

# Cytotoxic Potential of Ethanol Extract Akar Kedayan (*Aristolochia foveolata* Merr.) on Fertilized *Tripneustes gratilla* Eggs

## (Potensi Sitotoksik Akar Kedayan (*Aristolochia foveolata* Merr.) pada Sel Telur *Tripneustes gratilla* Terfertilisasi)

SYUHADA<sup>1\*</sup>, BENAZIR EVITA RUKAYA<sup>1</sup>

<sup>1</sup>Pharmacy Study Program, Politeknik Kaltara, Tarakan, North Kalimantan, 77113, Indonesia

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**Abstract:** The cytotoxicity test is a screening test used to evaluate the reaction of live cells to implants in cell culture tests. This study aims to determine the cytotoxic activity of akar kedayan in fertilized *Tripneustes gratilla* eggs, which have an impact on inhibiting the development of fertile cells. This study used an experimental method by comparing the inhibition of *Tripneustes gratilla* egg cell division in the ethanol extract group of akar kedayan at concentrations of 10, 100, and 1000 ppm, with negative control, then observing embryo cell division at 1, 2, 4, and 12 hours. After fertilization, a comparative statistical analysis was conducted using the ANOVA test, followed by the Tukey HSD Post Hoc Test. The results showed that the negative control and 10 ppm concentration did not inhibit the division of the fertile *Tripneustes gratilla* eggs ( $P\text{-value} > 0.05$ ), while the ethanol extracts of 100 and 1000 ppm significantly inhibited the division of *Tripneustes gratilla* eggs ( $P\text{-value} < 0.05$ ). This study concludes that the ethanol extract of akar kedayan has cytotoxic ability by inhibiting the development of *Tripneustes gratilla* fertile eggs with the optimum cytotoxic dose range at concentrations of 10-100 ppm.

**Keywords:** Akar kedayan, *Aristolochia foveolata*, cytotoxic, *Tripneustes gratilla*

**Abstrak:** Uji sitotoksitas merupakan tes skrining, yang digunakan untuk evaluasi reaksi sel hidup terhadap implan dalam tes kultur sel. Tujuan penelitian ini adalah untuk mengetahui kemampuan sitotoksik akar kedayan terhadap sel telur *Tripneustes gratilla* terfertilisasi. Penelitian ini menggunakan metode eksperimental dengan membandingkan kemampuan penghambatan pembelahan embrio *Tripneustes gratilla* antara kelompok kontrol negatif, konsentrasi 10, 100, dan 1000 bpj, kemudian dilakukan pengamatan pembelahan sel embrio pada jam ke 1, 2, 4, dan 12 sejak fertilisasi. Setelah pembuahan, analisis statistik komparatif dilakukan dengan menggunakan uji ANOVA, dilanjutkan dengan Uji Post Hoc Tukey HSD. Hasil penelitian menunjukkan bahwa kontrol negatif dan konsentrasi 10 ppm tidak menghambat pembelahan sel telur *Tripneustes gratilla* yang telah dibuahi ( $p\text{-value} > 0,05$ ), sedangkan ekstrak etanol 100 dan 1000 ppm secara signifikan menghambat pembelahan sel telur *Tripneustes gratilla* ( $p\text{-value} < 0,05$ ). Penelitian ini menyimpulkan bahwa ekstrak etanol akar kedayan memiliki kemampuan sitotoksik dengan menghambat perkembangan telur fertil *Tripneustes gratilla* dengan kisaran dosis sitotoksik optimum pada konsentrasi 10-100 ppm. Hasil penelitian menunjukkan hanya kelompok konsentrasi 100 ppm dan 1000 ppm memiliki kemampuan sitotoksik dibandingkan kontrol negatif ( $p\text{-value} < 0,05$ ), dan konsentrasi optimum aktivitas sitotoksik berada pada 10-100 bpj.

**Kata kunci:** Akar kedayan, *Aristolochia foveolata*, sitotoksik, *Tripneustes gratilla*

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\*Corresponding author  
e-mail: syuh\_a@yahoo.com

## INTRODUCTION

ARISTOLOCHIA is a genus of plants with more than 500 different species and has a prominent place in the history of medicine. Theophrastus (371-287 BC) first described the use of *Aristolochia clematitis*, known as birthwort, in treating snake bites, head wounds, insomnia, constipation, gynecological problems, and inflammation<sup>(1-3)</sup>.

