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Cytotoxicity Test of Bandotan Herbal Ethanol Extract (*Ageratum conyzoides* L.) Using the *Brine Shrimp Lethality* Test (BSLT) Method

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Abstract

Cytotoxic activity is a process that can induce cell death. One of the plants that has cytotoxic properties is bandotan (Ageratum conyzoides L.). The purpose of this study was to identify the class of secondary metabolites present in the ethanol extract of Bandotan herb and to evaluate its cytotoxicity by determining the LC_{50} value using the Brine Shrimp Lethality Test (BSLT) method. This research includes phytochemical screening and cytotoxicity testing of an ethanol extract of Herba bandotan using the BSLT method. BSLT is the first method for cytotoxicity testing using Artemia salina Leach larvae at the nauplii stage. Used in the BSLT method because it has similar responses with mammals, namely DNA-dependent RNA polymerase in Artemia salina Leach, similar to DNA-dependent RNA polymerase in mammals. By phytochemical screening, the secondary metabolites in Herba Bandotan (Ageratum conyzoides L.) are alkaloids, flavonoids, saponins, steroids, and tannins. Based on the results of the cytotoxicity test using the BSLT method, it shows that the ethanol extract of Herba bandotan (Ageratum conyzoides L.) has the potential to be anti-cancer because it has an LC_{50} value of 255.1915 g / mL, which is included in the toxic category and has the potential to be anti-cancer. Potential as an anticancer agent in the BSLT test if it has an LC_{50} value < 1000 µg / mL.

Keywords: Bandotan, metabolites, cytotoxicity, BSLT, LC_{50}

Introduction

Cancer is a disease characterized by the continuous abnormal growth of cells that proliferate to suppress normal cell growth (Nasution et al., 2023). The uncontrolled growth of cancer cells is then followed by invasion of surrounding tissues and metastasis to other tissues (Nurviani, 2020). Various intensive cancer treatment efforts, such as chemotherapy and radiation, as well as surgery, have been widely applied; however, up to now, the healing therapy with existing cancer drugs has not been satisfactory, apart from the significant side effects, expensive price, and difficulty in obtaining (Sepvina et al., 2022). As a result, many people turn to treatments with natural ingredients. This encourages the search for new sources of anticancer compounds in nature.

One of the traditional plants commonly used by the community is Bandotan (*Ageratum conyzoides* L.). In Indonesia, bandotan is a wild plant found in gardens and fields. The parts used in medicine include herbs, such as the above-ground parts and roots (Rumape et al., 2023). Empirically, Bandotan (*Ageratum conyzoides* L.) is used externally to heal wounds, leprosy, and boils, and as an antihaemorrhagic, antiseptic, and hemostatic agent. Bandotan is used as a traditional medicine because it contains flavonoid phytochemicals, which are secondary metabolites, namely polyphenolic compounds, which have anti-inflammatory and anti-tumor effects (Adelya et al., 2022)

This research was conducted as an initial step in cytotoxicity testing using the Brine Shrimp Lethality Test (BSLT) method with Artemia salina Leach sea shrimp larvae. Cytotoxic activity refers to the ability to cause cell death (Ridwanto et al., 2023). Artemia salina Leach at the nauplii stage is used as the test animal in the BSLT method because Artemia salina Leach at the nauplii stage exhibits responses similar to those of mammals. Specifically, DNA-dependent RNA polymerase in Artemia salina Leach is comparable to that in mammals (Farid et al., 2019).

The growth stage of shrimp larvae used in this study is the nauplius stage, as at this stage, Artemia salina Leach is in the most active phase of mitotic division, which is similar to that of cancer cells, which also divide mitotically (Farid et al., 2019).

This led to the development of the cytotoxicity test using the BSLT method. The BSLT method is used to assess the cytotoxicity effect on cells and is typically employed in preliminary tests for screening or evaluating the pharmacological

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activity of medicinal plants. It is also utilized for screening new anticancer compounds derived from plants. The results of toxicity tests using this method have been shown to have a positive correlation with the cytotoxicity of anticancer compounds. In addition, this method is easy to use, inexpensive, fast, and accurate. According to the BSLT method, an extract can be considered toxic if it has an LC50 value of ≤ 1000 µg/mL (Melati, 2021).

