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Anti-Cancer Effect of Phosphatidylcholine Containing Conjugated Linoleic Acid at *sn*-2 Position on MCF-7 Breast Cancer Cell Line

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ABSTRACT

Conjugated linoleic acid (CLA) exhibits anti-cancer effects; however, most studies have only investigated its free fatty acid forms (CLA-FFA). Here, we compared the anticancer effect of CLA with its lipid forms (FFA and phosphatidylcholine [PC]) on the breast cancer cell line MCF-7 with Soy-PC as a control. CLA-FFA, CLA-PC, and Soy-PC caused a dose- and time-dependent inhibition of MCF-7 growth. However, the lowest antiproliferative activity was observed with CLA-PC. These results were consistent with Hoechst staining and protein expression of apoptosis markers Bax and Bcl-2. Interestingly, CLA-PC uptake was four times lower than that of Soy-PC, which may have affected its antiproliferative effect. Many papers showing that phospholipid forms of functional compounds have superior activity than intact forms have been reported, however, our results suggest that the PC form of CLA was inferior to its FFA form in inhibiting MCF-7 cell growth. This is the first report showing that the phospholipid form is not always the superior form.

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1. INTRODUCTION

Conjugated linoleic acid (CLA) is the positional and geometric isomer of linoleic acid (LA, C18:2 [n-6]). CLA has been studied for its anticarcinogenic activity since its discovery in 1987 (Ha *et al*., 1987). Later, it was reported to exert many other physiological functions, such as anti-obesity, anti-diabetic, and antihypertensive effects. Thus, CLA can be effective in preventing lifestyle-related diseases and metabolic syndromes (Koba & Yanagita, 2004). The United States Food and Drug Administration has approved CLA as "generally regarded as safe" for human consumption (Basak & Duttaroy, 2020). CLA is currently one of the most popular and commercially available nutraceuticals.

The World Health Organization has reported that cancer is the leading cause of death worldwide, accounting for nearly 10 million deaths in 2020, with breast cancer being the most common cancer, accounting for 2.26 million cases (Sung *et al*., 2021). Even in Japan, breast cancer shows the highest incidence and more than fourteen thousand deaths in 2020 (Cancer Information Service, National Cancer Center, Japan, 2020).

The anticarcinogenic activity of CLA has been demonstrated in a chemically induced rat mammary tumor model with an effective dietary range of 0.1–1% in the diet (Ip *et al*., 1991). This CLA content is ordinarily found in ruminant meat and its dairy products (Schmid *et al*., 2006). Additionally, CLA has been shown to exert anticancer effects against breast, colon, and prostate cancer cell lines (Maggiora *et al*., 2004). Ip *et al*. (2003) suggested that CLA may be an excellent candidate for preventing breast cancer (Ip *et al*., 2003). Thus, a lot of concern with CLA to prevent breast cancer has got attention.

The mechanism of anticarcinogenic effects of CLA in breast cancer cells is mostly mediated by the apoptosis pathway (Majumder *et al*., 2002; Rakib *et al*., 2013; Islam *et al*., 2008). Islam *et al*. reported that different isomers of CLA exert different anticancer effects (Rakib *et al*., 2013). It has been reported that *t,t*-CLA (a mixture of 1.2% *t*7,*t*9-CLA; 4.1% *t*8,*t*10-CLA; 43.5% *t*9,*t*11-CLA; 42.4% *t*10,*t*12-CLA; 5.0% *t*11,*t*13-CLA; and 3.8% *t*12,*t*14-CLA) exerted stronger anticancer activity than *c*9,*t*11-CLA, *t*10,*c*12-CLA, and LA.

The *t,t*-CLA treatment effectively induced a cytotoxic effect in a time-and concentration-dependent manner and increased the levels of tumor suppressor proteins p53 and Bax, but suppressed the expression of Bcl-2 protein. Similarly, Roz et al. reported that *t*9,*t*11-CLA was more efficient than *c*9,*t*11-CLA, and *t*10,*c*12-CLA in decreasing MCF-7 proliferation and inducing apoptosis after 24 h treatment (Le *et al*., 2019).

The majority of the studies investigating the anticancer effects of CLA used the free fatty acid (FFA) form. Phosphatidylcholine (PC) is commonly used as an emulsifier in food processing and as a component of drug delivery systems. Additionally, soy lecithin-derived liposomes are commonly used as drug delivery systems in cancer treatment, brain targeting, and vaccinology (Gandola *et al*., 2014). Gandola *et al*. (2014) reported that PC nanoparticles exerted mitogenic effects on MCF-7 cells (Gandola *et al*., 2014). Moreover, PC can be combined with certain nutrients to provide further health benefits (Li *et al*., 2015).

