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In vitro anticancer potentials of *Lactobacillus plantarum* IIA-1A5 and *Lactobacillus acidophilus* IIA-2B4 extracts against WiDr human colon cancer cell line

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Abstract

Colorectal cancer is an emerging public health problem in Indonesia that urges more serious attempts to combat the disease. Previous in vitro studies indicated some probiotics offer promising avenues for the prevention and treatment of colorectal cancer. Earlier, two Indonesian meat-based probiotics of *Lactobacillus plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4, were isolated and exhibited promising functional properties in food fermentation. Nevertheless, their potential anticancer activity has never been examined. This study aims to determine the inhibitory properties of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 against the WiDr human colon carcinoma cell line. To address, extracellular and intracellular extracts were firstly harvested from both probiotics at the population of 8.22 and 9.13 log (cfu/mL) for *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4, respectively. The extracts from both bacteria were found to have similar pH and titratable acidity values, but different in their protein contents. Further, extra- and intracellular extracts of both strains displayed inhibitory activity towards WiDr cells in a dose-dependent response. The calculated IC50 values of extracellular extracts of both probiotics were significantly lower than that of the intracellular extracts. This activity is predicted to be partly contributed by some protein substances in the extract.

Keywords: probiotic; colorectal cancer; WiDr cancer cells; *Lactobacillus plantarum* IIA-1A5; *Lactobacillus acidophilus* IIA-2B4.

Practical Application: The current study confirmed the in-vitro activity of extra- and intracellular extracts of Indonesian probiotics *Lactobacillus plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 against WiDr human colon carcinoma cell line. The extracts are promising to be further explored and applied in cancer treatments.

1 Introduction

Colorectal cancer is one of the most common and deadly cancer in the world with about 1.93 million cases in 2020 (Xi & Xu, 2021). Unhealthy lifestyles such as the consumption of foods high in fat and low fiber content may increase the risk of developing colorectal cancer (Larsson & Wolk, 2006). There are three main types of treatment for cancer, namely surgery, radiotherapy, or chemotherapy (Hahn & Payne, 2003). Surgery is usually considered effective in the earlier stage, while radiation therapy has negative side effects and was reported to increase the risk for the development of other cancers. On the other side, chemotherapy treatment often uses drugs, mainly doxorubicin, that turn off cancer cells. Nevertheless, the sensitivity and specificity of Doxorubicin are challenging as treatment with the chemotherapy drug Doxorubicin (DOX) may lead to toxicities that affect noncancer cells, which further leads to undesirable side effects. Chemotherapy works by inhibiting cancer cells' proliferation and growth with limited toxicity, but they may also harm normal cells (Conklin, 2004). The discovery and identification of new anticancer natural products with minimal side effects have been the primary goal in many studies (Duraikannu et al., 2014).

Probiotics are live microorganisms that, when given in an adequate amount confer health benefits to the-host (Darsanaki et al., 2014; Roy & Trinchieri, 2017). Haggar & Boushley (2009) reported that the complex bacterial population residing in the colon (gut flora) plays a critical role in promoting the development of colorectal cancer. Consumption of probiotics could alter the environment of the gut flora and it seems to be one of the most interesting candidates for colorectal cancer treatment (Zhu et al., 2013). Lactic acid bacteria (LAB) are the most commonly used bacteria for probiotics. LABs have displayed anti-inflammatory activities and anti-tumor effects (Ding et al., 2018; El-Deeb et al., 2018; Tukenmez et al., 2019). Some studies have also identified that the consumption of LABs could stimulate the immune system, leading to the prevention of colorectal cancer (Chen et al., 2011; Akbari et al., 2016). Nami et al. (2014) identified that probiotic *Lactobacillus acidophilus* has anticancer activity and remarkable antimicrobial activity as well. *L. plantarum* was suggested by (Nandhini & Palaniswamy, 2013) as a food component to prevent cancer.

L. plantarum IIA-1A5 and *L. acidophilus* IIA-2B4 are indigenous lactic acid bacteria (LAB) isolated from Indonesian Ongole Crossbred Cattle (Arief et al., 2015). These bacteria displayed some probiotic characteristics based on *in vitro* and *in vivo* studies. *L. plantarum* IIA-1A5 exhibits strong antimicrobial activity against *Escherichia coli, Staphylococcus aureus,* and *Salmonella* typhimurium (Arief et al., 2010), while also having

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a high tolerance against low pH values (Arief et al., 2013). *L. acidophilus* IIA-2B4 showed the ability to prevent diarrhea in rats exposed to Enteropathogenic *Escherichia coli* (EPEC) and immunomodulatory effects (Astawan et al., 2011). However, the anticancer activity of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 against colorectal cancer is still unknown. In this study, the ability of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 to inhibit the growth of the WiDr cancer cells line is described. It is concluded that the study on anticancer activity of these Indonesian LABs is quite promising to be further developed as an alternative treatment for colorectal cancer.

