Acute toxicity test combination of binahong leaves extract (*Anredera cordifolia*) and catfish oil (*Pangasius micronema* Blkr.) in mice

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Abstract. Binahong (*Anredera cordifolia*) and catfish (*Pangasius micronema* Blkr.) plants have been proven to have pharmacological effects through various studies. However, there has been no research on the toxicity level of the combination of two ingredients. The purpose of this study was to determine the acute toxicity after administration of the combination of natural extracts, the LD_{50}, and the toxicity category of the compound. The parameters observed were symptoms of toxicity, changes in body weight, and changes in organ weight of mice for 14 days of observation. The combination of binahong extract with catfish oil was given orally in graded doses, namely group I (250 mg/kg catfish oil and 100 mg/kg binahong), group II (500 mg/kg catfish oil and 200 mg/kg binahong), and group III (1000 mg/kg catfish oil and 400 mg/kg binahong). The data obtained were then subjected to an ANOVA test to determine differences between the test groups. The results showed that the symptoms of toxicity that appeared, body weight testing, and organs of the liver, heart, and kidneys of mice obtained a significance value of $p>0.05$ so there is no significant difference between the weight of mice's hearts. The LD_{50} value in this study is a pseudo-LD_{50} because there is no death in all test animals. This shows that the combination of binahong extract and catfish oil is categorized as practically non-toxic with $LD_{50} > 5000$ mg/kg.

1 Introduction

Indonesia is a country rich in natural resources, particularly biological ones. It boasts over 30,000-40,000 species, of which only 2.5% are utilized or explored in traditional medicine [1]. At present, the world's attention to the use of natural materials has increased, especially in the realm of health. This approach is based on the content evidenced by numerous studies on the standardization of materials, including both preclinical and clinical tests on the content of secondary metabolites [2].

Binahong (*Anredera cordifolia*) is an example of a medicinal plant that contains bioactive compounds such as flavonoids, saponins, steroids, and kumari, with flavonoids having the highest antioxidant content [3]. Binahong's bioactive compounds, when

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combined with other natural ingredients like catfish oil, can enhance the desired therapeutic effect with its fatty acid content [3-4]. Catfish (*Pangasius micronema* Blkr.) is a type of low-fat fish with high protein content, which is a consumption choice for various groups. Catfish also contains important nutrients such as omega-3 fatty acids, selenium, and taurine, which play a role in stimulating the growth and development of brain cells, as well as abundant vitamin and mineral content when compared to other types of freshwater fish [5].

Using binahong extract as an antioxidant is expected to prevent the formation of radicals and can also inhibit oxidation reactions by binding free radicals and highly reactive molecules [6]. Meanwhile, catfish with high protein levels and all amino acids can be used as an anti-inflammatory by forming and maintaining body tissues [7]. Combining the two natural components should improve their pharmacological action, which synergizes with antioxidant and anti-inflammatory mechanisms. To determine the LD_{50} value and safety of a medication component, toxicity studies must be conducted due to each substance's biochemical constituents and characteristics.

2 Materials and method

2.1 Materials

The materials used in the study include binahong leaves (*Anredera cordifolia*), catfish (*Pangasius micronema* Blkr.) from lokal market in Surakarta city, 96% ethanol pharmaceutical grade (repacking by PT. Nathin-Naturafit), distilled water (repacking by Arkan Medika Solo), corn oil (Tropicana slim), Na CMC powder (repacking by Saba Kimia Solo, chloroform merck (EMSURE) and bentonite. Binahong leaves were collected from the determination in the B2P2TOOT Tawangmangu.

2.2 Research methods

2.2.1 Preparation of binahong extract

Binahong was extracted using a maceration method, utilizing 96% ethanol solvent in a ratio of 1:3 and stirring every 24 hours. We filtered the sample after 3 days of maceration to obtain the macerate. We then evaporate the macerate on a hotplate to obtain a thick extract and calculate the yield. The yield obtained was 3.25%.

