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Growth inhibition and apoptosis induction by all *trans* conjugated linolenic acids on human colon cancer cells.

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Keywords: Conjugated linolenic acid isomers, β-eleostearic acid, β-calendic acid, growth inhibition, apoptosis, Caco-2 cells,

Abstract. *Conjugated linolenic acids (CLN) are geometric and positional isomers of linolenic acid. We compared growth inhibition and apoptosis induction by α-eleostearic acid (c9,t11,t13-CLN), β-eleostearic acid (t9,t11,t13-CLN), α-calendic acid (t8,t10,c12-CLN), and β-calendic acid (t8,t10,t12-CLN). β-Eleostearic acid and β-calendic acid, which have all-trans-conjugated double bonds, exerted stronger growth inhibition and more DNA fragmentation, which is an indicator of apoptosis induction, in human colon cancer cells Caco-2 than α-eleostearic acid and α-calendic acid with cis configuration. Down-regulation of bcl-2 and up-regulation of bax mRNA by β-eleostearic acid was also greater than that by α-eleostearic acid. Interestingly, cytotoxic effects of β-eleostearic acid and β-calendic acid were not counteracted completely by α-tocopherol, whereas cytotoxic effects of α-eleostearic and α-calendic acids were lost in the presence of α-tocopherol. These results suggest that β-eleostearic acid and β-calendic acid have signaling pathways different from α-eleostearic and α-calendic acids to exhibit high potency for reducing cell viability on Caco-2.*

The term “conjugated fatty acids” is a generic descriptor of polyunsaturated fatty acids with conjugated double bonds in the molecule. Conjugated linoleic acids (CLAs) are known to have many health benefits as anti-cancer (1-3), anti-obesity (4,5) and anti-atherosclerosis effects (6,7). Conjugated linolenic acids (CLNs), which are geometric and positional isomers of linolenic acid, are also found in high concentrations in some kinds of plant seed. For example, α-eleostearic acid (*c*9,*t*11,*t*13-CLN) and α-calendic acid (*t*8,*t*10,*c*12-CLN) are contained in bitter gourd seed oil (BGO) and pot marigold seed oil, respectively (8). We recently demonstrated that dietary BGO, which contains α-eleostearic acid, remarkably inhibits the development of AOM-induced colonic aberrant crypt foci (ACF) (9) and adenocarcinoma in F344 rats (10). Furthermore, we and others have reported that free fatty acid prepared from BGO induced apoptosis in colon cancer cells (11, 12).

On the other hand, β-eleostearic acid (*t*9,*t*11,*t*13-CLN) and β-calendic acid (*t*8,*t*10,*t*12-CLN), with all-*trans*-conjugated double bonds, are also known to be contained in some seed oils as minor fatty acids (8). Furthermore all-*trans*-CLN isomers are found in mixtures of CLNs chemosynthesized by

alkaline isomerization of linolenic acid (13). Recently, Igarashi reported that β-eleostearic acid has a stronger antiproliferative effect than α-eleostearic acid (13). However, there has been no report on apoptosis induction by all-*trans*-CLN isomers. To characterize the anticancer effects of all-*trans*-CLN isomers in detail, we performed the first study on the apoptosis induction by β-eleostearic and β-calendic acids in comparison with α-eleostearic and α-calendic acids. Furthermore, we show that the effects of the antioxidant α-tocopherol on reducing cell viability on Caco-2 cells differ among all-*trans* CLN isomers and other isomers with the *cis* configuration.

Materials and Methods

Materials. α-Eleostearic acid (*c*9,*t*11,*t*13-CLN, >98% purity), β-eleostearic acid (*t*9,*t*11,*t*13-CLN, >97% purity), α-calendic acid (*t*8,*t*10,*c*12-CLN, >98% purity) and β-calendic acid (*t*8,*t*10,*t*12-CLN, >97% purity) (Fig. 1) were purchased from Larodan Fine Chemicals AB, Sweden. WST-1 (2-(4-Lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt) was purchased

from Wako Chemical Co. (Tokyo, Japan).

Cell culture. Human colon cancer cells line, Caco-2 (ATCC HTB-37) was obtained from the American Type Culture Collection (Rockville, MD, USA). Caco-2 cells were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acid, 100 U/mL penicillin and 100 μg/mL streptomycin. Cell cultures were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 ^{o}C.

