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EXPRESSION OF *BCL-2* AND *BAX* GENES INDUCED BY AN OVARIAN EXTRACT OF SINGKARAK LAKE PUFFERFISH (*Tetraodon leiurus*) IN BREAST CANCER CELL

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ABSTRACT

It is known that the ovarian extract of Singkarak Lake Pufferfish *(Tetraodon leiurus)* has the potential for chemoprevention in MCF-7 cells. However, the expression of *Bcl-2* and *Bax* genes was associated with cancer. Therefore, this study aimed to analyze the *Bcl-2* and *Bax* gene expression induced by the ovarian extract of Singkarak Lake Pufferfish *(Tetraodon leiurus)* in MCF-7. The method in this study was an in vitro experiment using MCF-7 control cells and MCF-7 cells induced by the ovarian extract of Singkarak Lake Pufferfish *(Tetraodon leiurus)*, and Real-time PCR was used to examine the expression of *Bcl-2* and *Bax* genes. The result showed that the expression of the *Bcl-2* gene had an insignificant decrease (p>0.05) by 15%, and the expression of the *Bax* gene had a significant increase (p<0.05) by 489% compared to the control. These results support that ovarian extract Singkarak Lake Pufferfish *(Tetraodon leiurus)* can be alternative cancer chemoprevention.

Key words: Cancer, chemoprevention, Jabuih fish, Lake Singkarak, MCF-7 cell line

INTRODUCTION

Cancer cells are resistant to apoptosis by various mechanisms, including overexpression of antiapoptotic genes, down-regulation or mutation of proapoptotic genes, and alteration of p53 or the PI3K/AKT pathway (Mansoori *et al*., 2017). Apoptotic resistance is caused by cancer cells overexpressing Bcl-2 (B-cell lymphoma 2) (Li *et al*., 2017) as antiapoptotic, as well as expressing Bax (Bcl-2 associated X-protein) as a proapoptotic in small amounts (Wang *et al*., 2019). Bcl-2 overexpression occurs in various types of cancer cells, including breast cancer (Lindeman & Visvader 2013), in addition, low expression of Bax was also found in breast cancer (Kholoussi *et al*., 2014). Bax dysregulation can cause apoptotic dysfunction that increases cancer incidence (Liu *et al*., 2015).

Apoptosis is a process of programmed cell death (Brown *et al*., 2014). The manipulation of apoptosis by decreasing antiapoptotic or increasing proapoptosis activity (Pistritto *et al*., 2016) is a target in the development of cancer chemoprevention (Jagadeeshan *et al*., 2018). Cancer chemoprevention uses natural compounds, synthetic drugs, or a combination of both to stop and prevent cancer (Grigolato *et al*., 2020). Natural compounds derived from animals, plants, and microorganisms (Mushtaq *et al*., 2018) have the potential for cancer chemoprevention because they show minimal side effects and toxicity *in vitro* and *in vivo* experiments compared to synthetic drugs (Ko & Moon, 2015).

One of the natural compounds that can be used for cancer chemoprevention is a toxin in pufferfish (Tetrodotoxin (TTX)/Saxitoxin (STX)). TTX and STX are the names of toxins in pufferfish, which are neurotoxins (Campbell & Haughey, 2014). TTX and STX have the same mechanism of action, can inhibit nerve and muscle conduction, and selectively block sodium channels, resulting in respiratory paralysis and death (Walker *et al*., 2012).

In Lake Singkarak, West Sumatra, pufferfish (*T. leiurus*) was found and known as Jabuih fish. Zhu *et al*. (2020), reported that the ovarian of *T. leiurus* in the Mekong River, Thailand, contains STX as the main toxin component. Walker *et al*. (2012), stated that STX has a mechanism of action by blocking and inhibiting Na_V (Voltage-gated sodium) channels in nerve cells and cancer cells. Akbora *et al*. (2019), explained that pufferfish toxin content could differ within and between species. These differences are influenced by seasonal changes, ecological food chains, geographic habitats, and bacterial accumulation.

In several studies, pufferfish toxin induces cancer cell death; this is supported by the research of Veeruraj *et al*. (2016), who reported that the

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ovarian extract of sea pufferfish (*Arothron stellatus)* is cytotoxic and antiproliferative in cervical cancer cells (HeLa). Untario *et al*. (2017), reported that TTX from the liver extract pufferfish (*T. fluviatilis*) caused apoptosis in HeLa cells. Hanif *et al*. (2021), wrote that the ovarian extract Singkarak Lake pufferfish (*T. leiurus*) is cytotoxic to breast cancer cells (MCF-7) and has the potential for cancer chemoprevention. Chen *et al*. (2020), provided experimental support for the apoptosis-enhancing effect of STX in zebrafish embryos by decreasing the expression of the *Bcl-2* gene and increasing the expression of the *Bax* gene.

