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Cytotoxic effect of proteinaceous postbiotic metabolites produced by *Lactobacillus plantarum* I-UL4 cultivated in different media composition on MCF-7 breast cancer cell

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ABSTRACT

Aims: *Lactobacillus* sp. has capability of producing an array of bioactive metabolites that exhibit probiotic effects. Therefore, the objective of this study was to determine the cytotoxicity effect of proteinaceous postbiotic metabolites (PPM) produced by *Lactobacillus plantarum* I-UL4 cultivated in different media composition on MCF-7 breast cancer cell.
Methodology and results: *L. plantarum* I-UL4 was cultivated in yeast extract and modified de Man, Rogosa and Sharpe broth containing Tween 80 (CRMRS+T80) or without Tween-80 (CRMRS-T80). Human breast adenocarcinoma cell (MCF-7) was employed as cancer cell in this study. Cytotoxicity and antiproliferative effects of PPM were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl Tetrazolium Bromide assay and Trypan Blue Dye Exclusion assay, whereas Acridine Orange/Propidium Iodide staining was employed to determine the cytotoxicity mechanism. PPM produced in CRMRS+T80 exerted cytotoxicity in a time and dose dependent manner that was selective towards MCF-7 cancer cell. Profound cytotoxicity with the lowest IC_{50} concentration of 10.83 µg was detected at 72 h of incubation, whereas the most potent antiproliferative effect revealed by the lowest viable cell population was observed at 24 h of incubation. PPM cultivated in CRMRS+T80 induced 80.87% of apoptotic MCF-7 cells at 48 h of incubation.
Conclusion, significance and impact of study: PPM of *L. plantarum* I-UL4 cultivated in different media composition induced different levels of MCF-7 cancer cell death. The percentage of apoptotic MCF-7 cells treated with PPM cultivated in CRMRS+T80 increased significantly ($p < 0.05$) from 24 to 48 h of incubation. The results obtained in this study have revealed the potential of PPM produced by *L. plantarum* I-UL4 as human health supplement and as anticancer preventive agent.

Keywords: Lactobacillus plantarum I-UL4, cytotoxic effect, proteinaceous postbiotic metabolites, media composition, breast cancer

INTRODUCTION

Lactobacillus plantarum I-UL4 is one of the Lactic acid bacteria (LAB) species isolated from Malaysian fermented food, *Tapai Ubi* (Moghadam *et al*., 2010) that is capable of producing bioactive metabolic products so called postbiotics. The postbiotics of LAB have been demonstrated to possess vast potential as food supplement for healthier intestinal homeostasis and as therapeutic aids in inflammatory bowel disease (IBD) (Silk *et al*., 2009). Tsilingiri *et al*. (2012) reported that postbiotics can be a safe alternative for treating patients with IBD in the acute inflammatory phase as it can protect healthy epithelium from highly infectious-agents such as *Salmonella* and down-regulate pro-inflammatory pathways in IBD tissue. LAB have been reported to have other beneficial properties, such as anti-allergic (Bae *et al*., 2009; Fujii *et al*., 2014), anti-inflammatory (Bowe *et al*., 2011), hypocholesterolemic (Jeun *et al*., 2010; Wang *et al*., 2014), anti-gastric, anti-viral (Sharma *et al*., 2012) and anti-cancer activities (Nandhini *et al*., 2013; Zhong *et al*., 2014).

Lactobacilli are among the most popular LAB employed for the production of fermented dairy products and have gained special attention as an important probiotic species (Tiwari *et al*., 2012). This was further

