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Antitumor potential of ovarian and skin extract Singkarak Lake pufferfish (*Tetraodon leiurus*)

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Abstract
Pufferfish contain toxins known as tetrodotoxin (TTX) and saxitoxin (STX). The toxins are highest in the ovaries and skin. Singkarak Lake's Pufferfish (*Tetraodon leiurus*), with the local name Jabuih fish, is a poisonous fish consumed after the poison is discarded. The purpose of this study was to test the potential of antitumors using ovarian and skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake. In addition, the effect of ovarian and skin extracts was tested on the viability of MCF-7 cells by the MTT assay method. Toxicity test showed that ovarian extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake were potentially chemoprevention with an IC_{50} value of 613,164 µg/ml (moderate cytotoxic) while skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake with an IC_{50} value of 1086.97 µg/ml (not cytotoxic).

Keywords: cytotoxicity; jabuih fish, MCF-7 breast cancer cell line; toxin

Introduction
Pufferfish (Tetraodontidae) is a member of the order Tetraodontiformes distributed in tropical to arctic regions [1]. Pufferfish are known for their toxic content, tetrodotoxin (TTX) and saxitoxin (STX). TTX and STX are non-protein neurotoxins, where their antidotes have not been found [2, 3], produced from food eaten by Pufferfish both in the sea and in freshwater. TTX in Pufferfish made from foods containing bacteria such as *Vibrio alginolyticus, Shewanella algae, Shewanella putrefaciens,* and *Alteromonas tetraodonis* [4]. STX is produced from foods containing sea dinoflagellates (*Alexandrium* sp., *Pyrodinium bahamense, Gymnodinium catenatum*) and freshwater cyanobacteria (*Anabaena, Aphanizomenon, Cylindrospermopsis, Lyngbya,* and *Planktothrix*) [3].
TTX and STX bind specifically to proteins in Pufferfish, which is saxitoxin tetrodotoxin binding protein (PSTBP). TTX and STX are detected in the ovaries, skin, liver, and intestines [5]. TTX can inhibit nerve and muscle conduction, selectively block sodium channels resulting in respiratory paralysis and causing death. STX also has the same mechanism of action as TTX [6]. This toxin is 10.000 times more deadly than cyanide; the fatal level of this toxin is 1-2 mg for humans [7].
Sea Pufferfish contain TTX, and freshwater Pufferfish have STX, which are highest in the ovaries and skin [8]. One of the Pufferfish, *Tetraodon leiurus,* was found living in Singkarak Lake, West Sumatra, known locally as Jabuih fish. This fish can be consumed after the toxin is discarded. However, not many people know how to process this fish properly. Fishers assume Pufferfish as trash fish, and thorns from these fish can damage fishing nets so that they are thrown back into the lake. Pufferfish are regarded as non-economic fish due to the lack of knowledge about the biology of this fish and its benefits.
Several researchers have researched the use of Pufferfish toxins. For example, the study of an antitumor using skin extract (*Arothron diadematus*) of the Red Sea in the EAC (Ehrlich Ascites Carcinoma) tumor model in mice [9]. The changes in the electrophysiological properties of MDA-MB-231 breast cancer cells at different TTX concentrations [10]. The comparison of sensitivity STX and STX Neuro-2a to Neuro-2a cells [11]. The research and utilization of freshwater Pufferfish are scanty. Therefore it is necessary to study toxins from the ovarian and skin extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake as an antitumor.

Materials and Methods
Sample Collection
The number of samples of adult Pufferfish collected as many as 20 from Ombilin River (outlet) of Singkarak Lake, Tanah Datar Regency, West Sumatra using nets. Pufferfish are dissected, and the ovaries and skin of Pufferfish are collected, put in a film bottle, and the film bottle is inserted into a liquid nitrogen tube.

Extraction of Toxins in the Ovaries and Skin of Pufferfish
Sample extraction refers to the Fouda method [8]. The ovarian and skin of Pufferfish (*Tetraodon leiurus*) Singkarak Lake was mixed with acidified methanol, then heated for 10 minutes. After that, centrifuge at a speed of 1000 rpm for 15 minutes. Supernatant extract (aq) is evaporated so that a deep liquid sample is obtained.

