



Acute Toxicity Test of Black Pomegranate Peel Extract (*Granati Fructus Cortex*) Against Larvae of Shrimp (*Artemia salina* Leach)

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Abstract: Toxicity test on red pomegranate has done, while in black pomegranate is not widely known. Black pomegranate peel (*Granati Fructus Cortex*) has some chemical ingredients such as saponins, flavonoids, tannins, and alkaloids. Alkaloids, saponins, and flavonoids are thought to be toxic at certain levels. This study aimed to determine the effects of toxic black pomegranate peel extract (*Granati Fructus Cortex*) against larvae of shrimp (*Artemia salina* Leach) indicated LC50 values below 1000 µg / ml. This study is purely experimental by using *Brine Shrimp Lethality Test* (BSLT). The experiments divided into five groups, namely ethanol extract of black pomegranate peel (*Granati Fructus Cortex*) with a concentration of 100, 150, 200, 250 ppm, and negative control (seawater). Mortality data percentage of *Artemia salina* Leach analyzed by probit analysis. Results showed that the extract of black pomegranate peel (*Granati Fructus Cortex*) has a toxic effect on larvae of *Artemia salina* Leach with LC50 values of 114.090 µg/ml. From these results, it can conclude that black pomegranate peel extract has a potential acute toxic to larval shrimp (*Artemia salina* Leach) with *Brine Shrimp Lethality Test* (BSLT) method.

Keyword: pomegranate black peel; acute toxicity test; brine shrimp lethality test; *Artemia salina* Leach.

INTRODUCTION

Indonesia is a rich country in flora and fauna, which widely spread in various regions; these natural resources have utilized by the population, which is more than 230 million people. This diversity has a significant impact on the development of biotechnology in the use of biodiversity by the community, one of which is the use of natural medicines or traditional medicines (Fitri, Oktiarni, & Arso, 2018). In developing of the health sector, conventional medication needs to be developed and gradually utilized based on a scientific foundation, so that it can be used in the issue of standard health services to the community (Roihatul, 2014).

There are various kinds of traditional medicines derived from plants and have studied in much chemical content and efficacy in them. But there are still many plants whose toxicity levels are not yet known, so it needs to be further investigated to determine safety ingredient (Abnaz, Zahra Dzakhirah & Jutti Levita, 2018). One of the natural ingredients that can utilize is black pomegranate skin (*Granati Fructus Cortex*). There are several types of pomegranate, namely white pomegranate, red pomegranate, and black pomegranate (Setiawati, 2014). Black pomegranate skin (*Granati Fructus Cortex*) has several chemical contents such as saponins, polyphenols, flavonoids, tannins, and alkaloids (Rizka & Saptarini, 2018). Flavonoids, polyphenols, tannins, and alkaloids have benefits as an anti-microbial

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and antioxidant. Alkaloid compounds, saponins, and flavonoids are assumed to be toxic at certain levels (Khotimah, 2016).

Toxicity tests are needed to assess the safety of the drug, or ingredients used as supplements or food (Makiyah & Tresnayanti, 2017). It is also to protect the community from potentially harmful effects. The effects of drug toxic are often seen in the liver because the liver plays a central role in the metabolism and extraneous materials that enter the body. The liver will change the structure of lipophilic to the hydrophilic drug, so it quickly removed from the body through urine or gallbladder (Syam, 2016). Excretion via gallbladder allows xenobiotics buildup in the liver to cause hepatotoxicity effects (Putri, 2016).

One of the methods used to test the toxicity is to use the larva of the shrimp type *Artemia salina* Leach. In this method, *A. Salina* Leach used as A bioindicator. This method is easy to work with, cheap, short time detection, and accountable (Sangi, Momuat, & Kumaunang, 2012). The toxicity test in red pomegranate has been done much, while the black pomegranate is not widely known. So, based on this background, the author is interested in doing a black pomegranate toxicity test on shrimp larva (*Artemia salina* Leach).

This study aims to determine the toxic effects of black pomegranate peel extract (*Granati Fructus Cortex*) on shrimp larvae (*Artemia salina* Leach) and to determine LC 50 value of shrimp larvae (*Artemia salina* Leach) after supplying of black pomegranate peel extract (*Granati Fructus Cortex*).