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described by Li *et al*. (2012) that *Lactobacillus rhamnosus* has the strongest effect in decreasing prevalence, severity and the duration of diarrhoea. Several researches reported that when *L. gasseri* was used together with immunoglobulin (Sakai *et al*., 2014) would improve host defence against mucosally transmitted pathogens and maintain the homeostasis of mucosal surface (Fagarasan, 2008). Most of the *Lactobacillus* species including *L. rhamnosus* CNCM I-3690 (Grompone *et al*., 2012) and *L. plantarum* MYL26 (Chui *et al*., 2013) are prominent for their anti-inflammatory effect, while *L. plantarum* CNRZ 1997 is the most pro-inflammatory strain against Influenza A virus infection (Kechaou *et al*., 2013). Vissers *et al*. (2010) reported that *Lactobacillus* strains have the competency to control the production of interleukin (IL)-Iβ, IL-10, interferon-γ and tumour necrosis factor-α that suppressed the inflammation found in mouse colitis model (Chen *et al*., 2012). Furthermore, Elmer (2001) reported that daily consumption of yogurt that contains several *Lactobacillus* species would reduce the severity of women affected by vaginal candidiasis. In addition, LAB have also been reported to possess antiviral effect (Ibrahem, 2013; Lakshimi, 2013).

LAB have anti-cancer properties in colon cancer by activating anticancer immune response and interfering the metabolic activity of cancer cells (Liong, 2008). The preventive effect of probiotics on intestinal carcinogenesis may be associated with the changes in the intestinal microbiota which in turn suppresses the growth of bacteria that promote the growth of cancer cells and resulted in the reduction of carcinogens in the intestine (Kumar *et al*., 2010). Breast cancer is the most commonly diagnosed cancer in women (American Cancer Society, 2013). Interestingly, several LAB such as *B. infantis*, *B. bifidum*, *B. animalis*, *L. acidophilus, L. paracasei* and *L. helveticus* R389 have been shown to have growth inhibitory effect on MCF-7 breast cancer cell line (Biffi *et al*., 1997; de Moreno and Perdigon, 2010).

Several *L. plantarum* strains that isolated from Malaysian foods have been demonstrated to play an important role in maintaining the overall health of rats and livestock (Loh *et al*., 2008, 2009 and 2010; Thanh *et al.,* 2009; Choe *et al*., 2012; Thu *et al*., 2011a). They were able to enhance overall growth performance, gut health, preventing or reducing the diarrhoea incidents and improve the general immune system. Moreover, accumulative studies have been conducted on rats, poultry, laying hens and piglets to reveal the beneficial effect of postbiotic produced by *L. plantarum* I-UL4 (Foo *et al*., 2003b; Loh *et al*., 2009; Thanh *et al*., 2010; Thu *et al*., 2011b; Choe *et al*., 2012). However, the cytotoxic effect of proteinaceous postbiotic metabolites (PPM) has not been reported elsewhere. Hence, an attempt was conducted to determine the cytotoxic effect of PPM produced by *L. plantarum* I-UL4 cultivated in different media composition on MCF-7 breast cancer cell line.

MATERIALS AND METHODS

Bacterial Growth Conditions

Lactobacillus plantarum I-UL4 was obtained from the Laboratory of Industrial Biotechnology, Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia (UPM) which was previously isolated from Malaysia traditional fermented food of *Tapai ubi* (Foo *et al*., 2003a). The stock culture of *L. plantarum* I-UL4 was kept at –20 °C in de Man, Rogosa and Sharpe (MRS) broth (Merck, Germany) containing 20% (v/v) of glycerol. The culture was revived according to the method described by Moghadam *et al*. (2010).

Cancer cell lines and growth conditions

An anchorage dependent cell line of MCF-7 (human mammary gland adenocarcinoma) was obtained from Animal Tissue Culture Laboratory, Department of Molecular Biology, Faculty of Biotechnology and Biomolecular Sciences, UPM. MCF-10A cell line that purchased from American Tissue Culture Collection (ATCC) was used as a control for normal glandular epithelium cell of human mammary gland. Both cell lines were cultivated and maintained in T*25 cell culture flask (TPP, Switzerland) containing 10 mL of complete growth media of RPMI-1640 (Sigma, USA) or DMEM (Sigma, USA) added with 100 IU/mL penicillin and streptomycin (Sigma, USA) and 10% (v/v) foetal bovine serum (FBS) (PAA, Austria). The cells were propagated at 37 °C in an environment supplied with 5% CO_2 and 95% humidified atmosphere. The growth medium was discarded after 24 h of incubation and replaced with fresh complete growth media during sub-culturing. Cells (4-5 × 10^5 cells/mL) were seeded and grown exponentially before each experiment was conducted.