Among the Dayak tribes in Indonesia, akar ke-dayan is a traditional medicine for skin diseases, toothaches, nosebleeds, diabetes, gout, and rheumatism. It neutralizes insect poisons, snake poisons, and various venomous animal bites and can be used to neutralize the intoxicating effects of alcoholic beverages<sup>(4-6)</sup>.

Between 2004 and 2011, more than eighteen *Aristolochia species* have been investigated worldwide for their chemical composition, and various constituents have been characterized. The secondary metabolites of aristolochia species include aristolochic acids and esters, aristolactam, aporphine, protoberberine, isoquinolines, benzyliso-quinolines, amides, flavonoids, lignans, biphenyl ethers, coumarins, tetralones, terpenoids, steroids, and others. Aristolochic acid is a host of phenanthrene derivative metabolites in which aristolactam also has a similar functional framework<sup>(7)</sup>.

Based on the latest research report, *Aristolochia foetida* Kunth. (Aristolochiaceae) demonstrated cytotoxic potential on MCF-7 cells and non-tumor cells obtained from bovine mammary epithelial cells (bMECs) with an IC<sub>50</sub> value of 45.9 mcg/mL. In healthy epithelial cells, cytotoxic effects were seen at higher doses<sup>(8)</sup>.

Preliminary testing of cytotoxic activity can utilize fertilized eggs, one of which comes from the marine animal *Tripneustes gratilla* which comes from the Echinoidea class and can produce eggs in large quantities. Fertilized *Tripneustes gratilla* eggs are very sensitive to the effects of interactions with chemicals, so they are more easily observed in cell toxicity studies<sup>(9)</sup>. Several advantages of the *Tripneustes gratilla* embryo system over cultured tumor cells for preclinical drug testing with a particular focus on telomerase and telomere interactive drugs. In standard tests, low concentrations of the potent drug must be administered to tumor cells over many cell cycles, often taking more than a month. In the case of *Tripneustes gratilla* embryos, the process of division occurs every hour through the first 8-10 divisions, allowing assays on a comparable number of cell cycles to be completed in a matter of hours<sup>(10)</sup>.

The process of fertilizing *Tripneustes gratilla* eggs is quite easy to do under in vitro conditions, making it easier to carry out preliminary studies of cytotoxic

activity in cells that develop in the early stages after fertilization<sup>(11)</sup>.

Identification of clinical toxicity of *Aristolochia sp.* started appearing in the 1990s. About 100 healthy Belgians have suddenly been diagnosed with chronic kidney failure requiring dialysis and a kidney transplant. Based on the research results, all of these people have long received dietary supplements containing *Aristolochia fangchi*, an ingredient in Traditional Chinese Medicine<sup>(12)</sup>.

Side effects associated with aristolochic acid are still widely reported, especially in Asia and the Balkans. Currently, aristolochic acid is listed as a group I carcinogen by the International Agency for Research on Cancer (IARC)<sup>(13)</sup>. The content of Aristolochic acids (AAs, I and II) in the aristolochiaceae family is thought to be responsible for the adverse effects on the body. Among them, damage to kidney function in high doses and long term causes anemia and induces the formation of tumors in the kidney, bladder, stomach, and subcutaneous tissue in experimental animals<sup>(14)</sup>. Based on the acute toxicity test, the administration of *Aristolochia sp.* aqueous extract for rats is relatively safe on acute oral exposure with LD<sub>50</sub> estimated to be greater than 10 g/kg, while its LD<sub>50</sub> on intraperitoneal administration was 407.38 mg/kg<sup>(15)</sup>.

The long history of using *Aristolochia sp.* in the world until the early 19th century also showed many perceived benefits, and it was challenging to get data on severe side effects such as reports and findings of the toxicity of *Aristolochia sp.* which began to appear in the late 19th century<sup>(2,3)</sup>. Some empirical data and cytotoxicity tests of aristolochia species show potential directions as anti-cancer. Thus, preliminary research efforts on the cytotoxic effects of various aristolochia species can be an initial study in the discovery and development of anti-cancer substances from natural ingredients<sup>(16)</sup>.

## MATERIALS AND METHODS

**MATERIALS.** *Aristolochia foveolata* Merr., Aquadest, 95% technical ethanol (Onemed®, Indonesia), KCl (Pudak®, Indonesia), seawater, *Tripneustes gratilla*.