This research aims to determine the class of secondary metabolite compounds present in the ethanol extract of bandotan herb and their cytotoxicity by examining the LC_{50} value using the Brine Shrimp Lethality Test (BSLT) method.

Methods

The tools used in this study include analytical balance (Sartonius CP224S), drying cabinet, mixer (Phillips), maceration vessel, microscope (Binocular), rotary evaporator (Heidolph), oven (Furnace), hot plate (Thermolyn), test tube rack, porcelain beaker, porcelain crucible, desiccator (Duran), water bath (Memmert), filter paper, vial, magnifying glass (Joyko), spatula, Artemia salina Leach egg drop vessel, aerator (Amara), aquarium lamp (Yamano), and laboratory glassware.

The materials used in this study include Herba bandotan (*Ageratum conyzoides* L.), Artemia Salina Leach eggs (Supreme Plus), sea salt, 96 % ethanol, distilled water (*E-Merck*®), anhydrous acetic acid (*E-Merck* ®), concentrated sulfuric acid (*E-Merck*®), bismuth nitrate (*E-Merck*®), chloroform (*E-Merck*®), toluene (*E-Merck*®), Mercury (II) Chloride (*E-Merck*®), Ferrous (III) Chloride (*E-Merck*®), Lead (II) Acetate (*E-Merck*®), Magnesium Powder (*E-Merck*®), Chloral Hydrate (*E-Merck*®), Sodium Hydroxide (*E-Merck*®), Concentrated Hydrochloric Acid (*E-Merck*®.

Preparation of extract

Bandotan herb extract is made using the maceration method. Put the Bandotan herb simplicia powder, of a suitable fineness, into a vessel. Then, pour 75 parts of 96% ethanol through a filter, cover it, and leave it for 5 days, protected from light, while stirring frequently. After that, mix and squeeze. Then the dregs are washed with 25 parts of filter fluid until 100 parts are obtained. Then transfer to a closed vessel, leave in a cool place, and protect from light for 2 days. After that, pour or strain it. The extraction results are then concentrated using a rotary evaporator (Robiatun et al., 2022).

Simplicia characterization

Simplicia characterization examination includes macroscopic and microscopic examination of simplicia powder, determination of water content, determination of soluble essence in water, determination of soluble essence in ethanol, determination of total ash content, and determination of acid-insoluble ash content (Mentari et al., 2020).

Phytochemical screening

Phytochemical screening of the ethanol extract of bandotan herb includes examination of alkaloids, flavonoids, saponins, tannins, triterpenoids/steroids, and glycosides (Pulungan et al., 2022).

Cytotoxicity test using the BSLT method

Making artificial seawater

Artificial seawater is made by dissolving 38 grams of salt without iodine in 1 liter of water and then stirring until the solution is homogeneous. Then, it was filtered with Whatman paper (Ridwanto et al., 2023).

Hatching of Artemia salina Leach Larvae

Egg hatching is carried out in a clear container containing artificial seawater. The container used is divided into two parts by a perforated partition, namely the dark part and the light part. The perforated partition allows the hatched larvae to move naturally towards the light. Then add a teaspoon of egg to the dark part. The dark part of the container is covered with aluminum foil or black duct tape. The bright part of the container is illuminated with lamp light so that the hatching temperature is maintained at 25-30 ˚C. Shrimp eggs are left to soak for 48 hours, during which time they hatch and develop. The eggs will hatch within 24-36 hours and will naturally move towards a bright area, separating the shrimp larvae from the egg or eggshell. Larvae that are actively moving are suitable for use as test animals in research (Fadli et al., 2019).

Making concentration solutions

The ethanol extract of bandotan herb (*Ageratum conyzoides* L.) was prepared into a stock solution of 2000 µg/mL by weighing 0.2 g of the extract and then dissolving it in 100 mL of artificial seawater. Then it was diluted into 10 concentrations to be used first as orientation, namely concentrations of 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 µg / mL, and one tube was used for negative control, each with three repetitions (Rani et al., 2022).

Cytotoxicity testing of the ethanol extract of the bandotan herb

Vials were prepared for each concentration level; each provided three vials and was replicated 3 times. Each vial is filled with an extract that has been diluted to 10 concentrations and then filled with artificial seawater to a total volume of 10 mL. A total of 10 Artemia salina Leach larvae were put into each vial containing the test compound. The negative control (blank) was treated the same as the test solution, except that no extract was added. The number of dead shrimp larvae in each vial was counted over 24 hours. The level of toxicity is

determined by counting the number of dead larvae using a magnifying glass. The standard criterion for assessing the death of shrimp larvae is if the shrimp larvae do not show movement for several seconds (Rani et al., 2022).

