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CONTENT OF ACTIVE COMPOUNDS, TOXICITY OF CRUDE EXTRACTS OF MANGROVE LEAVES (*RHIZOPHORA MUCRONATA*) AND ITS MODULATION OF TILAPIA (*OREOCHROMIS NILOTICUS*)

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Technology, Indonesia Open University, Indonesia*E-mail: ashari.fahrurrozi@gmail.com**ABSTRACT**

Limited cultivation unit land makes the increase of aquaculture from a widely developed into an intensive system. Intensive system with high density care in confined spaces has the potential to cause stress and disease in fish. Natural ingredients have good criteria such as environmentally friendly, cost-effective, and broad spectrum activities. Alternative natural ingredients in this study using coarse extract *Rhizophora mucronata*. This study aims to determine the content of active compounds, toxicity values (LC_{50}) and at what doses in such compounds can maximally modulate the immune response (leukocytes) in indigo (*Oreochromis niloticus*). In this study the extraction method used maceration with methanol solvent p.a. Analysis of biological components is performed by phytochemical test method, UV-Vis spectrophotometer and FT-IR. Phytochemical test results contain two compounds namely flavonoids (+) and alkaloids (+). UV-Vis tests showed the basic structure of flavonoid group compounds (tape I 282 nm, 236 nm and tape II 404 nm) and alkaloids (susceptible to 203 nm-230 nm) by strengthening the emergence of group absorption of functions typical of both compounds using FT-IR. LC_{50} indicates a dose of 66,364 mg.kg⁻¹. The most significant increase in total leukocytes in test fish (*Oreochromis niloticus*) was at a dose of 30 mg.kg⁻¹ (2.6×10^4 cells/mm³) with an influence of 91%.

KEY WORDS

Rhizophora mucronata, flavonoids, alkaloids, LC_{50} , total leucocytes, *Oreochromis niloticus*.

Tilapia (*Oreochromis niloticus*) is an important aquaculture commodity in Indonesia, with the last production report of 1,291,391 tons. The amount of production puts Indonesia second only to China, with a percentage of 18.76% of the world's total production [1]. In addition, tilapia production will continue to be increased from the world's total production with a range of 60% in 2025. However, given the limited land for aquaculture units, the increase in fish farming from extensively developed into an intensive system [2]. Intensive system with high density treatment in limited space has the potential to cause stress and disease in fish [3].

Improvements and innovations in dealing with pathogen infections using safe methods are an important concern. Approach to using antibiotics has unintended environmental consequences [4]. On the other hand, vaccines have good efficiency but not for all pathogens and the application is quite expensive [5]. Therefore, the traditional approach of natural ingredients becomes an alternative to control. In addition to being good for the environment, natural ingredients have effectiveness in cost and broad spectrum activities [6].

The advancement of the role of natural ingredients in the cultivation of tilapia (*Oreochromis niloticus*) is reported to have many properties. Especially in disease management, the effects of natural ingredients can be seen by the health index in the body of fish [7]. One of the natural ingredients that can be used and abundant in Indonesia is mangrove *Rhizophora mucronata*, because most of its parts are known to contain bioactive compounds or often called secondary metabolite compounds [8]. Secondary metabolite compounds contained in *R. mucronata* include flavonoids, alkaloids, terpenoids, tannins and saponins [9]. The content of compounds in *R. mucronata* is known to serve as a support or modulate the health of fish, especially in the increase of leukocytes [10].

Observations about immune stimulating compounds (immunomodulators) can reportedly be seen from the non-specific immune response of fish, one of which such as cells (leukocytes) can be found in the bloodstream or tissues (thymus, anterior kidney, spleen and lymphoid tissue associated with the intestines and mucus). The total increase in leukocytes can be used as an indicator for readiness to detect and fight any attack of foreign substances or pathogens when entering the body. Leukocytes themselves work with phagocytosis or through cytotoxic response to pathogens when attacking [3]. Based on this explanation, this study aims to find out the content of any active compounds contained in crude extracts of *Rhizophora mucronata*. In addition, at what dose the compound can make the fish population die 50% and the optimal dose in modulating the immune response (leukocytes) in tilapia (*Oreochromis niloticus*).

