

## Growth Inhibition and Apoptosis Induction by All-*trans*-conjugated Linolenic Acids on Human Colon Cancer Cells

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

The term "conjugated fatty acids" is generic for polyunsaturated fatty acids with conjugated double bonds in the molecule. Conjugated linoleic acids (CLAs) are known to have many health benefits such as anticancer (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 seeds. For example,  $\alpha$ -eleostearic acid (*c9,t11,t13-CLN*) and  $\alpha$ -calendic acid

(*t8,t10,c12-CLN*) are contained in bitter melon seed oil (BMO) and pot marigold seed oil, respectively (8). We recently demonstrated that dietary BMO, which contains  $\alpha$ -eleostearic acid, remarkably inhibited the development of azoxymethane-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 BMO induced apoptosis in colon cancer cells (11, 12).

On the other hand,  $\beta$ -eleostearic acid (*t9,t11,t13-CLN*) and  $\beta$ -calendic acid (*t8,t10,t12-CLN*), with all-*trans*-conjugated double bonds, are also known to be contained in some seed oils as minor fatty acids (8). All-*trans*-CLN isomers are also found in mixtures of CLNs chemosynthesized by the alkaline isomerization of linolenic acid (13). Recently, Igarashi and Miyazawa reported that  $\beta$ -eleostearic acid had a stronger antiproliferative effect than  $\alpha$ -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, the first study on apoptosis induction by  $\beta$ -eleostearic and  $\beta$ -calendic acids in comparison to  $\alpha$ -eleostearic and  $\alpha$ -calendic acids was performed here. Furthermore, it demonstrated that the effects of the antioxidant  $\alpha$ -tocopherol on reducing the cell viability of Caco-2 differ among all-*trans*-CLN isomers and other isomers with the *cis* configuration.

### Materials and Methods

**Materials.**  $\alpha$ -Eleostearic acid (*c9,t11,t13-CLN*, >98% purity),  $\beta$ -eleostearic acid (*t9,t11,t13-CLN*, >97% purity),  $\alpha$ -calendic acid (*t8,t10,c12-CLN*, >98% purity) and  $\beta$ -calendic acid (*t8,t10,t12-CLN*, >97% purity) (Figure 1) were purchased from Larodan Fine Chemicals AB, Sweden. WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium sodium salt) was purchased from Wako Chemical Co. (Tokyo, Japan).

**Cell culture.** The 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

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**Key Words:** Conjugated linolenic acid isomers,  $\beta$ -eleostearic acid,  $\beta$ -calendic acid, apoptosis, Caco-2 cells.

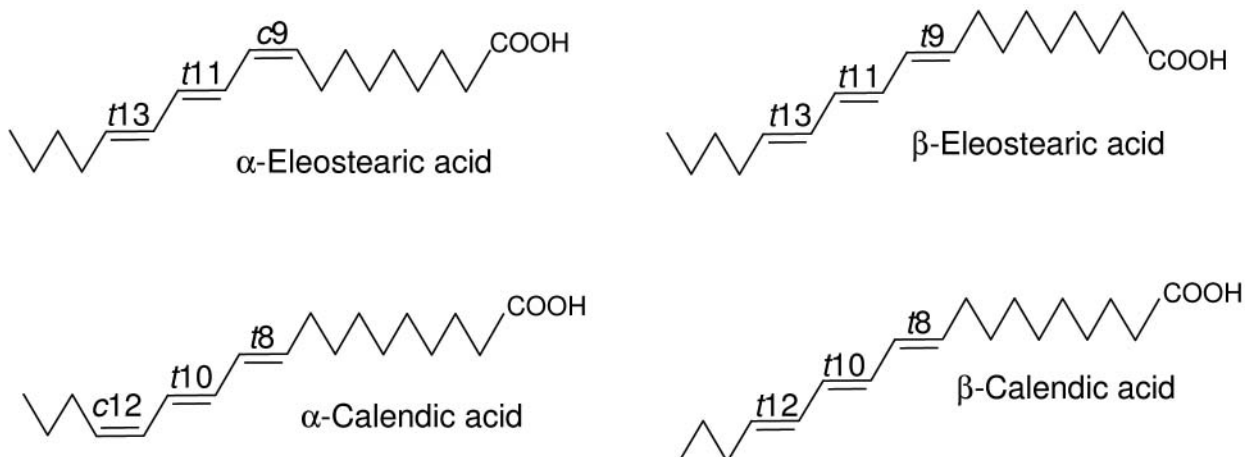


Figure 1. Structure of the conjugated linolenic acid isomers used in this study.