Production of proteinaceous postbiotic metabolites

Lactobacillus plantarum I-UL4 [1% (v/v)] was grown in three different media composition: reconstituted MRS broth with Tween 80 (CRMRS+T80), without Tween 80 (CRMRS-T80) and Yeast extract (C-Yeast) and incubated for 24 h at 30 °C. The CRMRS+T80 broth consisted of: 20 g/L glucose, 11.89 g/L Yeast extract, 1 g/L Tween 80, 5 g/L sodium acetate, 2 g/L di-potassium hydrogen phosphate, 2 g/L di-ammonium hydrogen citrate, 0.2 g/L magnesium sulphate tetrahydrate and 0.04 g/L manganese sulphate heptahydrate. The composition of CRMRS-T80 was similar to CRMRS+T80 but Tween 80 was excluded, whereas C-Yeast broth contained solely yeast extract. The PPM were prepared according to the method as described by Foo *et al.* (2003b). The bacterial cell was separated by centrifugation at 10,000 × *g* for 10 min and the cell free supernatant (CFS) was collected and used as PPM.

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Protein determination

Protein content of PPM was determined by Modified Lowry method (Miller, 1959), using bovine serum albumin as protein reference.

3-(4,5-Dimethylthiozol-2-yl)-2,5-diphenyl Tetrazolium Bromide assay

Both MCF-7 and MCF-10A cells (1×10^5 cells/mL) were seeded onto 96-well microplate containing 100 µL/well of complete growth media and incubated for 24 h at 37 °C in 5% CO_2 condition. A serial two-fold dilution of freeze dried PPM of *L. plantarum* I-UL4 was prepared in complete growth media to achieve the total volume of 200 µL per well. The diluted PPM that contained 3.275 µg to 200 µg of PPM were tested on both MCF-7 and MCF-10A cells and incubated for 24, 48 and 72 h respectively. After respective incubations, 20 µL of 3-(4,5-Dimethylthiozol-2-yl)-2,5-Diphenyl Tetrazolium Bromide (MTT) solution (5 mg/mL) (Sigma, USA) was added into each well and the plate was incubated in dark condition for additional 4 hours at 37 °C in 5% CO_2 atmospheric condition. A volume of 170 µL media was removed from each well and the violet formazan crystals were dissolved in 100 µL dimethylsulfoxide (Fischer Scientific, UK) and incubated further for 20 min. The completely solubilised formazan salt was quantified by using µ Quant ELISA reader (BIOTEK EL 340, USA) at 570 nm. Mean value and standard error from three times with triplicate samples were determined for each experiment. The absorbance readings of both the treated samples (cancer cells treated with two-fold diluted PPM in complete growth media) and untreated control treatments (untreated cancer cells) were subtracted from the blank which contained complete growth media. The following equation was used to calculate the percentage of cell viability:

Percentage of cell viability = $(A_{sample} - A_{blank})/ (A_{control} - A_{blank}) \times 100\%$

The inhibition concentration of 50% of the growth (IC_{50}) was determined from the graph of percentage of cancer cells viability versus concentration of PPM of *L. plantarum* I-UL4.

Trypan Blue Dye Exclusion assay

Trypan Blue Dye Exclusion assay was used to evaluate MCF-7 cell viability through cell membrane integrity. MCF-7 cells were seeded (2×10^5 cells/mL) in 6 well plates (Greiner Bio-One, Netherlands) and incubated for 24 h. The cells were then treated with 62 µg of PPM, which was approximately two-fold of IC_{50} concentration obtained in MTT assay conducted for 48 h of incubation. The treated cells were incubated at 37 °C for 24, 48 and 72 h in a 5% CO_2 humidified incubator. The adherent cells were treated with trypsin and collected by centrifugation at 200 × *g* for 10 min. The cell pellet was then suspended gently with 1 mL of growth media. A volume of 10 µL trypan blue (Sigma, USA) was added to an equal volume of cells and the cell mixture was left for 2 min at room temperature. The trypan blue positive (cells stained blue) and trypan blue negative (transparent) cells were observed and scored by using haemacytometer (Fisher Scientific, UK). The cell population was calculated based on the following equation:

