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Inhibition of Matrix Metalloproteinases-12 (MMP-12) and Anti-oxidant Effect of Xanthohumol from Hop (*Humulus lupulus* L.)

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Abstract – Xanthohumol was isolated from hops (*Humulus lupulus* L.), and then investigated anti-oxidant effect by AAPH-induced LLC-PK1 cell and oxygen radical absorbance capacity (ORCA) assays and MMP-12 inhibitory effect by direct MMP-12 inhibition assay. The treatment of xanthohumol protected LLC-PK1 cells from AAPH-induced cell damage such as cell viability, SOD and GSH-px reduction in a dose dependant manner (0.1, 1, and 5 μM), the SOD value was 2.98, 4.51, and 5.77 U/mg protein, and GSH-px value was 30.12, 49.32, and 60.11 U/mg protein. ORAC value of xanthohumol was showed as 4320, 12004, and 14209 μM TE/g at the concentration 0.1, 1, and 5 μM, respectively. The change of SOD and GSH-px values was significantly correlated with the results of ORAC assay, that is, AAPH-induced cell and ORCA assays. In addition, inhibition of MMP-12 that is known to play an important role in skin aging was 14%, 37%, 46%, and 79% at the concentration of 0.01, 0.1, 1, and 5 μM, respectively. On the basis of these results, xanthohumol from hops (*Humulus lupulus* L.) showed interesting biological and pharmacological activity such as anti-oxidant effect and anti-aging.
Keywords – Xanthohumol, Oxygen radical absorbance capacity, AAPH, MMP-12.

Introduction

Xanthohumol, which is classified as a prenylated chacone flavonoid from hops (*Humulus lupulus* L.), is used in the brewing industry to add flavor and bitterness to beer. Xanthohumol and related derivatives have a variety of biological activities. These compounds showed anti-proliferative activity in cancer cell lines derived from human breast cancer, colon cancer, and ovarian cancer in vitro (Miranda *et al*., 1999). The xanthohumol-enriched hop extract containing 8.4% xanthohumol displayed weak to moderate anti-viral activity against bovine viral diarrhea virus (BVDV), herpesviruses HSV-1 and HSV-2. The xanthohumol alone appeared to account for all of the anti-viral activity of the xanthohumol-enriched hop extract for the therapeutic index (TI) of xanthohumol against BVDV, HSV-1, and HSV-2 were similar to those of xanthohumol-enriched extract (Buckwold *et al*., 2004; Vogel and Heilmann, 2008). Xanthohumol is also an inhibitor of diacylglycerol acyltransferase (DGAT) that catalyses the reaction of triacylglycerol formation and involves in age-related metabolism (Tabata *et al*., 1997).

Skin aging can be divided into two basic processes, intrinsic aging, and photoaging (Gilchrest, 1989). The premature skin aging is induced by photoaging in chronically photodamaged skin. During photoaging process, free radicals are formed naturally through normal human metabolism and can cause coarse, deep, severe wrinkling, and pigmentary changes. On the other hand, intrinsic aging shows a general decrease in the extracellular matrix with reduced elastin and the disintegration of elastic fibers (Braverman and Fonferko, 1982; Mera *et al*., 1987; Montagna *et al*., 1989). During the natural skin aging process, the collagen content in the dermis decreases approximately 1% per year throughout adult life. The molecular mechanisms of collagen deficiency associated with natural skin aging are also the result of elevated MMP expression and a concomitant reduction in collagen synthesis (Varani *et al*., 2000).

Therefore, Matrix metalloproteinases (MMPs) are essential to the remodeling of the extracellular matrix (ECM) in human tissue. The ECM gives tissue its structural integrity and predominantly comprises of the fibrillar collagens, basement membrane, and elastin fibers composed of elastin and fibrillin (Philips *et al*., 2010). The upregulation of MMPs accelerates aging and cancer,

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even though it is essential to epidermal differentiation and the prevention of wound scars. These MMPs such as MMP-1, MMP-3, and MMP-9 degrade collagen, by causing chronic and repetitive damage, which finally results in a collagen deficiency, and leads to wrinkling (Seo and Chung, 2006). MMP-12 is a member of the MMP family, which is the most active MMP against elastin (Hautamaki *et al*., 1997).

