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Research Article

Acute toxicity test of water and ethanol extract from african leaf (*Gymnanthemum amygdalina* Del.) on zebra fish embrio (*Danio rerio*)

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Abstract

Bitterleaf or African Leaf is a herbal plant empirically used to relieve fever and kidney disease. Pharmacological research reports that the leaves have antibacterial, anti-inflammatory, and antidiabetic properties, and a toxicity test was also conducted to ensure their safety. Therefore, this experiment aimed to obtain LC_{50} of African Leaf (*Gymnanthemum amygdalina* Del.) ethanol and water extract with the ZFET (Zebra Fish Embryo Acute Toxicity) method and also to observe the toxicity effect of zebrafish (*Danio rario*) embryo's morphology after the induction of the extract. The experiment refers to the OECD No.236 of 2013 as a guideline, and it was shown that African Leaf's ethanol extract LC_{50} values were obtained at 6.3629 ppm. Furthermore, the water extract LC_{50} value was obtained at 25.0520 ppm, and the African Leaf extract was categorized in the toxic category. The leaf extract has a harmful effect on zebrafish embryos, resulting in malformations of the tail, notochord, pericardium, and yolk sac.

Introduction

Herbal medicine is a mixture of natural ingredients used traditionally based on experience. According to Mulyani et al. l. (2020), the African leaf (*Gymnanthemum amygdalina* Del.) is a plant commonly used in herbal medicine. Its secondary metabolite compounds include flavonoid, terpenoids, alkaloids, tannins, saponins, and glycosides (Alara et al., 2019). Research on the pharmacological activities reported antibacterial, anti-inflammatory (Setiani & Rusli, 2020), and antidiabetic properties (Alara et al., 2017). Safety in using herbal medicines is very important, and it is necessary to conduct testing to determine the toxicity value and obtain a threshold for using herbal medicines. Toxic effects resulting from the test preparations on animals can be reviewed based on biochemical, physiological, and pathological reactions correlated to humans (Badan Pengawas Obat dan Makanan, 2020). African leaf extract has an LC_{50} value of 123 ppm using the Brine Shrimp Lethality Test (BSLT) method, which belongs to the toxic category (Hasanah & Arnanda, 2020). However, the use of the BSLT method was not observed in the morphology and physiology of Artemia salina larvae exposed to active compounds. Therefore, using the Zebra Fish Embryo Acute Toxicity Test method, other test animals are needed to determine the effects that arise on morphology and physiology. The selection of

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toxicological tests utilizing zebrafish embryos was used as an initial screening. This is because of the short analysis time, embryo transparency, short life cycle, genetic similarity with humans in the morphology and physiology of the nervous, cardiovascular, and digestive systems and can produce many embryos (Chahardehi et al., 2020). The genetic similarity of zebrafish embryos with humans is 70-85% (Wijaya, 2020). Based on this background, this research was conducted on the acute toxicity of ethanolic and water extracts of African leaf zebrafish embryos.

Research methods

Time and place

The research was conducted from September to October 2021 in Pharmaceutical Laboratory, Faculty of Mathematics and Natural Sciences, Pakuan University, Bogor.

Instruments

The instruments used in the toxicity test process were aluminum foil (Klin Pak®), aquarium, aerator (Amara®), porcelain dish, petri dish, the crucible, Microwave (Samsung®), microscope (XSZ®), analytical balance (LabPRO DT224C®), oven, laboratory glassware (Pyrex®), infusion equipment, dropper, furnace (Daihan Scientific Furnace®), thermometer, vacuum dryer, and well plate-24.

Material

The materials used included African leaf obtained from Cariu Village, Bogor Regency, zebrafish embryos obtained from local fish farmers in the Cibinong area, distilled water, 2N hydrochloric acid (HCl), concentrated hydrochloric acid (HCl), Iron (III) Chloride ($FeCl_3$), 70% ethanol (CH_2OH), 96% ethanol (CH_2OH), gelatin, Liebermann-Bourchard reagent solution, Dragendorff reagent solution, Mayer reagent solution, Bouchard at reagent solution, Sodium Chloride (NaCl), Magnesium (Mg) powder, and Zinc (Zn) powder.

Preparation of african leaf extract infusion method

A total of 200 mL of water was heated in an infusion pan to 90^{0}C, and 20 g of simplicia powder was put into the infusion pan. Subsequently, the extraction process was repeated for 15-20 minutes until 200 g of simplicia powder was extracted, filtered to obtain an African leaf infusion, and vacuumed to form a dry extract.

