Analysis of Triterpenes in Foods and By-products, and Study of their Utilization as Functional Foods

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Chapter 1. General Introduction

Oleanolic acid (3β-hydroxy-olea-12-en-28-oic-acid; OA) and its isomer, ursolic acid (3β-hydroxy-urs-12-en-28-oic acid; UA), are triterpenoid compounds that exist widely in fruits and plants commonly used in human diets and in medicinal herbs, such as olive (Vázquez and Janer, 1969), apple (Jäger and Trojan, 2009), grape (Amico et al., 2004), clove (Jäger and Trojan, 2009), and pomegranate (*Punica granatum*) flowers (de Melo, C. L. et al., 2010). These triterpenoid compounds mainly exist in natural plants in the form of free acid, or as aglycones in the case of triterpenoid saponins (Price et al., 1987; Mahato et al., 1988; Wang and Jiang, 1992). Saponins can be chemically categorized depending on whether an aglycone is linked to one or more sugar chains. There are 2 groups of saponins; one contains a steroidal aglycone, and the other contains a triterpenoid aglycone (Price et al., 1987). Squalene is considered to be the most common precursor for the biosynthesis of both steroid and triterpenoid systems (Price et al., 1987). Like steroids, triterpenoids have many biological effects and research interest in triterpenoids is growing (Price et al., 1987; Mahato et al., 1988).

Occurrence in folk medicine

OA has been isolated from more than 120 plant species to date (Wang and Jiang, 1992), and has been shown to play an active role in several biological processes. The results of a partial survey on the use of OA and UA in folk medicine, and their biological activities are listed in Tables 1.1 and 1.2.

Table 1.1 Partial survey on medicinal plants containing OA as an active ingredient

Plant	Biological activity	References
Aralia chinensis L. var nuda Nakai (*Araliaceae*)	Hepatoprotection	Wang and Jiang, 1992; Liu et al., 1994b
Beta vulgaris L. var. cicla (*Chenopodiaceae*)	Hepatoprotection	Yabuchi et al., 1988
Calendula officinalis L. (*Compositae*)	Antifungal activity	Favel et al., 1994
Eugenia jambolana Lam. (Myrtaceae)	Inhibition of lipid peroxidation and protection against adriamycin toxicity; Anti-fertility activity	Balanehru and Nagarajan, 1991; Rajasekaran et al., 1988
Ganoderma lucidum Karst.	Anti-cariogenic activity	Hada et al., 1990
Sapindus mukorossi Gaertn (Sapindaceae)	Anti-inflammation	Takagi et al., 1980
Tinospora sagittata G. (Menispermaceae)	Anti-hyperglycemia	Hao, 1991

Table 1.2 Partial survey on medicinal plants containing UA as an active ingredient

Plant	Biological activity	References
Eriobotrya Japonica Lindl. (Rosaceae)	Inhibition of mutagenesis in bacteria	Young et al., 1994
Eucalyptus hybrid (Myrtaceae)	Hepatoprotection	Shukla et al., 1992
Melaleuca leucadendron L. (Myrtaceae)	Inhibition of histamine release	Tsuruga et al., 1991
Pyrola rotundifolia (Pyrolaceae)	Anti-inflammation	Kosuge et al., 1985
Solanum incanum L. (Solanaceae)	Hepatoprotection	Lin et al., 1988

The traditional uses of these OA- or UA-containing plants in folk medicine are variable, including anti-inflammatory, hepatoprotection, and cardiotonic, among others. Many of these therapeutic effects have been confirmed in contemporary scientific research.

Hepatoprotection perspective

OA and UA are well known for their hepatoprotective effects, and are currently used, both alone and in combination with other hepatoprotective ingredients, as oral medications (Liu, 2005). The hepatoprotective effect of OA was first reported in 1975 in a study of *Swertia mileensis*, a traditional herbal medicine used for hepatitis. Of the 3 compounds isolated from this herb, OA was found to be the most effective in protecting against CCl_4-induced liver injury in rats (Hunan Med. Inst., 1975). Subsequently, OA has been further demonstrated to decrease CCl_4-induced liver parenchymal cell necrosis, steatosis, and degeneration (Ma et al., 1982), and to prevent CCl_4 plus alcohol-induced chronic cirrhosis in rats (Han et al., 1981). OA protects against the hepatotoxicity produced not only by CCl_4, but also by acetaminophen, cadmium, bromobenzene, phalloidin, thioacetamide, furosemide, colchicine, and D-galactosamine plus endotoxin. However, it is ineffective in decreasing the hepatotoxicity produced by allyl alcohol, dimethylnitrosamine, α-amanitin, and chloroform (Liu et al., 1995a). OA was also shown to have hepatoprotective effects against carbon tetrachloride-induced hepatic injury, which was due, at least in part, to the decreased biotransformation of carbon tetrachloride by suppression of cytochrome P450 2E1 activity and expression (Jeong, 1999).

Hepatoprotective profiles have indicated that OA protects many, but not all hepatotoxicants, which suggests that multiple mechanisms may be involved in the hepatoprotective effect of OA.

UA, the isomer of OA, was also identified as an active hepatoprotective component in the preparation of *Sambucus chinensis* Lindl. (Ma et al., 1986), *Solanum incanum* L. (Lin et al., 1988), *Tripterospermum taiwanense* (Gan and Lin, 1988), and *Eucalyptus* hybrid (Shukla et al., 1992). In addition to its protection against CCl_4-induced liver injury, UA also protected against D-galactosamine-induced liver injury in rats, and prevented acetaminophen-induced cholestasis (Shukla et al., 1992). Furthermore, UA was shown to protect against ethanol-mediated experimental liver damage in rats (Saravanan et al., 2006). In fact, UA was found to be more potent than OA in decreasing chemically-induced liver injury in mice (Liu et al., 1994a).

Anti-hyperlipidemia perspective

The hypolipidemic and anti-atherosclerotic properties of triterpenoids, including UA and glycyrram, were first reported in 1979. UA fed to rabbits and rats resulted in prevention of experimental atherosclerosis, and lowered blood cholesterol (44%) and β-lipoprotein levels (50%) (Parfenteva, 1979; Vasilenko et al., 1981). Another 14 triterpenoid compounds were further tested, and all of them, including OA, were found to be effective in preventing hyperlipidemia in rabbits, guinea pigs, and rats (Vasilenko et al., 1982). Liu et al. (2007) revealed that OA exerted hypolipidemic effects by lowering serum total cholesterol and triglyceride levels, and that it could also protect against fatty liver disease. One possible mechanism of this effect might be related to reducing the expression of the acetyl-CoA acetyltransferase (ACAT) gene (a key enzyme regulating metabolic fatty acid), and thus decreasing intestinal absorption, synthesis, or storage of exogenous and endogenous cholesterol. OA did not affect blood lipoprotein levels in normal rabbits, but decreased elevated blood cholesterol levels and prevented lipid precipitation in the blood vessels and major organs of experimental hyperlipidemic rabbits. Following OA treatment, serum concentrations of high-density lipoprotein increased, whereas low-density lipoprotein concentrations decreased (Ma, 1986). The anti-hyperlipidemic effect of OA and UA has stimulated considerable clinical interest.

Anti-inflammatory activity

The anti-inflammatory effect is a common property of many triterpenoids (Price et al., 1987; Mahato et al., 1988). OA and UA are among the most notable triterpenoid compounds. The anti-inflammatory effect of OA was first reported in the 1960s. Gupta et al. (1969) reported the inhibitory effects of OA on carrageenan-induced rat paw edema and formaldehyde-induced arthritis. The anti-inflammatory effects of OA were further confirmed in subsequent studies (Takagi et al., 1980; Dai et al., 1989a; Singh et al., 1992). Additionally, OA has been shown to inhibit rat paw edema produced by dextran, and to suppress adjuvant-induced arthritis in rats and mice (Singh et al., 1992).

UA was identified as an active component of *Pyrola rotundifolia* L. in preventing carrageen-induced paw edema in rats, as well as acetic acid-induced writhing in mice (Kosuge et al., 1985). In medicinal preparations from *Rosmarinus officinalis* L. (Rosemary), UA was identified as one of the active components in preventing 12-o-tetradecanoylphorbol-13-acetate (TPA)-induced mouse ear edema (Hirota et al., 1990; Huang et al., 1994).

The mechanisms underlying the anti-inflammatory effects of OA and/or UA have been attributed to the following factors: (1) inhibition of histamine release from mast cells induced by the compound 48/80 and concanavalin A (Dai et al., 1989b; Rajasekeran et al., 1990; Tsuruga et al., 1991), or by Adriamycin (Balanehru, et al., 1994); (2) inhibition of lipoxygenase and cyclooxygenase activity (Simon et al., 1992; Najid et al., 1992), thus reducing some inflammatory factors produced during the arachidonic acid cascade; (3) inhibition of elastase, which is thought to play a role in the tissue inflammatory response in rheumatic diseases (Ying et al., 1991) (inhibition rates were similar between UA and OA with IC_{50} values of 4.4 μM and 6.4 μM, respectively); (4) inhibition of complement activity (Dai et al., 1989b), possibly through the inhibition of the C_3-convertase of the classical complement pathway (Kapil et al., 1994). In addition, high doses of OA can result in thymus atrophy (Dai et al., 1989a, 1989b).

Antitumor and chemotherapy perspective

Both tumor initiation and promotion are inhibited by OA and UA to various degrees. The most notable effect of these 2 triterpenoids is antitumor-promotion.

OA and UA were identified as active components of *Ligustrum lucidum* Ait. in inhibiting the mutagenicity produced by benzo[a]pyrene (B[a]P) in bacteria. The amount of OA and UA at 90% suppression in each solvent fraction was 65 μg and 30 μg, respectively (Niikawa et al., 1993). OA and UA were also shown to be active components of *Glechoma hederacea* L. in the inhibition of tumor-promoting effects by TPA, both *in vitro* (Ito et al., 1983; Ohigashi et al., 1986) and *in vivo* (Tokuda et al., 1986). TPA-induced Epstein-Barr virus (EBV)-associated activation in Raji cells was inhibited by OA and UA at an approximately 1000-fold molar ratio to TPA or to another tumor promoter, teleocidin B-4 (Ohigashi et al., 1986; Konoshima et al., 1987).

OA and UA have also been shown to inhibit tumor initiation and promotion at various stages of tumor development, and were also found to induce tumor cell differentiation and apoptosis. In a two-stage mouse skin carcinogenesis model, the protection of OA against TPA-promoted carcinogenesis was associated with inhibition of aberrant gene expression (Oguro et al., 1998). OA derivatives have also been proven effective for acute myeloid leukemia by inducing apoptosis of tumor cells (Konopleva et al., 2004). Furthermore, OA and its derivatives effectively inhibited angiogenesis, invasion of tumor cells, and metastasis, and have therefore emerged as a new class of chemotherapeutics (Ovesna et al., 2004).

Toxicity of OA

OA and UA are relatively non-toxic. After a single subcutaneous injection of 1.0 g/kg OA to mice or rats, no mortality was observed during the first 5-day period post-injection (Hunan Med. Inst., 1975; Singh et al., 1992). Furthermore, following multiple administration of 180 mg/kg *per os* OA for 10 days, no abnormalities were observed in the brain, heart, lung, liver, kidney, thyroid, testes, stomach, spleen, or intestine (Hunan Med. Inst., 1977). A 70-case clinical trial for acute jaundice hepatitis showed that 60-90 mg/day OA for 30 days was therapeutically effective with no apparent side effects (Xu and Wan, 1980). Long-term use of OA (> 3 months) in 188 cases of chronic hepatitis indicated that OA is safe (Xu, 1985).

Other pharmacological effects

A variety of novel pharmacological effects produced by OA and UA have been reported, including their beneficial effects on cardiovascular systems (Samova et al., 2003), interactions with cytochrome P450s (Kim et al., 2004), antimicrobial activities (Zaletova et al., 1986), and anti-ulcer effects (Gupta et al., 1981; Snyckers and Fourie, 1984; Wrzeciono et al., 1985).

All reported pharmacological effects are too numerous to be mentioned in detail here, which, in addition to their non-toxicity, have contributed to the growing research interest of these naturally occurring triterpenoids. Consequently, identifying suitable resources that are rich in these triterpenoids has also become increasingly important. Therefore, the objective of this study was to investigate resources that are rich in OA and UA.

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Chapter 2. Oleanolic Acid and Ursolic Acid in Dried Fruits

2.1 Introduction

Triterpenoids, such as OA and UA, are known to exist mainly in fruit skins (Zhang et al., 2004; Juan et al., 2006; Wang et al., 2011). However, because the skins of fresh fruits are often removed when eaten, fresh fruits may therefore not constitute a significant source of OA and UA intake for many people. Since the solubility of OA and UA to water is quite low, they also remain on the fruit surface following processing, including the washing and squeezing steps. However, unlike fresh fruits, dried fruits are usually eaten without removing the pericarp, and may therefore be a good source for the natural oral intake of OA and UA.

