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Superoxide radicals scavenging and xanthine oxidase inhibitory activity of magnesium lithospermate B from *Salvia miltiorrhiza*

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Abstract

In this study we investigated the superoxide radicals scavenging effect and xanthine oxidase inhibitory activity by magnesium lithospermate B, which was originally isolated from the roots of *Salvia miltiorrhiza* (also named Danshen or Dansham), an important herb in Oriental medicine. Superoxide radicals were generated both in β-NADH/PMS system and xanthine/ xanthine oxidase system. Magnesium lithospermate B significantly inhibited the reduction of NBT induced by superoxide radicals with an IC_{50} of 29.8 μg/mL and 4.06 μg/mL respectively in the two systems. Further study suggested that magnesium lithospermate B can directly inhibit xanthine oxidase and exhibits competitive inhibition. Magnesium lithospermate B was also found to have the hypouricemic activity *in vivo* against potassium oxonate-induced hyperuricaemia in mice. After oral administration of magnesium lithospermate B at doses of 10, 20 and 30 mg/kg, there was a significant decrease in the serum urate level when compared to the hyperuricemia control. In addition, magnesium lithospermate B significantly protected HL-60 cells from superoxide radicals-induced apoptosis in the xanthine/ xanthine oxidase reactions. This study provided evidence that magnesium lithospermate B exhibits direct superoxide radicals scavenging and xanthine oxidase inhibitory activity.

Keywords: *Magnesium lithospermate B, superoxide radicals scavenging, xanthine oxidase, inhibition, hypouricemic effect, apoptosis prevention*

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Introduction

Xanthine oxidase catalyzes the oxidation of xanthine and hypoxanthine to uric acid. During this reaction, superoxide radicals are produced according to the following equation [1,2]:

$$\text{Hypoxanthine} \xrightarrow{\text{xanthine oxidase}} \text{xanthine}$$

$$+ O_2 \xrightarrow{\text{xanthine oxidase}} \text{Uric acid} + O_2^- \qquad (1)$$

Superoxide radicals together with hydroxyl radicals and hydrogen peroxide are called reactive oxygen species (ROS). They can damage critical biomacromolecules and alter biological processes, including signal transduction and gene expression, causing mitogenesis, mutagenesis and cell death. There is evidence that several biological disorders, including neurodegenerative diseases, immune dysfunction, cancer, and inflammatory conditions, are related to the increase of ROS *in vivo* [3].

The over-activity of xanthine oxidase can induce the accumulation of uric acid and lead to hyperuricemia. The deposition of urate monohydrate crystals in joint and kidney results in gouty arthritis and uric acid nephrotithiasis [4]. The increased risk of hyperuricemia has been also associated with the development of hypertension, hyperlipidemia, cancer, diabetes and obesity [5].

The above suggests that a compound which can scavenge superoxide radicals and inhibit xanthine oxidase may have a beneficial effect in the treatment of

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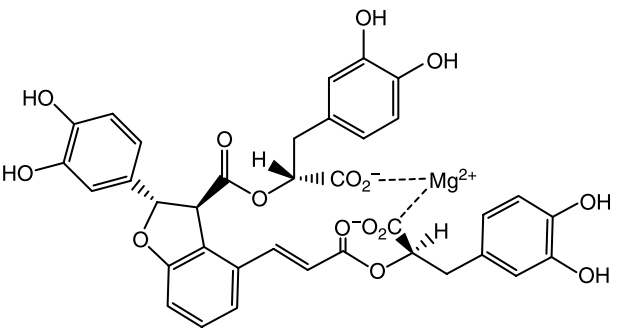

Figure 1. The structure of Magnesium lithopermate B.

hyperuricemia, but also in the alleviation of inflammation induced by gout and other diseases.

This study reports the inhibitory effect on xanthine oxidase, as well as the superoxide radicals scavenging activity and hypouricemic effects of magnesium lithospermate B (MLB) (Figure 1), a component of the root extract from Salvia miltiorrhiza.

Materials and methods

Materials

β-Nicotinamide adenine dinucleotide, reduced dipotassium salt (NADH), phenazine methosulfate (PMS), nitroblue tetrazolium chloride (NBT), xanthine, superoxide dismutase (SOD), potassium oxonate, and allopurinol were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Annexin V-FITC Apoptosis Kit was purchased from Jingmei Bio Tech Co. Ltd (Shanghai, China). Xanthine oxidase (XO) was purchased from Roche Co. Ltd. (Shanghai, China) and other analytical reagents were produced in China. Magnesium lithospermate B was provided by professor Zhu (State Key Laboratory of Drug Research, Institute of Materia Medica, Shanghai Institute for Biological Science, China).

