THE ENDOPHYTIC ACTINOBACTERIAL TOXICITY TEST OF GINGER (*Zingiber officinale* Roscoe) USED THE BSLT (Brine Shrimp Lethality Test) METHOD

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Abstract: Cancer is the biggest cause of death in Indonesia. Cancer treatment efforts have been made but it could harm cancer patients. It is necessary to find another alternative from nature as an anticancer. This study aims to see the ability of endophytic actinobacterial isolates from ginger (*Zingiber officinale*) as an anticancer tested using the BSLT method (Brine Shrimp Lethality Test) by calculating the values of LC_{50} and LT_{50}. The best value isolates from the BSLT method, then tested using phytochemical ethanol extract. The highest toxicity value was found in AJ2 isolates. LC_{50} and LT_{50} values of AJ2 isolates were 309.358 µg/mL and 11.879 hours. Phytochemical results of ethanol extract of AJ2 isolates were detected containing steroids, terpenoids, phenols, tannins, flavonoids, alkaloids, and saponins. AJ2 isolates which derived from ginger endophytic actinobacteria have potential as an anticancer because they have high toxic values that rised rapidly.

Keywords: anticancer, endophytic actinobacteria, BSLT, ginger

Abstrak: Kanker menjadi penyebab kematian terbesar di Indonesia. Upaya pengobatan kanker sudah dilakukan namun memberikan dampak negatif bagi penderita kanker. Perlu dilakukan penemuan alternatif lain berasal dari alam sebagai antikanker. Penelitian ini bertujuan untuk melihat kemampuan isolat aktinobakteri endofit asal jahe *(Zingiber officinale)* sebagai antikanker. 7 isolat aktinobakteri endofit jahe diuji menggunakan metode BSLT *(Brine Shrimp Lethality Test)* dengan menghitung nilai LC_{50} dan LT_{50}. Isolat nilai terbaik dari metode BSLT, selanjutnya diuji menggunakan fitokimia ekstrak etanol. Nilai toksisitas tertinggi terdapat pada isolat AJ2. Nilai LC_{50} dan LT_{50} isolat AJ2 adalah 309,358 µg/mL dan 11,879 jam. Hasil fitokimia ekstrak etanol isolat AJ2 terdeteksi mengandung steroid, terpenoid, fenol, tanin, flavonoid, alkaloid dan saponin. Isolat AJ2 yang berasal dari actinobacteria endofit jahe berpotensi sebagai antikanker karena memiliki nilai toksik yang tinggi dalam waktu singkat.

Kata kunci: antikanker, aktinobakteri endofit, BSLT, dan jahe

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Introduction

More than 10 million people worldwide were diagnosed with cancer in 2008. 5-10% of cancer cases are genetic defects, and 95-90% come from the environment and lifestyle (Pangastuti, Amin, Amin, Amin, 2016). Various efforts were made for cancer treatment, such as surgery, radiation, chemotherapy, and immunotherapy. However, the treatment process has side effects for cancer patients, such as a decrease in the number of blood cells (will return to normal about a week later), infection (marked by heat, sore throat, burning sensation). When urinating, shivering and redness, swelling and burning), anemia, bleeding such as nosebleeds, hair loss, dry and itchy skin, nausea and vomiting, dehydration, low blood pressure, constipation, diarrhea, and neurological disorders (Inthe, Tarman,& Safithri, 2014). As a result of the excess side effects of anticancer treatments that will harm the body, the community returns to traditional herbal medicine, which uses medicinal plants, ginger (*Zingiber officinale*). Many medicinal plants need biomass if used continuously. Another alternative is to use endophytic microbes isolated directly from the ginger rhizome plant, endophytic actinobacteria.

Brine Shrimp Lethality Test (BSLT) is a method for detecting bioactive compounds using aquatic crustacean species belonging to the Artemidea family (Mirzaei,& Mirzaei, 2013). The BSLT method is one of the methods for screening medicinal plants designed as an anticancer because it was cheaper, shorter, easier to developed, and there was no ethical rules in using test materials (Ningdyah, Alimuddin,& Jayuska, 2015). The Lethal Concentration (LC_{50}) value of the tested animal was 50% within a specific time interval. The LC_{50} did not react to specifically damaged tested organs from the total mortality of the tested animals. Accordingly, the LC_{50} value was used in the short-term test. LC_{50} is used to calculate the mortality rate of *Artemia,* given its uncomplicated digestive structure and high sensitivity (Ningdyah, Alimuddin,& Jayuska, 2015). The value of LT_{50} is a time calculation of chemicals with a certain concentration that can be canceled by 50% of animal tests. Therefore, the lower the value of LT_{50}, the higher the material values mortality. (Nurhaifah,& Sukesi, 2015).