Making african leaf extract with MAE (Microwave Assisted Extraction) method

The simplicia fine powder of African leaf was weighed as much as 200 g, and about 20 g was put into a 500 mL erlenmeyer with a solvent in a ratio of 1:10. The solvent used is 70% ethanol. The mixture of 70% ethanol and simplicia 20 g was put in a microwave with 600 watts of power for 4 minutes (Alara et al., 2018). Periodically, the solution was irradiated in the microwave to keep the temperature below 80^{0}C, and the extract was left at room temperature and filtered (Quan et al., 2006). The results obtained from the MAE was vacuumed until a dry extract was formed.

Determination of water content

Determination of water content was carried out by the gravimetric method, which was initiated by incandescent and crucible in an oven at 105^{0}C for 1 hour and then weighed. A total of ±2 g of powdered simplicia was weighed and put into a crucible that had been tempered, ignited at 105^{0}C for 5 hours, cooled in a desiccator, and weighed, which was then re-ignited. This was repeated until the weight became constant or the difference in weight was not more than 0.0025 g (Departemen Kesehatan Republik Indonesia., 2000), then the water content was calculated using the formula:

$$\text{Water Content} = \frac{w_1\text{-}w_2}{\text{the initial weight of the sample}} \times 100\%$$

Description:
W_1 = weight of the cup + sample before heating
W_2 = constant weight of the cup+sample before heating

Determination of ash content

A total of ±2 g of Simplicia powder was put into a silicate crucible that was ignited. Subsequently, it is flattened, then incandescent at a temperature of ±600^{0}C in a kiln until it becomes charcoal. The charcoal is then cooled in a desiccator and weighed. Hot water is added if

the ash still has not disappeared, anedd filtered through ash-free paper. The remaining ash-free filter paper in the same crucible is to be re-ignited, and the filtrate is put into a crucible to be evaporated, ignited, and weighed (Departemen Kesehatan Republik Indonesia., 2000), and the ash content is calculated using the formula:

$$\text{Ash Content (\%)} = \frac{w_1 - w_2}{\text{the initial weight of the sample}} \times 100\%$$

Description:
W_1 = crucible weight + ash untill
W_2 = crucible weight

Phytochemical testing of simplicia and african leaf extract

Alkaloid test

About 0.5 g of the sample was weighed and dissolved with 1 mL of 2N hydrochloric acid and 9 mL of distilled water, then heated in a water bath for 15 minutes. Samples that have been heated and cooled to be filtered with the filtrate are dropped on a watch glass before adding alkaloid reagents, such as Dragendorff, Mayer, and Bouchardat. The formation of a brown-orange precipitation indicates positive results in Dragendorff's reagent. In Bouchardat's reagent, the formation of brown indicates positive results for black deposits, and the formation of white deposits characterizes Mayer's reagent. (Hanani, 2015).

Flavonoid test

For the flavonoid test, 0.5 g of sample was weighed and dissolved with 5 mL of 96% ethanol, then evaporated until dry before adding 2 - 3 drops of ethanol. Each sample was added with magnesium and zinc powders, and 5 drops of concentrated hydrochloric acid were added and then heated in a bath. Positive results on magnesium powder are indicated by the formation of an orange color, which indicates the presence of flavonon, flavonol, flavononol, and dihydroflavonol compounds. In contrast, the zinc powder is indicated by the formation of pink color, showing dihydroflavonol compounds (Hanani, 2015).

Tannin test

About 0.5 g of the sample was extracted with 30 mL of 80% ethanol for 15 minutes, then filtered and evaporated over a water bath before adding hot distilled water that was stirred, cooled, and centrifuged. The upper liquid was separated by decantation, and the resulting filtrate was further identified for tannin compounds by the following test: (i) The filtrate was added to a 10% gelatin solution, and the formation of a white precipitate indicates a positive result. (ii) Sodium chloride and gelatin were then introduced to form a solution of 1% gelatin in 10% sodium chloride in a ratio (1:1). Meanwhile, the formation indicated a positive result of a white residue. (iii) The filtrate was added to a 3% solution of iron (III) chloride, and a positive result was indicated by forming a blue-green to black color (Hanani, 2015).