Animals

Male ICR mice (26 ~ 30 g) were purchased from Laboratory Animal Center (Shanghai, China) and maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room for 1 week prior to the experiment. All procedures were carried out in pursuance with Chinese legislation on the use and care of laboratory animals and were approved by the respective committees for animal experiments.

Evaluation of superoxide radicals scavenging activity by nonenzymatic method

Superoxide radicals which were generated by the NADH/PMS system were determined by a described procedure [6–8]. The reaction mixtures in the simple well consisted of β-NADH 156 μM, NBT 25 μM, PMS 10 μM and different concentrations of MLB (10, 20, 30, 40 and 50 μg/mL) and dissolved in distilled water. The reaction was conducted at room temperature for 2 min initiated by the addition of PMS. The superoxide radicals were estimated by the spectrophotometric measurement at 560 nm in the reaction product of NBT. SOD was taken as the positive control.

Evaluation of superoxide radicals scavenging activity by enzymatic system

Superoxide radicals were generated by the xanthine/xanthine oxidase system following a described procedure [9,10]. The superoxide radicals were estimated by the spectrophotometric measurement at 560 nm of the reaction product of NBT. The reaction mixture contained 80 mM sodium pyrophosphate buffer (pH 7.5), 120 mM xanthine, 0.1 U/mL xanthine oxidase, 100 μM NBT and MLB (2.5, 5, 10, 15 20 μg/mL). The reaction was started by the addition of xanthine oxidase. SOD was the positive control.

In vitro *xanthine oxidase inhibitory activity*

The *in vitro* inhibitory activity was assayed spectrophotometrically at 295 nm under aerobic condition as described elsewhere [11,12]. The reaction mixture contained 80 mM sodium pyrophosphate buffer (pH 7.5), 0.1 U/mL xanthine oxidase and several concentrations of xanthine (60, 120, 180, 240 and 300 μM). The reaction was started by the addition of xanthine oxidase and the formation of uric acid was detected by the absorption increments at 295 nm. Allopurinol was the positive control. In order to evaluate the type of inhibition using the Lineweaver-Burk plot, MLB were assayed for xanthine oxidase inhibitory activity at concentration of 0, 2, 4, 6 μg/mL respectively.

Hypouricemic activity in vivo

Mice were divided into 6 groups each consisting of 10 animals. Group I served as the control, which received normal saline. Group II served as hyperuricemic control, which received potassium oxonate (300 mg/kg body weight, i.p.) as described elsewhere [13,14]. Group III-V received MLB orally (10 mg/kg, 20 mg/kg, 30 mg/kg body weight, respectively). Group VI received the reference drug allopurinol orally (10 mg/kg body weight). Animals of groups II ~ VI were injected potassium oxonate (300 mg/kg, i.p.) 1 h before the MLB and allopurinol administration to increase the serum urate level. Blood was obtained from the mice via tail tip cuts 1 h after the

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MLB and allopurinol administration. Uric acid levels were determined by the phosphotungstic acid method.

Protection of HL-60 cells from superoxide radicals' injury

The method was similar to the one described by Lin et al. [15]. Briefly, human promyclocytic leukemic HL-60 cells were maintained in RPMI-1640 medium supplemented with 10% PBS in a humidified atmosphere of 5% CO_2 in air at 37°C 2×10^5/mL HL-60 cells were incubated with serum-free medium and pretreated with or without MLB, then treated with 120 μM xanthine and 0.1 U/mL xanthine oxidase for 4 h. Detection of apoptosis by flow cytometry was performed using the Annexin V-FITC/propiolium iodide (PI) Apoptosis detection kit. The staining was performed according to the producer's manual.

Statistical analysis

All data were expressed as mean ± standard error of the mean (S.E.M) for each group. They were analyzed for the significance of intergroup differences utilizing two-tailed, paired Students'-tests and $p < 0.05$ was considered as significant.

Results

Scavenging effect of MLB on superoxide radicals generated by β-NADH/PMS system

We first measured the scavenging activity of MLB on superoxide radicals generated by β-NADH/PMS system. In this study, MLB showed a stronger scavenging effect on superoxide radicals in a dose-dependent manner with an IC_{50} of 29.8 μg/mL (41.2 μmol/L) (Figure 2A).