MATERIALS AND METHOD

The research conducted at Animal Laboratory of Institute of Health Science Bhakti Wiyata Kediri. The ingredients used are black pomegranate peel extract, ethanol, distilled water, concentrated HCl, HCl 2N, Mayer, Wagner, Dragendorff, hexane, metal Mg, FeCl₃ 1%, acetic acid, concentrated sulfuric acid, *Artemia salina* Leach larvae, yeast as shrimp larvae feed and seawater. The tools used in this research are Philips blender HR 2116, triple beam balance MB-2610, Rotavapor R-100 Rotary Evaporator, Waterbath health type YNC-WBE-8L. Blender used to crush and smoothing black pomegranate skin, analytical scales for weighing black pomegranate skin that has become into powder. Rotary Evaporator is a powder stirrer black pomegranate that has macerated with ethanol 70% to obtained concentrated extracts. The function of the water bath is to remove ethanol from the black pomegranate powder.

Extraction

Black pomegranate peel used previously weighed, then dried and blended with a blender. Pomegranate peel powder as many as 100 grams put into Erlenmeyer 1000 mL; then, the dust was macerated with 70% ethanol repeatedly then concentrated with a rotary evaporator until crude extract obtained. Make an extract concentration solution of 500 ppm by dissolving 50 mg of extract sample dissolved with 100 mL of seawater. From 500 ppm solution, then make a solution with concentrations of 250, 200, 150, and 100 ppm by dilution. For control (0 ppm) is done without the addition of extracts (Rakhmawati, Qadriyati, & Wijayanti, 2011).

Phytochemical Test

The ethanol extract is further obtained by phytochemical testing to determine the content of its compounds. Tests conducted include flavonoids, saponins, tannins, alkaloids, and analysis, not ethanol (Simaremartye, 2014).

Selection of *Artemia salina* Leach eggs

The selection of shrimp eggs made by soaking the eggs in distilled water for one hour. Good eggs will settle while bad eggs will float (Filha Ferraz, Lombardi, Guzzo, & Guimarães, 2012).

Preparation of larvae *Artemia salina* Leach

Shrimp larvae are prepared by incubating *Artemia salina* Leach eggs for two days before testing. The hatching does by soaking the shrimp eggs into an aquarium container filled with seawater and given aeration. After 48 hours of soaking, the eggs hatch and produce larvae of *Artemia salina* Leach which are ready for use in a testing (Filha Ferraz et al., 2012).

Toxicity Test

Test solutions with concentrations of 250, 200, 150, and 100 ppm, each in a pipette of 10 mL, were inserted into a vial bottle, and ten tail shrimp larvae aged two days added. The observation was carried out for 24 hours on shrimp larvae death, where each concentration was carried out twice during repetition and compared with control. Consideration of the number of dead larvae counted within 6 hours, namely the 6th, 12th, 18th, and 24th hours. Shrimp larvae disclosed to be dead while they are motionless for 10 seconds. After observation for 24 hours later, the level of toxicity determined by calculating the number the dead larvae. LC50 value specified with a linear regression analysis in table 1 (Rampe & Toumbuku, 2015).

Table 1. Level of Toxicity Value LC50

No.	LC50 Value (µg/ml)	Toxicity Levels
1.	0-250	Very toxic
2.	250-500	Toxic
3.	500-1000	Intermediate
4.	>1000	Non toxic

Research data will be processed and presented in tabular form. LC50 calculation data analysis do by data % mortality transformed into a concentration log. Toxicity test data will be analyzed by Probit Analysis using *SPSS 16.0 for windows* to find out LC50 price.

RESULTS AND DISCUSSION

Yield Extract

Based on the examination results of black pomegranate peel extraction results are presented in Table 2.

Table 2. Results of Black Pomegranate Simplisa Powder Extraction Results (*Granati Fructus Cortex*).