Materials and Methods

Isolation of endophytic actinobacteria

The part of the medicinal plant used is the ginger rhizome. The first, 1 g of ginger rhizome was washed and carried out surface sterilization by soaking 70% alcohol for 5 minutes, followed by 1% hypochlorite solution for 5 minutes, then rinsed with sterile distilled water sterilized with 70% alcohol for 5 minutes. Next, the ginger rhizomes were then crushed and taken 0.1 mL of liquid sample and inoculated on agar HV media, then incubated for 14-30 days at room temperature.

Actinobacterial colonies that grow was purified using ISP-2 media (Taechowisan, Peberdy, & Lumyang, 2003).

Bacterial extraction

Isolates sample of endophytic actinobacteria from isolation from ginger plants isolation had been obtained from previous studies, which will be tested on anticancer activity. All isolates were inoculated into 250 mL ISP-2 liquid media, incubated for 10 days in an incubator shaker at the speed of 100 rpm at room temperature. The isolates were filtered with filter paper. The obtained supernatant was extracted using ethanol solvent. Extraction was done by adding solvents to the supernatant in a ratio of 1:1 and then homogenized with a shaker for 1 day to form a water fraction and solvent fraction. The solvent fraction then separated using a rotary evaporator until a concentrated fraction was obtained (Pujiyanto, 2012). This concentrated fraction was used for BSLT and phytochemical tests.

Anticancer activity test using the BSLT method (Brine Shrimp Lethality Test)

Preparation of larvae was done by taking 1 g of *Artemia salina* eggs. Hatching was done by soaking the eggs in 2 L of seawater given incandescent lamps and aerated for 48 hours (Muaja, Koleangan,& Rutuwene, 2013). The concentration of samples was made by calculating concentrations of 10, 50, 100, 500, and 1000 µg/mL and the time of death at 4, 8, 12, 16, 20, and 24 hours. Each of the concentration calculation was taken 1 mL samples in test tubes and added 1 mL of seawater. Each concentration was made in three tubes (triplo). 10 *Artemia* larvae were put into the test sample then added seawater to 5 mL of volume. The same treatment was carried out for the solvent of the test sample (buffer), which functionated as a negative control. The number of dead and living larvae was observed, calculated and the LC_{50} and LT_{50} values were determined using SPSS probit analysis after 24 hours (Ningdyah, Alimuddin,& Jayuska, 2015).

Phytochemical test of actinobacteria bacteria

The selected isolates were then extracted, and phytochemical tests were carried out.

Flavonoid test

The extract solution was added 2-4 drops of HCL solution and 2 pieces of Mg metal. The indicated flavonoid observed from color changes of dark yellow to orange (Mariana, Andayani,& Gunawan, 2013).

Alkaloid test

A solution of 2 mL test extract was evaporated on a porcelain plate until residue was obtained. The residue were then dissolved with 5 mL of HCl 2 N. The solution was divided into 3 test tubes. The first tube was added with 2 N HCl, which functions as a blank. The second tube was added with 3 drops of

Dragendorff reagent, and a third tube was added to the Mayer reagent with 3 drops. The formation of orange deposits in the second tube and white to yellowish deposits in the third tube shows alkaloids (Harborne, 1987).

Steroid/terpenoid test

The extract solution was tested using a Liebermann-Burchard reagent. First, the solution was dripped with a chloroform solution dissolved in acetic anhydride. Then pressed concentrated sulfuric acid. If there is a change in the color orange or purple indicates containing terpenoids. Finally, if it turns blue, it shows positive steroids (Parbuntari, Prestica, Gunawan, Nurman, & Adella, 2018).

Saponin test

The saponin test was carried out by the forth method by inserting 2 mL of the sample into the test tube, then adding 10 mL of distilled water and then shaking it for 30 seconds, observed the changes that occurred. Saponin's presence was indicated if a solid foam was formed under 30 second (Harborne, 1987).

Phenol/tannin test

The test extract solution was reacted with 10% iron (III) chloride solution, if dark blue, blackish-blue, or greenish-black color showed phenol and tannin compounds (Harborne, 1987).

