Ellagic Acid, a Component of Pomegranate Fruit Juice, Suppresses Androgen-Dependent Prostate Carcinogenesis via Induction of Apoptosis

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BACKGROUND. Ellagic acid (EA), a component of pomegranate fruit juice (PFJ), is a plant-derived polyphenol and has antioxidant properties. PFJ and EA have been reported to suppress various cancers, including prostate cancer. However, their chemopreventive effects on development and progression of prostate cancer using in vivo models have not been established yet.
METHODS. The transgenic rat for adenocarcinoma of prostate (TRAP) model was used to investigate the modulating effects of PFJ and EA on prostate carcinogenesis. Three-week-old male transgenic rats were treated with EA or PFJ for 10 weeks. In vitro assays for cell growth, apoptosis, and Western blot were performed using the human prostate cancer cell lines, LNCaP (androgen-dependent), PC-3 and DU145 (androgen-independent).
RESULTS. PFJ decreased the incidence of adenocarcinoma in lateral prostate, and both EA and PFJ suppressed the progression of prostate carcinogenesis and induced apoptosis by caspase 3 activation in the TRAP model. In addition, the level of lipid peroxidation in ventral prostate was significantly decreased by EA treatment. EA was able to inhibit cell proliferation of LNCaP, whereas this effect was not observed in PC-3 and DU145. As with the in vivo data, EA induced apoptosis in LNCaP by increasing Bax/Bcl-2 ratio and caspase 3 activation. Cell-cycle related proteins, $p21^{WAF}$, $p27^{Kip}$, cdk2, and cyclin E, were increased while cyclin D1 and cdk1 were decreased by EA treatment.
CONCLUSIONS. The results indicate that PFJ and EA are potential chemopreventive agents for prostate cancer, and EA may be the active component of PFJ that exerts these anti-cancer effects. *Prostate* © 2014 Wiley Periodicals, Inc.

KEY WORDS: **pomegranate; ellagic acid; prostate cancer; chemoprevention; apoptosis**

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INTRODUCTION

Prostate cancer is the most common non-skin cancer in men in the United States, where it is responsible for the second most male deaths [1]. Prostate carcinogenesis is a process over time involving cellular growth and division, therefore, inhibition of or delaying this process by dietary supplements can potentially prevent cancers from becoming clinically significant.

Ellagic acid (EA) is a polyphenol found in pomegranate fruit juice (PFJ) and other plant foods that has become known as a potent anti-carcinogenic/anti-mutagenic compound. EA not only induces apoptosis in several cancer cell lines but has been also shown to initiate cell cycle arrest, apoptosis and anti-tumorigenic activity in animal models [2–5]. EA is thought to modulate intracellular signaling pathways by various mechanisms, including regulation of cell cycle-related proteins such as cyclins and cyclin-dependent kinases (cdk) [2]. Previously, we reported that non-toxic dose of EA inhibits migration and invasion of prostate cancer cell lines [6]. However, a chemopreventive potential of EA and PFJ against prostate carcinogenesis, especially in early stage, is unclear.

To study the mechanisms involved in prostate carcinogenesis and to elucidate chemopreventive and chemotherapeutic compounds for prostate cancer, we established a transgenic rat for adenocarcinoma of prostate (TRAP) model which harbors a transgene encoded SV40 T antigen under the probasin promoter [7]. The TRAP develop androgen-dependent neoplastic lesions; prostatic intraepithelial neoplasias (PIN) and non-invasive adenocarcinomas were evident at 15 weeks of age, and microinvasive adenocarcinomas were observed at 35 weeks and older in all prostatic lobes [8]. Recently, we also reported that invasive adenocarcinomas with abundant collagenous stroma were efficiently induced by intermittent testosterone propionate administration in the prostate of TRAP within a shorter period of time (at 22 weeks of age) [9]. Therefore, in a short-term experimental study, the TRAP model could be a good tool to evaluate the chemopreventive action in the early stage of prostate carcinogenesis from PIN to non-invasive adenocarcinoma. We previously reported that phytochemicals such as resveratrol, nobiletin, auraptene, and purple corn color could inhibit prostate carcinogenesis in this TRAP model [10–12].

In animal models, free radicals have been proposed to play a role in cancer pathogenesis involving different organs such as breast, stomach, ovary, oral, and prostate [13–17]. Aydin et al. revealed an alteration in the lipid peroxidation index, with concomitant changes in the antioxidant defense system in patients with prostate cancer as compared to benign prostate hyperplasia [18]. Antioxidants including EA were recently shown to harbor preventive effects in the progression of prostate carcinogenesis [19–22].

In this study, we examined if PFJ can suppress the early stage of prostate carcinogenesis and if EA has similar effects to PFJ in the TRAP model. Moreover, we investigated whether or not the effects of EA on the proliferation of prostate cancer cell lines are through regulation of the cell cycle and induction of apoptosis.

MATERIALS AND METHODS

Cell Lines

Androgen-dependent human prostate cancer cell line, LNCaP and androgen independent cells, PC-3 and DU145 were purchased from The American Type Culture Collection (ATCC, Manassas, VA).

Cell Culture and Treatment

LNCaP, PC-3, and DU145 cells were grown in RPMI 1640 (Gibco, Carlsbad, CA) containing 10% fetal bovine serum (FBS) and supplemented with antibiotics (50 U/ml penicillin, 50 mg/ml streptomycin) in a humidified incubator with 5% CO_2 at 37°C. Cell culture medium was changed every 48 hr. EA (Sigma–Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO) at a maximum concentration 10 mM. Final concentrations of EA in cell culture medium were 125, 100, 75, 50, and 25 μM in RPMI adjusted to pH 6.5, while a maximum of 1% DMSO in RPMI pH 6.5 was used as a control. Cell proliferation was evaluated by soluble formazan formation using the WST-1 assay (Roche Diagnostics GmbH, Mannheim, Germany). The number of apoptotic cells was measured by staining with annexin V and propidium iodide followed by quantitation with flow cytometry (Guava Technologies, Inc., Hayward, CA)

Animals

Heterozygous male TRAP used in this study were established with a Sprague-Dawley (SD) genetic background, as described previously [7]. All experimental rats were housed 3 per plastic cage on wood-chip bedding in an air-conditioned specific pathogen-free (SPF) animal room at 22 ± 2°C and 55 ± 5% humidity with a 12 hr light/dark cycle, and fed soybean-free powdered basal diet (Oriental MF, Oriental Yeast Co., Tokyo, Japan), with or without test chemical, and water ad libitum. The Institutional Animal Care and Use Committee of Nagoya City University Graduate School of Medical Sciences specifically approved this study.

