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Brine Shrimp Lethality Bioassay of *Zingiber zerumbet* and *Z. cassumunar* Rhizomes Extracts.

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Abstract. Genus of Zingiber is the Indonesian medicinal plant that known have to various biological activities. However, the toxicity studies of Zingiber such as *Z. zerumbet* and *Z. cassumunar* were still limited. The aim of this study was to know the toxicity of the *Z. zerumbet* and *Z. cassumunar* rhizomes extracts using the brine shrimp lethality test (BSLT) method. The extraction of samples was performed by maceration using acetone solvent. The extract of acetone, then successively partitioned with *n*-hexane and ethyl acetate. Toxicity test results were expressed by a lethal concentration of 50 percent (LC_{50}). The toxicity assays were confirmed that extract of acetone, fractions of *n*-hexane and ethyl acetate of *Z. zerumbet show* LC_{50} value of 7.88 µg/mL, 23.90 µg/mL, and 25.31 µg/mL successively. Whereas the acetone extract, *n*-hexane and ethyl acetate fractions of *Z. cassumunar* show LC_{50} value of 34.42 µg/mL, 82.49 µg/mL and 22.11 µg/mL respectively. The screening of phytochemical showed that *Z. zerumbet* and *Z. cassumunar* extracts were contains polyphenols, flavonoids, terpenoids, and phytosterol.

INTRODUCTIONS

Traditional medicine based on biological natural resources, especially plants, has long been used in Indonesia. Therefore, it is not surprising that medicinal plants are the most interesting topic as an alternative to cure various diseases in Indonesia [1]. Indonesia as an area with a high diversity of plant species makes it possible to find the compounds that have important biological activities and pharmacological effects.

The genus of *Zingiber* (Zingiberaceae family) are the Indonesian medicinal plant that are used for food additive and treats several diseases. Zingiber plants have various physiological and pharmacological effects and widelly used as a concoction in traditional medicines. These rhizomes have been proven to be effective in the treatment of several conditions of medical including atherosclerosis, fever, cough, epilepsy, sore throat, bruises, wounds, arthritis, headaches, hyper cholesterol, muscular pains, ulcers, liver complaints and several digestion problems. Phytochemical investigation of the Zingiber species, have found the presence of bioactive compounds including gingerols, zerumbone, phenylbutenoids, diarylheptanoids, flavonoids, sesquiterpenoids and diterpenoids[2].

Zingiber zerumbet and *Z. cassumunar* are two species of this genus that have get more interest from scientists because of their high medicinal values. *Z. zerumbet* is noted by several names, for example, 'Lempuyang' (Indonesia and Malaysia), 'Ghatian' (India), and 'Hong qiu jiang" (China). The traditional usages of *Z. zerumbet* rhizome as herbal medicine such as the treatment of swelling, cough, fever, stomach ache, diarrhea, toothache, relieve pain, skin disease or edema, and diuretic [3]. Several studies reported that *Z. zerumbet* rhizome extract has various biological activities including antioxidant and antibacterial [4], antimicrobial [5], larvicidal activity against

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Aedes aegypti and *Anopheles nuneztovari* and toxic to *Artemia salina* Leach [6], anthelmic activity [7], immunomodulator [8], immunosuppressive [9], and therapeutic effect [10]. Phytochemical study was reported that the rhizome of *Z. zerumbet* contains two main compounds groups, namely terpenoids and polyphenol [11].

Z. cassumunar which is known as 'Bangle' in Indonesia has been used as a medicinal plant in folk remedies to cure various diseases, such as inflammatory, asthma, cough, infections, allergic, gastrointestinal disorders, relieve pain and rheumatism. Several types of compounds in *Z. cassumunar* rhizome have been identified such as essential oils containing monoterpenoids and sesquiterpenoids, curcuminoids, phenylbutenoids, and quinones [12]. The extracts of *Z. cassumunar* rhizome have variety biological activities, including antioxidant [13], and lipogenesis inhibition [14], anti-inflammatory [15], antibacterial and anticancer [16], neuroprotective [17], repellent, larvicidal and adulticidal activities [18].

The rhizomes of *Z. zerumbet* and *Z. cassumunar* are known to exhibit important biological activities, but studies on their toxicity are still limited. In this study, the toxicity of the rhizomes of *Z. zerumbet* and *Z. cassumunar* has been studied. Toxicity assay was carried out on *Artemia salina* larvae by the brine shrimp lethality test (BSLT) method. This method was based on the ability to kill (toxicity) of crude extracts and pure compounds on *Artemia salina*. This assay was a preliminary test to observe the pharmacological activity of sample, determine pesticide residues, mycotoxin, metal toxicity, carcinogenicity and seawater pollutants. [19]. Several advantages of this method were low cost, simple, and effective[20].