Research on the expression of *Bcl-2* and *Bax* genes induced by the ovarian extract Singkarak Lake pufferfish (*T. leiurus*) in MCF-7 has not been carried out. Therefore, this study was carried out to analyze the effect of the extract on the apoptosis of MCF-7 cells to utilize toxins from the ovarian extract Singkarak Lake pufferfish (*T. leiurus*) as cancer chemoprevention. Therefore, the expression of *Bcl-2* and *Bax* genes was the target of the observation.

MATERIALS AND METHODS

Sample collection

The pufferfish samples were taken from the Singkarak Lake outlet, namely Ombilin River, Tanah Datar Regency, West Sumatra, Indonesia. Samples were collected using the active method using a net of a minimum of twenty individuals. First, the sample was dissected, and the ovaries were taken, then the ovaries were put into a film bottle and stored in a liquid nitrogen tube.

Extraction of toxins in the ovarian of Singkarak Lake pufferfish (*T. leiurus*)

The method used in the extraction of the toxin on the ovarian of the pufferfish based on Fouda (2005), the ovarian sample was mixed with methanol and 1% acetic acid, then crushed to obtain a liquid sample, then heated for 10 min, centrifuged at 1000 rpm for 15 min, the supernatant extract (aq) were obtained and stored at -4 °C.

The MCF-7 cells induced extract

The MCF-7 cells grown in complete medium (DMEM added with 10% FBS and 1% penicillin-streptomycin) at a density of 5×10^5 cells/mL. Ovarian extract of the pufferfish was homogenized with the complete medium in a ratio of 1:1. The extract concentration was made at 250 µg/mL according to Hanif *et al*. (2021) and induced in MCF-7 cells and then was incubated for 24 h at 37 °C in a 5% CO_2 incubator.

RNA isolation of the MCF-7 cells and measurement of RNA concentration

According to the protocol, RNA isolation of the MCF-7 control cells and MCF-7 cells induced extract using TRIzol® reagent (Thermo Fisher Scientific, CA, USA). The MCF-7 cells were centrifuged at 5000 × *g* for 10 min, the supernatant was discarded, the pellet obtained was added 200 µL of TRIzol reagent, then 50 µL of chloroform was added, incubated for 3 min, and centrifuged at 12000 × *g* for 15 min. The results of centrifugation obtained aqueous phase and organic phase, transferred the aqueous phase to a microtube, added 150 µL of isopropanol, incubated for 10 min, and centrifuged 12000 *g* for 20 min. The supernatant obtained was discarded, added 150 µL of 70% ethanol, and centrifuged at 12000 *g* for 10 min. The supernatant obtained was discarded and the RNA pellet was vacuumed for 10 min, then 30 µL of RNAse free water was added and the RNA was stored in a -80 °C freezer. Total RNA isolated as much as 1 µL was quantified and assessed for purity with the average value between 1.8 and 2.0 at wavelengths of 260 nm and 280 nm using NanoDrop 2000 spectrophotometry. The blank used was RNAse-free water.

Synthesis of cDNA

The synthesis of cDNA using the Bioline SensiFAST cDNA Synthesis Kit (Cat. no. BIO-65053) was used according to protocol. Reactions were performed in a final volume of 20 µL, using n µL RNA total, 4 µL of 5× transAmp buffer, and 1 µL of reverse transcriptase. We were setting temperature and time on a thermal cycler with a program of 25ºC for 10 min (primary annealing), 42 °C for 15 min (reverse transcription), and 85ºC for 5 min (inactivation).