Experimental Protocol

A total of 53 male TRAP at 3 weeks of age were segregated into EA (Wako, Osaka, Japan), and PFJ (Kirin Beverage, Tokyo, Japan) and their respective control groups. Twenty-nine rats were fed a basal diet (n = 9), or phytoestrogen-low diet supplemented with 0.1% (n = 10) or 1% EA (n = 10) for 10 weeks. Separately, 12 rats were treated with 5% PFJ in the drinking water for 10 weeks with 12 control rats.

At the end of week 10, all rats were sacrificed under deep anesthesia. Each prostate was removed and half of the ventral prostate (VP) and lateral prostate (LP) lobes were immediately frozen in liquid nitrogen while the remainder of the prostates was fixed in 10% phosphate-buffered formalin. After formalin fixation for 48 hr the seminal vesicle and remaining VP and LP were trimmed and routinely embedded in paraffin for histopathological evaluation and immunohistochemistry. Testosterone and estrogen levels in the serum were analyzed by radioimmunoassay by SRL Inc. (Tokyo, Japan).

Assessment of Prostate Neoplastic Lesion Development

Neoplastic lesions of the prostate were classified as low grade PIN (LG-PIN), high grade PIN (HG-PIN) and non-invasive adenocarcinoma as previously described by Seeni et al. [11]. Importantly, adenocarcinoma was distinguished from HG-PIN by the presence of multiple cribriform glands with prominent nuclear atypia and focal necrosis, which is comparable to that in humans (Fig. 1A). The incidence of neoplastic lesions was scored in each VP and LP.

Western Blot Analysis

The harvested cells and frozen tissues were homogenized in RIPA buffer (150 mM NaCl, 0.5% sodium deoxycholate, 0.1% nonidet P-40, 0.1% SDS and 50 mM Tris) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride). Total cellular proteins were quantified by the Bradford procedure and equal amounts of proteins were mixed with Laemmli sample

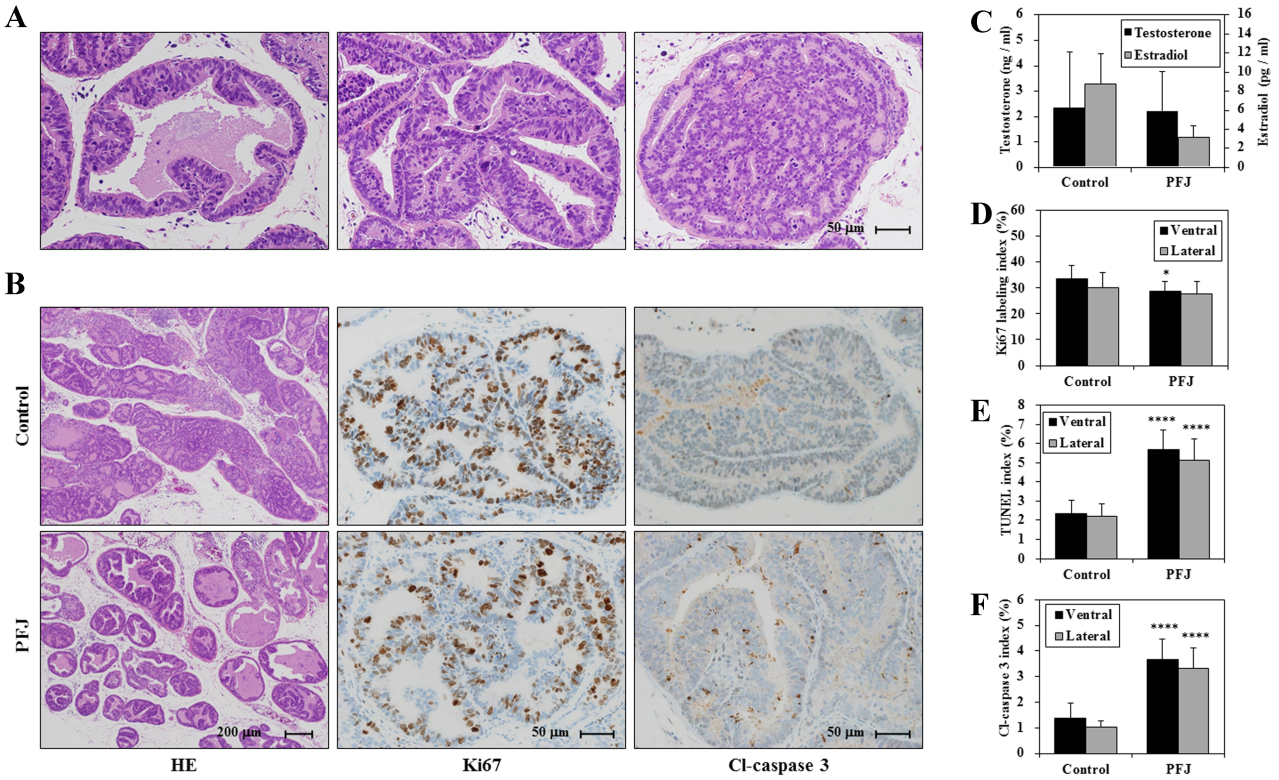

Fig. 1. PFJ supplementation suppressed progression of prostate carcinogenesis and induced apoptosis in the TRAP model. **A**: Representative histological findings for LG-PIN (**left**), HG-PIN (**middle**), and adenocarcinoma (**right**). **B**: HE, Ki67, and cl-caspase 3 expression in VP. **C**: Levels of serum testosterone and estradiol in TRAP treated with PFJ. Labeling indices for Ki67- (**D**), TUNEL- (**E**), and cl-caspase 3- (**F**) positive cells in VP and LP. $^{*}P < 0.05$, $^{****}P < 0.0001$ as compared to the control group.

buffer (BioRad Laboratories, Inc., Hercules, CA) and fractionated by gel electrophoresis in 8% or 12% polyacrylamide resolving gels with 0.1% sodium dodecyl sulfate (SDS). Proteins were transferred onto Hybond^{TM}-ECL^{TM} nitrocellulose membranes (Amersham, Freiburg, Germany), which were subsequently incubated overnight with primary antibodies in 5% non-fat dry milk. The primary antibodies used in this study were against cleaved caspase 3 (cl-caspase 3), caspase 3 (Cell Signaling, Boston, MA), androgen receptor (AR), Bax, cyclin D1, cyclin E, p21^{WAF}, PCNA (Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (Novocastra Laboratories, Newcastle, UK), cdk1, cdk2, p27^{KIP} (BD Biosciences, Franklin Lakes, NJ), and prostate specific antigen (PSA, Dako Cytomation, Glostrup, Denmark). Equal protein loading was ascertained by Western blotting with β-actin antibody (Sigma–Aldrich).

