Isolation, characterization and anticancer potential test of crude extract of L-asparaginase enzyme from siam weed leaf (Chromolaena odorata Linn): a novel source

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Abstract. L-asparaginase enzyme is potentially isolated from Siam weed (C. odorata L.) and also used as anticancer treatment. The point of this study is to isolate crude extract of L-asparaginase as well as its characteristics for anticancer. The activity of the enzyme was observed by Nessler Method and its potential for anticancer was tested by Brine Shrimp Lethality Test (BSLT) method. The results showed the specific activity by 4.8234 UI/mg. The enzyme worked maximally at pH of 8, 37 °C for 30 minutes with Na⁺ and K⁺ being activator and Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺, Mn²⁺ as inhibitor. Base on BSLT revealed an LC₅₀ value of 5.8063 μg/mL and proved that the enzyme has biotoxicity in high level. The results of this study denoted that C. odorata L. leaves able to be isolated and characterized by L-asparaginase enzyme and these reveals the potential to be developed as an anticancer in the future.

1. Introduction
The recent report of cancer cases predicts that there are at least 18.1 million new cases and 9.6 million deaths in 2018. Furthermore, the cancer diagnosis during the 5 years that are found of 43.8 million cancer patients in worldwide and more than 50% of deaths in 2018 that are occurred in Asia, especially in developing countries including Indonesia [1].

According to [2] that L-asparaginase is an enzyme that could inhibit or kill cancer cells through the mechanism of the hydrolysis reaction of L-asparagine which is a non-essential amino acid for protein synthesis and cell growth into L-aspartate and ammonia. Thus, the process of protein synthesis is disrupted and also inhibits the growth of cancer cells that ultimately results in the death of cancer cells.

The L-asparaginase enzyme able to be isolated from bacteria such as Enterobacter cloaceae but the drawback is that it requires a lot of substrates to produce it. Nevertheless, it could also be isolated from the extract of a plant that the process is more practical and simple. Some types of plants that contain
this enzyme are red onion, garlic, white turmeric rhizome, and tomatoes. However, this method must require many plant preparations so that it is very limited by season and plant population. Therefore, it takes a variety of plant that has an abundant population, is easily available and capable to be produced as an L-asparaginase enzyme [3,4].

One of potential plant to isolate L-asparaginase enzyme as an anticancer medicine is C. odorata L. This weed is classified as invasive weeds, originally found in South and Central America which is spread in tropical region of Asia, Africa, and the Pacific, fast-growing woody shrubs whose its presence could prevent the growth of other plant species and it has an allelopathic effect. This plant has a characteristic triangular leaf that there are three leaf bones every leaf that is clearly visible, as well as its leaf, has a very pungent odor when is squeezed. The other characteristics are that have face branching and compound inflorescence that looks remotely dirty white [5]. Base on the observation of [6] that C. odorata L. leaf contains the essential and non-essential amino acids every 100 grams of its protein which one of them is Aspartate of 8.51%. The researcher concluded that plant leaves have the potential to be developed as a natural anticancer medicine based on the high content of aspartic acid which is known through biosynthesis with the L-asparaginase enzyme so that it is assumed that siam weed leaves contain these enzymes.

One way to find out the potential of a compound as an alternative to a new drug is to carry out a biological toxicity test. The principle of toxicity testing is that bioactive components are always toxic when are given with high doses and they are medicinal when are given at low doses. Toxicity tests are carried out by the BSLT method which is a preliminary test to determine whether a compound has a bioactive content and has pharmacological activity. The method has several advantages, namely faster, cheaper, easier, does not require aseptic conditions and a 95% confidence level. The test animal used is Artemia salina Leach [7].

The results of this study indicate that the leaves of C. odorata L. capable to be isolated L-asparaginase enzyme with a large enough specific enzyme activity and its enzyme characterization which is approximately equal to the characterization of L-asparaginase enzymes in general and denotes the potential to be developed as an anticancer which could be seen from the data of BSLT.