100  $\mu\text{g}/\text{mL}$  streptomycin. The cell cultures were maintained in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  at 37°C.

**Cell viability assay.** Caco-2 cells ( $2 \times 10^3$  cells) were pre-incubated in 96-well plates with 100  $\mu\text{L}$  medium per well for 24 h. Each CLN isomer was dissolved in ethanol and was then added into the culture medium to a final concentration up to 50  $\mu\text{M}$ . The final concentration of ethanol was below 0.1% (v/v). The Caco-2 cells were incubated for 21 h to 45 h in culture medium containing CLN with/without  $\alpha$ -tocopherol at 2.5  $\mu\text{M}$  ~ 50  $\mu\text{M}$ . Then, 10  $\mu\text{L}$  of WST-1 solution (14) were added to each well and the culture plate was incubated for an additional 3 h. The number of viable cells were measured at 450 nm. Viability was expressed as a percentage of the viable cells of the control culture.

**Measurement of DNA fragmentation.** DNA fragmentation was measured as an indicator of apoptotic cells using a commercial kit (Cell Death Detection ELISA<sup>PLUS</sup>, 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. The cell culture conditions were the same as in the cell viability assay.

**Extraction of total RNA.** Caco-2 cells ( $5 \times 10^5$  cells) were cultivated in 100-mm tissue culture dish for 24 h and each CLN isomer in ethanol was then added to the culture dish. Total RNA was extracted from the Caco-2 cells by an acidic phenol method and further purified by using an RNeasy Mini Kit (Qiagen, Chatsworth, CA, USA), according to the manufacturer's instructions.

**Real-time quantitative RT-PCR analysis.** Total RNA was reverse-transcribed by a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. Then, 1  $\mu\text{L}$  of cDNA solution (adequate concentration) was mixed with 1.25  $\mu\text{L}$  TaqMan probe, 12.25  $\mu\text{L}$  TaqMan Master Mix, 10.25  $\mu\text{L}$  water and the quantitative RT-PCR reaction was performed in an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The thermal cycling conditions were as

follows: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 sec and annealing and extension at 60°C for 1 min. TaqMan probes, *bcl-2* (Hs99999903\_m1), *bax* (Hs00608023\_m1) and  $\beta$ -actin (Hs00180269\_m1), used in this study were purchased from Applied Biosystems.

**Statistical analysis.** The data are expressed as means  $\pm$  SD. The statistical analysis between two groups (Figure 2) was determined using the unpaired Student's *t*-test. Differences with  $p < 0.05$  were considered significant. Statistical analyses between multiple groups were determined by Holm's test ( $p < 0.05$ ) (Figures 3, 4, 5).

## Results

**Effect of CLN isomers on Caco-2 cell viability.** To compare the cytotoxic effects of the CLN isomers, Caco-2 cells were incubated in culture medium containing  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid and  $\beta$ -calendic acid (Figure 1). As shown in Figure 2, each CLN isomer exerted a strong cytotoxic effect against the Caco-2 cells in a dose- and time-dependent manner. The  $\beta$ -eleostearic acid and  $\beta$ -calendic acid isomers, in particular, which have the all-*trans* configuration, showed stronger cytotoxic effects than the  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid isomers, which have the *cis* configuration, at concentrations of 3.125  $\mu\text{M}$  to 12.5  $\mu\text{M}$  after 24-h incubation. At 48-h incubation,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid also remarkably reduced the cell viability compared to  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid at 3.125  $\mu\text{M}$  and 6.25  $\mu\text{M}$ .