2.2.2 Preparation of catfish oil

Catfish oil was prepared using the wet rendering method, where fresh meat is boiled and pressed using water to get oil. Boiling produces catfish juice, which we filter, add 1% bentonite to, and then separate with a magnetic stirrer to extract catfish oil.

2.2.3 Dilution of sample

The sample will be diluted using two different materials: binahong leaf extract will be diluted using Na CMC solution, and catfish oil will be diluted using corn oil.

2.2.4 Test animal preparation

This study used male mice strain wistar weighing 20-30 grams (2-3 months old), obtained from the Faculty of Medicine, Universitas Sebelas Maret. The control stage was carried out by dividing the test animals into four groups, each consisting of 5 mice. The test animals were acclimatized for 3 weeks because the body weight of the mice was below the average. The mice were acclimatized while controlling the frequency of feed and drinking water given. Body weight weighing of test animals was done once a week to control the growth, health, and readiness of the test animals. After the observation, mice are then dissected to observe the toxicity that appears by euthanizing them first using chloroform, then performing surgery to see signs of toxicity that appear in organs, especially the liver, heart, and kidneys.

2.2.5 Acute toxicity test

Before this study started the ethics clearance was obtained in Moewardi hospital with number 1.996/XI/HREC/2023. The grouped test animals were then treated where the control group was given the combination of 100 mg/kg binahong extract and 250 mg/kg catfish oil; group II was given a combination of 200 mg/kg binahong extract and 500 mg/kg catfish oil; group III was given a combination of 400 mg/kg binahong extract and 1000 mg/kg catfish oil. The dosage was obtained from the dose determination in the previous study. In the previous study, the dose used in toxicity testing did not cause death in 50% of the test animals. Therefore, further toxicity testing was carried out in this study by increasing the dose to twice the multiple of the initial dose to increase the toxic effect. Then, observations were made for the first 24 hours after the administration and the next 48 hours. Testing was then carried out for up to 14 days with observation parameters, including abnormal symptoms and mortality of test animals.

2.2.6 Determination of LD_{50}

The determination of the LD_{50} value in this study was carried out by the Thomson and Weil method, where the formula used is:

$$Log\ m = log\ D + d(f + 1) \quad (1)$$

where m : LD_{50} price, D : smallest dose used, d : log r (dose multiple), f : factor. Data were analyzed using a one-way ANOVA test using IBM SPSS Statistics.

3 Result and discussion

This study employs an acute toxicity test, periodic testing within the first 24 hours and once daily for the next 14 days. The acute toxicity test aims to ascertain a substance's LD_{50} (lethal dose). The LD_{50} is the dose at which animals statistically die in an experiment. Determination half of the LD_{50} method involves administering samples in varying doses to a group of test animals, each receiving a single dose [8]. Additionally, LD_{50} testing can reveal the specific toxic effects of the substance on the affected organs and assist in determining safe doses for long-term use. The study used the following parameters: toxicity symptoms, body weight changes, and organ weight changes in the test animals.

3.1 Toxicity observations

Toxicity observations were performed on test animals that displayed abnormal symptoms and died after receiving the extract combination. Signs of toxicity were observed visually in the first 24 hours after administration of the sample test and continued 48 hours later. Table 1 shows the results of mice's mortality observations.

Table 1. Mortality observations.

Group	Binahong dose	Catfish oil dose	Number of mice	Number of mortality
Control	0	0	5	0
I	100	250	5	0
II	200	500	5	0
III	400	1000	5	0

The table displays the mortality observations (death) in mice, observed for the first 24 hours and continued for the next 48 hours. We continued the observations for 14 days, and the results revealed no mouse deaths. If the test animals do not die at the highest dose, there is no need to continue using samples with higher doses [9]. In addition to observing mortality in mice, abnormal symptoms that arise after administering the sample test are also observed.

Observe common abnormal symptoms such as depression, shallow breathing, coma, and death. Additionally, the parameters of toxicity symptoms include the observation of activity and behavior patterns, sensitivity to pain, and sensitivity to touch. The parameters of toxicity symptoms are shown in Table 2.

