Fitoterapia 81 (2010) 214–218

Contents lists available at ScienceDirect

Fitoterapia

journal homepage: www.elsevier.com/locate/fitote

Anti-inflammatory and analgesic properties of *cis*-mulberroside A from *Ramulus mori*

Zuofa Zhang [a,b], Liangen Shi [a,*]

[a] College of Animal Science, Zhejiang University, Hangzhou 310029, China
[b] Institute of Horticulture, Zhejiang Academy of Agricultural Science, Hangzhou 310021, China

ARTICLE INFO

Article history:
Received 31 May 2009
Accepted in revised form 4 September 2009
Available online 13 September 2009

Keywords:
Cis-mulberroside A
Ramulus mori
Analgesic activity
Anti-inflammatory

ABSTRACT

This study examined the analgesic and anti-inflammatory actions of *cis*-mulberroside A isolated from *Ramulus mori* in several models of inflammatory pain in mice. *Cis*-mulberroside A (25 and 50 mg/kg) given by p.o. route 30 min before challenge produced a dose-dependent inhibition of the acetic acid-induced pain and Evans blue leakage in mice. In addition, this compound exhibited significant systemic anti-inflammatory activity in carrageenan-induced mouse paw edema in a concentration-related manner (33.1–68.5% inhibition), and similar results were achieved in formalin test. Suppressive effects of *cis*-mulberroside A on the production of NO and expression of inducible nitric oxide synthase (iNOS) in lipopolysaccharide (LPS)-stimulated macrophages were also assessed. Collectively, *cis*-mulberroside A showed high analgesic and anti-inflammatory activities. The above results will be the supporting evidence for the potential anti-rheumatoid activity of *R. mori* in Chinese traditional medicine.

1. Introduction

Mulberry is a fast-growing deciduous plant that grows under different climatic conditions (i.e., tropical, subtropical and temperate). Mulberry is valued for its foliage, which constitutes the most important feed for silkworms. *Ramulus mori* are the young twigs of mulberry. In traditional Chinese medicine, its actions are to expel wind, dredge the meridians, and ease joint pain. *R. mori* has been shown to possess pharmacological properties such as immunity, hypolipidemic effect and anti-inflammatory activity [1,2]. But in China, only a small part of the *R. mori* is used as the medicine, most of which acts as agricultural wastes, occupying much space and create greater environmental problems. On the other hand, most studies are mainly focused on the leaves and root cortices of mulberry, and little is known about the activity of *R. mori*.

We have isolated *cis*-mulberroside A (Fig. 1) from the *R. mori* with bioassay-guided methods [3]. *Cis*-mulberroside A is an oxyresveratrol glycoside where 4-OH and 3′-OH were substituted by two glucoses, and its protective function against ethanol-induced hepatic damage has been investigated as shown in [3]. To the best of our knowledge, its activities were seldom investigated. The aim of this study was to investigate the potential analgesic and anti-inflammatory activities of *cis*-mulberroside A in animals. We expect that it will support the evidence for the potential anti-rheumatoid activity of *R. mori*.

2. Experimental

2.1. Plant

R. mori was collected from the mulberry field of Zhejiang University on April, 2008. The sample was confirmed by Professor Zhiyi Ye (expert in cultivation of mulberry), College of Animal Science, Zhejiang University and a voucher specimen has been deposited to the herbarium of College of Animal Science, Zhejiang University, China under the accession number SZ-1086.

* Corresponding author. Tel.: +86 571 86971723.
E-mail address: slgsilk@zju.edu.cn (L. Shi).

0367-326X/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.fitote.2009.09.005

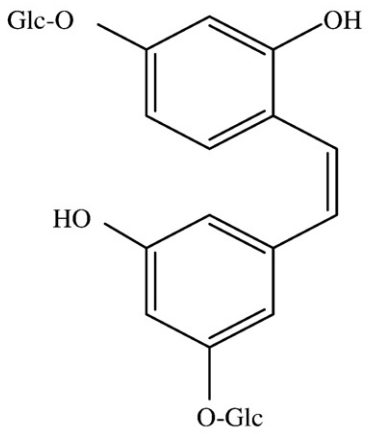

Fig. 1. The structure of *cis*-mulberroside A in *Ramulus mori.*

2.2. Isolation of cis-mulberroside A

The isolation of *cis*-mulberroside A was according to our previous article [3] and all spectra of this compound could be obtained from the corresponding author.