Steroid and terpenoid test

For steroid and terpenoid tests, 2 mL of the extract was taken and put into a test tube before adding the Liebermann-Bourchard reagent. Positive results from the sample are indicated by the formation of an orange-red and blue color, showing the presence of terpenoid and steroid compounds (Ergina et al., 2014).

Saponin test

About 0.5 g of the sample was weighed and put into a test tube containing 10 mL of hot water and shaken vigorously for 10 seconds. The formation of a stable foam indicates a positive result, and when hydrochloric acid is added, the foam does not disappear (Hanani, 2015).

Acute toxicity test

Acute toxicity testing was carried out using the fish embryo testing procedure as stated in the OECD Guidelines for Testing of Chemicals No. 236 (2013) using embryonic test animals from zebrafish. The use of these test animals has passed the ethical review submitted to the Pharmaceutical Pharmacology Ethics Review Team, FMIPA, Pakuan University, Bogor, with letter number 013/KEPHP-UNPAK/07-2021. Furthermore, Zebrafish embryos obtained from local fish farms in the Cibinong area are selected and tested by exposing the test materials to the embryos.

Control solution preparation

Internal control, also known as egg-water, is well water filtered with Whatman filter paper to remove big particles and other contaminants and then aerated with oxygen-rich air bubbles for 24 hours. In contrast, the control extract was a test solution containing African leaf extract tested on zebrafish embryos. The preparation of the test solution conducted a preliminary test to determine the initial concentration in the test solution. The preliminary test was performed by making a 4000 ppm mother liquor by dissolving 400 mg of the extract into 100 mL of water used in the internal control. The dilution obtained concentrations of 50 ppm, 500 ppm, 1000 ppm, 1500 ppm, and 2000 ppm. The concentrations of more than 50 ppm caused all embryos' deaths and then decreased. The concentrations used were 10 ppm, 20 ppm, 30 ppm, 40 ppm, and 50 ppm using 500 ppm mother liquor. Meanwhile, 500 ppm mother liquor was prepared from 50 mg of extract dissolved in 100 mL of water, internal control water.

Zebra fish embryo selection

Zebra fish embryos obtained from local fish farmers in the Cibinong area were placed in a container filled with internal control (egg-water), aerated using an aerator for 24 hours, and filtered with filter paper. A total of 30 embryos were transferred into Petri dishes, and their fertility was observed using a microscope. The characteristics of embryos are used are 8 hours after fertilization, transparent in color, and contained an amniotic sac. Unfertilized embryos are characterized by embryo coagulation, no somites formed, no release of the tail bud with the yolk, and no heartbeat.

Acute toxicity test on zebra fish embryo

According to Organisation For Economic Co-Operation and Developmen (OECD 236, 2013), acute toxicity testing begins with validation to obtain valid results. The parameters include (i) The fertilization rate of all eggs should be ≥ 70% in the test group. (ii) The water is controlled with a temperature of 25 – 27^{0}C during the test. (iii) Embryos in negative control should be ≥90% survival to 96 hours of exposure to the test material. (iv) Embryo mortality in positive controls should be ≥ 30% to 96 hours of exposure. (v) The hatching rate of eggs in negative controls should be ≥ 80% for up to 96 hours of exposure. (vi) After 96 hours of exposure, the dissolved oxygen concentration in the test and negative control should be ≥ 80% of saturation. After validation, this was continued by testing the toxicity of water and ethanolic extracts of African leaf with several concentrations. Each concentration used 10 embryos put into a multiwell to be tested and repeated three times. Using a microscope, morphology and physiology were assessed on embryos 24 hours, 48 hours, 76 hours, and 96 hours after exposure to the control extract. Parameters that determined the death of zebrafish embryos observed are embryo coagulation, somite formation, the release of the embryo's tail from the yolk, and heart rate.

Research ethics

The acute toxicity test of the ethanolic and water extract of African leaf (*Gymnanthemum amygdalina* Del.) on Zebra Fish (*Danio rario*) embryos has passed the ethical review submitted to the Pharmacology Ethical Committee Team, Faculty of Mathematics and Natural Sciences, Pakuan University, Bogor, with letter number 013/KEPHP-UNPAK/07-2021.