Immunohistochemistry

Deparaffinized sections were incubated with antibodies for AR (Santa Cruz Biotechnology) and Ki67 (Novocastra Laboratories). Apoptotic cells were detected by terminal deoxy nucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay as well as cl-caspase 3 (Cell Signaling) immunohistochemistry. TUNEL assay was performed using an in situ Apoptosis Detection Kit from Takara Bio Inc. (Otsu, Japan). The labeling indices of Ki67, TUNEL and cl-caspase 3 were determined by counting at least 1,000 HG-PIN cells under a microscope light microscopy at high magnification.

Quantitative Reverse Transcription-PCR (qRT-PCR) Analysis

Harvested cells and frozen tissues were lysed with 1 ml of ISOGEN reagent (Nippon Gene Co. Ltd., Tokyo, Japan), and then total RNA was prepared according to the manufacturer's protocol and treated with DNase I (Promega, Madison, WI) to remove DNA contamination. One microgram of total RNA was used to synthesize cDNA using AMV-reverse transcriptase (Promega) with oligo $(dT)_{15}$ primer (Promega). qRT-PCR was performed using SYBR Premix Ex Taq^{TM} Reagents (Promega) on a LightCycler^{TM} (Roche) with universal cycling conditions. The comparative threshold cycle (C_T) method was used to quantify data using β-actin and rat cyclophilin as the normalizing genes. Primers used for amplification of each mRNA were carefully designed to span an intron to prevent amplification of genomic DNA and were as follows: AR forward (5′-TGT CAA CTC CAG GAT GCT CTA CTT-3′); AR reverse (5′-TTC GGA CAC ACT GGC TGT ACA-3′); PSA forward (5′-TCT GCG GCG GTG TTC TG-3′); and PSA reverse (5′-GCC GAC CCA GCA AGA TCA-3′).

Lipid Hydroperoxide in VP

Oxidative stress in VP was examined by a Lipid Hydroperoxide assay kit (Northwest Life Science Specialty, Vancouver, WA). Briefly, homogenized VP (in 10% PBS w/v) were subjected to a lipid peroxide (LOOH) assay. Endogenous hydrogen peroxide (H_2O_2) was neutralized by incubation of 100 μl sample with catalase for 10 min at room temperature. Then 500 μl methanol-butyl hydroxytoluene (BHT) was added to prevent an antioxidative process in the reaction. After incubation for 1 hr at room temperature, 50 μl of xylenol orange-iron mixture was added, mixed and incubated for more than 1 hr. The reaction mix was centrifuged at 10,000*g* for 5 min then measured at an absorbance of 560 nm. The concentration of LOOH was calculated as per the product manual.

Statistical Analysis

Differences in the quantitative data, which are expressed as mean ± SD, between groups were compared by Student's *t*-test or one-way ANOVA and Dunnett's post-hoc test using Graph Pad Prism 5 (GraphPad Software, Inc., La Jolla, CA).

RESULTS

PFJ Suppressed Progression of Prostate Carcinogenesis by Augmentation of Apoptosis

Administration of PFJ in drinking water did not affect body weight (Table IA) and the relative weights of the liver and kidneys in the PFJ group (data not shown), and there were no significant changes in serum testosterone and estrogen levels (Fig. 1C). Absolute weight of the dorsolateral prostate in the PFJ treatment group was significantly reduced as compared to control group (Table IA). The effect of PFJ on prostate tumor progression in the TRAP model was investigated by histological analysis. The number of LG-PIN, HG-PIN and adenocarcinoma lesions in VP and LP was scored by microscopy and presented as percentage of lesions in each prostate as summarized in Table IB. In VP of non-treated TRAP, the lesions of acini were mostly HG-PIN and adenocarcinoma, 78.4 ± 6.9% and 13.1 ± 4.9%, respectively. Although rats that received PFJ exhibited a similar percentage of HG-PIN relative to the control group, the percentage of adenocarcinomas was significantly reduced in rats that received PFJ (4.2 ± 3.0%, $P < 0.0001$). In LP, the lesions of acini were mostly LG-PIN and HG-PIN

TABLE IA. Body and Prostate Weights in TRAP Treated With PFJ

	No. of rat	Body weight (g)	Total prostate (g)	Ventral prostate (g)	Dorsolateral prostate (g)
Control	12	454.3 ± 33.9	2.43 ± 0.25	0.264 ± 0.038	0.71 ± 0.04
PFJ	12	450.4 ± 24.7	2.33 ± 0.19	0.251 ± 0.040	$0.62 \pm 0.06^{***}$

Data are means $\pm$ SD, $^{***}P < 0.001$: significantly different from control group (Student's *t*-test).

TABLE IB. Incidence of Carcinoma and Quantitative Evaluation of Neoplastic Lesions in Prostates of TRAP Treated With PFJ

		Ventral prostate (VP)				Lateral prostate (LP)			
			% of lesions in prostate				% of lesions in prostate		
	No. of rat	Incidence of carcinoma	LG-PIN	HG-PIN	Adenocarcinoma	Incidence of carcinoma	LG-PIN	HG-PIN	Adenocarcinoma
Control	12	12 (100%)	8.6 ± 3.9	78.4 ± 6.9	13.1 ± 4.9	11 (92%)	15.3 ± 4.2	82.6 ± 4.2	2.1 ± 1.5
PFJ	12	12 (100%)	$16.2 \pm 7.3^{**}$	79.6 ± 9.0	$4.2 \pm 3.0^{****}$	5 (42%)**	$27.6 \pm 6.9^{****}$	$72.6 \pm 7.0^{***}$	$0.4 \pm 0.6^{**}$

Data are means $\pm$ SD, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$: significantly different from control group (Student's *t*-test).

