Assessment cytotoxic assay of Rhizophora plants mangrove using brine shrimp (Artemia salina L) model

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Abstract. Rhizophoraceae is the main family of mangroves as a source of bioactive compounds originating from the coast. Ethnopharmacologically Rhizophoraceae has been used in various traditional medicine. Natural sources as anticancer from the Rhizophoraceae family are interesting to know. This study aimed to determine the cytotoxic bioactivity of methanolic extracts of roots, bark, leaves, and fruit/hypocotyl from five species of Rhizophoraceae (Bruguiera cylindrica, B. gymnorrhiza, Ceriops decandra, Rhizophora apiculata, and R. mucronata) from the Langsa mangrove forest, Aceh. The method used in this study was the Brine Shrimp Lethality Test (BSLT) bioassay using Artemia salina Leach at extract concentrations of 1, 10, 100, 500, and 1000 μg/ml. Samples were extracted using the maceration method and methanol as the solvent. The cytotoxic activity of 20 Rhizophoraceae methanol extracts showed that 12 extracts were toxic with an LC50 range of 31.5 - 934.9 μg/ml (based on LC50 ≤ 1000 μg/ml). The two extracts of which the closest to highly toxic (based on LC50 ≤ 30 μg/ml) were C. decandra bark showed LC50 of 31.5 μg/ml, and R. mucronata bark showed LC50 31.8 μg/ml. This shows that Rhizophoraceae extract has potential as a natural anticancer agent. In the four rhizophoraceae species, C. decandra was the most active compared to other species. In the four plant parts, the bark was the most toxic.

1. Introduction
Rhizophoraceae is a family of flowering plants that have habitats growing on land and coast. Based on data from The Plant List 2013 Rhizophoraceae consists of 18 genera and 142 species that have been identified [1]. Four genera of them (Bruguiera, Ceriops, Kandelia and Rhizophora) live as true mangrove plants. True mangroves have special characteristics, that is plants growing on land and sea surfaces in tropical and subtropical latitudes, in conditions of high salinity, high tides, strong winds, high temperatures in the morning and afternoon, muddy soil and anaerobes [2]. Rhizophoraceae is the most dominating family in all mangrove forests around the world [3]. Rhizophoraceae is the most dominating family in all mangrove forests around the world [3]. Rhizophoraceae is an annual plant with...
a height of 3-15 m, with respiratory roots visible above the soil surface, the flowers grow into fruit and the hypocotyl as a means of reproduction, if the old hypocotyl falls to the ground, new plants will grow [4]. The root system has a unique shape, namely tunjang root, plank root, and knee root [5]. Rhizophoraceae is also often referred to as a mangrove tree.

All parts of the mangrove tree have been used by people in various countries as traditional medicine. The leaves are used to cure fever, snake bites, diabetes, hypertension [6][7][8], the bark is used to cure diarrhea [9], the fruit is used to treat malaria [10], the roots can be used to reduce fever, wound healing, and dysentery [11][12]. Furthermore, it is used as a portion of additional food, for example, the hypocotyl Bruguiera gymnorrhiza by the people of Sulawesi is used as an additional food in addition to the staple food [13]. Several studies have shown the phytochemical content of Rhizophoraceae are triterpenoids, flavonoids, alkaloids, tannins, saponins, and glycosides [14][11][15]. The active secondary metabolite compounds will have the potential for the development of natural medicinal ingredients such as anticancer, antihypertensive, rheumatic, antioxidant, diabetes mellitus, arthritis, antinociceptive, inflammation, and hepatoprotective agents [2].