Dried fruit is a popular snack worldwide, and is considered a delicacy due to its high nutritive value (66–90% carbohydrate) and shelf life. The production of dried fruits is currently widespread across the globe. Nearly half of all dried fruits in the international market are raisins, followed by dates, prunes, figs, apricots, peaches, apples, pears, and other fruits. A significant amount of sour cherries, cherries, pineapples, and bananas are also sold dried (Hui, 2006).

However, with the exception of raisins, little is known about the OA and UA content of dried fruits. Therefore, the objective of this chapter was to quantify and compare the levels of OA and UA found in a variety of dried fruits in order to identify rich sources of these triterpenoids.

2.2 Materials and Methods

2.2.1 Materials

The 17 types of commercially available dried fruits (apple, apricot, banana, blueberry, cherry, cranberry, green raisin, Kyoho raisin, Sultana raisin, mango, orange, pear, persimmon, pineapple, prune, fig, and strawberry) used in this study were purchased from Japanese markets (Table 2.1).

2.2.2 Preparation of sample

Dried fruits (20 g) were added to 100 mL ethanol and were homogenized by agitation for 1 h, followed by filtration through No. 2 filter paper (Advantec, Tokyo, Japan). After 3 repetitions of this procedure, the combined extracts were concentrated to dryness using a rotary evaporator, and were dissolved in 10 mL methanol for use in thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses.

2.2.3 Recovery yield

2mg of UA was added to 20 g of green raisin, and follow-up extraction and HPLC analysis were performed. Similarly, 2 mg of OA was added to 20 g of dried cranberry and analyzed.

Table 2.1 Dried fruits used in this study

Dried fruits	Materials	Skin	Country of origin
Apple	apple, sugar, citric acid, spice, bleaching agent	-	China
Apricot	apricot	+	Turkey
Banana	banana, sulfite	-	Thailand
Blueberry	blueberry, sugar, melon seed oil	+	USA
Cherry	cherry, sugar, melon seed oil	+	USA
Cranberry	cranberry, sugar, plant oil	+	USA
Green raisin	grape (white)	+	China
Kyoho raisin	grape (red), plant oil	+	Chile
Mango	mango, sugar, citric acid, sulfite, colorant	-	Thailand
Orange	Starch syrup, sugar, orange skin, lemon juice	+	Italy
Pear	pear, sugar, pH adjustment agent, vitamin C, sodium thiosulfate	-	China
Persimmon	persimmon, sulfur dioxide	-	Japan
Pineapple	pineapple, sugar, pineapple juice, citric acid, sulfite	-	Thailand
Prune	prune, plant oil, potassium sorbate	+	USA
Fig	fig	+	Turkey
Strawberry	strawberry, sugar, plant oil, citric acid, sulfite, colorant, spice	+	China
Sultana raisin	grape (red), plant oil	+	Turkey

The recovery yields of UA from green raisin and OA from cranberry in this extraction method were 98.2 ± 1.7% (n = 3) and 97.9 ±2.3% (n = 3), respectively.

2.2.4 TLC analysis

The presence of OA and UA were confirmed by TLC prior to HPLC analysis. Sample solutions (10 μL) were applied to a 70-plate silica gel (0.25 mm thickness, 20 × 20cm; Wako, Osaka, Japan), and separated with cyclohexane:acetone:ethyl acetate (4:2:1). After the silica gel plates were dried, samples were visualized by spraying the plates with 50% sulfuric acid and baking.

2.2.5 HPLC analysis

Quantitative analyses of OA and UA were carried out by HPLC according to a previously reported protocol (Ebisui, 2006) with some modifications. Separation was achieved using a 4.6 × 250 mm, 5 μm Wakosil II 5C 18 column (Wako) and L-2420 UV-VIS detector (Hitachi, Tokyo, Japan) set at 210 nm. The solvent was vacuum-filtered and degassed prior to use, and sample extracts were filtered through a 0.2 μm Dismic-13 filter (Advantec) before injection. Samples (7 μL) were injected onto the column following equilibration with acetonitrile:methanol:water: phosphoric acid (500:400:100:0.5), and were separated with the same solvent at a rate of 0.5 mL/min. Analyses were performed at 20°C, data were processed with a PC-based integrator (Chromato-Pro; Lablab

Company, Hachioji, Japan), and the amounts of OA and UA in each dried fruit were calculated from the calibration curves (concentration range: 10–2000 μg/mL) of OA (> 97%, Wako) and UA (> 95%, Wako) standards, respectively. The representative calibration curve of OA was $y = 1.55e^{6}x - 3.06e^{4}$ (x = mg/mL of OA; y = area), with a correlation coefficient of $9.994e^{-1}$, and the representative calibration curve of UA was $y = 0.12e^{7}x - 0.42e^{5}$ (x = mg/mL of UA; y = area), with a correlation coefficient of $9.998e^{-1}$. All data are presented as the means of 3 replicates with standard deviations calculated using Microsoft Excel (Microsoft, USA).

2.3 Results and Discussion

2.3.1 TLC analysis

OA and UA were not detected in dried fruits without skin, such as apple, banana, mango, pear, and pineapple (data not shown). A previous report demonstrated that triterpenic compounds such as OA and UA were mainly distributed in the skins of fruits (Frighetto et al., 2008; Orbán et al., 2009). These 5 kinds of dried fruit had their skins removed during processing (Table 2.1). The dried fruits without skin do not contain, or contained very little, OA and UA, which support results of previous studies.

In contrast, TLC analysis clearly detected OA and/or UA in the 3 varieties of raisin, persimmon, cranberries, and cherries (Fig 2.1).

However, we were unable to confirm the presence of OA or UA in prunes, oranges, figs, or strawberries, which were processed with skin intact under our TLC assay conditions (detection limit by TLC was 0.20 μg/spot). Because these results suggested that these fruits contained only trace amounts of UA and OA, we did not analyze them further using HPLC.

2.3.2 HPLC analysis

As OA and UA are isomers with similar characteristics, they cannot be separated by TLC (Fig 2.1). We therefore employed HPLC to separate and quantify the respective levels of OA and UA present in the dried fruits that were shown to contain these triterpenoids in the TLC analysis. Figure 2.2A shows a representative chromatogram for the HPLC separation of OA and UA, which showed complete separation of the OA and UA peaks under our chromatography conditions.

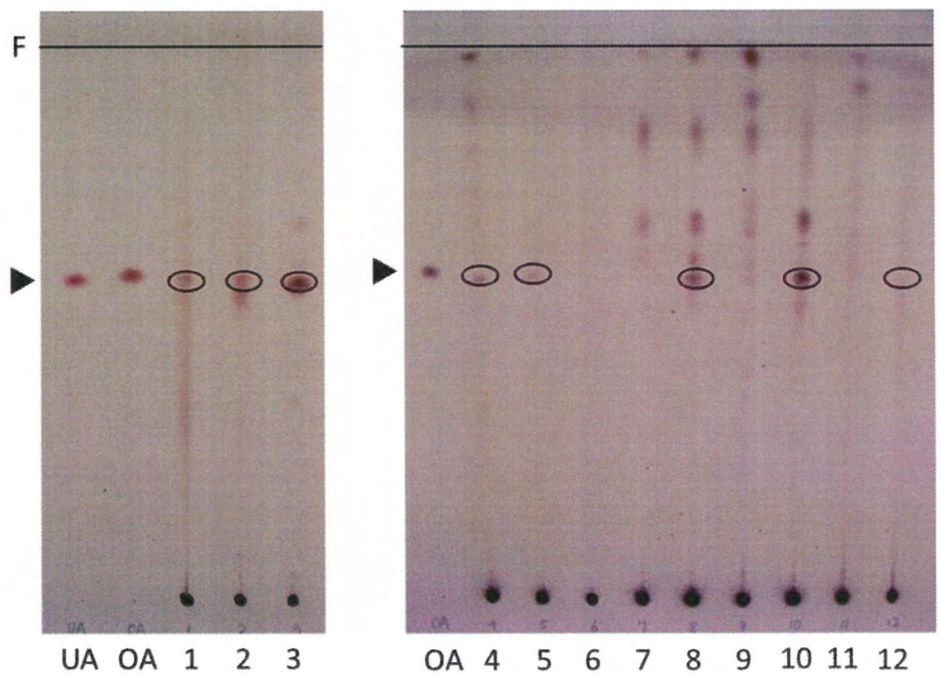

Fig 2.1 TLC profile of OA or UA fraction of dried fruits. OA or UA fraction was derived by cyclohexane : acetone : ethyl acetate (v:v:v=4:2:1) on silica gel plate, and was visualized by spraying the plates with 50% sulfuric acid and baking. Arrowheads and circles indicate the positions of OA and UA. The solid line indicated by F represents the front line of the TLC analysis. Lanes 1–12 are as follows: Sultana raisin (1), Kyoho raisin (2), green raisin (3), cherry (4), persimmon (5), orange (6), strawberry (7), cranberry (8), prune (9), blueberry (10), fig (11), and apricots (12).

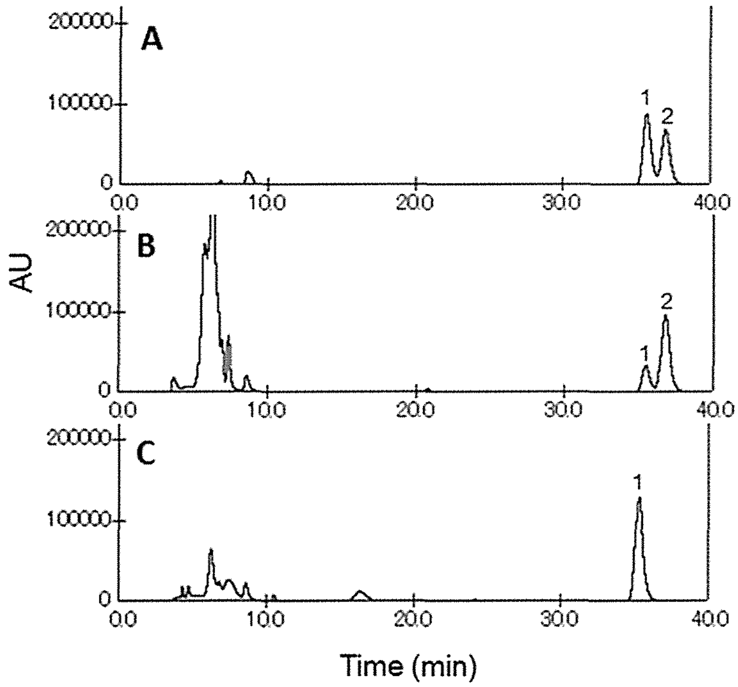

Fig 2.2 HPLC profile of OA and UA in OA and UA standard mix (A), cranberry (B), and green raisin (C). Analytical condition of HPLC was written in method. The column used was Wakosil II 5 C18, and the mobile phase was acetonitrile : methanol : water : phosphoric acid (v : v : v : v = 500 : 400 : 100 : 0.5). The longitudinal axis shows absorbance at 210 nm in arbitrary units (AU). Peaks: (1) OA; (2) UA.

All 3 varieties of raisin investigated in this study were found to contain higher levels of OA compared to all other dried fruits, with OA contents of 79.0 mg/100 g (green raisins), 65.9 mg/100 g (Sultana raisins), and 38.5 mg/100 g (Kyoho raisins) (Fig. 2.3). This corresponds to previous findings that have shown that the presence of OA in raisins is independent of whether the grapevine cultivar is red or white (Yunoki et al., 2008; Orbán et al., 2009). We were unable to confirm the reasons for the differences in OA content between the different raisin types investigated in this study, as the OA content in the raw materials and any changes that occurred during manufacturing were unknown. No UA was detected in any of the raisin varieties (Fig. 2.2C, Fig. 2.3).

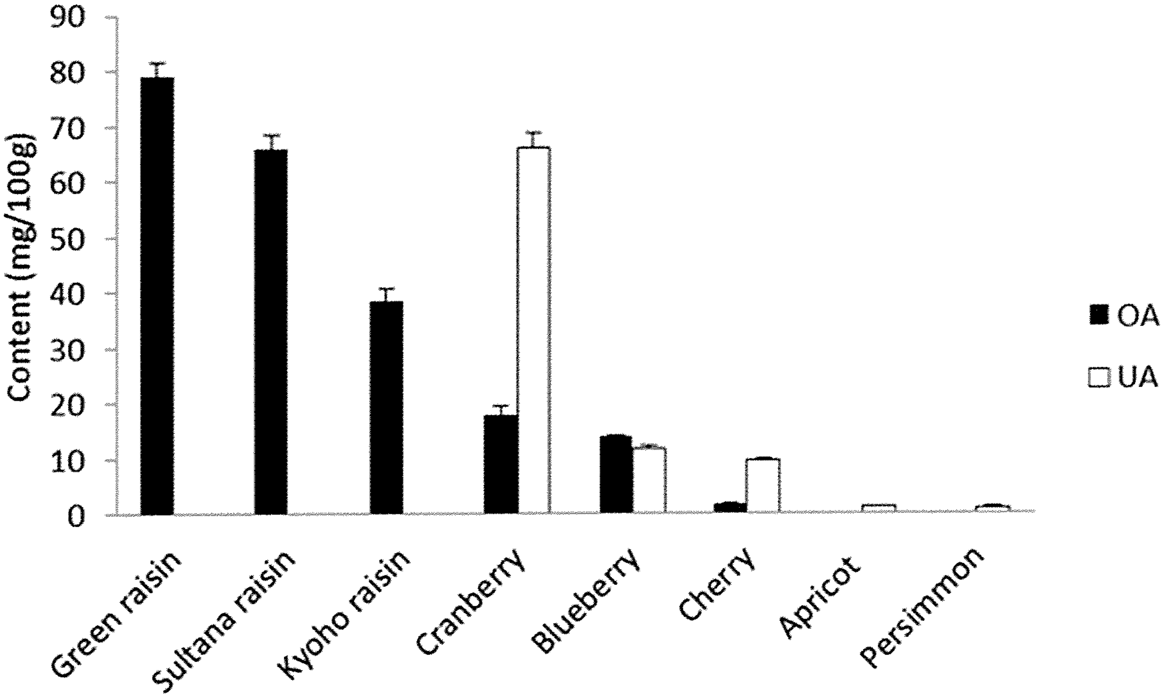

Fig 2.3 OA and UA contents in dried fruits. Bars indicate standard deviations (n = 3 independent extracts).