In the present study, we examined the anti-oxidant activity of xanthohumol by MMP-12 inhibitory assay and the cell damage assay under AAPH-induced cells.

Experimental

Materials and chemicals – Xanthohumol (> 96%, HPLC), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and 2,2'-azobis(2-amidinoproapne) dihydrochloride (AAPH) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's modified medium (D-MEM), F12 medium, and fetal bovine serum were purchased from Invitrogen Co. (Grand Island, NY, USA). All other chemicals were used as analytical grade. LLC-PK1 cell line was supplied from American Type Culture Collection (ATCC CRC-1392). MMP-12 assay kit was employed by Enzo Life Sciences International, Inc (Plymouth Meeting, PA, USA). ORAC Antioxidant Assay Kit was purchased from Zenbio (Research Triangle Park, NC USA)

Extraction and isolation of xanthohumol – Xanthohumol (MW 410, Fig. 1) was extracted and purified from the hop *H. lupulus,* which is granted from Development of Science and Technology for the Korean Agriculture and Beyond. Hop cones (8 kg) were exhaustively extracted with 70% ethanol (five times) at room temperature. Evaporated hop extract (5 L) was extracted with petroleum ether (10 L, five times) and EtOAc (10 L, five times), sequentially. EtOAc extract (380 g) was separated with silica gel column chromatography using a chloroform-methanol step wise gradient (100 : 2 v/v → 95 : 5 v/v → 90 : 10 v/v). The fractions containing xanthohumol with chloroform–methanol (98 : 2 v/v) were purified by repeated column chromatography (chloroform : EtOAc, from 90 : 10 to 80 : 20 v/v) to afford xanthohumol (3.6 g). Identification of hop-extracted xanthohumol was compared with xanthohumol standard, which is purchased from Sigma, using HPLC with DAD detector and LC-MS.

Cell culture – LLC-PK1 (porcine kidney epithelial cells) cell line was maintained at 37 °C in a humidified atmosphere of 5% CO_2 in culture plates with a 5% FBS-supplemented D-MEM/F-12 medium. After approximately

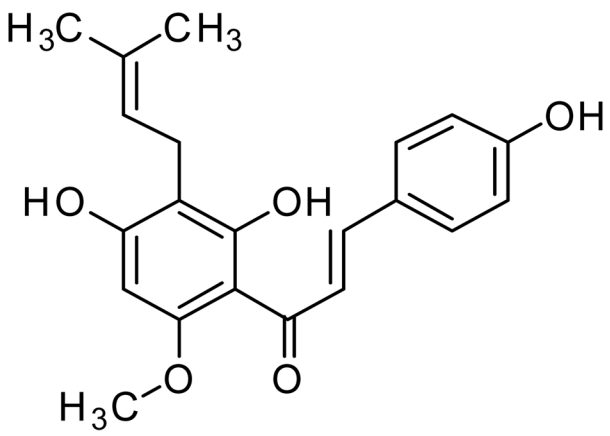

Fig. 1. Structure of xanthohumol.

80% confluence had been reached, the cells were seeded into 24 well plates (4×10^5 cells/well). When the cells is attached (after 3 hours later), 1 mM AAPH was added to each of the wells, pre-incubated for 24 h, and then incubated with xanthohumol between 0.01 μM and 100 μM for 24 h (Piao *et al*., 2004). The proper concentration of AAPH and the incubation time were determined by the preliminary experiment.