**Comparison of apoptosis induction in Caco-2 cells by CLN isomers.** The DNA fragmentation in Caco-2 cells incubated in culture medium containing CLN isomers was measured 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

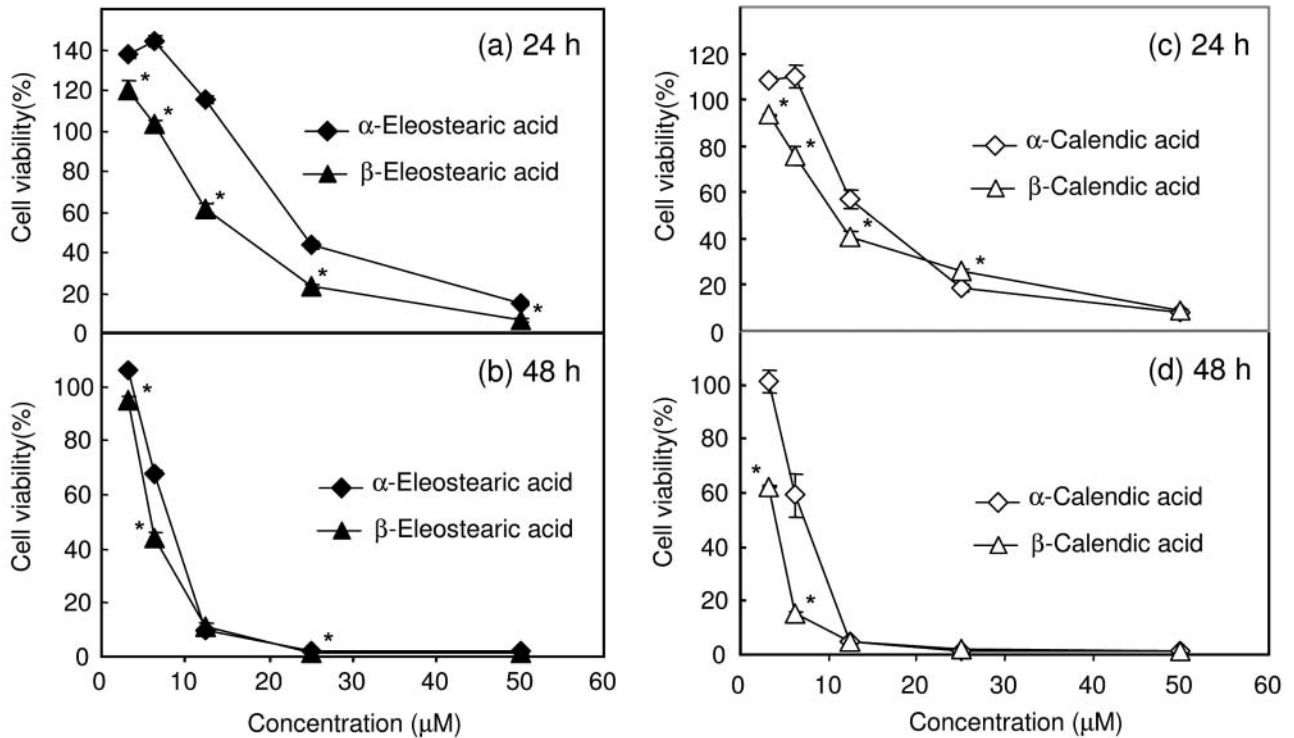


Figure 2. Effect of conjugated linolenic acid isomers on cell viability of Caco-2. Caco-2 cells were incubated in the medium with  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid, and  $\beta$ -calendic acid for 24 h or 48 h after 24 h of pre-incubation. The cell viability was measured by WST assay as described in Materials and Methods. The presented data are shown as cell numbers relative to control. All the data are expressed as means  $\pm$  SD of six experiments. \* $p < 0.05$  vs. Caco-2 cells treated with  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid at each concentration.