Results

The isolates obtained from 7 isolates from endophytic actinobacteria from ginger were coded AJ1, AJ2, AJ3, AJ4, AJ5, AJ6, and AJ7, which is a continuation of previous research (Rahayu, Fitri,& Ismail, 2019). All isolates were extracted to obtain concentrated fractions for BSLT and phytochemical tests. Lethal Concentration (LC_{50}) is a toxicity test based on the BSLT method. This test uses ethanol extract from the seven isolates. The data on table 1 showed that AJ1, AJ2, AJ4, and AJ6 were toxic while AJ3, AJ5, and AJ7 are non-toxic. One isolate was considered the most toxic of the four isolates. AJ2 isolate was isolated with the lowest LC_{50} value of the other three isolates with an LC_{50} value of 309.358 µg/mL. Isolate AJ2 showed that the extract concentration of 309.358 µg/mL was able to kill half the *Artemia salina* shrimp larvae population.

The Median Lethal Time (LT_{50}) is the time needed to cause the death of 50% of the test animal population. Table 2 shows the LT_{50} values in isolates AJ1 and AJ6, that the greater the concentration, the smaller the value. At a 1,000 µg/mL concentration, the isolate AJ1 had a value of LT_{50} of 21.986 hours, and isolate AJ6 had an LT_{50} value of 19.115 hours. The meaning that isolates AJ1 after 21.986 hours, isolates AJ6 after 19.115 hours had been able to cause 50% death of *Artemia salina* larvae. In isolates AJ2 and AJ4, the value of LT_{50} fluctuate at various concentrations. The lowest LT_{50} value was found at a

concentration of 500 µg/mL. It shows that the AJ2 and AJ4 isolates were able to kill 50% of the test larvae well at that concentration. The value of LT_{50} at 500 µg/mL isolates AJ2 was 11.879 hours, and isolates AJ4 were 17.569 hours. AJ3, AJ5, and AJ7 indicated non-toxic. AJ3 isolate only 10% of larvae died at a concentration of 1,000 µg/mL, this concentration was the highest in this test, but only 10% of larvae died with a value of LT_{50}, 141.344 hours. In AJ5 isolates, the larvae that died at concentrations of 1,000 µg/mL were only 40%, and due to abnormal data distribution, the value of LT_{50} was 0. The AJ7 only at concentrations had dead larvae of 3% in isolates, and no larvae died at 500 µg/mL other concentrations. Then the value of LT_{50} obtained at a concentration of 500 µg/mL is 356.353 hours. From LT_{50} value, AJ2 isolate can kill *Artemia salina* population in a fairly fast time compared to other isolates of 11.879 hours.

Based on LC_{50} and LT_{50} values from the BSLT method, AJ2 isolate is an isolate capable of killing test animals (*Artemia salina*) in a fast time (11.879 hours) with a not very high concentration (500 µg/mL). AJ2 isolates are then characterized by morphology.

Table 1. LC_{50} value

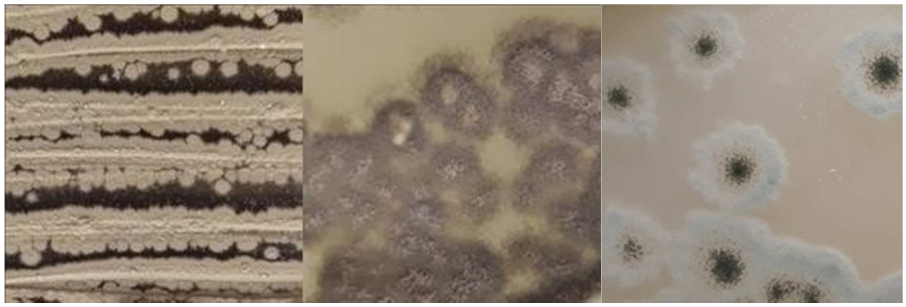
Isolate code	% larval death at 24 hours					LC50 µg/mL
	10 µg/mL	50 µg/mL	100 µg/mL	500 µg/mL	1,000 µg/mL	
AJ1	20	26	40	43	53	803.410
AJ2	3	10	10	76	93	309.358
AJ3	0	0	0	3	10	>1,000
AJ4	40	36	50	66	73	653.381
AJ5	0	0	0	0,3	40	>1,000
AJ6	26	33	43	50	60	832.535
AJ7	0	0	0	3	0	>1,000

