



Note

Apoptosis Induced by the Flavonoid from Lemon Fruit (*Citrus limon* BURM. f.) and Its Metabolites in HL-60 Cells

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The flavonoid from lemon fruit (*Citrus limon* BURM. f.) and its metabolites, particularly eriodictyol, 3,4-dihydroxyhydrocinnamic acid, and phloroglucinol had the function of DNA fragmentation in HL-60 cells when analyzed by flow cytometry. An apoptotic DNA ladder and chromatin condensation were observed in HL-60 cells when treated with these compounds. The caspase inhibitor prevented DNA fragmentation. These compounds are anticipated to be useful for medical purposes.

Key words: apoptosis; lemon fruit; flavonoids; eriodictyol; HL-60

Lemon fruit (*Citrus limon* BURM. f.) has been known as a typical healthy food for a long time, and it has been reported that lemon juice, which contains various biofunctional nutrients, has a desmutagenic effect¹⁾ and an antimutagenic effect.²⁾ In the previous study, Miyake *et al.* found that eriocitrin, one of the flavonoids in lemon fruit, had antioxidative activity.³⁾ It is now thought that dietary antioxidants may play an important role in preventing carcinogenesis and in extending the life span of animals.⁴⁾ The use of natural antioxidants in food can be expected to provide antioxidative activity *in vivo* and offer effective protection from peroxidative damage without side effects.

Many polyphenolic compounds such as flavonoids from plants are well-known as active oxygen radical scavengers⁵⁾ and chemotherapeutic agents.⁶⁾ Moreover, it has been reported that tea polyphenols showed growth inhibition and induced apoptosis in various cancer cell lines.^{7–9)} Apoptosis is a type of gene-directed cell death with distinct morphological and biochemical features when compared with necrosis and is induced by such stimuli as hormones, growth factor withdrawal, oxidative stress, DNA

damaging reagents, and antitumor drugs.¹⁰⁾ It is thought that the antitumor action of tea polyphenols may be related to their apoptosis-inducing activity. Many researches on the flavonoids derived from tea have been reported. However, there are no reports on the relationship between lemon flavonoids and apoptosis. In this study, we examine whether eriocitrin from lemon fruit and its metabolites by intestinal bacteria can induce apoptosis in the acute myelomonocytic leukemia cells, HL-60, which has been used as a model to study apoptosis.

HL-60 cells were cultured in RPMI 1640 medium (Gibco BRL Co., Grand Island, NY, U.S.A.) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Gibco BRL Co., lot US177246) at 37°C in a humidified atmosphere of 5% CO₂ in air, and were treated with 10–500 µM the flavonoid or its metabolites for the indicated time. Eriocitrin, 3,4-dihydroxyhydrocinnamic acid, and phloroglucinol were dissolved in ultrapure water, and eriodictyol was dissolved in ethanol. The solvent reached a concentration not greater than 1% in all experiments. In these conditions, we confirmed that apoptosis was not observed in HL-60 cells. Z-Asp-CH₂-DCB (a wide-spectrum caspase inhibitor) was purchased from Peptide Institute (Osaka, Japan). The cells were treated with 100 µM the inhibitor for 1 h before the treatment with the flavonoid or its metabolites.

Apoptosis assays were performed by the protocol described in ref. 11. Apoptosis was quantified by calculating the percentage of cells with hypodiploid DNA using FACSCalibur (Becton Dickinson Co., San Jose, CA, U.S.A.). To detect the DNA ladder, treated cells were suspended in lysis solution, then centrifuged. The resulting supernatant was treated

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Abbreviations: ERC, eriocitrin; ERD, eriodictyol; 3,4-DHCA, 3,4-dihydroxyhydrocinnamic acid; PHL, phloroglucinol; FCM, flow cytometry; Z-Asp-CH₂-DCB, benzyloxycarbonyl-Asp-CH₂OCO-2,6-dichlorobenzene

with RNase A (Sigma Chemical Co., St. Louis, MO, U.S.A.) and subsequently treated with proteinase K. DNA was electrophoresed in 2% agarose gel. Morphological changes were observed under a fluorescence microscope. The treated cells were fixed with 1% glutaraldehyde and stained with 1 mM Hoechst 33342 (Calbiochem Co. Cambridge, MA, U.S.A.). Other reagents used in this research were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries Co. (Osaka, Japan) unless otherwise specified.

