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Anticancer Activity of the 28.4 kDa Protein from *Pediococcus pentosaceus* SR6 in MCF-7 Breast Cancer Cell Line

I Wayan Suardana[1*], Hevi Wihadmadyatami[2] and Dyah Ayu Widiasih[2]

[1]Department of Preventive Veterinary Medicine, Veterinary Public Health Laboratory, The Faculty of Veterinary Medicine, Udayana University, Bali-Indonesia
[2]Department of Anatomy, The Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta-Indonesia
[3]Department of Veterinary Public Health, The Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta-Indonesia
***Corresponding author:** wayan_suardana@unud.ac.id

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ABSTRACT

A preliminary study of total protein from the lactic acid bacteria (LAB) strain *Pediococcus pentosaceus* SR6 was proven to have an anticancer role in T47D cells. On the other hand, studies on the specific protein of this strain regarding its anticancer effect have so far never been revealed. Based on these considerations, this research was conducted. Research activities began by cultivating of LAB *Pediococcus pentosaceus* SR6 isolates, analyzing protein fragments using SDS-PAGE, isolating and electroeluting specific proteins from SDS-PAGE results, preparing MCF-7 breast cancer cell cultures, cytotoxic tests using the MTT cell proliferation and cytotoxicity kit method, and analyzing cell morphology via observation using the DAPI and Acridine Orange/Propidium Iodide (AO/PI) tests. The test results were then analyzed statistically using the SPSS25 program and then displayed in the form of Tables or Figures. The research results showed the 28.4kDa protein from *Pediococcus pentosaceus* SR6 was known to have anticancer activity with an IC50 of 5.42ppm. Their anticancer activity is selective because it is less toxic to normal cells which require a higher toxic dose, namely 28.36ppm. The protein has an ability to stimulate MCF-7 cells' apoptosis and necrosis that is proved from the double staining with Acridine Orange/Propidium Iodide (AO/PI) and DAPI stainings. In conclusion, The 28.4kDa protein from LAB isolate *Pediococcus pentosaceus* SR6 was proven to be used as an breast anticancer compound with its selective ability against normal cells.

Key words: Apoptosis, *Pediococcus pentosaceus* SR6, MCF-7 cell line, Vero cells, IC_{50}

INTRODUCTION

Breast cancer is one type of cancer that has the highest prevalence. WHO data for 2020, recorded that breast cancer sufferers in Indonesia were at the forefront among other cancers, namely 65,858 cases or 16.6%, shifting the position of cervical cancer at 9.2% of the total cases. Breast cancer can occur in both men and women, but the prevalence in women is much higher (Anonymous 2021). Like in humans, epidemiological studies on 2,000 dogs in America showed that 23% of canine deaths were caused by cancer (Morris and Dobson 2001). Although the incidence of mammary tumors in dogs and cats is rare, they are fatal if not treated properly (Sorenmo et al. 2011). Recently it has been developed comparative oncology as a study of cancers in companion animals for the determination of their translational relevance to human cancers (Schiffman and Breen 2015). Human mammary tumor and canine mammary tumors are similar in various aspects, such as relative age of onset, hormonal dependence, metastasis pattern and role of environmental factors at the onset of the disease (Sultan and Ganaie 2018). The evidence shows that between human and canine mammary tumors have many pathological and molecular similarities (Sorenmo et al. 2009).

The management of cancer sufferers is usually carried out with a series of treatments. In general, some of those are surgery, radiation, chemotherapy, hormone therapy, and most recently immunological therapy (He and Xu 2021). Ideally, cancer treatment can eliminate tumors and metastases completely, but they are generally still have shortcomings such as requiring additional treatment or being less specific to the target which results in additional side effects (Pucci et al. 2019). The study found several

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side effects from chemotherapy for breast cancer including fatigue, nausea, no appetite, vomiting, joint pain, fever, diarrhea, difficulty swallowing, allergies, constipation, mouth ulcers, swollen right hand, and itching (Khairani et al. 2019). Moreover, cancer therapy generally damages of normal tissues and organs such as kidney, bone marrow, and oral mucosa, causes inflammation, hinders the normal metabolism, and secondary lymphedema (Dennert and Horneber 2006).