($15.3 \pm 4.2\%$ and $82.6 + 4.2\%$, respectively). TRAP that received PFJ showed a significantly lower percentage of adenocarcinomas ($0.4 \pm 0.6\%$, $P < 0.01$) as compared to the non-treated group ($2.1 \pm 1.5\%$), and a higher percentage of LG-PIN ($27.6 \pm 6.9\%$, $P < 0.0001$). Furthermore, the incidence of adenocarcinomas was significantly decreased in LP of PFJ-treated rats (42%, $P < 0.01$) as compared to the control group (92%).

The Ki67 index was slightly decreased by PFJ in VP (Fig. 1B and D). TUNEL assay indicated that the percentage of apoptotic cells was significantly increased in both VP and LP of TRAP treated with PFJ as compared to control (Fig. 1E). Immunohistochemical analysis showed that the percentage of cl-caspase 3-positive cells was clearly elevated in both VP and LP (Fig. 1B and F). These results indicated that PFJ suppressed prostate carcinogenesis by induction of caspase 3-dependent apoptosis and partially inhibited cell growth.

Anti-Proliferation and Apoptosis Induction Capabilities of EA in LNCaP Cells

Next, we determined whether or not EA, which is one of the major components of PFJ, has a suppressive effect on cell proliferation of prostate cancer cells by treating LNCaP, PC-3, and DU145 cells with EA. Treatment with EA at pH 6.5, which is equal in pH to 5% PFJ, resulted in reduction of relative cell number of LNCaP cells by up to 35% as compared to non-treated cells, and these effects were not observed in PC-3 and DU145 cells (Fig. 2A). On the other hand, at physiological pH 7.4, EA did not affect cell proliferation of any of these prostate cancer cells (data not shown). Therefore, all the subsequent in vitro studies were performed using EA at pH 6.5. To investigate whether EA induced apoptosis, EA-treated LNCaP cells were stained with annexin V and propidium iodide, and then evaluated by flow cytometry. The percentage of apoptotic LNCaP cells following treatment with EA was calculated and is shown in Figure 2B. At doses of 75 and 100 μM EA, the percentage of apoptotic cells was increased as compared to control at both 24 and 48 hr. By immunoblotting, we found that caspase 3 was activated after treatment with 75, 100 and 125 μM EA (Fig. 3A). Further, while the protein level of Bax was increased, that of Bcl-2 was slightly decreased. These results suggested that EA treatment resulted in induction of apoptosis in LNCaP cells as with PFJ intake in the TRAP model.

EA Inhibits Cell Growth Through Regulation of Cell Cycle Proteins

Since EA induced apoptosis in LNCaP, we examined the effects of EA on cell cycle regulatory molecules. The results in Figure 3B showed that EA increased the expression of $p21^{WAF}$ and $p27^{KIP}$, which regulate the entry of cells at G1-S phase checkpoint, whereas cyclin D1 and cdk1 were decreased. Conversely, the expression of cyclin E as well as cdk2, which are known to be regulated by $p21^{WAF}$ and $p27^{KIP}$, were slightly increased in EA-treated LNCaP cells. Collectively, these results suggested that EA

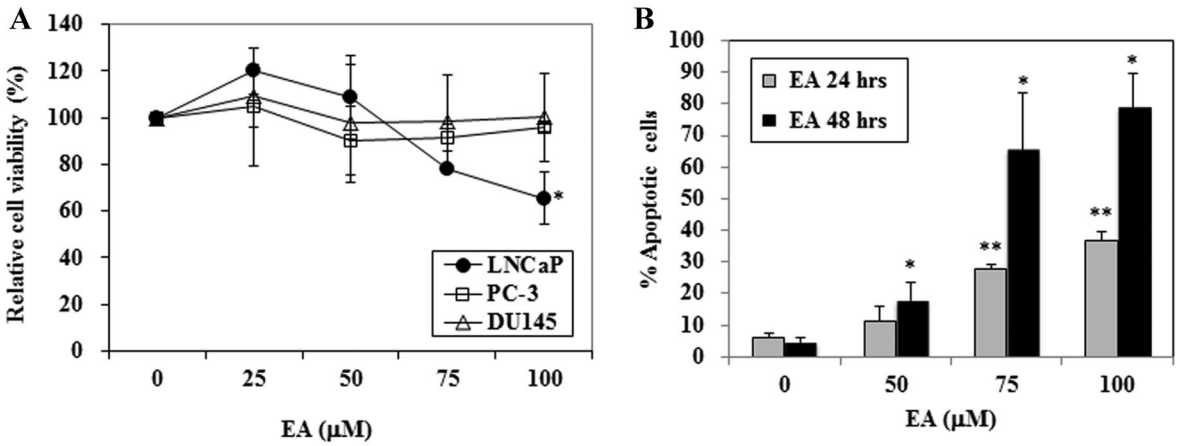

Fig. 2. **A**: Effect of EA treatment on cell growth of prostate cancer cell lines, LNCaP, PC-3, and DUI45, was determined by WST-I assay. The data are represented as relative cell viability of treated cell to vehicle control and shown as mean ± SD of three independent experiments. $^{*}P < 0.05$ as compared to control (EA 0). **B**: EA induced apoptosis in LNCaP cells. Apoptotic cells were labeled by annexin V and evaluated by flow cytometry. The percentage of apoptotic cells in each treatment is presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared to control (EA 0).

inhibited the growth of prostate cancer cells through induction of cell cycle arrest along with apoptosis.

EA Suppressed the Expression of AR in LNCaP Cells

AR, an essential mediator of androgen, is known to be involved in the development and progression of prostate cancer. LNCaP is an androgen-responsive prostate cancer cell line, therefore we examined the effect of EA on the protein and mRNA expression of AR (Fig. 4A and B). We found that AR protein was decreased in LNCaP cells treated with EA in a dose-dependent manner, and the level of AR mRNA was decreased within 6 hr after treatment of EA at doses of 50 and 75 μM. Moreover, the level of PSA mRNA was time-dependently decreased with 90% reduction within 24 hr after EA treatment (Fig. 4B). Because PSA is a responsive gene that reflects AR function, these results indicated that PSA level in LNCaP was decreased from the lowest dose of EA.