2.3. Animals

Experiments were performed on ICR male mice (body weight range, 22–25 g) from the Animal Experiment Center of Medical College, Zhejiang University, China. All procedures were conducted in accordance with the P.R. China legislation under NO. 8910MO047 on the use and care of laboratory animals and with the guidelines established by the Institute for Experimental Animals of Zhejiang University. Efforts were made to minimize animal suffering and to reduce the number of animals used. Mice were used once only. Animals were housed in a climate- and light-controlled room with a 12-h light/dark cycle. Twelve hours before experiments, food was withheld, but animals had free access to drinking water. At the end of the experiment, animals were euthanized in a CO_2 chamber. No side effects were observed in any of the studied animal groups.

2.4. Cell culture

Raw 264.7 cell line was from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The mammalian cells were cultured in Dulbecco's-modified Eagle's medium with 10% heat-inactivated fetal bovine serum (FBS), 25 mM HEPES (pH7.5), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were plated at a density of 1×10^6 and preincubated for 24 h at 37 °C, and maintained in a humidified atmosphere containing 5% CO_2. For all experiments, the cells were grown to 80–90% confluence, and subjected to no more than 20 cell passages.

The RAW 264.7 cells were incubated with LPS (1 μg/ml) in the presence or absence of *cis*-mulberroside A (25 and 50 μg/ml) for 24 h and then washed twice with ice-cold phosphate-buffered saline (PBS). The cells were lysed in a buffer containing 20 mM HEPES (pH 7.9), 0.1 M KCl, 0.3 M NaCl, 10 mM EDTA, 1% SDS, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin. Western blotting was performed as previously described [4]. For immunoblotting, anti-inducible nitric oxide synthase and anti-β-actin antibodies were used.

2.5. Acetic acid-induced abdominal constrictions and peritoneal capillary permeability in mice

The abdominal constrictions were induced according to procedures described previously [5] and resulted in contraction of the abdominal muscle together with a stretching of the hind limbs in response to an intraperitoneal injection of acetic acid (0.6%) at the time of the test. Firstly, in the beginning of the experiment mice were pre-treated intravenously with 2.5% Evans blue dye solution (10 ml/kg), used as peritoneal capillary permeability marker. Twenty minutes later, mice received the *cis*-mulberroside A (25 and 50 mg/kg) by oral routes 30 min before the acetic acid injection. In separate series of experiments, we also investigate the effect of indomethacin (10 mg/kg), used as positive control, 30 min before the acetic acid injection. Control animals received a similar volume of the appropriate vehicle (10 ml/kg) used to dilute the *cis*-mulberroside A. After the challenge, mice were individually placed into glass cylinders of 20 cm diameter and the abdominal constrictions were counted cumulatively over a period of 20 min. Analgesic activity was expressed as the reduction in the number of abdominal constrictions (the difference between control mice and animals pre-treated with the *cis*-mulberroside A or indomethacin). Immediately after the test, mice were sacrificed by cervical dislocation and peritoneal fluids were collected after washing with a saline (3 ml) solution of the peritoneal cavity and then centrifuged at 1000 rpm for 10 min. The concentration of Evans blue in the peritoneal cavity was determined by the absorbance at 630 nm in a spectrophotometer. The vascular permeability was represented in terms of the absorbance ($A_{630\ nm}$) which leaked into the cavity. Experiments were performed in triplicate.

2.6. Formalin test

Formalin (1%, 20 μl) was injected into the sub-plantar region of the right hind paw of the animals. The duration of paw licking was measured for 15–30 min after formalin administration [6]. Animals were treated with *cis*-mulberroside A, vehicle, or Aspirin (150 mg/kg) 30 min before. The percentage inhibition of licking was calculated by the formula:

$$[(C-T)/C]\times 100$$

where C represents the vehicle treated control group value and T represents the test sample treated group value [7].

2.7. Carrageenan-induced hind paw edema model

Carrageenan-induced hind paw edema model was used for determination of anti-inflammatory activity [8]. The difference in footpad thickness between the right and left foot was measured with a pair of dial thickness gauge calipers (Ozaki Co., Tokyo, Japan). Mean values of treated groups were compared with mean values of a control group and analyzed

using statistical methods. Sixty minutes after the oral administration of test sample or dosing vehicle, each mouse was injected with freshly prepared (0.5 mg/25 μl) suspension of carrageenan (Sigma, St. Louis, Missouri, USA) in physiological saline (154 nM NaCl) into sub-plantar tissue of the right hind paw. As the control, 25 μl saline solution was injected into that of the left hind paw. Paw edema was measured during 4 h after induction of inflammation. Mean values of treated groups were compared with mean values of a control group and analyzed using statistical methods. Indomethacin (10 mg/kg) was used as the reference drug.