On the other hand, recently researchers studied to seek an antitumor agent with minimal side effects, one of which is bacteriocins. Bacteriocins produced by lactic acid bacteria have been reported potential against various cancer cells (Molujin et al. 2022). Bacteriocin is a cationic peptide that is synthesized in ribosomes. Some bacteriocins have been indicated selectively in their cytotoxicity to the cancer cells compared with normal cells. This selectivity promises bacteriocins as a prime candidate for further investigation of cancer therapy and ideal to use in clinical trials (Wu et al. 2020). Some bacteriocins from LAB that are studied to have potency as an anticancer such as pediocin, colicin, pyocin, and mecrocin (Niamah 2018; An et al. 2019; Molujin et al. 2022). Moreover, the previous study of the LAB strain *Pediococcus pentosaceus* SR6 isolated from the rumen fluid of Bali cattle found that has great potential as a superior candidate for probiotics. It also has potency as an anticancer which is evidenced by its activity in the reduction of the number of living T47D cancer cells from 95.08 to 50.65% (Swacita et al. 2022). According to the results of the previous studies and considering there is no yet study about the anticancer activity of the specific protein from LAB strain 16, this study is interesting to present.

MATERIALS AND METHODS

Ethical approval

This study did not require Ethical approval because there was no use of live animals.

Cultivation of the SR6 lactic acid bacteria strain isolate

The pure culture of LAB isolate SR6 was taken from 30% gloserol stock which was stored at a storage temperature of -20°C. The isolate was then thawed at 4°C for 15 minutes before being planted at room temperature in sterile MRS broth media before being incubated at 37°C for 24 hours. The grown isolate was ready for further tests (Sukrama et al. 2017; Sukrama et al. 2020).

Protein fragments analysis of lactic acid bacteria SR6

Protein fragments of the isolate were analyzed with the Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) method. The LAB SR6 culture suspension was centrifuged at 3000rpm at 4°C for 10min followed by washing it with 0.1M PBS buffer pH 7.0 and then centrifuged for the same temperature, speed and time. Washing was done 3 times, and the protein was then broken down in a cooling time of 5 seconds using a sonicator for 30 seconds with 6 repetitions and 0.7 duty cycle amplitude. The cell mass then centrifuged at 13,000 rpm for 10min, and the supernatant was used as a protein sample. The protein content was measured using the Bradford method. The concentration of the electrophoresed protein sample was equalized by dilution. The dissolved protein profile was visualized using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) consisting of 12.5% resolving gel, and 5% stacking gel. A total of 15µL of protein (35µg/µL) was added to the wells, but the firts well was added with the protein marker Standard 20-220 kDa (Invitrogen LC5602) as a comparison. The gel was stained with coomassie brilliant blue 1%, and then destained until the protein band was visible with a mixture of methanol solution, glacial acetic acid, and distilled water in a ratio of 5:1:4. The protein bands of molecular weight were calculated as a retention factor/Rf by comparing the mobility of the protein molecule on a reference protein standard with the formula (Suardana et al. 2013):

$$Rf = \frac{\text{The distance of the moving protein band from its starting point}}{\text{The distance of the moving tracker color from its starting place}}$$

Isolation, purification and electroelution of the specific proteins

The unstained SDS-PAGE gel was cut along the specified bands. Each gel piece was put into a cellophane bag and soaked with 1-2mL of 0.2mM phosphate buffer (PB). Then put it in an electro-elution chamber containing 0.1mM phosphate buffer. The next step was electroelution in a cool chamber with a power of 220V, 20mA overnight. The eluted protein can be determined by staining the acrylamide gel piece with commasie blue staining for 20min. Then destaining was added, if there was no band, it means the protein has eluted. The liquid containing the protein contained in the cellophane bag was removed and then precipitated and purified with 1:1 absolute ethanol to obtain protein that was ready for further testing (Suardana et al. 2013).