Cell population = Total cell counted × dilution factor × 10^4

Determination of cell death mode by fluorescent microscopy using Acridine Orange/Propidium Iodide staining assay

MCF-7 cells (2×10^5 cells/mL) were seeded in 6-well plate for 24 h prior to treatment with 62 µg of PPM. Treated MCF-7 cells were incubated in 5% CO_2 atmospheric condition at 37 °C at three different incubation times (24, 48 and 72 h). After each incubation period, the detached cells in the medium were collected and mixed with adherent cells treated with trypsin. The treated cells were collected by centrifugation at 200 × *g* for 5 min and washed with PBS. The cell pellet was suspended with 50 µL of growth media. A volume of 8 µL of the cell suspension was mixed with 2 µL of Acridine Orange/Propidium Iodide (AO/PI, 50 µg/mL) (Sigma, USA) and left for 1 min before visualising under fluorescent microscope (Nikon FC-35DX, Japan) using a combination of excitation filter and barrier filter at 450 – 490 nm and long pass filter of 520 nm. The percentage of viable (green intact cells), apoptotic (green shrinking cell with condensed or fragmented nucleus), and late apoptotic and necrotic (red cells) were determined (Gorman *et al*., 1996). The stained cells were determined from more than 200 cells to be statistically significant. Results were presented as a proportion of the total number of cell observed. All experiments were repeated three times with triplicate samples.

Statistical analysis

Results were expressed as mean ± S.E.M. The significant difference between each test variable was determined by Grand Linear Model (GLM), a two-way analysis of variance (ANOVA). The statistical analysis was conducted by using SAS Statistical Software at differences of $p < 0.05$.

RESULTS AND DISCUSSIONS

Cytotoxic effect on breast cancer cell lines

Different protein contents (4.84 µg/µL to 5.67 µg/µL) were detected for various PPM (Tables 1 and 2), indicating different PPM was harvested after culturing *L. plantarum* I-UL4 in different media composition. The PPM was then examined for their cytotoxic activity on MCF-7 cancer cells and MCF-10A normal cells at different incubation time. The cytotoxicity results expressed as IC_{50} values for 24, 48 and 72 h of incubation times are shown in Table 1 and

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Table 2 for MCF-7 cancer cells and MCF-10A normal cells respectively. PPM produced by *L. plantarum* I-UL4 cultivated in CRMRS+T80 exerted cytotoxicity in a time and dose dependent manner. Significant ($p < 0.05$) reduction of IC_{50} concentration was observed when MCF-7 cells were treated with PPM cultivated by using CRMRS+T80 from 24 to 72 h of incubation, whereby profound cytotoxicity with the lowest IC_{50} concentration of 10.83 µg was detected at 72 h of incubation. Nevertheless, the IC_{50} concentrations of CRMRS-T80 cultivated PPM were reduced slightly from 29.83 µg to 26.87 µg throughout the incubation time. The least potent cytotoxicity effect was shown by the PPM cultivated by C-Yeast, whereby the IC_{50} value of 94.00 µg was only detected after 48 hours of incubation and no IC_{50} values were detected at 24 h and 72 h of incubations respectively.

The IC_{50} values were increased from 51.9% to 86.0% for MCF-10A normal cell line as compared to MCF-7 breast cancer cells when treated with similar PPM at the respective incubation time. The IC_{50} values for MCF-10A normal cell line were only detected at 48 and 72 h of incubation for both CRMRS+T80 and CRMRS-T80 cultivated PPM. However, no IC_{50} values were detected for CRMRS+T80 and CRMRS-T80 cultivated PPM for 24 h of incubation. Similarly, no IC_{50} values were detected for 24 to 72 h of incubation for C-Yeast cultivated PPM. The cytotoxicity results demonstrated that PPM produced by *L. plantarum* I-UL4 cultivated in different media compositions was selective towards MCF-7 breast cancer cells. In comparison to the effect of probiotic cells, Motevaseli *et al*. (2013) and Nami *et al*. (2014) reported that probiotic *Lactobacilli* were selectively cytotoxic to tumour cell but not to normal cells.