Cell viability assay. Caco-2 cells ($2x10^3$ cells) were preincubated in 96-well plates with 100 μL medium per well for 24 h. Each CLN isomers were dissolved in ethanol and were then added into the culture medium at final concentration up to 50 μM. The final concentration of ethanol was below 0.1% (v/v). Caco-2 cells were incubated for 21 h to 45 h in the cultured medium containing CLN with/without α-tocopherol at 2.5 μM~50 μM. Then, 10 μL of WST-1 solution (14) was added into each well, and the culture plate was

incubated for additional 3 h. A number of viable cells were measured at 450 nm. Viability was expressed as a percentage to the viable cells of control culture.

Measurement of DNA fragmentation. DNA fragmentation was measured as an indicator of apoptotic cells using a commercial kit (Cell Death Detection ELISA^{PLUS}, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. The assay is based on a quantitative sandwich enzyme immunoassay to detect the histone-associated DNA fragments produced during apoptosis. Cell culture conditions were the same as in cell viability assay.

Extraction of total RNA. Caco-2 cells ($5x10^5$ cells) were cultivated in 100 mm tissue culture dish for 24 h, and each CLN isomer in ethanol was then added into culture dish. Total RNA was extracted from Caco-2 cells by an acidic phenol method and further purified by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA) according to the manufacture's instructions.

Real time quantitative RT-PCR analysis. Total RNA was reverse-transcribed by High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Then, one microliter of cDNA solution (adequate concentration) was mixed to 1.25 μL TagMan prob, 12.25 μL TaqMan Master Mix, 10.25 μL water and quantitative RT-PCR reaction was performed in an ABI PRIZM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal cycling conditions were as follows: 48 °C for 30 min to prevent carrying over of DNA, an initial denaturation of 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and an annealing temperature of 55 °C for 1 min. TaqMan probes, *bcl-2* (Hs99999903_m1), *bax* (Hs00608023_m1) and *β-actin* (Hs00180269_m1), used in this study were purchased from Applied Biosystems (Foster City, CA, USA).

Statistical analysis. Data are expressed as means ± SD. Statistic analysis between two groups (Fig. 2) was determined using the unpaired Student's *t*-test. Differences with $P<0.05$ were

considered significant. Statistic analyses between multiple groups were determined by Holm's test (P<0.05) (Fig. 3,4,5).

Results

Effect of CLN isomers on cell viability of Caco-2. To compare the cytotoxic effects of CLN isomers, Caco-2 cells were incubated in culture medium containing α-eleostearic acid, β-eleostearic acid, α-calendic acid, and β-calendic acid (Fig. 1). As shown in Fig. 2, each CLN isomer exerted strong cytotoxic effect against Caco-2 cells in a dose- and time-dependent manner. Especially, β-eleostearic acid and β-calendic acid isomers, which have the all-*trans* configuration, showed stronger cytotoxic effects than α-eleostearic acid and α-calendic acid isomers, which have the *cis* configuration, at concentrations of 3.125 μM to 12.5 μM after 24 h incubation. Also at 48 h incubation, β-eleostearic acid and β-calendic acid remarkably reduced cell viability compared to α-eleostearic acid and α-calendic acid at 3.125 μM and 6.25 μM.

Comparison of apoptosis induction in Caco-2 cells by CLN isomers. We measured DNA fragmentation in Caco-2 cells incubated in culture medium containing CLN isomers as an indicator of apoptosis induction. All of the CLN isomers used in this study induced DNA fragmentation in Caco-2 cells in a dose-dependent manner during 48 h incubation (Fig. 3). Relative DNA fragmentation, induced by 6.25 μM and 12.5 μM β-eleostearic acid, increased to 3.5-fold and 6.9-fold level of the control after 48 h incubation, while DNA fragmentation by α-eleostearic acid was 1.5-fold and 4.9-fold level of the control, respectively. β-Calendic acid also induced more DNA fragmentation in Caco-2 cells than did α-calendic acid at 6.25 μM, although DNA fragmentation induced by 12.5 μM α-calendic acid and β-calendic acid were the same level. Thus, β-eleostearic acid and β-calendic acid induced more DNA fragmentation than did α-eleostearic acid and α-calendic acid, respectively. This greater fragmentation corresponds to the reduction of cell viability by each CLN isomer. Further, down-regulation of *bcl-2* mRNA, which is one of anti-apoptotic genes, was observed in Caco-2 cells treated with 10 μM β-eleostearic acid for 24 h (Fig. 4). β-Eleostearic acid also up-regulated pro-apoptotic *bax* mRNA up to 1.4-fold in Caco-2 cells, while

α-eleostearic acid did not affect *bax* mRNA expression. The greater DNA fragmentation and regulation of apoptosis-related genes corresponded to the reduction of cell viability by each CLN isomer.

