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Chemopreventive Effect of Xanthorrhizol from *Curcuma xanthorrhiza*

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Xanthorrhizol is a sesquiterpenoid isolated from *Curcuma xanthorrhiza* that has been known to possess anti-inflammatory and anti-carcinogenic effects. In this work, we have found that xanthorrhizol reduced superoxide anion generation by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in differentiated HL-60 cells and exerted a dose-related inhibition of *His*$^+$ reversion in *Salmonella typhimurium* TA102 induced by *tert*-butylhydroperoxide (*t*-BOOH) and hydrogen peroxide. Pretreatment of xanthorrhizol significantly decreased the incidence and the multiplicity of mouse skin tumors stimulated by TPA for 19 weeks after a single application of dimethylbenz[*a*]anthracene (DMBA), and mitigated the expression of epidermal ornithine decarboxylase in the skins. These results demonstrate that xanthorrhizol may be a potent cancer chemopreventive agent.

Key Words: *Curcuma xanthorrhiza*, Xanthorrhizol, Chemopreventive agent

INTRODUCTION

The development of a malignant tumor involves complex interactions between several factors, both exogenous (environmental) and endogenous (genetic, hormonal, and immunological). Carcinogenesis often proceeds through multiple discernible but overlapping stages.[1)] These include initiation, promotion, progression and their further evolution to tumors with increasing degrees of malignancy. The transitions between successive stages can be enhanced or inhibited by various agents. Several types of evidence indicate that 50～80% of human cancer is potentially preventable because its causation is largely exogenous.[2)] Therefore, recent studies have been focused on a new alternative approach referred to as cancer chemoprevention, which is defined as the use of specific chemical compounds to prevent, inhibit, or reverse carcinogenesis.[3,4)] Natural products, particularly dietary substances-green and black tea polyphenols,[5)] lycopene,[6)] soy isoflavones,[7)] curcumin,[8)] phenethyl isothiocyanate (PEITC),[9)] indol-3-carbinol,[10)] and perillyl alcohol,[11)] have played an important sole...

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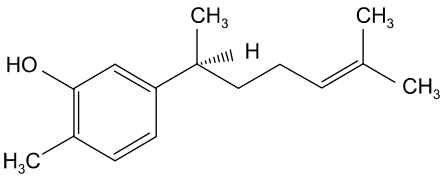

Fig. 1. The structure of xanthorrhizol isolated from *Curcuma xanthorrhiza.*

other chronic diseases. The majority of naturally occurring compounds which possess marked antioxidative and anti-inflammatory properties retain chemopreventive or chemoprotective activities as well.[12)]

Xanthorrhizol (Fig. 1) isolated from the rhizoma of *Curcuma xanthorrhiza* Roxb (Zingiberaceae) is a compound structurally related to curcumene, the phenolic sesquiterpenoid hydrocarbon. *C. xanthorrhiza* has been used in Indonesian folk medicine as cholagogues, aromatic stomachics, analgesics and a rheumatic remedy.[13,14)] Antiinflammatory,[15,16)] antitumor[17)] and hepatoprotective[18)] activities of the methanolic extract of *C. xanthorrhiza* have also been reported. Although many pharmacological effect of the crude extract of *C. xanthorrhiza* have been reported, little attention has been paid to the biological activity of xanthorrhizol, one of its active principles. We reported previously that xanthorrhizol exhibited a potent inhibition of cyclooxygenase-2 and inducible nitric oxide synthase activity in lipopolysaccharide-stimulated RAW 264.7 cells.[19)] The present study was carried out to elucidate the chemoprotective effect of xanthorrhizol. We investigated its effects on (a) superoxide anion generation by TPA in differentiated HL-60 cells, (b) oxidant-induced bacterial mutagenesis and, (c) tumor formation and the expression of ornithine decarboxylase (ODC) in two- stage mouse skin carcinogenesis model.