Table 2. Value of LT_{50} at various concentrations

Isolate code	Consentration (µg/mL)	% Time death						LT_{50} (hours)
		4 hours	8 hours	12 hours	16 hours	20 hours	24 hours	
AJ1	10	0	0	0	0	13	20	35.511
	50	0	0	6	10	20	26	34.511
	100	0	0	3	10	30	40	25.855
	500	0	3	10	23	36	43	25.368
	1,000	0	6	16	26	50	53	21.986
AJ2	10	0	0	0	0	3	3	70.768
	50	0	0	10	10	10	10	93.832
	100	0	3	10	10	10	10	141.344

Isolate code	Consentration (µg/mL)	% Time death						LT_{50} (hours)
		4 hours	8 hours	12 hours	16 hours	20 hours	24 hours	
	500	0	30	70	70	76	76	11.879
	1,000	0	33	40	60	86	93	12.397
AJ3	10	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
	500	0	0	3	3	3	3	356.353
	1,000	0	3	10	10	10	10	141.344
AJ4	10	0	0	23	33	40	40	24.398
	50	0	0	6	16	30	36	27.443
	100	0	3	13	23	40	50	23.730
	500	0	3	36	43	60	66	17.569
	1,000	0	6	13	26	46	73	19.470
AJ5	10	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
	500	0	0	0	0	3	3	0
	1,000	40	40	40	40	40	40	0
AJ6	10	0	0	3	13	26	26	30.996
	50	0	3	16	23	26	33	32.498
	100	0	0	16	23	40	43	24.557
	500	0	3	13	30	46	50	22.420
	1,000	0	20	26	36	56	60	19.115
AJ7	10	0	0	0	0	0	0	0
	50	0	0	0	0	0	0	0
	100	0	0	0	0	0	0	0
	500	0	0	3	3	3	3	356.353
	1,000	0	0	0	0	0	0	0

The characteristics of AJ2 isolates on the ISP-2 media have the color of aereal celium and light brown substrate, do not have soluble pigments, and are convex shaped. In the ISP-3 media, dark brown aereal mycelium and substrate mycelium are yellow, do not have pigments, and are convex. In the ISP-4 media, the isolate has the color of green and white aerial mycelium, and the substrate mycelium is green, does not have soluble pigments, and is convex (Figure 1). On microscopic observation, the AJ2 isolate has a flexous spore chain (figure 2). The morphological characteristics of AJ2 use media ISP-2, ISP-3, and ISP-4 as media (Kim, Tomoda, Iizima, Fukuda, Matsumoto, Takahashi & Omura, 2003).



(a) (b) (c)

Figure 1. Morphology of endophytic ginger actinobacterial isolates AJ2 colonies on media (a). ISP-2; (b). ISP-3; (c). ISP-4.

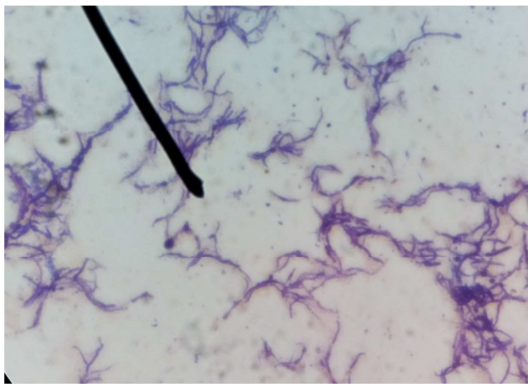


Figure 2. Isolate AJ2 microscopic with 1000x magnification.

Based on the BSLT test, the selected isolates were AJ2 isolates tested for phytochemical ethanol extract. AJ2 isolate is an isolate that has the highest toxicity and has the potential as an anticancer. The results of phytochemical tests (Table 3) showed that AJ2 isolates were detected containing steroids/ terpenoids, phenols, tannins, flavonoids, alkaloids, and saponins. The main fraction in ginger is divided into two, namely, volatile and non-volatile fractions.

Table 3. Phytochemicals of AJ2 isolates and Ginger Rhizomes

Compound	AJ2 isolate	Ginger (Rhizome Agustina, Ruslan, &Wiraningtyas, 2016)
Steroids	+	+
Terpenoids	+	+
Phenols	++	+
Tannins	++	-
Flavonoids	+	+
Alkaloids	+	+
Saponins	++	+

Description: - : Not detected
+ : Detected
++ : Medium detected
+++ : Many detected