Cancer is one of the deadly diseases that is a health problem for people in developed and developing countries. Cancer is an abnormal cell growth resulting from the continuous proliferation and differentiation of individual cells in multicellular organisms which are characterized by changes in basic control at the molecular and cellular level [16]. Based on the International Agency for Research on Cancer, every year there is an increase in the number of cases and deaths from cancer from 8.2 million deaths in 2012 to 17 million deaths in 2030 from an estimated 26 million new cancer cases that appear [17]. Meanwhile in Indonesia, based on Basic Health Research data, the prevalence of tumors/cancer in Indonesia showed an increase from 1.4 per 1000 population in 2013 to 1.79 per 1000 population in 2018 [18]. Cancer treatment using radiation, surgery and chemotherapy over time becomes less effective and has the risk of being toxic to normal tissues and causing resistance in cancer cells [19]. Therefore, investigation for natural materials that are secure and show anticancer activity can be an alternative cancer treatment.

Screening of bioassays on plant families is an interesting study to follow, especially in finding new sources of materials that have the potential to be further developed because their activities have been tested. Bioassays in the form of toxicity tests on plant extracts were carried out as a basic study to search for active ingredients for anti-cancer [20]. The Brine Shrimp Lethality Test (BSLT) method has been used as a general bioassay capable of detecting the spectrum of bioactivity in plant extracts [17]. The use of Artemia salina Leach in this approach provides a toxicity response similar to that of the mammalian system, since A. salina has a metabolic system similar to that of mammals [21]. This was first done by Michael 1956, followed by Meyer et al, 1982 who determined toxicity by estimating the lethal concentration of plant extracts against A. salina at a concentration of LC 50 using probit analysis [22][23] and proved by Anderson, 1991 that the test BSLT has high accuracy in screening for antitumor/anticancer activity in vivo [24]. This method has a simple procedure, inexpensive, does not require a long time, can be carried out in a laboratory with standard equipment, and this test provides high confidence in specific anticancer tests. The BSLT assay is considered an adequate method to provide preliminary screening of the action of bioactive substances, which can be further supported by more specific bioassay methods after the active compounds have been isolated [25]. Bioassay studies in the form of cytotoxicity in one family of mangrove plants, namely Rhizophoraceae, are very interesting to study because ethnopharmacologically Rhizophoraceae have been used as natural ingredients for traditional medicines by the community since ancient times. However, among the parts of the plant, it is not known that the most potential as natural anticancer ingredients. The purpose of this study was to evaluate the cytotoxic activity of methanol extracts of 5 species of Rhizophoraceae, namely Bruguiera cylindrica, B. gymnorrhiza, Ceriops decandra, Rhizophora apiculata, and R. mucronata originating from the Langsa mangrove forest, Aceh against A. salina larvae as part of an anticancer screening from natural ingredients.
2. Materials and methods

2.1 Preparation and drying of plant samples
A total of 5 species from 3 genera of Rhizophoraceae were taken from Langsa Mangrove Forest. The plant parts used included roots, bark, leaves, fruit, and hypocotyl so that 20 samples were obtained. The following parts of the plant are used and their names:

- Root of B. cylindrica (RBc)
- Root of B. gymnorrhiza (RBg)
- Root of C. decandra (RCd)
- Root of R. apiculata (RRa)
- Root of R. mucronata (RRm)
- Bark of B. cylindrica (BBc)
- Bark of B. gymnorrhiza (BBg)
- Bark of C. decandra (BCd)
- Bark of R. apiculata (BRa)
- Bark of R. mucronata (BRm)
- Leave of B. cylindrica (LBc)
- Leave of B. gymnorrhiza (LBg)
- Leave of C. decandra (LCd)
- Leave of R. apiculata (LRa)
- Leave of R. mucronata (LRm)
- Hypocotyl of B. cylindrica (HBc)
- Hypocotyl of B. gymnorrhiza (HBg)
- Hypocotyl of R. decandra (HBr)
- Fruit of C. decandra (FCd)
- Fruit of R. apiculata (FRa)
- Fruit of R. mucronata (FRm)

The plant parts are washed with running water. Once the plant parts are clean, chop as thinly as possible. The plant parts are dried by shade drying (avoiding direct sunlight) and assisted by a fan. Drying is carried out until the plant parts become stiff and brittle and the weight shrinks to ± 80% - 90%. The dried roots and bark, fruit, and hypocotyl were ground to a coarse powder.