Culture preparation of MCF-7 and vero cells

The cells used in this research originated from the laboratory of Parasitology at the Gadjah Mada University, Yogyakarta. A total of 1mL of MCF-7 cell culture or Vero cells stored in liquid N2 in a frozen state (cryotube) was thawed. Cells were washed with DMEM medium by centrifuging at 3000rpm for 5min in condition 4°C. The precipitate was washed again at the same time and temperature before added with 10mL of complete media (DMEM media contain 10% Newborn Calf Serum (Sigma N4887), 100mg/mL streptomycin, 100IU penicillin/ml, and 50 µg fungison (Fisher Scientific, BW17-745H). Cells were then incubated at 37°C, 5% CO_2 atmosphere. The growth medium was replaced after 24 hours and the monolayer cells were observed within 3–7 days (Liu and Pan 2010; Suardana et al. 2018).

Cytotoxicity assay

Cytotoxicity testing of LAB specific proteins in MCF-7 cell cultures and Vero cells was carried out by calculating of the cytopathic effect (CPE) formation. 50µL monolayer of MCF-7 cells and Vero cells with density of $5x10^4$cells/well were coated in two 96-well culture plates and then incubated overnight at 37°C, with an atmospheric humidity of 5% CO_2. In each well, 10µL of the electroeluted specific protein pellet was added to the culture plate containing monolayer MCF-7 cells or Vero

cells. After 24 hours of incubation, the liquid was discarded, the cell monolayers were washed two times with DMEM media. Furthermore, 100µL of complete growth medium were added to the cells and incubated for 24 hours. Cells were observed under a phase contrast microscope. Positive results were indicated by the loss of cells 24 hours after inoculation (Liu and Pan 2010). The toxicity of the cells were measured by using the MTT cell proliferation and cytotoxicity kit (Cyto-M, MTT-1200). The media from the plate was discarded (after seeing the presence of loose cells/CPE in the microscope). A total of 5mg of MTT was dissolved in 1000µL of sterile PBS and then vortexed and mixed into 10ml of complete DMEM media. 100µL of the MTT mixture was added to each well, then incubated at 37°C, 5% CO_2 atmosphere for 4 hours. Observation of living cells was carried out under a phase contrast microscope. The reaction was stopped by adding each well with 100µL of mixture containing 10% SDS and 0.01N HCl. The culture plate was placed in a plastic container with a tissue paper cover and incubated overnight. Living cells were read with an ELISA reader (Model 680 XR) with a λ of 550nm. The CPE was calculated by comparing the OD value average of the treatment with the control (Liu and Pan 2010; Suardana et al. 2018).

Acridine Orange/Propidium Iodide (AO/PI) stainings

Cell morphology was detected by Acridine Orange-Propidium Iodide double staining according to the method of Liu et al. (2015) with slight modifications. MCF-7 cells ($5x10^4$cells/well) were spread in coverslips in a 24-well plate filled with DMEM growth medium until the density reached 50-60%. Cells were then incubated with 50µL of specific protein (IC50 concentration) for 6 hours. After that, the growth medium of each well was aspirated and the coverslips were washed with sterile PBS pH 7.2 for 3 times. Cells were permeabilized by incubating the coverslips with 0.1% Triton X-100 (Sigma) in PBS followed by washing to remove Triton X-100 with PBS for 3 times. Cells were then covered with 15µL PBS containing 0.05% acridine orange (Sigma)-Propidium Iodide (Sigma). The coverslips were then attached to an object glass, and the slides were then checked with a fluorescence microscope (Pinatih et al. 2021; Suardana et al. 2022).

DAPI staining

The DAPI staining of the cells were done according to the method described by the previous study with slight modifications. MCF-7 cells ($5x10^4$cells/well) were spread in coverslips in a 24-well plate filled with DMEM growth medium until the density reached 50-60%. Cells were then incubated with 50µL of specific protein (IC50 concentration) for 6 hours. Growth medium was aspirated followed by washing of the coverslips for 3 times with sterile PBS pH 7.2. Cells were the permeabilized by incubating with 0.1% Triton X-100 (Sigma) in PBS. Triton was removed and the coverslips were washed for 3 times with PBS. Cells were then stained using DAPI which was dissolved in PBS (2.5 g/mL) and left for 20 minutes in the dark room. The coverslips were then attached to an object glass, and the slides were then viewed under a fluorescence microscope (Papi et al. 2013).

