelation method can provide stable nanoparticle formation, there are still some

limitations that need to be considered, one of the main challenges is the stability of nanoparticles over time, where particles can experience aggregation or changes in size due to environmental conditions such as pH, temperature and ionic strength of the storage medium. Control of particle size and distribution is still currently an obstacle, because parameters such as the ratio of polymer (chitosan, alginate) to crosslinking agent (NaTPP) can produce variability in nanoparticle characteristics. In addition, the potential for toxicity is also a concern, especially if nanoparticles are used in biomedical applications where chemical residues such as acetic acid or excess crosslinking agents (NaTPP) can affect their biocompatibility. The efficiency of encapsulation and release of active substances is not always optimal, this still depends on the physicochemical properties of the materials used, so it can affect the performance of drug delivery. Therefore, further research is needed to improve the stability, safety and effectiveness of nanoparticle-based systems produced through the ionic gelation method [63].

## 5. Conclusions

The research findings lead to the following conclusions: Surian Leaf Extract (SLE) and Ethyl Acetate Fraction Surian Leaves (EAFSL) demonstrate superior inhibition percentage against the elastase enzyme compared to the Water Fraction Surian Leaves (WFSL) and n-Hexane Fraction Surian Leaves (nHFSL). The Relative Fluorescence Unit (RFU) value is related to the activity of the elastase enzyme, where the higher the RFU value, the higher the activity of the elastase enzyme and vice versa. If the RFU value decreases, the inhibitory power of the extract against the elastase enzyme increases, due to the IC50 value for SLE of 12.351 ± 0.092 and the IC50 value for EAFSL of 15.5865 ± 0.546 in addition to the total flavonoid content of Surian Leaf Extract (SLE) of 33.19 mg / g and the total phenolic content of EAFSL of 365 mg / g which can reduce the ability of elastase to break down fluorescent substrates, so that the resulting fluorescence signal becomes lower and vice versa the RFU value increases because the inhibitory power of the extract against the elastase enzyme decreases, so that more fluorescent substrates are broken down and produce stronger and more fluorescence signals. Based on the inhibition percentage against elastase, SLE underwent further development for nanoparticle fabrication using the ionic gelation method with 1% chitosan and 1% alginate polymers, in addition to the cross-linker NaTPP. The resulting characteristics of the Surian Leaves Extract Nanoparticles (SLENP) and Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP) are outstanding and align with requirements, encompassing particle size (173.00 nm and 186.00 nm), PDI (1.40 and 1.95), zeta potential (-22.10 mV and -16.70), and %EE (97.31% and 98.50%). This implies that SLENP and EAFSLNP can amplify anti-aging activities, potentially leading to reduced required dosages for achieving anti-aging effects due to heightened active substance absorption. Subsequent research is encouraged to explore the inhibition percentage of SLENP and EAFSLNP against the elastase enzyme and determine the necessary drug loading dose for creating anti-aging nanohydrogel cosmetic formulations. Reducing the dose of Surian Leaf Extract (SLE) in the manufacture of nanoparticles is based on initial findings where Surian Leaf Extract (SLE) 2.5% has an IC50 of 12,351 ppm, this antioxidant activity works synergistically with the ability to stabilize the role of Reactive Oxygen Species (ROS) in the photoaging process, so that with the development of a nanoparticle technology system in the form of Surian Leaves Extract Nanoparticles (SLENP) a small dose and concentration of Surian Leaf Extract (SLE) is required with a dose/concentration of 0.14% but has a very high potential for inhibition of the elastase enzyme of 39% compared to Surian Leaf Extract (SLE) of only 30.18%. Likewise, with Ethyl Acetate Fraction Surian Leaves Nanoparticles (EAFSLNP), a small dose and concentration of Ethyl Acetate Fraction Surian Leaves (EAFSL) is required with a dose/concentration of 0.18% but has a very high inhibitory potential against the elastase enzyme of 87.3% compared to Ethyl Acetate Fraction Surian Leaves (EAFSL) of only 22.42%.

## Data Availability

I state the datasets used and/or analysed during the current study available from the corresponding author on reasonable request. (corresponding author: muhaimin@unpad.ac.id)

**Additional information**

In this experimental research that authors comply with the IUCN Policy Statement on Research Involving Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora.

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