EXPERIMENTAL

Materials and Instruments

The fresh rhizomes of *Z. zerumbet and Z. cassumunar* were collected from Purwokerto, Central Java, Indonesia. All chemicals used for extractions (acetone, *n*-hexane, methanol, and ethyl acetate) were of technical grade, whereas dimethyl sulfoxide was analytical grade from Merck. The equipment used includes macerations chamber, rotary evaporator, separating funnel and hatching chamber equipped with aerator and lamp.

Extraction of Samples

The fresh rhizomes of (2 kg) were washed then chopped into small size. After that the samples were dried and grinded to powder. The powders (250 g) were maserated three times with acetone for 3 days at room temperature. The filtrates of *Z. zerumbet* and *Z. cassumunar* rhizomes then evaporated to give acetone extract without solvents. Asetone extracts was partitioned by separating funnel using *n*-hexane and methanol (1:1). The fraction that soluble in *n*-hexane was accommodated, then the fraction that soluble in methanol was partitioned with ethyl acetate and water (1:1), then the fraction that soluble in ethyl acetate was accommodated. The fractions of *n*-hexane and ethyl acetate were evaporated, until there are no more solvents [21]. Acetone extracts, *n*-hexane and ethyl acetate fractions of *Z. zerumbet* and *Z. cassumunar* rhizomes were tested for their toxicity against brine shrimp, respectively.

Brine Shrimp Lethality Assays

A. salina eggs were hatched in sea water in small chamber, with an aerator and as oxygen and a light source (lamp). After 24 hours, yeast solution (0.05%) was added to the hatching chamber for every liter of sea water, to feed the larvae. The nauplii that used for bioassay, were 48 hours old.

The stock solution was served by dissolving 10 mg of samples in 10 mL of dimethyl sulfoxide. The solution with concentration series of 200, 100, 50, 10, 5 and 1 µg/mL were served by diluting the stock solution. Six test tubes (1, 2, 3, 4, 5 and 6) were prepared, then 1 mL each solution dilutions were poured into each test tubes that already contains 14 mL of seawater and 10 nauplii. For each tube, the number of live nauplii was calculated after 24 hours, then determined the percentage of nauplii deaths. The percentage lethality of the nauplii in control solution was also calculated. For each sample, the treatments were carried out in three replicates.

$$\%\ Death = \frac{\text{number of dead nauplii}}{\text{number of dead nauplii} + \text{number of live nauplii}}\ x\ 100$$

The median lethal concentration (LC_{50}) was determined by a plot of % death nauplii to the log concentration of samples. The values of LC_{50} were assigned using analysis of probit, it was analyzed with SPSS 20.0 for Windows with confidence intervals of 95%.

Phytochemical Screening

Phytochemical analysis were carried out for all samples used the common methods for screening phytochemical [22].

- **Alkaloid**: each extract and fractions were dissolved in dilute hydrochloric acid, then filtered. The filtrates were added small amount of Dragendorff's reagent (solution of potassium bismuth iodide), and if alkaloids were presents, an orange-brownish precipitate will appear.
- **Flavonoids**: each extract and fractions were added a few drops solution of sodium hydroxide. The existence of flavonoids was confirmed by formation a deep yellow solution, when diluted acid was added, it was colorless.
- **Polyphenols**: each extract and fractions were added with few drops of ferric chloride solution. The positive reactions will give of bluish black colors.
- **Sterols (Liberman Burchard's test)**: each extract and fractions were dissolved with chloroform, then filtered. The filtrates were added small amounts of acetic anhydride, then heated to boiling and cooled. The phytosterols were indicated by formation of brown solution.
- **Terpenoids**: each extract and fractions were added chloroform (2 mL), then concentrated sulfuric acid (3 mL) carefully. The presence of terpenoids were indicated by the formation of brownish red coloration.

RESULTS AND DISCUSSION

Extractions of *Z. zerumbet* dan *Z. cassumunar* Rhizomes

Extraction of the samples were used macerations method. Maceration is a simple extraction process, which immersing the sample in a suitable solvent at room temperature for a define time, so that the components damage can be minimized. Maceration of the dried powder of *Z. zerumbet* and *Z. cassumunar* (@ 250 mg) rhizomes used acetone were yielded the dark brown pasta of 20.6 g and 21.16 g, successively. The fractionation by liquid-liquid extractions of *Z. zerumbet* acetone extract with *n*-hexane: methanol, and ethyl acetate: water successively, were yielded *n*-hexane fraction of 5.68 g, and ethyl acetate fraction of 9.50 g. The fractionation with the same methods for *Z. cassumunar* acetone extract were obtained *n*-hexane fraction of 6.63 g and ethyl acetate fraction of 11.01 g. respectively. The yields of ethyl acetate fractions of *Z. zerumbet* and *Z. cassumunar* were more than the *n*-hexane fractions, these indicated that most of components in the acetone extract was soluble in ethyl acetate. The previous study notified that *n*-hexane fraction of *Z. zerumbet* and *Z. cassumunar* rhizome contains the essential oils which consist of the mayor components was monoterpene and sesquiterpene, whereas the ethyl acetate fractions of *Z. zerumbet* rhizomes have main component was flavonoids, whereas curcuminoids was the main component of *Z. cassumunar* ethyl acetate fraction [2]

Brine Shrimp Lethality Assays

Brine shrimp lethality assays is a method that commonly used for preliminary studies of the biological activities of plant extracts or isolated compounds. This method is very useful for monitoring the toxicity levels of the samples, although the mechanism of action of this methods has not provided any sufficient information [19].