Data analysis

Research data were presented descriptively in the form of average and deviation standards. The OD value of the CFE was measured to determine the IC50 value. Detection of apoptosis and cell necrosis was determined based on the results of 3 field observations of 100 cells per observation. The average value was then analyzed using the General Linear Model and further tested with Duncan test in the SPSS version 25 program (Santoso 2018).

RESULTS

Protein fragments analysis of lactic acid bacteria SR6

Stock isolate of lactic acid bacteria SR6 was cultivated by growing in MRS broth media. The bacteria that have grown were then cultured again in a larger volume for protein analysis, and the results of protein analysis with SDS-PAGE are shown in Fig. 1.

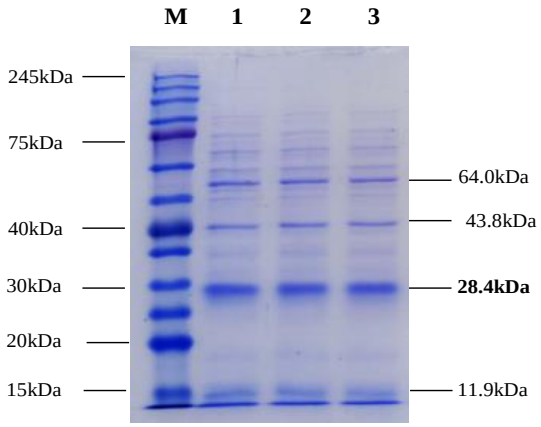

Fig. 1: The SDS-PAGE results of LAB SR6 isolate-protein with 5% stacking gel and 12% resolving gel. M: Marker (PM5100). No: 1-3 isolate SR6 15µL.

In Fig. 1, the protein band with a molecular weight (MW) of 28.4kDa is seen with thick and clear bands. These bands were then cut and collected to obtain sufficient quantities and continued with electroelution. The electroeluted protein was obtained in a volume of 3ml with a concentration of 0.085 µg/µL or 85 ppm. This protein was then used as a treatment in the cytotoxic test for MCF-7 breast cancer cells.

Cytotoxic test using the MTT cell proliferation

The results of the toxicity test for the 28.4kDa protein from the LAB strain SR6 on MCF7 cell culture after hybridization for 24 hours with serial concentrations before and after MTT are shown in Fig. 2.

Fig. 2 shows that a number of MCF-7 cells died especially when treated with a 50ppm of 28.4kDa protein concentration and opposite with the controls as well as for the protein concentration of 0.78ppm. Complete calculation results based on the OD value at λ 550nm at various concentrations show that the percentage of cancer cells that died at protein concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78ppm were 88.67, 90.29, 85.84, 76.65, 67.94, -16.73 and -14.75%, respectively. The research showed that the toxic effects of protein were visible up to 3,125ppm concentration. Furthermore, the value of Infectious Culture 50 (IC50) on the formation of

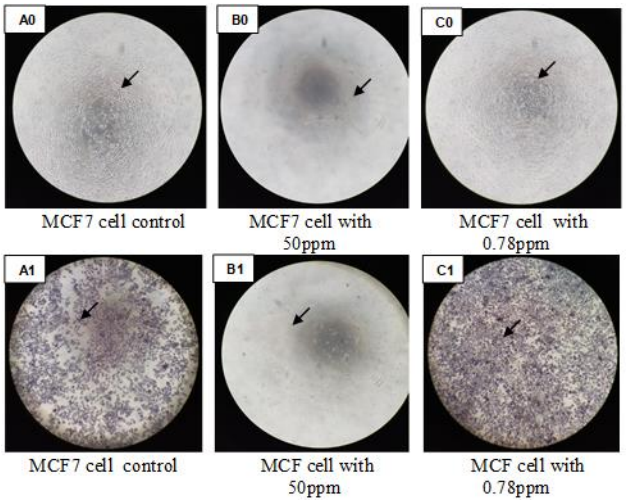

Fig. 2: MCF-7 cells treated with 28.4kDa proteine at various concentrations before and after MTT. Arrows (⟶) indicate the number of viable cells. A0, B0 and C0: MCF7 cells before MTT; A1, B1 and C1: MC cells after MTT. The images were captured with a fluorescence microscope at 100x magnification.

