Shiga-Like Toxin Produced by Local Isolates of *Escherichia coli* O157:H7 Induces Apoptosis of the T47 Breast Cancer Cell Line

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ABSTRACT

PURPOSE: It has been suggested that Shiga-like toxins produced by *Escherichia coli* O157:H7 could be used as novel therapeutic agents against malignant tumors. In addition, the antitumor potency of local isolates from Indonesia, which are known to be less toxic than the control isolate ATCC 43894, has not yet been tested. The study aimed to analyze local strains of *E. coli* O157:H7 as a proapoptosis agent on the T47 breast cancer cell line.

METHODS: As many as 30 culture cells of T47D breast cancer cell line were subjected to purified extracts of Shiga-like toxin originating from 5 local isolates of *E. coli* O157:H7: KL-48(2), SM-25(1), SM-7(1), DS-21(4), and 1 isolate ATCC 43894 which was used as a control. Toxin production of each isolate was detected using a sandwich enzyme-linked immunosorbent assay, and the treatment of cell lines was observed for 24 hours, with 2 replications; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide tests and acridine orange/ethidium bromide double staining assays were used for detection and analyses of apoptosis.

RESULTS: The study showed 2 local strains of *E. coli* O157:H7 (codes KL-48(2) and SM-25(1)) had toxins positive at titer 5 and 10 µg/100 µL. These titers were lower than the control isolate ATCC 43894, but they had a necrosis effect higher ($P < .05$), ie, 80.3%, than control isolate, ie, 63.3%. Other local strain SM-25(1) also had a good necrosis effect. It has a nondifferent necrosis effect ($P > .05$) with the control isolate ATCC 43894, ie, 13.0% from 13.3%.

CONCLUSION: This study concludes that the Shiga toxin produced by *E. coli* O157:H7 local isolate (Indonesia) has potential as a proapoptotic and/or necrotic agent for treating T47 breast cancer cell lines, as effectively as ATCC 43894 control isolates.

KEYWORDS: Breast cancer, T47 cell line, *E. coli* O157:H7, proapoptosis, Shiga-like toxin

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Introduction

Breast cancer is the most common malignancy affecting women in the Western world. Several single-agent and combination chemotherapeutic treatments have been shown to be effective in the management of the metastatic disease.[1] However, these often have substantial side effects. Besides surgery, endocrine treatment, radiation, and chemotherapy treatment, immunotherapy has become an established part of systemic therapy.[2,3] Researchers always search for new effective agents with as small side effects as possible to treat cancer.

Furthermore, Shiga or Shiga-like toxins as immunotherapeutic agents may be used against tumor malignancies, either independently or in combination with chemotherapy or radiotherapy.[4,5] The high specificity and apoptosis-inducing properties of Shiga or Shiga-like toxins suggest these may potentially be used for the treatment of Gb3-expressing cancer.[5,6] Apoptosis induced by Shiga-like toxins can be associated with enhanced expression of pro-apoptotic protein and inhibits expression of antiapoptosis.[7] Their capacity to target specific signaling pathways might reduce side effects to normal tissue and be an approach to generate specific antitumor agents.[8] Alternatively, Gb3 as a receptor of Shiga-like toxin has been reported to increase on the face of several tumor cells such as ovarian cancer, hematological malignancies, and breast cancer.[5,8] Furthermore, a previous study by Suardana et al[9] found the toxicity of 2 local strains of Shiga-like toxin produced by *Escherichia coli* O157:H7 (KL-48(2) and SM-7(1)) was less than ATCC 43894. These local strains produced cytopathic effect (CPE) on Vero cell lines 18.64% and 23.41%, respectively, but ATCC 43894 was 62.91%. However, both local strains had similar genetics, such as toxin sequences, regulatory elements of the toxin, and also 16S rRNA gene sequences, with

ATCC 43894.[10] This specific effect among isolates of the Vero cell line prompts the need for further research to assess the effectiveness of Shiga-like toxin produced by *E coli* O157:H7 local isolates as novel agents for enhancing apoptosis in breast cancer cells.