In contrast, dried cranberries, blueberries, and cherries were all found to contain both OA and UA (Fig. 2.3). Of the dried fruits analyzed in this study, the highest amount of UA (65.9 mg/100 g) was detected in cranberries, which also contained 17.8 mg/100 g OA. Blueberries contained similar levels of OA and UA, at 13.9 mg/100 g and 11.8 mg/100 g, respectively. The level of UA in cherries (9.7 mg/100 g) was similar to that of blueberries, although OA in cherries was detected at significantly lower levels (1.72 mg/100 g). Low levels of UA were also found in apricots and persimmon, although no OA was detected in either of them. Zhou et al. (2010) previously reported that both OA and UA were present in most of the cultivars of persimmon that they investigated. However, we detected only very low amounts of UA (1.03 mg/100 g) and no OA in dried persimmons. This discrepancy might be explained by the lack of skin present on the dried persimmons used in our study, whereas the UA that was detected might have originated from the calyces that were retained on the fruit tops.

Some folk medicine and herbal plants contain remarkable quantities of OA and UA, and their therapeutic effects may be partially due to these triterpenes. For instance, 18.2 mg/100 g fresh weight (FW) OA and 63.8 mg/100 g FW UA were detected in *Ziziphus jujuba* fruits (Guo et al., 2009), while the OA and UA contents in Chinese hawthorn (*Crataegus pinnatifida*) fruits were shown to be 14.7 mg/100 g FW and 95.2 mg/100 g FW, respectively (Cui et al., 2006). Therefore, the OA and UA contents detected in dried fruits in the present study were lower than those detected in these medicinal fruits. Nevertheless, the results of the present study show that OA and UA can be readily absorbed from raisins and dried cranberries as part of daily food consumption. As raisins and dried cranberries also contain various functional flavonoids (Zhao and Hall, 2007; Vinson et al., 2008), the combination of flavonoids with the triterpenes OA and UA provided by these fruits might be particularly beneficial for human health.

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Chapter 3. Oleanolic Acid and Ursolic Acid in Tropical Fruits and Their Anti-cancer Effects

3.1 Introduction

In the Global COE program of Obihiro University of Agriculture and Veterinary Medicine, I followed the Pacific Food Resource Search Unit (Asian) and visited Samoa and Indonesia. During the visit, I had the opportunity to visit market where I collected some tropical fruit samples for my research.

Fruit resources are particularly abundant in tropical areas. According to the local residents of Samoa and Indonesia, almost all fruits grown locally were used for export, making juice, or for direct consumption. To the best of my knowledge, no report has yet indicated that tropical fruits are also utilized for making wine and vinegar (Maldonado et al, 1975).

To date, little is known about the triterpenoid levels in tropical fruits, and few studies have reported triterpenoid levels in these fruits, including OA and UA. In order to identify rich sources of OA and UA, and to expand the utilization of tropical fruits, OA and UA levels were quantified from Indonesian and Samoan fruit samples. In addition, some commercial products of the tropical fruit noni were also measured, and triterpenoid levels were compared to those of natural fruits. Finally, the anti-cancer effect of triterpenoid extracts from tropical fruits was measured.

3.2 Materials and Methods

3.2.1 Materials and reagents

Several tropical fruits were collected from Samoa (Fai Papalagi, Fai Samoa, papaya, coconut, immature noni, and ripe noni) and Indonesia (ripe noni).

Noni products (Table 3.1), including noni extract (DHC, Japan), noni-dama (ASK, Japan), and 2 kinds of noni juice (noni Premium 100 (Okinawa Biken, Japan), Tahitian noni (Tahitian Noni Inc TB, Japan)), were purchased from a Japanese supermarket.

Table 3.1 Noni products used in this study

Noni products	Materials
Noni extract	olive oil, noni extract, gelatin, glycerin, VC, bee's wax, plant oil (contains VE), niacin, calcium pantothenate, VB_6, VB_2, VD_3, VB_1, VB_{12}
Noni-dama	noni powder
Noni premium 100	noni
Tahitian noni	Noni (produced in French Polynesia), grape, blueberry

OA (> 97%) and UA (> 95%), ethanol (> 99.5%), chloroform, methanol (> 99.8%, HPLC grade), acetonitrile (> 99.8%, HPLC grade), and ethyl acetate (> 99.8%, HPLC grade) were purchased from Wako Pure Chemical Industries, Ltd. (Japan). Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA solution were purchased from Sigma. Fetal bovine serum (FBS) was obtained from Biowest. Penicillin/streptomycin was obtained from GIBCO. The Dojindo cell counting kit-8 was used.

3.2.2 Sample preparation

Tropical fruits were separated into skin, flesh, and seed by hand, and were then freeze-dried.

The skin, flesh, and seed of dried tropical fruits, after homogenizing, were added to ethanol along with noni products (noni extract and noni-dama) at a weight to volume ratio of 1:20, and was then homogenized by agitation for 1 h, followed by filtration through No. 2 filter paper (Advantec, Japan). After 3 repetitions of this operation, the combined extracts were concentrated to dryness using a rotary evaporator, and were dissolved in methanol with a final concentration of 10 mg/mL for use in TLC and HPLC analyses.

Noni-products, noni premium 100 and Tahitian noni, and 2 kinds of noni juice were extracted using the Bligh-Dyer method (Bligh and Dyer, 1959). Noni juices were mixed with chloroform and methanol (v/v V = 0.8:1:2) for 2 h in a separating funnel. The same volume of chloroform and water was added and mixed overnight. The bottom phase was filtered out and concentrated to dryness using a rotary evaporator. Finally, methanol was added to the dried product at a final concentration of 10 mg/mL for use in TLC and HPLC analysis.

3.2.3 TLC analysis

The same TLC analysis method was applied as described in Chapter 1.

3.2.4 HPLC analysis

Quantitative analyses of OA and UA concentrations were carried out using the same HPLC analysis method as described in Chapter 1. The amounts of OA and UA in tropical fruits and noni products were calculated from the calibration curves of OA and UA standards, respectively. The representative calibration curve of OA was $y = 2.847e^{-7}x + 1.552e^{-2}$ (y = mg/mL of OA; x = area), with a correlation coefficient of $9.995e^{-1}$, and the representative calibration curve of UA was $y = 3.311e^{-7}x + 1.938e^{-2}$ (y = mg/mL of UA; x = area), with a correlation coefficient of $9.998e^{-1}$. All data are presented as the means of 3 replicates with standard deviations calculated using Microsoft Excel (Microsoft, USA).

3.2.5 Cell culture

Caco-2 cells (derived from human colon carcinoma) were maintained as monolayer cultures in DMEM supplemented with 10% FBS and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin) under a humidified atmosphere of 5% CO_2 at 37°C.

3.2.6 Cell proliferation assay

The effect of test compounds on cell proliferation was assayed in 96-multiwell culture plates seeded with 100 μL/well at a density of 5×10^4 cells/mL. Ninety-six hours after plating, the medium was discarded, and fresh medium containing test compounds at different concentrations (20 μM, 50 μM, 75 μM and 100 μM) was added. After 48 h of incubation with test compounds, cell viability was evaluated using a commercial kit (cell counting kit-8, Dojindo, Kumamoto, Japan). The optical density was read at 450 nm (absorbance) and 630 nm (reference absorbance) using the Original Multiskan JX (Thermo Electron Corporation). The results were expressed as cell viability (%), which was calculated using the following formula: cell viability (%) = [(mean absorbance of the sample − reference absorbance)/(mean absorbance of the control) × 100] (Zhang et al., 2009)

3.2.7 Identification of apoptotic cell

Caco-2 cells were seeded on a Lab-Tek II chamber slide W/Cover in an 8-well configuration and incubated with test compounds at 37°C with 5% CO_2 for 48 h. Apoptosis was assessed by a terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assay with a commercial kit (TACS™ 2 TdT Fluorescein In Situ Apoptosis Detection Kit) according to manufacturer instructions. Cells were incubated with DAPI for nuclei staining. The percentage of apoptotic cells was calculated as the ratio of TUNEL positive cells to the total number of cells (nuclei staining with

DAPI), from a count higher than 500 cells per slide under a fluorescence microscope.

3.2.8 Statistical analysis

Data were presented as means $\pm$ SD. Student's t test was used to assess the significance of differences. The value of $p < 0.05$ was considered statistically significant.

3.3 Results and Discussion

3.3.1 TLC analysis

As mentioned in Chapter 2, OA and UA are known to mainly exist in fruit skin. Similar results were found in the present study. OA and UA were not detected in the seed or flesh parts of tropical fruits assessed in this study. In the skin part, only noni skin (Samoa immature noni, Samoa ripe noni, and Indonesia ripe noni) were found to contain OA or UA (Fig 3.1), whereas the skin of Fai Papalagi, Fai Samoa, papaya, and coconut did not contain any traces of OA and UA. The synthesis of OA and UA requires α-amyrin and β-amyrin (Boar et al., 1970), respectively. Therefore, it is possible that OA and UA were not detected in the skin of some tropical fruits because these fruits lack either α-amyrin and β-amyrin or other enzymes that are related to OA and UA synthesis.

The TLC analysis also showed that the OA or UA content in Samoa noni skin extract was higher than that in Indonesia noni skin extract. This variation of OA or UA content in noni fruits might be due to substantial differences in geographical and/or environmental factors such as soil, sunlight, temperature, moisture, and air effects.

Among the noni commercial products assessed in this study, OA or UA was detected in noni-dama and Tahitian noni (Fig 3.1), but were not detected in noni extract or in the Noni premium 100. This difference might be due to specific production processes or differences in raw materials.

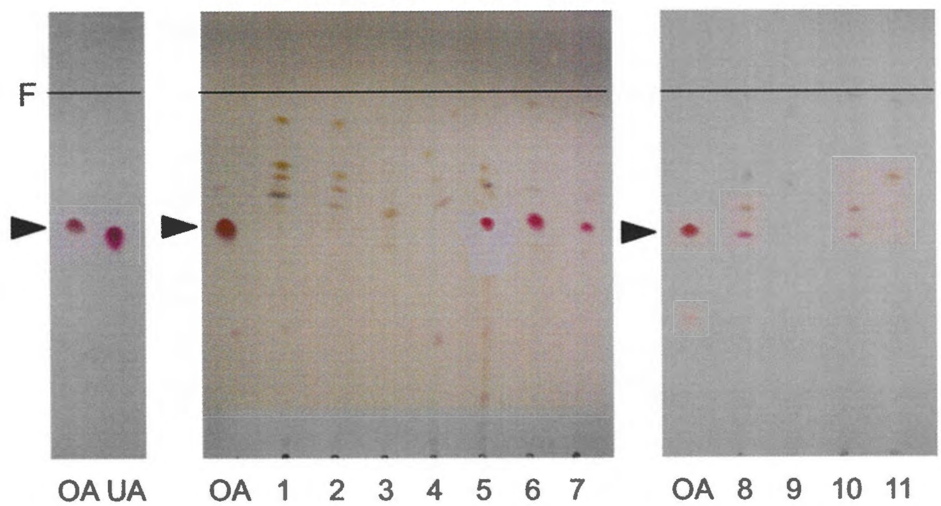

Fig. 3.1 TLC profile of OA and UA fraction of noni fruits (left) and noni products (right). OA or UA fraction was derived by cyclohexane : acetone : ethyl acetate (v:v:v=4:2:1) on silica gel plate, and was visualized by spraying the plates with 50% sulfuric acid and baking. Arrowheads and circles indicate the positions of OA and UA. The solid line indicated by F represents the front line of the TLC analysis. Lanes 1–11 are as follows: Fai Papalagi (1), Fai Samoa (2), papaya (3), coconut (4), Samoa immature noni (5), Samoa ripe noni (6), Indonesia ripe noni (7), and extracts of noni products: noni-dama (8), noni ex (9), Tahitian noni juice (10), and Noni 100 (11).