2.2 Plant extraction
Samples that were dried in a total of 100 grams each were put into a glass jar and then macerated using methanol for 24 hours. The filtrate obtained was then filtered using Whatman filter paper no.1. The filtrate obtained was dried using a vacuum rotary evaporator to obtain a thick methanol extract. Next, the thick extract was dried on a water bath at 50°C to dry. The dried extract was covered with aluminum foil and dried again at room temperature until completely dry.

2.3 Larva preparation of Artemia salina L.
The BSLT method used in this experiment refers to the Meyer method, 1982 with some modifications with Purnama et al., 2021 [23,26]. Shrimp eggs were weighed as much as 0.5 g per liter of water. Brine shrimp eggs A. salina L are placed in hatching vessels containing seawater and equipped with an aerator and lighting. The eggs will hatch approximately 24 hours after sowing (the eggs that have hatched we refer to as naupii). The hatchery is given dark and light conditions. Good larvae will swim towards a bright place because the larvae are phototropic. Naupii/ larvae are ready to be used for testing after 48 hours.

2.4 Brine shrimp lethality test
The dry extract of the Rhizophoraceae plant was weighed as much as 0.05 g in a beaker. Next, the extract was given 2 drops of 5% DMSO solution and dissolved in seawater up to 25 ml. For the insoluble extract, sonication was carried out. Based on this treatment, a stock solution with a concentration of 2000 μg/ml was obtained. The test solutions were made with concentrations of 1 μg/ml, 10 μg/ml, 100 μg/ml, 500 μg/ml, and 1000 μg/ml. Then the stock solution will be pipetted into test tubes (test tubes) each of them put of 2,5 μl; 25 μl; 250 μl; 1250 μl; dan 2500 μl using a micropipette. Next, the tube was filled with 10 A. salina larvae and then the volume of the tube was made up to 5 ml using seawater. The tube is placed under a lamp for 24 hours. After 24 hours, the number of live A. salina larvae was counted. Mortality at each concentration of the test solution was recorded. Then the percentage of mortality was calculated by the formula % of mortality (Equation 1). Then, probit analysis, LC50 calculations, linear equations, and linear equation graphs were carried out using the Excell program. An extract is active if the LC50 value obtained is ≤ 1000 μg/ml.

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\text{% Mortality} = \frac{\text{Number of death} - \text{number of control deaths}}{\text{number of early larvae}} \times 100\% \ [23]
\]
3. Results and discussion

The Rhizophoraceae family used in this study consisted of 5 plant species and 4 organs were taken from each plant, namely roots, bark, fruit/hypocotyl, and leaves. Thus obtained 20 parts of the plant. The four parts were taken because they are easy to take and do not cause death in plants with good harvesting techniques and are the main parts of plant growth. Roots are useful as a place for absorption of food juices in plants that come from growing media, stem bark is an organ that distributes water from roots and photosynthetic results from leaves to all plants, leaves are organs where photosynthesis takes place, and fruit is a place to distribute food juices from the leaves and used for the continuation of plant reproduction. These four parts have a very important role in a plant. The secondary metabolites contained in the four parts can have different compositions and activities. One plant family, usually have similar morphology but there are differences in secondary metabolite activity. This is what we want to observe in the mangrove family Rhizophoraceae. One of the bioassay tests is a cytotoxic test on plant extracts.

Cytotoxic BSLT is a simple and inexpensive bioassay to test the effectiveness of secondary metabolites found in plant extracts. The Brine Shrimp Lethality Test method was used in this study to evaluate the cytotoxic activity of 20 methanolic extracts of Rhizophoraceae extract using the larval test of *A. salina*. The results of the cytotoxic test of each of these extracts are shown in Table 1 below.