Data analysis

The research data obtained will be analyzed using probit analysis in Microsoft Office Excel 2010 to examine the linear relationship between probit values and concentration logs. The LC_{50} value can be calculated by entering the value 5 (50 % probability of test animal death) as y, so that x is produced as the log concentration value. The antilog of the x value is the LC_{50} value (Fadli et al., 2019).

Results and Discussion

An examination of the characterization of a simplicia is necessary to ensure the quality and accuracy of the simplicia. This characterization examination was conducted by the procedures established by the Indonesian Materia Medika and adheres to the requirements of the Indonesian Materia Medika and the Indonesian Herbal Pharmacopoeia.

Table 1. Results of characterization of the Bandotan herb simplicia

No	Parameters	Result (%)	Condition FHI (%)
1.	Water content	6	≤ 10
2.	Water-soluble essence content	20.28	≥ 11.4
3.	Solubility of essence in ethanol	17.07	≥ 15.4
4.	Total ash content	10.87	≤ 12.3
5.	Acid insoluble ash content	1.47	≤ 3.1

Information: FHI: Indonesian Herbal Pharmacopoeia.

The determination of water content is carried out to provide a maximum limit or range of water content contained in Bandotan herb simplicia. High water content will affect the enzymatic reaction, allowing the Bandotan herb simplicia to easily grow fungus or mold during storage, thereby decreasing the quality of the simplicia. The results of the characterization of the bandotan herb simplicia showed that the results of determining the water content were 6 %, so this is by the requirements of the MMI, namely no more than 10%, which is the maximum limit of % water content that is allowed about purity and contamination that may occur. The results of characterizing the water-soluble essence content of the Simplicia herba Bandotan powder yielded a percentage content of 20.28%. For testing the ethanol-soluble essence content of the Simplicia herba Bandotan powder, the percentage content was determined to be 17.7%. The results of determining the juice content showed that the bandotan herb simplicia contained more compounds that were soluble in water than those that were soluble in ethanol.

In determining the total ash content of Simplicia herba bandotan powder, the percentage content was found to be 10.87%. For determining the acid-insoluble ash content, the percentage content was 1.47%. The determination of total ash content aims to provide an overview of the internal and external mineral content throughout the entire process, from the initial process to the formation of the extract. In contrast, the determination of acid-insoluble ash content aims to determine the level of impurities mixed in the powder during simplicia preparation (Nurmazela et al., 2022).

Phytochemical screening was conducted to determine the content of chemical compounds in the simplicia or bandotan herb extract.

Table 2. Phytochemical screening of the simplicia and ethanol extract of the bandotan herb

No	Parameters	Result	
		Simplicia	extract
1	Alkaloids	+	+
2	Flavonoids	+	+
3	Tannin	+	+
4	Saponin	+	+
5	Steroids	+	+
6	Glycosides	-	-

Information:
(+) Positive: It contains a group of compounds
(-) Negative: Does not contain any compound class

Phytochemical screening tests on the bandotan herb revealed that it contains alkaloids, flavonoids, tannins, saponins, and steroids. The anti-cancer activity of the bandotan herb is influenced by the presence of alkaloid and flavonoid compounds. Flavonoids and alkaloids have anti-cancer mechanisms because flavonoids inhibit the larvae's ability to eat. Apart from that, several theories exist regarding the mechanism by which flavonoids act as anti-cancer agents and antioxidants, including the mechanism by which they activate the apoptotic pathway in cancer cells. Alkaloids derived from plants have a cytotoxic mechanism, namely acting as tubulin inhibitors.

Cytotoxicity is the degree to which a substance damages cells. The cytotoxic test on Artemia salina Leach shrimp larvae, also known as the brine shrimp lethality test (BSLT), is a preliminary test used to search for anti-cancer compounds by determining the LC_{50} value after exposing the larvae to the extract solution for 24 hours (Melati, 2021). Artemia salina Leach, which can be used for cytotoxic testing, is in the form of naupli or larvae. The larvae used were 48 hours old because larvae at this age are in a sensitive state. At 48 hours of age, the organs in Artemia salina Leach are fully formed (Ningsih et al., 2023). With the formation of a mouth, Artemia salina Leach can ingest artificial seawater that has been supplemented with test extracts at various concentrations. So the

death was caused by treatment with the ethanol extract of the bandotan herb (Rani et al., 2022).