Material and Methods

Cultivation of E. coli O157:H7 isolates

Five isolates of *E. coli* O157:H7: KL-48(2), SM-25(1), SM-7(1), and DS-21(4) were cultivated according to previous methods,[11,12] while the control isolate (ATCC 43894) was obtained from stock isolates (stored in 30% glycerol with a storage temperature of –20°C in the laboratory of Veterinary Public Health, Faculty of Veterinary Medicine, Udayana University). Isolates were cultured on lactose broth medium (LB) at 37°C, followed by incubation aerobically overnight, before which they were reconfirmed using *E. coli* O157 latex agglutination test (Oxoid, DR120M).[13-15]

Isolation and purification of toxin

The bacterial toxin was isolated by culturing isolates on *Luria Bertani*/LB broth (Sigma, L3022) and incubating at 37°C for 24 hours, followed by centrifugation at 2000 r/min, for 40 minutes at 4°C. Ammonium sulfate (5.97 g) was gradually added to 15 mL of the supernatant (Sigma, A4418) to obtain 65% saturation. The solution was recentrifuged at 2000 r/min for 40 minutes, the supernatant removed, the precipitate diluted with 3 mL of sterile physiological saline, and then dialyzed at 4°C overnight. The toxin was then sterilized by Millipore filtration using 0.22-µm filters (Corning, 431 219). The concentration of the toxin was measured with a solution of Bio-Rad Protein Assay Standard II (Biorad, 500-007 and Biorad, 500-0006) kits on spectrophotometric absorbance at a wavelength of 595 nm.[9]

Detection of Shiga-like toxin

The Shiga-like toxin was detected using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (DRG, EIA-4452) according to the manufacturer's instructions. The reaction was measured by reading the optical density (OD) in the ELISA reader at λ 450 nm. The interpretation of results was based on a cutoff value of the OD negative control + 0.20. The results were considered negative if they were less than the cutoff, and positive if they were greater than the cutoff.[9,16]

Preparation of T47D cancer cells

As much as 1 mL of T47D cancer cells maintained under standard cell culture conditions were grown as a monolayer culture in Dulbecco's Modified Eagle Medium (Sigma, D6046) and supplemented with 10% Newborn Calf Serum (Sigma N4887), 100 IU penicillin/mL, 100 mg/mL streptomycin, and 50-µg Fungizone (Fisher Scientific, BW17-745H). The cells were incubated at 37°C, in a humidified atmosphere containing 5% CO_2.

Cytotoxic assay

As much as 50 µL of T47D cancer cells were implanted into 96-well microplates (Merck) and incubated at 5% CO_2 for 24 hours to obtain confluent growth at a density of 5×10^4 cells/well. Subsequently, media were replaced using 50 µL of purified toxin with serial dilutions. After 15-minute incubation at room temperature, the purified toxin was removed and monolayer cells were washed twice. Complete growth medium (100 µL) was added to the cells before they were incubated at 37°C, 5% CO_2 for 24 hours. The positive test was shown by the number of cancer cell lyses after incubation.[10,17] Cytotoxicity of the cancer cells was determined using a 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) test. At the end of incubation, media were removed, and the cells washed with a solution of phosphate-buffered saline (PBS). Then, 100 µL of culture media and 10 µL of MTT reagent 0.5% were added to each well, incubated again for 4 to 6 hours in 5% CO_2 at 37°C to form formazan. The reaction was stopped by 100 µL of MTT reagent stopper (sodium dodecyl sulfate). The cells were incubated overnight at room temperature, and then analyzed using an ELISA reader at λ 550 nm.[10,17,18]