MATERIALS AND METHODS

1) Chemicals

Xanthorrhizol was supplied from Dr. Jae-Kwan Hwang of Department of Biotechnology, Yonsei University (Seoul, Korea). $NADP^+$, DMBA, TPA, *tert*-butylhydroperoxide (*t*-BOOH), dimethylsulfoxide (DMSO), glucose 6-phosphate, cytochrome c were purchased from Sigma Chemical Co. (St. Louis, MO, USA). RPMI 1640, fetal bovine serum (FBS), gentamicin were obtained from Gibco BRL (Grand Island, NY, USA). All other chemicals and solvents used were commercial products of analytical grade.

2) Animals

Female ICR mice (about 6 weeks of age) were supplied from DAEHAN Biolink, Co., Ltd. (Chungbuk, Korea). Mice were fed *ad libitum* and kept at temperature of 22±2°C with a 12 h light/dark cycle.

3) Measurement of superoxide generation in differentiated HL-60 cells

HL-60 cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 5 g/ml gentamicin as described previously.[20)] The cells were suspended at a density of 5×10^5 cells/ml in RPMI medium containing 10% fetal bovine serum and 1.25% DMSO, and incubated for 6 days at 37°C in a humidified atmosphere of 5% CO_2. The cells were harvested by centrifugation, washed with and then resuspended in phosphate-buffered saline (1×10^6 cells/ml). The cells were preincubated for 10 min with or without xanthorrhizol. Reactions were stopped 40 min after the addition of TPA (8μM) and cytochrome *c* (160μM). Control cells received PBS instead of TPA. The samples were centrifuged to remove the cells before analysis of the supernatants at 550 nm.

4) Ames/Salmonella mutagenicity assay

The bacterial mutagenicity assays were conducted with *Salmonella typhimurium* TA102 by using the liquid pre-incubation method developed by Maron and Ames.[21)] Briefly, *S. typhimurium* TA102 were grown in Oxoid nutrient broth medium. 100μl of an 11-h culture of *S. typhimurium* TA102 was added to 600μl of the mixture containing hydrogen peroxide (50

g/plate) and *t*-BOOH (100 g/plate) in the presence or absence of a given xanthorrhizol. After pre-incubation for 30 min at 37°C in a rotary shaker, the mixture was transferred to 2 ml top agar containing 0.5 mM histidine-biotin (all kept warm at 45°C) and poured onto minimal glucose plates. The plates were incubated for 48 h at 37°C and the number of *His*$^+$ revertant colonies was counted.

5) Two-stage mouse skin carcinogenesis

The dorsal regions of 6-week-old female ICR mice (30 per group) were shaved with an electric clipper. The mice were treated topically with a single dose of 0.2μmol DMBA, and one week later, 7.5 nmol of TPA in 0.2 ml of acetone was topically applied three times weekly for 19 weeks. When necessary, 2μmol and 6μmol of xanthorrhizol in 0.2 ml of acetone-DMSO (85 : 15, v/v) were applied topically 30 min prior to TPA treatment. Tumors of at least 1 mm in diameter were counted and recorded biweekly. The results are expressed as the average number of tumors per mouse and percentage of tumor-bearing mice. The mice were sacrificed by cervical dislocation at 19 week and the skin was excised. The skin was stored at -80°C for molecular analyses.

6) Western blot analysis of mouse epidermal ODC

The frozen skin described above was pulverized with mortar in liquid N_2 after the fat removed. The pulverized mouse skin was lysed in 300μl of ice-chilled lysis buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycero-phosphate, 1 mM Na_3VO_4, 1 g/ml leupeptin] for 1 hr. Lysates were centrifuged at 15,000 rpm for 10 min, and aliquots of supernatant containing 20μg protein were boiled in SDS sample loading buffer for 5 min before electrophoresis on a 10% SDS-polyacrylamide gel. Blots were transferred from SDS-polyacrylamide gel to PVDF membrane (Amersham, Arlington Heights, IL), blocked with 5% fat-free dry milk-PBST (phosphate buffered saline containing 0.1% Tween-20) buffer for 2 hr at room temperature and then washed in PBST buffer. Membrane was incubated overnignt at 4°C with a 1 : 1,000 dilution of mouse ODC monoclonal antibody (Sigma-Aldrich). Blots were rinsed with PBST, incubated overnight with a 1 : 5,000 dilution of anti-mouse horseradish peroxidase-conjugated secondary antibody at 4°C and then washed again three times in PBST buffer for 5 min. Transferred proteins were visualized with an ECL detection kit (Santa Cruz Biotechnology, Santa Cruz, CA), according to the manufacturer's procedures.