2.8. Nitrite analysis

Accumulated nitrite (NO_2^-) in the culture media obtained from murine macrophage RAW264.7 cells was spectrophotometrically determined based on the Griess reaction [9]. 100 μl *cis*-mulberroside A (25 and 50 μg/ml) was incubated with 100 μl Griess reagent (6 mg/ml) at room temperature for 10 min, and then $NO2^-$ concentration was determined by the absorbance at 540 nm. The standard curve was constructed using the known concentrations of sodium nitrite.

2.9. Statistical analysis

The results are expressed as mean ± S.E.M., the data were analyzed for statistical significance using Student's *t*-test. *P*-values less than 0.05 were considered to be significant.

3. Results

Fig. 2 showed that *cis*-mulberroside A (25 and 50 mg/kg), 30 min beforehand, produced a dose-related inhibition of acetic acid-induced abdominal constrictions in mice. The inhibitions were 29.0% and 42.3% for the treated doses of 25 and 50 mg/kg, respectively. *Cis*-mulberroside A in the same range of doses and treatment mentioned above also reduced, in a concentration-dependent manner, the Evans blue dye diffusion induced by acetic acid. The inhibitions were 53.5% and 69.8% for the treated doses of 25 and 50 mg/kg, respectively. The treatment of mice with indomethacin (10 mg/kg, i.p.) also produced marked inhibition of acetic acid-induced writhing response and Evans blue leakage. The inhibitions were 47.5% (writhing movement) and 76.7% (Evans blue leakage), respectively.

Cis-mulberroside A produced inhibition on formalin-induced pain responses (inflammatory pain) in mice (Fig. 3). The analgesic effect of this compound occurred predominantly after 15 min of formalin treatment. It significantly attenuated formalin-induced pain with the percentage inhibition reaching 54.6% at the dose of 50 mg/kg, which was comparable to the positive control drug, Aspirin (10 mg/kg, 59.8%).

Carrageenan-induced hind paw edema model was employed for anti-inflammatory assessment, *cis*-mulberroside A exhibited statistically significant inhibition, ranging between 33.1 and 68.5% at the dose of 25 mg/kg and 50.1–68.2% at the dose of 50 mg/kg, and the results were quite comparable to indomethacin (42.5–51.9% inhibition) at the dose of 10 mg/kg, a reference agent (Table 1).

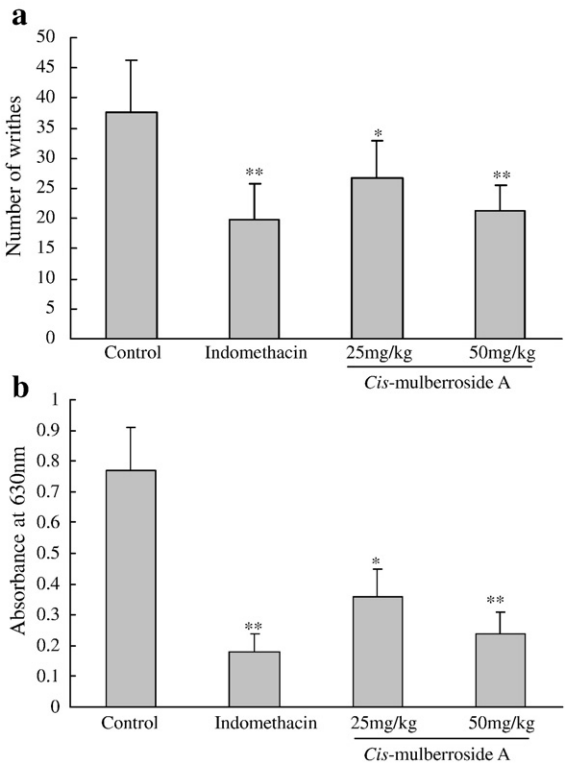

Fig. 2. Effects of oral administration of *cis*-mulberroside A from *Ramulus mori* and indomethacin (10 mg/kg) on acetic acid-induced writhing movements (a) and Evans blue leakage (b) in mice. Data are expressed as means ± S.E. $N = 8$ mice per group. $^*P < 0.05$, $^{**}P < 0.01$ compared with vehicle control.