2. Materials and methods
2.1 Materials
C. odorata L. leaves from Paccing village (Bone regency, Indonesia), buffer Tris-HCl, L-asparagine (Merck), BSA (Bovine Serum Albumin), TCA (Trichloracetic acid) (Merck), Nessler reagent, (NH₄)₂SO₄ (Ammonium Sulphate) (Merck), Lowry reagent, aquadest, A. salina Leach, seawater and metals (Na⁺, K⁺, Ca²⁺, Zn²⁺, Mg²⁺, Cu²⁺, Co²⁺, Mn²⁺).

2.2 Methods
2.2.1 Enzyme isolation
Fresh siam weed leaves about 250 g were cut into pieces then they were added 500 mL of buffer Tris-HCl pH 8 and blended for 20 minutes. Furthermore, the mixture was left for 1.5 hours at 4°C then it was filtered. Then, the filtrate was centrifuged at 5000 rpm for 30 minutes. The supernatant was called crude extract [8].

2.2.2 Protein Level Test
2.2.2.1 Determination of Maximum Wavelength. Lowry C solution about 2 mL plus 0.1 mL of BSA solution 0.3 mg/mL and incubated at 37 °C for 30 minutes. Then, 0.2 mL Lowry D was added and re-incubated for 10 minutes at room temperature while occasionally shaking. then the absorbance of the solution was measured at the wavelength (λ) 600-700 nm and 10 nm intervals with a UV-Vis spectrophotometer [8].

2.2.2.2 Making BSA Standard Curves. Lowry C solution about 2 mL was added 0.1 mL BSA solution (0.1; 0.2; 0.3; 0.4 and 0.5 mg / mL) and incubated at 37°C for 30 minutes. Then 0.2 mL Lowry D was
added and re-incubated for 10 minutes at room temperature while occasionally shaking. Then the absorbance of the solution was measured at the optimum BSA wavelength (\( \lambda \)) with a UV-Vis spectrophotometer. Protein levels were determined by linear regression on the BSA standard curve [8].

2.2.1.3 Sample Analysis. Lowry C solution about 2 mL plus 0.1 mL of crude enzyme extract (0.1; 0.2; 0.3; 0.4 and 0.5 mg/mL) and incubated at 37°C for 30 minutes. Then 0.2 mL Lowry D was added and re-incubated for 10 minutes at room temperature while occasionally shaking. As a control, the enzyme solution was replaced with aquadest, then the absorbance of the solution was measured at the optimum wavelength (\( \lambda \)) of BSA with a UV-Vis spectrophotometer. Protein contents were determined by linear regression on the BSA standard curve [8].

2.2.3 Enzyme Activity Test
2.2.3.1 Determination of the Maximum Wavelength. The standard solution of 0.3 mg/mL ammonium sulfate was piped as much as 0.5 mL then added 8.5 mL aquadest and 1 mL Nessler reagent, then the absorbance of the solution was measured by a UV-Vis spectrophotometer at a maximum wavelength of 400-500 at 10 nm intervals [8].

2.2.3.2 Making a standard Ammonium Sulphate Curve. Standard solutions of ammonium sulfate with the concentration of 0.1; 0.2; 0.3; 0.4 and 0.5 mg/mL were pipetted and then 8.5 mL aquadest and 1 mL Nessler reagent were added, then the absorbance of the solution was measured by a UV-Vis spectrophotometer at the maximum wavelength [8].

2.2.3.3 Sample Analysis. L-asparagine solution 0.01 M about 1 mL, 0.2 mL of the crude enzyme extract and 0.8 mL of 0.2 M Tris-HCl buffer pH 8 were put into a test tube and incubated at 37 °C for 30 minutes. Then 0.2 mL of 1.5 M TCA was added and centrifuged at 13000 rpm for 30 minutes. As a control, 0.2 mL of the enzyme was removed (heated, cooled and added 1 mL TCA 1.5 M), then added 0.8 mL of 0.2 M Tris-HCl buffer pH 8 and 1 mL of L- asparagine solution. A total of 0.5 mL of filtrate was taken and then added 8.5 mL aquadest and 1 mL Nessler reagent, then the absorbance of the solution was measured by a UV-Vis spectrophotometer at the maximum wavelength and analyzed with a standard solution of ammonium sulfate [8]. L-asparaginase activity was calculated using the formula of [9]:

$$\text{Enzyme activity (IU/mL)} = \frac{(Y-b)}{a} \times \frac{V_{\text{total}}}{V_{\text{analysis}}} \times \frac{1}{V_{\text{enzyme}}} \times \frac{1}{T_{\text{incubation}}}$$