MCF-7 Cell Culture
The MCF cells in this study were obtained from the Biomedical Laboratory, Faculty of Medicine, Andalas University. MCF-7 cells are grown in a medium DMEM which had added 10% FBS and 1% pen-strep. MCF-7 cells are cultured using a culture flask in a 5 % CO_2 incubator at 37 ºC.

Cytotoxicity Test against the MCF-7 Cells
Cytotoxicity test is performed by MTT method *(3-[4, 5-*

dimethylthiazol-2yl]-2, 5-diphenyl tetrazolium bromide). Ovarian and skin extract with six variations in the concentration of 25; 50; 100; 150; 200; 250 µg/ml as much as 100 µl in medium DMEM and two types of controls: cell control (100 µl), medium control (100 µl) with three replications in 96-well plate that have contained MCF-7 cells as many as 10^4 cells.

The 96-well plate is incubated at 37ºC in a 5% CO_2 incubator for 24 hours. After being set for 24 hours, the medium in the well is discarded. Then added 100 µl PBS and discarded. A total of 100 µl DMEM and 20 µl MTT are added to each well, then incubated for 4-6 hours in a 5% CO_2 incubator at 37ºC. The MTT reaction was stopped by adding DMSO 100 µl, and then the 96-well plate was re-incubated for 30 minutes in a dark room at room temperature. The absorbance of each well is measured with a spectrophotometer microplate reader at a wavelength of 570 nm.

Data Analysis

Data obtained from absorbance measurements using spectrophotometers is converted to a percentage of cell viability with the formula:

$$\% \text{ Viability cell} = \frac{\text{Absorbance samples} - \text{Absorbance medium control}}{\text{Absorbance control cell} - \text{Absorbance medium control}} \times 100\%$$

Variations in extract concentration and percentage of cell viability are displayed in figures, and the IC_{50} value is determined using linear regression analysis in Microsoft Excel [12].

Results and Discussion

Extraction of toxins in ovarian and skin Pufferfish (*Tetraodon leiurus*) Singkarak Lake

The results of research on ovarian and skin extract of Pufferfish (*T. leiurus*) Singkarak Lake are shown in figure 1.

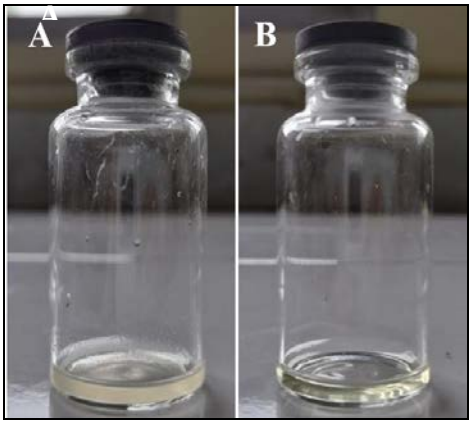

Fig 1: Pufferfish extract of Pufferfish (*T. leiurus*) Singkarak Lake. A. Ovarian extracts; B. Skin extract.

Figure 1 shows that the crude extract obtained in the form of a concentrated, odorless liquid does not form sediment, clear white in ovarian extract, and clear yellow in skin extract of Pufferfish (*T. leiurus*) Singkarak Lake. The toxin in Pufferfish is odorless, tasteless, and colorless [13]. Skin extract of Pufferfish (*T. leiurus*) Singkarak Lake is yellow due to the yellow pigment on the skin of Pufferfish dissolved in acidified methanol. In the dermis part of the skin pufferfish, a chromatophore of xanthophores type serves to give the yellow or brown color to the skin of Pufferfish [14]. Xanthophores can be extracted in polar solvents such as methanol [15], and chromatophores can dissolve in acidified methanol [16].

Morphology MCF-7 Cells

The result of the morphological of MCF-7 cells control and after being treated is shown in Figure 2.

