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Modifying Effects of Fungal and Herb Metabolites on Azoxymethane-induced Intestinal Carcinogenesis in Rats

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Modifying effects of a fungal product, flavoglaucin, and four plant-derived chemicals, shikonin, gingerol, oleanolic acid and paeoniflorin, on intestinal carcinogenesis were examined in a rat model using azoxymethane (AOM). A total of 280 male F344 rats, 6 weeks old, were divided into 12 groups. Group 1 (30 rats) was given two subcutaneous injections of 15 mg/kg of AOM at the start of the experiment. Groups 2 (30 rats), 3 (20 rats), 4 (20 rats), 5 (30 rats) and 6 (30 rats) received a test chemical (flavoglaucin, shikonin, gingerol, oleanolic acid or paeoniflorin, respectively) in the diet at a concentration of 0.02% for 3 weeks, during which time AOM was applied, and then kept on basal diet until the end of experiment (one year). Groups 7–11 (each 20 rats) were given a test chemical corresponding to Groups 2–6, respectively. Group 12 (20 rats) served as a control. The incidence and average number of intestinal tumors in Group 2 (47%, 0.57±0.68) were significantly less than in Group 1 (74%, 1.07±0.87) ($P<0.05$, respectively). Multiplicity of intestinal neoplasms of Group 3 (0.55±0.60) or 4 (0.47±0.51) was also significantly smaller than that of Group 1 ($P<0.05$ and $P<0.01$, respectively). These results suggest that flavoglaucin, shikonin and gingerol might be promising chemopreventive agents for intestinal neoplasia.

Key words: Chemoprevention — Fungal metabolite — Herb — Intestinal carcinogenesis — Blocking effect

Chemoprevention is based on the idea that non-carcinogenic synthetic or naturally occurring products can inhibit the process of carcinogenesis.[1-5] A number of agents have proved effective against chemical carcinogenesis in different organs and have been classified into three categories according to the stage of the carcinogenic process at which they are effective: (1) compounds preventing formation of the ultimate carcinogen from the precursor, (2) agents blocking the initiation phase and (3) agents suppressing the post-initiation phase.[2] However, the modes of action of chemopreventive compounds are not yet well understood.

Since large bowel cancer has one of the highest incidences and mortality rates of human neoplasms, the discovery of effective chemopreventive agents, especially in the natural environment, is an important aim. Certain agents exerting inhibitory effects on intestinal carcinogenesis are already known.[6-12] In the present study, we evaluated the effects of one fungal metabolite, flavoglaucin, and four plant-derived chemicals, shikonin, gingerol, oleanolic acid and paeoniflorin, on carcinogen-induced intestinal carcinogenesis in rats, since these chemicals are widely distributed in the natural environment and some of them have been reported to exert anti-tumor promoting activity.[13,14] Flavoglaucin, a quinol compound, is widely produced by species of *Aspergillus* which have been used in a fermentation process for the manufacture of *katsuobushi* (dried bonito), a traditional marine food product in Japan. Shikonin is contained in some medicinal plants such as *Lithospermum erythrorhizon* Sieb et Zucc. Gingerol is a major component of *Zingiber officinale* Roscoe (ginger). Oleanolic acid is mainly contained in an anti-inflammatory Japanese-Chinese medicinal plant, *Glechoma hederaceae L.* Paeoniflorin is a representative compound contained in a herbal medicine, Shakuyaku-Kanzo-To. Since the plants containing these chemicals are widely used as herbal remedies or as foods in Japan, it is important to examine the modifying effects of the chemicals on carcinogenesis.

MATERIALS AND METHODS

Animals A total of 280 male Fischer 344 rats, six weeks old, from Shizuoka Laboratory Animal Center (Shizuoka) were used. Animals were housed in wire cages and given diet and water *ad libitum* under controlled conditions of humidity (50±10%), lighting (12 h light and dark cycle) and temperature (23±2°C).

Chemicals Azoxymethane (AOM[5]) was purchased from Sigma Chemical Co. (St. Louis, MO). Flavoglaucin was

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[5] Abbreviations: AOM, azoxymethane; MAM, methylazoxymethanol; SCC, squamous cell carcinoma.