Scavenging effect of MLB on superoxide radicals generated by xanthine/xanthine oxidase system

MLB was also studied for its ability to scavenge superoxide radicals generated by the xanthine/xanthine oxidase system. The amount of generated superoxide radicals was determined by measuring the reduction of NBT. Under our experimental conditions, MLB significantly inhibited the reduction of NBT in a dose-dependent manner. IC_{50} was 4.06 μg/mL (5.62 μmol/L) (Figure 2B). The reduction of NBT was almost completely inhibited by SOD both in β-NADH/PMS system and xanthine/xanthine oxidase system.

Inhibition of the activity of xanthine oxidase by MLB

We found MLB could inhibit the reduction of NBT both in β-NADH/PMS system and xanthine/xanthine oxidase system. The effect of MLB on the xanthine oxidase activity must be examined, because an inhibitory effect on the enzyme itself would also lead to a decrease of NBT reduction. So we have to determine the inhibitory effect on the xanthine oxidase through the measurement of the formation of uric acid.

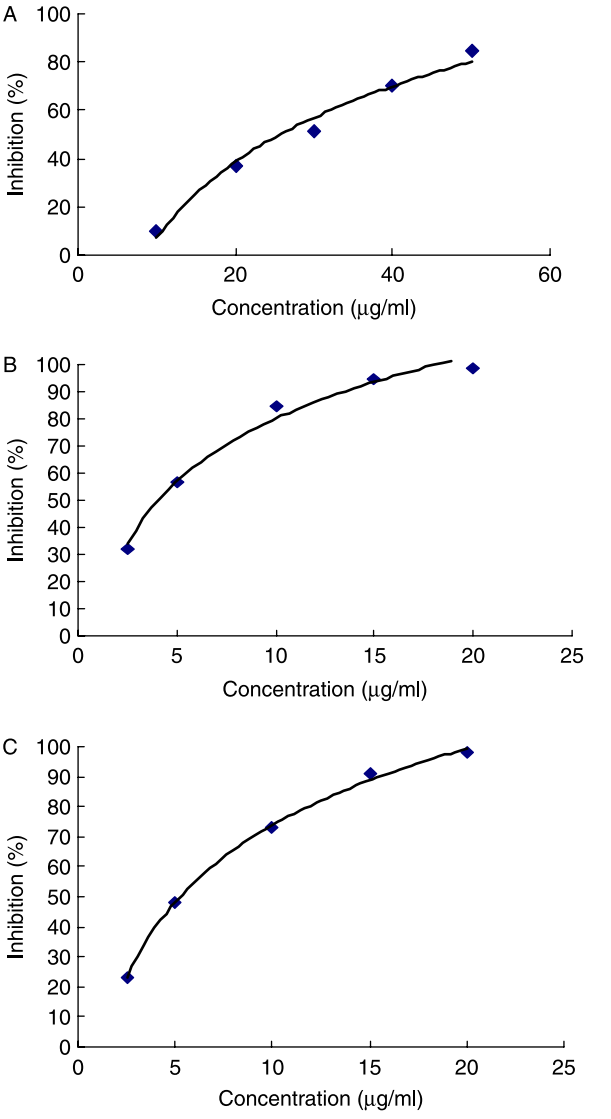

Figure 2. Effect of MLB on (A) NBT reduction induced by superoxide radicals generated in an NADH/PMS system (B) NBT reduction induced by superoxide radicals generated in xanthine/xanthine oxidase system and (C) xanthine oxidase activity. Values show mean ± S.E.M from four experiments performed in triplicate.

MLB inhibited the formation of uric acid under the assay condition with an IC_{50} of 5.2 μg/mL (7.20 μmol/L) (Figure 2C).

To further characterize the binding region on xanthine oxidase, Lineweaver-Burch double reciprocal plots were shown in Figure 3. MLB was exhibited to be a competitive inhibitor of xanthine oxidase. The Ki was 0.9484 $(\mu g/mL)^{-1}$.