Real-time PCR

The expression of genes was detected using cDNA as the template by Real-time PCR assay using Bioline SensiFAST SYBR No-ROX Kit (Cat. no. BIO-98002). In this study, the primer sequences for the *GAPDH* gene were F:5'-TCGCCAGCCGAGCCACATC-3' and R:5'-CGTTCTCAGCCTTGACGGTGC-3' (Wang *et al*., 2020), for the *Bcl-2* gene were F:5'-GTGGATGACTGAGTACCTGAAC-3' and R:5'-GAGACAGCCAGGAGAAATCAA-3' (Godfrey *et al*., 2017), and the *Bax* gene were F:5'-CCCGAGAGGTCTTTTTCCGAG-3' and R:5'-CCAGCCCATGATGGTTCTGAT-3' (Borhani *et al*., 2014). Real-time PCR mix formulation with a final volume of 10 µL consisting of 5 µL 2× SensiFAST SYBR® No-ROX Mix, 0.5 µL forward primer (10 µM), 0.5 µL reverse primer (10 µM), 2 µL template, 2 µL H_2O. Temperature and time settings on the thermal cycler with a 2-step cycling program are 95 °C for 2 min (polymerase activation), 95 °C for 5 s (denaturation), and the annealing/extension temperature for the *GAPDH* gene primer was 57.1 °C for 30 s, for the *Bcl-2* gene primer was 53 °C for 20 s and the *Bax* gene primer was 60.4 °C for 30 s.

Data analysis

Data obtained from Real-time PCR in the form of C_T (cycle threshold) values, determined by the ΔC_T value, through the equation:

ΔC_T experimental = C_T experimental target gene - C_T experimental reference gene

ΔC_T Control = C_T target gene in control - C_T reference gene in control

$\Delta\Delta C_T = \Delta C_T$ experimental - ΔC_T Control

Gene relative expression (R (Relative Expression)) is obtained by using the equation:

$R = 2^{-\Delta\Delta C_T}$

Analysis of the R-value was carried out using the Paired Sample T-Test to determine the significance value (Li *et al*., 2017).

RESULTS AND DISCUSSION

Extraction of toxins in the ovarian of Singkarak Lake pufferfish (*T. leiurus*)

The ovarian extract of Singkarak Lake pufferfish *(T. leiurus)* in Figure 1 is a crude extract, extracted using methanol mixed with 1% acetic acid. The characteristics of the ovarian extract are clear white color, no precipitate formed, odorless and tasteless. According to Kosker *et al*. (2016), the toxin in pufferfish is very polar and dissolves in methanol and acidic environments, and is tasteless, odorless, and colorless. Chen *et al*. (2012), also explain that crude extract can be used for cancer chemoprevention. It states that natural compounds regardless of crude extracts or isolated active compounds had drawn growing attention as an agent in cancer therapy and chemoprevention due to their ability to modulate apoptosis.

Fig. 1. The ovarian extract of Singkarak Lake pufferfish *(T. leiurus)*.

The MCF-7 cells morphology induced by ovarian extract of Singkarak Lake pufferfish (*T. leiurus*)

Morphological changes in MCF-7 cells induced by ovarian extract of Singkarak Lake pufferfish *(T. leiurus)* were observed under a microscope. The cells exhibited a prominent effect after extract was induced, shown in Figure 2. The microscopic MCF-7 control cells in Figure 2a are characterized by flattened epithelial shape, and the boundary between the membrane and the growth medium is visible. The microscopic results are those described by Lam *et al*. (2020), which state that the morphology of living cells resembles the shape of epithelial cells, seen as the boundary between the membrane and the growth medium. Figure 2b shows the MCF-7 cells induced by an ovarian extract pufferfish *(T. leiurus)*; there are cells that experience death characterized by cell membranes that look cracked, round cells, and there are also cells detached from the bottom of the plate. Istifli *et al*. (2019), described the morphology of these dead cells, where the cells that experience death have a round cell morphology with damaged cell membranes. Vijayarathna and Sasidharan (2012), also described the morphology of cell death; the cells exhibiting a mechanism of cell death have the characteristics of becoming more rounded, smaller, and showing signs of detachment from the well surface.

These results showed the mortality of MCF-7 cells induced by the ovarian extract pufferfish (*T. leiurus*) was caused by the extract was thought to contain STX causing the cells to death, and STX can cause oxidative stress in the MCF-7 cells. The same result was reported by Melegari *et al*. (2012), where the neurotoxin STX showed the mechanism of oxidative stress triggered by its toxic effect, causing cell death in Neuro 2A cells. Cao *et al*. (2018), also explained that STX exposure induces oxidative stress, cell damage, and immunotoxicity in oysters and scallops.