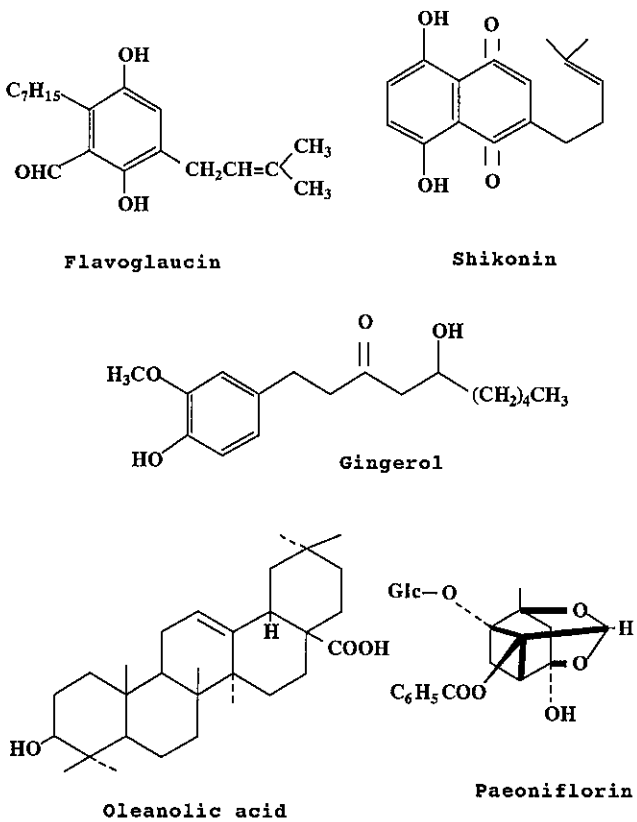

Fig. 1. Molecular structures of the five examined chemicals.

isolated from the dried mycelium of *Aspergillus chevalieri* (Mangin) by one of the authors.[15] Shikonin, gingerol, oleanolic acid and paeoniflorin were also extracted, isolated and purified from *Lithospermum erythrorhizon* Sieb et Zucc, ginger, *Panax japonicus* G.A. Meyer and *Paeonia lactiflora* Pall, respectively, by one of the authors.[16] The purity of each chemical was confirmed by high-performance liquid chromatography or thin layer chromatography (>99.5%, in each case). The mixture of each chemical (0.02%) showed no detectable spectral alteration after storage for 1 month at 4°C, indicating analytical purity and stability in the diet. The molecular structures of the examined chemicals are shown in Fig. 1.

Treatment The experimental protocol is illustrated in Fig. 2. Two hundred and eighty male F344 rats, 6 weeks old, were divided into 12 groups. Group 1 (30 rats) was given two subcutaneous injections of 15 mg/kg body weight of AOM in the shoulder at 1 and 2 weeks from the commencement of the experiment. Groups 2 (30 rats), 3 (20 rats), 4 (20 rats), 5 (30 rats) and 6 (30 rats) each received a test chemical (Group 2; flavoglaucin, Group 3; shikonin, Group 4; gingerol, Group 5; oleanolic acid and Group 6; paeoniflorin) in the diet at a concentration of 0.02% for 3 weeks during which AOM was applied, and then kept on basal diet, CE-2 (CLEA Japan Inc., Tokyo) until the end of the experiment (one year). Groups 7–11 (each 20 rats) were each given a test compounds corresponding to Groups 2–6, respectively. The dose and