Thus, because phospholipid form has many advantages, we have studied phosphatidylation of functional compounds, such as terpenes, ricinoleic acid, and panthenol. Anti-cancer effects of terpenes such as geraniol, perillyl alcohol, myrtenol, and nerol were increased by phosphatidylation (Yamamoto *et al*., 2008).

The anti-inflammatory effects of ricinoleic acid and panthenol were also increased by phosphatidylation (Yamamoto *et al*., 2019;

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Yamamoto *et al*., 2020). However, no study has been reported that is showing a negative effect of the phosphatidylation.

Considering the use of PC in cancer treatment, we investigated the effects of the molecular form of CLA. We compared the individual effects of CLA in the FFA (CLA-FFA) form and PC-conjugated form (CLA-PC; with CLA at the *sn*-2 position) on proliferation and apoptosis in MCF-7 breast cancer cells, using commercially available Soy-PC as a control.

2. METHOD

2.1. Materials

CLA-FFA was commercially available (CLA Gold Gym™, Tokyo, Japan), whereas CLA-PC was prepared by phospholipase A_2-catalyzed esterification as reported previously (Fauziah *et al*., 2020). Both samples contained CLA as a mixture of *c*9,*t*11-CLA, and *t*10,*c*12-CLA at similar concentrations. Soy-PC and glycerophosphocholine (GPC) were purchased from Holstein Co., Ltd. (Tokyo, Japan). Choline and LA were purchased from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan).

Tocopherols in the samples were removed using activated charcoal. The fatty acid compositions of CLA-FFA, CLA-PC, and Soy-PC were analyzed using gas chromatography as reported previously (Dachev *et al*., 2021) and are summarized in **Table 1**. All solvents and other chemicals used were of analytical grade.

2.2. Cell Culture and Sample Treatment

The MCF-7 breast cancer cell line was obtained from the Japanese Collection of Research Bioresources (JCRB0134, Osaka, Japan) and cultured in Eagle's Minimum Essentials Media (EMEM, Sigma Aldrich, Osaka, Japan) supplemented with essential amino acids (Nacalai Tesque, Kyoto, Japan), solution of 100 U/mL penicillin and 100 mg/mL streptomycin (Fujifilm Wako Pure Chemical Corp.), and 10% fetal bovine serum (FBS, Sigma Aldrich, St Louis, MO, USA) at 37 °C in a humidified 5% CO_2 incubator.

Briefly, cells were grown to 80% confluence and subcultured following detachment with 0.1% trypsin-EDTA (Denka Seiken, Tokyo, Japan). The cells were subcultured or treated with the samples for further analysis. Samples were dissolved in ethanol and the final ethanol concentration in the culture medium was ≤0.1%. Soy-PC was used as the control for CLA-PC. Cells were treated with the sample after 24 h preculture.

2.3. Analysis

For easy understanding, a flowchart of the experiment in this study is illustrated in **Figure 1**.

Table 1. Fatty acid composition of cla-ffa, cla-pc, and soy-pc used in this study.

Fatty acid	CLA-FFA (%)	CLA-PC (%)	Soy-PC (%)
Palmitic acid	5.1 ± 0.6	36.1 ± 0.9	13.6 ± 0.1
Stearic acid	1.5 ± 0.1	12.1 ± 0.4	3.1 ± 0.0
Oleic acid	12.9 ± 0.6	8.1 ± 0.5	6.9 ± 0.0
LA	1.3 ± 0.4	ND	68.5 ± 0.1
*c*9,*t*11-CLA	36.6 ± 0.3	19.6 ± 0.8	ND
*t*10,*c*12-CLA	36.9 ± 0.7	16.6 ± 0.6	ND
Linolenic acid	ND	ND	7.9 ± 0.0
n.i.	5.7 ± 0.9	7.5 ± 1.1	ND

Abbreviations: n.i., not identified; CLA, conjugated linoleic acid; FFA, free fatty acid; PC, phosphatidylcholine; LA, linoleic acid. Data are expressed as the mean ± SD (n = 3).