Influence of α-tocopherol on reducing cell viability of Caco-2 by CLN isomers. In previous studies, we and others have reported that the cytotoxic effect of and apoptosis induction by α–eleostearic acid are induced through intercellular lipid peroxidation (12, 15, 16). To investigate the mechanisms underlying the cytotoxic effects by all-*trans*-CLN isomers, we incubated Caco-2 cells with CLN isomers and α-tocopherol. When 25 μM α-tocopherol was added to culture medium, the reduction of cell viability by 50 μM α-eleostearic acid disappeared completely, as found in previous reports (Fig. 4) (12, 16). On the other hand, the cytotoxic effect of 50 μM β-eleostearic acid was observed even in the presence of 25 μM α-tocopherol, although the viability of Caco-2 cells was partially restored, to 50% from 0.7%, by the addition of α-tocopherol (Fig. 5).

Furthermore, we examined the effect of α-tocopherol on the growth inhibition effects of CLN isomers in detail (Fig. 6 (a)). The viability of Caco-2

cells treated with 25 μM β-eleostearic acid increased as the α-tocopherol concentration increased. However, cell viability was plateau at 70% and the cytotoxic effect of β-eleostearic acid was not restored completely by the addition of α-tocopherol. The cytotoxic effect of 25 μM β-calendic acid also remained in the presence of α-tocopherol (Fig. 6 (b)). In contrast, cytotoxic effects induced by 25 μM α-eleostearic acid and α-calendic acid were lost completely by the addition of α-tocopherol (Fig. 6 (a, b)).

Discussion

The present study demonstrated that two all-*trans*-CLN isomers, β-eleostearic and β-calendic acids, have more-potent cytotoxic effects and higher levels of apoptosis induction in Caco-2 cells than do α-eleostearic and α-calendic acids, which are CLN isomers possessing the *cis* configuration. Furthermore, β-eleostearic acid and β-calendic acid showed cytotoxic effects even in the presence of α-tocopherol, whereas α-tocopherol counteracted the cytotoxic effects of α-eleostearic acid and α-calendic acid.

CLNs are found at high concentrations in the oils of certain kinds of seeds

(8). α-Eleostearic and α-calendic acids are major fatty acids of bitter gourd seed oil and pot marigold seed oil, respectively. In addition, β-eleostearic acid and β-calendic acid, which have all-*trans*-conjugated double bonds, are found in some kinds of seed oils (8). Moreover, all-*trans*-CLN isomers are produced by alkaline isomerization of linolenic acid (13). In previous studies, we and others have reported that α-eleostearic acid and α-calendic acid exhibit strong cytotoxic effects on human monocytic leukemia cells (U-937) (15) and colon cancer cells (12, 16). Recently, Igarashi et al. reported that β-eleostearic acid inhibits the growth of human tumor cell lines, and that its effect is stronger than that of α-eleostearic acid (13). However, there are no detailed reports about the antiproliferative effect and apoptosis induction by all-*trans*-CLN isomers. In the current study, we demonstrated for the first time that two all-*trans*-CLN isomers, β-eleostearic acid and β-calendic acid, induce apoptosis in Caco-2 cells, and their apoptosis activities are higher than those of α-eleostearic acid and α-calendic acid, which have the *cis* configuration. Furthermore, down-regulation of *bcl-2* mRNA and up-regulation of *bax* mRNA in Caco-2 cells by β-eleostearic acid were greater than those by α-eleostearic acid. Bcl-2 and Bax proteins are known to play as

anti-apoptotic and pro-apoptotic factors in mitochondria, respectively (17). These results indicate that the configuration of conjugated double bonds is important in cytotoxic effect and apoptosis induction against Caco-2 cells. In particular, all-*trans*-CLN isomers were more effective chemotherapeutic compounds.