3.3.2 HPLC analysis

HPLC analysis was further applied in order to separate and quantify the independent levels of OA and UA present in samples found to contain these triterpenoids by TLC analysis (Fig 3.1),

As shown in Figure 3.2, the UA content was higher than the OA content in each sample tested. Samoa ripe noni skin was found to contain the higher levels of OA and UA (1.58 mg/g dry weight (DW) and 7.29 mg/g DW, respectively) compared to those of immature noni skin (1.30 mg/g DW and 6.18 mg/g DW, respectively), which suggested that the maturity of fruits might also affect the OA and UA content. OA and UA levels were also higher in Samoa ripe noni (OA 0.88 mg/g DW and UA 4.57 mg/g DW, respectively) compared to Indonesia ripe noni. These differences might reflect different cultivars or different growing environments.

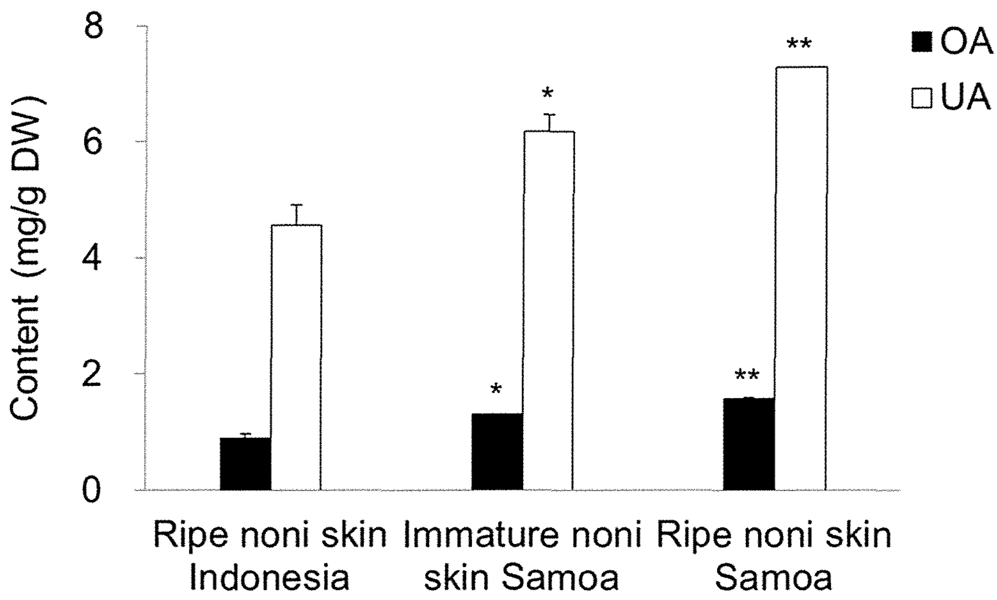

Fig. 3.2 OA and UA contents in noni skin extracts. Error bars indicate standard deviations (n = 3 independent extracts). *, $p < 0.05$, **, $p < 0.01$, compared with ripe noni skin Indonesia.

OA and UA contents in noni products (noni-dama and Tahitian noni) were also analyzed by HPLC (data not shown). According to the suppliers, in general, most people drink 100 mL juice or eat 3 tablets at once. Therefore, in this study, OA and UA contents were compared among 100 mL Tahitian noni, 3 tablets of 300 mg noni-dama, and 300 mg noni skin extract (Fig. 3.3). The UA content was found to be consistently higher than the OA content. One hundred milliliters of Tahitian noni contained higher levels of OA (0.18 mg) and UA (0.74 mg) than 3 tablets of noni-dama (0.05 mg OA and 0.19 mg UA), which suggested that people could intake more OA and UA from 100 mL Tahitian noni than from 3 tablets of noni-dama. However, these amounts of OA and UA were still much lower when compared with similar amounts of noni skin extract (0.47 mg OA and 2.19 mg UA), which suggests that noni skin is a good source of OA and UA.

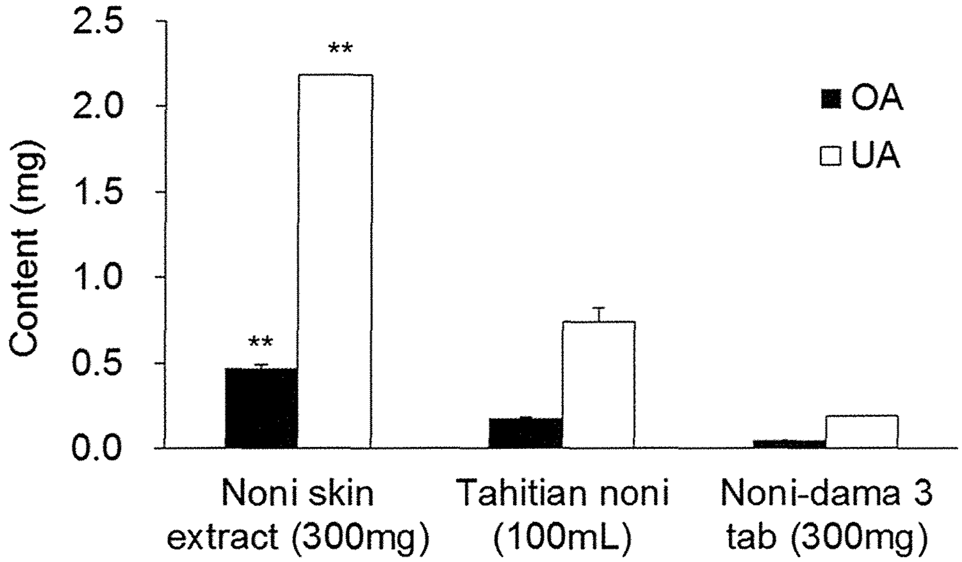

Fig. 3.3 OA and UA contents in Tahitian noni, noni-dama and noni skin extract. Error bars indicate standard deviations (n = 3 independent extracts). **, $p < 0.01$, compared with 100 mL Tahitian noni.

According to local residents of Samoa and Indonesia, many pomaces remain after noni juice production, which are usually used for foraging. The quantification results showed that noni skin is rich in OA and UA, which indicates that the noni pomace (especially the skin) could be used as raw material for making functional food or medicine.

3.3.3 Effect of OA, UA, and noni skin extract on cell proliferation

In order to select the optimal concentrations of OA, UA, and noni skin extract (expressed as total content of OA and UA in noni skin) that could be used effectively in this study, the effects of OA, UA, and noni skin extract on cell proliferation were evaluated using a commercial kit (cell counting kit-8). Four different concentrations (20 μM, 50 μM, 75 μM, and 100 μM) were applied. When Caco-2 cells were incubated with OA, UA, and noni skin extracts for 48 h, cell viability significantly decreased in a dose-dependent manner (Fig. 3.4). This result indicated that OA, UA, and noni skin extract have high cytotoxicity and inhibit cell proliferation of Caco-2 cancer cells, while OA and UA are relatively nontoxic to normal cells (Liu, 1995). Among the 4 different concentrations used in this study, concentration of 100 μM showed the strongest effect on the suppression of cell proliferation significantly ($p < 0.001$), and this concentration was therefore used in the following experiments.

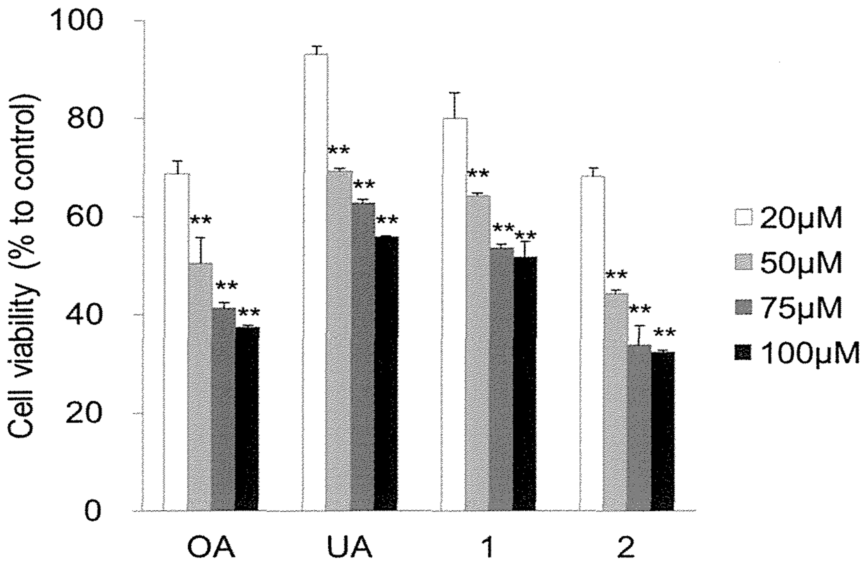

Fig 3.4 Effect of OA, UA, and noni skin extract on cell viability (expressed as % of control) of Caco-2 cells. 1: Samoa noni skin extract; 2: Indonesia noni skin extract. Concentration of noni skin extract was expressed in total concentration of OA and UA. Error bars indicate standard deviations ($n = 3$). **, $p < 0.01$, compared with concentration of 20μM experiment group.

3.3.4 Effect of OA, UA, and noni skin extract on apoptosis

The ability of OA, UA, and noni skin extracts to induce apoptosis was investigated using the TUNEL assay. Results of the assay are shown in Figure 3.5, in which DAPI-stained cells indicate nucleus staining and TUNEL negative cells. All compounds significantly induced apoptosis in Caco-2 cells (Fig. 3.6).

Apoptotic cells increased significantly from 1.80% in the control to 50.31%, 39.30%, 40.17%, and 47.83% in cells treated with 100 μM of OA, UA, Samoa noni skin extract, and Indonesia noni skin extract, respectively (Fig. 3.6). These results demonstrated the anti-cancer properties of OA, UA, and noni skin extract on Caco-2 cells. Compared with UA treatment group, OA showed significantly higher apoptosis induction. Similar results were found in an analysis of human hepatoma HuH7 cells (Shyu et al., 2010). Additionally, OA and UA were also shown to induce apoptosis in HL-60 leukemia cells, B16F-10 melanoma cells, MCF-7 breast cancer cells, and DU145 prostate cancer cells (Zhang et al., 2007; Manu et al., 2008; Kassi et al., 2009; Zhang et al., 2009). Cipak et al. (2006) suggested that OA and UA could inhibit the growth of HL60 leukemia cells, and that their IC_{50} values were 70 μM and 10 μM, respectively. Li et al. (2002) indicated that the IC_{50} values of OA and UA on HCT15 human colon carcinoma cells were 60 μM and 30 μM, respectively. In the present study, the IC_{50} values of OA and UA on Caco-2 cells were both approximately 100 μM. OA and UA have similar molecular structures, but have different methyl group sites on the E ring: if the methyl group at C19 of UA is moved to C20, it

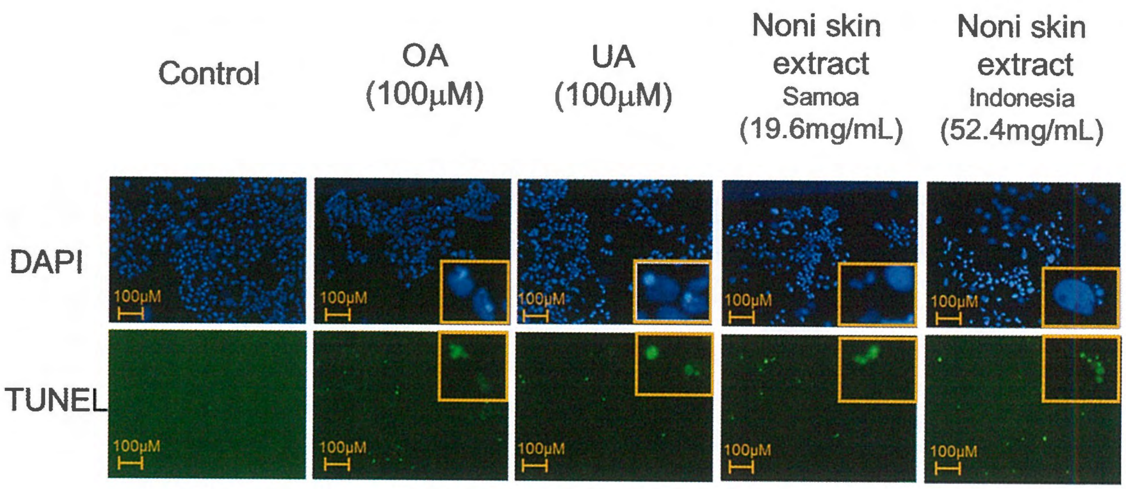

Fig. 3.5 Effect on apoptosis of 100 µM of OA, UA, Samoa noni skin extract, and Indonesia noni skin extract as measured by the TUNEL assay. DAPI staining was used for identifying nuclei, and TUNEL staining was used for identifying apoptotic cells.