Acridine orange/ethidium bromide double staining assay

An apoptosis assay was performed using double staining acridine orange (Sigma, 158550)-ethidium bromide (AO/EB) (Sigma, E 1510) at a concentration of IC_{50}. The T47 cancer cells (5×10^4 cell/well) were implanted in a 24-well microplate using coverslips and incubated at a 5% CO_2 incubator for 24 hours to obtain confluent growth of 50% to 60%. The cells were then incubated with a 50-µL crude toxin (concentration IC_{50}) for 6 hours. The media were removed and the cells were washed with PBS solution. A total of 15 µL of AO-EB solution was added to each well and allowed to stand for 5 minutes, and then observed under a fluorescent microscope. Living cells showed green fluorescence and dead cells, orange fluorescence.[10,17,19]

Data analyses

The treatment was made twice, and OD of CPE to measure the IC_{50} value was determined using Microsoft Excel. Detection of apoptosis and necrosis cells for each treatment was worked out by the average of 3 fields of view, with 100 cells in each observation.[20] The change in cell shape was analyzed using analysis of variance and followed by Duncan multiple range test and estimated mean. The chart was created in the set of a high-low box plot in the SPSS 24 program.

Table 1. Detection of Stx 2 production among *Escherichia coli* O157:H7 strains using *sandwich* ELISA.

E. COLI STRAINS	SOURCE	OD (λ 450 NM) VALUE WITH VARIOUS CONCENTRATIONS (μG/100 μL)			
		10	5	2.5	1.25
ATCC 43894	Human, United States	0.553 (+)	0.433 (+)	0.394 (+)	0.305 (–)
KL-48(2)	Human, Indonesia	0.472 (+)	0.332 (–)	0.348 (–)	0.295 (–)
SM-25(1)	Cattle feces, Indonesia	0.472 (+)	0.377 (+)	0.329 (–)	0.296 (–)
SM-7(1)	Cattle feces, Indonesia	0.367 (+)	0.258 (–)	0.250 (–)	0.274 (–)
DS-21(4)	Beef, Indonesia	0.336 (–)	0.291 (–)	0.242 (–)	0.240 (–)

Abbreviations: ELISA, enzyme-linked immunosorbent assay; OD, optical density; Stx, Shiga-like toxins.
OD values in Table 1 were average of 2-time repetition.
OD negative control = 0.155.
Cutoff (OD negative control + 0.2) = 0.355.