We first measured the ratio of DNA fragmentation in HL-60 cells that had been treated with the flavonoid from lemon fruit and its metabolites at various concentrations by FCM analysis. Eriodictyol had a

particularly strong function of DNA fragmentation in HL-60 cells, the ratio of DNA fragmentation finally resulting in more than 70% after the treatment with this compound at 250 μ M for 24 h. 3,4-Dihydroxyhydrocinnamic acid and phloroglucinol produced a DNA fragmentation ratio of about 40% after a treatment at 500 μ M for 24 h (Fig. 1). However, eriocitrin had a little effect. To investigate whether DNA fragmentation induced by the flavonoid and its metabolites was the result of apoptosis, we performed agarose gel electrophoresis of DNA extracted from HL-60 cells that had been treated with these compounds. As shown in Fig. 2, an apoptotic DNA ladder was only observed in HL-60 cells when treated with these compounds. Furthermore, we observed any morphological change of nuclei with a

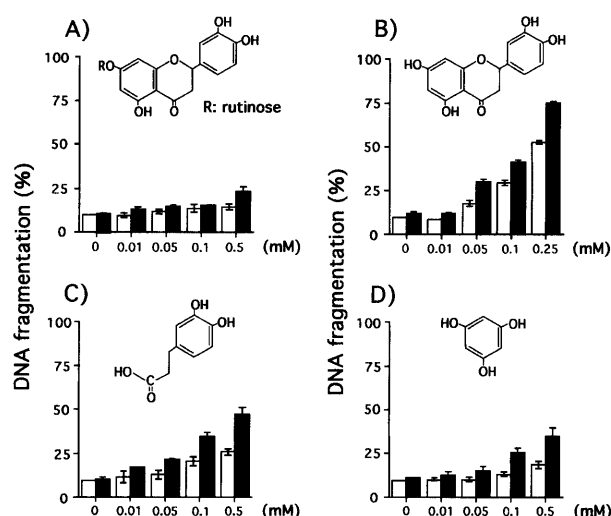


Fig. 1. The Percentage of DNA Fragmentation Induced by the Flavonoid from Lemon Fruit and Its Metabolites.

HL-60 cells were treated with the lemon flavonoid or its metabolites at various concentrations for 12 h (□) and 24 h (■). The percentage of DNA fragmentation was measured by FCM as described in ref. 11. Results are presented as the mean \pm SD from four independent experiments. Some SD values were less than the size of the symbols.

A) eriocitrin; B) eriodictyol; C) 3,4-dihydroxyhydrocinnamic acid; D) phloroglucinol.

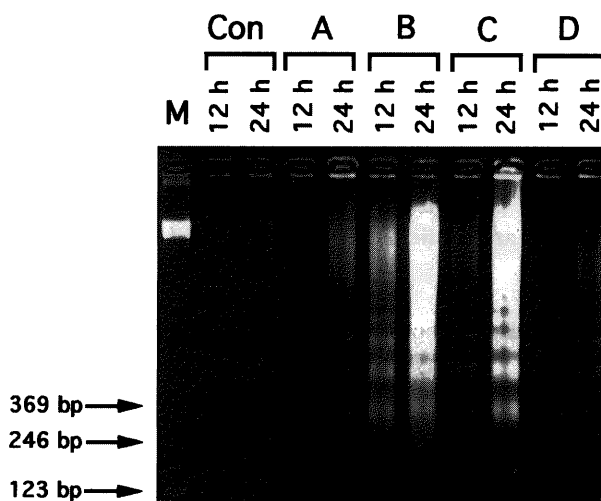


Fig. 2. Agarose Gel Electrophoresis of DNA Extracted from HL-60 Cells Treated with the Flavonoid from Lemon Fruit and Its Metabolites.