Cell viability – The MTT assay of cell viability was experimented following a modified method (Carmichael *et al*., 1987). Cells were plated in 24-well plates at a number of 4×10^5 cells per each well. After the end of culture, 100 μL of MTT solution (5 mg/ml in PBS) was added to each well containing 1 ml medium. After 4 h of incubation, the media were removed and formazan crystals were solubilized with 300 μL DMSO. The absorbance of each well was then read at 540 nm using a microplate reader.

AAPH-induced cell damage assay – AAPH-induced cell damage assay is used by the modification of Jimenez's method (Jimenez *et al*., 2000). In brief, before adding to all of the wells in microplate, cells (5×10^6 cells/dish) were preincubated for 2 h 1 mM of AAPH in 10 mm dishes. AAPH-induced cells were added to the wells, and pre-incubated for 24 h. After the pre-incubation of cells, xanthohumol was treated with or without the indicated concentrations of xanthohumol for 24 h. The medium was discarded and the cells washed twice with PBS. 1 ml of 50 mM potassium phosphate buffer with 1 mM EDTA (pH 7.0) was added, and then cells were scraped. Cell suspensions were sonicated on ice three times for 5 sec each time and then sonicated cell was centrifuged at 10,000 × *g* for 20 min at 4 °C. Cell supernatants were measured at 540 nm for anti-oxidant effect by glutathione peroxidase (GSH-px) activity (Lawrence

and Burk, 1976). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nM of NADPH per minute. SOD activity was determined by monitoring the method of auto-oxidation of pyrogallol (Marklund and Marklund, 1974).

Oxygen radical absorbance capacity (ORAC) assay – Anti-oxidant capacity of xanthohumol was measured using the ORAC method (Prior *et al*., 2003). In brief, Trolox, a water-soluble analogue of vitamin E, was used as a control standard. The experiment was conducted at 37 ºC under pH 7.4 condition with a blank sample in parallel. The analyzer was programmed to record the fluorescence of FL every minute after addition of AAPH. All fluorescent measurements are expressed relative to the initial reading. The final results were calculated using the differences of areas under the FL decay curves between the blank and a sample and were expressed as micromole Trolox equivalents (TE) per gram (μM TE/g).

Matrix metalloproteinase (MMP) assay – MMP-12 assay was performed as follows (Weingarten and Feder, 1985). 20 μL of MMP was added to each well containing 2 μL of test sample and then 68 μl of assay buffer was added. Plates mixed the MMP and the test samples were incubated for 60 min at 37 ºC. After incubation, 20 μL of thiopeptolide substrate was inoculated in each well and reacted for 60 min at 37 ºC. The absorbance of each well at 405 nm was measured using a Microplate reader Model 3550 (Bio-Rad, Richmond, California). Comparing prototypic inhibitor was used by N-isobutyl-N-(4-methoxyphenylsulfonyl)-glycyl-hydroxamic acid (NNGH) as 1.3 μM of final concentration.

Statistical analysis – Data were presented as mean ± SD. All data were analyzed using analysis of variance (ANOVA) followed by tukey-kramer test for comparison. Statistical significance was defined as $p < 0.05$.

Results and Discussion

Cytotoxicity – For the purpose of an optimum range to test antioxidant activity using AAPH-induced cell, xanthohumol was assayed at the concentration of between 0.01 μM and 100 μM against LLC-PK1 cells and AAPH-induced cells. In these results, xanthohumol was not significantly cytotoxic to AAPH-induced LLC-PK1 cells and non-induced cells at the range of between 0.1 and 10 μM (Fig. 2), even though weak cytotoxicity was shown at 100 μM. Therefore, the optimum concentration was available to set down the range of between 0.1 and 5 μM because it could not be affected the growth of this cells at this range.