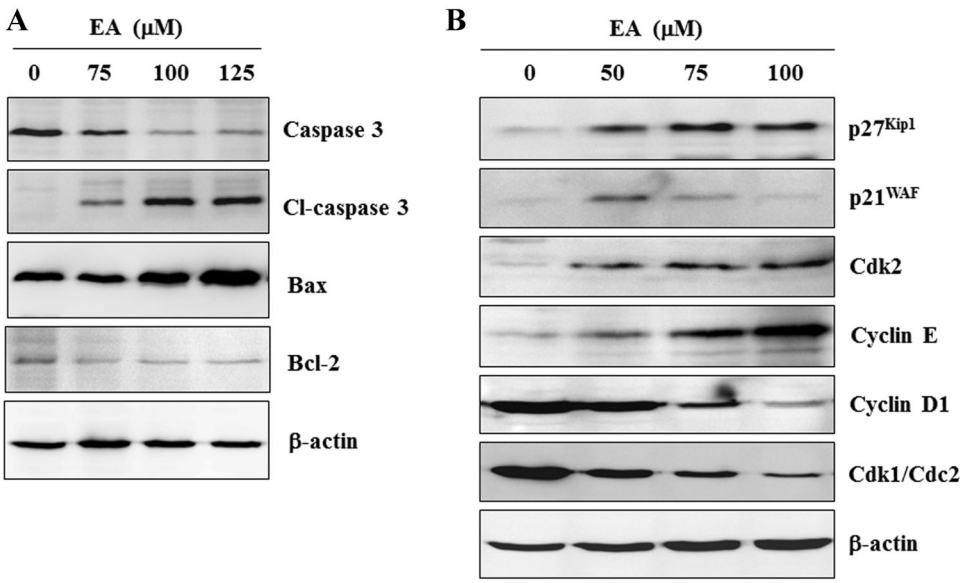

Fig. 3. Protein changes as assessed by Western blotting analysis of LNCaP cells after incubation with EA for 48 hr. **A**: Apoptosis-related proteins: cleaved and non-cleaved caspase-3, Bax and Bcl-2; and (**B**) cell cycle-related proteins: $p21^{WAF}$, $p27^{KIP}$, cdk2, cyclin E, cyclin DI, and cdkI were normalized by β-actin. The data are representative of three independent experiments with similar results.

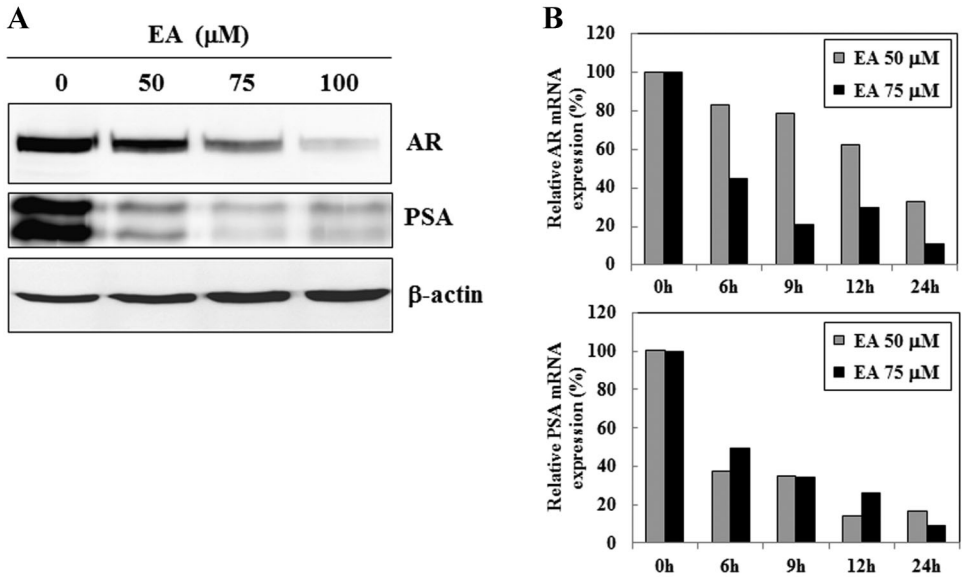

Fig. 4. EA suppressed AR and downstream PSA expression in LNCaP cells. **A**: AR and PSA protein levels were detected in LNCaP cells treated with EA for 48 hr by Western blot analysis and normalized by β-actin. qRT-PCR showed the relative quantification of AR (**B**) and PSA (**C**) mRNA in LNCaP cells at 0, 6, 9, 12, and 24 hr after treatment with 50 and 75 μM of EA. The data are expressed as ratio to control cells and normalized with β-actin as an endogenous control gene.

EA Inhibited Prostate Carcinogenesis in TRAP via Activation of Caspase Signaling

Finally, to investigate the effects of EA on prostate carcinogenesis, an in vivo study was conducted by treating TRAP with EA. Body weight (Table IIA), relative organ weights (testes, liver and kidneys, data not shown) and food consumption were not affected by administration of EA in the diet to TRAP. Dorsolateral prostate weights in the 0.1% and 1% EA treatment group were significantly decreased compared with the control group (Table IIA). Since EA reduced AR expression in vitro, we examined whether or not AR expression was down-regulated by EA treatment in vivo. Immunohistochemical analysis, Western blotting and qRT-PCR showed that there were no significant changes in AR expression at the protein and mRNA levels by EA, as well as in serum testosterone and estrogen levels (Fig. 5A–D).

Both doses of EA treatment significantly suppressed the progression of prostatic lesions from LG-PIN to HG-PIN or adenocarcinoma in LP. In VP, the percentage of adenocarcinomas was significantly decreased by 1% EA (Table IIB, $11.6 \pm 5.2\%$, $P < 0.05$). To confirm that EA induced apoptosis in prostate tissue as in the in vitro study, the percentage of apoptotic cells in HG-PIN among each group were evaluated by TUNEL staining. Apoptotic indices were significantly increased in VP and LP of animals given EA as compared to control (Fig. 5E). Immunohistochemical staining showed increased caspase 3 activation in both VP and LP of rats treated with EA (Fig. 5A and F). On the other hand, the Ki67 labeling index was not affected by EA (Fig. 5G).