Table 1: IC_{50} of proteinaceous postbiotic metabolites produced by *Lactobacillus plantarum* I-UL4 cultivated in different media composition on MCF-7 cancer cell for 24, 48 and 72 h of incubation.

Media composition	Protein content (µg/µL)	IC_{50} (µg)		
		24 h	48 h	72 h
CRMRS+T80	5.33 ± 0.32^{a}	56.13 ± 0.68^{f}	39.50 ± 4.61^{agk}	10.83 ± 0.87^{a}
CRMRS-T80	4.84 ± 0.30^{a}	29.83 ± 2.33^{e}	29.57 ± 2.53^{ck}	26.87 ± 1.07^{b}
C-Yeast	5.67 ± 0.28^{a}	N.D.	94.00 ± 3.46^{i}	N.D.

Notes: MCF-7, human mammary gland adenocarcinoma cell line; CRMRS+T80, cultured reconstitute MRS broth with Tween 80; CRMRS-T80, cultured reconstitute MRS broth without Tween 80; C-Yeast, cultured yeast extract; IC_{50} value, inhibition concentration that caused 50% of cell viability. N.D., no IC_{50} value was determined up to concentration of 200 µg. Values shown represent mean ± SEM. Values within the same column with different superscripts indicated significant different at $p < 0.05$.

Table 2: IC_{50} of proteinaceous postbiotic metabolites produced by *L. plantarum* I-UL4 cultivated in different media composition on MCF-10A cell for 24, 48 and 72 h of incubation.

Media composition	Protein content (µg/µL)	IC_{50} (µg)		
		24 h	48 h	72 h
CRMRS+T80	5.33 ± 0.32^{a}	N.D.	82.12 ± 0.18^{b}	77.63 ± 0.31^{b}
CRMRS-T80	4.84 ± 0.30^{a}	N.D.	85.24 ± 0.50^{a}	80.11 ± 0.06^{a}
C-Yeast	5.67 ± 0.28^{a}	N.D.	N.D.	N.D.

Notes: MCF-10A, non-malignant human glandular epithelium cells; CRMRS+T80, cultured reconstitute MRS broth with Tween 80; CRMRS-T80, cultured reconstitute MRS broth without Tween 80; C-Yeast, cultured yeast extract; IC_{50} value, inhibition concentration that caused 50% of cell viability; N.D., no IC_{50} value was determined up to concentration of 200 µg. Values shown represent mean ± SEM. Value within the same column with different superscripts indicated significant different at $p < 0.05$.

The total viable cell numbers of treated MCF-7 cells incubated for 24, 48 and 72 h of incubation with PPM cultivated with different medium compositions are shown in Figure 1. PPM produced in CRMRS+T80 exerted antiproliferative effects in time dependent manner, whereby the viable cell population of treated MCF-7 cells increased significantly ($p < 0.05$) from 24 to 48 h of incubation. However, the viable cell population of treated MCF-7 cells with CRMRS+T80 cultivated PPM was not significantly different ($p > 0.05$) from 48 to 72 h of incubation. Meanwhile, the cell viability of MCF-7 cells treated with PPM cultivated in CRMRS-T80 showed gradual increase (significantly different at $p < 0.05$) from 24 to 72 h of incubation. Although both PPM cultivated with CRMRS+T80 and CRMRS-T80 improved the cell numbers throughout the three incubation times, but these PPM demonstrated significant decrease ($p < 0.05$) in cell population when compared with untreated control for 24 to 72 h of incubation.

On the other hand, C-Yeast exhibited no significant different ($p > 0.05$) on cell viability as compared to untreated control at 24 h. However, at 48 hours of incubation time, there was a decrease in the cell population from 24 to 48 h and then increased at 72 h. The cell population at 48 and 72 h were significantly ($p < 0.05$) lower than untreated control for PPM cultivated with