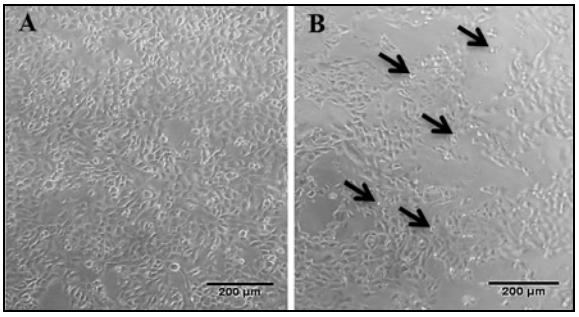

Fig 2: Microscopic MCF-7 cells. A. Control; B. Treatment ovarian extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake concentration 250 µg/ml. Arrow (➔) indicates the cell that experienced death.

Figure 2 A shows the morphology of control MCF-7 cells, which living cells are characterized by cells with epithelial-shaped flattened and visible boundaries between membranes and mediums. Figure 2 B shows that some MCF-7 cells death after being treated with extracts. Cell death is characterized by a round cell with a broken or somewhat faint cell membrane, and some cells have detached from the base of the plate. The morphology of living cells resembles the shape of epithelial cells, visible the boundary between the membrane and the growth medium [17]. In comparison, the dead cell is a round cell with a broken cell membrane [18]. Toxins in Pufferfish have a mechanism to blocking Na_V channels in nerve cells [19]. Na_V is signal conduction in nerve cells and is also found in cancer cells [20]. TTX and STX bind to P-glycoproteins expressed by the MDR1 gene from the Na_V channel, thus preventing cancer cells from obtaining enough Na^+ ions for intracellular function and cell integrity [21]. The same mechanism also occurs in breast cancer cells [10] and EAC tumors [9].

Cytotoxicity of MCF-7 Cells

The cytotoxic test aims to determine the IC_{50} value from the linear regression equation. The percentage of MCF-7 cell viability is obtained through the MCF-7 cell absorbance data of the MTT test results. The absorbance data were converted to determine the percentage of MCF-7 cell viability against variations in the concentration ovary and skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake (Table 1).

Table 1: Variations in the concentration ovary and skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake and the percentage of viability of MCF-7 cells

Concentration (µg/ml)	% Viability of MCF-7 cells ± SD	
	Ovarian extract	Skin extract
25	100^a ± 0,026	$98,171^a$ ± 0,016
50	$99,361^a$ ± 0,027	$97,206^a$ ± 0,022
100	$89,196^b$ ± 0,067	$97,46^a$ ± 0,023
150	$85,311^b$ ± 0,01	$95,886^b$ ± 0,036
200	$84,832^b$ ± 0,014	$89,842^b$ ± 0,03
250	$82,118^b$ ± 0,015	$88,979^b$ ± 0,017

Notation: Different superscripts show significant differences ($p < 0.05$).

Table 1 shows the effect of variation of extract concentration on the percentage of viability of MCF-7 cells. The lowest percentage of MCF-7 cell viability was obtained at a concentration of 250 µg/ml is 82.161 % for ovarian extract and 88.979 % for skin extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake. The viability of cells is affected by the extract's concentration; the greater concentration, the higher number of cell deaths, and the lower the cell viability [22].

The percentage viability MCF-7 cells (Table 1) treatment of ovarian extract concentrations 25 and 50 µg/ml (notation a) has a significant difference with concentrations of 100; 150; 200 and 250 µg/ml (notation b) and skin extract treatment concentration 25; 50 and 100 µg/ml (notation a) have significant differences with a concentration of 100; 150; 200 and 250 µg/ml (notation b). The ovarian extract has a higher ability than skin extracts in inhibiting the growth of MCF-7 cells. The same results were reported that the ability of ovarian extract is higher than skin extract of *Takifugu oblongus* in causing cell degeneration in the test mice; the high value of ovarian extract is thought to have more varied proteins than proteins in the skin [23]. The specific protein in Pufferfish, PSTBP, is involved in the difference in toxification in Pufferfish [24]. PSTBP is detected in the ovaries, skin, liver, and intestines. Ovaries containing vitellogenic lyocytes, ovarian walls, and vitelline envelopes contain PSTBP, while on the skin, only the dermis contain this protein [5]. PSTBP and analogs are responsible for the accumulation of toxins in Pufferfish [25]. The result of cell viability percentage shown in Figure 3 and Figure 4).