Reduction of Oxidative Stress in VP of TRAP That Received EA

Oxidative stress in VP was evaluated by the level of lipid peroxidation. The level of LOOH in VP was significant decreased in TRAP treated with 1% EA as compared to non-treated rats (Fig. 5H).

DISCUSSION

The present study demonstrated significant suppression of adenocarcinoma development in TRAP by

TABLE IIA. Body and Prostate Weights in TRAP Treated With EA

	No. of rat	Body weight (g)	Total prostate (g)	Ventral prostate (g)	Dorsolateral prostate (g)
Control	9	408.6 ± 41.8	2.39 ± 0.33	0.26 ± 0.05	0.72 ± 0.08
0.1% EA	10	425.8 ± 26.0	2.21 ± 0.28	0.25 ± 0.03	$0.62 \pm 0.08^*$
1% EA	10	424.6 ± 20.3	2.09 ± 0.23	0.24 ± 0.04	$0.64 \pm 0.06^*$

Data are means ± SD, $^*P < 0.05$: significantly different from control group (one-way ANOVA and Dunnett's post-hoc test).

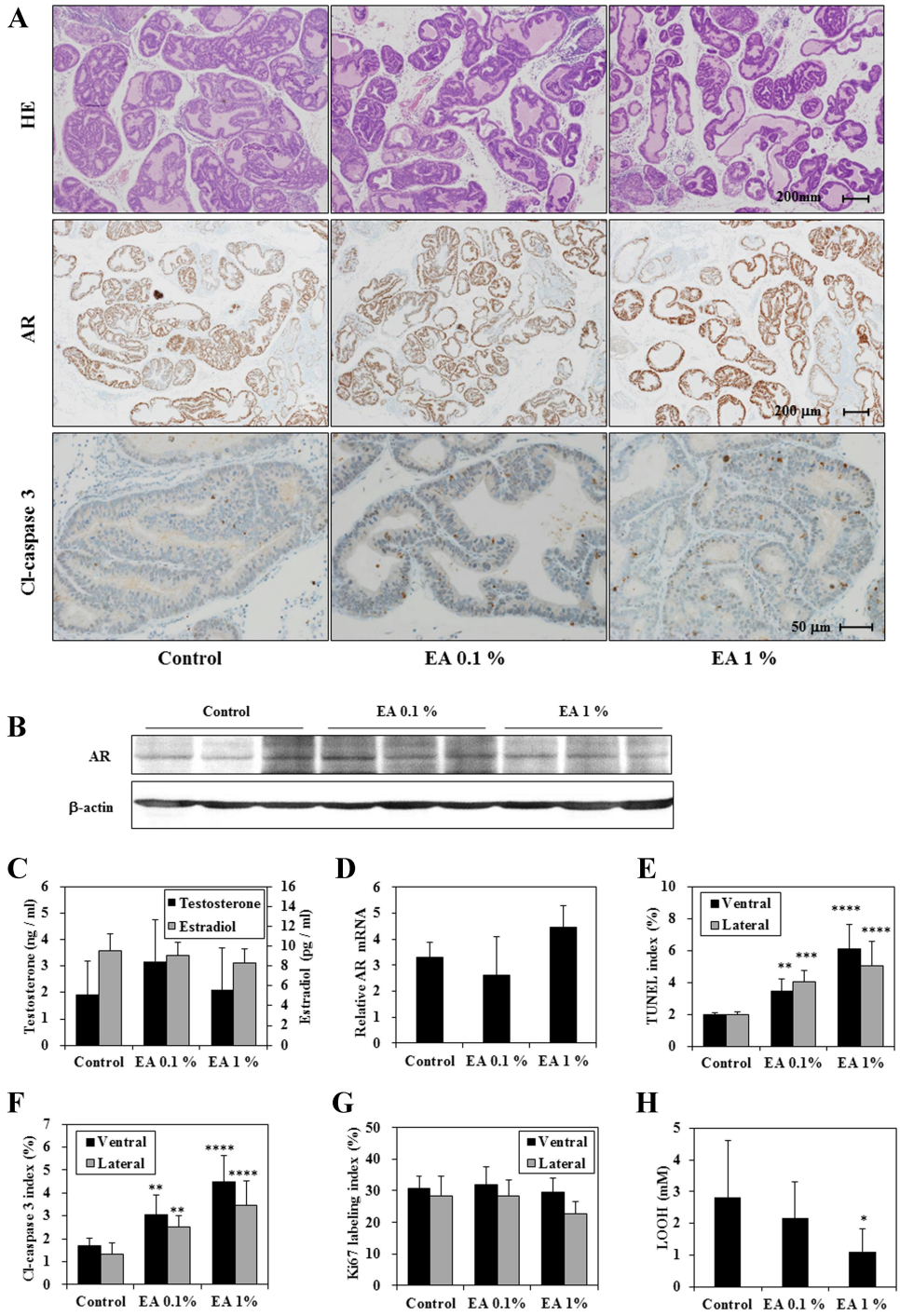

Fig. 5. EA inhibited prostate carcinogenesis by induction of apoptosis without down regulation of AR expression in the TRAP model. **A**: HE and immunohistochemical stainings for AR and cl-caspase 3 in VP. **B**: Western blotting analysis of AR in ventral prostates of TRAP rats treated with EA. **C**: Levels of serum testosterone and estradiol in TRAP treated with EA. **D**: AR mRNA level in VP as detected by qRT-PCR. The average results were calculated from 5 rats per group. Labeling indices of TUNEL- (**E**), cl-caspase 3- (**F**), and Ki67- (**G**) positive cells in VP and LP. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ as compared to the control group. **H**: The level of lipid peroxide (LOOH). $^{*}P < 0.05$ as compared to the control group.

daily intake of PFJ and its component EA, suggesting a potential for chemoprevention of human prostate cancer. PFJ contains a mixture of flavonoids, anthocyanins, tannins, and polyphenols, such as EA [23]. Chemopreventive effects of PFJ in prostate cancer were previously shown by Malik et al. [24] using the androgen-independent cell line, PC3. Pantuck et al. [25] also reported that PSA doubling time significantly

TABLE IIB. Incidence of Carcinoma and Quantitative Evaluation of Neoplastic Lesions in Prostates of TRAP Treated With EA