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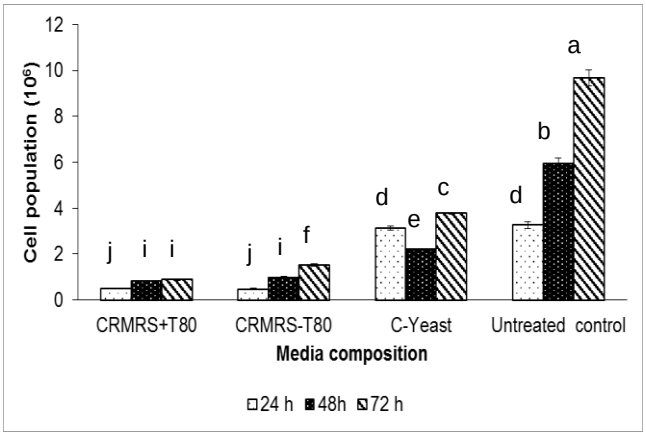
C-Yeast. The treated cell population of MCF-7 cells by CRMRS+T80 and CRMRS-T80 cultivated PPM was cultivated in CRMRS+T80 and CRMRS-T80 have potent anti-proliferation effect on MCF-7 cells as compared to PPM cultivated in C-Yeast.



Notes: CRMRS+T80, cultured reconstitute MRS broth with Tween 80; CRMRS-T80, cultured reconstitute MRS broth without Tween 80; C-Yeast, cultured yeast extract. Mean values with same subscripts are not significantly different at $p > 0.05$.

Figure 1: Total viable cell number of MCF-7 cancer cells treated with 62 µg proteinaceous postbiotic metabolites produced by *L. plantarum* I-UL4 cultivated in different media composition that determined by Trypan Blue Dye Exclusion assay for 24, 48 and 72 h of incubation.

According to the results obtained in MTT assay and Trypan Blue Dye Exclusion assay, the PPM cultivated with CRMRS+T80 induced the highest cytotoxicity and antiproliferative effects on MCF-7 cells than the other two media compositions at 72 h of incubation, which could be attributed to the protein content of CRMRS+T80 cultivated PPM. The protein content of the PPM (Tables 1 and 2) cultivated in CRMRS+T80 (5.33 µg/µL ± 0.32) was higher than the protein content of PPM cultivated in CRMRS-T80 (4.84 µg/µL ± 0.30), implying different media compositions have an impact on the production of different PPM by *L. plantarum* I-UL4. The effect of growth media compositions on the cytotoxicity of *Steptomyces anulatus* has been reported by Hirvonen *et al.* (2001). *L. plantarum* I-UL4 required a complete medium composition (CRMRS+T80) for the fermentation process to produce potent PPM possessing prominent cytotoxicity and antiproliferative effects on MCF-7 breast cancer cells.

Cell death mode of treated MCF-7 cells

AO/PI staining assay was used to determine the cytotoxicity mode of PPM produced by *L. plantarum* I-UL4 by observing the morphological changes in cell membrane and chromatin (Hanahan *et al*., 2000). According to Chuah *et al*. (2010), Choudhari *et al*. (2013) and Lay *et al*. (2014), the morphological changes of apoptotic cells include chromatin condensation, cell shrinkage, membrane blebbing and formation of apoptotic bodies. The percentage of apoptotic, necrotic and viable cells were determined by AO/PI assay and scored under fluorescent microscope. PPM that produced by *L. plantarum* I-UL4 using different types of culture medium exerted potent apoptotic cell death of MCF-7 cells throughout 24, 48 and 72 h of incubation (Table 3). The percentage of apoptotic MCF-7 cells increased significantly ($p < 0.05$) from 24 to 48 h after the treatment of 62 µg PPM cultivated with CRMRS+T80. However, there was no significant difference ($p > 0.05$) for the percentage of apoptotic MCF-7 cells treated for 48 to 72 h. Similar results were obtained for the MCF-7 cells treated with PPM cultivated with CRMRS-T80.

Table 3: The mode of cell death of MCF-7 cancer cells treated with 62 µg proteinaceous postbiotic metabolites produced by *L. plantarum* I-UL4 cultivated in different media composition for 24, 48 and 72 h of incubation.