Toxicity assays were carried out on acetone extract, *n*-hexane and ethyl acetate soluble fractions of *Z. zerumbet* and *Z. cassumunar* rhizomes. The toxicity levels are expressed by the LC_{50} values. Then was compared with Meyer's and Clarkson's toxicity index. Based on index of Meyer's toxicity, sample with LC_{50} less than 1000 µg/mL was classified as toxic, in another hand was a non-toxic if sample have LC_{50} more than 1000 µg/mL [23]. Clarkson's toxicity criterion for the toxicity levels, are classified as follows: samples with LC_{50} more than 1000 µg/mL are non-toxic, LC_{50} of 500 - 1000 µg/mL are low toxic, samples with LC_{50} of 100 - 500 µg/mL are medium toxic, and samples with LC_{50} of less than 100 µg/mL are highly toxic [19]

In this study, the toxicity of the extracts and fractions of *Z. zerumbet* and *Z. cassumunar* rhizomes shown in Table 1. Based on the Meyer's toxicity index, all the samples are categorized as toxic, because they have LC_{50} value less than 1000 µg/ml. While refers to Clarkson's toxicity criterion, all the samples showed highly toxic because they have LC_{50} of less than 100 µg/mL

TABLE 1. Toxicity of *Z. zerumbet* and *Z. cassumunar* rhizomes extracts and fractions

Samples	LC_{50} (µg/mL)
Z. zerumbet	
Acetone extract	7.88
n-Hexane fraction	23.90
Ethyl acetate fraction	25.31
Z. cassumunar	
Acetone extract	34.42
n-Hexane fraction	82.49
Ethyl acetate fraction	22.11

Phytochemical Screening

Phytochemical analysis was carried out on acetone extracts as well as *n*-hexane and ethyl acetate fractions of *Z. zerumbet* and *Z. cassumunar* respectively. The analysis was based on standard method for detecting secondary metabolite compounds. The phytochemical screening result were presented in Table 2.

TABLE 2. Screening phytochemical of extracts and fractions of *Z. zerumbet* and *Z. cassumunar* rhizomes

Sample	Alkaloid	Polyphenols	Flavonoid	Terpenoids	Phytosterols
Z. zerumbet					
Acetone extract	-	+	+	+	+
n-Hexane fraction	-	-	-	+	+
Ethyl acetate fraction	-	+	+	-	-
Z. cassumunar					
Acetone extract	-	+	+	+	+
n-Hexane fraction	-	-	-	+	+
Ethyl acetate fraction	-	+	+	-	-

In this study showed that the phytochemical screening of *Z. zerumbet* and *Z. cassumunar* rhizomes, the presence of polyphenols, flavonoid, terpenoids and phytosterols, in acetone extracts were observed. This shows that acetone dissolves nonpolar, semi polar and polar components of plants. Acetone is volatile, water miscible, and has a weak toxicity in bioassay treatment. [22]. In this studies, lipophilic or nonpolar components such as terpenoids and sterol are dissolved in the non-polar solvents *n*-hexane, meanwhile the more polar components such as polyphenols and flavonoids were dissolved in the semipolar solvent ethyl acetate. However, the polarity of the components was not only depend on the type of carbon skeleton or compound groups, but also influenced by the functional groups of the compounds.

CONCLUSION

The extracts and fractions of *Z. zerumbet* and *Z. cassumunar* rhizomes were highly toxic on *A. salina* Leach, with LC_{50} value 0-100 µg/mL. The toxicity assays were confirmed that the extract of acetone, and fractions of *n*-hexane and ethyl acetate of *Z. zerumbet* show LC_{50} value of 7.88 µg/mL, 23.90 µg/mL, and 25.31 µg/mL successively. Whereas the acetone extract, *n*-hexane and ethyl acetate fractions of *Z. cassumunar* show LC_{50} value of 34.42 µg/mL, 82.49 µg/mL and 22.11 µg/mL successively. The screening of phytochemical was showed the existence of polyphenols and flavonoid in *n*-hexane fractions, meanwhile terpenoids and phytosterols were contained in ethyl acetate fractions, in both plants.

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