Supplementary material

Effects of lobetyolin on xanthine oxidase activity *in vitro* and *in vivo*: weak and mixed inhibition

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ABSTRACT

Lobetyolin (LBT), a general marker compound mainly found in *Codonopsis* plants including *C. pilosula*, *C. tubulosa*, and *C. lanceolata*, exhibits antitumor, antiviral, anti-inflammatory, mucosal protective, and antioxidant activities. Xanthine oxidase (XO) catalyzes the formation of uric acid from xanthine, a critical metabolic pathway related to hyperuricemia and gout. The aim of this study was to investigate the effect of LBT on XO activity and its mechanism using *in vitro* enzyme assay system and *in vivo* potassium oxonate-induced hyperuricemic mice. LBT was found to weakly inhibit XO activity via a mixed type mechanism. Consistently, the impact of 1-week oral LBT treatment on serum XO activity *in vivo* is limited in hyperuricemic mice. However, oral LBT at 50 mg/kg significantly reduced hepatic XO activity *in vivo*. To the best of our knowledge, this is the first study to report effects of LBT on XO activity and its inhibition mechanism.

Keywords: Lobetyolin, Xanthine oxidase, Hyperuricemic mouse, Mixed inhibition

Experimental

Materials. LBT (Lot No. CFS201602) was purchased from ChemFaces Biochemical Co. (Hubei, China). Allopurinol, XO, sodium carboxymethylcellulose (CMC-Na), and potassium oxonate were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Other chemicals and solvents were of reagent grade.

Determination of XO activity *in vitro*. XO inhibitory activity was assessed by measuring uric acid formation in an enzymatic reaction system *in vitro* as described previously (Yoon et al. 2017b). The assay system consisted of 0.6 mL phosphate buffer (100 mM; pH 7.4), 0.1 mL sample, 0.1 mL XO (0.2 U/mL), and 0.2 mL xanthine (1 mM; dissolved in 0.1 N NaOH). The reaction was initiated by adding the enzyme and stopped by adding a 0.2 mL aliquot of 1 N HCl. Changes in absorbance of the mixture at wavelength of 290 nm were monitored for 15 min compared to the absorbance of blank reagent. Allopurinol was used as a positive control.

Animals. Male ICR mice (4 weeks old) were purchased from Orient Bio Co. (Sungnam, South Korea).

These mice were housed in a clean room at a temperature of 20–25˚C with 12-h light (07:00–19:00) and dark (19:00–07:00) cycles and a relative humidity of 50 ± 5%. They were placed into ventilated mice cages (Tecniplast, West Chester, PA, USA) under filtered and pathogen-free air with food (Agribrands Purina Korea, Inc., Seoul, South Korea) and water available *ad libitum*. All animal experiments were conducted in line with the guidelines of the Animal Investigation Committee of Jeonnam Bioindustry Foundation (Naju, South Korea) (approval no. JINR1517).

Pretreatment and hyperuricemia induction in mice. Potassium oxonate-induced hyperuricemic mice were prepared as described previously (Yoon et al. 2017a; Yoon et al. 2016; Yoon et al. 2017b). Briefly, LBT was dissolved in 0.3% CMC-Na aqueous solution. Five groups of mice (n = 5 for each group) were pretreated once daily for 7 days as follows. Mice in two negative control groups (NOR and HU groups) received 0.3% CMC-Na aqueous solution. Mice in a positive control group (HU+ALP group) received ALP solution at a dose of 10 mg/kg. Mice in HU+LBT5 and HU+LBT50 groups received the extract solution at doses of 5 and 50 mg/kg, respectively. Potassium oxonate (dissolved in phosphate-buffered saline (PBS); 250 mg/kg) was administered intraperitoneally to all mice except for those in the NOR group at 1 h before the last pretreatment on the 7th day. Mice in the NOR group received PBS instead of potassium oxonate. At 1 h after the last pretreatment on the 7th day, approximately 0.5 mL blood was collected via the tail vein, allowed to clot for 1 h at 4°C, and centrifuged at 10000×g for 15 min to obtain serum. Serum samples were stored at –80°C until further analysis.