Data analysis

The analysis to obtain the LC_{50} value used the Microsoft Office Excel method by using a straight line equation between the log concentration of the extract used, denoted as x, and the probit value denoted as y, where y = a + bx. Furthermore, the value 5 (the probit used to give 50% mortality in the test animals) is entered as y from the straight-line equation, while the x is the log value of the concentration. The LC_{50} value is obtained using the antilog of the x value, and using a randomized block design, the data obtained were analyzed by ANOVA (Analysis of Variance). The ANOVA analysis results showed a significant difference, and further analysis was performed using Duncan's test. The data processing used the IBM SPSS Statistics 24 program for windows.

Results and discussion

Results of making african leaf extract

Extracts were made using the MAE method (Microwave Assisted Extraction) with 70% ethanol solvent and the infusion method with distilled water as solvent. Each extracted filtrate was dried to form a dry extract using a vacuum dryer, and the ethanol extract was greenish with a characteristic odor, while the water extract was brownish. The weights of ethanol and water extract were 17.8 g and 15.7 g, with 8.9% and 7.85% yields, respectively. The difference in yield percentage is because there are microwaves that can produce electromagnetic wave in the method used. Therefore, it increases the temperature to be hotter and evaporates the water in the simplicia powder cell for bioactive compounds to easily come out and be extracted (Kristanti et al., 2019). In the infusion method, extraction was assisted by heating and stirring, hence, a diffusion process occurs from the simplicia powder to the solvent.

Results of simplicia water content and african leaf extract

Water content was determined to maintain the quality of simplicia and extracts. It was conducted using the gravimetric method with the principle of evaporating water by heating the extract to a constant weight. The results of determining the water content of simplicia powder, ethanol extract, and water extract can be seen in Table 1.

Table 1. Determination of the water content of simplicia and african leaf extract

Sample	Water Content Results (%)	Condition (%)
Ethanol Extract	3.2	≤5
water extract	3.5	≤5

Results of simplicia ash content and african leaves extract

The determination of ash content has the principle that the sample is heated at a high temperature to obtain an overview of the mineral and inorganic content (DepKes RI, 2000) in two repetitions or duplicates. The results of determining the ash content of Simplicia powder, ethanol extract, and water extract can be seen in Table 2.

Table 2. Determination of ash content of simplicia and african leaf extract

Sample	Ash Content Results (%)	Condition (%)
Ethanol Extract	4.65	≤10%
water extract	4.95	≤10%

Phytochemical test results of simplicia and african leaf extract

The results of the phytochemical test of simplicia powder, ethanol extract, and water extract of African leaf reported the presence of alkaloids, flavonoids, terpenoids, tannins, and saponins. The results of the phytochemical test of simplicia powder, ethanol extract, and water extract can be seen in Table 3.

Table 3. Phytochemical test results of simplicia powder and african leave extract

Compound Identification	Reagent	Parameter	Description	
			Ethanol Extract	Water Extract
Alkaloids	*Dragendorff*	↓brown	+	+
	Mayer	↓white	+	+
	Bouchardat	↓brown	+	+
Flavonoids	Zn+HCl P powder	Red	++	+
	Mg+HCl P powder	Orange	++	+

continued Table 3

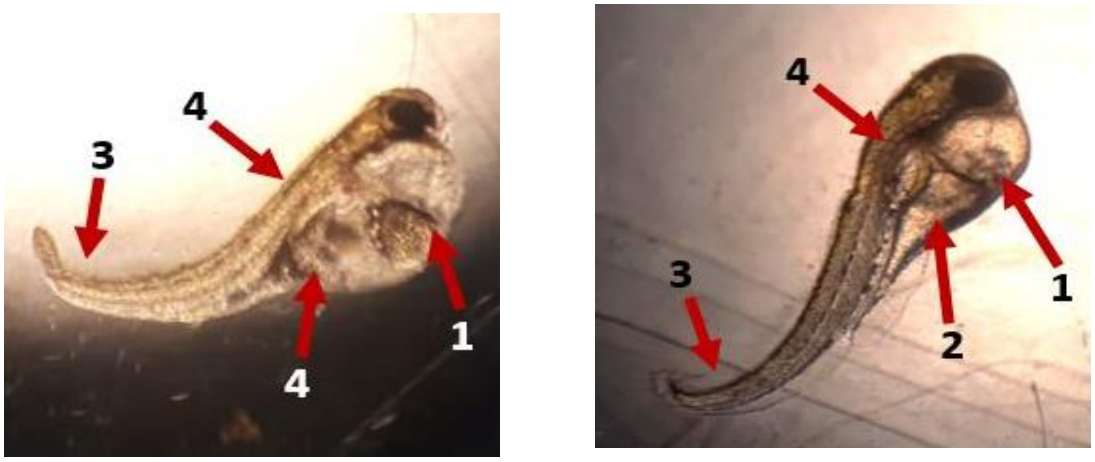
Compound Identification	Reagent	Parameter	Description	
			Ethanol Extract	Water Extract
Tannins	10% Gelatin	↓white	+	+
	Nacl+gelatin	↓white	+	+
	$FeCl_3$	Dark blue	++	+
Terpenoids	*Liebermann-Bourchard*	Orange	++	+
Saponins		foaming	++	+