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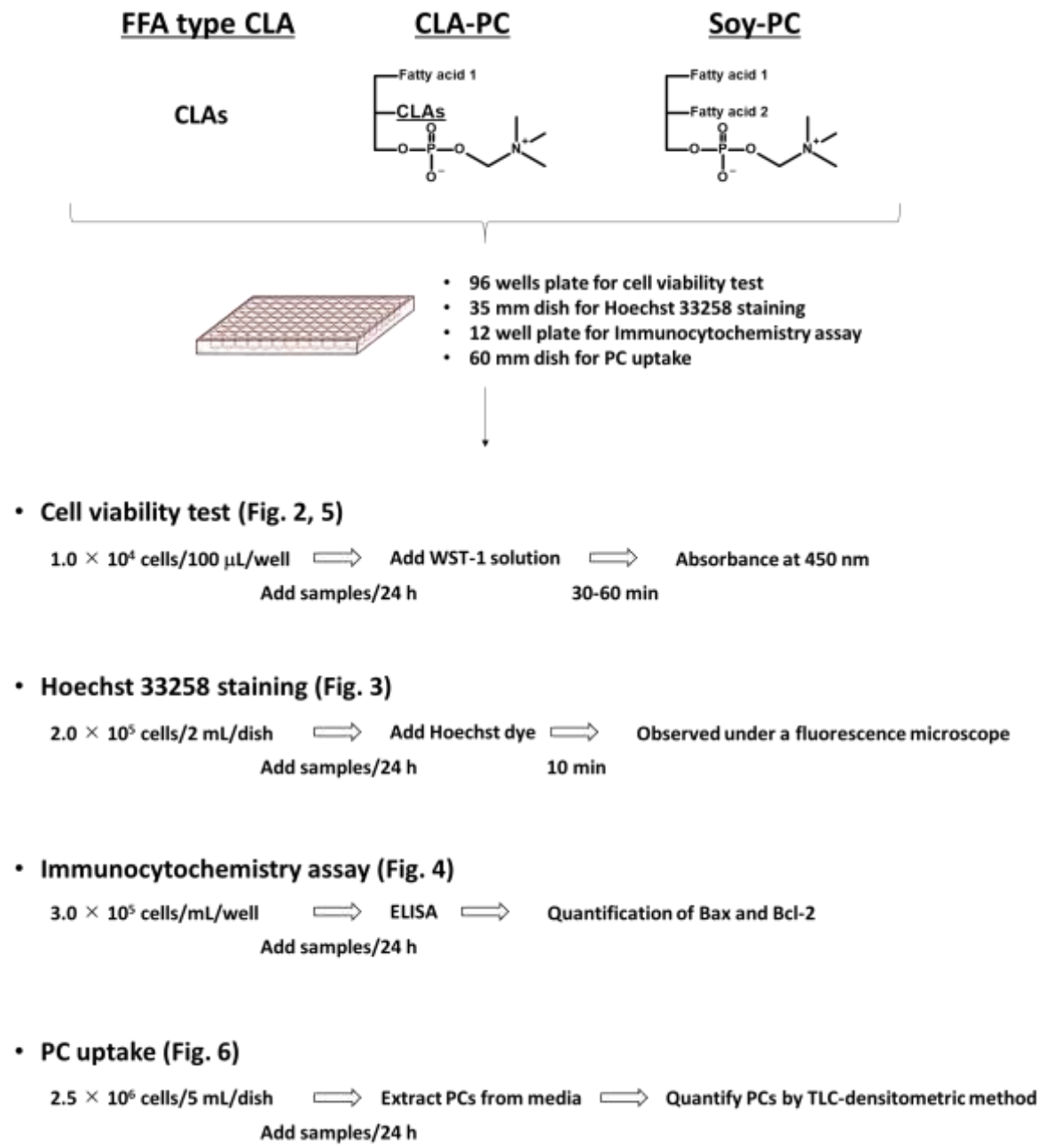

Figure 1. Flowchart of experimental of this study.

2.4. Cell Viability Test

Cell viability was assessed using 2- (4-Iodophenyl) -3- (4-nitrophenyl) -5- (2,4 disulfophenyl)-2H-tetrazolium sodium salt (WST-1, Fujifilm Wako Pure Chemical). MCF-7 cells were seeded at a density of 1.0×10^4 cells/well in 96-well microplates and cultured in 100 µL medium/well for 24 h. The samples (CLA-FFA, CLA-PC, and Soy-PC) were dissolved in ethanol at the desired concentrations and 10 µL of the ethanoic solution was added to each well. After 24 h incubation at 37 °C in a humidified 5% CO_2 incubator, 10 µL of WST-1 solution was added to each well and the plate was incubated for an additional 30–60 min. Cell viability was measured at 450 nm using a microplate reader (Varioskan, Thermo Fisher Scientific, Waltham, MA, USA) and calculated as a percentage of the control.

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2.5. Hoechest 33258 Staining

Treated cells were also stained with Hoechst 33258 to observe apoptosis. The cell density was 2.0 × 10^5 cells/2 mL/dish. Following 24 h treatment, the media was aspirated and cells were stained with 5 µg/mL Hoechst dye (Dojindo Laboratories, Kumamoto, Japan) for 10 min and incubated at 37 °C and 5% CO_2. The stain was aspirated and the cells were washed three times with D-PBS (Fujifilm Wako Pure Chemical Corp.) and observed under a fluorescence microscope (BZ-X700 Series; Keyence, Itasca, IL, USA).