| Table 1. Cytotoxic test results of 20 Rhizophoraceae methanol extracts using *A. salina*. |
|---------------------------------------------------------------|
| **Extracts** | **Percent of death at 24 hr (%)** | **LC₅₀ (μg/ml)** | **linear equation** | **R²** |
|----------------|-----------------------------|-----------------|-----------------|------|
|                | 1 μg/ml | 10 μg/ml | 100 μg/ml | 500 μg/ml | 1000 μg/ml |
| 1 BCd          | 13      | 20      | 63      | 83      | 97     | 31.5 | y=0.963x + 3.556 | 0.9202 |
| 2 BRm          | 23      | 30      | 67      | 77      | 77     | 31.8 | y= 0.558x + 4.162 | 0.9466 |
| 3 LCd          | 20      | 27      | 63      | 83      | 83     | 32.1 | y=0.668x + 3.994 | 0.9557 |
| 4 FRm          | 23      | 33      | 40      | 77      | 90     | 35.7 | y=0.629x + 4.023 | 0.8343 |
| 5 RCd          | 13      | 27      | 63      | 83      | 83     | 40.7 | y=0.750x+ 3.793 | 0.9838 |
| 6 FCd          | 13      | 13      | 37      | 73      | 83     | 105.1 | y=0.731x + 3.521 | 0.8833 |
| 7 BRa          | 13      | 17      | 40      | 53      | 70     | 238.6 | y=0.542x + 3.710 | 0.9423 |
| 8 BBg          | 13      | 13      | 33      | 60      | 67     | 281.8 | y=0.566x + 3.613 | 0.8978 |
| 9 HBC          | 3       | 17      | 20      | 57      | 73     | 311.9 | y= 0.764x + 3.095 | 0.9224 |
| 10 RRa         | 13      | 20      | 27      | 50      | 60     | 608.6 | y=0.446x + 3.578 | 0.9194 |
| 11 LBC         | 7       | 13      | 23      | 40      | 67     | 691.7 | y=0.574x + 3.369 | 0.8926 |
| 12 Bbc         | 7       | 17      | 27      | 37      | 60     | 934.9 | y=0.507x + 3.494 | 0.9353 |
| 13 RRm         | 17      | 27      | 27      | 37      | 60     | 1,457.10 | y=0.316x + 4 | 0.7627 |
| 14 HBG         | 3       | 13      | 20      | 33      | 50     | 1,828.70 | y=0.564x + 3.161 | 0.9625 |
| 15 Rbg         | 3       | 17      | 20      | 27      | 50     | 2,514.90 | y=0.515x + 3.793 | 0.8828 |
| 16 RBC         | 20      | 23      | 27      | 43      | 47     | 7,978.50 | y=0.236x + 4.080 | 0.8783 |
| 17 LBC         | 13      | 13      | 20      | 37      | 50     | 3,989.70 | y=0.369x + 3.673 | 0.8229 |
| 18 LRa         | 7       | 20      | 20      | 27      | 37     | 19,539.70 | y=0.321x + 3.621 | 0.8818 |
| 19 FRA         | 10      | 13      | 23      | 23      | 33     | 113,739.97 | y=0.621x + 3.680 | 0.9232 |
| 20 LRM         | 7       | 13      | 13      | 20      | 33     | 139,561.20 | y=0.304x + 3.438 | 0.8605 |

Based on Table 1, there were 12 extracts that were toxic with an LC₅₀ range of 31.5 - 934.9 g/ml sequentially, the toxic levels were methanol extract of *C. decandra* bark (BCd), *R. mucronata* bark (BRm), *C. decandra* leaves (LCd), *R. mucronata* fruit (FRm), *C. decandra* root (RCd), *C. decandra*...
fruit (FCd), *R. apiculata* bark (BRa), *B. gymnorrhiza* root (BBg), *B. cylindrica* hypocotyl (HBc), roots *R. apiculata* roots (RRa), *B. gymnorrhiza* leaves (LBg), and *B. cylindrica* bark (BBc). According to Meyer 1982, the minimum concentration that can state that the extract has activity that is causing the death of 50% of shrimp larvae is LC$_{50} \leq 1000$ μg/ml. While the methanol extract of Rhizophoraceae which is close to very toxic activity is the methanol extract of the stem bark of *C. decandra* (BCd), the bark of *R. mucronata* (BRm) which showed LC$_{50}$ 31.5 and 31.8 based on LC$_{50} \leq 30$ mg/ml.