Anti-oxidant enzyme activity against AAPH-induced cells – Cells are protected from activated oxygen species by endogenous anti-oxidant enzymes such as SOD and GSH-px. When AAPH is pretreated in LLC-PK1 cells, free radicals generated from AAPH cause cell damages. In this test, the effects of xanthohumol on those anti-oxidant enzyme activities are shown in Table 1. Pretreatment with 1 mM AAPH for 24 h significantly decreased SOD activity of LLC-PK1 cells compared with untreated cells. The treatment of xanthohumol increased in SOD values against 1 mM AAPH-treated cells alone. In case of the treatment of xanthohumol, the SOD activity was elevated by the dose-dependent manner that, in detail,

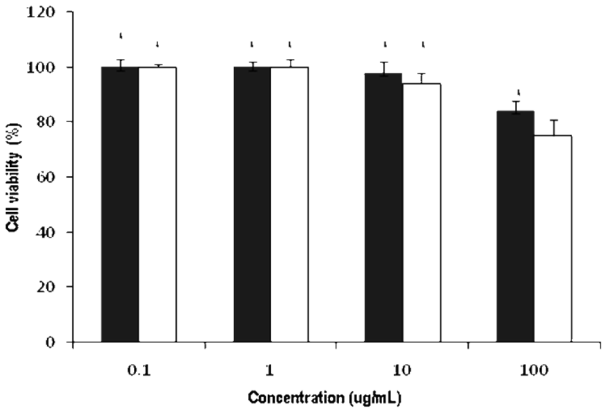

Fig. 2. Cytotoxicity of xanthohumol on LLC-PK1 cell and AAPH-induced cell. Values are expressed as mean ± SD (n = 3) (*: $p < 0.05$).

Table 1. Effect of xanthohumol on superoxide dismutase (SOD) and glutathione peroxidase (GSH-px) activity in AAPH 1 mM (1 mM)-induced LLC-PK1 cell line

Treatment (μg/ml)	SOD (U/mg protein)	GXH-px (U/mg protein)
Normal	7.42 ± 0.13*	83.33 ± 5.71*
1 mM AAPH	2.47 ± 0.92*	29.19 ± 2.53*
0.1	3.02 ± 0.09*	34.27 ± 5.01*
Xanthohumol 1	4.49 ± 0.11*	59.33 ± 4.41*
5	6.01 ± 0.53*	63.47 ± 7.37*

All values are expressed as mean ± SD (n = 5) (*: $p < 0.05$).

Table 2. Inhibitory rate (%) of matrix metallo-proteinase (MMP)-12 by xanthohumol

Concentration (μM)				IC_{50} (μM)
0.01	0.1	1	5	
14	37	46	79	3.4

Inhibition rate of NNGH (1.3 μM) as a control inhibitor was 50% in MMP-12.

each treatment exhibited the SOD activity of 2.98, 4.51, and 5.77 U/mg protein at the concentration of 0.1, 1, and 5 μM. In specific, the treatment of 5 μM of xanthohumol improved SOD activity by around normal level (6.32 U/mg protein). GSH-px activity in LLC-PK1 cells treated with 1 mM AAPH was significantly decreased to 30.11 U/mg protein compared with normal level of 80.31 U/mg protein in untreated cells. Treatment of xanthohumol resulted in the increase of GSH-px activity on AAPH-pretreated cells at the concentration of 1 and 5 μM, as shown by 49.32 and 60.11 U/mg protein. However, GSH-px activity in 0.1 μM of xanthohumol was not significantly increased in AAPH-pretreated LLC-PK cells, as shown by 30.12 U/mg protein. In AAPH-pretreated LLC-PK1 cell system, xanthohumol showed a potential increase on SOD and GSH-Px activities, in specific, at the dose of 1 and 5 μM. Therefore, these results show that xanthohumol significantly contribute to anti-oxidant activity from SOD and GSH-px activation.

Oxygen radical absorbance capacity (ORAC) – The ORAC assay is a well-established test system to identify peroxyl and hydroxyl radical scavengers. According to the antioxidant capacity value determined by the ORAC assay, antioxidant capacity of xanthohumol was showed as 4320, 12004, and 14209 μM TE/g (Fig. 3) at the concentration 0.1, 1, and 5 μM, respectively.