Description: (+) the result contains compounds; (−) the results do not contain compounds; (↓) a precipitate is formed

The phytochemical test of ethanol and water extract of African leaf showed the same positive results. These results are the same as Rispita's (2018), a research that came from Medan, which positively contains alkaloid compounds, flavonoids, terpenoids, and tannins. The positive results formed were suspected because the compounds in the extract were polar. Meanwhile, the extract used polar solvents of water and ethanol. Phytochemical testing of the ethanol extract gave brighter results and more residue than the water extract of African leaf. This is due to differences in the process, where extraction using the MAE method can attract more secondary metabolites (Utami et al., 2020).

African leaf extract acute toxicity test results

The acute toxicity test results showed that the longer the exposure time, the lower the concentration, and the higher the number of dead zebrafish embryos. The long exposure time caused the embryo to absorb more extracts, resulting in toxic effects. Abnormalities due to toxic effects on the morphology were seen from 48 to 96 hours, namely edema in the pericardium and yolk sac accompanied by abnormalities in the tail and spine seen in Figure 1. Abnormalities are suspected to be due to high concentrations of secondary metabolite compounds. However, there have been no further research of compounds in African leaf extract that can cause abnormalities and deaths in zebrafish embryos.



(a) Ethanol extract (b) Water extract

Figure 1. Abnormalities in Zebra Fish After Exposure to African Leave Extract
Description: (1) Edema of the yolk sac; (2) Pericardial edema; (3) Tails; (4) Spine

In addition to causing toxic effects, exposure to extracts with high concentrations in the long term will result in death. Zebrafish embryo mortality was calculated using the probit of mortality during exposure to obtain an LC_{50} value. The results were obtained from the total number of embryos used in each repetition, and the LC_{50} value obtained can be seen in Table 4.

Table 4. LC_{50} Value of ethanol extract and african leave water extract

Exposure time	LC_{50} value (ppm)		Average
	Ethanol extract	Water extract	
24	43.25	113.08	78.1637[a]
48	10.37	68.37	39.3721[b]
72	8.60	36.32	22.4598[c]
96	6.36	25.05	15.7075[d]

Description: The difference in superscript letters in different columns shows that they do not have the same effect. The ethanol extract and LC_{50} values at the 48th to 96th hours were in the range of 1 to 100 ppm, classified into the toxic category. However, at the 24th hour, there were in the range of 100 to 200 ppm and categorized as moderately toxic. The toxicity category of this test material is based on the LC_{50} value from Hinwood et al., (1994) seen in Table 5.

Table 5. Toxicity level category

No.	Category	LC_{50} value (µg/mL)
1	Very Toxic	< 1
2	Toxic	1 – 100
3	Moderate	100 – 1000
4	Slightly Toxic	1000 – 10.000
5	Almost Non-Toxic	10.000 – 100.000
6	Non-Toxic	>100.000

The data analysis obtained a p-value of 0.00, where p is less than 0.05, there is a significant difference, and Duncan's further test is performed. The results showed that the LC_{50} value of ethanol and water extract at 24, 48, 72, and 96 hours did not have the same effect.

Conclusion

The LC_{50} value of the african leaf ethanol and water extract is 6.3629 ppm and 25.0520 ppm, which can be classified into the toxic category. However, the LC_{50} value of the ethanol extract was more toxic than the water extract. Toxic effects on zebrafish embryos after exposure to African leaf extract showed abnormalities in the tail, spine, and swelling (edema) in the pericardium and yolk sac.

Author's declaration

Authors declare that there is no conflict of interest. LAS and IYW conducted field experiments, recorded and analyzed field data, and prepared the manuscript. LAS and AMA supervised the experiment and conducted manuscript proofreading before submission. Two authors read and approved the final version of the manuscript.

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