**Tools.** Aerator, micropipette (Hospital & Home Care®), microscope (Svbonny SV065®, China), digital microscope camera, Hayear® Software, 3 cc syringe (Onemed®, Indonesia).

**METHODS. Sampling and Processing.** The research sample in the form of akar ke-dayan stems was taken from the indigenous Dayak community at the Inai Traditional Market, Kuala Lapang Village, West Malinau, Malinau Regency, North Kalimantan.

Based on the evaluation results at the Materia Medica Batu Herbal Laboratory with number 074/559/102.7-A/2021, akar kedayan was identified as *Aristolochia foveolata* Merr.<sup>(17,18)</sup>. Stem samples of *Aristolochia foveolata* Merr. which had been cleaned, then rinsed with water until clean. The clean samples were then cut into small pieces.

**Sample Extraction.** Stem *Aristolochia foveolata* Merr. 0.5 kg put into a glass container. The maceration process was carried out by adding 95% ethanol until the simplicia was submerged entirely and left for about 3 times 24 hours in a closed container and protected from light while stirring occasionally, the extract obtained was then filtered. The residue was macerated again with a new solvent. This procedure is carried out 3 times. The extract was then evaporated to obtain a thick extract.

**Retrieval of Test Animals.** *Tripneustes gratilla* is obtained from the coast of Derawan Island, East Kalimantan, and is shipped using a container with an aerator. Before treatment, *Tripneustes gratilla* was adapted to a new environment for 24 hours.

**Preparation of KCl Solution (10% w/v).** Weigh 10 grams of KCl and put it into a 100 mL volumetric flask, then add distilled water slowly, shake the KCl solution and make up the volume to 100 mL.

***Tripneustes gratilla* Egg Fertilization.** Eggs and sperm were obtained by induction of male and female *Tripneustes gratilla* by injecting 1 mL of 10% KCl into the gonads. Visually, the sperm has a milky white appearance, while the egg looks like a golden yellow viscous liquid. Sperm and egg cells were collected in glass cups filled with protozoa-free seawater. After that, put it in the refrigerator with a temperature of less than 5°C. Fertilization is carried out by mixing a ratio of 1 mL of sperm cells with 4 mL of egg cells in an erlenmeyer glass containing 45 mL of protozoa-free seawater.

**Sample Testing.** The negative control was 900 µL seawater free from protozoa plus 100 µL zygotes. Stock solution (10,000 ppm) was prepared by weighing 50 mg of the ethanol extract of *Aristolochia sp.* and dissolved in ethanol until the volume reached 5 mL. Prepare a test sample with a concentration of 1000 ppm by pipetting 100 µL of the stock solution into an eppendorf tube, then adding 800 µL of protozoa-free seawater and 100 µL of liquid containing zygotes. A test sample with a concentration of 100 ppm was prepared by pipetting 10 µL of the stock solution into an eppendorf tube, then adding 890 µL of protozoa-free seawater and 100 µL of liquid containing zygotes. The concentration of the 10 ppm test sample was prepared by pipetting 1 µL of the stock solution into an Eppendorf tube, then adding 899 µL of seawater

free from protozoa and 100 µL of liquid containing zygotes. Replication was carried out 4 times for each test and control sample and stored between 15 to 20°C with occasional shaking.

**Observation of Cytotoxic Activity.** Cytotoxic activity of the ethanol extract of *Aristolochia foveolata* Merr. concentrations of 10, 100, and 1000 ppm were tested on fertilized *Tripneustes gratilla* eggs. The cytotoxic activity was observed at 1; 2; 4; 12 hours at room temperature between 20-25°C. The observation process is only 12 hours, because embryo division occurs every hour through the first 8-10 divisions, so observations of cell development can be carried out in a matter of hours<sup>(15)</sup>.

Observation of cytotoxic activity was carried out by looking at the condition of the cells using a microscope with a magnification of 100x. Observations were made by viewing and taking pictures using a digital microscope camera with Hayear<sup>®</sup> software. Observation of each sample with 3 different fields of view for each sample replication. Data from microscopic observations were calculated based on the condition of dividing, non-dividing, and damaged cells.

## RESULTS AND DISCUSSION

**Preparation for Fertilization.** Egg and sperm collection was carried out by induction of *Tripneustes gratilla* gonads with 10% KCl. K<sup>+</sup> ions in KCl molecules can depolarize the muscles on the outer surface of the ovaries, alveoli, and testes. Sperm or eggs are forcibly ejected through five gonopores on the aboral (opposite to the mouth) surface. Electrical shocks (6–10V) can also contract the gonads and force the gametes out<sup>(19,20)</sup>.