2 Materials and methods

2.1 Culture propagation

Lyophilized cultures of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 were obtained from the Department of Animal Production and Technology laboratory, Faculty of Animal Science, IPB University. Each strain was inoculated in De Man, Rogosa and Sharpe Broth (MRSB) medium with 5% yeast extract followed by incubation at 37 °C for 24 h. After 2 successive transmissions, approximately 10% (vol/vol) of activated culture were transferred to 10-mL aliquots of MRS broth and incubated at 37 °C for 24 h. The cell population of each strain was assessed using MRS agar (Oxoid) and the pour-plate technique. The assessment was done in duplicate after incubation at 37 °C for 36 h. Plates having colonies within the countable range of 25 to 250 colonies were enumerated and the colony-forming units (cfu) were calculated.

2.2 Preparation of intracellular and extracellular compound extract

Preparation of the extracts was performed based on Ningtiyas et al. (2021). Briefly, *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 cultures were performed and centrifuged at 6,000 x g at 4 ˚C for 10 min to isolate both pellets and supernatants. Intracellular extracts were derived from the pellets while the extracellular extracts were derived from cell-free medium (supernatants). Isolated pellets were suspended in PBS solution with 10 mM EDTA. Approximately 16 µL of lysozyme (40 mg/mL) were added to the suspension followed by incubation at 37 °C for 1 h. The suspensions were subjected to sonication with 5 cycles for 2 min run and 1 min rest. After sonication, the suspensions were centrifuged at 10 000 x g at 4 °C for 20 min, and the pellets were discarded to obtain the intracellular compound extracts. Isolated supernatants from the initial centrifugation were filtered using Sartorius Minisart 0.22 µm membrane filter and dialyzed against 0.02 M potassium phosphate buffer (pH 6.2) with 10% of 10 mM EDTA. The cell-free medium obtained was also subjected to dialysis using Wako dialysis membrane size 20 for 24 h at 8 °C in 0.02 M potassium phosphate buffer (pH 6.2) with 10% of 10 mM EDTA. The product of dialysis is the extracellular compound extracts. The intracellular extracts of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 are designated as Lp-I and La-I, respectively. Meanwhile, the extracellular extracts of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 are designated as Lp-E and La-E, respectively.

2.3 pH and titratable acidity measurement

The pH of the extracts was recorded by using Ionix Instruments pH5 Spear pH Tester (Robinson, Singapore). The pH meter was calibrated prior to the measurement according to the manufacturer's protocol. Meanwhile, the titratable acidity of the extract was measured according to Kosikowski (1977). Briefly, a mixture of samples containing 20 mL milk or yogurt and 1 mL of 2% w/v solution of phenolphthalein was prepared in an Erlenmeyer flask. The mixture was then titrated with 0.01 N NaOH solution until the faint pink color is observed and stable for at least 2 min. The percentage of titratable acidity was then calculated based on the formula of Carić et al. (2000).

2.4 Protein concentration analysis

The Lowry method for protein quantitation (Walker, 2009) was used to determine the protein concentration within the sample. Bovine serum albumin (BSA) protein ranging from 10-2000 µg/mL was used for standard. To 0.1 mL of samples, 0.1 mL of 2 N NaOH were added and then hydrolyzed at 100 ˚C for 10 min. The hydrolysates were cooled to room temperature and added with 1 mL of complex reagent composed of 2% Na_2CO_3, 1% $CuSO_4 \cdot 5H_20$, and 2% Na. The solution was kept at room temperature for 10 min. Then 0.1 mL of Folin reagent were added and kept the solution at room temperature for 30-60 min. Perkin Elmer Lambda 25 UV/Visible Spectrophotometer (MA, USA) was used to read the absorbance of each mixture at 550 nm. The absorbance was calculated to determine the protein concentration.