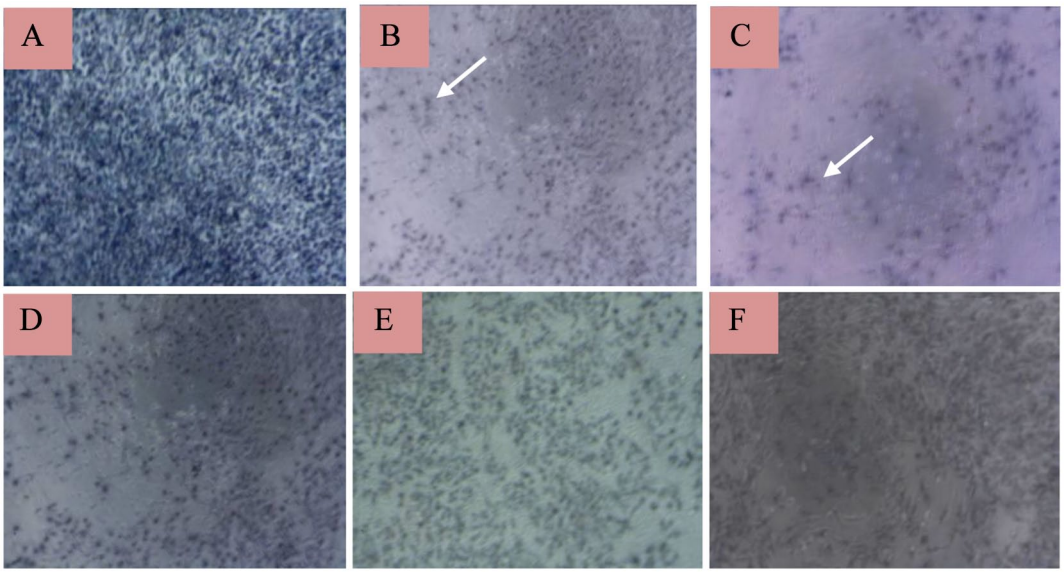

Figure 1. Staining of T47D cells with MTT 24 hours after treatment. The arrow (⟶) indicates live cells stained. (A) Control cells without treatment. (B) Cells treated with the toxin *Escherichia coli* ATCC 43894 as a positive control. (C) Cells treated with toxin KL-48(2). (D) Cells treated with toxin SM-25(1). (E) Cells treated with toxin SM-7(1). (F) Cells treated with toxin DS-21(4) as a negative control. MTT, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyltetrazolium bromide.

Results

Shiga-like toxin production

Shiga-like toxin production of 2 local strains of *E. coli* O157:H7 (KL-48(2) and SM-7(1)) was lower than that of the control isolate ATCC 43894. The ELISA test showed ATCC 43894 was positive until the lowest dilution of 2.5 μg/100 μL. There were contrary results among local strains, with SM-25(1) having a minimal positive concentration of 5 μg/100 μL, while KL-48(2) and SM7(1) were only positive to 10 μg/100 μL (Table 1).

Cytotoxic assay

Cytotoxic assays were preceded by the preparation of T47D cell culture followed by treatment with various concentrations of Shiga-like toxin. The development of T47D cell lines for each treatment after colored with MTT was variable (Figure 1), and the data minimum concentration of infectious culture 50 (IC_{50}) of isolates on the formation of CPE in T47D cell line after 24-hour observation (Supplemental Additional File: Data S1) is summarized in Table 2

Based on the descriptions given in Figure 1, the MTT assays show the differences in both cytotoxicity and cell viability. This assay principally determines cell viability through the determination of the mitochondrial function of cells by measuring the activity of mitochondrial enzymes such as succinate dehydrogenase, which can be quantified by light absorbance at a specific wavelength. This method is so applicable. It is easy to use, safe, has high reproducibility, and widely used to determine both cell viability and cytotoxicity tests. Hence, the MTT assay is used in cytotoxicity studies for screening new anticancer compounds because of its accuracy and relative simplicity.[21]

Table 2. The minimum concentration of infectious culture 50 (IC_{50}) of isolates on the formation of cytopathic effect in T47D cell lines after 24-hour observation.

ISOLATE	SOURCE	IC_{50} CONCENTRATION (μG/μL)
ATCC 43894	Human, United States	1.85
KL-48(2)	Human, Indonesia	1.88
SM-25(1)	Cattle feces, Indonesia	2.30
SM-7(1)	Cattle feces, Indonesia	7.51
DS-21(4)	Beef, Indonesia	5.88

IC_{50}: The minimum concentration required to kill 50% of the sustainable T47D cells. Values in Table 2 are the average of 3-time observations per visual field of 100 cells.