2.6. Immunocytochemistry Assay of BAX and Bcl-2

Immunofluorescence analysis was performed to analyze the expression of Bax and Bcl-2 proteins using the Human Bax SimpleStep ELISA Kit and Bcl-2 Human ELISA Kit (both from Abcam, Cambridge, UK), respectively, as per the manufacturer's instructions. Cell culture conditions were similar to those used for WST-1 cell viability assay, except that the cells were at a density of 3 × 10^5 cells/mL/well in 12-well plates and treated with 200 µM of the samples.

2.7. Phosphatidylcholine (PC) Uptake

PC uptake analysis was performed to evaluate the availability of samples in the different PC forms. Cell culture conditions were similar to those used for WST-1 cell viability assay, except that the cells were seeded at a density of 5 × 10^5 cells/mL in 5-mL Petri dishes (60 mm) and treated with 200 µM of the samples.

PC content in the media was measured before and after 24 h treatment. PC in the media was extracted using chloroform and methanol at chloroform:methanol:water ratio of 3:10:5 (v/v/v) and until PC separated and evaporated into the chloroform layer. PC content was then measured using silica gel thin-layer chromatography (TLC), followed by quantification using the ImageJ software (https://imagej.nih.gov).

First, a standard curve was prepared using Soy-PC and oleic acid (C18:1, Fujifilm Wako Pure Chemical Corp) as the internal standards in TLC plates (Merck, Darmstadt, Germany). Then, an equation with the weight ratio (Soy-PC/C18:0) and area ratio (Soy-PC/C18:1) was defined: y = 0.4973x + 0.1528, R^2 = 0.9944. Chloroform:methanol:water (65:25:4, v/v/v) was used as the mobile phase, and I_2 vapor was used for detection. The resulting spots were converted to gray-colored 16-bit images and their densities were quantified. Finally, the PC uptake was quantified using Eq. (1):

PC uptake (µg/plate) = Blank – remained PC (1)

where "Blank" is the PC content obtained from the media-only with added PCs.

2.8. Statistical Analysis

All experiments were performed in triplicates. Data are expressed as the mean ± standard deviation (SD). Significances were determined using Scheffe's test ($P < 0.01$).

3. RESULTS AND DISCUSSION

3.1. Growth Inhibition of Treatments on MCF-7 Cells

The inhibitory effects of the different molecular forms of CLA on cell growth were evaluated at various concentrations (50–200 µM). All samples inhibited the growth of MCF-7 cells in a dose- and time-dependent manner (**Figure 2**). Although Soy-PC showed a similar inhibitory effect to that observed with 200 µM CLA-FFA, CLA-FFA was more effective at inhibiting cell growth than Soy-PC. The lowest inhibitory effect was observed with CLA-PC. It was surprising that CLA-PC, which is phosphatidylated CLA, showed a lower effect than CLA-FFA, which is before phosphatidylation. As mentioned in the introduction section, several reports have shown that phosphatidylated functional compounds exert higher physiological activity than that before phosphatidylation

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(Yamamoto *et al*., 2008; Yamamoto *et al*., 2019; Yamamoto *et al*., 2020). The reason why phospholipid form shows superior activity have supposed that phospholipid form has higher solubility to the media and affinity to the cell membrane (Takami and Suzuki, 1994). However, the results obtained by this study showed the opposite effect.