(1)

Note:
\( Y \) = Absorbantion
\( a \) = Slope
\( b \) = Intercept
\( V_{\text{total}} \) = Enzyme volume + substrate + buffer + TCA
\( V_{\text{analysis}} \) = Total analyzed volume
\( V_{\text{enzyme}} \) = Total analyzed volume
\( T_{\text{incubation}} \) = 30 minutes

Specific activity (IU/mg) = \( \frac{\text{Enzyme activity (IU/mL)}}{\text{Protein level (mg/mL)}} \) (2)

Relative activity (IU/mg) = \( \frac{\text{L-asparaginase activity incubate } "x" \text{ minutes of Enzyme activity (IU/mL)}}{\text{Protein level (mg/mL)}} \times 100\% \) (3)

One L-asparaginase unit (IU) is defined as the amount of L-asparaginase enzyme that catalyzes the formation of one \mumol ammonia per minute under test conditions [11].
2.2.4 Characterization of L-asparaginase enzyme

2.2.4.1 Effect of pH on L-asparaginase enzyme activity. L-asparagine solution 0.01 M of 1 mL, 0.2 mL of the crude enzyme extract and 0.8 mL of 0.1 M potassium phosphate buffer (pH 6.0-7.0) and Tris-HCl (pH 8.0-10.0) were put into a test tube and incubated at 37 °C for 30 minutes. Then 0.2 mL of 1.5 M TCA was added and centrifuged at 13000 rpm for 15 minutes. Then, 0.5 mL of filtrate was taken and added 8.5 mL of aquadest and 1 mL of Nessler reagent. Then, the absorbance of the solution was measured by a UV-Vis spectrophotometer with a standard solution of ammonium sulfate [9].

2.2.4.2 Effect of temperature on L-asparaginase enzyme activity. L-asparagine solution 0.01 M about 1 mL, 0.2 mL of the crude enzyme extract and 0.8 mL of 0.1 M Tris-HCl buffer at optimum pH previously obtained were put in a test tube. Then the enzyme was incubated at temperatures of 30, 37, 40 and 45 °C for 30 minutes. Then 0.2 mL of 1.5 M TCA was added and centrifuged at 13000 rpm for 15 minutes. Furthermore, 0.5 mL of filtrate was taken and added 8.5 mL of aquadest and 1 mL of Nessler reagent. Then the absorbance of the solution was measured by a UV-Vis spectrophotometer with a standard solution of ammonium sulfate [9].

2.2.4.3 Effect of incubation time on L-asparaginase enzyme activity. L-asparagine solution 0.01 M about 1 mL, 0.2 mL of the crude enzyme extract and 0.8 mL of 0.1 M Tris-HCl buffer at optimum pH were put in a test tube. Then, the enzyme was incubated at the optimum temperature obtained previously where Pre-incubation was carried out for 120 minutes and L-asparaginase activity was tested every interval of 30 minutes at the optimum temperature. Furthermore, the solution was added 0.2 mL of 1.5 M TCA and centrifuged at 13000 rpm for 15 minutes. Then, 0.2 mL of 1.5 M TCA was added and centrifuged at 13000 rpm for 15 minutes. Furthermore, 0.5 mL of filtrate was taken and added 8.5 mL of aquadest and 1 mL of Nessler reagent. Then the absorbance of the solution was measured by a UV-Vis spectrophotometer with a standard solution of ammonium sulfate [9].

2.2.4.4 Effect of metals on L-asparaginase enzyme activity. L-asparagine solution 0.01 M about 1 mL, 0.2 mL of the crude extract enzyme, 0.1 mL of metals (K+, Na+, Ca2+, Co2+, Cu2+, Zn2+, Mg2+, Mn2+) and 0.7 mL 0.1 M Tris-HCl buffer at optimum pH previously obtained were put in a test tube. Then the enzyme was incubated at optimum incubation time and at the optimum temperature obtained previously. Then 0.2 mL of 1.5 M TCA was added and centrifuged at 13000 rpm for 15 minutes. Furthermore, 0.5 mL of filtrate was taken and added 8.5 mL of aquadest and 1 mL of Nessler reagent. Then the absorbance of the solution was measured by a UV-Vis spectrophotometer with a standard solution of ammonium sulfate [9].