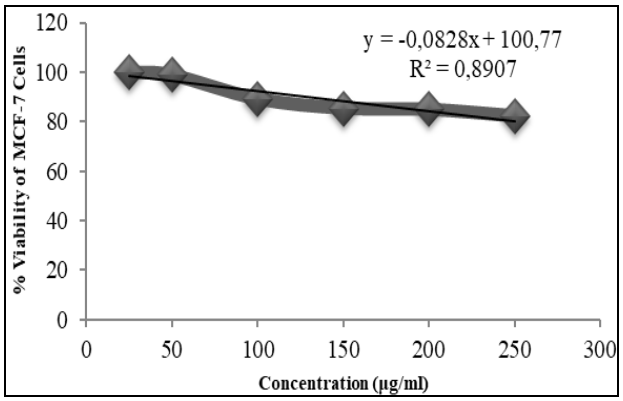

Fig 3: Effect of variations in the concentration ovary extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake on the viability of MCF-7 cells

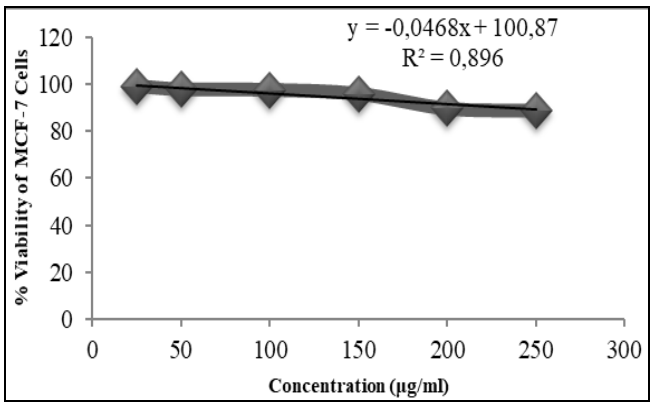

Fig 4: Effect of variations in the concentration skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake on the viability of MCF-7 cells

Figure 3 shows the effect of variations in the concentration ovary and skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake on the viability of MCF-7 cells with linear equations y = -0,0828x + 100,77. Based on the linear equation, the IC_{50} value ovary extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake is 613,164 µg/ml (moderate cytotoxic). Figure 4 shows the effect of variations in the concentration of skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake on the viability of MCF-7 cells with linear equations y = -0,0468x + 100,87. The IC_{50} value skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake is 1086, 97 µg/ml (not cytotoxic). The value of cytotoxicity categorized into three groups, the first group of potential cytotoxic if IC50 < 100 µg/ml, the second group of cytotoxic moderate if 100 µg/ml < IC50 < 1000 µg/ml, and the third is not cytotoxic if IC50 > 1000 µg/ml [26, 27]. The concentration of extract with potential toxicity can be used as anticancer agents, while the moderate cytotoxicity for chemoprevention [28]. Based on several aspects that have been tested prove that ovary extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake has the potential for chemoprevention that can prevent and inhibit the growth of MCF-7 cells.

The ovarian and skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake tested in this study are crude extracts that are still not pure and contain other compounds such as dissolved proteins. Low cytotoxic activity due to the addition of artifact compounds in the extract [29]. These artifact compounds are dissolved proteins and organic acids that can interfere with identifying and quantifying anti-proliferation components with cytotoxic potential [30].

Increased expression of Na_V channels in cancer cells causes changes in cell membrane architecture, so that cell membranes become abnormal [31]. Abnormal cell membranes result in reduced anticancer binding on the surface of cells [32]. Based on the characteristics of these cancer cells, presumed toxins from the ovary and skin extract of Pufferfish (*Tetraodon leiurus*) Singkarak Lake cannot bind to the Na_V channel of MCF-7 cells.

Conclusion

Toxicity tests of MCF-7 cells showed that ovarian extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake (IC_{50} value of 613,164 µg/ml) were potentially chemoprevention with moderate cytotoxic. While skin extracts of Pufferfish (*Tetraodon leiurus*) Singkarak Lake with an IC_{50} value of 1086.97 µg/ml have not cytotoxic quality.

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