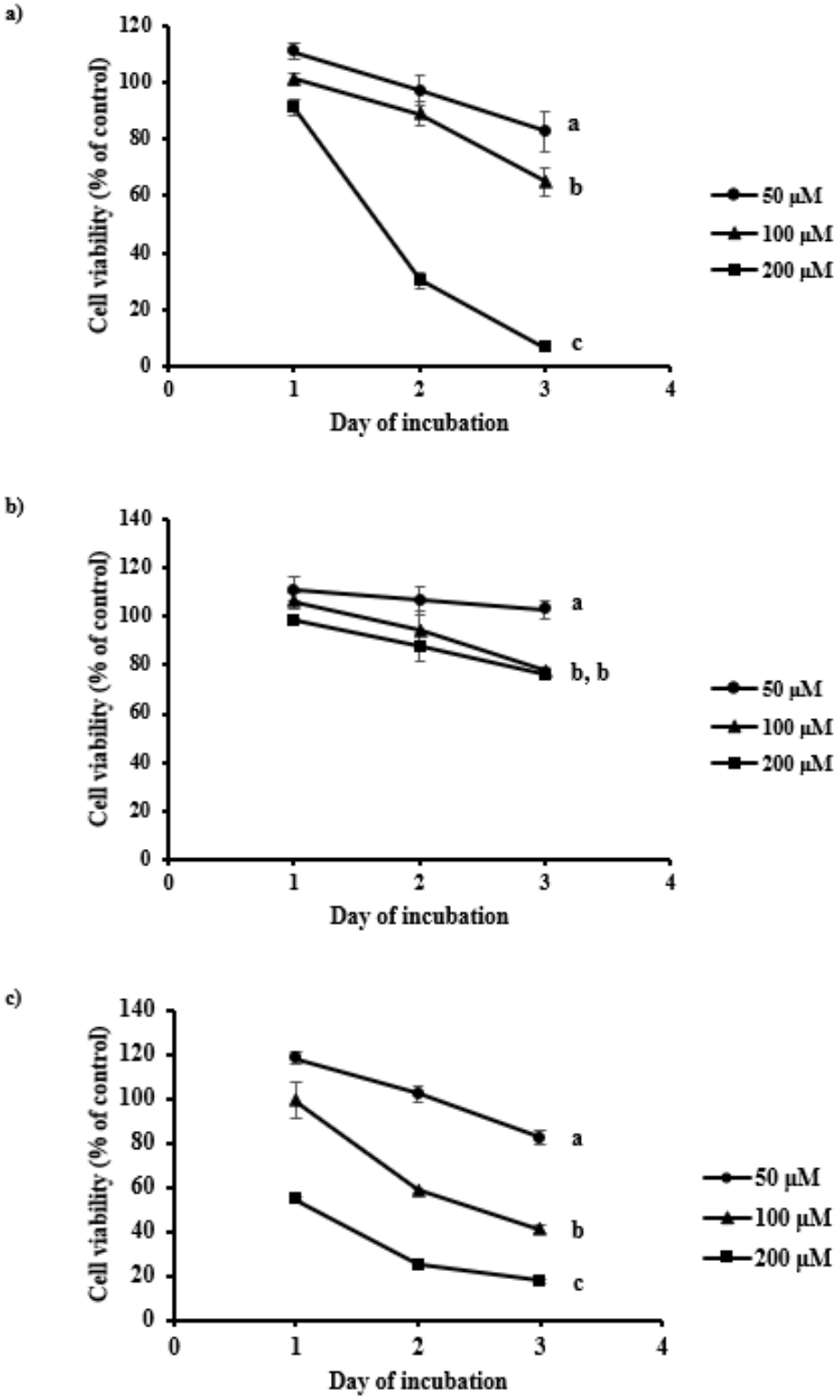

Figure 2. Dose- and time-dependent effect of (a) conjugated linoleic acid (CLA)- free fatty acid (FFA), (b) CLA- phosphatidylcholine (PC), and (c) Soy-PC on the growth of MCF-7 cells. Values are presented as a percentage (%) of the control. Different letters represent significant difference between the groups ($P < 0.01$, Scheffe's test).

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3.2. Induction of Cell Apoptosis

Apoptosis is one of the most common mechanisms underlying the anticarcinogenic effect of CLA. The mechanism of apoptosis-mediated growth inhibition of MCF-7 cells by CLAs was illustrated in **Figure 3**.

Hoechst staining was performed to observe apoptosis following treatment with CLA-FFA, CLA-PC, and Soy-PC. Hoechst stain would be intact with the DNA of the cell, so when apoptosis occurred, the cells shrunk and condensed nuclei appeared under the fluorescence microscope as blue light (Islam *et al.*, 2008). The highest number of apoptotic cells was observed in CLA-FFA-treated cells, followed by Soy-PC- and CLA-PC-treated cells (**Figure 4**).

To further investigate apoptosis, an immunocytochemistry assay was performed to analyze the protein expression of the prop-apoptotic Bax and anti-apoptotic Bcl-2. The expression of Bax and Bcl-2 proteins affects apoptosis in MCF-7 cells (Kudo and Murakami, 1999). The reciprocal expression of these proteins is used to determine whether the observed cell death is due to apoptosis (Majumder *et al.*, 2002; Rakib *et al.*, 2013; Islam *et al.*, 2008).

In the present study, CLA-FFA showed the highest Bax and lowest Bcl-2 expression (**Figure 5**). This is consistent with our finding that CLA-FFA is the most effective in inhibiting the growth of MCF-7 cells compared to CLA-PC and Soy-PC. Similarly, Islam et al. reported that *t,t*-CLA treatment increased the expression of Bax, but suppressed the expression of Bcl-2 (Islam *et al.*, 2008).