2.2.5 Test the potential for anticancer L-asparaginase enzyme from the leaves of C. odorata L. by the BSLT method

The eggs of A. salina Leach were put into a place filled with seawater. The eggs were then aerated and left under the lighting for 48 hours so that the hatch is perfect. The hatched Eggs of A. salina Leach become larvae and were used for cytotoxicity tests. 10 salts of A. salina Leach larvae were inserted into the test plate which was filled with sea water. Furthermore, the L-asparaginase enzyme extract was added with variations in concentrations of 1, 10, and 100 μg/mL, while for the control no enzyme extract solution was added. The treatments were three replications at each extract and control concentration, then observed after 24 hours by calculating the number of dead larvae from the total larvae entered to obtain percent mortality. The LC50 value was obtained using the probit LC50 analysis at a 95% confidence interval using the SPSS (Statistical Package for the Social Sciences) program [7].

3. Results and discussion

3.1 Isolation of the L-asparaginase enzyme from the leaves of C. odorata L.

L-asparaginase was extracted from the leaves of C. odorata L. by using a homogenization method to obtain the crude extract of the enzyme. The results in Table 1 show enzyme and enzyme-specific
activity of crude L-asparaginase in *C. odorata* L. leaf extract respectively 13.6743 IU/mL and 4.8234 IU/mg. These results prove that *C. odorata* L. leaves have the potential to be processed as the newest source for producing the L-asparaginase enzyme. Besides, the comparison between the results of this study with previous studies which was not as good as *Phyllanthus emblica* or *Vigna unguiculata* [10,11]. However, these results outperform several other sources such as *Phyllanthus amarus*, *Phyllanthus niruri*, *Phyllanthus urinarii* when viewed from the result of specific enzyme activities and the other advantages are abundant sample populations because it is an invasive weed species capable of growing in various types of environmental conditions and is a host for other plants [4,10,11].

| Sample          | Protein level (mg/mL) | Enzyme activity (IU/mL) | Specific activity (IU/mg) |
|-----------------|-----------------------|-------------------------|--------------------------|
| Crude extract   | 2.8350                | 13.6743                 | 4.8234                   |

L-asparaginase activity was determined by the Nessler method. The amount of the produced ammonia from L-asparaginase amino acid substrate is catalyzed by the L-asparaginase enzyme that is expressed as its activity. Ammonia was released by the hydrolysis of the L-asparaginase enzyme reacts with the Nessler reagent. Then the levels are determined by using the standard ammonium sulfate based on formula equation of [9] using the optimum wavelength obtained in this study (480 nm). One L-asparaginase unit (IU) is defined as the amount of L-asparaginase enzyme which catalyzes the formation of one μmol ammonia per minute under test conditions [11,12].

The protein levels contained in the enzyme solution was determined by the Lowry method [8]. In this method, the Folin Ciocalteau reagent was used which able to react with protein and give a strong dark blue color. The measured absorbance uses the maximum wavelength obtained (660 nm) then processed with the help of the BSA standard curve so that the protein content in the crude extract was obtained. Therefore, specific enzyme activities which are comparisons between enzyme activity units hence the protein levels can be determined.

### 3.2 Characterization of the L-asparaginase enzyme from the leaves of *C. odorata* L.

The characterization of enzymes is needed to determine the nature and characteristics of L-asparaginase enzyme. The characteristics of the free and immobilized L-asparaginase enzymes that was studied in this research including the effect of pH, temperature, incubation time and metal ions.

#### 3.2.1 Effect of pH on the L-asparaginase enzyme from the leaves of *C. odorata* L.

![Figure 1](image_url)  
*Figure 1.* Effect of pH on the L-asparaginase enzyme from the leaves of *C. odorata* L.
The enzyme has a unique optimum pH, which is a pH that causes maximum activity. The activity profile of the enzyme pH describes the pH of the enzyme when the important proton giver and receiver groups on the catalytic site of the enzyme are in the desired ionization state [9]. L-asparaginase activity increased with increasing pH until optimum at pH 8 with enzyme activity 13.6743 IU/mL, then enzyme activity decreased after pH passes 8 as shown in Figure 1. The results of this observation have similarities with some previous studies but of different sources [3,9,11].