The mechanism underlying the cytotoxic effect and apoptosis induction by α-eleostearic acid is supposed to involve lipid peroxidation, because the antioxidant α-tocopherol diminished the growth inhibition and apoptosis induction by α-eleostearic acid on colon cancer cell lines. In the present study, we also observed that the cytotoxic effect and apoptosis induction by α-eleostearic acid and α-calendic acid were completely suppressed by the addition of α-tocopherol in the culture medium, as found in previous reports (*12, 14, 15*). It is noteworthy that the cytotoxic effect of β-eleostearic acid and β-calendic acid, which have all-*trans*-conjugated double bonds, were not counteracted completely in the presence of α-tocopherol (Fig. 5, 6). These results suggest that β-eleostearic acid and β-calendic acid reduce cell viability of Caco-2 *via* another pathway in addition to the pathway of lipid peroxidation. On the other hand, the cytotoxic effect of β-eleostearic acid on

DLD-1, another colon cancer cell line, was lost by the addition by α-tocopherol (data not shown). Thus, the metabolic and signal transduction systems in Caco-2 cells may be important to exhibit different anticancer effects among CLN isomers. Further investigations are required for a better understanding of the specific mechanisms underlying the cytotoxic effect and apoptosis induction by β-eleostearic and β-calendic acids.

In summary, two all-*trans*-CLN isomers, β-eleostearic and β-calendic acids, markedly reduced cell viability and induced apoptosis in Caco-2 cells. Their effects were stronger than those of α-eleostearic and α-calendic acids, which have the *cis* configuration. Furthermore, since the cytotoxic effect of β-eleostearic and β-calendic acids on Caco-2 cells were not suppressed completely by α-tocopherol, CLN isomers with the all-*trans* configuration are suggested to exert anticancer effects through signaling pathways different from that of the CLN isomers having the *cis* configuration.

Acknowledgment

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Figure legends

Fig. 1. Structure of CLN isomers used in this study.

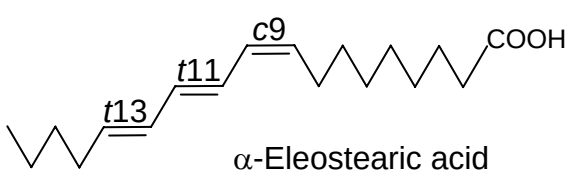
Fig. 2. Effect of CLN isomers on cell viability of Caco-2. Caco-2 cells were incubated in the medium with α-eleostearic acid, β-eleostearic acid, α-calendic acid, and β-calendic acid for 24 h or 48 h after 24 h preincubation. Cell viability was measured by WST assay as described in Materials and Methods. Presented data are shown as cell numbers relative to control. All data are expressed as means ± SD of six experiments. $^{*}P<0.05$ vs. Caco-2 cells treated with α-eleostearic acid and α-calendic acid at each concentration.

Fig. 3. DNA fragmentation in Caco-2 cells treated with CLN isomers. Caco-2 cells were incubated in culture medium with α-eleostearic acid, β-eleostearic acid, α-calendic acid, and β-calendic acid at 6.25 μM or 12.5 μM for 48 h after 24 h preincubation. DNA fragmentation was measured by a sandwich enzyme immunoassay using anti-histone antibody and anti-DNA antibody. DNA fragmentation is relative to the assigned control value of 1.0. Values are means ± SD, n=3. The values with different letters were significantly different from each other. $P<0.05$ (Scheffe's F-test)

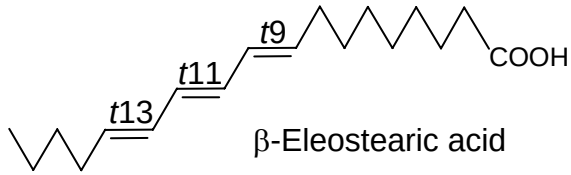
Fig. 4. Expression level of *bcl-2* and *bax* mRNA in Caco-2 cells treated with α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 10 μM α-eleostearic acid or β-eleostearic acid for 24 h, respectively. The expression level of *bcl-2* and *bax* mRNA was estimated by real time RT-PCR. Data from three independent experiments were normalized to β-actin mRNA level and are shown as the means ± SD. The values with different letters were significantly different from each other. $P<0.05$ (Scheffe's F-test)

Fig. 5. Effect of α-tocopherol on antiproliferation by α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 50 μM CLN isomers with/without 25 μM α-tocopherol for 48 h. Cell viability was measured by WST assay described in Materials and Methods. Data represent cell viability expressed as a percentage of the control, which was taken to be 100%. Values are means ± SD, n=6. The values with different letters were significantly different from each other in no α-tocopherol group and 25 μM α-tocopherol group. $P<0.05$ (Scheffe's F-test)

Fig. 6. Comparison of antiproliferation by CLN isomers in the presence of α-tocopherol. (a) Caco-2 cells were treated with 25 μM α-eleostearic acid, β-eleostearic acid, and α-tocopherol after 24 h preincubation. Values are means ± SD, n=6. (b) Caco-2 cells were treated with 25 μM α-calendic acid, β-calendic acid and α-tocopherol after 24 h preincubated. Presented data are shown as cell numbers relative to control. Values are means ± SD, n=6.