Table 2. Parameters of toxicity symptoms

Toxicity symptoms	Dose			
	Control	**Group I**	**Group II**	**Group III**
Activity and behavior patterns	-	+	+	++
Cardiovascular	-	+	+	++
Itching	-	+	++	++
Fur	-	+	++	++

Description:
(-) = Asymptomatics
(+) = Mildly symptomatics
(++) = Moderately symptomatics
(+++) = Severely symptomatics

These observations are seen from the increase or decrease in the activity of test animals from their normal activities [9]. Based on the observations, it is known that after giving distilled water as a control, no toxic symptoms appeared. After administering the sample test, mice continued to make active movements and show normal behavior. Toxic symptoms began to appear in group I, where there was a decrease in motor activity, increased cardiovascular rate, itching, and standing upright fur. However, these symptoms were only mild and lasted the first 30 minutes after administration of the sample, after which the mice returned to normal. At doses of group II and group III, signs of toxicity escalated, resulting in moderate symptoms. These abnormal symptoms appear up to 1–2 hours after administering the sample and do not cause death in the entire test animal.

3.2 Weight measurement

The mice's weight is measured every week, namely the first week before and the second week after the administration of the sample test. This was done to see the effect of giving the sample on changes in mice's body weight in 14 days [10]. Table 3 showed the mice's body weight during the study.

Table 3. The mice's body weight between groups

Groups	Mean (SD) (grams)		
	Day 0	Day 7th	Day 14th
Control	27.30 (1.49)	27.90 (1.61)	30.38 (1.07)
Group I	29.28 (3.52)	30.98 (3.20)	27.88 (5.43)
Group II	28.28 (2.03)	28.34 (3.17)	27.98 (4.79)
Group III	28.22 (2.40)	31.16 (3.82)	29.06 (3.54)

Significant changes in body weight are an early indicator of a test sample's toxic effects. If a compound causes a change in body weight of more than 20% from before the administration of the sample test, we can declare it to have a toxic effect [11]. The absence of a change in body weight of more than 20% in the test animals indicates that the administration of the combined sample of binahong leaf extract and catfish oil did not affect the body weight of the test mice. Several factors, such as the mice's growth and cell development, influence changes in body weight. In addition, food intake, stress response due to treatment, environmental conditions, and cage cleaning processes can also be factors that cause changes in body weight in mice [12].

3.3 Organ weight measurement

After 14 days of observation, mice that were still alive were euthanized using chloroform and dissected to take part of the liver, heart, and kidney organs. Organs taken are then weighed to determine the average weight of each mice organ. The organs weight of mice can be seen in Table 4.

Table 4. The mice's organs weight between groups

Groups	Mean (SD) (grams)		
	Liver	Heart	Kidney
Control	1.39 (0.16)	0.13 (0.01)	0.16 (0.04)
Group I	1.13 (3.52)	0.13 (3.20)	0.18 (5.43)
Group II	1.05 (2.03)	0.13 (3.17)	0.16 (4.79
Group III	1.05 (2.40)	0.12 (3.82)	0.15 (3.54)

The results showed a decrease in the average weight of the liver, heart, and kidney organs in all test groups of mice. Observations of each organ are shown in Figure 1.