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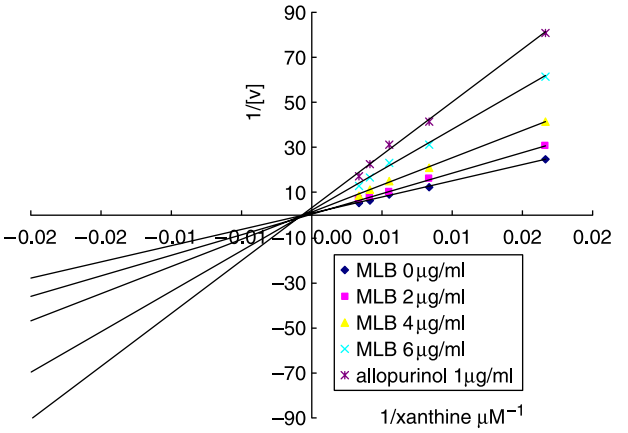

Figure 3. Lineweaver-Burk plots of the inhibition of xanthine oxidase by MLB. Enzyme activity was measured using 60, 120, 180, 240 and 300 μM of xanthine as a substrate. The data represented the mean ± SEM from four experiments performed in triplicate.

In vivo *hypouricemic activity of MLB*

Xanthine oxidase is a major target to decrease the uric acid level *in vivo*. So we attempted to detect the *in vivo* hypouricemic effect of MLB on potassium oxonate-pretreated mice. The uric acid levels in non-hypouricemic, vehicle-treated mice were 1.505 mg/dl. The intraperitoneal injection of potassium oxonate markedly increased the serum uric acid levels. The animals treated with MLB showed a significant decrease in the serum urate level (Figure 4).

Protection of HL-60 cells from superoxide radicals induced apoptosis by MLB

The prevention of apoptosis of HL-60 cells induced by superoxide radicals was analyzed. The superoxide radicals were generated in the xanthine/xanthine oxidase system. The HL-60 cells were treated with xanthine and xanthine oxidase, and cell apoptosis was

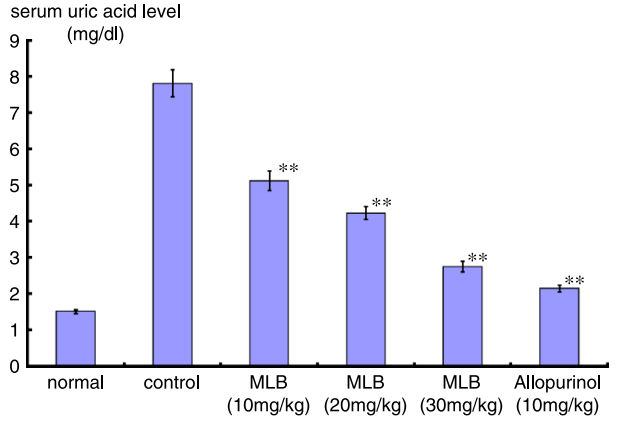

Figure 4. Effects of MLB on serum urate level of oxonate hyperuricemia in mice. Values were mean ± S.E.M (n = 10). ** $P < 0.01$ when compared with the hyperuricemic control. All drugs were given orally except potassium oxonate which was injected *i.p.*

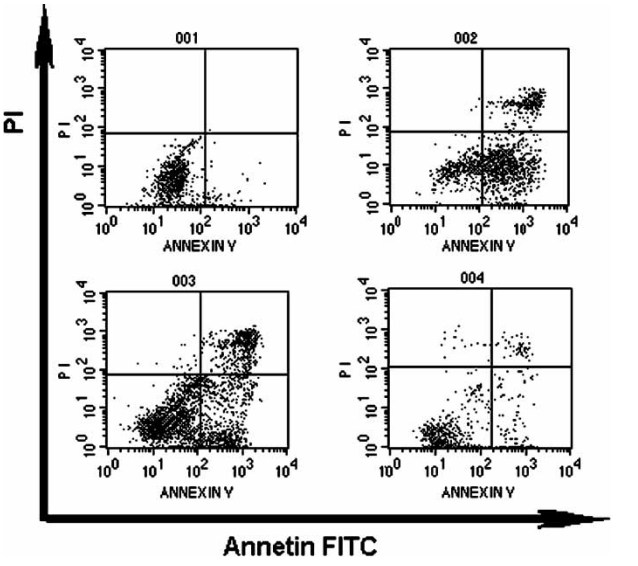

Figure 5. Protective effect of MLB on HL-60 cells apoptosis detected by Annexin V- PI. File001: Normal control cell; File002: Cell apoptosis induced by superoxide radicals without MLB; apoptosis rate is 62.16 ± 5.84%; File003: Cell apoptosis induced by superoxide radicals pretreated with 3 μg/mL MLB; apoptosis rate is 37.42 ± 4.32%; File004: Cell apoptosis induced by superoxide radicals pretreated with 6 μg/mL MLB; apoptosis rate is 15.44 ± 1.87%.

