

## Zapotin Prevents Mouse Skin Tumorigenesis During the Stages of Initiation and Promotion

MURIEL CUENDET<sup>1</sup>, CAROL P. OTEHAM<sup>1</sup>, ARUP MAITI<sup>1</sup>, BRUCE A. CRAIG<sup>2</sup>,  
MARK CUSHMAN<sup>1</sup>, RICHARD C. MOON<sup>1</sup> and JOHN M. PEZZUTO<sup>1</sup>

<sup>1</sup>*Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and  
Pharmaceutical Sciences, College of Pharmacy, Nursing, and Health Sciences,*

<sup>2</sup>*Department of Statistics, College of Science, Purdue University, West Lafayette, IN 47907, U.S.A.*

**Abstract.** *Background:* Zapotin, a flavonoid associated with *Casimiroa edulis*, was isolated as part of a program to discover natural inhibitors of carcinogenesis. Zapote blanco, the fruit of *Casimiroa edulis*, is consumed in many parts of the world. Zapotin is a non-toxic inducer of cellular differentiation, apoptosis and cell cycle arrest with cultured HL-60 promyelocytic cells. *Materials and Methods:* An efficient chemical synthesis for zapotin was devised. Using this synthetic material, activity was examined in the two-stage mouse skin carcinogenesis model. *Results:* Topical zapotin significantly inhibited 7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate-induced mouse skin tumorigenesis, using the anti-initiation and anti-promotion protocols. *Conclusion:* Encouraging results from previous and current *in vivo* studies warrant further investigation of the chemopreventive activity of zapotin.

Zapotin is a polymethoxylated flavone (Figure 1) that was isolated from *Casimiroa edulis* Llave & Lex (*Rutaceae*) as part of a program to discover natural inhibitors of carcinogenesis (1). The medicinal value of zapote blanco, the fruit of *C. edulis* that is consumed in many parts of the world, was first discovered by the Aztecs, and crude plant extracts of the seeds or leaves of *C. edulis* were later found to affect blood pressure (2), cardiac activity (2) and aortic muscular tone (3), and to possess anticonvulsant activity (4). Zapotin has been found to inhibit 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase (ODC) activity in T24 cells and to display promising inhibition of TPA-induced NF- $\kappa$ B activity in

HepG2 cells (5). It is also a non-toxic inducer of cellular differentiation with cultured HL-60 promyelocytic cells (6) and membrane phenotype analysis showed zapotin induced a pattern of expression similar to that produced by macrophage inducers, with down-regulation of CD15 (granulocytic marker) and up-regulation of CD13 and CD11b (granulocytic/monocytic markers). In addition, zapotin induced apoptosis and cell cycle arrest in lymphoma and colon cancer cells (5-7). In mice, zapotin showed activity in azoxymethane-induced aberrant crypt foci studies (7).

The two-stage mouse skin carcinogenesis protocol has been one of the best-studied models and most informative with regard to understanding mechanisms and identifying chemopreventive agents (8). Skin tumors can be readily induced by the sequential application of a subthreshold dose of carcinogen, referred to as the initiation stage, followed by repetitive treatment with a noncarcinogenic tumor promoter, referred to as the promotion stage. The initiation stage, usually accomplished by a single application of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA), results in a small subset of keratinocytes carrying a mutation in critical genes. The promotion stage requires repeated application of noncarcinogenic tumor-promoting agents, such as TPA, which cause the initiated cells to proliferate, eventually producing papillomas, some of which spontaneously progress to squamous cell carcinomas. In this study, we investigated the cancer chemopreventive efficacy of zapotin in this mouse skin carcinogenesis model.

### Materials and Methods

*Animals and reagents.* Female CD-1 mice were obtained from Charles River Breeding Laboratories (Portage, MI) at 4 weeks of age. Zapotin was synthesized from commercially available 2-hydroxy-6-methoxyacetophenone and 2,6-dimethoxybenzoyl chloride (5). The synthesis resulted in 44% overall yield using a novel method that avoids the Baker-Venkataraman rearrangement. TPA was from LC Laboratories (Woburn, MA, USA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

*Correspondence to:* John M. Pezzuto, University of Hawaii at Hilo, College of Pharmacy, 34 Rainbow Drive, Hilo, HI 96720, U.S.A. Tel: +1 8089332909. Fax: +1 8089332981, e-mail: pezzuto@hawaii.edu

*Key Words:* Zapotin, two-stage mouse skin carcinogenesis, anti-initiation, anti-promotion.