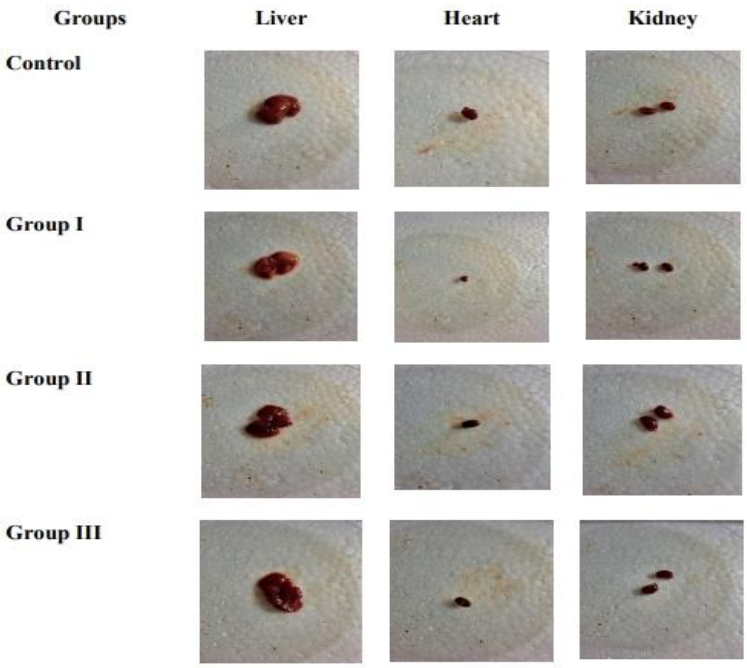

Fig. 1. Figure of each organs.

The weight of mice's organs lost are a sign of modification in organ cells due to chemical exposure [10]. The standard weight of the mice's liver ranges from 2 to 3 grams [13], but in the study's results, there was a decrease in the weight of the mice's liver organs, which may be due to the inability to metabolize and detoxify compounds in the combination of binahong extract and catfish oil [14].

The standard weight of mice's hearts is approximately 0.6% of their body weight [15]. The average weight of mouse heart organs is lower than the standard, but the significance value is $p>0.05$, so there is no significant difference between the weight of mice's heart. It is potentially due to disruptions in the body's nutrient circulation system, leading to reduced heart weight. Observations on the kidney organ showed a decrease in weight from the standard of 0.16-0.22 grams [16]. Alkaloid and tannin compounds in binahong may trigger an inflammatory process, leading to the decrease in kidney weight in mice [3]. In addition, flavonoid content as an antioxidant can reduce oxidative stress, decreasing kidney weight [10]. Although there were weight changes in mice's liver, heart, and kidney organs, these changes were not significant, indicating that the combination of binahong extract and catfish oil did not have a toxic effect on mice's organs.

3.4 Determination of LD_{50}

In this study, no deaths were observed in the test animal group during the 14 days of observation. All researchers concur that if the test animals receive the maximum dose without experiencing death, we refer to the LD value as pseudo-LD or not the actual value [17]. If there is no death in the test animals with a single dose, the highest dose of the test sample is used to determine the LD_{50} value. Therefore, this study's apparent LD_{50} value for the binahong leaf extract and catfish oil combination is greater than >1000 mg/kg, meeting the non-toxic criteria [9].

4 Conclusion

The research results indicate that the acute toxicity of the combination of binahong leaf extract and catfish oil did not result in death in all test animals. However, several toxicity symptoms appeared shortly after sample administration, including those in the eyes, activity and behavior patterns, cardiovascular system, allergies (itching), and fur. The LD_{50} value in this study cannot be determined because it did not cause death in 50% of the test animals, so the LD_{50} value is expressed as pseudo- LD_{50} value. This study can be used for further research to determine the level of toxicity of the combination of binahong leaf extract and catfish oil by conducting quantitative research through histopathological testing of mice organs.