Simplisa Powder Weight (g)	Solvent	Extract Weight (g)	Yield Percentage (% b/b)
100,0	Etanol 70%	20,676	20,676

Table 1 shows the results of black pomegranate peel powder extraction (*Granati Fructus Cortex*). The removal produces a yield of 20,676% black pomegranate peel extraction. The concentrated extract tested free of ethanol, so it

did not affect the treatment of experimental animals, in other words, the cause of death of *Artemia salina* Leach larvae was not due to ethanol but due to the active compound contained in the extract. Ethanol-free test results presented in table 3.

Table 3. Results of Ethanol Free Test for Black Pomegranate Peel Extract (*Granati Fructus Cortex*)

Ethanol Free Test	(+) Literatur Results	Testing Resultd
/Extract + H ₂ SO ₄ concentrated + CH ₃ COOH heated	There is no specific eter smell	No specific eter smell

Phytochemical Test

Phytochemical results test observations of black pomegranate (*Granati Fructus Cortex*) extract can be seen in table 4.

Table 4. Phytochemical Test Results of Black Pomegranate Peel Extract (*Granati Fructus Cortex*)

Chemical Content	Testing Results	Description
Flavonoid	Orange reddish forming	+
Saponin	Foam forming	+
Tanin	Blackish blue colour forming	+
Alkaloid	No sediment forming	-

The results of the test positive of flavonoids. A reddish-orange color characterizes the positive outcome of the flavonoid test. Liquid flavonoids consist of phenols that commonly found in many vascular plants. Flavonoids show biochemical activities such as antioxidant, anti-viral, anti-bacterial, and anti-cancer. Flavonoids can inhibit the proliferation of cancer cells with a cell cycle inhibitory mechanism and induce apoptosis. Maryati's Research (2006) proves that the flavonoids are isolated from the ethyl acetate fraction of the ethanol extract of Life (*Gynura procumbens* (Lour.) MERR) has cytotoxic activity with IC₅₀ of 98 µg/mL against breast cancer cells T47D (Rampe & Toumbuku, 2015).

The next test is the saponin test, saponin test on black pomegranate extract shows positive results, this is due to the formation of a stable foam when black pomegranate extract is added to water and heated, then cooled and shaken to form foam. Extracts and water heated to increase the solubility of saponins in water. The formation of foam caused by glycosides contained in black pomegranate peel extract so froth formed in water and will hydrolyze into glucose and other compounds (Muthmainnah, 2017). Saponin is a compound in the form of glycosides that are widespread in high-level plants as well as some marine animals and are a diverse group of compounds in the structure, physicochemical properties, and biologic effects (Yanuartono, Purnamaningsih, Nururrozi, & Indarjulianto, 2017). Saponins are hypocholesterolemic, Immunostimulatory, and Anticarcinogenic. Anticarcinogenic mechanisms of saponins include direct antioxidant and cytotoxic effects on cancer cells (Permana, Husni, & Budhiyanti, 2016).

The tannin test on black pomegranate peel extract also showed positive results. Tannin is a phenolic compound that is water-soluble and usually has a high molecular weight. The tannins test on black pomegranate extract also showed

positive results. Tannins are phenolic compounds that are water-soluble and typically have high molecular weight. Tanin has a toxic effect on other organisms because it can bind to proteins so that the mixture can have toxic effects and bioactivities such as antimicrobial (Alfarabi & Widyadhari, 2018).

Alkaloid test results show negative results. Based on the literature, pomegranate skin contains an alkaloid, pelletriene, but the phytochemical screening results of pomegranate peel extract do not contain alkaloids. This is due to the small alkaloid content, which will be difficult to detect with the reagents used (Sopyan Iyan, Rosa Apriana, 2017).

Toxicity Test

The results of the toxicity test using *Artemia salina* shrimp larvae on an ethanol extract of black pomegranate peel (*Granati Fructus Cortex*) shown in table 5. The number of larvae in each vial used in this study was 10, with a total of 100 larvae from 2 replications. Total larval deaths were obtained by adding up dead larvae at each concentration, while the average mortality was obtained by dividing the total deaths at each level against the number of replications performed. Then calculate the percentage of mortality from the average mortality at each concentration.