2.5 Anticancer activity analysis

Anticancer activity investigations were performed by determining the viability of the cell cancer using the 3-(4, 5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay method (Mosmann, 1983). Samples of bacterial protein extraction were prepared in different concentrations and diluted into Dulbecco's Modified Eagle Medium (DMEM) and buffer based on Nandhini & Palaniswamy (2013). The concentration of samples used for treatment were: 0 µg/mL, 15 µg/mL, 50 µg/mL, 100 µg/mL, and 200 µg/mL. Doxorubicin with the same concentration was also used for treatment as a positive control. Each sample was inoculated into individual wells in a 96-well microplate containing WiDr cancer cells. The microplates were incubated for 48 h at 37 °C before MTT solution was added into the culture. MTT was converted to formazan by metabolically viable cells and the absorbance was measured using a Microplate Reader BIOBASE-EL 10A (Shandong, China) at a wavelength of 595 nm to determine the inhibitory activity. IC_{50} values were calculated using Graphpad Prism 8 by comparing the normalized absorbance percentage with the log of concentration used.

2.6 Statistical analysis

The results are expressed as mean ± standard of deviation of three independent experiments, where each of the experiments consisted of three replications. One-way analysis of variance (ANOVA) and post-hoc Tukey's test was used to determine

the differences among the means. The values of $P < 0.05$ and $P < 0.01$ were statistically significant and very significant, respectively.

3 Results and discusion

3.1 Bacterial preparation

The population of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-1A5 used in this study is presented in Table 1. Each strain was harvested after the incubation at 37 °C for 36 h. The population of both strains was statistically comparable ($P > 0.05$). Notable, the population of both strains were higher than the minimum population of the probiotic to use in fermented foods (Rezac et al., 2018). This indicated that the population used in this study met the minimum requirement of probiotic population in the fermented products. Nevertheless, Molska & Regula (2019) proposed the minimum dose of Lactobacillus genus to reduce the risk of colon cancer was in the range of 10-11 log cfu/mL. This concentration is slightly higher than the population of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B42 used in this study. However, we believe that it is acceptable as some studies on the anticancer properties of LAB strains were shown that the anticancer activities were observable at the population lower than 1010-1011 log cfu/mL (Tiptiri-Kourpeti et al., 2016; Nami et al., 2014). In addition, in this current study, the bacterial cells were not used to challenge the cancer cells *vis-à-vis*, instead, the metabolite products of the bacteria were used in the test.

3.2 pH and titratable acidity

Figure 1 showed the pH and titratable acidity values of the extracts from *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4, which demonstrated both pH and titratable acidity values among the extracts were considerably comparable ($P > 0.05$). Notable, the extracts were obtained from the bacteria harvested after 36 h incubation (fermentation) time. During the fermentation, *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 produced organic acids as the main product of glucose fermentation. The presence of organic acids should lower the pH value of the extracts. Figure 1 showed no differences in the pH value and titratable acidity among the extracts, which indicated that the organic acids produced by fermentation of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 were considered comparable. To note, pH and titratable value are theoretically in an inverse relationship, whereby the low pH (high acids) sample should have a high titratable acidity value as more acids in the sample are titrated (more NaOH needed to titrate the acids). Nevertheless, Figure 1 indicated that the inverse relationship between both values was not obviously observed due to insignificant differences among the values. Arief et al. (2015) previously has identified *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 as homofermentative bacteria, which produced the lactic acid as the predominant organic acid, there is a possibility for these bacteria to also have other organic acids as by-products. Accordingly, the pH and titratable acidity values of the extracts in this study do not necessarily correlate to lactic acid as the only organic acid product. The values may also be due to the presence of other organic acids in the extract. The type of organic acids produced by *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4, nevertheless, remain to be experimentally identified. In general, the presence of organic acids (particularly lactic acid) is preferable for fermentation products due to their health effects. Garrote et al. (2015) indicated that lactate could regulate critical functions of some immune system components such as macrophages and dendritic cells. Lactate and fatty acids derived from the fermentative metabolism of lactobacilli have been shown to downregulate proinflammatory responses in intestinal epithelial cells and myeloid cells (Iraporda et al., 2015). To note, the pH values of the extracts were close to the neutral. The intracellular and extracellular extracts were dialyzed against 0.02 M potassium phosphate buffer (pH 6.2) following the harvesting. This step might account for the nearly neutral pH of extracts.