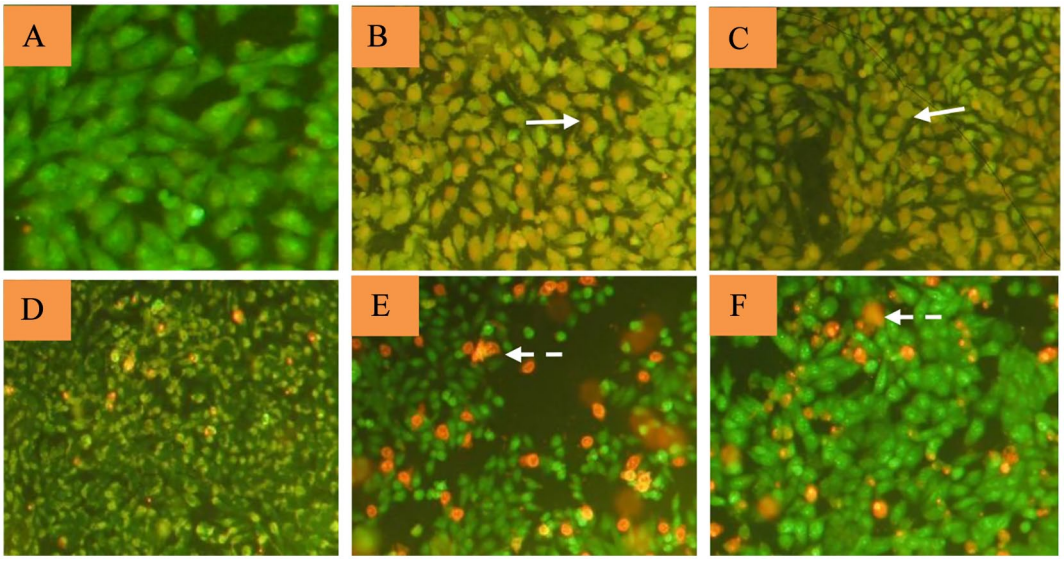

Figure 2. The staining of acridine orange/ethidium bromide on T47D cells 24 hours after treatments. The images were taken using a phase-contrast microscope (Olympus, type IMT-2/605029) with a magnification of 10 × 40. The arrow (⟶) indicates apoptotic cells and the broken line arrow (- - ▶) indicates necrotic cells. (A) Control cells without treatment. (B) Cells treated with toxin *Escherichia coli* ATCC 43894 as a positive control. (C) Cells treated with toxin KL-48(2). (D) Cells treated with toxin SM-25(1). (E) Cells treated with toxin SM-7(1). (F) Cells treated with toxin DS-21(4).

Based on the data provided in Table 2, it appears that local isolates of *E. coli* O157:H7 (strain KL-48(2), as well as SM-25(1)) have IC_{50} values higher than those of the control isolate ATCC 43894. The data showed that the minimum concentration of each isolate, ie, 1.88 and 2.30 μg/μL of isolates KL-48(2) and SM-25(1), respectively, has been able to kill 50% of the T47D cell line. This contrasts with isolates SM-7(1) and DS-21(4), which required higher concentrations to kill 50% of cells, ie, 7.51 and 5.88 μg/μL, respectively.

Acridine orange/ethidium bromide double staining assay

Detection of cytotoxicity (apoptosis and necrosis) caused by each isolate of *E. coli* O157:H7, to confirm data provided in Table 2, is presented in Figure 2.

Double staining assays as analyses of the cytotoxic effect on T47D cells showed the staining of cells posttreatment by AO will reduce green fluorescence and increase the incidence of orange fluorescence compared with normal cells (Figure 2). This is believed to be a result of the breakdown of DNA fragments into a single strand of nucleic acid that is more easily bound to AO dye.[22] Acridine orange penetrates into living and dead cells emitting green fluorescence (when intercalated into DNA, and red fluorescence when bound with single-stranded nucleic acid, such as RNA). Ethidium bromide emits red fluorescence by intercalation into DNA when cells have altered cell membranes.[23] The color changes are associated with an increasing amount of AO binding to the sugar-phosphate backbone of DNA as a result of DNA denaturation.[24,25]

Moreover, the summary of the T47D cell lines with their percentages of normal cells, necrosis, and apoptosis, after being treated with a Shiga-like toxin *E. coli* O157:H7 (Supplemental Additional File: Data S2), is presented in Table 3 and their chart is shown in Figure 3.

The data in Table 3 and illustrated in Figure 3 shows local isolates of *E. coli* O157:H7, strain KL-48(2) and SM-25(1), showed a toxic effect on T47 cell lines. Strain KL-48(2) had a necrosis effect higher ($P < .05$) than that of the control isolate

Table 3. Percentage of normal cells, apoptosis, and necrosis of T47D cell lines after 24-hour treatment with Shiga-like toxin *Escherichia coli* O157:H7.