The ethanol extract of the bandotan herb was prepared into a stock solution at a concentration of 2000 µg/mL, achieved by weighing 0.2 g of the ethanol extract and dissolving it in 100 mL of artificial seawater. Then, the stock solution is diluted to several concentrations, namely 1000, 900, 800, 700, 600, 500, 400, 300, 200, and 100 g/mL, which will be used for the concentration orientation test first. Additionally, a negative control was prepared, consisting only of seawater and 10 Artemia salina Leach larvae. The negative control aims to determine the effect of adding Bandotan herb ethanol extract on larval death rates. This experiment was conducted with three replications to minimize data errors. Observations on test larvae were carried out 24 hours after treatment. The calculation of larval death is done by observing the larvae for a few seconds. Larval death was calculated when there was no further larval movement within a few seconds. The cytotoxicity orientation test of Bandotan herb ethanol extract and the cytotoxicity test results of Bandotan herb ethanol extract are presented in **Tables 3** and **4.**

Based on the research results obtained, larval mortality varied at each concentration. Once the total number of dead larvae is known, the data is then used to calculate the mortality rate at each treatment concentration. From the mortality percentage data, the concentration used for cytotoxicity is the percentage of larval death between 20% and 80%, as this death percentage provides a more linear curve, allowing the LC50 value obtained in the BSLT test to describe the actual results accurately.

Based on Table 4, the percentage of larval death falls within the range of 20–80%, specifically at concentrations of 100-600 g/mL. Meanwhile, the blank does not provide mortality for larvae. This is under the theory that the higher the extract concentration, the greater the number of larvae that die. Apart from that, from the percentage of larval deaths, it can be concluded that the higher the extract concentration, the higher the number of larval deaths.

The way the toxic compound works, which causes death in Artemia salina Leach larvae, is by acting as a stomach poison. This toxic compound can disrupt the digestive system and inhibit taste receptors in the larva's mouth area. This is what causes the larvae to fail to receive a taste stimulus, rendering them unable to recognize their food and ultimately leading to their death.

The mechanism of larval death is thought to be related to the function of flavonoid and alkaloid compounds. Flavonoids and alkaloids have anti-cancer mechanisms because flavonoids inhibit the larvae's ability to eat. Apart from that, several theories exist regarding the mechanism by which flavonoids act as anti-cancer agents and antioxidants, including the mechanism by which they activate the apoptotic pathway in cancer cells. The mechanism of cell apoptosis in this theory is due to DNA fragmentation. This fragmentation begins with the release of the proximal DNA chain by reactive oxygen compounds such as hydroxyl radicals. Another effect is that flavonoids act as inhibitors of tumor or cancer proliferation, one of which is by inhibiting protein kinase activity, thereby inhibiting the signal transduction pathway from the membrane to the cell nucleus. Flavonoids inhibit receptor tyrosine kinase activity because increased receptor tyrosine kinase activity plays a role in the malignant growth of cancer cells. Flavonoids also function to reduce tumor resistance to chemotherapy agents.

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Alkaloids derived from plants have a cytotoxic mechanism, namely acting as tubulin inhibitors. During the cell cycle process, alkaloids bind to tubulin, a protein that forms microtubules. The binding of tubulin to alkaloids hinders the polymerization of proteins into microtubules, thereby preventing the formation of the mitotic spindle and causing the cell cycle to stall at metaphase. Because they cannot carry out cell division, the cells will then undergo apoptosis (Rani et al., 2022).

The data obtained in **Table 4** were then analyzed using a probit analysis table to obtain the LC_{50} value. The LC_{50} value can be calculated using a straight-line regression equation by entering the probit value of 50% death of test animals as the y-axis and the log concentration value as the x-axis.

After conducting the probit analysis, it is evident that the linear regression equation is $Y = 1.8605x + 0.5219$, with a correlation coefficient of $R^2 = 0.9949$. This indicates that the concentration of the extract explains 99.49% of the variation in the probit value. The R2 correlation coefficient value is categorized as strong if the R2 value is ≥ 0.67,

moderate if > 0.33 and < 0.67, and weak if > 0.19 and < 0.33. After conducting the probit analysis.