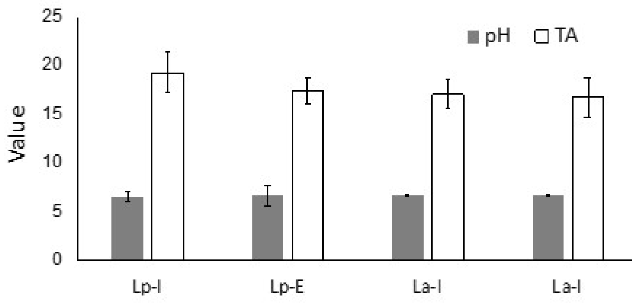

Figure 1. pH and titratable acidity (TA) values of the extracts from *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4. (Lp-I) = *L. plantarum* IIA-1A5 intracellular extract; (Lp-E) = *L. plantarum* IIA-1A5 *extracellular* extract, (La-I) = *L. acidophilus* IIA-2B4 intracellular extract; (La-E) = *L. acidophilus* IIA-2B4 extracellular extract.

Table 1. Bacterial populations of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4.

Sample	Population (log cfu/mL)
L. plantarum IIA-1A5	8.22 ± 0.29
L. acidophilus IIA-2B4	9.13 ± 0.04

3.3 Protein concentration

The protein concentration of the extracts was then determined using the Lowry method (Table 2). Protein concentration from extracellular extracts of both strains has a higher value ($P < 0.05$) than their intracellular counterparts. While Nogueira et al. (2012) reported that the secreted proteins in bacteria were about 5-6% of the total proteins only, the range of concentration is believed to be different depending on the type of bacteria, growth rate,

Table 2. Protein concentration analysis.

Sample	Protein concentration (μg/mL)
Lp-I	264.56 ± 44.72[a]
La-I	367.46 ± 101.59[a]
Lp-E	1,113.00 ± 149.43[b]
La-E	1,078.79 ± 259.39[b]

[a-b]Means in the same column with different superscripts differ ($P < 0.05$).

medium, and temperature. Some extracellular proteins include some enzymes, toxins, as well as antimicrobial peptides, where the concentration of these proteins is considerably different for each of the bacteria. Secreted enzymes are used to scavenge nutrients. Secreted toxins are involved in defense against protozoa, virulence towards humans, and antagonistic interactions with other bacteria (Garcia-Garcera & Rocha, 2020). Sánchez et al. (2010) also reported that extracellular proteins secreted by probiotic bacteria were found to be important for the attachment into the host mucosal cells.

3.4 Anticancer activity

MTT assay was performed to determine the inhibitory activity of a compound. The result (Table 3) showed all extracts displayed inhibitory activity towards WiDr cells in a dose-dependent response, yet with a different magnitude. At the concentration of 15 μg/mL, all the extracts inhibited WiDr cells, yet with lower inhibition activity than Doxorubicin. Lp-E and La-E exhibited comparable inhibition activity with Doxorubicin at the minimum concentration of 50 μg/mL. Meanwhile, a minimum concentration of 100 μg/mL is required for Lp-I or La-I to have comparable inhibition activity with Doxorubicin. This indicated that extracellular extract of both bacteria had better inhibition activity against WiDr cells than the intracellular cells. Interestingly, at any concentration used, Lp-E and La-E showed comparable inhibition activity against WiDr cells. A similar phenomenon was also observed for Lp-I and La-I at any concentration. Further, up to a concentration of 200 μg/mL, none of the extracts exhibited higher inhibition activity than Doxorubicin.

To support, the viability of WiDr Cells was also confirmed microscopically as shown in Figure 2. It is clear that the addition of the extracts induced the cell to death as observed under the microscope. Negative control without any treatment showed normal WiDr cells (A). WiDr cells which have been subjected to 100 ppm of sample treatment displayed a shrinkage and difference in color due to cell death. The compound can cause death to cancer cells by different mechanisms such as apoptosis induction, necrosis induction, anti-proliferation, inhibition of cell cycle, and inhibition of angiogenesis (Ren et al., 2003).

Table 3. Inhibitory activity of different combination of treatments on WiDr cells.

Sample	Dose (μg/mL)				
	0	15	50	100	200
Lp-I	0^d	64.62 ± 14.00^b	96.02 ± 0.52^a	96.32 ± 1.15^a	97.04 ± 1.08^a
Lp-E	0^d	31.57 ± 4.03^c	54.44 ± 4.72^b	87.20 ± 8.43^a	95.56 ± 1.65^a
La-I	0^d	52.39 ± 16.38^b	91.98 ± 4.33^a	95.79 ± 2.05^a	97.16 ± 2.23^a
La-I	0^d	28.10 ± 6.87^c	57.79 ± 9.67^b	85.21 ± 7.80^a	96.68 ± 2.11^a
Doxorubicin	0^d	93.18 ± 0.74^a	94.46 ± 1.09^a	94.63 ± 0.83^a	93.95 ± 0.59^a

$^{a-d}$Means in the same row with different superscripts differ ($P < 0.05$). L.p. = Lactobacillus plantarum; L.a. = Lactobacillus acidophilus; I = Intracellular; E = Extracellular.