62.16 ± 5.84%. The cell apoptosis was significantly prevented when cells were pretreated with 3.0, 6.0 μg/mL MLB (apoptosis rate is 37.42 ± 4.32% and 15.44%, respectively, $p < 0.01$ when compared with the no-MLB group) (Figure 5). These results suggested that MLB was not only a potent inhibitor of xanthine oxidase but also has a direct superoxide radicals scavenging effect and was able to prevent cell apoptosis induced by xanthine/xanthine oxidase reaction.

Discussion

MLB is a caffecic acid tetramer and was a major component of *S. miltiorrhiza* ("Danshem" in Korean and "Danshen" in Chinese), an important herb in Oriental medicine, which has been used to treat cardiovascular diseases. Previous studies demonstrated MLB has many therapeutic effects including ameliorating renal cortical microperfusion in rats [16], inhibitory activity on Na^+,K^+-ATPase [17], inhibition of aldose reductase [18] and a hydroxyl radial-scavenging action [19]. However, no report has been issued on the effects of MLB on xanthine oxidase activity. In the present study, we found MLB could prevent the reduction of NBT both in the NAPH/PMS and the xanthine/xanthine oxidase system. In the NAPH/PMS system, IC_{50} is 29.8 μg/mL. This result suggests that MLB has a direct scavenging effect on superoxide radicals. In the xanthine/xanthine oxidase system, IC_{50} is 4.06 μg/mL. The IC_{50} in xanthine/xanthine oxidase system is even

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lower than in the NAPH/PMS system. This result suggests that the possible mechanism of inhibition effect was through inhibiting the xanthine oxidase but not directly superoxide radicals- scavenging action. So we observed the inhibitory effect on xanthine oxidase by MLB through measurement of the formation of uric acid. The results suggested that MLB inhibited the xanthine oxidase in a dose-dependent manner; the enzyme dynamic results showed that it is a competitive inhibitor of xanthine oxidase.

In vivo, xanthine and hypoxanthine are oxidized into uric acid by the activity of xanthine oxidase. The overproduction of uric acid can cause gout [20,21]. Therefore the inhibitor on xanthine oxidase can decrease the uric acid level. Allopurinol is the most common, and perhaps the only inhibitor of xanthine oxidase being used in clinical practice, but allopurinol has severe adverse effects on some patients, including nephatitis, nephropathy and allergic reactions [22,23]. Thus, the development of new hypouricemic agents of greater effectiveness and safety is highly warranted. MLB not only inhibits the xanthine oxidase *in vitro*, but also has a hypouricemic effect on potassium oxonate-pretreated mice. So we concluded that the hypouricemic effect of MLB is mainly through inhibiting the xanthine oxidase.

The occurrence ratio of gout increases as the living standard increases. Nowadays the treatments of gout in clinic are mainly by decreasing the serum uric acid level and anti-inflammation. But none of the clinical drugs has the two effects at the same time. Through our study, we found MLB could not only decrease uric level, but also directly eliminate superoxide radicals. Since the superoxide radicals are important factors in the inflammation, the therapeutic effect of MLB in the gouty inflammation is worthy of further studies.

In conclusion this study provides evidences that MLB exhibits superoxide radicals scavenging activity, xanthine oxidase inhibitory effect *in vitro* and hypouricemic effect *in vivo*.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Note

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References

[1] McKee Trudy, McKee James R. Biochemistry: An introduction. 2nd ed. New York: McGraw-Hill Companies; 1999.

[2] Xia M, Dempski Robert, Hille Russ. The Reductive Half-reaction of Xanthine Oxidase.reaction with aldehyde substrates and identification of the catalytically labile oxygen. J Biol Chem 1999;274(6):3323–3330.

[3] Wishman H, Halliwell B. DNA by reactive oxygen and nitrogen species: Role in inflammatory disease and progression to cancer. Biochem J 1996;313(Pt 1):17–29.

[4] Kramer Holly Mattix, Curhan Gary. The association between gout and nephrolithiasis: The national health and nutrition examination survey III, 1988-1994. Am J Kidney Dis 2002;40(1):37–42.

[5] Lin KC, Lin HY, Chou P. The interaction between uric acid level and other risk factors on the development of gout among asymptomatic hyperuricemic men in a prospective study. J Rheumatol 2000;27(6):1501–1505.

[6] Zhang HY, Lu CS. A study of the SOD-like activity of some copper(II)-small peptide and amino acid complexes. Acta biochimica et biophysica Sinica 1990;22:593–594.