cytopathic effect (CPE) as a result of regression line equation with the formula Y = 27.931 ln (x) + 2.8075 with a value of R2 = 0.7517, the Infectious Culture 50 (IC50) value for the formation of cytopathic effect (CPE) is 5.42 ppm. The IC50 value obtained was slightly higher than the similar protein tested on T47D breast cancer cells which required a much higher dose, namely 484.5 µL/ml (Swacita et al. 2022), As a control for their selectivity against normal cells, the toxicity on the Vero cells line is shown in Fig. 3.

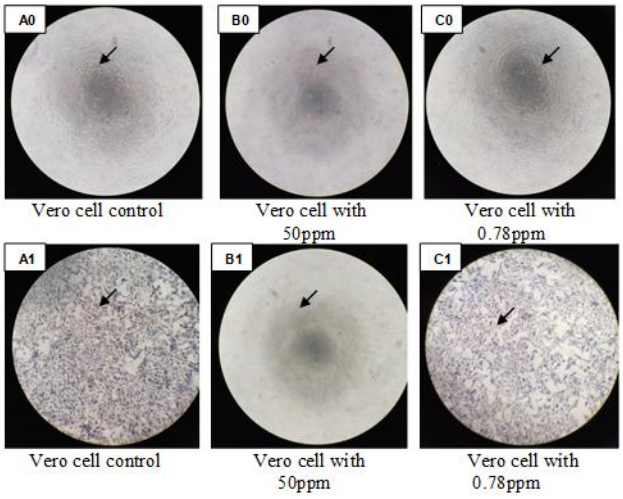

Fig. 3: Vero cells treated with 28.4kDa protein at various concentrations before and after MTT. Arrows (⟶) indicate the number of viable cells. A0, B0 and C0: Vero cells before MTT; A1, B1 and C1: Vero cells after MTT. The images were captured with afluorescence microscope at 100x magnification.

Fig. 3 also shows that some MCF-7 cells died specifically when treated with a 50ppm 28.4kDa protein concentration. Furthermore, MCF-7 cells on the treated with 0.78ppm show almost viable cells like the control. Complete calculation results based on the OD value at λ 550nm show that the percentage of Vero cells that died at protein concentrations of 50, 25, 12.5, 6.25, 3.125, 1.56, and 0.78 ppm were 51.30, 30.75, 29.44, 52.61, -137.44, -153.02, and -158.51 %, respectively. The results showed that toxic effects were visible only up to a concentration of 6.25ppm, different from effects in MCF-7 cells that were needed at 3,125ppm. The calculation of Infectious Culture 50 (IC50) on the formation of cytopathic effect (CPE) by the calculation results from the regression line equation Y = 59.945 ln (x) - 150.52 with a value of R2 = 0.7674, the value of Infectious Culture 50 (IC50) is 28.36ppm.

Acridine Orange/Propidium Iodide (AO/PI) staining test

The AO/PI staining test to detect the morphological change of the MCF-7 cells after being treated with 28.4KDa protein is presented in Fig. 4.