Discussion

To our knowledge, research on the endophytic actinobacteria of toxicity test of ginger used the BSLT method as an is the first. In the testing using the BSLT method, the highest LC_{50} value was owned by AJ2 isolates, 309.358 µg/mL. The higher the toxicity of secondary metabolites of a compound, the more potential the compound is represented by, the smaller LC_{50} value. According to Meyer criteria, an extract is considered very toxic if it has an LC_{50} value below 30 µg/mL, is considered toxic if it has an LC_{50} value of 30 to 1,000 µg/mL, and is considered not toxic if it has an LC_{50} value exceeding 1,000 µg/mL (Meyer, Ferrigni, Putman, Jacobsen, Nichols, & McLauglin, 1982). Based on the criteria for the LC_{50} value, according to Clarkson, AJ2 isolate was moderately toxic with an LC_{50} value of 309.358 µg/mL. LC_{50} values at a concentration of 100-500 ppm are moderate toxic (Hardiyanti, Marpaung, Adnyana,& Simanjuntak, 2019). It is consistent with the research conducted by Sukandar to determine the anticancer activity of fragrant pandan (*Pandanus amaryllifolius)* leaf extract, which showed toxic ethyl acetate extract with an LC_{50} value of 288.4 ppm (Sukandar, Hermanto,& Lestari, 2009). Oratmangun conducted a toxicity test of fracture plant extracts (*Euphorbia tirucalli* L.) as a preliminary anticancer study, the results showed that fracture methanol and chloroform extracts were toxic with LC_{50} values of 332.2489 µg/mL and 240.6432 µg/mL respectively (Oratmangun, Fatimawati,& Budhi, 2004).

In LT_{50} testing, AJ2 isolates were able to kill Artemia larvae within 11.879 hours. Research conducted by Dondong about a series of 3-aryl propionic esters and their analogs designed and evaluated for acaricidal activity in vitro against Psoroptes cuniculi, scabies mites. The results showed that 6 compounds had very good activities with the LC_{50} of 0.17-0.24 µg/mL, and the LT_{50} value was 1.5-2.9 hours (Dongdong, Ye, Mingxuan, Xinyuan, Ding, Xinjuan,& Le, 2018). Of the seven isolates, the lowest value of LT_{50} was found in AJ2 isolates at a 500 µg/mL concentration, 19.115 hours. This value indicates that this concentration is very toxic in this isolate because it can kill *Artemia salina* larvae in a short time than others. Based on the LC_{50} and LT_{50} tests, it was found that the selected isolates were AJ2 isolates with LC_{50} values of 309.358 µg/mL. In testing $LT_{50,}$ the isolate showed a good value of LT_{50} at a concentration of 500 µg/mL. The LT_{50} value of the AJ2 isolate was 11.879 hours. The results of the correlation analysis show that the significance value <0.05 is correlated. The isolate is perfectly correlated with the Pearson correlation value of 1,000. This value indicates that the AJ2 isolate has a correlation based on the values of LT_{50} and LT_{50} produced.

Based on the morphological and microscopic observations, it was suspected that AJ2 isolates were thought to belong to the *Streptomyces* genus group. *Streptomyces* is the dominant genus, most commonly isolated as endophytic actinomycetes (Golinska, Wypij, Agarkar, Rathod, Dahm, Rai, 2015).

Endophytic actinobacteria can produce secondary metabolites by inducing them in the host plant, causing physiological and biochemical changes. Endophytic actinobacteria have immunosuppressant, antibiotic, anti-cholesterol, and anticancer compounds (Diness, Srinivasan, Sheaja, Anandraj, Srambikkal, 2017).

Flavonoids have important effects on chemoprevention and cancer chemotherapy. Flavonoids play a major role in many molecular action mechanisms to prevented cancer by interacting between various types of genes and enzymes (Chahar, Sharma, Dobhal,& Joshi, 2011). Cellulase enzymes through the roots and colonizes in the roots. One endophytic bacteria is actinobacteria. Actinobacteria can produce secondary metabolites with its host, such as producing antimicrobial, antidiabetic, other compounds, and anti-cancer (Golinska, Wypij, Agarkar, Rathod, Dahm,& Rai, 2015).

Conclusion

Endophytic actinobacteria from ginger can produce toxic compounds that have the potential as anticancer is isolate AJ2. Based on the results of the BSLT method test. It was evidenced by the isolates of AJ2 who had toxic values which were high from the seven isolates resulting from isolation of endophytic ginger actinobacteria. AJ2 isolation can kill the *Artemia salina* population in a fairly fast time of 11.879 hours at a 500 µg/mL concentration. The flavonoids which acted as cancer prevention were detected in AJ2 isolates from phytocemical test results.

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