Table 5. Toxicity Test Results for Black Pomegranate Peel Extract (*Granati Fructus Cortex*) using *Artemia salina* shrimp larvae

Replication	Control	Total Mortality of Larvae each Concentration				Volume of Final Media (ml)
	0 ppm	100 ppm	150 ppm	200 ppm	250 ppm	
1	0	4	7	9	10	10
2	0	5	8	9	10	10
Total Mortality	0	9	15	18	20	
Average		4,5	7,5	9	10	
%		45	75	90	100	

From this table, it can seem that the various concentrations of ethanol extract of black pomegranate peel (*Granati Fructus Cortex*) show different effects on the percentage of *Artemia salina* Leach larvae mortality. Pearson correlation test was conducted to determine the relationship between concentration and the rate of larval deaths. In the correlation matrix obtained a significant value <0.01 that is 0.001 and the correlation value is positive with a number close to 1 that is 0.991, so it can interpret that there is a correlation between concentration of black pomegranate peel extract with percentage of *Artemia salina* Leach death, the higher level of black pomegranate peel extract, the increasing percentage of *Artemia salina* Leach larvae mortality.

The results of probit analysis between ethanol extract concentrations of black pomegranate peel (*Granati Fructus Cortex*) to the percentage of *Artemia salina* Leach larvae mortality obtained LC50 price of ethanol extract of black pomegranate peel (*Granati Fructus Cortex*) that was 114.090 $\mu\text{g/ml}$. Ethanol extract of black pomegranate peel (*Granati Fructus Cortex*) is included in a very toxic category because it has an LC50 price of 0-250 $\mu\text{g/ml}$. Based on previous research,

if a plant extract is toxic according to LC50 estimates by the BSLT method, then the plant can be developed as an anticancer drug (Kramy Prayogi, Triawanti, 2017). In the past decade, numerous studies on pomegranate constituents have published. The results suggest that pomegranate components have antioxidant, anti-carcinogenic, and anti-inflammatory ingredients, which are active in the prevention and treatment of cancer and other chronic and infectious diseases (Sharma, McClees, & Afaq, 2017).

The Use of the pomegranate juice, peel, and oil has been indicated that pomegranate has anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion, and angiogenesis (Khwairakpam et al., 2018). Pomegranate possesses antioxidant, anti-inflammatory, anti-proliferative, anti-angiogenic, anti-invasive, and anti-metastatic properties, and induces apoptosis (Ozbay & Nahta, 2011). It also down-regulates various signaling pathways such as NF- κ B, PI3K/AKT/mTOR, and Wnt, and down-regulates the expression of genes that are responsible in cancer development, such as anti-apoptotic genes, MMPs, VEGF, cmet, cyclins, Cdks, and pro-inflammatory cytokines (Jahromi, 2018).

Pomegranate Peel has ellagitannins, ellagic acid, gallic acid, hydroxybenzoic acids such as ellagic acid, gallagic acid, and ellagic acid glycosides. Punicalagin is the major bioactive component of pomegranate peel. Anthocyanidins mainly contained cyanidin, pelargonidin, and delphinidin14 and flavonoids such as kaempferol, luteolin, and quercetin (Seidi, Esfahlan, Abasi, & Abbasi, 2016).

Ozbay et al. (2011) reported that delphinidin presents growth inhibitory activity in breast cancer cells of different molecular subtypes but elevates potential drug antagonism when used in combination with existing targeted therapies in HER2-overexpressing breast cancer. Therefore, the inclusion of pomegranate products such as black pomegranate peel extract (*Granati Fructus Cortex*) in diet would assist in a healthy life protected from cancer and also act as an effective chemotherapeutic with no toxic side effects.

CONCLUSION

Black pomegranate peel extract (*Granati Fructus Cortex*) contains active compounds, including flavonoids, saponins, and tannins. Black pomegranate peel extract (*Granati Fructus Cortex*) has an acute toxic potential against *Artemia salina* Leach larvae, which is indicated by LC50 values $<1000\mu\text{g} / \text{ml}$. LC50 value of black pomegranate peel extract (*Granati Fructus Cortex*) is $114,090\mu\text{g} / \text{ml}$, thus proving the existence of anticancer activity according to the Brine Shrimp Lethality Test (BSLT) method.

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