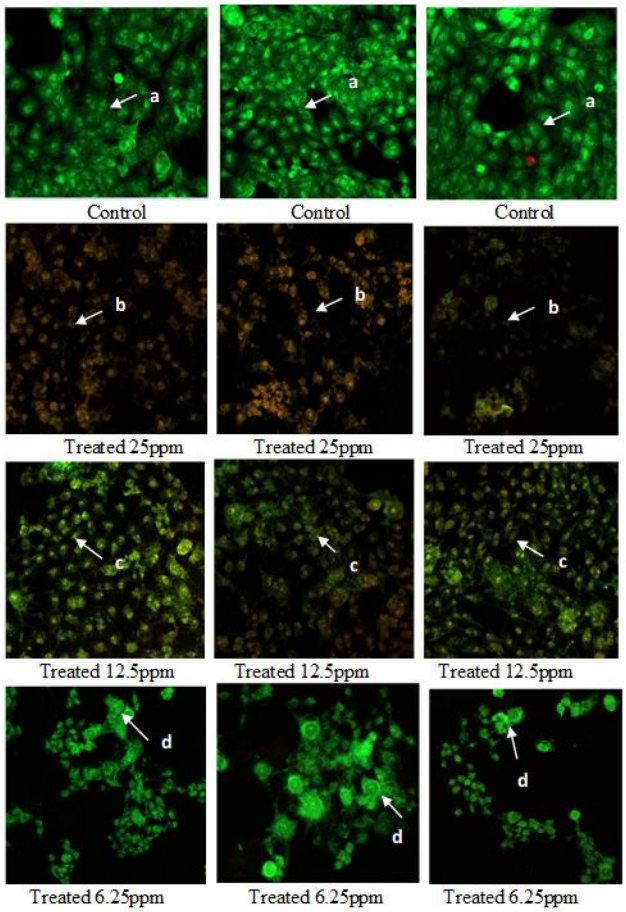

Fig. 4: Results of staining MCF-7 cells with Acridine Orange-Propidium Iodide (AO/PI) 24 hours after treatment. a: normal cells; b: necrotic cells; c: late apoptotic cells and d: early apoptotic cells. The images were captured with a fluorescence microscope at 100x magnification.

Fig. 4 shows most of the cells are still alive in green color, especially in the control, and several cells are orange specifically for the cells treated with 25ppm and 12.5ppm protein concentrations, which indicates that the cells have undergone apoptosis or necrosis. Furthermore, the summary of morphological changes of MCF-7 cells staining that trated with 28.4kDa protein from *Pediococcus pentosaceus* SR6 are shown in Fig. 5.

Fig. 5 shows the correlation of the MCF-7 cell line that enters the stages of necrosis or apoptosis stages with the concentration of the 28.4kDa protein from *Pediococcus pentosaceus* SR6. The higher of the concentration the higher of the cell entered to the necrosis or apoptosis stages.

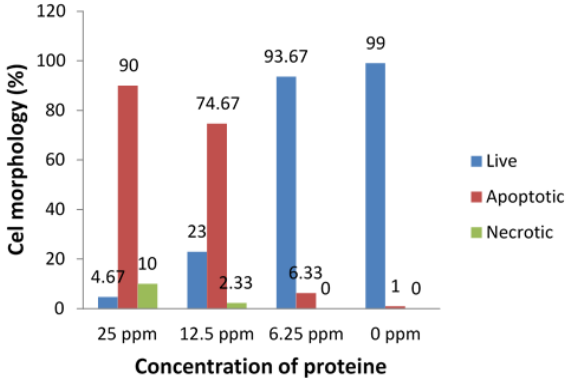

Fig. 5: Percentage morphology of MCF-7 cells treated with various concentrations of the 28.4kDa protein from *Pediococcus pentosaceus* SR6. The graph was created based on the average of 3 observational fields of view in 100 cells.