The apoptotic DNA ladder is shown at 12 and 24 h after treatment with or without the lemon flavonoid or its metabolites at 250 or 500 μ M.

Marker (M): 123-bp ladder marker; Con: control; A) eriocitrin; B) eriodictyol; C) 3,4-dihydroxyhydrocinnamic acid; D) phloroglucinol.

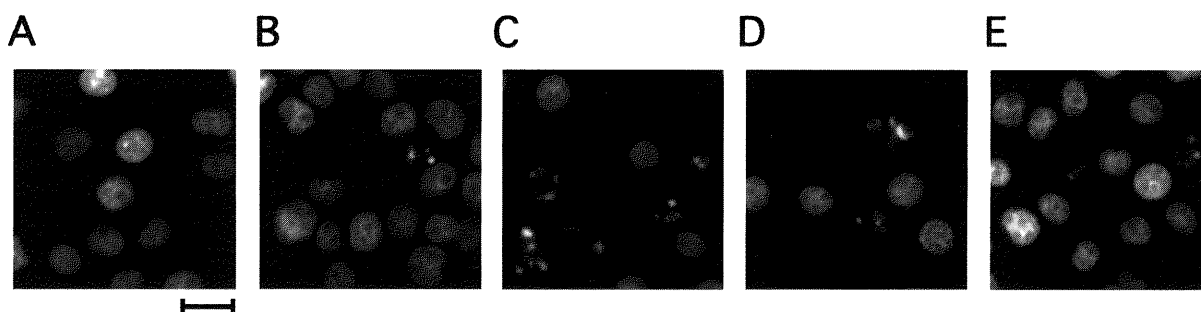


Fig. 3. Morphological Change of HL-60 Cells Treated with the Flavonoid from Lemon Fruit or Its Metabolites.

HL-60 cells were treated with each of these compounds at 250 or 500 μ M for 24 h. Apoptotic chromatin condensation was observed as described in ref. 11. A) control; B) eriocitrin; C) eriodictyol; D) 3,4-dihydroxyhydrocinnamic acid; E) phloroglucinol.

The scale bar shows 10 μ m.

fluorescence microscope in HL-60 cells (Fig. 3). Chromatin condensation was only observed in the cells when treated with these compounds. Consequently, we conclude that the flavonoid and its metabolites induced apoptosis in HL-60 cells. The order of potency as an inducer of apoptosis was eriodictyol, 3,4-dihydroxyhydrocinnamic acid, phloroglucinol, and eriocitrin.

It is well-known that activation of the cascade composed of various caspases occurs in apoptosis signal transduction and execution.¹²⁾ Therefore, we examined whether the cascade of caspases participated in the apoptosis induced by these compounds. We investigated the effect of a caspase inhibitor on the ratio of DNA fragmentation induced by the flavonoid and its metabolites. As a result, Z-Asp-CH₂-DCB, which is known as a wide-spectrum caspase inhibitor, completely prevented the DNA fragmentation induced by these compounds (Fig. 4). We also examined whether Z-Asp-CH₂-DCB blocked the morphological change of the apoptosis induced by these compounds (data not shown). Consequently, the activity of caspases participated in the apoptosis induced by the flavonoid and its metabolites. Our results suggest that these compounds induced apoptosis via caspase pathways. It would be interesting to investigate what are the death substrates cleaved by caspases and to clarify which caspases play a crucial role in the apoptosis induced by these compounds. Moreover, we want to investigate the death mechanism for the activation of caspases and the execution of apoptosis.