Determination of XO activity *in vivo*. Residual activity of XO in mouse liver and plasma were determined by measuring the formation of uric acid from xanthine as described previously (Lemos Lima Rde et al. 2015). Briefly, mouse livers (0.5 g) were homogenized in a 1 mL aliquot of 50 mM sodium phosphate buffer (pH 7.4). Homogenates were centrifuged at 3000×g for 10 min at 4°C. After removing the lipid layer, the residual supernatant was centrifuged at 10000×g for 60 min at 4°C. The resultant supernatant was then used for determining XO residual activity and total protein concentration. A 0.12 mL aliquot of xanthine solution (250 mM) was added to a test tube containing 10 μL liver homogenate and 0.54 mL potassium oxonate solution (1 mM) in 50-mM sodium phosphate buffer (pH 7.4) previously incubated at 35°C for 15 min. The reaction was stopped after 0 and 30 min by adding a 0.1 mL aliquot of 1 N HCl. Thereafter, the test tube was centrifuged at 3000×g for 5 min and absorbance of the supernatant was measured at wavelength of 295 nm using a UV/VIS spectrophotometer (Biochrom Libra S12; Biochrom Ltd., Cambridge, UK). Total protein concentration was determined by the Bradford method (Bradford 1976). XO activity was expressed as micromoles of uric acid formed per minute (U) per milligram protein.

Data analysis. IC_{50} of LBT for inhibiting XO activity was determined by nonlinear regression analysis using GraphPad Prism (version 5.01; GraphPad Software, San Diego, CA, USA) according to a four-parameter logistic equation as shown below:

$$Y = \mathrm{Min} + \frac{\mathrm{Max}-\mathrm{Min}}{1+(X/IC_{50})^{-P}} \qquad \text{(Equation S1)}$$

where X and Y were inhibitor concentration and response, respectively. Max and Min were initial and final Y values, respectively. Power P was Hill coefficient. Control samples (without inhibitor) were assayed in each

analytical run (CV = 5.07%). The inhibition type of LBT on XO activity was determined graphically from a double reciprocal plot. K_i of LBT for the inhibition of XO activity was determined by nonlinear regression analysis using GraphPad Prism according to a mixed-model enzyme inhibition equation:

$$Y = \frac{V_{max} \cdot X}{K_m \cdot \left(1 + \frac{I}{K_i}\right) + X \cdot \left(1 + \frac{I}{\alpha \cdot K_i}\right)} \quad \text{(Equation S2)}$$

where X, Y, and I were substrate concentration, enzyme activity, and inhibitor concentration, respectively. V_{max}, K_m, and K_i were the maximum enzyme activity (metabolic velocity) without inhibitor, Michaelis–Menten constant, and inhibition constant, respectively. Parameter α is also indicative of inhibition type. The mixed model is a general equation that includes competitive, uncompetitive, and noncompetitive inhibition as special cases. When α equals 1, the mixed model is identical to noncompetitive inhibition. When α is very large ($\alpha \to \infty$) or very small ($\alpha \to 0$), the mixed model becomes identical to competitive inhibition or uncompetitive inhibition, respectively. In other cases ($\alpha \neq 1$), the mixed model describes mixed inhibition.

Statistical analysis. A *p*-value less than 0.05 was considered to be statistically significant using a *t*-test between unpaired two means or using analysis of variance (ANOVA) with a post-hoc Tukey's Honestly Significant Difference (HSD) test among unpaired three or more means (Statistical Package for the Social Sciences software; version 12.0, IBM Co., Armonk, NY, USA). All data were expressed as mean ± standard deviation and rounded to two decimal places.

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Table S1. Inhibition constants and inhibition type of LBT on XO activity.

Parameter	Value
IC_{50} (μM)	2985
α	1.87
K_i (μM)	4512
Type	Mixed

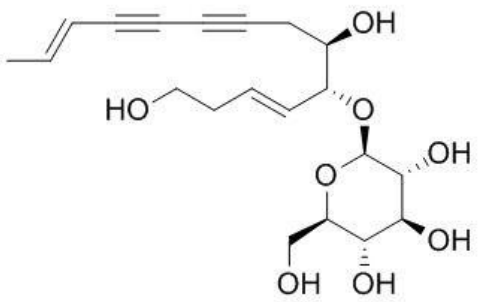

Figure S1. Chemical structure of LBT.

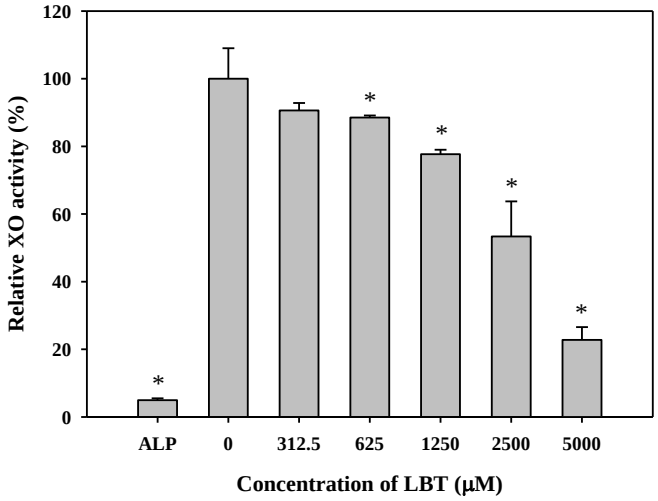

Figure S2. XO inhibitory activity of allopurinol (ALP) at a concentration of 125 μM and LBT at various concentrations ranging from 0 (serving as control) to 5000 μM *in vitro*. The rectangular bars and their error bars represent the means and standard deviations, respectively (n = 5). The asterisk represents a value significantly different from that of the control group ($p < 0.05$, ANOVA *a posteriori* Tukey's HSD test).