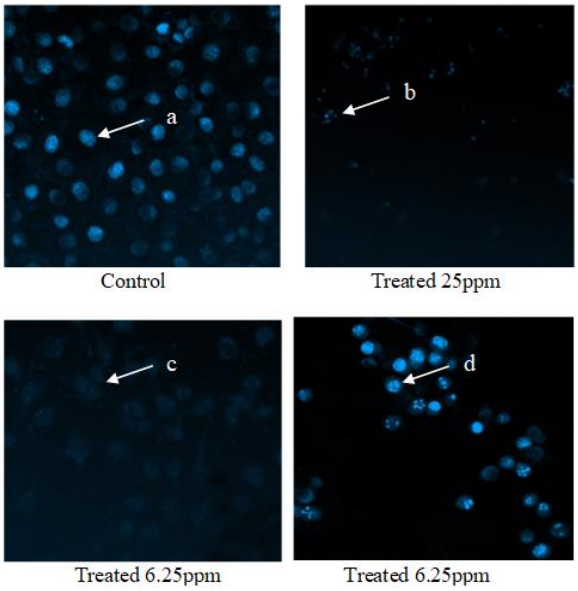

Fig. 6: Staining of MCF-7 cells with DAPI (4',6-diamidino-2-phenylindole) 24 hours after treatment. a: normal cells (normal nuclei); b: cell necrosis (fragmented nuclei); c: apoptotic cells (chromatin condensation) and d: normal cells (normal nuclei) but in more limited numbers. The images were captured with afluorescence microscope at 100x magnification.

DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) staining test

DAPI is a cell-permeable fluorescent dye for differentiating the living and dead cells. DAPI binds to AT-rich regions in double-stranded DNA of the cells. The DAPI-DNA complex showed a light blue fluorescence color with an excitation light of 364nm. Normal cells show intact nuclear staining results. On the other hand, cells that have undergone apoptosis or necrosis will have their nuclei damaged or ruptured. The DAPI staining results as confirmation of the AO/PI double staining test are shown in Fig. 6.

Fig. 6 shows the control cells with DAPI staining with normal cell nuclei, whereas when treated with 24.8kDa protein, brightly colored apoptotic cells were seen with typical characteristics such as nuclear fragmentation, as well as chromatin condensation and aggregation of apoptotic bodies. Many nuclei also looked normal in the treatment with a concentration of 6.25ppm, but their number was decreased compared with the control.

DISCUSSION

At present, several researchers are constantly using extracelluler protein or bacteriocine produced lactic acid bacteria (LAB) as one of the research interests to explore cancer therapies with few side effects. This study confirms the potential of LAB as an anticancer agent specifically in breast cancer cells. Bacteriocin is known to have activities that are selective for normal and cancer cells, considering that the concentration required to cause toxic effects on normal cells is higher than cancer cells. These results follow the statement of LAB has antagonistic effects against pathogenic bacteria, immuno-modulating effects, antimutagenic effects, and anti-carcinogenic effects (Albuquerque et al. 2020). The anti-carcinogenic effect of LAB was strengthened by the previous studies that found reducing of MTT value on viable Hela cells treated with goat's milk fermented with *Lactobacillus plantarum* and *Lactobacillus paracasei* than control. These results in line with an increase of the concentration of goat's milk hydrolyzate given between 30-240 µg (Nandhini and Palaniswamy 2013). The anti-cancer activity of LAB was also confirmed both in vitro and in animal models against cancer cells from various malignancies (Garbacz 2022).

The anti-cancer effect of the 28.4 protein of *Pediococcus pentosaceus* SR6 was confirmed by the results of acridine orange propidium iodide staining. Theoretically, all nucleated cells show green fluorescence produced by Acridine orange (AO) staining, while red fluorescence resulted by the staining Propidium iodide (PI) that can only enter dead cells with weak membrane integrity (Bank 1988). Acridine orange (AO) will emit green fluorescence after the stain bound to double-stranded DNA, while red fluorescence after being bound to single-stranded DNA or RNA. Based on their activity, Acridine orange can be used in apoptosis studies. Depending on the degree of membrane integrity loss, apoptotic cells will stain orange or red, but in the early apoptosis still show green color (Mascotti et al. 2000; Leles et al. 2013; Hussain et al. 2019). Staining cells with acridine orange and ethidium bromide or propidium iodide can provide information about nuclear morphology of the cells such as nuclear shrinkage, perinuclear chromatin condensation, and fragmentation of the cell nucleus. Therefore, acridine orange and propidium iodide/ethidium bromide staining helps detect four main types of the cells: (a) Viable cells with uniform green nuclei and organized chromatin structure (b) Early apoptotic cells with unstructured green nuclei regular but condensed chromatin as apoptotic bodies or green spots/fragments (c) Late apoptotic cells, with orange or red nuclei with condensed or fragmented chromatin, and (d) Necrotic cells with uniform red-orange or red nuclei with organized structure due to loss of membrane integrity (Mascotti et al. 2000; Leles et al. 2013; Hussain et al. 2019). The results obtained also support the results of previous research which stated the role of bacteriocins produced by lactic acid bacteria as anticancer compound (Kaur and Kaur 2015) as has been also demonstrated in rectal colon cancer (Zhong et al. 2014). The percentage of morphological change in Fig. 5 supports the results of previous research (Swacita et al. 2022) that proved the SR6 strain of lactic acid bacteria identified as *Pediococcus pentosaceus* can be toxic to T47D cancer