In this study, it was shown that the percentage of the mortality rate of *A. salina* was directly proportional to the increase in the concentration of the extract. In the control which contained only sea water, after 24 hours of observation all shrimps survived in the control [27]. In this procedure we assumed that any *A. salina* larvae that survived at a certain dose would also survive at a lower dose, and that any cells that died at a certain dose would also die at a higher dose [28].

Based on the test conducted by Anderson, 1991 the extract which is toxic in the BSLT test means that it is also active against tumor cells/cancer cells [24]. The BSLT test is a representative test for further tests because it has high accuracy for the next test. This is the easiest cytotoxic test, because *A. salina* is sensitive to various chemical compounds and can be used widely. This assay is considered a useful method for assessing the cytotoxicity of chemical compounds in extracts [21]. Rhizophoraceae methanol extract has toxic properties as a crude extract, at this stage it is the most basic stage and it is recommended to carry out further evaluation of the partitioned methanol extract with polar and non-polar solvents such as n-hexane and ethyl acetate and filtering more complex compounds such as toxic properties of pure compounds to the direct test of extract toxicity using cancer cells using the microculture tetrazolium salt (MTT) method [29]. The crude extract stage when the extract often contains a mixture of various cytotoxic compounds, which may obscure observations of selective cytotoxicity.

Based on the extract of each plant part, the extract of the most toxic part of the plant root was indicated by the root extract of *C. decandra* (Figure 1) while the extract of the most toxic part of the bark was *C. decandra* and *R. mucronata* (Figure 2).

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**Figure 1.** Cytotoxic effect of *Rhizophoraceae* root extract.

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Based on Figure 3 and Figure 4, the leaf extract of the Rhizophoraceae plant was the most toxic, indicated by the leaf extract of *C. decandra* (Figure 3), while the most toxic hypocotyl fruit/plant extracts were *R. mucronata* and *C. decandra* hypocotyl fruit (Figure 4.).
Based on the four figures above, it shows the extract of 4 parts of the plant, overall \textit{C. decandra} species is the most toxic compared to other species. In addition, \textit{R. mucronata} has 2 parts that are the most toxic compared to 3 other species, namely \textit{B. cylindrica}, \textit{B. gymnorrhiza}, and \textit{R. apiculata}.

![Figure 5. Percentage of toxicity of plant parts of all samples of Rhizophoraceae methanol extract.](image)

Based on the cytotoxic properties, the highest toxic properties of the Rhizophoraceae methanol extract were the bark of 33.3\%, fruit/hypocotyl by 25\%, and roots and leaves of 16.6\% (Figure 5). Based on these results, the most active Rhizophoraceae species were the bark.

4. Conclusions
A total of 12 Rhizophoraceae methanol extracts, including \textit{C. decandra} bark (BCd), \textit{R. mucronata} bark (BRm), \textit{C. decandra} leaves (LCd), \textit{R. mucronata} fruit (FRm), \textit{C. decandra} root (RCd), fruit \textit{C. decandra} (Fcd), \textit{R. apiculata} stem bark (BRa), \textit{B. gymnorrhiza} stem bark (BBg), hypocotyl \textit{B. Cylindrica} (HBc), root of \textit{R. apiculata} (RRa), leaves of \textit{B. gymnorrhiza} (LBg), and the bark of \textit{B. gymnorrhiza} is active against the BSLT. The activity values of the 12 extracts showed LC\textsubscript{50} 31.5 - 934.9 μg/ml was at LC\textsubscript{50} ≤ 1000 μg/ml. in the five rhizophoraceae species, \textit{C. decandra} was most toxic activity compared to other extracts. Based on the four plant parts (roots, bark, leaves, and fruit/hypocotyl) Rhizophoraceae, the methanol extract of the stem bark was the most toxic.

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