# Toxicity Test of Toman Fish (*Channa Micropeltes*) against Cultured Baby Hamster Kidney-21 Fibroblasts

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**Abstract**

Consuming Toman fish (Channa micropeltes) to accelerate wound healing has been a famous South Kalimantan's folklore especially among diabetics. Although recent study has shown the clinical benefit following administration of Toman fish extract in diabetic rats, its toxicity on animal cells is yet unknown. This study aimed to evaluate the *in vitro* toxicity of Toman fish extract against cultured Baby Hamster Kidney-21 (BHK-21) fibroblasts as a tissue culture model.

The fish meat was steamed at 70-80oC for 30 min and hydraulically pressed to produce its extract. Subsequently, the confluent culture of BHK-21 fibroblasts in Dulbecco's Modified Eagle's medium was treated with two-fold serial dilutions of Toman fish extract containing dimethyl sulfoxide (DMSO) ranging from 210.94 to 27000 µg/ml for 24 hours. The Eagle's medium, BHK-21 fibroblasts in Eagle's medium and DMSO solutions served as controls. Meanwhile, Methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay was used to determine the cell viability.

Our result showed that the half maximal inhibitory concentration ($IC^{50}$) value for Toman fish extract was at 4745.238 µg/ml. This finding found that the Toman fish extract was not toxic on cultured BHK-21 fibroblasts and may suggest safe usage as wound healing agent in diabetic animal models.

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## Introduction

Indonesia is the seventh country in the world with the highest diabetes mellitus prevalence with 7.6 million sufferers and it has been predicted that there will be a continuous increase at 6% annually.[1] In hindsight, it is well acknowledged that diabetes mellitus can cause several manifestations in the oral cavity including burning mouth, taste disturbance, xerostomia, dental caries, gingivitis, increased tendency to oral infections, and poor wound healing.[2] As diabetes is a multicausal disease characterized by increased blood glucose (hyperglycemia) and causes disruption of carbohydrate metabolism due to insufficiency in the secretion and work of insulin[3], the control of the disease has been most challenging to the healthcare professionals as well as the public in general. The people of South Kalimantan empirically believed that consumption of Haruan fish (*Channa striata*) could help accelerate the healing of diabetic wounds.[4] However, Haruan fish is difficult to cultivate compared to Toman fish (*Channa micropeltes*), which has more rapid growth than the former.[5]

Recent study showed that both Haruan fish and Toman fish from Channidae family have high albumin content (4.5% and 5.3%, respectively).[6] Albumin, as an antioxidant serves as a radical scavenger that can repair damage

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caused by excessive Reactive Oxygen Species (ROS).[7,8] Nutrients such as zinc, omega-3, omega-6 and albumin in these fish, particularly in Toman, are shown to be useful for the formation and growth of body tissues and were found to accelerate the tissue healing process.[4,6,9] More recent studies have also shown accelerated wound healing in Wistar rats following the administration of Toman fish extract at 16 ml/kg weight of the Wistar rat.[9,10]

Although the properties of the Toman fish extract have been investigated, the discovery of its potential in clinical benefit much is yet unknown about its safety and efficacy on living cells.[10] Thus, this *in vitro* study was designed to evaluate the toxicity of Toman fish extract on Baby Hamster Kidney-21 (BHK-21) fibroblasts, as a groundwork for tissue culture model, using Methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay.

## Materials and methods

### Ethical clearance and Research design

Ethics approval was obtained from the Ethics Committee of the University (No. 148/KEPKG-FKGULM/EC/I/2019) before the conduct of this study. This research was an original experimental study designed with a post-test only control-group design for toxicity test using the Methylthiazol-2-yl-2,5-diphenyl tetrazolium bromide (MTT) assay. The research population was the Baby Hamster Kidney-21 (BHK-21) fibroblasts which were cultured on Dulbecco's Modified Eagle's medium at the Veterinaria Farma Central Laboratory (PUSVETMA) Surabaya. The assay involved 10 experimental groups, each of which consisted of Toman fish (*Channa micropeltes*) extract in concentrations of 210.94 µg/ml, 421.88 µg/ml, 843.75 µg/ml, 1687.5 µg/ml, 3375 µg/ml, 6750 µg/ml, 13500 µg/ml, 27000 µg/ml and two controls, which are BHK-21 cells in Eagle's medium plus DMSO solutions and Dulbecco's Modified Eagle's medium, in quadruplicates for each treatment based on Federer formula.