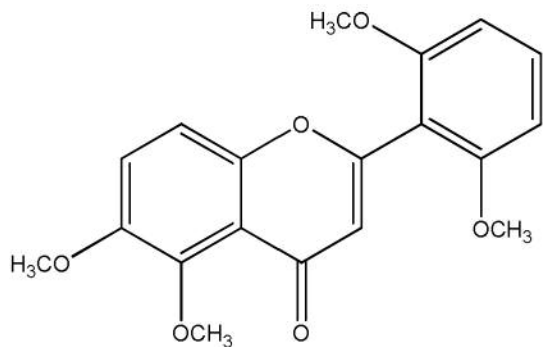


Figure 1. Structure of zapotin.

**Skin tumorigenesis study.** Female CD-1 mice (4 weeks of age) were housed under standard conditions at a temperature of  $21\pm 1^\circ\text{C}$ , relative humidity of 40%, and subjected to a 12 h light/dark cycle. Animals were allowed access to water and Teklad 4% mouse/rat diet *ad libitum*. After one-week acclimation, the dorsal sides of the mice were shaved using electric clippers and only mice with no hair growth during the next 72 h were used in the experiment. Mice were randomized by weight into groups of 20 animals. Initiation for all groups was with a single dose of 200 nmol DMBA in 0.2 ml acetone on the dorsal, shaved surface. Promotion began 1 week after initiation; 5 nmol TPA in 0.2 ml acetone were applied to the dorsal surface twice weekly for 15 weeks. Different zapotin concentrations (1, 5 and 10  $\mu\text{mol}/\text{mouse}$ ) were applied in 0.2 ml acetone 1 h prior to treatment with TPA. Group 1 was the DMBA/TPA control group. Groups 2-4 (anti-initiation protocols) were treated with zapotin twice during the week before initiation, once on the day of initiation, 1 h prior to DMBA, and twice during the week between initiation and promotion. Groups 5-7 (anti-promotion protocols) received zapotin beginning 1 week after DMBA, two-times per week, until the end of the study. Groups 8-10 (anti-initiation/anti-promotion protocols) received zapotin twice during the week before DMBA initiation and application was continued thereafter two-times per week for the duration of the study. Body weights, tumor incidence and multiplicity were recorded on a weekly basis. The duration of the study was 15 weeks.

**Statistical analysis.** Data are expressed as means $\pm$ SD. All analyses were carried out using the SAS statistical package (SAS Institute, Cary, NC, USA). All of the tests were two-sided, and, unless otherwise specified, a *p*-value of less than 0.05 was considered to be significant.

The number of tumors per mouse was analyzed through a one-factor analysis of variance (ANOVA) model with repeated measures. A square-root transformation was used to stabilize the variance. Pairwise comparisons with the control were made using Dunnett's multiple comparison procedure. Incidence between treatment and control was compared at each observation day using Fisher's exact test and a Bonferroni multiple comparison adjustment. The latency was also analyzed using a one-factor ANOVA model with the observation time as the time of tumor development. Similar results were obtained when these data, by treatment, were fit to generalized gamma survival distributions and the mean or median was compared.

## Results

As illustrated in Table I and Figure 2, utilization of the two-stage DMBA/TPA protocol generated a high incidence of skin tumors in mice. When zapotin was given prior to DMBA treatment, using an anti-initiation protocol, it failed to influence the percentage of tumor-bearing mice, except at day 71, where mice treated with 5 and 10  $\mu\text{mol}$  zapotin had a significantly lower tumor incidence compared to the control (Figure 2A). The number of tumors per mouse became significantly lower than the control starting at day 71 in the three groups of mice pretreated with zapotin. At the end of the study, the control group averaged  $16.75\pm 6.70$  tumors per mouse *versus*  $10.80\pm 6.20$  in the 1  $\mu\text{mol}$  group,  $11.10\pm 6.03$  in the 5  $\mu\text{mol}$  group, and  $6.90\pm 4.87$  in the 10  $\mu\text{mol}$  group (Figure 2B). Tumor latency was unaffected by zapotin in this anti-initiation protocol.