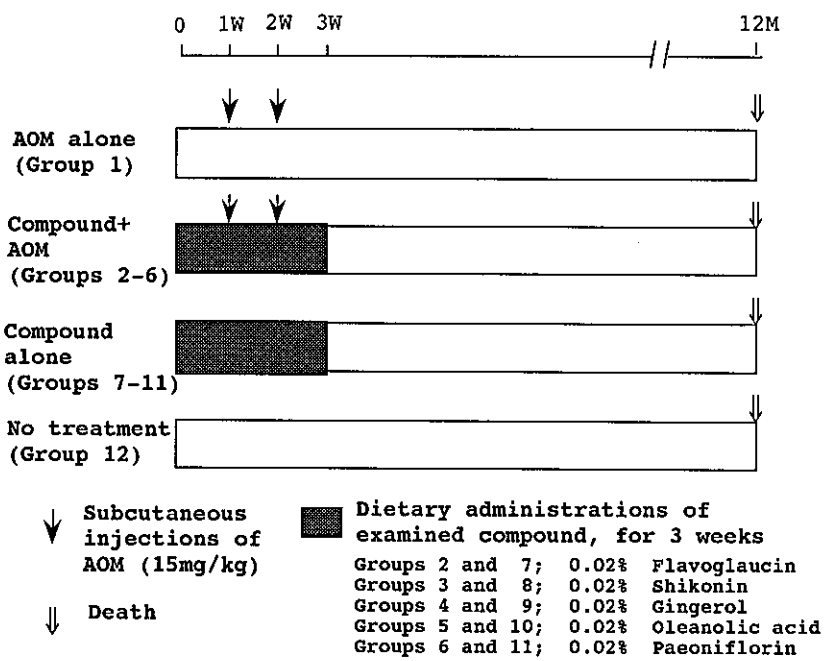

Fig. 2. Experimental protocol.

Table I. The Body and Liver Weights at Termination of the Experiment on Intestinal Carcinogenesis in Rats

Groups	Treatments	No. of rats	No. of effective rats	Body weight (g)	Liver weight (g)	Ratio[a]
1	AOM alone	30	27	329±45[b]	11.5±2.2	3.5±0.4
2	Flavoglaucin[c]+AOM	30	30	300±45	11.2±2.7	3.7±0.5
3	Shikonin[c]+AOM	20	20	316±47	10.8±1.9	3.4±0.4
4	Gingerol[c]+AOM	20	17	330±35	11.5±2.0	3.5±0.5
5	Oleanolic acid[c]+AOM	30	29	297±46	10.2±2.6	3.4±0.6
6	Paeoniflorin[c]+AOM	30	28	303±46	10.8±2.6	3.6±0.6
7	Flavoglaucin[c]	20	20	319±50	13.4±2.9	4.2±0.5
8	Shikonin[c]	20	20	314±35	11.2±1.9	3.5±0.4
9	Gingerol[c]	20	20	321±27	12.3±1.3	3.8±0.2
10	Oleanolic acid[c]	20	20	341±48	14.8±3.1	4.2±0.6
11	Paeoniflorin[c]	20	20	292±40	11.5±2.8	3.9±0.6
12	No treatment	20	20	301±70	12.9±2.3	4.1±0.6

a) Ratio=liver weight/body weight×100.
b) Mean±SD, no significant difference between the corresponding groups.
c) Each compound was given at the level of 0.02% in the diet.

Table II. The Incidences of Neoplasms in Small and Large Intestines of Rats of Each Group

Groups	Treatments	No. of effective rats	No. of intestinal tumor-bearing rats (%)	No. of rats with neoplasms at: small intestine			large intestine		
				Total	AD	ADC	Total	AD	ADC
1	AOM alone	27	20 (74)	6	0	6	17	6	13
2	Flavoglaucin[a]+AOM	30	14 (47)[b]	5	0	5	10	4	6
3	Shikonin[a]+AOM	20	10 (50)	1	0	1	9	4	6
4	Gingerol[a]+AOM	17	8 (47)	0	0	0	8	5	3
5	Oleanolic acid[a]+AOM	29	15 (52)	3	0	3	13	8	6
6	Paeoniflorin[a]+AOM	28	17 (61)	8	0	8	13	9	5
7–11	Examined compound alone[a]	20	0	0	0	0	0	0	0
12	No treatment	20	0	0	0	0	0	0	0

Abbreviation: AD, adenoma; ADC, adenocarcinoma.
a) Each compound was given at the level of 0.02% in the diet.
b) Significantly different from AOM alone by χ^2-test ($P<0.05$).

duration of administration of the test chemicals were determined on the basis of a previous study of ours.[8] Group 12 (20 rats) was kept on the basal diet during the experiment and served as an untreated control.

At the termination of the experiment, complete autopsies were performed. The intestines were carefully inspected. The tissues were fixed with 10% buffered formalin, processed for histological examinations and stained with hematoxylin and eosin.

The intestinal tumors were diagnosed according to the criteria described by Ward.[17] The statistical analysis of the incidence or multiplicity of the lesions was performed by using the chi-squared test or Welch's method, respectively.

RESULTS

There were no clear toxic changes in the liver of rats in the groups given test chemicals and/or AOM. Body, liver and relative liver weights (liver wt./100 g body wt.) of rats in all groups are shown in Table I; no significant differences were recognized between the groups.