MATERIALS AND METHODS OF RESEARCH

This study used *R. mucronata* leaves and tilapia (*Oreochromis niloticus*) with a size of 10-15 cm ($\pm$ 18 grams / tail). First of all the maceration extract using methanol solvent tested the active compounds in it using compound detection reagents (phytochemical tests) and spectrophotometers (UV-Vis and FTIR). Furthermore, in the test of toxicity and modulation activity, the design of this study was conducted by experimental methods. This is because experimental methods aim to determine the cause of administration of one or more treatments rather than controls.

Toxicity test (LC_{50}) was conducted at doses from 10-100 mg.kg⁻¹ with observation for 96 hours. While leukocyte modulation test is conducted with several doses below LC_{50} result value, as well as control (without extracting). Treatment as much as 3 repetitions. Blood retrieval is performed on the 1st day before the injection of crude extract of *R. mucronata* leaves and the 4th day after injection of the extract.

Analysis is performed to determine the content of active compounds using descriptive analysis. Test toxicity (LC_{50}) of crude extract of *Rhizophora mucronata* mangrove leaves using probity analysis. As for the immune response test (leukocytes) using one-way ANOVA with further tests in the form of Tukey to find out the average difference. Then the analysis is done using repeated measures to determine changes in the immune response (leukocytes) after the administration of extracts. In addition, the regression analysis aims to determine how much influence is generated.

RESULTS AND DISCUSSION

Phytochemical test results of crude extract of *Rhizophora mucronata* with methanol solvent can be seen in Table 1. Methanol solvent is a polar solvent [11], so that it can attract secondary compounds or metabolites that are polar in nature such as flavonoids [12], alkaloids [13], tannins [14] and saponins [15]. However, phytochemical test results on crude extracts of *R. mucronata* with methanol solvents can only attract flavonoid compounds and alkaloids. The result is characterized by a change in color or texture produced. Qualitative analysis showed that the crude extract of *R. mucronata* was positive for flavonoids because of the orange and alkaloid color formed by the formation of brown deposits. While the compounds tannins and saponins are not contained in the extract because of the absence of blackish brown color and permanent foam.

Table 1 – Phytochemical analysis of crude extracts of *Rhizophora mucronata*

No	Compound Identification	Parameters [16]	Results	Description
1.	Flavonoids	Orange/red		Positive (+)
2.	Alkaloids	Chocolate precipitate		Positive (+)
3.	Tannins	Blackish brown/blackish blue		Negative (-)
4.	Saponins	Permanent foam		Negative (-)

Source: Field Data.

UV-Vis spectrophotometer is a technique for the initial analysis of compound structure in a very useful extract [17]. Analysis of the UV-Vis spectrum shows that *Rhizophora mucronata* crude extract using methanol solvents contains flavonoid compounds and alkaloids as evidenced in Figure 1. The determination of compounds contained in crude extract *R. mucronata* because specifically the absorption of flavonoid compounds in UV-Vis generally has two ribbons. In band I the wavelength is between 300-500 nm and on tape II it is between 250-285 nm [18]. While the alkaloid compounds maximum wavelength is between 203 nm-230 nm [19]. This indicates that the compounds contained in the extract are alkaloid compounds because they appear at wavelength absorption of 204.9 nm, 208.1 nm, 210 nm, 212 nm, 215 nm, 217 nm, 228 nm and 230 nm. In addition, there are flavonoid compounds, as they appear at wavelength absorption of 404 nm for band I and 282 nm for tape II. While at absorption 236 nm is also a flavonoid compound because flavonoid compounds of the isoflavone group have a wavelength absorption range between 236 nm-260 nm [20].

Figure 1 – Analysis of UV-Vis Crude Extract *Rhizophora mucronata* (Source: Field Data)

The active compounds contained in a sample, in addition to being analyzed using phytochemical tests with some typical regen and UV-Vis spectrums are also performed with FT-IR or infrared spectrum analysis for compound affirmation [21]. Analysis using FT-IR aims to determine the function group contained in a sample [22]. Based on FT-IR analysis in Figure 2, it shows that *Rhizophora mucronata* crude extract contains flavonoid active compounds with the onset of absorption typical for some function groups grouped in Table 1. Such absorption is at a wavelength of 3424 cm^{-1} which indicates the presence of widened absorption as an O-H tendril vibration, and reinforced by the vibration of C-O alcohol bends in the area of 1251 cm^{-1} and 1062 cm^{-1} . Vibration at wave number 2927 cm^{-1} indicates the absorption of C-H ulur. Another characteristic that supports is the presence of aromatic rings C=C aromatic at absorption 1539 cm^{-1} . In addition, the C=C cluster at sharp absorption in the wavelength area of 1631 cm^{-1} also indicates the presence of the C=O cluster.