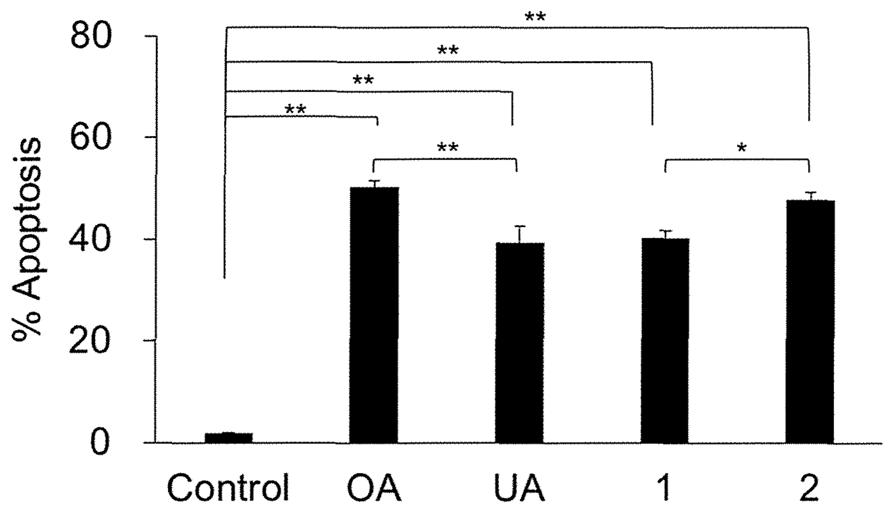

Fig. 3.6 Percentage of apoptosis induced by 100 μM of OA, UA, Samoa noni skin extract (1), and Indonesia noni skin extract (2). The percentage of apoptotic cells was calculated as the ratio of TUNEL positive cells to the total number of cells (nuclei staining with DAPI), from a count higher than 500 cells per slide under a fluorescence microscope. *, $p < 0.05$, **, $p < 0.01$.

becomes OA (Es-saady et al., 1994). This small difference in structure might result in the different magnitudes of effects observed on cancer cells. In addition, differences in cancer cell types and laboratory conditions could also produce different results across studies.

Indonesia noni skin extract showed significantly higher suppression of cell viability than did Samoa noni skin extract, despite containing similar amounts (100 μM) of OA and UA. In 100 μM noni skin extracts, the percentage of OA in Indonesia noni skin extract (24.6%) was higher than that in Samoa noni skin extract (22.1%). This difference may have contributed to their different effects on Caco-2 cells. Furthermore, other compounds in noni skin extracts such as scopoletin and quercetin (Deng et al., 2010) may have also contributed to the observed variation.

3.4 Conclusion

Results of this study demonstrated that only noni skin contained OA and UA among all parts of the fruit. Samoa ripe noni skin contained higher amounts of OA and UA compared to immature noni skin, which were even higher than those of Indonesia ripe noni skin. OA showed a greater effect on Caco-2 cells than did UA. When containing similar amounts of OA and UA, Indonesia noni skin extract had a greater effect on apoptosis of Caco-2 cells.

Among the 4 kinds of noni commercial products analyzed, only noni-dama and Tahitian noni were found to contain OA and UA. When similar amounts of noni skin extract and noni commercial products (noni-dama and Tahitian noni) were compared, noni skin extract contained higher levels of OA and UA, suggesting that noni skin extract is a particularly good source of OA and UA.

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Chapter 4. Synergistic Effect of Oleanolic Acid and Polyphenols from Wine Pomace on Cancer Cells

4.1 Introduction

Grape production has developed into the most important fresh fruit crop in the world, 70% of which is used for wine production (Jackson, 1993). Wine making produces grape pomace as a by-product in an estimated amount of 13% by weight of grapes (Torres et al., 2002). These wine making by-products, wine pomace, consist of the skins, seeds, and stems of grapes, and should be treated as a special solid waste due to its high levels of residual phenolic compounds, which may have adverse environmental impacts, mainly due to the inhibition of germination properties of polyphenols (Morthup et al., 1998). Consequently, wine producers' costs will increase for waste treatment. On the other hand, wine pomace is also known to be rich in many kinds of bioactive compounds (Amico et al., 2004). Wine pomace has been tested for use as compost, as a supply source of polyphenols, and as a raw material of grape seed oil (Baran et al., 2001; Lo Curto and Tripodo, 2001; Negro et al., 2003; Palenzuela et al., 2004; Gambuti et al., 2004). Besides polyphenols, wine pomace is also rich in OA (Amico et al., 2004), which was confirmed in our laboratory (Yunoki et al., 2008). Therefore, the costs associated with wine pomace treatment can be reduced by extracting and using its bioactive compounds such as OA. However, after OA extraction, other bioactive compounds remain in the residues, such as polyphenols. Whether or not it might be worth also extracting these polyphenols from residues after OA extraction is an open question as the specific amount of polyphenols remaining in these residues is unknown. To address these problems, and to make full use of these wine pomaces, we extracted and quantified polyphenols from residues after OA extraction. The polyphenols in OA extracts were also quantified. The results of Chapter 3 showed that OA inhibited Caco-2 cell proliferation; however, if the residues are also rich in polyphenols, the residual polyphenols might also have an effect on Caco-2 cells. Therefore, in the current study, experiments were performed to determine whether OA and residue polyphenol extracts from pomace might have synergistic effects on Caco-2 cells?

4.2 Materials and Methods

4.2.1 Materials

Frozen wine pomaces were obtained from the Tokachi-Ikeda Research Institute for Viticulture and Enology, Ikeda, Hokkaido, Japan.

4.2.2 Preparation of sample

4.2.2.1 Preparation of pomace ethanol extract

Wine pomaces were vacuum freeze-dried with the vacuum freeze drying machine (Yamato, Japan). Two grams of dried pomace (stem:seed:skin = 0.1:0.9:1.5) were agitated with 40 mL of ethanol for 1 h either after homogenization treatment (crushing in a grinder for 2 min, with 15 s rest periods at 15 s intervals to avoid overheating the samples) or no homogenization, and were then filtered using No. 2 filter paper (Advantec, Tokyo, Japan). After 3 repetitions of similar procedures, the combined extracts were concentrated to dryness using a rotary evaporator. The dry matter was weighed to obtain the pomace ethanol extract (PEE) quantity, and was then dissolved in methanol at a final concentration of 10 mg/mL for use in HPLC analysis.

4.2.2.2 Preparation of residual polyphenol

After PEE extraction, with or without homogenization, the residues were de-oiled to remove lipids as suggested by Jayaprakasha et al. (2001), because the lipids remained in residues will disturb the polyphenol extraction. The powdered pomace (m) was extracted with hexane (v) with a ratio of m:v = 1:5 by gentle shaking in a mild mixer (Taitec, Japan) for 1 h at room temperature. The mixtures were then filtered and the residues were air-dried.

The conditions of polyphenol extraction (time, temperature, liquid-to-solid ratio, and number of extractions) were chosen based on data from the literature (Cacace and Mazza, 2003; Shi et al., 2003; Nawaz et al., 2006; Bucić-Kojić et al., 2007). The polyphenol was extracted from de-fatted residues at room temperature (~25°C) for 40 min using either acetone/water (ace/w), ethanol/water (eth/w), or methanol/water (meth/w) at 1:1, v/v ratios. The extraction was carried out 3 times using a liquid to solid ratio of 10:1, the combined extracts were freeze-dried and dissolved in water with a final concentration of 20 mg/mL, and were then stored at 4°C for further processing and analysis.

4.2.3 HPLC analysis

The quantification of OA was carried out using the same HPLC method described in Chapter 2. The amount of OA in wine pomace was calculated from the calibration curve: $y = 2.929e^{-7}x + 1.239e^{-2}$ (y = mg/mL of OA; x = area), with a correlation coefficient of $9.998e^{-1}$. All data are presented as the means of 3 replicates with standard deviations calculated using Microsoft Excel (Microsoft, USA).

4.2.4 Polyphenol analysis

The polyphenol content was determined spectrophotometrically using the Folin-Ciocalteu method. This test is based on the oxidation of phenolic groups by phosphomolybdic and phosphotungstic acids (FC reagent). The use of this reagent is based on the Slinkard and Singleton (1997) method, and the early work of Singleton and Rossi (1965), which is a colorimetric oxidation/reduction method for phenolic compounds. The products of the metal oxide reduction exhibit blue coloration that emits a broad light absorption with a maximum at 765 nm. The intensity of light absorption at this wavelength is proportional to the concentration of phenols. Briefly, a 20 μL sample of either calibration standard or blank was added to 1.58 mL distilled water for dilution (80 times), and then 100 μL of FC reagent was added. After 3 min, 300 μL of saturated sodium carbonate solution (25%) was added and incubated 2 h at room temperature. The absorbance was measured at 765 nm. The calibration curve was constructed with various concentrations of gallic acid solutions ranging from 0.05 to 2 mg/mL, and the results were expressed as gallic acid equivalents (GAE) (mg GAE/g pomace).

4.2.5 Cell culture

As in Chapter 3, Caco-2 cells (derived from human colon carcinoma) were used in this study.

4.2.6 Effect on cell proliferation

The effect of test compounds (ethanol solution) on Caco-2 cell proliferation was assayed using the cell counting kit-8 (Dojindo) as described in Chapter 3.

4.2.7 Effect on apoptosis

The effects of PEE, residual polyphenol, and their combination on apoptosis were assessed with a TUNEL assay using a commercial kit (TACS™ 2 TdT Fluorescein In Situ Apoptosis Detection Kit) following manufacturer instructions with the same methods described in Chapter 3.

4.2.8 DNA microarray analysis

To investigate the mechanism of Caco-2 cancer cell apoptosis induced by 100 μM OA, 0.16 mg/mL PEE, 1 mg/mL residual polyphenol, and the combination of 0.16 mg/mL PEE and 1 mg/mL residual polyphenol, a DNA microarray analysis was performed using the Affymetrix system. Total RNA was extracted from cells treated with each compound using Trizol reagent (Ambion) following the RNA isolation protocol (Abcam), and its quality was checked based on absorbance at 260 nm and 280 nm and by electrophoresis. Equal amounts of RNA from each RNA extract replicate (n = 3) of each compound treatment group were pooled to normalize individual differences. Using Affymetrix GeneChip 3' IVT Express Kits, 500 ng of total RNA of each compound treatment group was used to synthesize double-stranded cDNA by reverse transcription, which were subsequently converted to biotin labeling cRNA, purified, and fragmented. Aliquots of 10 μg of fragmented cRNA were hybridized to a Human Genome U133A 2.0 GeneChip (Affymetrix) at 45°C for 16 h. After hybridization, the gene chips were washed and stained using a GeneChip Fluidics Station 450 (Affymetrix), and were then scanned with the GeneChip scanner (Affymetrix). Data analyses were performed with ArrayStar software (DNASTAR). The identification of genes that were up- or down-regulated by 100 μM of OA, 0.16 mg/mL of PEE, 1 mg/mL of residual polyphenol, or the combination of PEE (0.16 mg/mL) and residual polyphenol (1 mg/mL) was performed by comparing gene expressions in Caco-2 cells to those of control treatment group. We selected only those genes showing more than 2-fold changes (either up-regulated or down-regulated) in expression relative to controls. Moreover, differentially expressed genes were categorized using DAVID tools and were mapped to KEGG pathways.

4.2.9 Statistical analysis

Data were presented as means + SD. Student's t test was used to assess the significance of differences. The value of $p < 0.05$ was considered statistically significant.

4.3 Results and Discussion

4.3.1 OA in PEE

The quantification of OA in PEE (homogenized or without homogenization) was achieved using HPLC analysis, and the result was expressed as mg/g pomace. Results showed that OA contents in homogenized and not homogenized PEE were 12.76 mg/g pomace and 11.75 mg/g pomace, respectively. The homogenization treatment increased the yield of OA in PEE. Yunoki et al. (2008) determined the OA contents in several types of wine pomace, which was found to be 0.25 to 1.26% different than that of dried pomace. Amico et al. (2004) measured the constituents of wine pomace from the Sicilian cultivar 'Nerello Mascalese', and found that the OA content was 0.158% of that of pomace dry weight. These differences in the quantity of OA might be caused by different laboratory conditions, wine pomace cultivars, and other environmental factors.

4.3.2 PEE inhibited cancer cell proliferation

In Chapter 3, the results showed that OA could inhibit Caco-2 cell proliferation in a dose-dependent manner. As the results described in section 4.3.1 above showed that PEE contained OA, the effect of PEE on Caco-2 cell proliferation was further investigated. As shown in Figure 4.1, similar to OA, PEE also inhibited the proliferation of Caco-2 cells in a dose-dependent manner, and a concentration of 100 μM showed the strongest effect. Furthermore, PEE showed a stronger effect on cell proliferation than OA. This difference may have been caused by other compounds present in PEE, such as polyphenols. These results suggested the hypothesis that polyphenol was one of the key compounds involved in PEE's inhibitory effects on Caco-2 cell proliferation.