incubation (Figure 3). Relative DNA fragmentation, induced by 6.25  $\mu$ M and 12.5  $\mu$ M  $\beta$ -eleostearic acid, increased to 3.5-fold and 6.9-fold the level of the control after 48 h of incubation, while DNA fragmentation by  $\alpha$ -eleostearic acid was 1.5-fold and 4.9-fold the level of the control, respectively.  $\beta$ -Calendic acid also induced more DNA fragmentation in Caco-2 cells than did  $\alpha$ -calendic acid at 6.25  $\mu$ M, although the DNA fragmentation induced by 12.5  $\mu$ M  $\alpha$ -calendic acid and  $\beta$ -calendic acid was of the same level. Thus,  $\beta$ -eleostearic acid and  $\beta$ -calendic acid induced more DNA fragmentation than did  $\alpha$ -eleostearic acid and  $\alpha$ -calendic acid, respectively. This greater fragmentation corresponds to the reduction of cell viability by each CLN isomer. Furthermore, the down-regulation of *bcl-2* mRNA, which is an anti-apoptotic gene, was observed in the Caco-2 cells treated with 10  $\mu$ M  $\beta$ -eleostearic acid for 24 h (Figure 4). In addition,  $\beta$ -eleostearic acid up-regulated pro-apoptotic *bax* mRNA up to 1.4-fold in Caco-2 cells, while  $\alpha$ -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  $\alpha$ -tocopherol on reducing Caco-2 cell viability by CLN isomers.* In previous studies, we and others have reported that the cytotoxic effect of and apoptosis induction by  $\alpha$ -eleostearic acid are induced through intercellular lipid peroxidation (12, 15, 16). To investigate the mechanisms underlying the cytotoxic effects by all-*trans*-CLN isomers, Caco-2 cells were incubated with CLN isomers and  $\alpha$ -tocopherol. When 25  $\mu$ M  $\alpha$ -tocopherol was added to the culture medium, there was no reduction of cell viability by 50  $\mu$ M  $\alpha$ -eleostearic acid, as found in previous reports (Figure 4) (12, 16). On the other hand, the cytotoxic effect of 50  $\mu$ M  $\beta$ -eleostearic acid was observed even in the presence of 25  $\mu$ M  $\alpha$ -tocopherol, although the viability of the Caco-2 cells was partially restored, to 50% from 0.7%, by the addition of  $\alpha$ -tocopherol (Figure 5).

Furthermore, the effect of  $\alpha$ -tocopherol on the growth inhibition effects of CLN isomers was examined in detail (Figure 6 a). The viability of the Caco-2 cells treated with 25  $\mu$ M  $\beta$ -eleostearic acid increased as the  $\alpha$ -tocopherol concentration increased. However, cell viability reached a plateau at 70% and the cytotoxic effect of  $\beta$ -eleostearic acid was not restored completely by the addition of  $\alpha$ -tocopherol.