The absence of flavonoid compounds in *R. mucronata* extract is O-H is a bond that supports the presence of flavonoid compounds and C=C is a typical chromophore group of flavonoids in the conjugated bonding system. In addition, the infrared spectra results of an extract indicate an overlap between the C=C and C=O bonds at about 1600 cm^{-1} [23]. C=O bonds that appear in low wave numbers are caused by resonance in the α,β -unsaturated carbonyl structure and the formation of intermolecular hydrogen bonds. C=O bonding is also a hallmark of flavonoid compounds [24]. The presence of the function groups O-H, C-H, C=C and C-O, indicates that the crude extract of *R. mucronata* contains flavonoid compounds [25]. This is reinforced that flavonoid compounds resulting from the analysis of the FTIR spectrum showed a typical peak in the presence of outstretched O-H function groups of phenolic, C-H alkenes, C=C aromatic rings and C-O [26].

Figure 2 – FTIR Analysis (Source: Research documentation)

The results of infrared spectrum analysis (FT-IR) crude extract *R. mucronata*, which can be seen in Table 1, may also contain alkaloid compounds in the presence of several function groups such as N-H at wave numbers 3424 cm^{-1} . This absorption is supported by the emergence of absorption at wave numbers of 1449 cm^{-1} and 1383 cm^{-1} indicating the presence of C-H groups, as well as the presence of bands at wavelengths of 1251 cm^{-1} indicating the presence of C-N clusters. The absence of alkaloid compounds is due to the function groups N-H, C-H and C-N are characteristic of alkaloid compounds [27]. Moreover, alkaloid compounds from *Rhizophora mucronata* mangrove leaf extract based on FT-IR spectroscopic analysis is suspected to contain N-H, C-N, C-H function groups [28].

Table 2 – Grouping of flavonoid and alkaloid compound function groups

No	Frequency of crude extract absorption tape <i>Rhizophora mucronata</i> (cm ⁻¹)	Frequency of absorption bands (cm ⁻¹) [29]	Types of compounds	Function cluster (Bond)
Flavonoids				
1	3424	3200-3600	Phenols, alcohol monomers, hydrogen bond alcohol	O - H
2	2927	2850-2970	Alkanes	C - H
3	1631	1610-1680	Alkenes	C = C
4	1539	1500-1600	Aromatic rings	C = C
5	1251	1050-1300	Alcohol, ether, carboxylic acid, esters	C - O
6	1062	1050-1300	Alcohol, ether, carboxylic acid, esters	C - O
Alkaloids				
1	3424	3300-3500	Amina, amide	N - H
2	1449	1340-1470	Alkanes	C - H
3	1383	1340-1470	Alkanes	C - H
4	1251	1180-1360	Amina, amide	C - N

Source: Field Data.

Based on LC₅₀ tests that have been conducted on crude extracts of mangrove leaves *R. mucronata* using methanol solvents, with doses of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg.kg⁻¹ in tilapia (*O. niloticus*). The test results, using a probit analysis seen in Appendix 5, showed the LC₅₀ value was in the range of 95,625 mg.kg⁻¹ with an upper limit of 153,110 mg.kg⁻¹ and a lower limit of 66,364 mg.kg⁻¹. LC₅₀ value obtained is then used as a reference for the next test stage (optimization of the dose) to become the active ingredient immunomodulator against tilapia, with a dose of < 66,364 mg.kg⁻¹ at the lower limit LC₅₀.