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References

1. Cahyaningsih, R., Magos Brehm, J., and Maxted, N. Setting the priority medicinal plants for conservation in Indonesia, Genetic resources and crop evolution, **68**(5), 2019-2050 (2021).
2. Aware, C. B., Patil, D. N., Suryawanshi, S. S., Mali, P. R., Rane, M. R., Gurav, R. G., and Jadhav, J. P. Natural bioactive products as promising therapeutics: A review of natural product-based drug development, South African Journal of Botany, **151**, 512-528 (2022).
3. Alba, T. M., Tessaro, E., and Sobottka, A. M. Seasonal effect on phenolic content and antioxidant activity of young, mature and senescent leaves from *Anredera cordifolia* (Ten.) Steenis (*Basellaceae*). Brazilian Journal of Biology, **84**, e254174 (2022).
4. Sasongko, H., Rizki, F., Fatihah, A.Y.D., Sholikin, A.A., Kristiyanti, A.A., Ningsih, F.A.V., Sari, S.W.S., Cahyadi, M. and Sutarno, Hypoglycemic and hypolipidemic activity of a catfish oil and Andrographis paniculata herb extract combination in diabetic rat model. Food Research, **8**(1), 174 – 180 (2024).
5. Ikhsan, A. N., Rohman, A., Mustafidah, M., Martien, R., and Lestari, L. A. The implementation of fourier transform infrared spectroscopy combined with chemometrics for the authentication of patin (*Pangasius micronema*) fish oil emulsion. Indonesian Journal of Pharmacy. **34**(2), 174–181 (2023).
6. Feriyani, F., Maulanza, H., Lubis, R. R., Balqis, U., and Darmawi, D. Effects of binahong (*Anredera cordifolia* (Tenore) Steenis) extracts on the levels of malondialdehyde (MDA) in cataract goat lenses. The Scientific World Journal, **2021**(1), 6617292 (2021).
7. Akunne, T. C., Okafor, S. N., Okechukwu, D. C., Nwankwor, S. S., Emene, J. O., and Okoro, B. N. Catfish (*Clarias gariepinus*) slime coat possesses antimicrobial and wound healing activities, Pharmaceutical and Biosciences Journal, **4(3)**, 81-87. (2016).
8. Sulastra, C. S. and Khaerati, K. Toksisitas akut dan lethal dosis (LD_{50}) ekstrak etanol uwi banggai ungu (*Dioscorea alata* L.) pada tikus putih (*Rattus norvegicus*). Jurnal Ilmiah Medicamento, **6**(1), 10-14 (2020).

9. BPOM RI, Peraturan Badan Pengawas Obat dan Makanan Nomor 10 Tahun 2022 tentang Pedoman Uji Toksisitas Praklinik secara In Vivo. Jakarta, Kepala Badan Pengawas Obat dan Makanan Republik Indonesia (2022).
10. Malik, M. K., Bhatt, P., Singh, J., Kaushik, R. D., Sharma, G., and Kumar, V., Preclinical safety assessment of chemically cross-linked modified mandua starch: Acute and sub-acute oral toxicity studies in Swiss albino mice. ACS omega, **7**(40), 35506-35514 (2022).
11. Sujana, D., Winda Suwandi, D., Rusdiana, T., and Subarnas, A., Acute toxicity test of ethanol extract of pakis tangkur (*Polypodium feei* Meet) root from Talaga Bodas Mountain on Swiss Webster Mice, Jurnal Ilmiah Farmako Bahari, **11**(2), 167-179 (2020).
12. Wittek, L., Touma, C., Nitezki, T., Laeger, T., Krämer, S., and Raila, J. Reduction in cold stress in an innovative metabolic cage housing system increases animal welfare in laboratory mice. Animals, **13**(18), 2866 (2023).
13. Rogers, A. B., and Dintzis, R. Z. Hepatobiliary system, In comparative anatomy and histology, Academic Press, 229-239 (2018).
14. Fitriani, R. N., Sitasiwi, A. J., and Isdadiyanto, S. Struktur hepar dan rasio bobot hepar terhadap bobot tubuh mencit (*Mus musculus* L.) jantan setelah pemberian ekstrak etanol daun mimba (*Azadirachta indica* A. Juss). Buletin Anatomi dan Fisiologi, **5**(1), 75-83 (2020).
15. Mboro, Y. M., Dima, A. O. M., and Ati, V. M. Profile of growth and percentage of organ weight internal mice (*Mus musculus* L.) male giving moringa leaf extract (*Moringa oleifera* Lamk.). Jurnal Biotropikal Sains, **15**(1), 57-73 (2018).
16. Melisa, E., and Yuliawati, Y. Uji toksisitas akut ekstrak etanol daun sungkai (*Peronema cenescens* Jack) terhadap fungsi ginjal mencit putih betina *(Mus musculus* Linn.). Majalah Farmasi dan Farmakologi, **26**(1), 32-37 (2022).
17. Loomis TA Essentials of Toxicology, 3rd Ed. Lea & Febiger, Philadelphia, PA 1–12 (1978).