Administration of zapotin during the promotion phase resulted in a significant decrease of tumor incidence with some of the doses between days 64 and 85 (Figure 2C). The number of tumors per mouse became significantly lower than the control starting at day 71 in the three groups of mice treated with zapotin. At the end of the study, the control group averaged  $16.75\pm 6.70$  tumors per mouse *versus*  $6.95\pm 5.37$  in the 1  $\mu\text{mol}$  group,  $5.85\pm 3.96$  in the 5  $\mu\text{mol}$  group, and  $6.15\pm 6.29$  in the 10  $\mu\text{mol}$  group (Figure 2D). Moreover, tumor latency in the anti-promotion protocol was significantly extended by treatment with 5  $\mu\text{mol}$  zapotin and marginally extended by the two other doses tested (Table II).

Administration of zapotin during both the initiation and promotion phases had no effect on the percentage of tumor-bearing mice (Figure 2E) or on latency. The number of tumors per mouse was significantly lower than the control starting at day 85 in the three groups of mice treated with zapotin. At the end of the study, the control group averaged  $16.75\pm 6.70$  tumors per mouse *versus*  $9.20\pm 4.44$  in the 1  $\mu\text{mol}$  group,  $9.10\pm 6.16$  in the 5  $\mu\text{mol}$  group, and  $8.15\pm 5.71$  in the 10  $\mu\text{mol}$  group (Figure 2F).

At the end of the study, survival was 100% in all the groups and body weight was not affected (data not shown). Furthermore, tumor incidence and tumor multiplicity was not statistically significantly different between the various zapotin treatment groups.

## Discussion

Dietary flavonoids and other polyphenolic food components have over many years been suggested to have preventative properties both at the initiation and the promotion stages of chemically induced carcinogenesis (9-10). Initiation events induce carcinogen activation, specifically by enhancing the cytochrome P450 system and by inhibiting selective enzymes in the carcinogen detoxification pathway. Central to protective