3.2.2 Effect of temperature on the L-asparaginase enzyme from the leaves of C. odorata L.

![Figure 2. Effect of temperature on the L-asparaginase enzyme from the leaves of C. odorata L.](image)

Chemical reactions including enzyme catalysis reactions could be influenced by temperature. The chemical reaction takes place slowly at low temperature, while the reaction takes place faster at higher temperatures. Besides that, this is because enzymes are a protein, the increase in temperature able to cause denaturation which damages the active site of the enzyme and decreases its activity [9]. The activity of L-asparaginase enzyme was obtained at an optimum temperature of 37 °C with the enzyme activity of 13.6743 IU/mL, these could be seen in Figure 2. The results of this research were similar to several previous studies but of different sources [3,10,11].

3.2.3 Effect of incubation time on the L-asparaginase enzyme from the leaves of C. odorata L.

![Figure 3. Effect of incubation time on the L-asparaginase enzyme from the leaves of C. odorata L.](image)
Enzyme activity is also influenced by incubation time. The incubation time shows the optimum time the active site of an enzyme reacts or interacts with the substrate which this study is the L-asparaginase enzyme with L-asparagine as its substrate. Based on data from Figure 3, the optimum incubation time of the L-asparaginase enzyme from *C. odorata* L. leaves was 30 minutes with the enzyme activity of 13.6743 IU/mL, then the activity decreased with increasing incubation time after 30 minutes of incubation. This is due to the inability of the enzyme to maintain its conformation so that the active site of the enzyme is by the substrate and is unable to maintain its activity at certain time conditions [9]. The results of this study have similarities with several previous studies but of different sources [13].

### 3.2.4 Effect of metal ions on the L-asparaginase enzyme from the leaves of *C. odorata* L.

**Table 2.** The effect of metal additions on the L-asparaginase enzyme from the leaves of *C. odorata* L.

| Ion metal | Enzyme activity (IU/mL) | Specific activity (IU/mg) | Relative activity (%) |
|-----------|-------------------------|---------------------------|----------------------|
| Control   | 13.6743                 | 4.8234                    | 100.00               |
| K⁺        | 19.2846                 | 6.8023                    | 141.03               |
| Na⁺       | 16.1934                 | 5.7120                    | 118.42               |
| Ca²⁺      | 11.3331                 | 3.9976                    | 82.88                |
| Zn²⁺      | 7.3566                  | 2.5949                    | 53.80                |
| Mg²⁺      | 12.9238                 | 4.5587                    | 94.51                |
| Cu²⁺      | 4.6172                  | 1.6286                    | 33.77                |
| Co²⁺      | 8.0635                  | 2.8443                    | 58.97                |
| Mn²⁺      | 9.3007                  | 3.2807                    | 68.02                |

**Figure 4.** Effect of metals on enzyme activity and relative activity of the L-asparaginase enzyme from the leaves of *C. odorata* L.

The activity of an enzyme is also influenced by the presence of metals. These metals are minerals commonly found in the human body, such as those used in this study. These metals are effectors that capable to increase or decrease the activity of an enzyme. Generally, some enzymes do not require additional components to reach their full activity. However, others also require non-protein molecules called activators to bind to enzymes and become active. Some enzymes do not require additional
components to reach their full activity. But some require non-protein molecules called activators to bind to enzymes and become active. There are also non-protein molecules that can reduce the activity of an enzyme because it becomes a disruption of the active site of the enzyme to its substrate, in this study L-asparaginase with L-asparagine as its substrate. The results were obtained in this study are monovalent metals (Na\(^+\) and K\(^+\)) as activators and K\(^+\) was as the strongest activator with relative activity of 141.03\% which means the increase of enzyme activity by 41.03\%. Whereas the divalent metals (Ca\(^{2+}\), Zn\(^{2+}\), Mg\(^{2+}\), Cu\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\)) as an inhibitor and Cu\(^{2+}\) was as the strongest inhibitor about relative activity of 33.77\% which means that the enzim activity decreases by 66.23\% as for the data could be seen in Table 2 and Figure 4. The results of the observation have some similarities with some previous studies but with different sources [3,14].