Macroscopically, intestinal tumors were found only in the groups treated with AOM. Other tumors also occurred only in rats treated with AOM, i.e., one cutaneous squamous cell carcinoma (SCC) in a rat of Group 1, 1 ear duct SCC in a rat and mammary adenoma in another animal of Group 2, malignant mesenchymoma of the abdominal cavity in one rat of each of Groups 3 and 4,

Table III. Multiplicities of Neoplasms in Small and Large Intestines of Each Group with Tumors

Groups	Treatments	No. of effective rats	Average number[a] of neoplasms at:		
			entire intestine	small intestine	large intestine
1	AOM alone	27	1.07±0.87[b]	0.22±0.42	0.85±0.80
2	Flavoglaucin[c]+AOM	30	0.57±0.68[d]	0.20±0.48	0.37±0.56[e]
3	Shikonin[c]+AOM	20	0.55±0.60[d]	0.05±0.22	0.50±0.61
4	Gingerol[c]+AOM	17	0.47±0.51[f]	0	0.47±0.51
5	Oleanolic acid[c]+AOM	29	0.66±0.72	0.10±0.31	0.55±0.69
6	Paeoniflorin[c]+AOM	28	1.04±1.14	0.32±0.55	0.71±0.98

Abbreviations: AD, adenoma; ADC, adenocarcinoma.
a) The number of effective rats without neoplasms was calculated as 0.
b) Mean±SD.
c) Each compound was given at the level of 0.02% in the diet.
d, e, f) Significantly different from AOM alone by Welch's method ($P<0.05$, $P<0.02$ and $P<0.01$, respectively).

1 ear duct SCC in a rat of Group 5, and mesothelioma in the abdominal cavity in a rat of Group 6.

Histologically, intestinal tumors were adenomas or adenocarcinomas. The incidence and average number of the intestinal tumors in each group are shown in Tables II and III. The values in Group 2 (47%, 0.57±0.68) were significantly less than in Group 1 (74%, 1.07±0.87) ($P<0.05$, respectively). The multiplicity of intestinal neoplasms of Group 3 (0.55±0.60) or 4 (0.47±0.51) was also significantly smaller than that of Group 1 ($P<0.05$ and $P<0.01$, respectively). Oleanolic acid or paeoniflorin did not show any effect on the AOM-induced intestinal carcinogenesis.

Concerning the difference of the intestinal sites of development of the tumors, the multiplicity of large bowel neoplasm in Group 2 (0.37±0.56) was significantly smaller than that of Group 1 (0.85±0.80) ($P<0.02$). However, no significant differences in the development of tumors in the small and large intestines were found between Group 1 and any of Groups 3–6.

DISCUSSION

In this study, the multiplicity and the incidence of intestinal tumors in the group given flavoglaucin together with AOM were significantly less than those of the group exposed to the carcinogen alone. The average number of intestinal tumors in the groups treated with shikonin or gingerol and AOM was also significantly smaller than that of the group given AOM alone. Since these chemicals were administered to rats during the initiation phase in this protocol, these chemicals can be regarded as blocking agents.

Flavoglaucin is the major pigment formed by a rice mold, *Aspergillus chevalieri,* causing the stored rice to take on a yellowish color.[13] However, it is noteworthy that *Aspergillus glaucus* also produces flavoglaucin[18] and has been used in the manufacture of *katsuobushi,* one of the most popular flavorings for Japanese food.

Shikonin is a naphthoquinone and has been used as stypticin in Japanese-Chinese medicine. It has been reported that naphthoquinone derivatives exert anti-inflammatory and anti-tumor effects.[13, 19, 20]

Ginger is believed to possess anti-ulcer, anti-inflammatory and antiemetic actions in Japanese-Chinese traditional medicine.[16] Koshimizu *et al.*[14] have reported that gingerol has an anti-tumor promoting activity in an *in vitro* short-term assay system, although it has been demonstrated to exhibit some mutagenicity.[21, 22]

The mechanism of the inhibitory effects of flavoglaucin, shikonin and gingerol in the present animal model is obscure. Ishikawa *et al.*[23] reported that flavoglaucin has an antioxidative activity and acts synergistically with tocopherol. The antioxidative activity of some foods is considered to provide protection against oxidative damage to DNA.[24] Thus, these antioxidative activities may be important for the biological functions of flavoglaucin. In fact, the metabolic change of AOM to methylazoxymethanol (MAM) involves oxidation,[25, 26] although the mechanism of intestinal carcinogenesis by AOM or MAM remains unclear. Flavoglaucin might prevent formation of the ultimate carcinogen from the precursor compound.[2] It is also accepted that natural antioxidants such as phenol derivatives are inhibitors of aging or carcinogenesis on the basis of currently available evidence.[27] It is therefore possible that flavoglaucin would have inhibitory effects in other animal models using different carcinogens such as 1,2-dimethylhydrazine.[12]