Mixing of egg and sperm cells is carried out immediately after the collection and identification of cells are carried out. The stages and data on the number of observation cells in the study are shown in Figure 1.

**Cytotoxic Test Results.** The stages of development of the *Tripneustes gratilla* cell embryo begin with the formation of the fertilization membrane, early division, morula, blastula, gastrula, prism, and pluteus larvae<sup>(21)</sup>. The condition of the cells at the time of fertilization and division can be seen in Figure 2.

Fertilization results between *Tripneustes gratilla* sperm and egg cells showed quite good results, with a low level of cell damage during incubation, even in group 100 ppm samples found no damage in the observation area. The average incidence of cell damage during observation in the negative control group 10 ppm, 100 ppm, and 1000 ppm, respectively, were 1.09%, 1.18%, 0%, and 1.10%. This condition

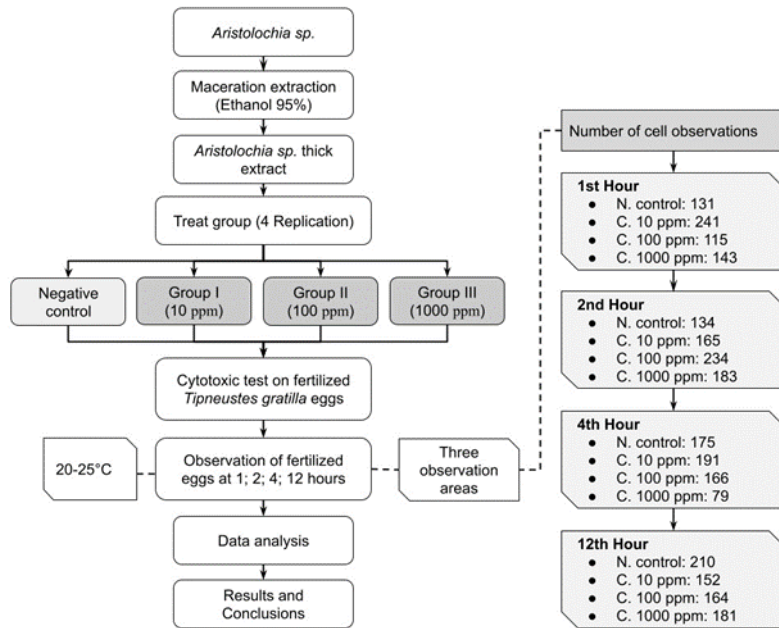


Figure 1. Stages and observation data on the number of cells in the study. C: concentration.

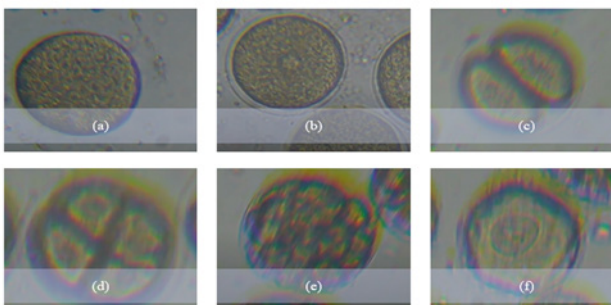


Figure 2. Early development of fertilized *Tripneustes gratilla* eggs; (a) ovum cells (b) formation of the fertilization membrane; (c) 2-cell embryo; (d) 4-cell embryo; (e) morula stage; (f) blastula stage.

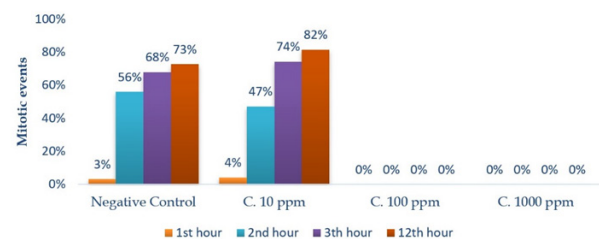


Figure 3. Mitotic events of fertilized *Tripneustes gratilla* eggs.

shows that the development process and condition of *Tripneustes gratilla* eggs in this study were going very well, with minimal cell damage that occurred.