T47D CELL CONDITION	ISOLATES					MEAN
	ATCC 43894	KL-48(2)	SM-25(1)	SM-7(1)	DS-21(4)	
Normal cells	23.3^{Db}	13.7^{Eb}	36.7^{Cb}	70.0^{Aa}	55.7^{Ba}	39.9^{b}
Apoptosis	63.3^{Ba}	80.3^{Aa}	50.3^{Ca}	6.7^{Ec}	21.3^{Db}	44.4^{a}
Necrosis	13.3^{Bc}	6.0^{Cc}	13.0^{Bc}	23.3^{Ab}	23.0^{Ab}	15.7^{c}

Values with the same letters to the row (uppercase) and to the column (lowercase) show no significant difference ($P > .05$). Values with the different letter to the row (uppercase) and to the column (lowercase) show significant differences ($P < .05$).

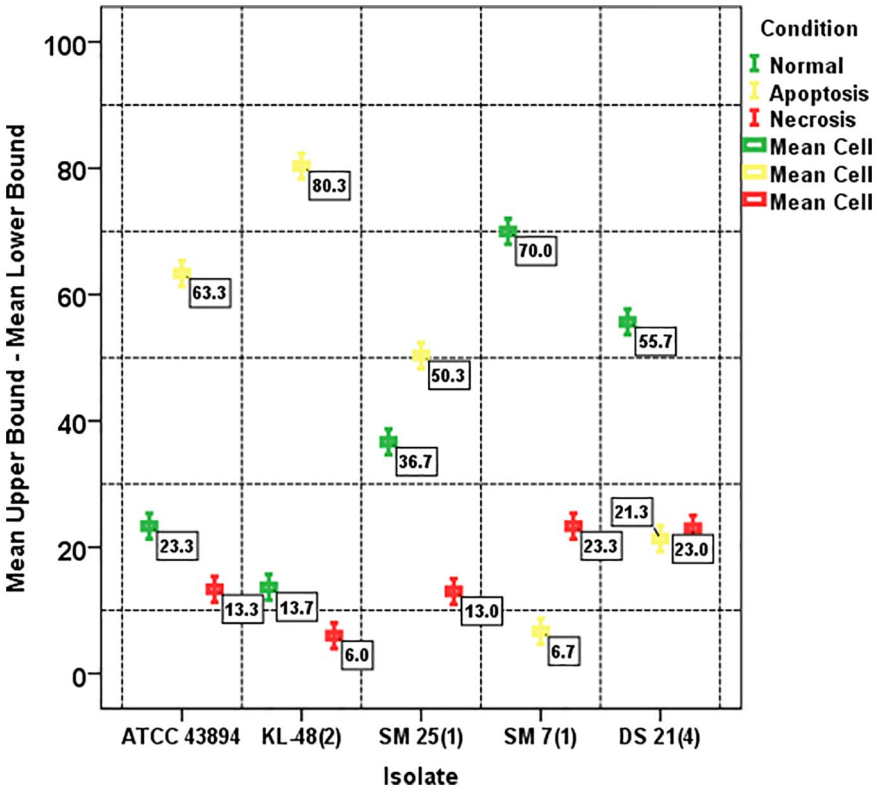

Figure 3. The chart of normal cells, apoptosis, and necrosis of T47D cell lines after 24-hour treatment with Shiga-like toxin *Escherichia coli* O157:H7.

ATCC 43894, ie, 80.3% than 63.3%, although its necrosis effect was lower ($P < .05$) ie, 6.0% than 13.3%. Local strain SM-25(1) had a nondifferent necrosis effect ($P > .05$) with the control isolate ATCC 43894, ie, 13.0% from 13.3%. This strain also showed its apoptosis effect, although it was lower ($P < .05$) ie, 50.3% than the control isolate ATCC 43894 as much as 63.3%.