RAW 264.7 macrophages were stimulated with LPS for 24 h in order to induce NO synthesis. The production of NO was estimated from the accumulation of nitrite, which is a stable product of the NO metabolism, in the medium using the Griess reagent. After the treatment with LPS, the nitrite content markedly increased (Fig. 4). When the macrophage cells were treated with 25 and 50 μg/ml *cis*-mulberroside A, NO production induced by LPS was significantly suppressed in a dose-dependent manner.

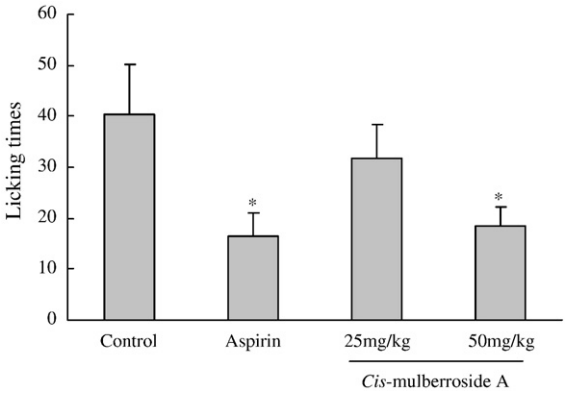

Fig. 3. Analgesic properties of the *cis*-mulberroside A from *Ramulus mori* and Aspirin (150 mg/kg) for 15–30 min after formalin administration. The values represent the means ± S.E. $N = 8$; $^*P < 0.05$ compared with vehicle control.

Table 1
Effect of *cis*-mulberroside A from *Ramulus mori* on carrageenan-induced paw edema in mice.

Material	Dose (mg/kg)	Mean swelling thickness (mm) ± S.E.M. (% inhibition)			
		30 min	60 min	120 min	240 min
Control	–	0.66 ± 0.18	0.54 ± 0.22	0.38 ± 0.12	0.56 ± 0.24
Indomethacin	10	0.48 ± 0.17(42.5)	0.26 ± 0.14(51.9) *	0.21 ± 0.09(44.8) *	0.31 ± 0.18(44.7) *
Cis-mulberroside A	25	0.33 ± 0.08(50.0) *	0.36 ± 0.27(33.1) *	0.12 ± 0.05(68.5) *	0.37 ± 0.12(33.9) *
Cis-mulberroside A	50	0.21 ± 0.04(68.2) *	0.27 ± 0.13(50.1) *	0.15 ± 0.08(61.5) *	0.26 ± 0.09(53.6) *

The values represent the means ± S.E. $N = 8$.
* $P < 0.05$ compared with vehicle control.

The effect of *cis*-mulberroside A on the LPS-induced expression of iNOS protein in RAW 264.7 macrophages was determined to identify the anti-inflammatory mechanism. The western blot experiments showed the induction of the iNOS protein in the cells 4 h after the LPS treatment. *Cis*-mulberroside A significantly suppressed the expression of the iNOS protein in a dose-dependent manner (Fig. 5).

4. Discussion

This is the first study investigating the anti-inflammatory and analgesic activities of *cis*-mulberroside A in two inflammation models: carrageenan-induced hind paw edema and acetic acid-induced peritoneal capillary permeability as well as formalin-induced licking model and acetic acid-induced writhing model for assessing analgesic activity. In addition, the NO production and the expression of iNOS were also investigated.

The acetic acid-induced writhing reaction in mice has long been used as a screening tool for the assessment of analgesic or anti-inflammatory properties of new agents, and is described as a typical model for visceral inflammatory pain [10]. The most important transmission pathways for inflammatory pain are that comprising peripheral polymodal nociceptors sensitive to protons, such as ASICs (acid sensitive ion channel) and to algogen substances, such as bradykinin, prostaglandin and cytokines. These receptors signal to the central nervous system via sensory afferent C fibers entering the dorsal horn [11]. The *cis*-mulberroside A from *R. mori* produced a dose-related inhibition of both the number of abdominal constrictions and Evans blue leakage, resulting from an inflammatory exudative reaction in the peritoneal cavity elicited by acetic acid which indicated the anti-inflammatory and analgesic activities of this active compound.