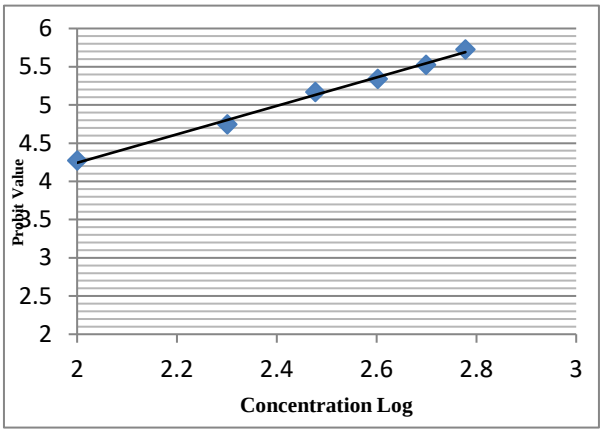

Figure 1. Linear regression curve between the log concentration of bandotan herb ethanol extract and probit value

The linear regression equation is Y = 1.8605x + 0.5219, with a correlation coefficient of R^2 = 0.9949. This indicates that the concentration of the extract explains 99.49% of the variation in the probit value. The R2 correlation coefficient value is categorized as strong if the R2 value is ≥ 0.67, moderate if > 0.33 and < 0.67, and weak if > 0.19 and < 0.33.

The graph shows the log of concentration against the probit value obtained from the percentage of larval deaths. Then enter the Y value, namely the probit value for 50 % of the test animals, so that the value x = 2.406866226 is obtained and the antilog LC_{50} value of 2.406866226 is 255.1915 µg / mL.

A compound is considered toxic and potentially anti-cancer in the BSLT test if it has an LC50 value of 1000 µg/mL. The LC_{50} value obtained was smaller than 1000 µg/mL, specifically 255.1915 µg/mL. Therefore, it can be concluded that the ethanol extract of the bandotan herb is categorized as toxic but has the potential to be anticancer. The LC_{50} value obtained reflects the toxicity of the material to test animals. The greater the LC_{50} value, the lower the toxicity, and conversely, the lower the LC50 value, the higher the toxicity (Melati, 2021).

Table 3. Cytotoxicity orientation test of ethanol extract of bandotan herb (*Ageratum conyzoides* L.)

No	Concentration	Number of dead larvae			Total	Average larval mortality	% Mortality
		P1	P2	P3			
1	Blank	0	0	0	0	0	0
2	100	3	2	2	7	2.33	23.3
3	200	5	4	3	12	4	40
4	300	6	5	6	17	5.66	56.6
5	400	7	6	6	19	6.33	63.3
6	500	8	7	6	21	7	70
7	600	8	8	7	23	7.66	70.6
8	700	8	9	8	25	8.33	83.3
9	800	9	9	8	26	8.66	86.6
10	900	10	10	10	30	10	100
11	1000	10	10	10	30	10	100

Table 4. Cytotoxicity Test Results of the Ethanol Extract of Bandotan Herb (*Ageratum Conyzoides* L.)

No	Concentration	Number of dead larvae			Total	Average larval mortality	% Mortality
		P1	P2	P3			
1	Blank	0	0	0	0	0	0
2	100	3	2	2	7	2.33	23.3
3	200	5	4	3	12	4	40
4	300	6	5	6	17	5.66	56.6
5	400	7	6	6	19	6.33	63.3
6	500	8	7	6	21	7	70
7	600	8	8	7	23	7.66	70.6
8	700	8	9	8	25	8.33	83.3
9	800	9	9	8	26	8.66	86.6
10	900	10	10	10	30	10	100
11	1000	10	10	10	30	10	100

Conclusions

The secondary metabolite compounds contained in the bandotan herb (*Ageratum conyzoides* L.) were identified using a phytochemical screening method, which revealed the presence of alkaloids, flavonoids, saponins, steroids, and tannins. Based on the results of the cytotoxicity test using the BSLT (Brine Shrimp Lethality Test) method, the ethanol extract of bandotan herb exhibits cytotoxicity, as indicated by an LC50 value of 255.1915 µg/mL, which falls within the toxic category.

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