α-Eleostearic acid



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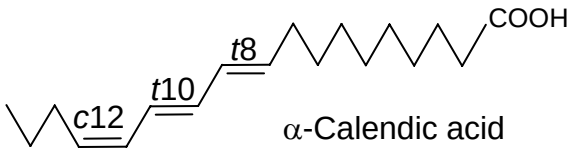
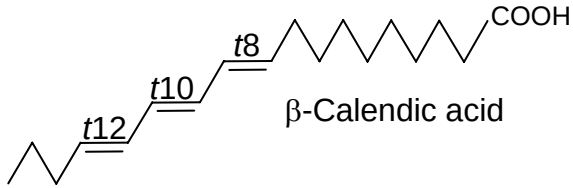
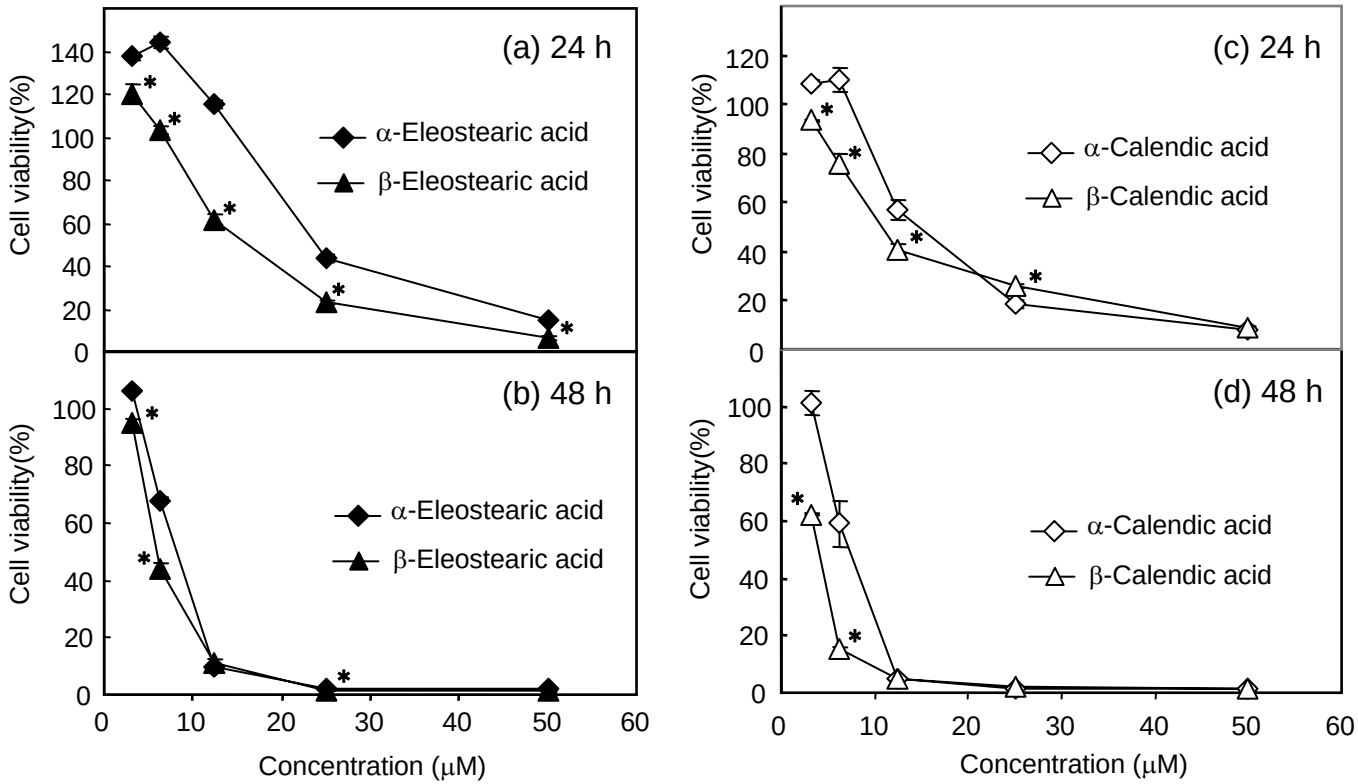


α-Calendic acid



β-Calendic acid

Fig. 1 Yasui et al.