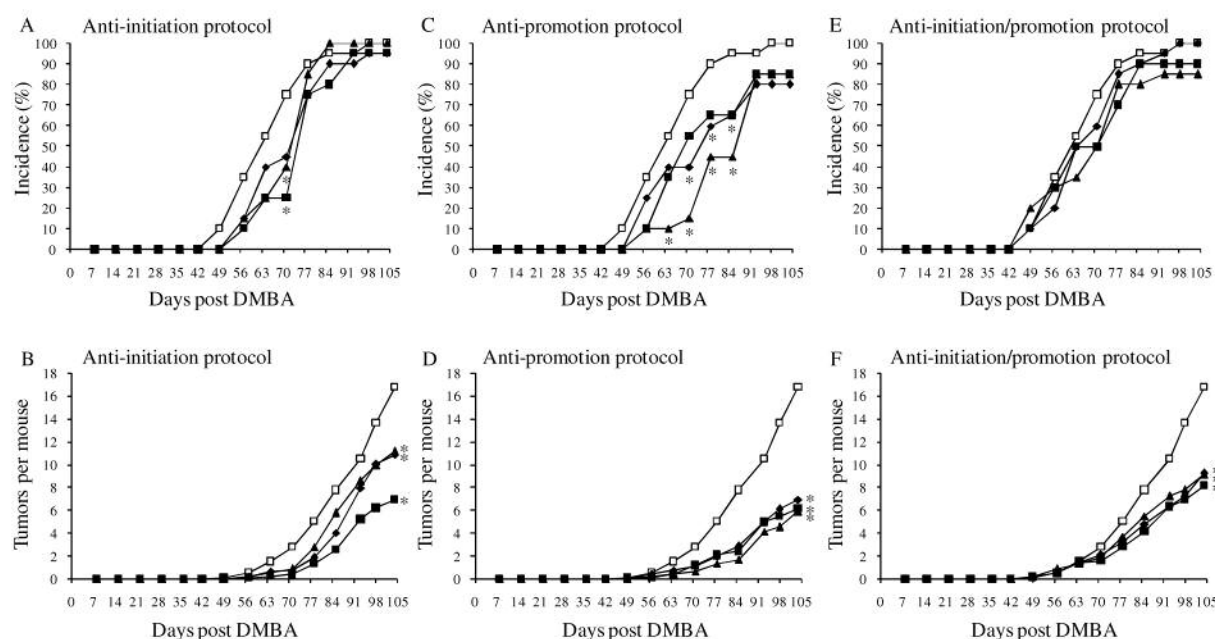


Figure 2. Effect of zapotin on DMBA/TPA-induced skin tumorigenesis. Panels A and B, anti-initiation protocol: mice were treated with zapotin starting 1 week prior and continuing until 1 week after DMBA initiation. Panels C and D, anti-promotion protocol: mice were treated with zapotin starting 1 week after DMBA initiation until the end of the study. Panels E and F, anti-initiation/anti-promotion protocol (combined): mice were treated with zapotin starting 1 week prior to DMBA initiation and continued throughout the study. For additional details see the Materials and Methods section. Top panels, effect of zapotin on the percentage of tumor-bearing mice. Bottom panels, effect of zapotin on the number of tumors per mouse. Open squares, DMBA/acetone control/TPA; closed diamonds, DMBA/1  $\mu$ mol zapotin/TPA; closed triangles, DMBA/5  $\mu$ mol zapotin/TPA; closed squares, DMBA/10  $\mu$ mol zapotin/TPA. \*Significantly different from control group ( $p < 0.05$ ).

Table 1. Effect of zapotin on tumor multiplicity.

Group	Schedule <sup>a</sup>	Treatment	Total tumor number	Average tumor number ( $\pm$ SD)
1	-	Control	335	16.8 $\pm$ 6.7
2	Anti-initiation	1 $\mu$ mol zapotin	216 <sup>b</sup>	10.8 $\pm$ 6.2
3	Anti-initiation	5 $\mu$ mol zapotin	222 <sup>b</sup>	11.1 $\pm$ 6.0
4	Anti-initiation	10 $\mu$ mol zapotin	138 <sup>c</sup>	6.9 $\pm$ 4.9
5	Anti-promotion	1 $\mu$ mol zapotin	139 <sup>c</sup>	7.0 $\pm$ 5.4
6	Anti-promotion	5 $\mu$ mol zapotin	117 <sup>c</sup>	5.9 $\pm$ 4.0
7	Anti-promotion	10 $\mu$ mol zapotin	123 <sup>c</sup>	6.2 $\pm$ 6.3
8	Anti-initiation/promotion	1 $\mu$ mol zapotin	184 <sup>c</sup>	9.2 $\pm$ 4.4
9	Anti-initiation/promotion	5 $\mu$ mol zapotin	182 <sup>c</sup>	9.1 $\pm$ 6.2
10	Anti-initiation/promotion	10 $\mu$ mol zapotin	163 <sup>c</sup>	8.2 $\pm$ 5.7

<sup>a</sup>Anti-initiation protocol: mice were treated with zapotin starting 1 week prior and continuing until 1 week after DMBA initiation. Anti-promotion protocol: mice were treated with zapotin starting 1 week after DMBA initiation until the end of the study. Anti-initiation/promotion protocol (combined): mice were treated with zapotin starting 1 week prior to DMBA initiation and continued throughout the study. <sup>b</sup> $p < 0.05$ , relative to group 1; <sup>c</sup> $p < 0.001$ , relative to group 1.

effects by dietary flavonoids and other polyphenols at the promotion stage of chemically induced carcinogenesis is the ability to inhibit cell proliferation; the damage that the carcinogens have inflicted on cellular DNA during the initiation stage is propagated into a new cell population. Human beings

are exposed to a variety of carcinogens that may act to both initiate and promote tumorigenesis. Furthermore, initiation of carcinogenesis may occur many years before promotion. For these reasons, it would be preferable if a chemopreventive agent could block multiple steps or stages in the carcinogenic process.