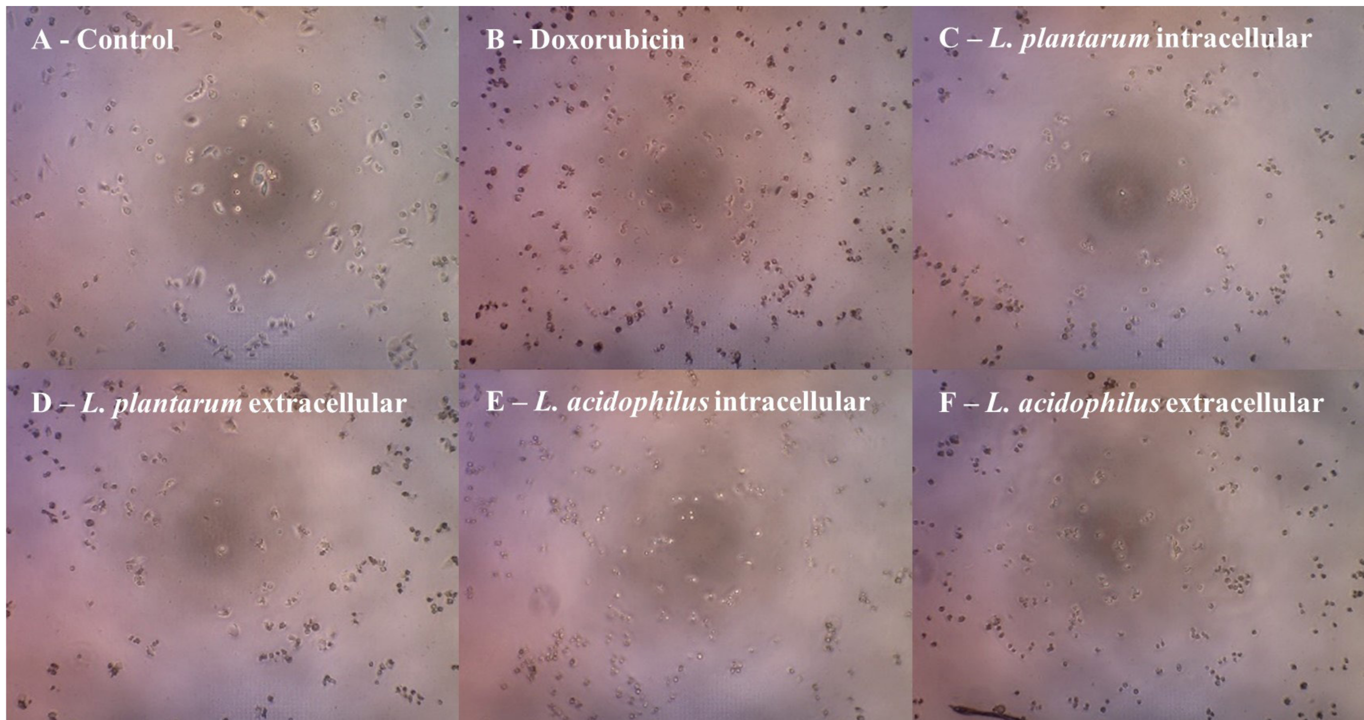

Figure 2. WiDr cells morphology with and without the treatments (100 ppm of Doxorubicin or Lp-E, La-E, Lp-I extracts La-I extracts).