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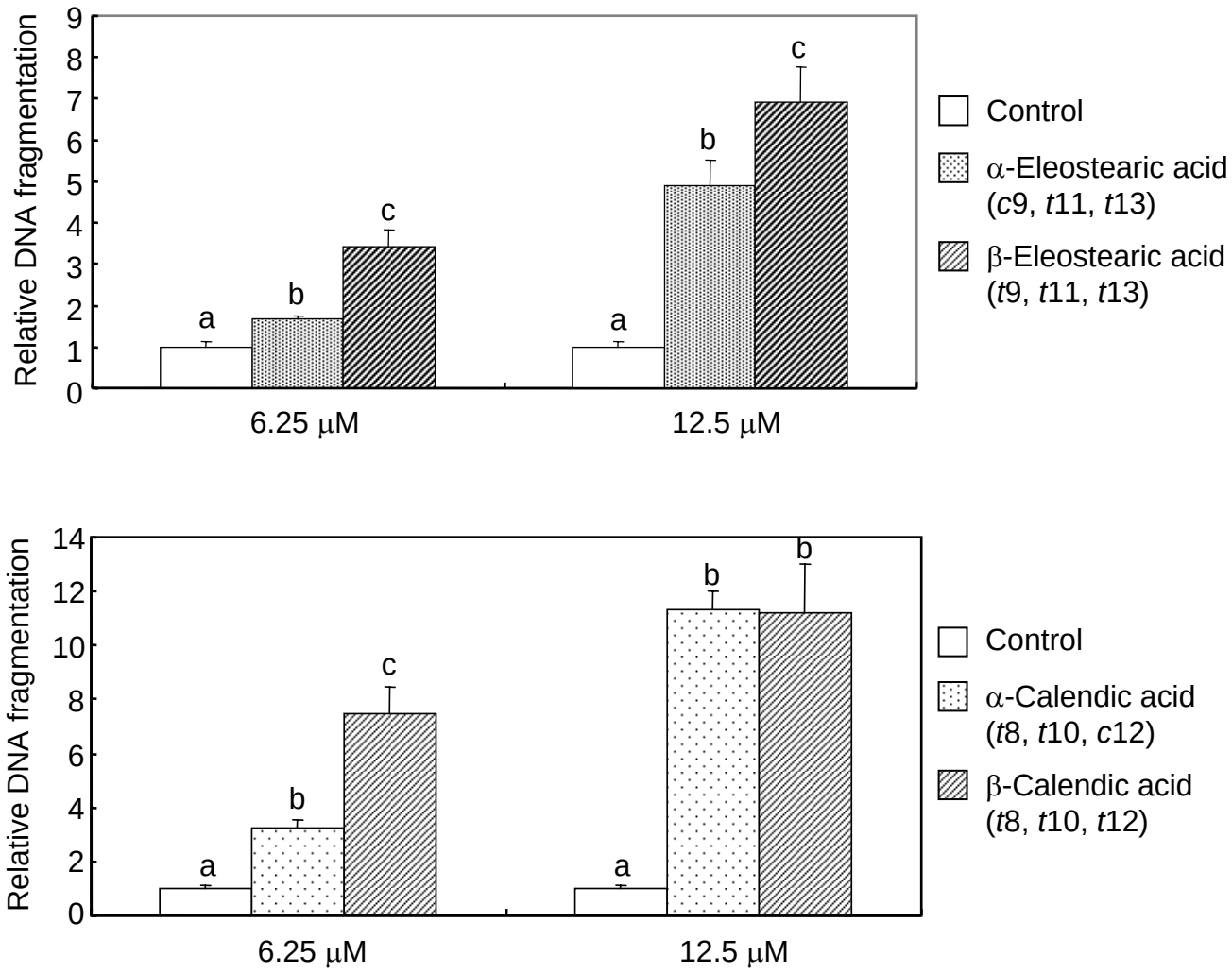