		Ventral prostate (VP)				Lateral prostate (LP)			
			% of lesions in prostate				% of lesions in prostate		
	No. of rat	Incidence of carcinoma	LG-PIN	HG-PIN	Adenocarcinoma	Incidence of carcinoma	LG-PIN	HG-PIN	Adenocarcinoma
Control	9	9 (100%)	10.3 ± 3.4	72.1 ± 5.7	17.6 ± 5.7	7 (78%)	15.5 ± 4.0	82.8 ± 3.3	1.7 ± 1.2
0.1% EA	10	10 (100%)	9.1 ± 6.0	74.1 ± 6.2	16.8 ± 4.0	3 (30%)	$24.5 \pm 6.9^{*}$	$75.0 \pm 6.7^{*}$	$0.4 \pm 0.7^{**}$
1% EA	10	10 (100%)	16.2 ± 8.5	72.2 ± 8.0	$11.6 \pm 5.2^{*}$	3 (30%)	$27.2 \pm 10.4^{**}$	$72.5 \pm 10.3^{**}$	$0.2 \pm 0.4^{**}$

Data are means $\pm$ SD, $^{*}P < 0.05$, $^{**}P < 0.01$: significantly different from control group (one-way ANOVA and Dunnett's post-hoc test).

increased with PFJ treatment in a phase II clinical trial. However, there was little evidence of the effects of PFJ on the early phase of prostate cancer, mostly characterized by androgen-dependent proliferation. In the present study, PFJ supplementation resulted in significant decreases in weight of dorsolatelal prostate, and incidence and progression of prostate cancer that were clearly through induction of apoptosis by activation of caspase 3 in the TRAP model, which indicated a new aspect of anti-cancer efficacy of PFJ.

Interestingly, the in vitro study using LNCaP cells indicated that EA also inhibited prostate cancer cell proliferation through induction of apoptosis via activation of caspase 3. In contrast, these effects were not induced in PC-3 and DU145 cells as we recently reported [6]. Although a paper demonstrated that growth of PC-3 was inhibited by EA at a concentration that was similar to the ones used in the present study, the concentration of DMSO in the non-treated control (EA 0) was different between their study and ours [26]. This may have affected the results. EA also suppressed progression of prostate cancer without any toxic effects and induced caspase 3-dependent apoptosis in both VP and LP in the TRAP model (Fig. 5). These findings clearly show that EA and PFJ inhibit the early stage of prostate carcinogenesis through a similar mechanism, which indicates that EA is likely the active compound of PFJ that exerts these anti-cancer effects. To our knowledge, this is the first study to demonstrate suppressive effects of EA on prostate tumor progression in an in vivo model. The EA concentrations that exerted chemoprevention effects in LNCaP cells in this study were of similar range to those reported in previously published documents [2,4,27]. According to a report by Seeram et al. that showed a relation between EA dose and its serum level in mice, oral intake of 0.1% EA seemed to reflect its effective dose in vitro in the present study [27].

EA treatment resulted in down-regulation of AR signaling in LNCaP cells (Fig. 4), which may be a complementary mechanism in addition to apoptosis induction for inhibition of cell proliferation, since these cells propagate in an androgen-dependent manner. In contrast, there were no changes in serum testosterone level or expression of AR mRNA and protein in prostate tissues of rats treated with EA in vivo. Additionally, PFJ supplementation also did not affect serum hormonal levels in the TRAP model. These results suggest that the anti-proliferative effect of EA in vivo is possibly due to a non-androgen-mediated pathway. EA is ionized at physiological pH, however, its aqueous solution is a weak acid, and the equilibrium of ionization is changed [28]. Such behavior of EA may result in the pH-specific differences in anti-proliferative effect in vitro and the discrepancy between our in vitro and in vivo results.

Oxidative stress is involved in tumor formation and cell proliferation of various cancers, including prostate cancer [29]. As recently reported, oxidative stress is also responsible for triggering apoptosis in tumors [30], and antioxidants are showing promise in prevention and therapy of prostate cancer [19,31]. In this study, oxidative damage as shown by the LOOH assay was decreased in VP of TRAP that received EA as compared to the control group, which was consistent with a previous in vitro study [22]. These results suggested that oxidative damage in the prostate was neutralized by the antioxidant activity of EA which may have retarded prostate carcinogenesis.

CONCLUSIONS

We presented novel findings in the present study that show that PFJ and EA exert chemopreventive effects against prostate carcinogenesis in the TRAP model, and EA also inhibits cell proliferation of the human prostate cancer cell line, LNCaP, possibly via induction of apoptosis by activation of caspase 3. These results suggest that PFJ and EA are efficacious for chemoprevention against early stage prostate cancer.