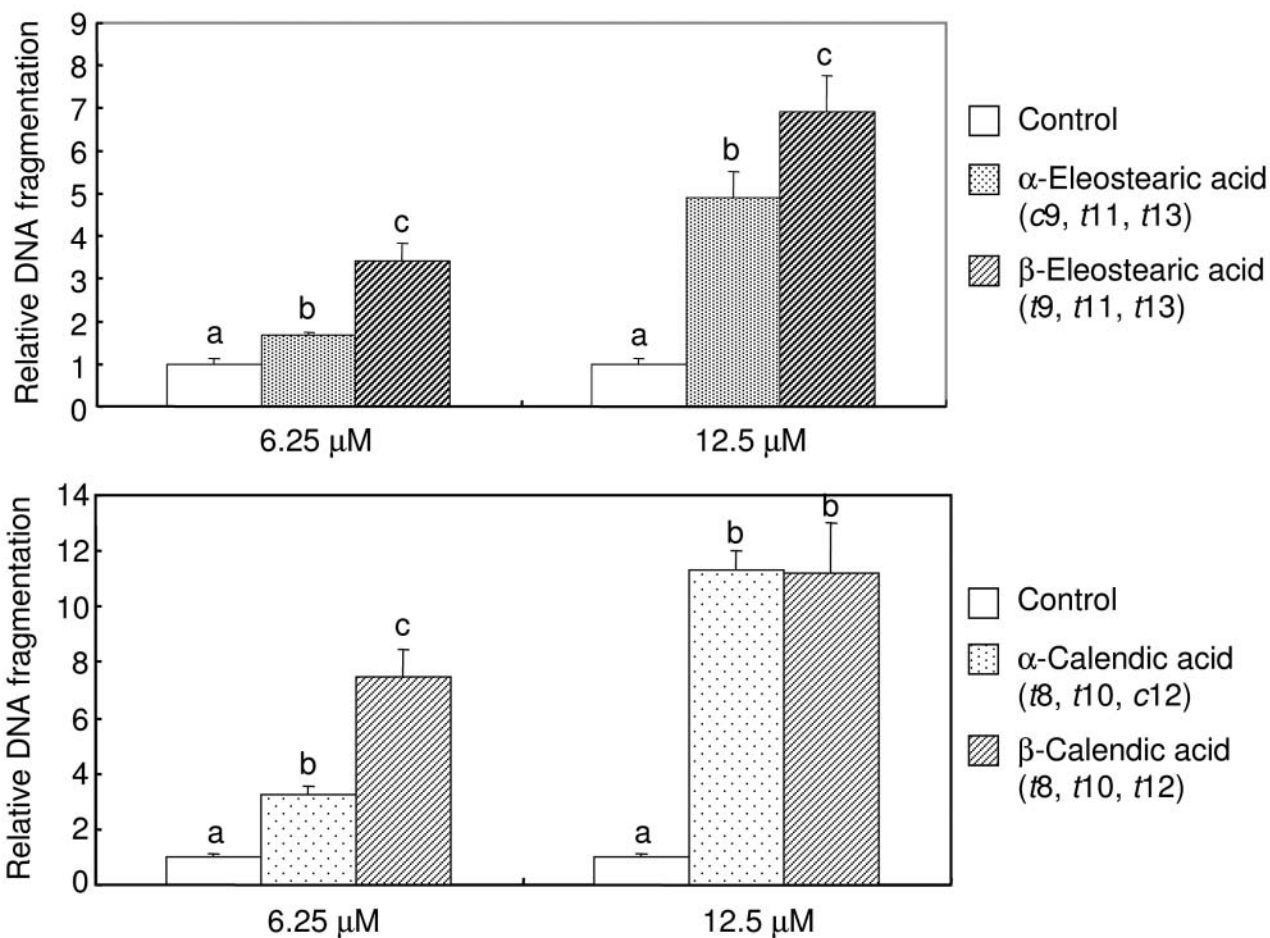


Figure 3. DNA fragmentation in Caco-2 cells treated with CLN isomers. Caco-2 cells were incubated in culture medium with  $\alpha$ -eleostearic acid,  $\beta$ -eleostearic acid,  $\alpha$ -calendic acid and  $\beta$ -calendic acid at 6.25  $\mu$ M or 12.5  $\mu$ M for 48 h after 24 h of pre-incubation. 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  $\pm$  SD, n=3. The values with different letters were significantly different from each other.  $p < 0.05$ .

The cytotoxic effect of 25  $\mu$ M  $\beta$ -calendic acid also remained in the presence of  $\alpha$ -tocopherol (Figure 6 b). In contrast, the cytotoxic effects induced by 25  $\mu$ M  $\alpha$ -eleostearic and  $\alpha$ -calendic acids were lost completely by the addition of  $\alpha$ -tocopherol (Figure 6 a, b).