4.3.3 Polyphenol in wine pomace

After PEE is extracted from wine pomace, the residues still contain some other bioactive compounds such as polyphenols. In order to determine the specific quantity of polyphenols remaining in residues, and whether they might be worth extracting to increase the efficient utilization of wine pomaces, polyphenol contents were quantified. As above, homogenization and no homogenization treatments were also applied in this experiment. Figure 4.2 shows the distribution of polyphenols in wine pomace. The homogenization treatment increased polyphenol yield in PEE by almost 3 times compared to those without the homogenization treatment.

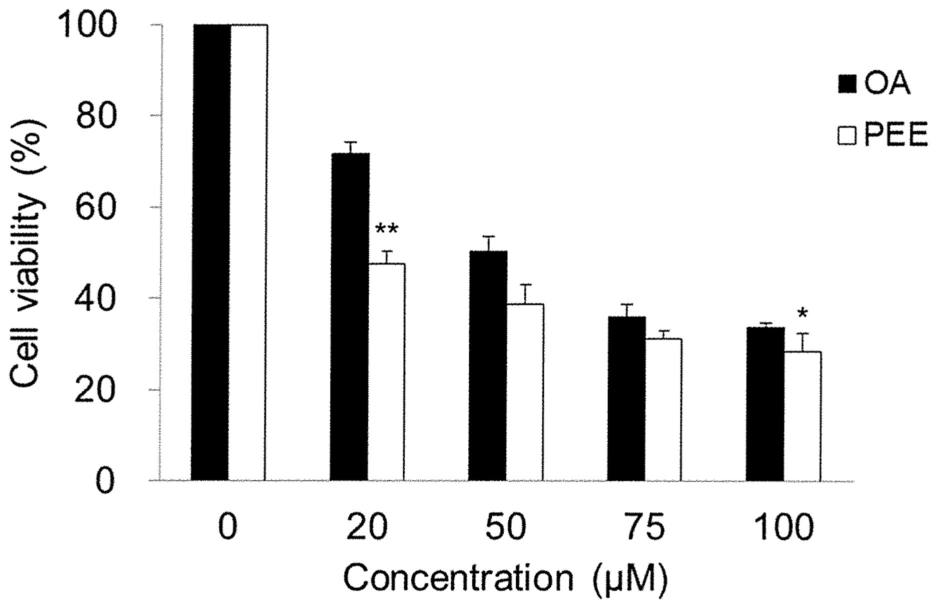

Fig 4.1 Effect of OA and PEE on Caco-2 cell proliferation. Concentration of PEE was expressed as that of OA in PEE. Error bars indicate standard deviations (n = 3). *, $p < 0.05$, **, $p < 0.01$, compared with OA treatment group in same concentration.

Regardless, polyphenol almost always remained in residues, and the quantities were found to be 4.90 mg GAE/g pomace in homogenized residues of the non-homogenized wine pomace treatment group and 4.48 mg GAE/g pomace in the homogenized wine pomace treatment group. Similarly, the homogenization treatment increased the polyphenol yield in residues. Together, these results demonstrate that it would indeed be valuable to extract polyphenols from residues after PEE extraction, which will enable more efficient utilization of wine pomaces.

4.3.4 Polyphenol inhibited cancer cell proliferation

The results described above in section 4.3.3 demonstrated that wine pomace residues were rich in polyphenols. To confirm whether these polyphenols might also have effects on cancer cell proliferation, Caco-2 cells were incubated with 0.1, 0.5, 1, and 3 mg/mL residual polyphenols for 48 h, and then cell viability was measured using the cell counting kit-8.

As shown in Figure 4.3, residual polyphenols inhibited Caco-2 cell proliferation in a dose-dependent manner, and a concentration of 3 mg/mL showed the highest effect. Ramos (2008) also demonstrated cell type- and dose-dependent effects of polyphenols. Furthermore, residual polyphenol

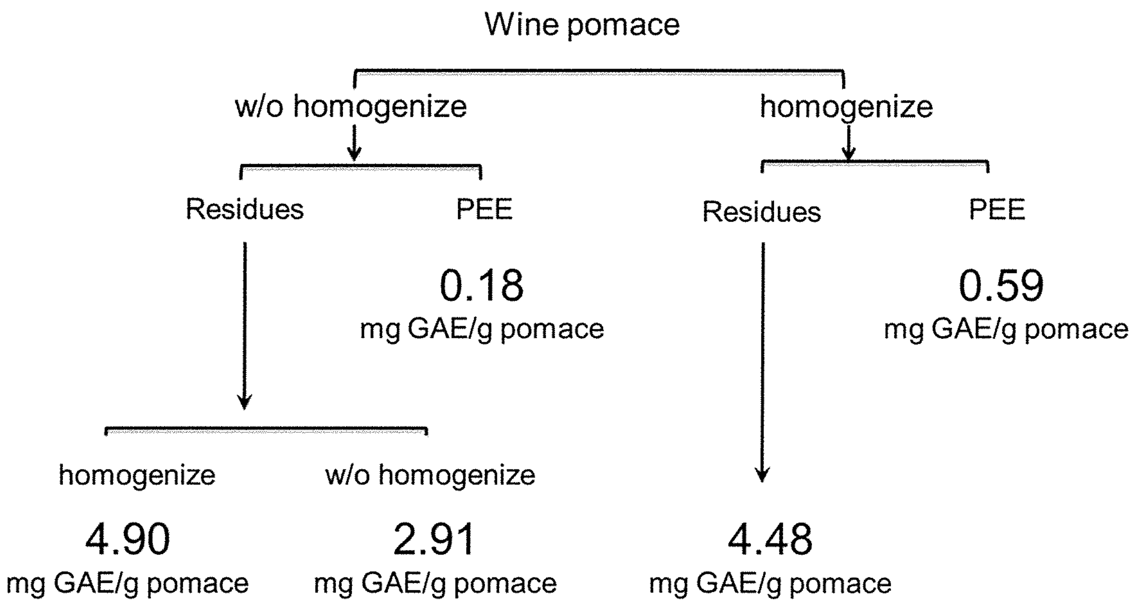

Fig. 4.2 Polyphenol distribution in wine pomace

from the non-homogenized treatment group showed a stronger effect on cell proliferation than did those from the homogenized treatment group. Concentration of 1 mg/mL in non-homogenized treatment group showed most significantly stronger effect. Besides increasing the yield, homogenization treatment might also affect the species of polyphenols in polyphenol extracts. Different polyphenols have been shown to have different effects on cancer cells. Polyphenols such as quercetin, EGCG, ellagic acid, and resveratrol have been shown to induce apoptosis (Manson, 2003; Surh, 2003), whereas rutin and epicatechin did not show any significant growth inhibition effects on cancer cells (Wu et al., 2007).

Together with the results of section 4.3.3, these results confirmed the hypothesis that polyphenols might help increase the effects of OA on inhibiting Caco-2 cell proliferation. This hypothesis was further examined in the following experiments, which used only residual polyphenol extracts from the non-homogenized treatment group.

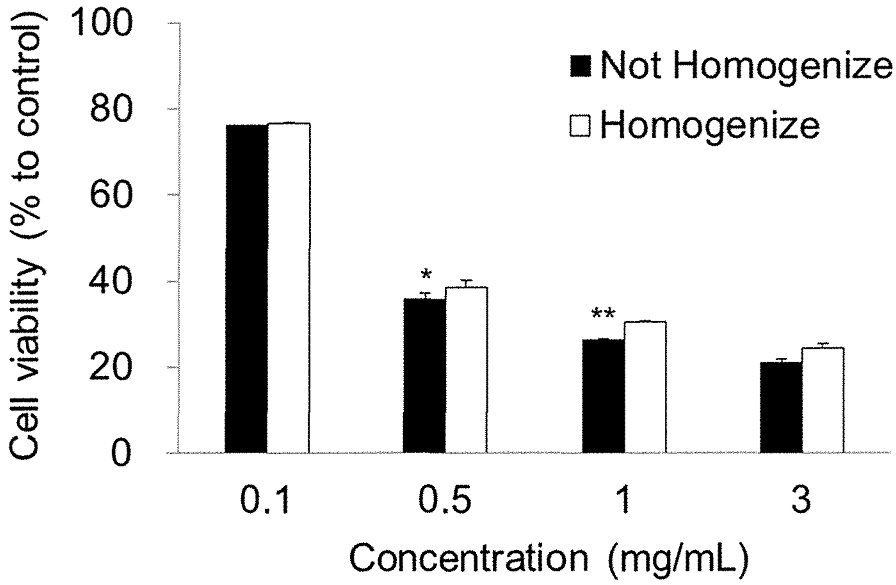

Fig. 4.3 Effect of polyphenols in homogenized or non-homogenized residues from the non-homogenized wine pomace treatment group on Caco-2 cell proliferation. Error bars indicate standard deviations ($n = 3$). *, $p < 0.05$, **, $p < 0.01$, compared with homogenized treatment group.

4.3.5 Synergistic effect of PEE and residual polyphenol on cancer cell proliferation

To confirm whether PEE and residual polyphenol extracts have a synergistic effect on inhibiting cancer cell proliferation, 0.16 mg/mL of PEE (~75 μM OA) and residual polyphenol extract from the non-homogenized wine pomace non-homogenized residual treatment group were used. Caco-2 cells were incubated with 0.16 mg/mL PEE and 0.1 mg/mL, 0.5 mg/mL, 1 mg/mL, or 3 mg/mL residual polyphenol for 48 h, and then cell viability was measured using the cell counting kit-8.

Results are shown in Figure 4.4. The addition of polyphenol increased the inhibitory effect of PEE on Caco-2 cell proliferation compared to PEE alone; the best combination of PEE and polyphenol with respect to inhibiting Caco-2 cell proliferation was 0.16 mg/mL and 1 mg/mL, respectively (Fig. 4.4C). Compared to controls, cell viability was increased by 34.32% (0.16 mg/mL PEE-treated cells), 25.71% (1 mg/mL polyphenol-treated cells), and 20.82% (0.16 mg/mL PEE- and 1 mg/mL polyphenol-treated cells).

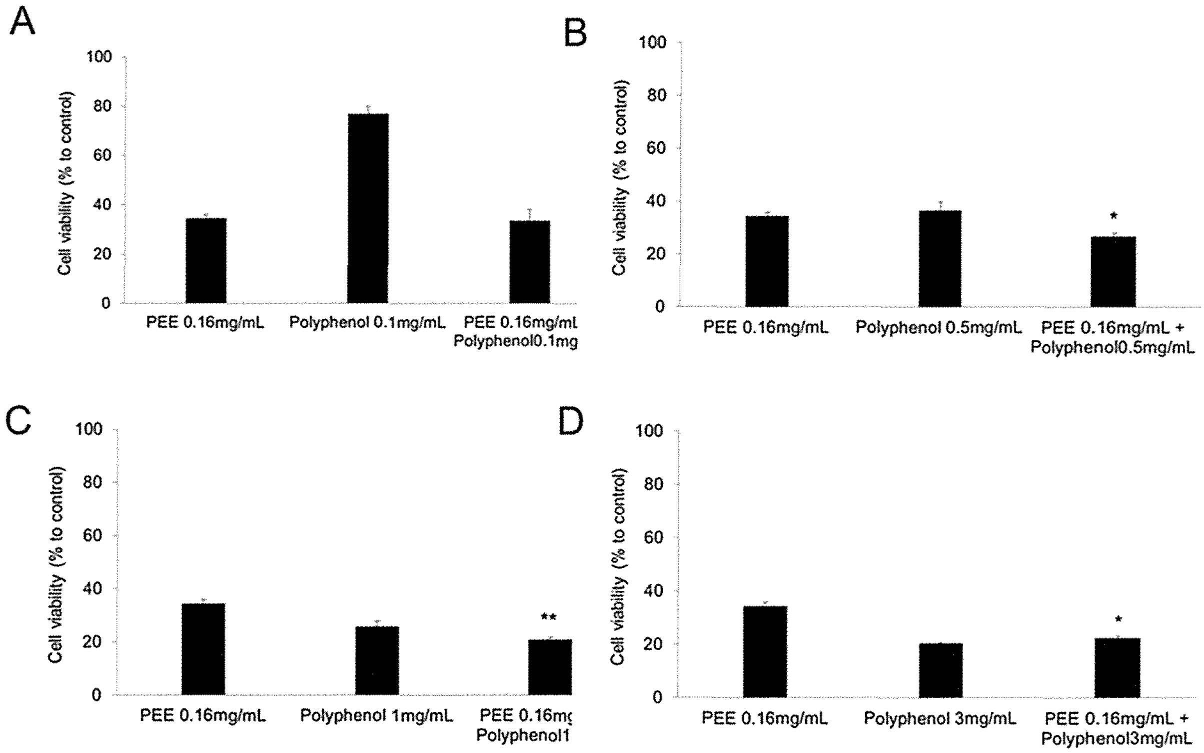

Fig. 4.4 Synergistic effect of PEE (0.16 mg/mL) and residual polyphenols (0.1 mg/mL, A; 0.5 mg/mL, B; 1 mg/mL, C; 3 mg/mL, D) on Caco-2 cell proliferation. Error bars indicate standard deviations (n = 3). *, $p < 0.05$, **, $p < 0.01$, compared with 0.16 mg/mL PEE treatment group.