RESULTS AND DISCUSSION

To meet the desired goal of widespread implementation of chemoprevention of cancer, progress will need to occur in four major areas: enlargement of basic scientific knowledge of mechanisms of carcinogenesis, clinical studies to validate specific pharmacological agents for chemoprevention of human cancer, development of new and better agents for eventual use for chemoprevention in men and women, and a more broadly based educational effort by both physicians and society to achieve better understanding and compliance with the goals of chemoprevention.[22] Recently, considerable attention has been focused on naturally occurring phytochemicals which exist in medicinal or edible plants as good sources to derive new chemopreventive agents. The phenolic compounds with antioxidative and anti-inflammatory activities has anticipated to exert chemopreventive effects in carcinogenesis, particularly in the promotion stage.

Reactive oxygen species (ROS) from both endogenous and exogenous sources can cause inflammation, oxidative DNA damage and dysregulated cell signaling, which are involved in the multistage process of carcinogenesis such as tumor initiation, promotion and progression. TPA-induced tumor promotion is best correlated with epidermal ODC activity and inflammation reactions such as hyperplasia, neutrophil infiltration and H_2O_2 formation in

mouse skin.[23] Among inflammatory cells, neutrophils are particularly adept at generating and releasing ROS such as superoxide anion, hydrogen peroxide, hypochlorous acid, singlet oxygen and hydroxyl radical. Excessive ROS released from neutrophils is closely associated with the metabolic activation of proximate carcinogens,[24] increase the levels of oxidized DNA bases[25] and enhance ODC activity.[26] Thus, the regulation of ROS from activated leukocytes is proposed to be one of the most promising strategies for cancer control. Although antioxidants inhibit tumor initiation by procarcinogens, they can more effectively suppress tumor promotion associated with inflammation by inhibiting leukocyte-derived ROS generation.[27]

As shown in Fig. 2, xanthorrhizol reduced the generation of superoxide anion by TPA stimulation in differentiated HL-60 cells in a dose dependent manner. Xanthorrhizol significantly blocked *His*$^+$ reversion in *S. typhimurium* TA102 induced by 50μg of hydrogen peroxide (Table 1) and 100μg of *t*-BOOH (Table 2), which is an organic hydroperoxide that generates oxygen radicals under physiological conditions and acts as a tumor promoter in mouse

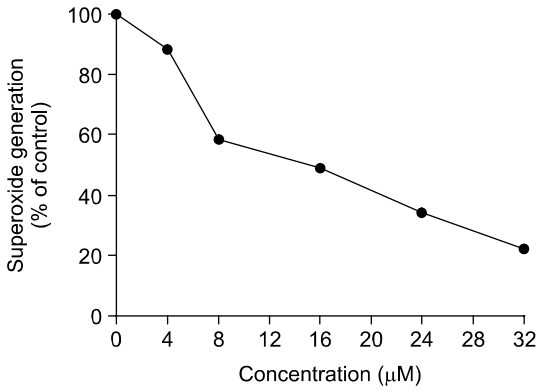

Fig. 2. Inhibitory effects of xanthorrhizol on TPA-stimulated superoxide anion generation in differentiated HL-60 cells. Xanthorrhizol was preincubated at the indicated concentration for 10 min with the cells differentiated with 1.25% DMSO prior to addition of TPA (8μM). Superoxide anion generation was determined 30 min after the TPA treatment.