## Discussion

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

CLNs are found at high concentrations in the oils of certain kinds of seeds (8).  $\alpha$ -Eleostearic and  $\alpha$ -calendic acids are major fatty acids of bitter melon seed oil and pot marigold seed oil, respectively. In addition,  $\beta$ -eleostearic acid and  $\beta$ -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 the alkaline isomerization of linolenic acid (13). In previous studies, we and others have reported that  $\alpha$ -eleostearic and  $\alpha$ -calendic acids exhibited strong cytotoxic effects on human monocytic leukemia cells (U-937) (15) and colon cancer cells (12, 16). Recently, Igarashi *et al.* reported that  $\beta$ -eleostearic acid inhibits the growth of human tumor cell lines and that its effect is stronger than that of  $\alpha$ -eleostearic acid (13). However, there are no detailed reports about the antiproliferative effects and apoptosis induction by all-*trans*-CLN isomers. In the current study, we demonstrated, for



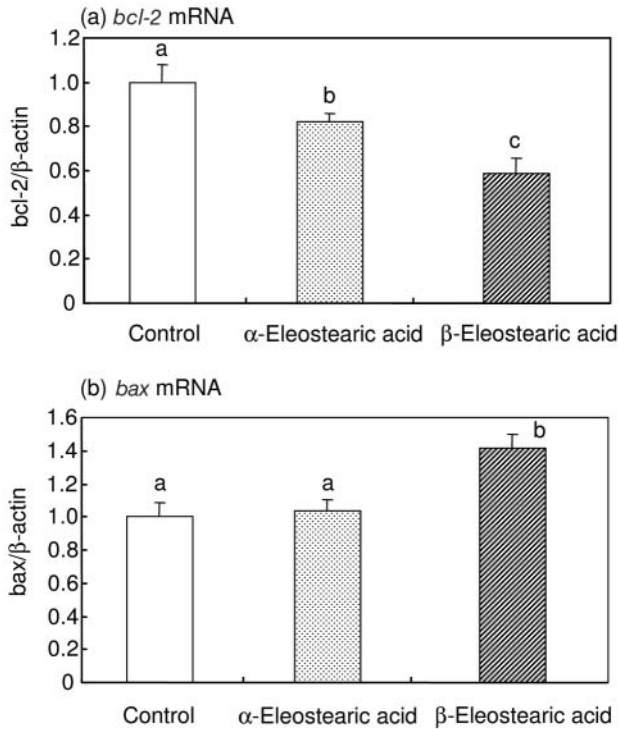


Figure 4. Expression level of *bcl-2* and *bax* mRNA in Caco-2 cells treated with α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 10 μM α-eleostearic acid or β-eleostearic acid for 24 h. The expression levels of *bcl-2* and *bax* mRNA were estimated by real-time PCR. Data from three independent experiments were normalized to the β-actin mRNA level and are shown as the means ±SD. The values with different letters were significantly different from each other.  $p < 0.05$ .

the first time, that two all-*trans*-CLN isomers, β-eleostearic acid and β-calendic acid, induced apoptosis in Caco-2 cells, and that their apoptosis activities were higher than those of α-eleostearic acid and α-calendic acid, which have the *cis* configuration. Furthermore, the down-regulation of *bcl-2* mRNA and the 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 function as anti-apoptotic and pro-apoptotic factors, respectively, in mitochondria (17). These results indicate that the configuration of conjugated double bonds is important with respect to the cytotoxic effect and apoptosis induction in Caco-2 cells. In particular, the all-*trans*-CLN isomers were more effective chemotherapeutic compounds.

The mechanism underlying the cytotoxic effect and apoptosis induction by α-eleostearic acid involves lipid peroxidation, since the antioxidant α-tocopherol diminished the growth inhibition and apoptosis induction by α-eleostearic acid on the colon cancer cell lines. In the present study, we also observed that the cytotoxic effects