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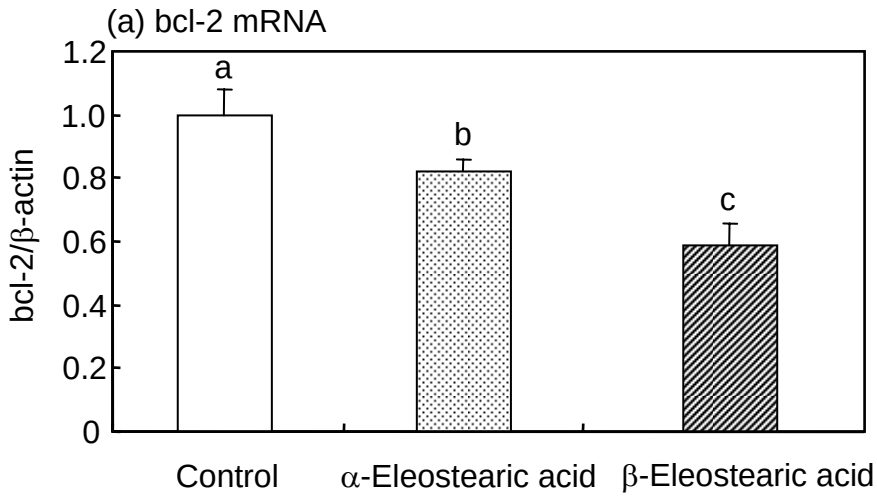