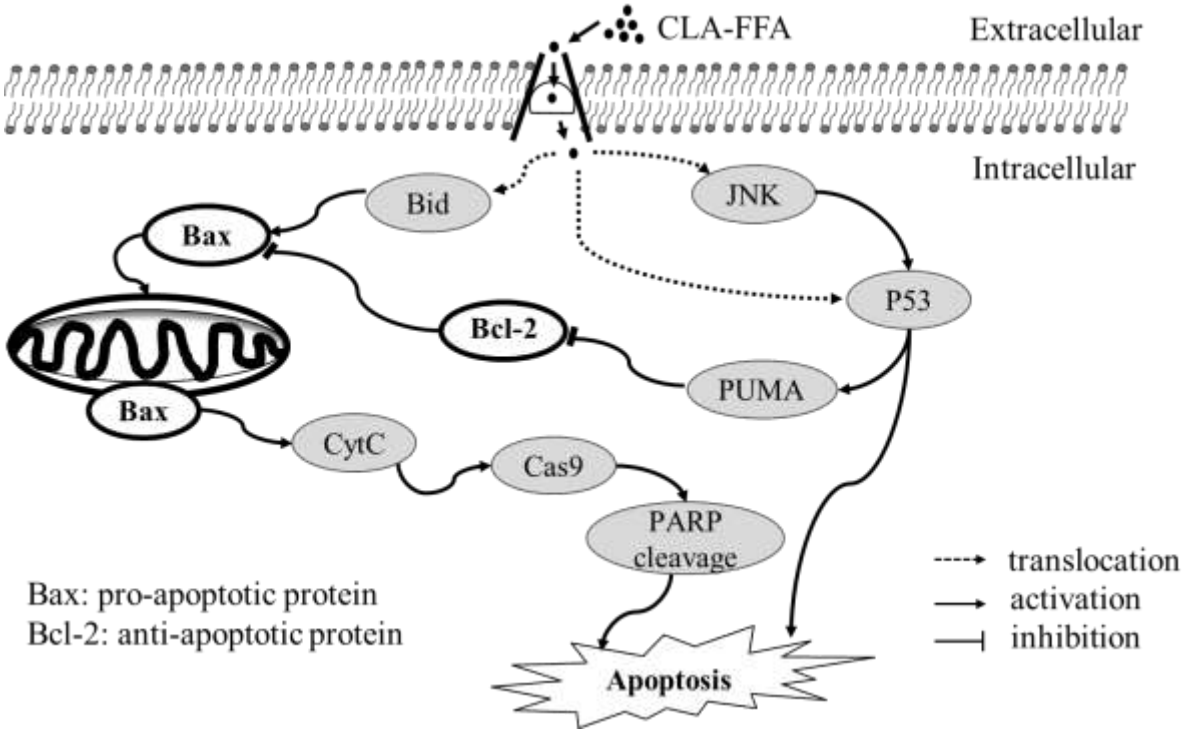

Figure 3. Mechanism of apoptotic-mediated growth inhibition of MCF-7 cells by CLAs (modified from Koronowicz *et al.*)

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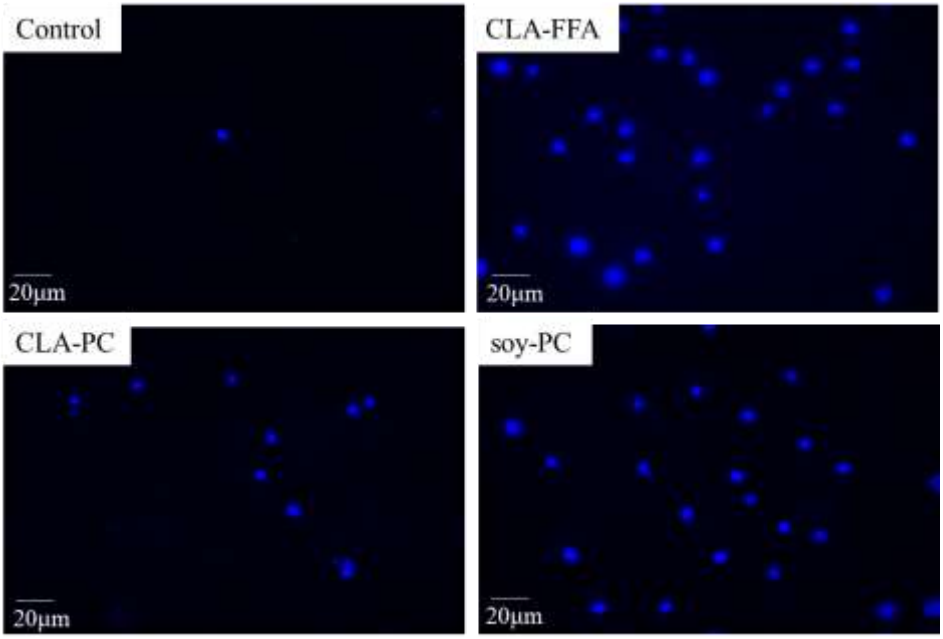

Figure 4. Hoechst stained MCF-7 cells treated for 24 h with 200 µM CLA-FFA, CLA-PC, and Soy-PC. Abbreviations: CLA, conjugated linoleic acid; FFA, free fatty acid; PC, phosphatidylcholine.