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013;63(1):11–30.
2. Li TM, Chen GW, Su CC, Lin JG, Yeh CC, Cheng KC, Chung JG. Ellagic acid induced p53/p21 expression, G1 arrest and apoptosis in human bladder cancer T24 cells. Anticancer Res 2005; 25(2A):971–979.
3. Mertens-Talcott SU, Percival SS. Ellagic acid and quercetin interact synergistically with resveratrol in the induction of apoptosis and cause transient cell cycle arrest in human leukemia cells. Cancer Lett 2005;218(2):141–151.
4. Narayanan BA, Geoffroy O, Willingham MC, Re GG, Nixon DW. p53/p21(WAF1/CIP1) expression and its possible role in G1 arrest and apoptosis in ellagic acid treated cancer cells. Cancer Lett 1999;136(2):215–221.
5. Paivarinta E, Pajari AM, Torronen R, Mutanen M. Ellagic acid and natural sources of ellagitannins as possible chemopreventive agents against intestinal tumorigenesis in the Min mouse. Nutr Cancer 2006;54(1):79–83.
6. Pitchakarn P, Chewonarin T, Ogawa K, Suzuki S, Asamoto M, Takahashi S, Shirai T, Limtrakul P. Ellagic acid inhibits migration and invasion by prostate cancer cell lines. Asian Pac J Cancer Prev 2013;14(5):2859–2863.
7. Asamoto M, Hokaiwado N, Cho YM, Takahashi S, Ikeda Y, Imaida K, Shirai T. Prostate carcinomas developing in transgenic rats with SV40 T antigen expression under probasin promoter control are strictly androgen dependent. Cancer Res 2001;61(12): 4693–4700.
8. Cho YM, Takahashi S, Asamoto M, Suzuki S, Inaguma S, Hokaiwado N, Shirai T. Age-dependent histopathological findings in the prostate of probasin/SV40 T antigen transgenic rats: Lack of influence of carcinogen or testosterone treatment. Cancer Sci 2003;94(2):153–157.
9. Sato S, Suzuki S, Naiki-Ito A, Komiya M, Long N, Kato H, Sagawa H, Yamashita Y, Shirai T, Takahashi S. Establishment of an invasive prostate cancer model in transgenic rats by intermittent testosterone administration. J Toxicol Pathol 2014;27(1): 43–49.
10. Long N, Suzuki S, Sato S, Naiki-Ito A, Sakatani K, Shirai T, Takahashi S. Purple corn color inhibition of prostate carcinogenesis by targeting cell growth pathways. Cancer Sci 2013;104(3): 298–303.
11. Seeni A, Takahashi S, Takeshita K, Tang M, Sugiura S, Sato SY, Shirai T. Suppression of prostate cancer growth by resveratrol in the transgenic rat for adenocarcinoma of prostate (TRAP) model. Asian Pac J Cancer Prev 2008;9(1):7–14.
12. Tang M, Ogawa K, Asamoto M, Hokaiwado N, Seeni A, Suzuki S, Takahashi S, Tanaka T, Ichikawa K, Shirai T. Protective effects of citrus nobiletin and auraptene in transgenic rats developing adenocarcinoma of the prostate (TRAP) and human prostate carcinoma cells. Cancer Sci 2007;98(4):471–477.
13. Srivastava DSL, Mittal RD. Free radical injury and antioxidant status in patients with benigh prostate hyperplasia and prostate cancer. Indian J Clin Biochem 2005;20(2):162–165.
14. Ray G, Batra S, Shukla NK, Deo S, Raina V, Ashok S, Husain SA. Lipid peroxidation, free radical production and antioxidant status in breast cancer. Breast Cancer Res Treat 2000;59(2):163–170.
15. Zheng QS, Sun XL, Wang CH. Redifferentiation of human gastric cancer cells induced by ascorbic acid and sodium selenite. Biomed Environ Sci 2002;15(3):223–232.
16. Ghalia AA, Rabboh NA, el Shalakani A, Seada L, Khalifa A. Estimation of glutathione S-transferase and its Pi isoenzyme in tumor tissues and sera of patients with ovarian cancer. Anticancer Res 2000;20(2B):1229–1235.
17. Sabitha KE, Shyamaladevi CS. Oxidant and antioxidant activity changes in patients with oral cancer and treated with radiotherapy. Oral Oncol 1999;35(3):273–277.
18. Aydin A, Arsova-Sarafinovska Z, Sayal A, Eken A, Erdem O, Erten K, Ozgok Y, Dimovski A. Oxidative stress and antioxidant status in non-metastatic prostate cancer and benign prostatic hyperplasia. Clin Biochem 2006;39(2):176–179.
19. Fleshner NE, Kucuk O. Antioxidant dietary supplements: Rationale and current status as chemopreventive agents for prostate cancer. Urology 2001;57(4 Suppl 1):90–94.
20. Hwang ES, Bowen PE. Effects of lycopene and tomato paste extracts on DNA and lipid oxidation in LNCaP human prostate cancer cells. Biofactors 2005;23(2):97–105.
21. Kampa M, Hatzoglou A, Notas G, Damianaki A, Bakogeorgou E, Gemetzi C, Kouroumalis E, Martin PM, Castanas E. Wine antioxidant polyphenols inhibit the proliferation of human prostate cancer cell lines. Nutr Cancer 2000;37(2):223–233.
22. Seeram NP, Adams LS, Henning SM, Niu Y, Zhang Y, Nair MG, Heber D. In vitro antiproliferative, apoptotic and antioxidant activities of punicalagin, ellagic acid and a total pomegranate tannin extract are enhanced in combination with other polyphenols as found in pomegranate juice. J Nutr Biochem 2005; 16(6):360–367.
23. El Kar C, Ferchichi A, Attia F, Bouajila J. Pomegranate (Punica granatum) juices: Chemical composition, micronutrient cations, and antioxidant capacity. J Food Sci 2011;76(6):C795–C800.
24. Malik A, Afaq F, Sarfaraz S, Adhami VM, Syed DN, Mukhtar H. Pomegranate fruit juice for chemoprevention and chemotherapy of prostate cancer. Proc Natl Acad Sci U S A 2005;102(41):14813–14818.
25. Pantuck AJ, Leppert JT, Zomorodian N, Aronson W, Hong J, Barnard RJ, Seeram N, Liker H, Wang H, Elashoff R, Heber D, Aviram M, Ignarro L, Belldegrun A. Phase II study of pomegranate juice for men with rising prostate-specific antigen following surgery or radiation for prostate cancer. Clin Cancer Res 2006; 12(13):4018–4026.
26. Malik A, Afaq S, Shahid M, Akhtar K, Assiri A. Influence of ellagic acid on prostate cancer cell proliferation: A caspase-dependent pathway. Asian Pac J Trop Med 2011;4(7):550–555.
27. Seeram NP, Aronson WJ, Zhang Y, Henning SM, Moro A, Lee RP, Sartippour M, Harris DM, Rettig M, Suchard MA, Pantuck AJ, Belldegrun A, Heber D. Pomegranate ellagitannin-derived metabolites inhibit prostate cancer growth and localize to the mouse prostate gland. J Agric Food Chem 2007;55(19):7732–7737.
28. Hasegawa M, Terauchi M, Kikushi Y, Nakao A, Okubo J, Yoshinaga T, Hiratsuka H, Kobayashi M, Hoshi T. Deprotonation process of ellagic acid in solution and solid state. Monatshefte fur Chemie 2003;134:811–821.
29. Khandrika L, Kumar B, Koul S, Maroni P, Koul HK. Oxidative stress in prostate cancer. Cancer Lett 2009;282(2):125–136.
30. Wang J, Yi J. Cancer cell killing via ROS: To increase or decrease, that is the question. Cancer Biol Ther 2008;7(12):1875–1884.
31. Meyer F, Galan P, Douville P, Bairati I, Kegle P, Bertrais S, Estaquio C, Hercberg S. Antioxidant vitamin and mineral supplementation and prostate cancer prevention in the SU. VI. MAX trial. Int J Cancer 2005;116(2):182–186.