Our results have shown that the flavonoid from lemon fruit and its metabolites induced apoptosis in HL-60 cells in a dose- and time-dependent manner. It

is well-known that the tea polyphenols, (–)-epigallocatechin-3-gallate (EGCG) and (–)-epigallocatechin, show strong growth-inhibitory effects and apoptosis-inducing activity against various human cancer cell lines. Otsuka *et al.* have reported that more than 50% of DNA synthesis was reduced in various human leukemic cell lines, including HL-60 cells, in the presence of 50 μ M EGCG and that the dead cells showed characteristics of apoptosis.⁸⁾ Saeki *et al.* have reported that polyphenolic compounds derived from tea catechins induced apoptosis in U937 cells at 200 μ M.⁹⁾ In particular, eriodictyol effectively induced apoptosis in HL-60 cells at higher than 50 μ M in our study. We consider that the apoptosis-inducing activity of eriodictyol might be almost equal to the activity of these tea catechins.

Kawaii *et al.* have investigated the antiproliferative activities of *Citrus* flavonoids against several cancer cell lines.¹³⁾ Eriodictyol particularly inhibited the growth of several cancer cell lines at about 10 μ M (IC₅₀). We suggest that this observation may be related to the apoptosis-inducing activity of eriodictyol. Moreover, their results showed that the flavonoid glycosides were not generally effective. We also found that eriocitrin did not effectively induce apoptosis in HL-60 cells. We suggest that this result may be related to the low permeability of flavonoid glycosides through the cell membrane. In the previous study, Miyake *et al.* have reported that eriocitrin was hydrolyzed to eriodictyol, its aglycon, which was then converted to 3,4-dihydroxyhydrocinnamic acid and phloroglucinol by human intestinal bacteria.¹⁴⁾ Moreover, these metabolites, especially eriodictyol, also preserved the strong antioxidative activity. Our results suggest that eriodictyol, 3,4-dihydroxyhydrocinnamic acid, and phloroglucinol may have induced apoptosis more effectively *in vivo* because these compounds were formed by intestinal bacteria. We therefore want to examine whether these compounds can induce apoptosis in various cell lines, and it will be necessary to confirm how antioxidants in food are metabolized *in vivo* to achieve this. Moreover, it is important to elucidate the structure-activity relationship in various flavonoids from lemon fruit and their related compounds as inducers of apoptosis.

Hirano *et al.* have reported that *Citrus* flavone tangeretin exhibited the growth inhibition of HL-60 cells partially through the induction of apoptosis.¹⁵⁾ Interestingly, they also found that tangeretin showed no cytotoxicity against normal human lymphocytes. We need to investigate the effect of the flavonoid and its metabolites on normal cells, and further study will also resolve the mechanism for apoptosis induced by these compounds in HL-60 cells. The flavonoid from lemon fruit and its metabolites may subsequently be found useful to develop antitumor therapeutics with no harmful side effects.

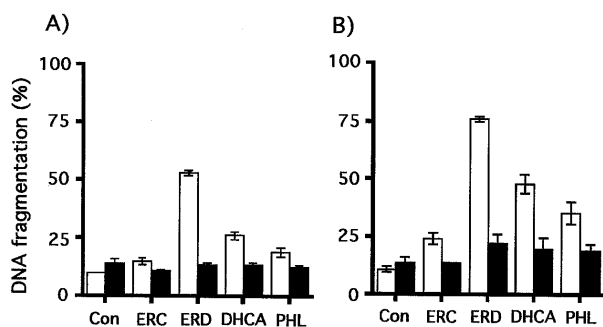


Fig. 4. Effect of the Caspase Inhibitor on DNA Fragmentation in HL-60 Cells Treated with the Flavonoid from Lemon Fruit and Its Metabolites.

HL-60 cells were previously incubated with the caspase inhibitor at 100 μ M for 1 h, and were then treated with each of these compounds at 250 or 500 μ M for 12 h (A) and 24 h (B). DNA fragmentation was quantified by FCM as described in ref. 11. □ no inhibitor; ■ Z-Asp-CH₂-DCB. Results are presented as the mean \pm SD from four independent experiments. Some SD values were less than the size of the symbols.

ERC: eriocitrin; ERD: eriodictyol; DHCA: 3,4-dihydroxyhydrocinnamic acid; PHL: phloroglucinol.

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