Table II. *Effect of zapotin on tumor latency.*

Group	Schedule <sup>a</sup>	Treatment	Time to first tumor (days) <sup>b</sup>
1	-	Control	67.0±12.3
2	Anti-initiation	1 µmol zapotin	75.4±16.0
3	Anti-initiation	5 µmol zapotin	73.5±9.20
4	Anti-initiation	10 µmol zapotin	78.7±15.0
5	Anti-promotion	1 µmol zapotin	81.5±23.3 <sup>c</sup>
6	Anti-promotion	5 µmol zapotin	87.5±17.6 <sup>d</sup>
7	Anti-promotion	10 µmol zapotin	80.9±21.9 <sup>c</sup>
8	Anti-initiation/promotion	1 µmol zapotin	70.2±13.2
9	Anti-initiation/promotion	5 µmol zapotin	76.0±24.2
10	Anti-initiation/promotion	10 µmol zapotin	74.1±21.0

<sup>a</sup>Anti-initiation protocol: mice were treated with zapotin starting 1 week prior and continuing until 1 week after DMBA initiation. Anti-promotion protocol: mice were treated with zapotin starting 1 week after DMBA initiation until the end of the study. Anti-initiation/promotion protocol (combined): mice were treated with zapotin starting 1 week prior to DMBA initiation and continued throughout the study. <sup>b</sup>The first day tumors appeared (after initiation) was averaged among the 20 animals in each group. <sup>c</sup> $p < 0.10$ , relative to group 1; <sup>d</sup> $p < 0.05$ , relative to group 1.

In this study, using a classic two-stage carcinogenesis protocol, where effects on initiation are separated from effects on promotion, significant inhibitory responses during the stages of initiation and promotion were observed. This is the first report to demonstrate inhibition of these stages of tumorigenesis by zapotin. With anti-initiation protocols, at doses of 5 and 10 µmol, and with anti-promotion protocols, at doses of 1, 5, and 10 µmol, significant protection was conferred against skin tumors, and no toxic effects were observed. Although zapotin administration reduced tumor incidence and multiplicity in the anti-promotion and combined stages, the differences between the treatment groups were not statistically significant. The lack of difference between the respective treatment groups may be due to the prolonged treatment with zapotin in the anti-promotion and combination groups and the prolonged binding of DMBA to the DNA of target cells, which has been shown to persist in some cells for as long as 42 days after DMBA administration (11-12).

The mechanisms involved in mouse skin tumor promotion have not been fully characterized but events such as hyperplasia, inflammation and production of reactive oxygen species, amongst others, have been shown to be important (13). Pre-treatment of murine skin with natural antioxidants suppressed the oxidative stress, activity of ODC, cell proliferation and ultimately inhibited skin tumor promotion (14). Zapotin has previously been reported to suppress TPA-induced ODC activity in T24 cells and to inhibit TPA-induced NF-κB activity in HepG2 cells (5). Moreover, zapotin is a non-toxic inducer of cellular differentiation, as

well as an inducer of apoptosis and cell cycle arrest in cultured HL-60 promyelocytic cells (6). When administered at different stages of tumorigenesis, zapotin not only lowers the carcinogenic activity of DMBA but also modulates the effect of the promoter. Differentiation-inducing agents have been shown to suppress cancer cell self-renewal selectively from normal stem cell renewal by inducing terminal differentiation followed by apoptosis. In addition, inducers of terminal differentiation, such as the retinoid and deltanoid (vitamin D3 derivatives) classes of compounds, have shown promising chemopreventive activity as suppressing agents that act during the promotion-progression stages of carcinogenesis (15-16).

The methoxylated flavones, a subclass of flavonoids, may have chemopreventive properties superior to the more common unmethylated flavonoids or polyphenols. The methylated flavones showed a better oral bioavailability, as well as more potent cancer chemopreventive properties at both the initiation and promotion stages (17). The methoxyflavone nobiletin inhibited tumor multiplicity of skin tumors in a dose-dependent manner during the stage of promotion using the two-stage mouse skin carcinogenesis model (18). The mechanism underlying the chemopreventive action of zapotin is not clear. The chemopreventive efficacy of zapotin in the mouse skin model may be comparable to that of resveratrol, which inhibited tumor formation at the dose range of 1-25 µmol (19). Together with a previous report showing *in vivo* activity of zapotin in preventing the formation of aberrant crypt foci, the present results suggest zapotin warrants further investigation as a chemopreventive agent.

## Acknowledgements

This work was supported by program project P01 CA48112 funded by the National Cancer Institute, NIH, Bethesda, MD, USA.