Media composition	24 h			48 h			72 h		
	A (%)	N (%)	V (%)	A (%)	N (%)	V (%)	A (%)	N (%)	V (%)
CRMRS+T80	70.66 ± 1.31^{d}	3.66 ± 0.13^{gh}	25.68 ± 1.27^{d}	80.87 ± 0.84^{abc}	4.93 ± 0.09^{de}	14.20 ± 0.86^{e}	82.80 ± 1.09^{a}	9.56 ± 0.43^{a}	7.64 ± 0.72^{g}
CRMRS-T80	69.51 ± 1.32^{d}	3.94 ± 0.07^{fg}	26.55 ± 1.32^{d}	81.20 ± 0.93^{ab}	5.13 ± 0.12^{d}	13.67 ± 0.91^{ef}	82.24 ± 0.81^{ab}	8.92 ± 0.25^{a}	8.84 ± 0.69^{g}
C-Yeast	64.07 ± 1.07^{e}	4.46 ± 0.11^{ef}	31.47 ± 1.08^{c}	77.97 ± 1.37^{c}	6.21 ± 0.09^{c}	15.82 ± 1.36 ^{e}	79.77 ± 0.84^{bc}	8.06 ± 0.10^{b}	12.17 ± 0.81^{f}
Untreated control	5.21 ± 0.29^{h}	3.30 ± 0.26^{h}	91.41 ± 0.42^{a}	6.38 ± 0.47^{g}	4.42 ± 0.26^{ef}	89.20 ± 0.50^{ab}	7.70 ± 0.39^{f}	4.41 ± 0.44^{ef}	87.88 ± 0.58^{b}

Notes: CRMRS+T80, cultured reconstitute MRS broth with Tween 80; CRMRS-T80, cultured reconstitute MRS broth without Tween 80; C-Yeast, cultured yeast extract. A %, Apoptotic cell percentage; N %, Necrotic cell percentage; V %, Viable cell percentage. Values shown represent mean ± SEM. Values within the same column and row of a particular cell type sharing a same superscript are not significantly different at $p > 0.05$.

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In comparison, the percentage of apoptotic MCF-7 cells was significantly lower ($p < 0.05$) when treated with PPM cultivated with C-Yeast throughout the 24, 48 and 72 h of incubation. Generally, the percentage of viable cells decreased significantly ($p < 0.05$) when the incubation time prolong from 24 to 72 h. The lowest percentage of viable cells was found on MCF-7 cells treated with 62 µg of PPM cultivated with CRMRS+T80, CRMRS-T80 and C-Yeast at 72 h of incubation time, implying apoptotic cell death pathway was induced as compared to necrotic cell death pathway when MCF-7 cells were treated with 62 µg of different PPM produced by culturing *L. plantarum* I-UL4 in different media compositions. Among the PPM, 62 µg of PPM cultivated with CRMRS+T80 achieved the highest apoptotic cell percentage at 72 h of incubation. These results indicated that the cytotoxicity of PPM produced by *L. plantarum* I-UL4 has the capability to induce MCF-7 cancer cells death by apoptosis in time-dependent manner. This is in agreement with the results reported by Chuah (2010), who has demonstrated the postbiotic metabolites of *L. plantarum* I-UL4 has the ability to cause the MCF-7 cancer cells death via apoptosis in dose and time-dependent manner. This assay demonstrated that treated groups have significantly higher ($p < 0.05$) apoptotic cells percentage than untreated control.

CONCLUSIONS

In conclusion, the PPM cultivated with CRMRS+T80 exhibited profound time- and dose-dependent cytotoxic effect on MCF-7 cells in comparison to PPM cultivated with CRMRS-T80 and C-yeast media. According to the results of AO/PI staining assay, the percentage of apoptotic MCF-7 cells treated with PPM cultivated with CRMRS+T80 increased from 24 to 72 h of incubation, inferring the PPM produced by *L. plantarum* I-UL4 induced MCF-7 cancer cells death by apoptosis in time-dependent manner. Generally, the PPM that produced by *L. plantarum* I-UL4 using different culture media have limited cytotoxicity on non-maglinant MCF-10A cell as compared to MCF-7 cancer cells. The results obtained in this study have revealed the potential of PPM produced by *L. plantarum* I-UL4 as human health supplement and as anticancer preventive agent.

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