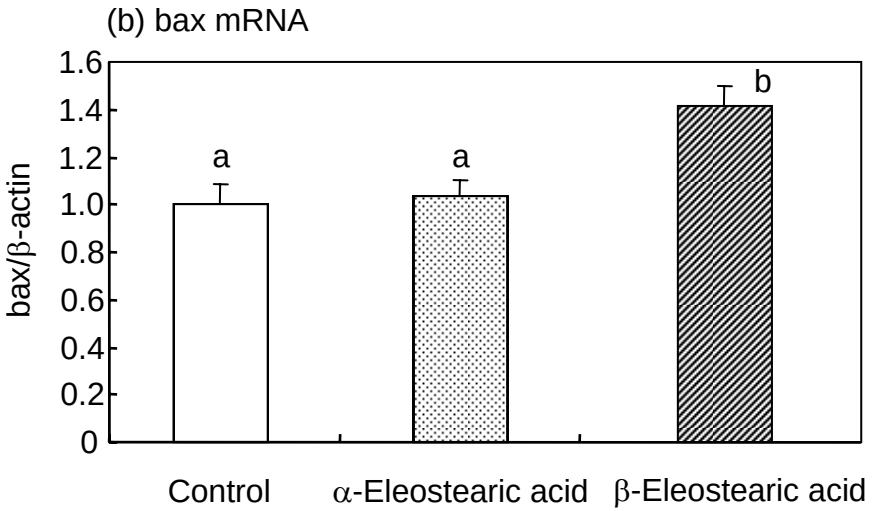

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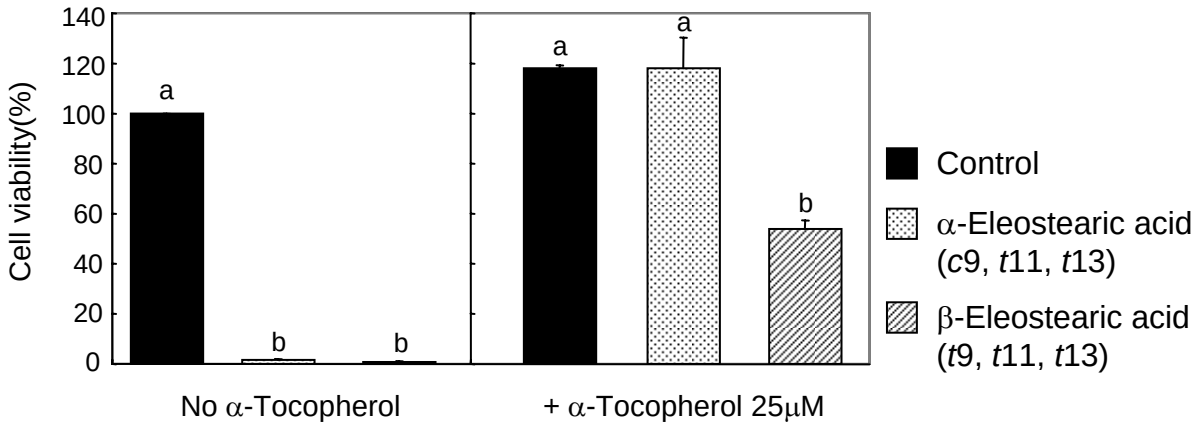
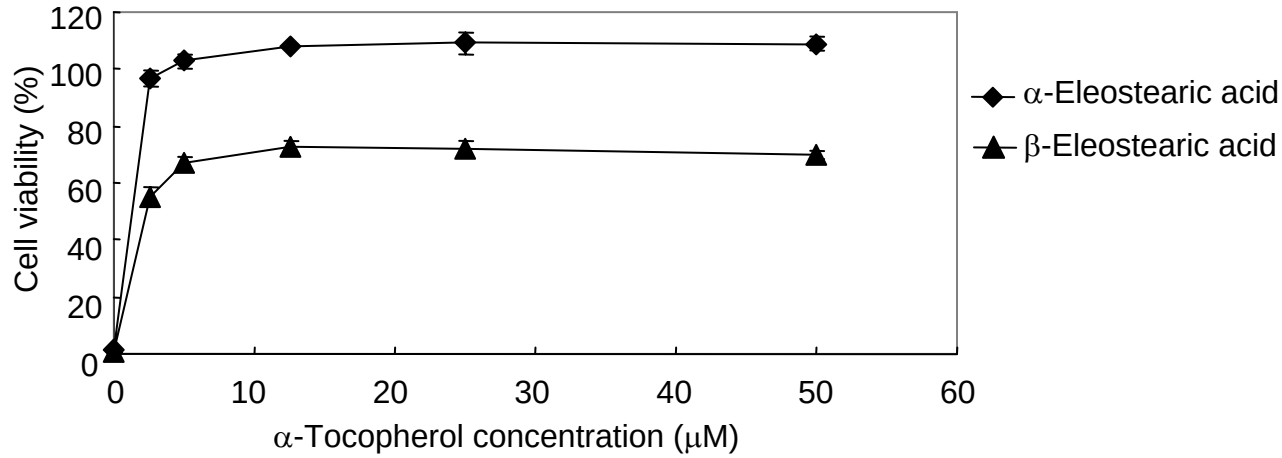


Fig. 5 Yasui et al.

(a)



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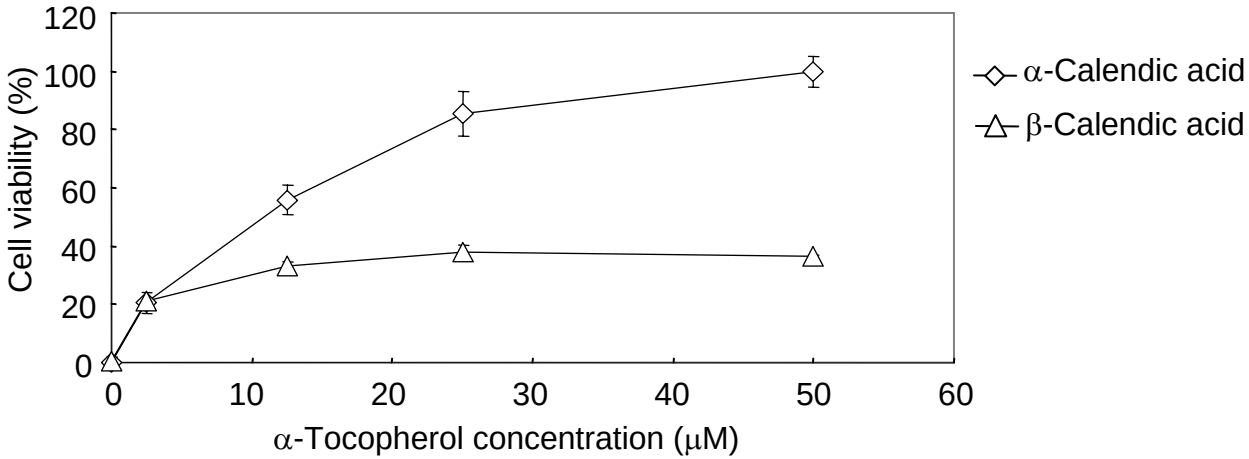

Fig. 6 Yasui et al.