Further, IC50 values of the extracts were calculated based on relationship between the concentrations of each extract and cell viability as shown in Figure 3. The calculated IC50 values of the extract, as well as the Doxorubicin are shown in Table 4. These values refer to the required concentration of samples (µg/mL) to inhibit 50% of cell viability. Accordingly, the higher IC50 value obtained should reflect lower inhibition activity. Table 4 showed that IC50 values of extracellular fractions were significantly higher than that of intracellular fractions. This confirmed that the extracellular extract of both bacteria has better inhibition activity against WiDr cells than the intracellular cells. Further, no significant differences between IC50 values of Lp-E and La-E or Lp-I and La-I. Unfortunately, the current study was not extended to the identification of compounds responsible for the inhibition properties against WiDr cells. Nevertheless, it is interesting to note that extracellular extracts (Lp-E and La-E) not only exhibited higher IC50 values but also higher protein content than the intracellular extracts (Lp-I and La-I). Accordingly, we assumed that the protein compounds of the extract are one of the bioactive compounds that are responsible for the inhibition properties. This hypothesis is supported by Liu et al. (2021) who previously indicated that protein is one of the anticancer substances from LAB. Two major anticancer proteins of BAL are S-layer protein (Zhang et al., 2020; Wu et al., 2019) and bacteriocin (Paiva et al., 2012; Norouzi et al., 2018; Hosseini et al., 2020; Prince et al., 2019). To note, both proteins were commonly found in extracellular fractions which might be explained why the extracellular extracts of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 exhibited higher inhibition activity against WiDr cells than the intracellular extracts. Nevertheless, non-protein substances may also contribute to the inhibition activity. Liu et al. (2021) and Choi et al. (2006) reported that non-protein substances of extracellular polysaccharides, nucleic acid, and peptidoglycan exhibited anticancer activity. Nevertheless, the anticancer substances that dominantly contributed to the anticancer activity of *L. plantarum* IIA-1A5 and *L. acidophilus* IIA-2B4 remain to be further identified and studied.

Table 4. IC_{50} value of different types of extracts.

Sample	IC_{50} µg/mL
Lp-I	12.01 + 1.89[a]
Lp-E	29.58 + 2.09[b]
La-I	13.99 + 0.94[a]
La-I	31.25 + 1.63[b]
Doxorubicin	5.74 + 0.37[c]

L.p. = *Lactobacillus plantarum*; *L.a.* = *Lactobacillus acidophilus*; I = intracellular; E = extracellular.

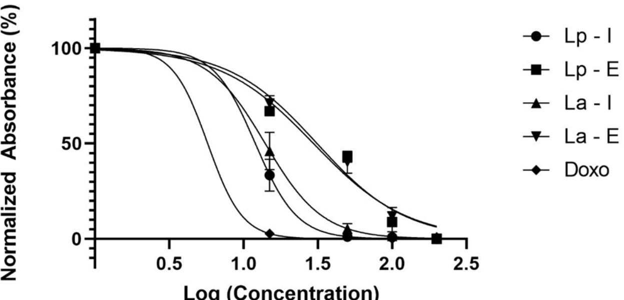

Figure 3. Relationship between normalized absorbance and log of concentration. (Lp-I) = *L. plantarum* intracellular extract; (Lp-E) = *L. plantarum* extracellular extract, (La-I) = *L. acidophilus* intracellular extract; (La-E) = *L. acidophilus* extracellular extract; (Doxo) = doxorubicin.

The ability of *L. plantarum* IIA-1A5 or *L. acidophilus* IIA-2B4 extracts to inhibit the WiDr cancer cells is in good agreement with the previous studies on anticancer activity of other BAL. Earlier, cell-free supernatant (extracellular extracts) of Lactobacilli were reported also to inhibit colorectal cancer cells (Kahouli et al., 2015; Rabiei et al., 2020). In addition, Atani et al. (2018) also reported about inhibitory effects of the cell wall (extracellular fraction) of *Lactobacillus reuteri* on HCT116 cell proliferation. Further, while we have confirmed that extracellular extracts exhibited higher activity against WiDr cells, it leads to the possibility of the food products fermented by *L. plantarum* IIA-1A5 or *L. acidophilus* IIA-2B4 to contain the anticancer substances secreted by these bacteria. This assumption is supported by previous studies on some fermented food products exhibiting anticancer activity. Camel milk fermented by indigenous LAB Lc.K782 exhibited antiproliferative activities against Caco-2, MCF-7, and HeLa cancer cell lines (Ayyash et al., 2018). Finally, Elfahri et al. (2016) observed that bovine milk fermented with selected *Lactobacillus helveticus* strains could inhibit the growth of HT-29 colon cancer cells.

4 Conclusion

In this study, extracellular and intracellular extracts of two Indonesian probiotics of *Lactobacillus plantarum* IIA-1A5 and *Lactobacillus acidophilus* IIA-2B were obtained and used to inhibit the WiDr colon cancer cells through *in vitro* approach. The extracts were found to have comparable pH and titrable acidity, yet with different protein concentrations. Further, both extracts exhibited anticancer activity against WiDr colon cancer cells in a concentration-dependent manner. Intracellular compound extracts, especially that of *L. plantarum* IIA-1A5, have a high rate to inhibit the growth of cancer cells, comparable to that of doxorubicin. Altogether, the bacteria and extracts are promising to be further studied for the development of anticancer prevention and treatments.

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