Mangrove *Rhizophora mucronata* is known to contain active compounds that are sedative or hypnotic and toxic to fish [9]. Therefore LC₅₀ testing is important to know the safe limits for its utilization. On the other hand, the LC₅₀ test results that have been conducted in this study which states that *R. mucronata* extract can cause death as much as 50-95% in tilapia (*O. niloticus*) at a dose of 50-100 ppm. However, with the results of the probit analysis the concentration value showed 64 ppm to be the limit of *R. mucronata* extract is safe and non-toxic to tilapia [9]. This is supported by the statement that if the extract has an LC₅₀ value of 30-200 mg/l it has activity to fight pathogens, although the LC₅₀ value < 1000 mg/l has toxic activity [30].

Dose optimization refers to the lower limit value of LC₅₀ which is 66,364 mg.kg⁻¹, then used several doses below it for optimization of 10, 20, 30, 40, 50 and 60 mg.kg⁻¹. The observations in Figure 3, showed leukocyte values both before and after treatment were in the normal range. This is because the range of leukocytes or total leukocytes of normal tilapia is at 2.0-15.0 (x 10⁴) cells/mm³ [31].

The observations using a one-way analysis of ANOVA, which was further tested using Tukey showed significant different notations (p<0.05). The difference can be seen in the table value after treatment (day 4) in Figure 3. In addition, to find out the three most significant increased doses were analyzed using repeated measures. The value of the results of the analysis showed the dose of 30 mg.kg⁻¹ had the highest and significant increase (p< 0.05) by 2.6 x10⁴ cell/mm³, compared to dose 10, 20, 40, 50 and 60 mg.kg⁻¹ (1.5; 2.3; 1.7; 1.3 and 1.0 (x10⁴) cells/mm³). While at the control value (without extracting) there was an increase in leukocytes by 0.03 x10⁴ cells/mm³. However, the value showed that the control treatment did not change leukocytes during the study (day 1 to day 4) because it did not differ markedly or insignificantly (p> 0.05). In addition, the results of the regression analysis showed a coefficient of determination of R² of 0.91. In other words, the administration of extracts affects 91% of the total leukocytes produced.

A significant increase in total leukocytes indicates that the active compound content of flavonoids and alkaloids in the coarse extract of *R. mucronata* leaves may affect the immune response of fish. Mechanism of flavonoids in the improvement of leukocytes by activating the process of signaling DC cells (dendritic cells) in lymphocytes and T-helper lymphocytes (Th lymphocytes). Flavonoids are also known to activate interleukin secretion processes (IL) -2,

specific Th proliferation, and signaling of Th1 and Th2 against other cells in the fight against pathogens [32]. While alkaloids are reported to improve immune response (leukocytes) by stimulating the hemopoietic system, improving bone marrow cellularity and positive cell esterase with an influence on stem cell proliferation in animals [33].

Figure 3 – Analysis of extract modulation against leukocytes (Source: Field Data)

The active compound alkaloids can also increase the total leukocytes by stimulating bone marrow activity [34]. The increase in total leukocytes in the treatment of extracts (immunomodulators) showed a positive impact on tilapia (*O. niloticus*). Because the increase indicates that the body defense system of fish also increases. This is due to the increasing total number of leukocytes followed by phagocytosis activity and will provide protection to fish if pathogens infect [35].

Based on the previous explanation, it can be concluded that the dose of 30 mg.kg⁻¹ with an increase of 2.6×10^4 cells/mm³ and the average value of the 4th day $7.8 \pm 0.2 \times 10^4$ cells/mm³ is the optimal dose. In this case the dose of the active ingredient is crude extract of mangrove leaves *Rhizophora mucronata*, in stimulating an increase in the total leukocytes to defend themselves against pathogenic attacks. The leukocyte changes that occur are indicators of the impact of the provision of active ingredients on fish. The indicator is used as a reference because the increase in the number of white blood cells (leukocytes) indicates the presence of the body's resistance response to foreign bodies (disease-causing agents) [36].

CONCLUSION

Crude extract of *Rhizophora mucronata* results of phytochemical tests corroborated by UV-Vis and FT-IR analysis showed the presence of flavonoid and alkaloid compound content. Toxicity results show (LC₅₀) crude extract *R. mucronata* is at a value of 66.364 mg.kg⁻¹. The compounds in the extract can modulate the immune system of fish, one such as leukocytes in the body of tilapia (*Oreochromis niloticus*). In all treatments (of the 6 doses), the treatment dose of 30 mg.kg⁻¹ is the maximum dose for immunomodulators with an influence of 91%.

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