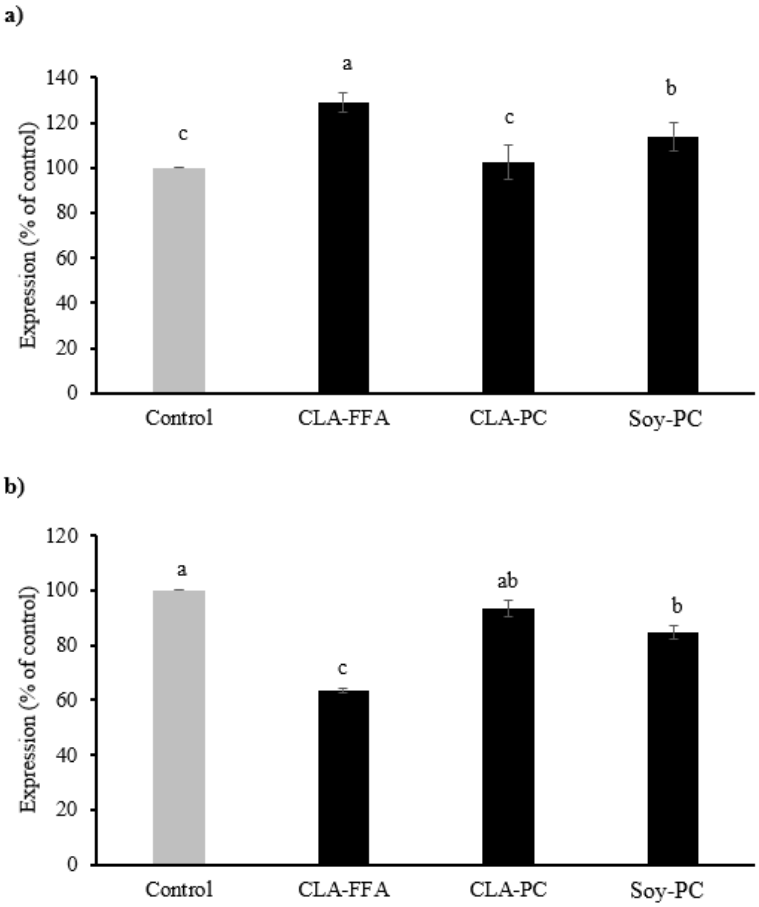

Figure 5. Protein expression of (a) Bax and (b) Bcl-2 in MCF-7 cells treated with 200 µM CLA-FFA, CLA-PC and Soy-PC. Values are presented as a percentage (%) of the control. Different letters represent significant difference between the groups ($P < 0.01$, Scheffe's test). Abbreviations: CLA, conjugated linoleic acid; FFA, free fatty acid; PC, phosphatidylcholine.

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3.3. Effect of PC Component on MCF-7 Cells Growth

To determine which component of Soy-PC caused the observed inhibition of MCF-7 growth, the effect of individual PC components, choline, glycerol, GPC, and LA, on MCF-7 cell growth was investigated. CLA-FFA, CLA-PC, and Soy-PC were also used for comparison. As shown in **Figure 6**, choline, glycerol, and GPC did not significantly inhibit MCF-7 cell growth, and only LA, the major fatty acid in Soy-PC, inhibited MCF-7 growth; however, the inhibition was not as strong as that of CLA-FFA. It was suggested that Soy-PC, even though it did not contain CLA, could inhibit MCF-7 growth because it contained LA, which is the component that inhibits MCF-7 cell growth. Because Soy-PC was more easily taken up by the cell than CLA-PC (as shown later), the effect of LA, a component of Soy-PC, is stronger than that of CLA, a component of CLA-PC. LA might be released from PC at cell/perinuclear membranes by cytosolic/Ca^{2+}-independent phospholipase A_2-mediated hydrolysis (Kudo and Murakami, 1999).

3.4. PC Uptake by MCF-7 Cells

Inhibition of MCF-7 cell growth was higher with Soy-PC treatment than with CLA-PC treatment (**Figure 2, 5**). To gain insight into this phenomenon, PC uptake in MCF-7 cells treated with CLA-PC and Soy-PC was monitored. Richard and Richard reported that PC itself is supposed to be incorporated into the cell membrane followed by internalization through endocytosis and localized in the endoplasmic reticulum and Golgi apparatus in the experiment using Chinese hamster fibroblasts (Richard & Richard, 1984).

Further, Villa et al. also reported that PC is incorporated into the cell membrane, internalized, and localized in the endoplasmic reticulum of MCF-7 cells using a PC-fluorescent derivative as a sample (Villa *et al.*, 2005). Interestingly, the uptake in CLA-PC-treated cells was significantly lower (four times lower) than that in Soy-PC-treated cells (**Figure 7**). Therefore, it can be assumed that CLA-PC exerts lower inhibition than Soy-PC on MCF-7 growth because of lower CLA-PC.