Several studies on different cancer cells have shown that many anticancer agents trigger apoptosis by introducing oxidative stress, thereby reducing the viability of cancer cells (Sezer *et al*., 2019). Furthermore, according to Hata *et al*. (2015), the use of anticancer agents directly induces apoptosis by targeting the members Bcl-2 protein family as apoptotic mediators, such as antiapoptotic and proapoptotic. To investigate the effect of the ovarian extract of Singkarak Lake pufferfish *(T. leiurus)* on the MCF-7 cell death, an analysis of *Bcl-2* and *Bax* gene expression was performed to determine the effect of the extract on the MCF-7 cell apoptosis.

Relative expression of *Bcl-2* and *Bax* genes induced by ovarian extract Singkarak Lake pufferfish (*T. leiurus*) in the MCF-7 cells.

The relative expression of *Bcl-2* and *Bax* genes induced by ovarian extract Singkarak Lake pufferfish *(T. leiurus)* using Real-time PCR. The test results showed significant and insignificant differences between control and extract induction (Table 1 & Figure 3). The expression of the *Bcl-2* gene was decreased ($p>0.05$) by 15%, although not statistically significant, while the expression of the *Bax* gene significantly increased ($p<0.05$) by 489% compared to the control (Table 1 & Figure 3). The insignificant decrease in *Bcl-2* gene expression in the MCF-7

cells was due to other antiapoptotic genes more involved in the mechanism of apoptosis induced by the ovarian extract Singkarak Lake pufferfish (*T. leiurus*). The same result was also reported by Cos *et al*. (2002), where the MCF-7 cells treated with 100 nM 1,25-dihydroxyvitamin D3 experienced an insignificant decrease in Bcl-2 expression and a significant decline in Bcl-X_L expression. Adefolaju *et al*. (2015), reported that 0.5 % DMSO treatment on the MCF-7 cells experienced an insignificant decrease in Bcl-2 expression. Fiebig *et al*. (2009), stated that in the MCF-7 cells, Bcl-X_L expression was ten times more active in suppressing apoptosis than Bcl-2. Bah *et al*. (2014), reported that decreased Bcl-X_L expression and not Bcl-2 expression caused breast cancer cells to be sensitive to apoptosis.

In the MCF-7 cells, *Bax* gene expression was small, so the MCF-7 cells induced by the ovarian extract Singkarak Lake pufferfish *(T. leiurus)* significantly increased *Bax* gene expression (Table 1 and Figure 3). According to Fani *et al*. (2016), Bax expression in the MCF-7 cells was low and increased significantly after treatment with anticancer.

The ovarian extract Singkarak Lake pufferfish (*T. leiurus*) is thought to cause P53 to bind to the promoter region of the *Bcl-2* and *Bax* gene, which causes an apoptotic mechanism. According to Chen *et al*. (2007), P53 as a transcription factor inhibits the expression of Bcl-2 and induces the expression of Bax by binding to the promoter of the *Bcl-2* and *Bax* gene. Ku *et al*. (2011), explained that the Bax peptide is responsible for binding to the BH3-binding groove of Bcl-2 by forming an amphipathic α -helix. The interaction between the Bax peptide and Bcl-2 involves five intermolecular salt bridges (Glu61, Arg64, Asp68, Glu69, & Arg78). Asp68 and Glu69 are conserved in the BH3 domains, and Glu61, Arg64, and Arg78 are paired with Arg139, Asp140, and Glu200 of Bcl-2.

The effect of ovarian extract Singkarak Lake pufferfish (*T. leiurus*), which is thought to contain STX causes an increase in ROS (Reactive Oxygen

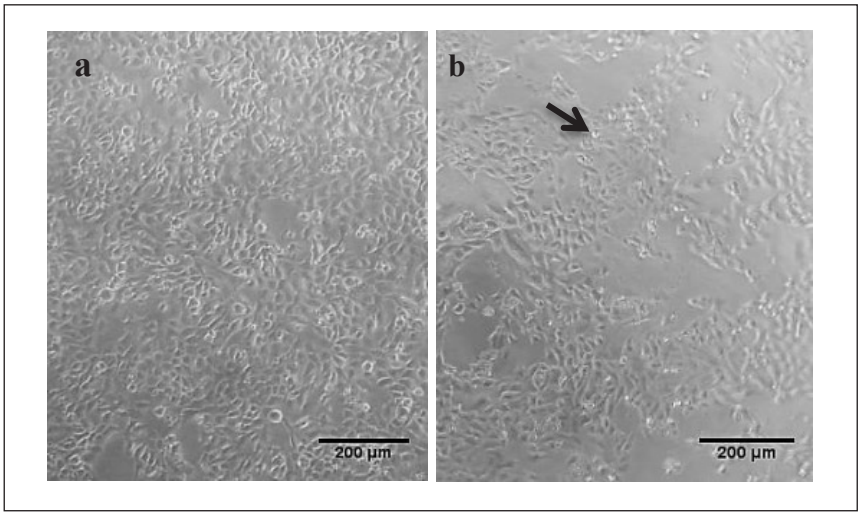

Fig. 2. Microscopic cells. (a), MCF-7 control cell; (b) MCF-7 cell induced extract. Arrow (➔) indicates the cell that experienced death.