The development of the fertilized egg cell division events can be seen from the pattern of division events in the negative control group. Cell division began to appear at the 1<sup>st</sup> hour, then increased drastically at the 2<sup>nd</sup> hour, then increased slowly at the 4<sup>th</sup> and 12<sup>th</sup> hours. The same pattern as the negative control was also shown in the 10 ppm concentration group. In contrast to the negative control and 10 ppm groups, the 100 ppm and 1000 ppm groups showed inhibition of embryonic cell division from the 1<sup>st</sup> to the 12<sup>th</sup> hour after fertilization. Data on the incidence of *Tripneustes gratilla* egg division based on incubation time is shown in Figure 3.

**Analysis of Research Results.** Statistical tests showed significant differences between the treatment groups at all observation times. Data on the inhibition of egg cell division in each treatment group can be seen in Table 1.

Based on statistical tests on *Tripneustes gratilla* egg division data 12 hours after fertilization, this study showed that the negative control and 10 ppm concentration groups were not significantly different, indicated by a P-value >0.05. Likewise, the 100 ppm and 1000 ppm concentration groups also showed conditions that were not significantly different and identical to the P value=1. Post Hoc Test statistical test data can be seen in Table 2.

The cytotoxicity test is considered a screening test used to evaluate the reaction of living cells to implants in cell culture tests, including cell viability and growth ability<sup>(22)</sup>. This study's results indicate a cytotoxicity ability in the ethanol extract of *Aristolochia foveolata* Merr. by inhibition of division of the fertilized *Tripneustes gratilla* egg. In addition, the appearance of egg cell morphology in all treatment groups was under normal conditions after incubation at 12 hours. Another finding in this study was the inhibitory concentration of akar kedayan ethanol extract on egg cell



**Table 1. Statistical test (ANOVA) of data between treatment groups at all observation times.**

Incubation Time (hours)	Mitotic Inhibition Group (Mean±SD)				p-value
	Negative Control	C. 10 ppm	C. 100 ppm	C. 1000 ppm	
1 <sup>st</sup>	98±3.58	96±3.83	100±0.00	100±0.00	0.006*
2 <sup>nd</sup>	38±19.07	51±10.11	100±0.00	100±0.00	0.000*
4 <sup>th</sup>	27±18.49	22±14.76	100±0.00	100±0.00	0.000*
12 <sup>th</sup>	25±11.77	19±8.72	100±0.00	100±0.00	0.000*

\*) P-value&lt;0.05

**Table 2. Statistical test (Tukey HSD) data on *Tripneustes gratilla* egg division 12th hour after fertilization.**

Groups	p-value	
Negative control	C. 10 ppm	0.201
	C. 100 ppm	0.000*
	C. 1000 ppm	0.000*
C. 10 ppm	Negative control	0.201
	C. 100 ppm	0.000*
	C. 1000 ppm	0.000*
C. 100 ppm	Negative control	0.000*
	C. 10 ppm	0.000*
	C. 1000 ppm	1.000
C. 1000 ppm	Negative control	0.000*
	C. 10 ppm	0.000*
	C. 100 ppm	1.000

\*) p-value&lt;0.05

division as an indicator of optimal cytotoxic activity at concentrations of 10-100 ppm.

The ability to inhibit cell division and development is closely related to the function of the cell regulatory system. The compounds in the plant family Aristolochiaceae that have an important role in cell regulation include aristolochic acid derivatives, aristolamide II, and kaempferol. Aristolochic acid F and Aristolochic acid G have cytotoxic activity on LLC-PK1 cells<sup>(23)</sup>. Aristolamide II inhibits superoxide anion generation and elastase release by human neutrophils in response to N-Formylmethionine-leucyl-phenyl alanine (fMLP)<sup>(24)</sup>. Kaempferol, with prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) inhibitory activity<sup>(25)</sup>.

The weakness of this study is the limited variation in the concentration of extract groups in the concentration range of 10-100 ppm. Inhibition data at different concentration levels can help calculate the extract's inhibitory concentration, including the IC<sub>50</sub> value. In addition, it is necessary to carry out further research on the ethanol extract of the stem of *Aristolochia foveolata* Merr. with the ability of cytotoxicity in human cells in vitro.

## CONCLUSION

The findings of this study indicate that the cytotoxic properties of the ethanol extract of akar kedayan can inhibit the growth of *Tripneustes gratilla* fertile eggs. The optimal dose range for cytotoxicity was between 10-100 ppm. For further research, we suggest isolat-

ing the pure compound *Aristolochia foveolata* Merr. having cytotoxic activity.

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