Additionally, as shown in **Table 1**, the concentration of CLA in CLA-FFA and LA in Soy-PC was approximately 70%, whereas the concentration of CLA in CLA-PC was only 36.2%; i.e., CLA content in CLA-PC was half of that in CLA-FFA and half of the LA content in Soy-PC. This difference may have affected the effectiveness of MCF-7 growth inhibition.

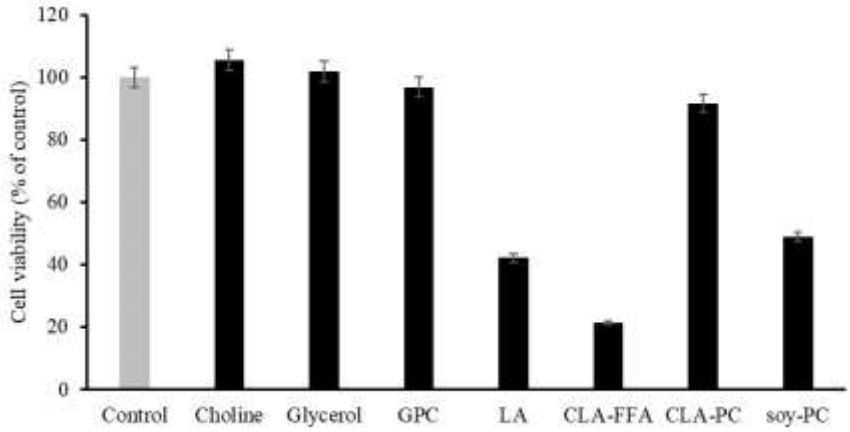

Figure 6. Cell viability of MCF-7 cells treated with PC components (Choline, Glycerol, GPC, and LA) compared to treatment with 200 µM CLA-FFA, CLA-PC, and Soy-PC. Values are presented as the mean ± SD, a percentage (%) of the control. Different letters represent significant difference between the groups ($P < 0.01$, Scheffe's test). Abbreviations: CLA, conjugated linoleic acid; FFA, free fatty acid; PC, phosphatidylcholine.

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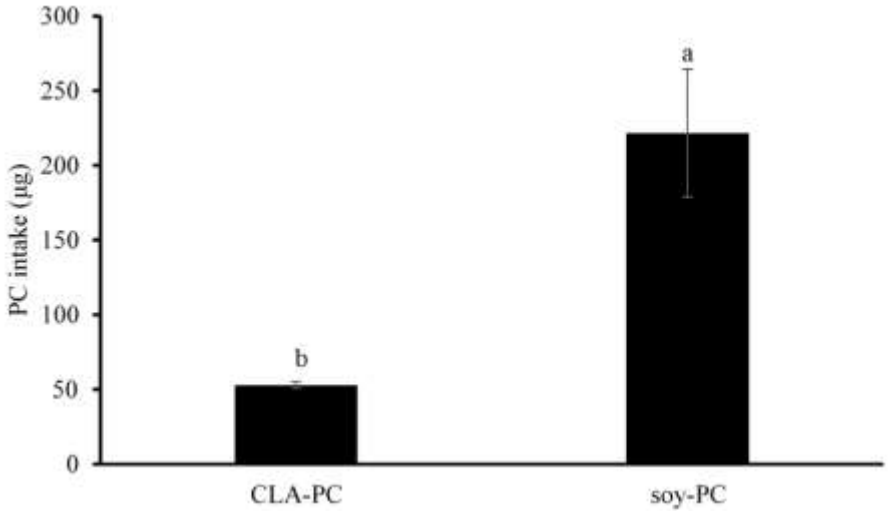

Figure 7. PC uptake in MCF-7 cells treated with 200 µM CLA-PC and Soy-PC. Values are presented as a percentage (%) of the control. Different letters represent significant difference between the groups ($P < 0.01$, Scheffe's test). Abbreviations: CLA, conjugated linoleic acid; PC, phosphatidylcholine.

4. CONCLUSION

In the present study, treatment with CLA-FFA, CLA-PC, and Soy-PC inhibited the growth of MCF-7 cells in a dose- and time-dependent manner. CLA and its phosphatidylated form, CLA-PC exerted an antiproliferative effect on MCF-7 cells. Although the underlying mechanism was the same, the degree of the effect varied; CLA-FFA was more effective than CLA-PC in inhibiting MCF-7 cell growth, as CLA-PC was associated with lower cellular uptake. Further studies are required to elucidate why the cellular uptake of CLA-PC is lower than CLA-FFA in MCF-7 cells.

5. ACKNOWLEDGMENTS

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6. AUTHORS' NOTE

The author(s) declare(s) that there is no conflict of interest regarding the publication of this article. The authors confirmed that the data and the paper are free of plagiarism.

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