[7] Schuldt EZ, Farias MR, Ribeiro-do RM, Ckless K. Comparative study of radial scavenges activities of crude extract and fractions from Cuphea carthagenensis leaves. Phytomedicine 2004;11:523–529.

[8] Valentao P, Fernandes E, Carvalho F, Andrade PB, Seabra RM, Bastos ML. Antioxidant activity of Centourium erythraea infusion evidenced by its superoxide radial scavenging and xanthine oxidase inhibitory activity. J Agri Food Chem 2001;49(7):3476–3479.

[9] Behera BC, Adawadkar B, Makhija U. Inhibitory activity of xanthine oxidase and superoxide-scavenging activity in some taxa of the lichen family Graphidaceae. Phytomedicine 2003;10:536–543.

[10] Cimanga K, De Bruyne T, Hu JP, Cos P, Apers S, Pieters L, Tona L, Kambu K, Vanden Berghe D, Vlietinck AJ. Constituents from *Morinda morindoides leaves* as inhibitors of xanthine oxidase and scavengers superoxide anions. Pharm Pharmacol Commun 1999;5(6):419–424.

[11] Komoriya K, Osada Y, Hasegawa M, Horiuchi H, Kondo S, Couch RC, Griffin TB. Hypouricemic effect of allopurinol and the novel xanthine oxidase inhibitor TEI-6720 in chimpanzees. Eur J Pharmol 1993;250(3):455–460.

[12] Fujimoto Yohko, Sakuma Satoru, Tagami Takaomi, Ichikawa Reiko, Fujita Tadashi. N-ethyimaleimide inhibits xanthine oxidase activity with no detectable change in xanthine dehydrogenase activity in rabbit liver. Life Sci 2000;68: 517–524.

[13] Osadu Yoshio, Tsuchimoto Masahiro, Fukushima Hisashi, Takahashi K, Kondo S, Hasegawa M, Komoriya K. Hypouricemic effect of the novel xanthine oxidase inhibitor, TEI-6720, in rodents. Eur J Pharmacol 1993;241(2–3): 183–188.

[14] Wang Y, Zhu JX, Kong LD, Yang C, Cheng CH, Zhang X. Administration of procyanidins from grapes seeds reduces serum uric acid levels and decreases hepatic xanthine dehydrogenase/oxidase in oxonate-treated mice. Basic Clin Pharmacol Toxicol 2004;94(5):232–237.

[15] Lin CM, Chen CS, Chen CT, Liang YC, Lin JK. Molecular modeling of flavonoids that inhibits xanthine oxidase. Biochem Biophys Res Commun 2002;294(1):167–172.

[16] Chen CG, Wang YP. Magnesiun lithopermate B ameliorates renal cortical microperfusion in rats. Acta Pharmacologica Sinica 2006;27(2):217–222.

[17] Tzen JT, Jinn TR, Chen YC, Li FY, Cheng FC, Shi LS, She HKh, Chen BC, Hsieh V, Tu ML. Magnesium lithospermate B possesses inhibitory activity on Na + , K + -ATPase and neuroprotective effects against ischemic stroke. Acta Pharmacologica 2007;28(5):609–615.

[18] Jung M, Lee HC, Ahn CW, Park W, Choi S, Kim H, Cho D, Lee GT, Li HR. Effective isolation of magnesium lithopermate B and its inhibition of aldose reductase and fibronectin on mesangial cell line. Chem Pharm Bull 2002;50(8):1135–1136.

[19] Yokozawa T, Chung HY, Dong E, Oura H. Confirmation that magnesium lithopermate B has a hydroxyl radial-scavenging action. Exp Toxicol Pathol 1995;47(5):341–344.

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[20] Umamaheswari M, AsokKumar K, Somasundaram A, Sivashanmugam T, Subhadradevi V, Ravi TK. Xanthine oxidase inhibitory activity of some Indian medical plants. J Ethnopharmacol 2007;109:547–551.
[21] Chen GL, Wei W, Xu SY. Effect and mechanism of total saponin of dioscorea on animal experimental hyperuricemia. Am J Chinese Med 2006;34(1):77–85.
[22] Kong LD, Cai Y, Huang WW, Christopher HK, Cheng RX Tan. Inhibition of xanthine oxidase by some Chinese medicinal plants used to treat gout. J Ethnopharmacol 2000;73:199–207.
[23] Burke A, Smyth E, FitzGerald GA. Analgesic-antipyretic agents; pharmacotherapy of gout. The pharmacological basis of therapeutics. 11th ed. New York: McGraw-Hill Medical Publishing Division; 2001.

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