Discussion

The results of the study indicate that Shiga-like toxin originating from the local Indonesian strain of *E. coli* O157:H7 might induce toxicity, as effective as the control isolate ATCC 43894, against the T47D cancer cell line (Figure 1). This study reinforces the statement of Johansson et al, in 2009 who states that bacterial toxins such as verotoxin are known therapeutic agents against malignant tumors. Cancer therapy with toxins is known to be very effective and can reduce the side effects on normal tissue due to the mechanism of action targeting very specific signaling pathways.[6]

Shiga and the Shiga-like toxins (Stx) are related protein toxins produced by *Shigella dysenteriae* and certain strains of *E. coli* such as *E. coli* O157:H7. These toxins (Stx) are composed of 2 noncovalently attached, modular parts: the A moiety (StxA) containing the enzymatically active A1 fragment and the nontoxic, pentameric binding moiety (StxB). The Stx binds specifically to the glycosphingolipid globotriaosylceramide (Gb3) at the surface of target cells and is then internalized by endocytosis. Subsequently, in toxin-sensitive cells, the Stx/Gb3 complex is transported in a retrograde manner via the Golgi apparatus to the endoplasmic reticulum, where the enzymatically active part of Stx is translocated to the cytosol, enabling it to irreversibly inhibit

protein synthesis via modification of ribosomal 28S RNA.[5,26] Furthermore, several researchers have found that the Gb3 receptor shows relatively restricted expression in normal human tissues, but has been reported to be highly expressed in many types of cancers. LaCasse et al[27] stated 8 of 10 (80%), and Johnson et al[6] stated 17 of 25 (68%) breast cancer cell lines were targeted by Stx. The enhanced expression of Gb3 in primary breast cancer and lymph node metastases can be targeted by the B-subunit of Shiga-like toxin.[28]

In addition, there are different patterns of cell death that can occur, namely apoptosis and necrosis. Apoptosis is characterized by the occurrence of cell shrinkage, chromatin condensation, and plasma membrane swelling 1 ± 4. The incidence of apoptosis results in the activation of protease as an effector of cell death (caspase), which will later attack the substrate of the cytoplasm and nucleus. DNA divides into nucleosome size and larger fragments by activation of caspase endonucleases and continues on the change in membrane phospholipids with the release of phosphatidylserine.[29] These changes are considered characteristic of apoptosis.

Specifically, apoptosis is activated within a few hours of exposure to various activation signals, including DNA damage, lack of growth factors, activation of death receptor signaling, radiation, and various chemotherapy drugs.[30] Furthermore, necrosis is known as the result of damage from most parts of the cell and is generally characterized by swelling of mitochondria, chromatin condensation, and the loss of cell integrity.[30,31] The use of AO/EB staining to detect apoptosis has been successfully used previously in a study by Kasibhatla et al in 2006. The authors cited this method as a reliable test to detect toxic effects on cell lines.[19] Likewise, this study shows that Shiga-like toxins originating from local Indonesian strains of *E. coli* O157:H7 have potential as a treatment of breast cancer, based on their ability to affect apoptosis and necrosis on T47 cell lines. These results are preliminary and must be studied in vivo before human clinical trials.

Conclusions

The expression level of Shiga-like toxins from Indonesian *E. coli* O157:H7 isolates was lower than that of control ATCC43894. They showed good potency (as effective as control ATCC 43894). The toxins induce apoptosis and necrosis in the T47D cell line and have the potential to treat breast cancer.

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Author Contributions

KJPP, IWS, and DAW conceived and designed the study. IWS performed most assays. KJPP cultivated all isolates, DAW detected Shiga-like toxin production, and IWS also performed cytotoxic assays, apoptotic detection, and double staining assays. The article was prepared by IWS, KJPP, and DAW. HS checked the final article. All authors read, gave advice, and approved the article.

Ethical Approval

The approval from the Institutional Ethics Committee to perform this study was not required as no invasive technique was used.

Supplemental Material

Supplemental material for this article is available online.

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