## References

- 1 Ito A, Shamon L, Yu B, Mata-Greenwood E, Lee S, van Breemen R, Mehta RG, Farnsworth N, Fong H and Pezzuto JM: Antimutagenic constituents of *Casimiroa edulis* with potential cancer chemopreventive activity. *J Agr Food Chem* 46: 3509-3516, 1998.
- 2 Garcia Gonzalez M, Freer Bustamante E and Morales Matamoros O: Effects of *Casimiroa edulis* (Rutacea) on blood pressure and heart rate in albino rats. *Rev Biol Trop* 42: 115-119, 1994.
- 3 Magos GA and Vidrio H: Pharmacology of *Casimiroa edulis*; Part I. Blood pressure and heart rate effects in the anesthetized rat. *Planta Med* 57: 20-24, 1991.
- 4 Garzon-De la Mora P, Garcia-Lopez PM, Garcia-Estrada J, Navarro-Ruiz A, Villanueva-Michel T, Villarreal-de Puga LM and Casillass-Ochoa J: *Casimiroa edulis* seed extracts show anticonvulsive properties in rats. *J Ethnopharmacol* 68: 275-282, 1999.

- 5 Maiti A, Cuendet M, Kondratyuk T, Croy VL, Pezzuto JM and Cushman M: Synthesis and cancer chemopreventive activity of zapotin, a natural product from *Casimiroa edulis*. *J Med Chem* 50: 350-355, 2007.
- 6 Mata-Greenwood E, Ito A, Westenburg H, Cui B, Mehta RG, Kinghorn AD and Pezzuto JM: Discovery of novel inducers of cellular differentiation using HL-60 promyelocytic cells. *Anticancer Res* 21: 1763-1770, 2001.
- 7 Murillo G, Hirschelman WH, Ito A, Moriarty RM, Kinghorn AD, Pezzuto JM and Mehta RG: Zapotin, a phytochemical present in a Mexican fruit, prevents colon carcinogenesis. *Nutr Cancer* 57: 28-37, 2007.
- 8 DiGiovanni J: Multistage carcinogenesis in mouse skin. *Pharmacol Ther* 54: 63-128, 1992.
- 9 Middleton E Jr, Kandaswami C and Theoharides TC: The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. *Pharmacol Rev* 52: 673-751, 2000.
- 10 Pervaiz S: Resveratrol: from grapevines to mammalian biology. *FASEB J* 17: 1975-1985, 2003.
- 11 Janss DH, Moon RC and Irving CC: The binding of 7,12-dimethylbenz(a)anthracene to mammary parenchyma DNA and protein *in vivo*. *Cancer Res* 32: 254-258, 1972.
- 12 Brookes P and Lawley PD: Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin: relation between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. *Nature* 202: 781-784, 1964.
- 13 Agarwal R, Katiyar SK, Zaidi SI and Mukhtar H: Inhibition of skin tumor promoter-caused induction of epidermal ornithine decarboxylase in SENCAR mice by polyphenolic fraction isolated from green tea and its individual epicatechin derivatives. *Cancer Res* 52: 3582-3588, 1992.
- 14 Sultana S and Saleem M: *Salix caprea* inhibits skin carcinogenesis in murine skin: inhibition of oxidative stress, ornithine decarboxylase activity and DNA synthesis. *J Ethnopharmacol* 91: 267-276, 2004.
- 15 Kelloff GJ, Boone CW, Crowell JA, Steele VE, Lubet R and Sigman CC: Chemopreventive drug development: perspectives and progress. *Cancer Epidemiol Biomarkers Prev* 3: 85-98, 1994.
- 16 Mehta RG: Stage-specific inhibition of mammary carcinogenesis by 1-alpha-hydroxyvitamin D5. *Eur J Cancer* 40: 2331-2337, 2004.
- 17 Walle T: Methoxylated flavones, a superior cancer chemopreventive flavonoid subclass? *Semin Cancer Biol* 17: 354-362, 2007.
- 18 Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, Kuwahara S, Takahashi Y, Ogawa K, Yano M, Tokuda H, Nishino H, Mimaki Y, Sashida Y, Kitanaka S and Ohigashi H: Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. *Cancer Res* 60: 5059-5066, 2000.
- 19 Jang M, Cai L, Udeani GO, Slowing KV, Thomas CF, Beecher CW, Fong HH, Farnsworth NR, Kinghorn AD, Mehta RG, Moon RC and Pezzuto JM: Cancer chemopreventive activity of resveratrol, a natural product derived from grapes. *Science* 275: 218-220, 1997.

Received July 22, 2008

Revised September 17, 2008

Accepted October 2, 2008