Inhibition of matrix metalloproteinase-12 – MMP-12 inhibitory activity of xanthohumol was measured at the dose of between 0.01 and 5 μM using MMP-12 enzyme ELISA kit. Inhibition of MMP-12 activity was 14%, 37%, 46% and 79% at the concentration of 0.01, 0.1, 1, and 5 μM, respectively. IC_{50} value of xanthohumol was 3.4 μM against the inhibition of MMP-12 (Table 2).

SOD is responsible for the elimination of cytotoxic activated oxygen by catalyzing the dismutation of the superoxide radical to oxygen and hydrogen peroxide (Husain and Somani, 1998). The superoxide anion radical scavenging activity of SOD is effective only when it is followed by the actions of GSH-px, because the activity of SOD generates hydrogen peroxide, which needs to be further scavenged by GSH-px. GSH-px catalyses the reduction of hydrogen peroxide to water, with the simultaneous conversion of reduced glutathione to oxidized

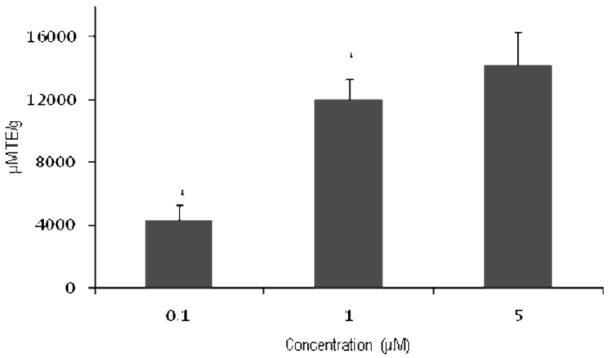

Fig. 3. Anti-oxidant capacity of xanthohumol determined by ORAC (Oxygen Radical Absorbance Capacity) method (n = 3, mean ± SD, * $p < 0.05$).

glutathione (Alptekin *et al*., 1996). Our results also showed a significant decrease of anti-oxidant enzyme activities such as SOD and GSH-px in LLC-PK1 cells treated with AAPH compared with untreated cells. Xanthohumol of 0.1, 1, and 5 μM increased dose-dependently the SOD activity.

MMP-12, human macrophage metalloelastase, is a member of the matrix metalloproteinase family, which is the most active MMP against elastin (Shapiro *et al*., 1993). It has been reported that MMP-12 mRNA and protein are induced by UV or TPA (12-O-tetradecanoyl phorbol-13-acetate) in fibroblasts, but not in normal human epidermal keratinocytes (Chung *et al*., 2002). Therefore, MMP-12 may play an important role in the development of skin aging by solar elastosis and heat. In this study, xanthohumol significantly showed the MMP-12 inhibitory effect at the concentration of between 0.01 and 5 μM. According to our results, it was shown that the action of MMP-12 was blocked by xanthohumol in this ELISA assay. In recent articles, it was reported that xanthohumol showed direct inhibition against elastase and MMP-1, MMP-2, and MMP-9 enzymes (Philips *et al*., 2010).

In conclusion, anti-oxidant activity and MMP-12 inhibition of xanthohumol have been assayed by AAPH-induced cell, ORAC, and MMP-12 assays. On the basis of our knowledge of antioxidant chemistry, it is concluded that the SOD and GSH-px value, which was through

AAPH-induced cell assay, and ORAC value reflect the peroxyl radical scavenging activity of xanthohumol. In addition, MMP-12, which is known as an enzyme that plays an important role in skin aging, showed significant effect on the inhibition of MMP-12 action at all tested doses. According to our results, xanthohumol is useful to block skin aging occurring by radicals and MMP-12 production and it needs to be developed to more effectively prevent skin aging.

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