cells, especially extracellular proteins at a concentration of 50% of the total volume.

Furthermore, the results of DAPI staining confirmed that cells treated with the 24.8kDa protein of *Pediococcus pentosaceus* SR6 experience apoptosis or necrosis in line with increasing concentrations of the protein agent as is the case with cells stained with AO/PI double dye. The apparent correspondence between the results of AO/PI staining and DAPI staining supports the results of previous research that carried out the same study, namely testing the inhibition of growth and necrosis in cancer cells by phenolic compounds from *Acacia hydaspica* (Afsar et al. 2016). The use of DAPI, a fluorescent DNA-binding agent, together with acridine orange/ethidium bromide staining to observe and ensure cell death and cellular morphological changes involved in apoptosis has been also proved. These methods were used to investigate the antiproliferative and apoptotic effects of *Sesbania grandiflora* leaves in human cancer cells (Pajaniradje et al. 2014).

As an anti-cancer theraphy, protein products of lactic acid bacteria have been studied by some researchers previously. Probiotic *Pediococcus acidilactici* strain isolated from tomato pickle alleviates gut inflammation and anti-cancer activity in-vitro. The cytotoxicity of the *Pediococcus acidilactici* TMAB26 also showed significant effect on the Caco-2 (92.63±0.63%) and HT-29 (94.91±1.27%) (Barigela and Bhukya 2021). The anticancer effect of the bacteriocin produced by Lactococcus and Streptococcus species has been proved recently. Nisin exhibited a significant effect on the inhibition growth for the both cell lines, HuH-7, and SNU182 through the apoptosis mechanism (Balcik-Ercin and Sever 2022). Nisin could suppressed metastatic process via down-regulation of CEAM6, MMP2F, CEA, and MMP9F genes (Norouzi et al. 2018). These results were in line with the statement that the utilization of bacteria has emerged as a new therapeutic approach in the treatment of various cancers (Fathizadeh et al. 2021). This phenomenon resulted from their anti-cancer product of bacteria such as antibiotics, enzymes, peptides, bacteriocins, and toxins that have been proven to have anti-cancer activity. The use of bacteriocin as an anti-cancer theraphy is also important because of its safety in clinical treatment (Liu et al. 2021). Based on this study's results, and in line with the previous study by some researchers, it confirms the potency of protein as an anti-cancer with its advantages. Further study on the mechanisms and expression of genes associated with the anti-cancer activity of the 28.4kDa protein from *Pediococcus pentosaceus* SR6 as an anticancer is in progress.

Conclusion

The specific protein from the LAB isolate of *Pediococcus pentosaceus* SR6 with a molecular weight (MW) of 28.4kDa is known to have anticancer activity in MCF-7 cells with an IC50 of 5.42ppm. Its anticancer activity is selective to the normal cells because it is less toxic to Vero cells which require a higher toxic dose, namely 28.36ppm. The resulting toxicity was proven by its ability to stimulate MCF7 cell apoptosis and necrosis from the results of double staining with acridine orange/propidium iodide and also strengthened by DAPI staining.

Authors' contributions

I Wayan Suardana: Conceptualized and designed the study and collected data. Hevi Wihadmadyatami: Conceptualization, methodology, data curation, resources, project administration, and writing-review and editing. Dyah Ayu Widiasih: Designed and formulated material in the laboratory and supervised data. All authors have read, reviewed, and approved the final manuscript.

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Competing interests: The authors declare there are no conflicts of interest.

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