Table 1. The inhibitory effects of xanthorrhizol on the mutagenesis induced by hydrogen peroxide in *S. typhimurium* TA102

Xanthorrhizol (nmol/plate)	*His*$^+$ revertants/plate[a]	% inhibition
0	219±30	
2	188±23	14
4	174±27[b]	21
8	133±9[c]	39
10	79±16[c]	64

[a]Each data represent the mean±SE from triplicate plates. *Significantly different from the plates treated with hydrogen peroxide without xanthorrhizol ([b]$P<0.05$, [c]$P<0.01$)

Table 2. The inhibitory effects of xanthorrhizol on the mutagenesis induced by *t*-BOOH in *S. typhimurium* TA102

Xanthorrhizol (nmol/plate)	*His*$^+$ revertants/plate	% inhibition
0	594±29	
10	309±78[b]	48
20	234±2[c]	61
40	23±14[c]	96
60	7±7[c]	99

[a]Each data represent the mean±SE from triplicate plates. *Significantly different from the plates treated with *t*-BOOH without xanthorrhizol ([b]$P<0.05$, [c]$P<0.00005$)

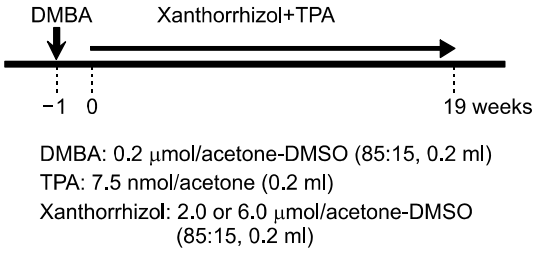

Fig. 3. Experimental protocols for evaluation of the chemoprotective effect against DMBA-induced mouse skin carcinogenesis. Female ICR mice were given 0.2μmol DMBA in 0.2 ml of acetone-DMSO (85 : 15) followed by topical application of 7.5 nmol TPA in 0.2 ml of acetone-DMSO (85 : 15), twice a week for 19 weeks. Some animals received 2 and 6 μmol xanthorrhizol in 0.2 ml of acetone-DMSO (85 : 15) before TPA treatment.

Table 3. The protective effects of xanthorrhizol against DMBA-initiated and TPA-promoted two-stage mouse skin carcinogenesis

Treatment[a]	No. of tumors/mouse	Tumor-bearing mice (%)
DMBA-TPA	15.5±2.3	100
DMBA-XT (2μmol)-TPA	6.9±1.1[b]	80
DMBA-XT (6μmol)-TPA	4.0±1.1[b]	57

[a]2 or 6μmol of xanthorrhizol (XT) was applied topically to female ICR mice 30 min prior to topical application of 7.5 nmol TPA for 19 weeks.
*Significantly different from the values observed in the group given TPA alone ($P < 0.005$)

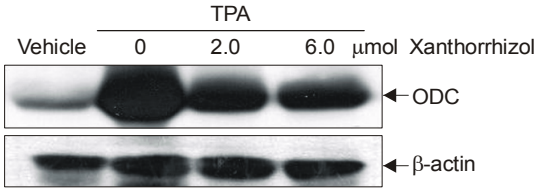

Fig. 4. Effect of xanthorrhizol on ODC expression in mouse skin treated with TPA for 19 weeks after a single topical dose of DMBA. Dorsal skins of female ICR mice were treated with 0.2 ml of acetone-DMSO (85 : 15, lane 1) and 7.5 nmol TPA in 0.2 ml of acetone (lane 2). For some animals, 2μmol (lane 3) and 6μmol (lane 4) of xanthorrhizol was given topically 30 min before TPA (7.5 nmol) for 19 weeks. ODC protein was derermined by immunoblot analysis, as described under Material and Methods.

skin.[28] These antioxidative and antimutagenic activities of xanthorrhizol were more excellent than those of curcumin (data not shown).

All of the mice treated with 7.5 nmol TPA three times weekly for 19 weeks after initiation with a single dose of 0.2μmol DMBA developed the average number of 15.5 skin tumors per animal (Fig. 3 and Table 3). Mice given topical application of 2 and 6 μmol xanthorrhizol during tumor promotion developed an average of 6.9 and 4.0 skin tumors per animal with 80% and 57% tumor incidences, respectively. ODC expression (Fig. 4) was markedly reduced in mice applied topically by xanthorrhizol.

Taken together, xanthorrhizol is a good ROS scavenger and a potent inhibitor on oxidant-induced bacterial mutagenesis. Moreover, xanthorrhizol significantly suppressed tumor promotion and ODC expression in mouse skin stimulated with TPA. These results demonstrate that xanthorrhizol possesses substantial chemopreventive activity.

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