4.3.6 Synergistic effect of PEE and residual polyphenol on cancer cell apoptosis

The ability of PEE, residual polyphenol, and their combination to induce apoptosis of Caco-2 cells was also assessed with the TUNEL assay (Fig. 4.5). Cells stained with DAPI indicated total cells. All compounds significantly induced apoptosis in Caco-2 cells. The percentage of apoptosis were found to be approximately 63.2% (0.16 mg/mL PEE-treated cells), 70.4% (1 mg/mL residual polyphenol-treated cells), and 73.4% (combination of PEE and residual polyphenol-treated cells) (Fig. 4.6). Residual polyphenol showed a synergistic effect with PEE on inducing Caco-2 cell apoptosis, and their combination induced apoptosis to a greater extent than residual polyphenol or PEE alone.

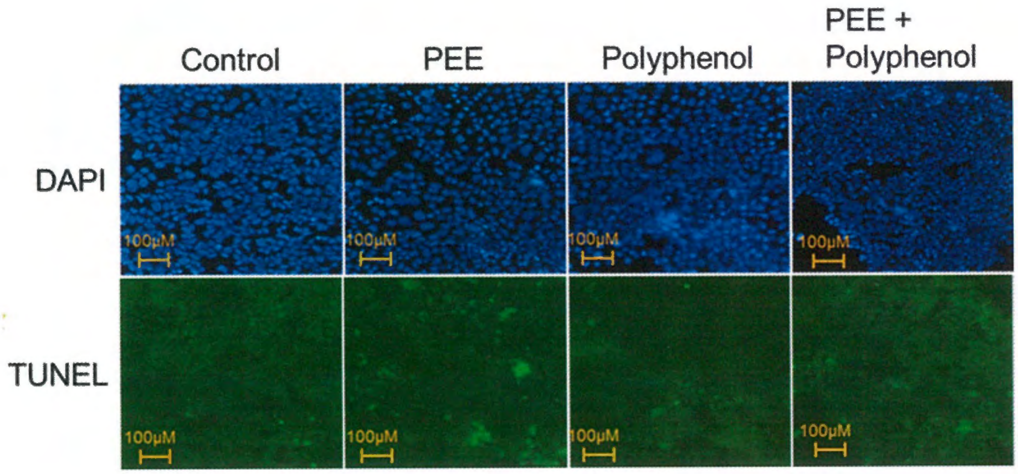

Fig. 4.5 Effect of 0.16 mg/mL PEE, 1 mg/mL residual polyphenol, and their combination on apoptosis as measured by the TUNEL assay. DAPI staining was used for identifying nuclei, and TUNEL staining was used for identifying apoptotic cells.

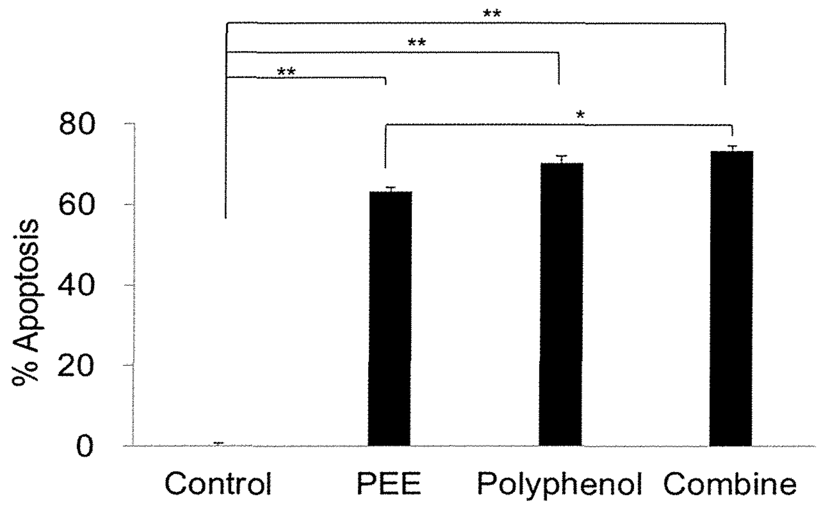

Fig. 4.6 Percentage of apoptosis induced by 0.16 mg/mL PEE, 1 mg/mL residual polyphenol, and their combination. The percentage of apoptotic cells was calculated as the ratio of TUNEL positive cells to the total number of cells (nuclei staining with DAPI), from a count higher than 500 cells per slide under a fluorescence microscope. *, $p < 0.05$, compared between PEE and combine treatment group, **, $p < 0.01$, compared with control group.

Some previous reports have shown synergistic effects of triterpenoids and polyphenols or between different polyphenols on cancer cells. Thyagarajan et al. (2007) demonstrated synergistic effects of green tea extract (polyphenol) with *Ganoderma lucidum* extract (triterpene) on inhibiting cancer cell proliferation, while Seeram et al. (2005) found that different polyphenols had synergistic effects of the inhibition of proliferation in human tumor cell lines.

4.3.7 Gene expression analyses

Expression changes of genes in Caco-2 cells were analyzed using DNA microarray analysis. Genes whose expression fold changes were over 2 compared to control were selected out. As shown in Table 4.1, PEE affected more genes (64) than OA (36) to regulate apoptosis. A total of 393 genes were commonly affected by 100 μM OA and 0.16 mg/mL PEE. After functional annotation clustering using DAVID tools, 25 of these genes (P = 0.0035) were found to be related to apoptosis regulation. These 25 genes are listed in Table 4.2. The pathway of apoptosis induced by 100 μM OA and 0.16 mg/mL PEE was shown in Fig 4.7. OA and PEE could commonly

Table 4.1 Number of genes those altered by 100 μM OA, 0.16 mg/mL PEE or commonly.

	Genes altered	Apoptosis					
		positive		negative		others	total
		Up	down	up	down		
OA	559	6	8	8	6	8	36
PEE	866	15	13	10	12	14	64
Common	393	6	7	4	3	5	25

Table 4.2 Alterations in apoptosis-related gene expression in Caco-2 cells of genes commonly affected by 100 μM OA and 0.16 mg/mL PEE.

Probe Set ID	Gene symbol	Gene name	P/N[a]	Fold Change	
				OA	PEE
205780_at	BIK	BCL2-interacting killer (apoptosis-inducing)	P	2.68↑	3.39↑
201849_at	BNIP3	BCL2/adenovirus E1B 19kDa interacting protein 3	P&N	7.96↓	4.74↓
221479_s_at	BNIP3L	BCL2/adenovirus E1B 19kDa interacting protein 3-like	P&N	4.59↓	4.34↓
211725_s_at	BID	BH3 interacting domain death agonist	P	2.10↑	2.43↑
212501_at	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta	P	2.80↑	2.02↑
204203_at	CEBPG	CCAAT/enhancer binding protein (C/EBP), gamma	P	2.30↑	2.10↑
204490_s_at	CD44	CD44 molecule (Indian blood group)	P	3.69↑	3.39↑
212935_at	MCF2L	MCF.2 cell line derived transforming sequence-like	P	2.27↓	2.27↓
211341_at	POU4F1	POU class 4 homeobox 1	P	2.32↓	2.99↓
204005_s_at	PAWR	PRKC, apoptosis, WT1, regulator	P	2.42↑	3.40↑
221617_at	TAF9B	TAF9B RNA polymerase II,	N	2.70↓	3.29↓

		TATA box binding protein (TBP)-associated factor, 31kDa			
201000_at	AARS	alanyl-tRNA synthetase	N	3.13↑	2.45↑
202834_at	AGT	angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	P&N	2.19↓	2.19↓
205047_s_at	ASNS	asparagine synthetase (glutamine-hydrolyzing)	N	9.05↑	7.29↑
207300_s_at	F7	coagulation factor VII (serum prothrombin conversion accelerator)	N	2.34↓	2.79↓
203139_at	DAPK1	death-associated protein kinase 1	P	2.44↓	2.28↓
214240_at	GAL	galanin prepropeptide	P	2.76↑	3.18↑
201631_s_at	IER3	immediate early response 3	N	2.66↑	5.81↑
201596_x_at	KRT18	keratin 18	N	2.89↑	2.85↑
207414_s_at	PCSK6	proprotein convertase subtilisin/kexin type 6	N	2.93↓	2.93↓
203125_x_at	SLC11A2	solute carrier family 11 (proton-coupled divalent metal ion transporters), member 2	—[b]	3.60↓	5.58↓
201009_s_at	TXNIP	thioredoxin	P	5.10↓	5.62↓

		interacting protein			
204924_at	TLR2	toll-like receptor 2	P	2.23↓	2.39↓
202431_s_at	MYC	v-myc myelocytomatosis viral oncogene homolog (avian)	P&N	2.28↑	2.94↑
217512_at	KNG1	kininogen 1	P	2.10↓	2.18↓

a — Positive regulation of apoptosis/negative regulation of apoptosis.
b — not identified with DAVID tools.

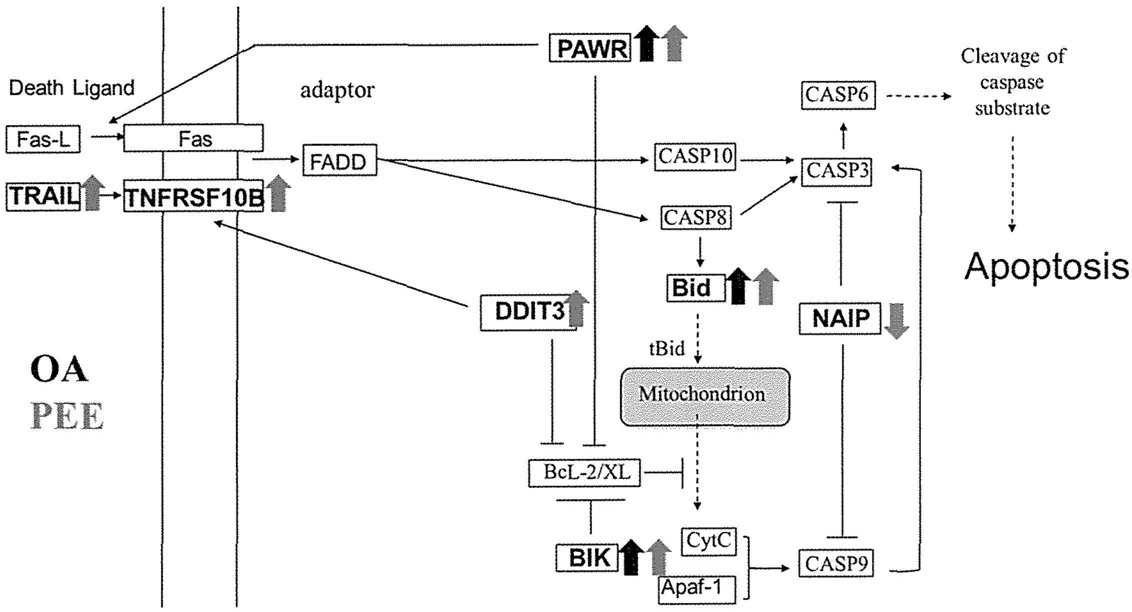

Fig. 4.7 Pathway of apoptosis induced by 100 μM OA and 0.16 mg/mL PEE.

up-regulate genes PAWR (apoptosis regulator), then the Fas pro-death pathway will be activated, and CASP8, 10, 3, 6 will be activated subsequently, and finally apoptosis will be induced. OA and PEE could also up-regulate gene BID (BH3 interacting domain death agonist), through mitochondrial pathway induce apoptosis. Mitochondrial pathway can be inhibited by anti-apoptotic proteins Bcl-2/XL, however, OA and PEE could up-regulate gene BIK (BCL2-interacting killer), and help to induce apoptosis. Except these genes, different from OA, PEE could also up-regulate TRAIL (death ligand), TNFRSF10B, DDIT3 (DNA-damage-inducible transcript 3), and down-regulate gene NAIP (apoptosis inhibitor) to induce apoptosis, Fig 4.7 also can explain why PEE showed stronger effect on cancer cell proliferation inhibition than OA.

On the other hand, residual polyphenol affected more genes (149) than PEE (64) to induce apoptosis (Table 4.3). A total of 715 genes were commonly affected by 0.16 mg/mL PEE and 1 mg/mL residual polyphenol. After functional annotation clustering using DAVID tools, 51 of these genes ($P = 1.4E^{-5}$) were found to be related to apoptosis regulation. The pathway of apoptosis induced by 0.16 mg/mL PEE and 1 mg/mL residual polyphenol was shown in Fig 4.8. Except up-regulation of genes PAWR, BID, BIK, DDIT3, TNFRSF10B and down-regulation of gene NAIP to induce apoptosis, residual polyphenol could also up-regulate gene FADD (death adaptor) and Apaf-1 (apoptotic protease activating factor 1) to induce apoptosis. Fig 4.8 also helped to explain why PEE and residual polyphenol have synergistic effect to induce more apoptosis.