### Toman fish extraction

Toman fish collected at the Martapura Market, South Kalimantan, Indonesia was cleaned and processed to obtain a total weight of 3 kg of fish meat using the methods by Nicodemus et al. (2014).[9] The raw material was steamed in a pan for 30 minutes at 70-80°C, then wrapped in a flannel cloth and put in a hydraulic press for pressing (Figure 1). The produced liquid was measured (7.5 ml), then transferred into a centrifuge tube (Pyrex) and centrifuged at 6000 rpm for 15 minutes (Centrifuge PLC Series, Taiwan). Subsequently, a total of the separated supernatant (700 ml) and sediment (50 ml) were collected. Finally, the collected supernatant (Toman fish extract) was stored in dark glass bottles and covered with a clean aluminum foil and stored in a refrigerator (15°C) for subsequent use.


**Figure 1.** Preparation of Toman fish extract, where the meat of Toman fish was boiled and pressed using a hydraulic press (a) and the centrifuged supernatant was kept in the dark glass bottles (b) for further use.

### Preparation of extract and BHK-21 fibroblasts for assay

Prior to the assay, the Toman fish extract was thawed to the room temperature followed by dissolving of the extract with dimethyl sulfoxide (DMSO) solution to produce two-fold serial dilution starting from concentrations of 27000 µg/ml, 13500 µg/ml, 6750 µg/ml, 3375 µg/ml, 1687.5 µg/ml, 843.75 µg/ml, 421.88 µg/ml and 210.94 µg/ml.[11]

The fibroblast cell-line used in this study was taken from Baby Hamster Kidney Strain 21 (BHK-21) cells. The BHK-21 cells were cultured in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum (FBS) in the roux bottles and incubated at 37°C for 48 hours with 5% $CO_2$ for cell proliferation. Once the cells were confluent, the Eagle's medium and FBS solutions were carefully removed from the roux bottles, and cells were rinsed three times with phosphate-buffered saline (PBS, pH 7.4) to remove the remaining serum. Then, 0.5 ml of 0.05% trypsin-versene was added to release cells from the bottle walls and separate the

bonds between cells so that they were not clustered. It was done by tapping the bottle until the roux bottle wall was clean. The cells were transferred to a 96-well microplate according to the number of samples and controls using a micropipette multichannel for subsequent assay.

**Toxicity test on Toman fish extract using MTT assay**

Each Toman fish extract concentration was inserted into a well containing BHK-21 fibroblast cell and incubated at 37°C in 5% $CO_2$ for 24 hours. Following this, the medium was carefully removed, and the cells were rinsed three times with PBS. Subsequently, a fresh supply of Dulbecco's Modified Eagle's medium supplemented with 10% FBS was added to each well. Then, 10 µl of the yellow 3-(4,5-dimethylthiazol-zyl)-2,5-diphenyltetrazolium bromide (MTT, 0.1 mg/ml) was added to each cell and the microtiter plate was incubated again at 37°C in 5% $CO_2$ for 4 hours. After incubation, the reaction between MTT and the cell was stopped using the DMSO stopper solution (100 µg/ml), and the plate was shaken for 5 minutes to ensure that the reaction stopped evenly so that it releases formazan. Then, the absorbance of the dissolved dark purple formazan was measured at an optical density (OD) of 620 nm wavelength using a microplate ELISA reader (Thermo Scientific, USA). The BHK-21 fibroblasts in DMSO and Dulbecco's Modified Eagle's medium served as control wells and assumed as 100% confluent, while Dulbecco's Modified Eagle's medium served as the negative control. The assay was performed in quadruplicates.

**Data analysis**

The percentage of cell viability was calculated using Freshney's (2000)[12] formula: [($OD_{620}$ of treated cells and reagent – $OD_{620}$ of reagent without cell) / ($OD_{620}$ control cells and reagent – $OD_{620}$ of reagent without cell)] × 100. After this, the calculation of the half maximal inhibitory concentration ($IC_{50}$) value i.e., 50% cell growth inhibition by the test compound using Probit analysis was done using SPSS 23.0 for Windows software. Results were expressed as mean ± standard deviation and the significance of the differences between groups was determined using One way-ANOVA with a value of $P< .05$ set at a significant level.

## Results

Results from the MTT assay showed there was a distinctive darker purple change at concentrations of 210.94 µg/ml, 421.88 µg/ml, 843.75 µg/ml, and 1687.5 µg/ml compared to other concentrations, indicating a higher percentage of living BHK-21 fibroblasts. Meanwhile, staining at concentrations of 3375 µg/ml, 6750 µg/ml, 13500 µg/ml, and 27000 µg/ml resulted in a slightly brighter purple color change, indicating the lesser percentage of viable cells. The result was supported by the calculation of the optical density formula, whereby the extract was not toxic if the percentage of living cells was more than 60%. Therefore, the result displayed in Table 1 indicated that Toman fish extract was not toxic to BHK-21 fibroblasts at concentrations of 210.94 µg/ml, 421.88 µg/ml, 843.75 µg/ml, 1687.5 µg/ml and 3375 µg/ml, but significantly toxic at 6750 µg/ml, 13500 µg/ml and 27000 µg/ml, respectively.