Oleanolic acid did not show clear modifying effects in the present study, although this agent slightly decreased both of the incidence and multiplicity of the intestinal

tumors and has been reported to show an anti-promoting effect in an *in vitro* short-term assay[14, 28] or in *in vivo* assay with mouse skin.[29] No modifying effect on the carcinogenesis was demonstrated by paeoniflorin. This chemical is present in a traditional herbal medicine, Shakuyaku-Kanzo-To, and is reported to inhibit steroid binding.[30]

In the present study, modifying effects of several natural products on intestinal carcinogenesis were examined by using a protocol for blocking agents. However, the protocol used may not detect all the beneficial effects of these chemicals. Another long-term experiment using a post-initiation method is now under way. Nevertheless, flavoglaucin, shikonin and gingerol were revealed to have inhibitory effects on intestinal carcinogenesis in this rat model. These natural products may be promising chemopreventive agents for human large bowel neoplasia.

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REFERENCES

1) Sporn, M. B. and Newton, D. L. Chemoprevention of cancer with retinoids. *Fed. Proc.*, **38**, 2528–2534 (1979).
2) Wattenberg, L. W. Chemoprevention of cancer. *Cancer Res.*, **45**, 1–8 (1985).
3) Weinstein, I. B. Cancer prevention: recent progress and future opportunities. *Cancer Res.*, **51**, 5080s–5085s (1991).
4) Tanaka, T. Cancer chemoprevention. *Cancer J.*, **5**, 11–16 (1992).
5) Cullen, J. W. The National Cancer Institute's intervention trials. *Cancer*, **62**, 1851–1864 (1988).
6) Reddy, S., Mori, H. and Nicolais, M. Effect of dietary wheat bran and dehydrated citrus fiber on azoxymethane-induced intestinal carcinogenesis in Fischer 344 rats. *J. Natl. Cancer Inst.*, **66**, 553–557 (1981).
7) Narisawa, T., Sato, A., Tani, M., Kudo, T. and Takahashi, T. Inhibition of development of methylnitrosourea-induced rat colon tumors by indomethacin treatment. *Cancer Res.*, **41**, 1954–1957 (1981).
8) Mori, H., Tanaka, T., Shima, H., Kuniyasu, T. and Takahashi, M. Inhibitory effect of chlorogenic acid on methylazoxymethanol acetate-induced carcinogenesis in large intestine and liver of hamsters. *Cancer Lett.*, **30**, 49–54 (1986).
9) Reddy, B. S., Sugie, S., Maruyama, H., El-Bayoumy, K. and Marra, P. Chemoprevention of colon carcinogenesis by dietary organoselenium, benzylselenium, benzylselenocyanate, in F344 rats. *Cancer Res.*, **47**, 5901–5904 (1987).
10) Reddy, B. S. and Sugie, S. Effect of different levels of Omega-3 and Omega-6 fatty acids on azoxymethane-induced colon carcinogenesis in F344 rats. *Cancer Res.*, **48**, 6642–6647 (1988).
11) Tanaka, T., Shinoda, T., Yoshimi, N., Niwa, K., Iwata, H. and Mori, H. Inhibitory effect of magnesium hydroxide on methylazoxymethanol acetate-induced large bowel carcinogenesis in male F344 rats. *Carcinogenesis*, **10**, 613–616 (1989).
12) Wattenberg, L. W. and Fiala, E. S. Inhibition of dimethylhydrazine-induced neoplasia of the large intestine by carbon disulfide. *J. Natl. Cancer Inst.*, **60**, 1515–1517 (1978).
13) Konoshima, T., Kozuka, M., Koyama, J., Okatani, T., Tagahara, K. and Tokuda, H. Studies on inhibitors of skin tumor promotion, VI. Inhibitory effects of quinones on Epstein-Barr virus activation. *J. Nat. Prod.*, **52**, 987–995 (1989).
14) Koshimizu, K., Ohigashi, H., Tokuda, H., Kondo, A. and Yamaguchi, K. Screening of edible plants against possible anti-tumor promoting activity. *Cancer Lett.*, **39**, 247–257 (1988).
15) Kawai, K., Mori, H. and Kitamura, J. The uncoupling effect of flavoglaucin, a quinol pigment from *Aspergillus chevalieri* (MANGIN), on mitochondrial respiration. *Toxicol. Lett.*, **19**, 321–325 (1983).
16) Yamahara, J., Mochizuki, M., Rong, H. Q., Matsuda, H. and Fujimura, H. The anti-ulcer effect in rats of ginger constituents. *J. Ethnopharmacol.*, **23**, 299–304 (1988).
17) Ward, J. M. Morphogenesis of chemically induced neoplasms of the colon and small intestine in rats. *Lab. Invest.*, **30**, 505–513 (1974).
18) Turner, W. B. "Fungal Metabolites," pp. 141 (1971). Academic Press Inc., London and New York.
19) Honda, G., Sakakibara, F., Yazaki, K. and Tabata, M. Isolation of deoxyshikonin, antidermatophytic principle from *Lithspermum erythrorhizon* cell cultures. *J. Nat. Prod.*, **51**, 152–154 (1988).
20) Tanaka, S.. Tajima, M., Tsukada, M. and Tabata, M. A comparative study on anti-inflammatory activities of the enantiomers, shikonin and alkanin. *J. Nat. Prod.*, **49**, 466–469 (1986).
21) Nagabhushan, M., Amonkar, A. J. and Bhide, S. V. Mutagenicity of gingerol and shogaol and antimutagenicity of zingerone in *Salmonella*/microsome assay. *Cancer Lett.*, **36**, 221–233 (1987).
22) Nakamura, H. and Yamamoto, T. The active part of the [6]-gingerol molecule in mutagenesis. *Mutat. Res.*, **122**, 87–94 (1983).
23) Ishikawa, Y., Morimoto, K. and Hamasaki, T. Flavoglaucin, a metabolite of *Eurotium chevalieri*, its antioxidation and synergism with tocopherol. *JAOCS, J. Am. Oil Chem. Soc.*, **61**, 1864–1868 (1984).