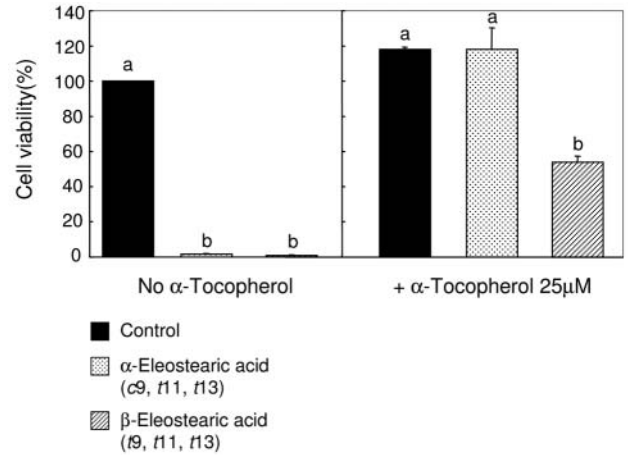


Figure 5. Effect of α-tocopherol on antiproliferation by α-eleostearic acid and β-eleostearic acid. Caco-2 cells were treated with 50 μM CLN isomers with/without 25 μM α-tocopherol for 48 h. The cell viability was measured by the WST assay described in Materials and Methods. The data represent cell viability 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 the no α-tocopherol group and the 25 μM α-tocopherol group.  $p < 0.05$ .

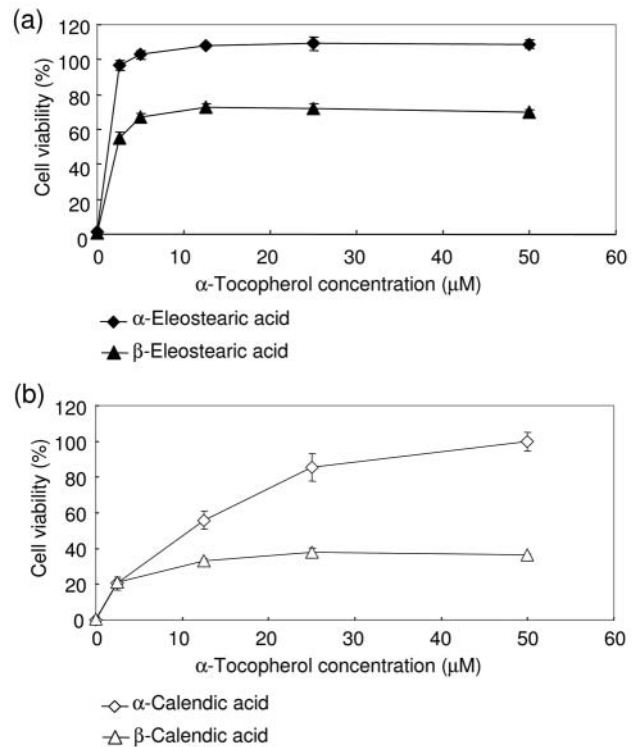


Figure 6. Comparison of antiproliferation by CLN isomers in the presence of α-tocopherol. (a) Caco-2 cells were treated with 25 μM α-eleostearic acid, β-eleostearic acid and α-tocopherol after 24 h of pre-incubation. Values are means ±SD,  $n = 6$ . (b) Caco-2 cells were treated with 25 μM α-calendic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

and apoptosis induction of  $\alpha$ -eleostearic and  $\alpha$ -calendic acids were completely suppressed by the addition of  $\alpha$ -tocopherol to the culture medium, as previously reported (12, 14, 15). It is noteworthy that the cytotoxic effect of  $\beta$ -eleostearic acid and  $\beta$ -calendic acid, which have all-*trans*-conjugated double bonds, were not counteracted completely in the presence of  $\alpha$ -tocopherol (Figures 5, 6). These results suggest that  $\beta$ -eleostearic acid and  $\beta$ -calendic acid reduce the cell viability of Caco-2 *via* another pathway in addition to the pathway of lipid peroxidation. On the other hand, the cytotoxic effect of  $\beta$ -eleostearic acid on DLD-1, another colon cancer cell line, was lost by the addition by  $\alpha$ -tocopherol (data not shown). Thus, the metabolic and signal transduction systems in Caco-2 cells may be important for different anticancer effects among CLN isomers. Further investigations are required for a better understanding of the specific mechanisms underlying the cytotoxic effects and apoptosis induction by  $\beta$ -eleostearic and  $\beta$ -calendic acids.

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

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