Table 4.3 Number of genes those altered by 0.16 mg/mL PEE, 1 mg/mL residual polyphenol (PP) or commonly.

	Genes altered	Apoptosis					
		positive		negative		others	total
		Up	down	up	down		
PEE	866	15	13	10	12	14	64
PP	2459	29	38	22	25	35	149
Common	715	9	14	12	5	11	51

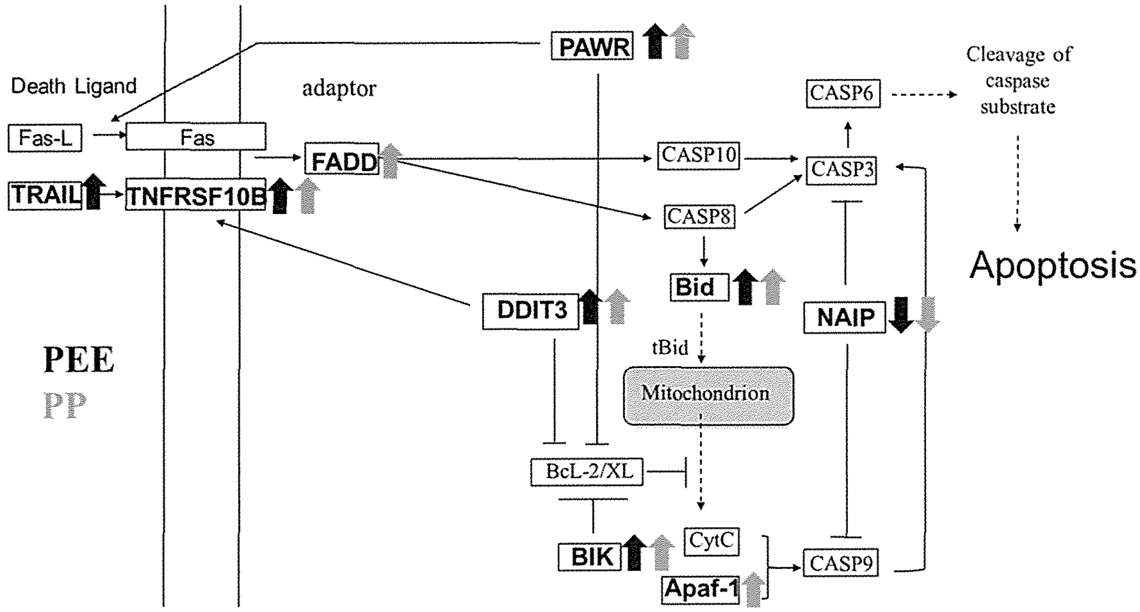

Fig. 4.8 Pathway of apoptosis induced by 0.16 mg/mL PEE and 1 mg/mL residual polyphenol.

Apoptosis, or programmed cell death, is a highly regulated process that involves the activation of a series of molecular events. Several studies have indicated that anti-cancer drugs cause the death of tumor cells through the induction of apoptosis (Robertson et al., 2000; Herr et al., 2001), and 2 major routes, including the death receptor pathway and the mitochondrial pathway, have been identified to be associated with drug-induced apoptosis (Thornberry et al., 1998). Our results suggest that OA, PEE, residual polyphenol also induced apoptosis of Caco-2 cells through the death receptor pathway (Fas pro-death pathway) and the mitochondrial pathway (Fig. 4.7, Fig 4.8). Bcl-2 family proteins play a central role in the regulation of the mitochondrial apoptosis pathway (Breckenridge et al., 2003; Daniel et al., 2003). Members of the Bcl-2 family of proteins can be divided into 2 subfamilies: anti-apoptotic proteins such as Bcl-2 and Bcl-XL, and pro-apoptotic proteins such as Bax, Bad, and Bid (Robertson et al., 2000). Our results showed that OA, PEE and residual polyphenol induced apoptosis through the up-regulation of pro-apoptotic BID, and indirectly down-regulated anti-apoptotic Bcl-2 and Bcl-XL by up-regulating BIK. Some previous reports have shown that NF-κB and Caspase-3, caspase-8, and caspase-9 were targets of OA for its anticancer activities (Deeb et al., 2008; Deeb et al., 2010; Yan et al., 2010). In the present study, the DNA microarray analysis results showed that NF-κB, and Caspase-3, caspase-8, and caspase-9 were differentially affected by OA and PEE. However, the fold changes compared to controls were too low (approximately 1.0 to 1.4) to be selected out.

4.4 Conclusion

In this chapter, we mainly studied the efficient utilization of wine pomace. Considering its anti-cancer activity efficiency, OA was extracted out (11.75 mg/g pomace) without homogenization, and polyphenols were subsequently extracted from residues (2.91 mg GAE/g pomace). PEE induced Caco-2 apoptosis in a dose-dependent manner, with which a concentration of 100 μM (approximately 0.20 mg/mL PEE) showed the strongest effect. Similarly, residual polyphenols also showed apoptosis inducing effects in a dose-dependent manner, with which 3 mg/mL expressed the strongest effect. PEE and residual polyphenol had a synergistic effect on inhibiting Caco-2 cell proliferation and inducing apoptosis, for which the best combination was 0.16 mg/mL PEE and 1 mg/mL residual polyphenol. According to the DNA microarray analysis, OA (100 μM), PEE (0.16 mg/mL) and residual polyphenol (1 mg/mL) appeared to induce Caco-2 cell apoptosis through 2 pathways: the death receptor pathway and the mitochondrial pathway. The apoptosis-related genes PAWR, BID, BIK, DDIT3, TRAIL, TNFRSF10B, FADD, Apaf-1 and NAIP were differentially regulated by the compounds to induce apoptosis, and among them, only NAIP was down-regulated.

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Chapter 5. General Discussion

OA and UA are known to possess many bioactive properties (see Chapter 1), suggesting that these triterpenoids are promising compounds for the development of new multi-targeting bioactive agents. Therefore, identifying good sources of these triterpenes has become increasingly important.

Considering that OA and UA are mainly present in fruit skin, dried fruits were our first target for identifying oral intake triterpenoid resources (Chapter 2). OA has been reported to be the most abundant and most effective compound among all grape skin compounds (Zhang et al., 2004). In Chapter 2, we found that green raisins contained the highest level of OA among the 17 different types of dried fruits analyzed. Other previous studies have also demonstrated the presence of triterpenoids in raisins, including OA (Gift et al., 1992; Seebacher et al., 2003; Wu, 2009). Although we did not conduct a functional study of raisins in the present work, there are some functional reports related to raisins, which have mostly focused on antimicrobial effects. Rivero-Cruz et al. (2008) fractionated and identified antimicrobial compounds (including OA) from raisins that are capable of suppressing growth and/or virulence properties of oral pathogens. *In vivo* studies revealed that raisins possessed a moderate to high cariogenic potential in laboratory rats (Mundorff et al., 1990), and Utreja et al. (2009) investigated the effects of raisins and raisin bran cereal on *in vivo* plaque acidogenicity in young children. Some *in vitro* studies have also demonstrated the antimicrobial effect of OA (Sasazuka et al., 1995; Kozai et al., 1999; Herrera et al., 2006). However, to the best of my knowledge, no study has yet investigated raisins with respect to anti-cancer effects. As an oral intake resource, the bioavailability of OA should be first considered. Jeong et al. (2007) found very low oral bioavailability of OA (0.7%) in rats due to poor absorption and extensive metabolic clearance.

Another good oral intake resource of OA and UA that was detected in Chapter 2 was dried cranberry. Some previous studies have also demonstrated the multiple functions of cranberries. Cranberries might have anti-cancer activity as a result of their inhibition on tumor cell growth (Yan et al., 2002) via a mechanism that might include apoptosis and G1 phase arrest (Sun et al., 2006). Cranberries have also been suggested to be used in the prevention of atherosclerosis and for the promotion of cardiovascular health (Milner, 2002; Neto, 2007).

In Chapter 3, noni skin was found to be a good resource of OA and UA, which were not detected in the seed or flesh parts of the fruit. However, UA was previously shown to be present in noni seeds (Yang et al., 2009; West et al., 2011), which contrasts with our results. Before transporting noni fruits from Samoa and Indonesia, samples were first cut into halves and frozen. When they were received, however, the samples were almost completely thawed, and they were washed and refrozen before experiments. Therefore, one possible reason for the lack of UA detected in seeds is that UA might have been lost happened during the cutting, washing, and transportation period. Furthermore, UA in the seeds might have been rinsed off with water during the thawing and washing procedures. Another reason for these different results may have been related to different cultivars, or that the UA level in the noni seeds of our collected samples might have been too low to be detected in the analysis.

In order to investigate the anti-cancer activity, we applied cancer cell experiments because of their convenience and time-saving properties. Previous studies have conducted similar investigations using many different types of human cancer cells such as the human liver cancer cell lines HepG2, HuH7, and HA22T (Yan et al., 2010), human lung cancer A549 cells (Hsu et al., 2004), the human breast cancer cell line MCF-7 (Suh et al., 1999), and the colon cancer cell lines HCT15 (Li et al., 2002), HT-29 (Juan et al., 2006), and Caco-2 (Ramos et al., 2010). We chose the Caco-2 human colon cancer cell line for our study because colon cancer has been reported to be one of the main causes of mortality in the western world, and was the second most common cancer in Europe in 2006 (Ramos et al., 2010). Furthermore, unlike other cancers, drug therapy for colon cancer can be more challenging as medicines need to be digested before reaching the colon. In our study, 4 different concentrations of OA and UA were applied. Their IC_{50} values for the inhibition of Caco-2 cells were approximately 50 μM and 100 μM, respectively. However, He et al. (2012) found that the IC_{50} values of OA and UA for anti-proliferative activities against Caco-2 cells were 22.6 ± 3.4 mg/L (~ 49.5 μM) and 20.1 ± 3.0 mg/L (~ 44 μM). Although the OA effect was similar, the UA effect differed between our study and that of He et al. (2012). This difference might have been caused by several factors, including individual experiment techniques and laboratory conditions.

As in wine pomace (Chapter 4), noni skin also contains polyphenols, although this was not discussed in Chapter 3. Besides OA and UA, noni skin ethanol extracts were also found to contain 0.64 mg/g polyphenol and 5.14 mg/g polyphenol was detected in residual noni skin after triterpenoid extraction; therefore, most polyphenols remained in residues as was observed for wine pomace. These results suggest that noni pomace could also be utilized efficiently. Unfortunately, the noni utilization study could not be continued due to limited time. Although tropical and subtropical areas are rich in fruit resources, reports on triterpenoids in tropical fruits are relatively rare, and we cannot effectively compare our results of OA and UA contents in tropical fruits to other studies. This field therefore merits further investigation.

In Chapter 4, the synergistic effect of polyphenols and triterpenoids was investigated. Yunoki et al. (2008) suggested that polyphenol had a synergistic effect with OA on plasma lipid lowering; 2% polyphenol and 11% OA were detected in PEEs that showed lipid lowering effects. Our study confirmed this synergistic effect with respect to anti-cancer activity. The combination of PEE and residual polyphenol showed the best anti-cancer effects, which should prove useful for functional wine pomace production.

Finally, the mechanism underlying the effects of OA, polyphenol, and their extracts on inducing Caco-2 apoptosis was investigated, which contributed increased knowledge about triterpenoids' anticancer activities. The human organism is a very large and complicated system that comprises a large number of genes. These genes interact with each other, are regulated by intrinsic or extrinsic factors, and control human diseases through different pathways. A previous study showed that many pathways are involved in cancer regulation, including the NF-κB signaling pathway, the MAPK pathway, the mitochondrial pathway, and the Akt signaling pathway, among others (Aggarwal et al., 2006). Results of the present analysis (Chapter 4) showed that OA, PEE, residual polyphenol, and their combination appeared to induce Caco-2 apoptosis through the mitochondrial and death receptor pathways. The different pathways activated might be related to different cancer cell types. In addition, the specific dose of the compound might affect which pathway is activated to induce apoptosis.

OA and UA are known to have similar functions. Shyu et al. (2010) demonstrated that although OA and UA have similar chemical structures, they differ with respect to the position of a methyl group in the E loop; if the methyl group on C19 of UA is moved to C20, the compound becomes OA. Although we did not analyze the independent effects of OA and UA on apoptosis induction, it is likely that they may show differences with respect to anticancer activities or other bioactivities.

In summary, this 3-year study revealed that noni skin and wine pomaces are good sources of OA and UA, and green raisin and dried cranberry are 2 good oral intake resource of OA and UA. Furthermore, the anti-cancer effects and apoptosis-inducing mechanism of these triterpenoids were demonstrated and clarified. However, further work is needed for confirmation of these results, including the difference in the anti-cancer effect mechanism between OA and UA, the efficient utilization of noni skin (pomace), and determining which polyphenol component in residual polyphenols shows the highest effect on Caco-2 cells. Although identifying OA and UA resources is important, their application is even more relevant. The results of this research, especially the efficient utilization study of wine pomace, should prove useful and valuable for functional food production.

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