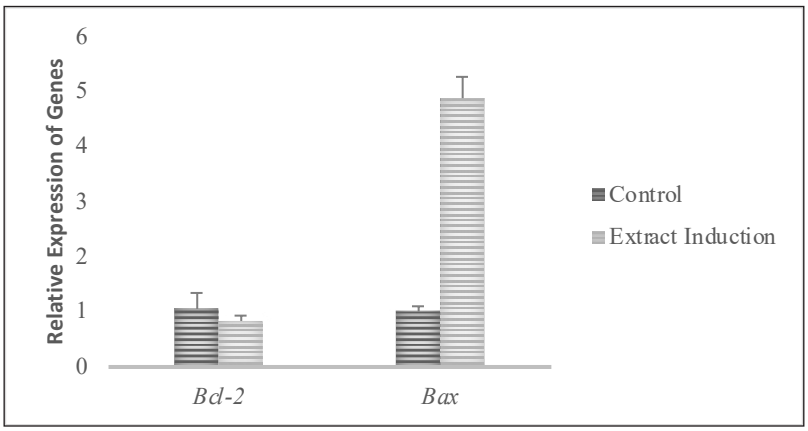

Fig. 3. Relative expression of *Bcl-2* and *Bax* genes induced by ovarian extract Singkarak Lake pufferfish (*T.leiurus*) in MCF-7 cells. Data shows mean ± SD of 3 replicates (n=3). The Paired Sample T-Test (*p<0.05).

Table 1. Relative expression of *Bcl-2* and *Bax* genes induced by ovarian extract Singkarak Lake pufferfish (*Tetraodon leiurus*) in MCF-7 cells

Samples	Relative Expression of Genes ± SD	
	Bcl-2	*Bax*
Control	$1.06^{a} \pm 0.49$	$1.01^{a} \pm 0.185$
Extract Induction	$0.85^{a} \pm 0.087$	$4.89^{b} \pm 0.38$

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

Species) which causes a decrease in the *Bcl-2* gene expression and an increase in *Bax* gene expression, causing apoptosis. Following Chen *et al*. (2020), STX accumulates upregulation of ROS, thereby inducing decreased expression of Bcl-2 and increased expression of Bax. Dutordoir and Bates (2016), reported that ROS could activate JNK (c-Jun N terminal kinase) and P53, and activate Bax, Bak, Puma, Noxa, and Bad as proapoptotic Bcl-2 proteins which can inhibit the function of Bcl-2, Bcl-$X_{L,}$ and the antiapoptotic Bcl-2 proteins other.

The ovarian extract Singkarak Lake pufferfish (*T. leiurus*), which is thought to contain STX, has a mechanism by inhibiting Na_V channels in cancer cells, causing a decrease in Na^+ concentration and causing an increase in Ca^{2+} concentration, which induces apoptosis. Boitano *et al*. (1997), reported a decline in the concentration of Na^+, causing an increase in the concentration of Ca^{2+} from intracellular storage. Guo *et al*. (2009), wrote that the release of Ca^{2+} from the endoplasmic reticulum is the main mechanism that regulates the intrinsic pathway of apoptosis, increased cytosolic Ca^{2+} from the endoplasmic reticulum can bind to the enzymes calpain and calcineurin, which can activate the proapoptotic protein group such as Bax which causes the release cytochrome c and triggers the apoptotic response.

The present study findings suggest that the ovarian extract Singkarak Lake pufferfish *(T. leiurus)* can decrease the expression of the *Bcl-2* gene and increase the expression of the *Bax* gene at the mRNA level by influencing the binding to the promoter region of the gene.

CONCLUSION

The ovarian extract Singkarak Lake pufferfish (*T. leiurus*) increased the expression of the *Bax* gene. These results support that ovarian extract Singkarak Lake pufferfish (*T. leiurus*) may be alternative cancer chemoprevention.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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