24) Osawa, T., Namiki, M. and Kawakishi, S. Role of dietary antioxidants in protection against oxidative damage. *In* "Antimutagenesis and Anticarcinogenesis Mechanisms II," ed. Y. Kuroda, D. M. Shankel and M. D. Waters, pp. 139–153 (1990). Plenum Press, New York.
25) Fiala, E. S., Kulakis, C., Christiansen, G. and Weisburger, J. H. *In vivo* and *in vitro* metabolism of the colon carcinogen azoxymethane (AOM). *Proc. Am. Assoc. Cancer Res.*, **18,** 105 (1977).
26) Zedeck, M. S. Hydrazine derivatives, azo and azoxy compounds, and methylazoxymethanol and cycasin. *In* "Chemical Carcinogens (Vol. 2)," ed. C. E. Searle, pp. 915–944 (1984). ACS, Washington DC.
27) Fujita, Y., Yamane, T., Tanaka, M., Kuwata, K., Okuzumi, J., Takahashi, T., Fujiki, H. and Okuda, T. Inhibitory effect of (−)-epigallocatechin gallate on carcinogenesis with N-ethyl-N′-nitro-N-nitrosoguanidine in mouse duodenum. *Jpn. J. Cancer Res.*, **80,** 503–505 (1989).
28) Ohigashi, H., Takamura, H., Koshimizu, K., Tokuda, H. and Ito, Y. Search for possible antitumor promoters by inhibition of 12-O-tetradecanoylphorbol-13-acetate-in-duced Epstein-Barr virus activation: ursolic acid and oleanolic acid from an anti-inflammatory Chinese medicinal plant, *Glechoma hederaceae* L. *Cancer Lett.*, **30,** 143–151 (1986).
29) Tokuda, H., Ohigashi, H., Koshimizu, K. and Ito, Y. Inhibitory effects of ursolic and oleanolic acid on skin tumor promotion by 12-O-tetradecanoylphorbol-13-acetate. *Cancer Lett.*, **33,** 279–285 (1986).
30) Tamaya, T., Sato, S. and Okada, H. Inhibition by plant herb extracts of steroid bindings in uterus, liver and serum of the rabbit. *Acta Obstet. Gynecol. Scand.*, **65,** 839–842 (1986).