Finally, the $IC_{50}$ calculated resulted in a value of 4745.238 µg/ml with a lower bound of 2176.816 µg/ml and an upper bound of 16210.777 µg/ml. This suggested that Toman fish extract was not toxic to BHK-21 fibroblasts. The toxicity standard analyzed by the Probit test showed that the toxicity level of Toman fish extract was determined at a cell concentration of ≥ 60% (Figure 2; Table 2).

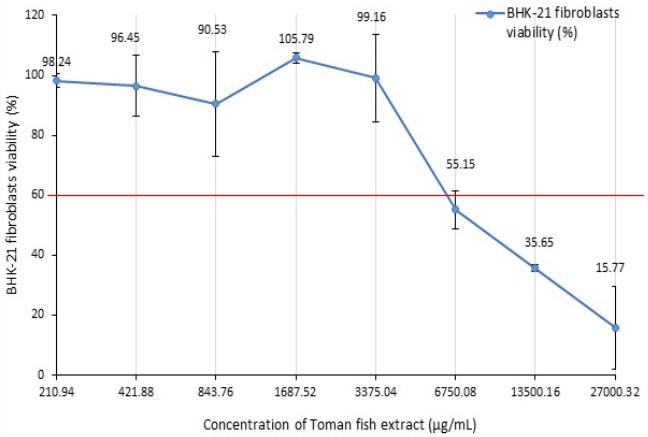


**Figure 2**. Percentage of BHK-21 fibroblasts viability after treatment with different concentration of Toman fish extract.

| Concentration of Toman fish extract (µg/mL) | Cell viability (mean % ± SD) | *P* value |
|---|---|---|
| Cell control | 100.00 | |
| 210.94 | 98.24 ± 2.19 | .772 |
| 421.88 | 96.45 ± 10.25 | .994 |
| 843.75 | 90.53 ± 17.42 | .942 |
| 1687.5 | 105.79 ± 1.87 | .056 |
| 3375 | 99.16 ± 14.52 | 1.00 |
| 6750 | 55.15 ± 6.46 | .006* |
| 13500 | 35.65 ± 1.17 | .000* |
| 27000 | 15.77 ± 13.97 | .009* |

**Table 1.** Percentage of cell viability calculated from optical density formula.
* statistically significant *P* < .05

| $IC_{50}$ | Category |
|---|---|
| 10 µg/mL ($10^6$ cell/mL < $IC_{50}$) | Very Toxic |
| 10 µg/mL < $IC_{50}$ < 100 µg/mL | Toxic |
| 100 µg/mL < $IC_{50}$ < 1000 µg/mL | Moderate |
| $IC_{50}$ > 1000 µg/mL | Not Toxic |

**Table 2.** Toxicity standard for Toman fish extract on BHK-21 fibroblasts based on Probit Test.

## Discussion

The use of natural extracts as alternative medicine is increasingly popular and has been shown to benefit society for many generations. Nonetheless, the scientific evidence of their properties is insufficient to provide merits to use them clinically. Proof of efficacy, safety, and quality standards must be put forward before a product can be recommended as an alternative drug. To measure the toxic properties of an extract against cells, a toxicity test must initially be carried out using experimental animal *in vivo* or using cell culture *in vitro.* The $IC_{50}$ value which refers to the concentration value that results in 50% of the cell growth inhibition of the population and shows the potential toxicity of a compound to the cell, must be determined.[13] The greater $IC_{50}$ value indicates that a material is increasingly non-toxic.[14]

The Toman fish has a cylindrical body shape, flathead, scaly exactly like a snakehead with light or white abdomen and a line slightly reddish black on the body. It has a protractile mouth shape with a snout rather pointed, and sharp fangs. Our results showed that Toman fish extract at higher concentrations did not have a toxic effect on BHK-21 fibroblasts and had an $IC_{50}$ value of 4745.238 µg/ml. Previous studies stated that the Toman fish extract contains non-toxic albumin to BHK-21 fibroblasts with the concentration of 210.94 µg/ml, 421.88 µg/ml, 843.75 µg/ml, 1687.5 µg/ml, 3375 µg/ml and 6750 µg/ml, respectively. It was also found that Toman fish extract at 16 ml/kg weight of the Wistar rat is effective for wound healing.[10] From these studies, it was suggested that the wound healing property may be due to the high albumin contents in the fish extract. Further investigation on the mechanism of action of the Toman fish extract on living cells is essential to support the scientific basis of the use of this material as an alternative medicine for wound healing, especially in diabetic patients.

## Conclusion

Our study found that the Toman fish extract (*Channa micropeltes*) was not toxic ($IC_{50}$ value 4745.238 µg/ml) on cultured BHK-21 fibroblasts, suggesting safe use for future *in vitro* and *in vivo* studies.

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## Declaration of Interest

The authors report no conflict of interest.

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