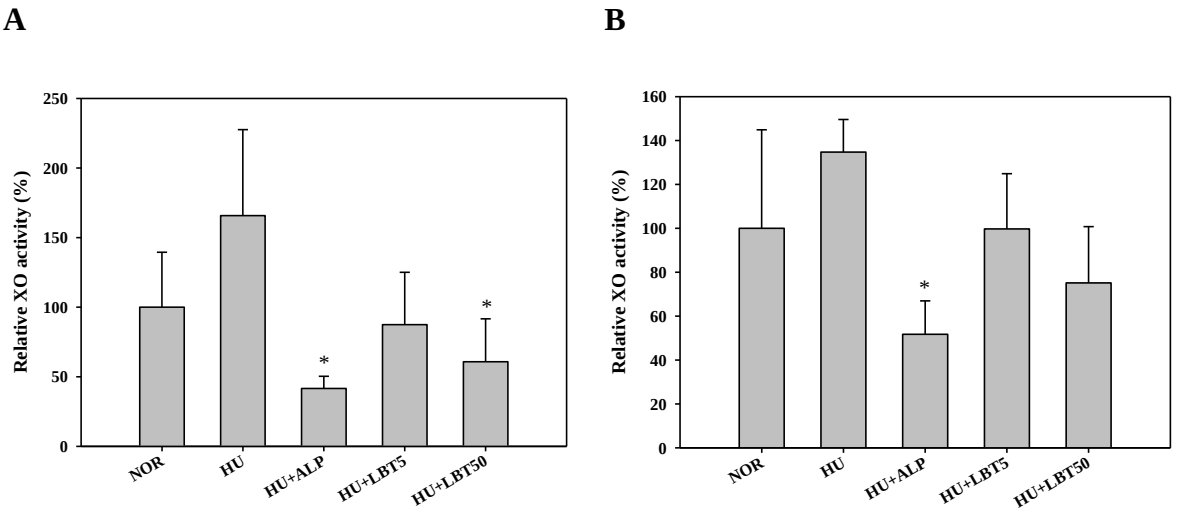

Figure S3. Hepatic (A) and serum (B) XO activity after the oral administration of saline in normal mice (NOR) or after the oral administration of saline (HU), allopurinol at a dose of 10 mg/kg (HU + ALP), or LBT at a dose of 5 (HU + LBT5) or 50 mg/kg (HU + LBT50) during 7 days prior to inducing hyperuricemia in mice. The rectangular bars and their error bars represent the means and standard deviations, respectively (n = 5). The asterisk represents a value significantly different from that of the HU group (p < 0.05, ANOVA *a posteriori* Tukey's HSD test).

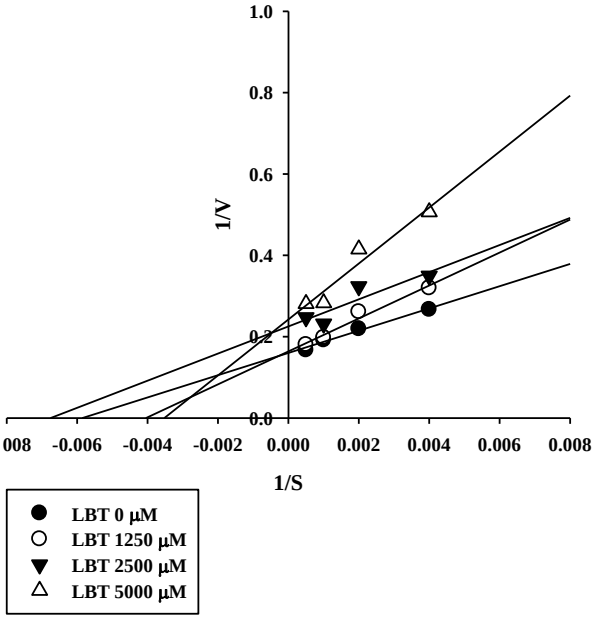

Figure S4. Double reciprocal plot for the inhibitory effects of LBT on XO-mediated metabolic activity in vitro. The S represents the concentration of xanthine, and the V represents the velocity for the formation of uric acid. The bullet symbols represent the concentration of lobetyolin; 0 (●), 1250 (○), 2500 (▼), and 5000 (Δ) μM.