3.3 Test the potential for anticancer L-asparaginase enzyme from the leaves of C. odorata L. by the BSLT method

One way to find out the potential of a compound as an alternative to a new medicine that is to carry out a biological toxicity test. The principle of the toxicity test is that bioactive components are always toxic if given with high doses and are medicinal if given at low doses. Toxicity tests using the BSLT method can be used as a preliminary test in studies that lead to cytotoxic tests. The test animals used are A. salina Leach. The A. salina Leach is commonly called shrimp larvae that has similarities with mammals such as the type of DNA-dependent RNA polymerase (DNA that directs the RNA transcription process). This causes a compound or extract that has activity on the system can be detected through this method. The correlation between this acute toxicity test and the cytotoxic test is when the mortality of A. salina Leach caused has a price of LC\(_{50}<1000\ \mu g/mL\). This method has several advantages, namely faster, cheaper, easier, does not require aseptic conditions and a 95\% confidence level. The mechanism of larval death is related to the function of the contained compounds in their cells that able to inhibit the feeding power of larvae [7].

| Table 3. Testing results of L-asparaginase enzyme from the leaves of C. odorata L. using the BSLT method |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Sample          | Conc. (\(\mu g/mL\)) | Axis X (Log [Conc.]) | % Larval death - Control | Axis Y (Probit value) | LC\(_{50}\) (\(\mu g/mL\)) |
| Crude extract   | 1 0.00           | 10               | 3.72               | 5.8063           |
|                 | 10 1.00          | 70               | 5.52               | 5.8063           |
|                 | 100 2.00         | 97               | 6.88               | 5.8063           |

Note. Conc. = Concentration

![Figure 5. The curve of the relationship between the probit value and the log [sample]](image-url)
The result of BSLT testing of the L-asparaginase enzyme from the leaves of *C. odorata* L. that was 5.8063 μg/mL. These results prove that the L-asparaginase enzyme from the leaves of *C. odorata* L. has the potential as an anticancer because of the price of LC\textsubscript{50} < 1000 μg/mL which means the sample is very toxic [7]. Therefore, the L-asparaginase enzyme from the leaves of *C. odorata* L. could be continued with cytotoxicity testing using cancer cells to see the anticancer ability of the L-asparaginase enzyme from the leaves of *C. odorata* L. against the theory of cancer cell activity that the action mechanism of this enzyme is through working to inhibit cancer cell protein synthesis without damaging normal cells. L-asparaginase will catalyze the hydrolysis reaction of L-asparagine to L-aspartate and ammonia, hence the process of protein synthesis is disrupted and inhibits the growth of cancer cells which ultimately results in the death of cancer cells, as shown in figure 6 [1]. Nevertheless, it should be done in stages to purify the L-asparaginase enzyme from the leaves of *C. odorata* L. to ensure that the obtained anticancer activity is purely produced by the extracts of L-asparaginase enzyme from the *C. odorata* L. leaves.

**Figure 6.** L-asparagine hydrolysis reaction by L-asparaginase [1]

4. Conclusion
Based on the results of the research, it could be concluded that the L-asparaginase enzyme capable to be isolated from the leaves of *C. odorata* L. with the specific activity of the crude extract of the L-asparaginase enzyme at 4.8234 IU/mg and the results of its characteristic, pH, temperature and optimum incubation time respectively pH 8, 37 °C and 30 minutes while the characterizing of the metal ion effects namely Na\textsuperscript{+} and K\textsuperscript{+} metals as activators while Ca\textsuperscript{2+}, Zn\textsuperscript{2+}, Mg\textsuperscript{2+}, Cu\textsuperscript{2+}, Co\textsuperscript{2+}, Mn\textsuperscript{2+} as inhibitors. The anticancer potential test results that the BSLT method showed an LC\textsubscript{50} value of 5.8063 μg/mL. It means that the enzyme extract has a very high toxicity level. The results of this study prove that the L-asparaginase enzyme from the leaves of *C. odorata* L. reveals the potential to be developed as an anticancer in the future.

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