The formalin-induced nociception is a well-described model and can be consistently inhibited by typical analgesic and anti-inflammatory drugs, including morphine, indomethacin and dexamethasone [12]. The licking response induced by formalin results from a combination of peripheral input and spinal cord sensitization, and the injection of formalin releases prostaglandin E_2, NO and kinins in the spinal cord [13–15]. Considering the inhibitory property of *cis*-mulberroside A on the formalin test, we might suggest an anti-inflammatory action of this compound, and the analgesic activity of *cis*-mulberroside A could be dependent on either peripheral or central sites of action [16].

Carrageenan-induced hind paw edema is the standard experimental model of acute inflammation. It is believed to be biphasic; the first phase (1 h) involves the release of serotonin and histamine and the second phase (over 1 h) is mediated by prostaglandins, cyclooxygenase products, and the continuity between the two phases is provide by kinins [17]. The *cis*-mulberroside A from *R. mori* used in our study inhibited edema induced by carrageenan in a dose-dependent manner, and its activity was comparable to indomethacin, a known cyclooxygenase inhibitor indicating the anti-inflammatory of this active compound.

In order to examine the mechanism underlying these potentially beneficial effects, this study examined whether or not *cis*-mulberroside A inhibited the expression of iNOS and

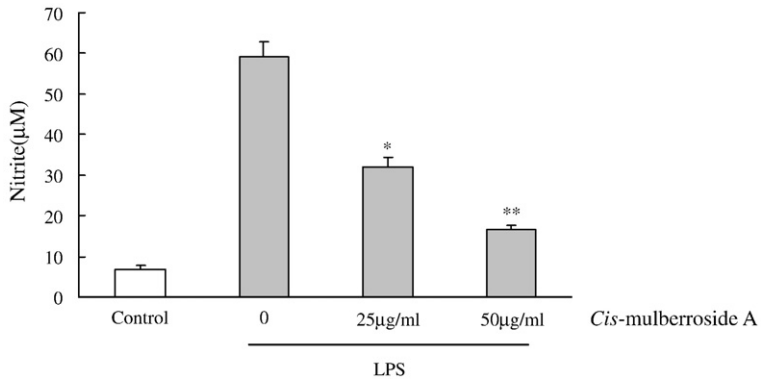

Fig. 4. Effect of *cis*-mulberroside A from *Ramulus mori* on NO production in murine macrophage RAW264.7 cells. The cells were treated with *cis*-mulberroside A at the indicated doses for 30 min and then left unstimulated (control) or stimulated with 1 μg/ml LPS. The nitrite concentration in the culture media was monitored through the Griess reaction 24 h after stimulated with LPS. The values represent the means ± S.E. from three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$ compared with the vehicle control.

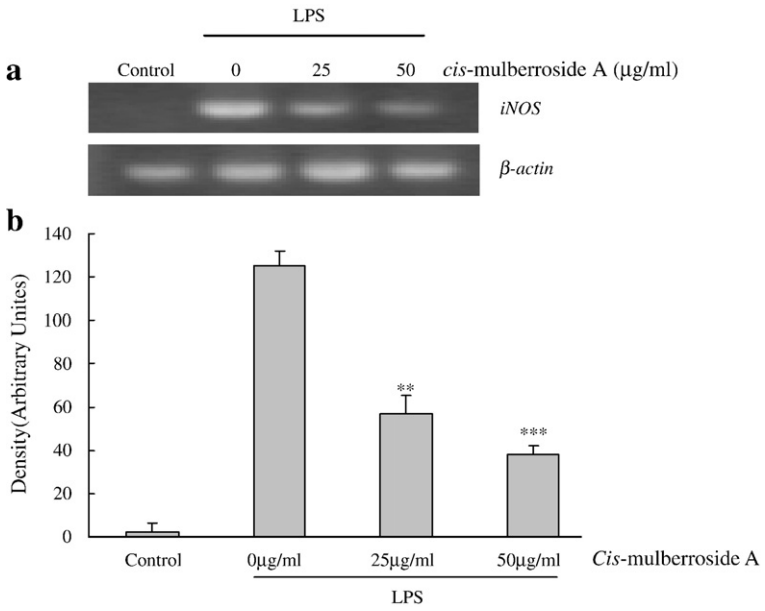

Fig. 5. Effect of *cis*-mulberroside A from *Ramulus mori* on iNOS expression in murine macrophage RAW264.7 cells. (a): The result of iNOS expression, (b): The relative density of iNOS expression (the density of control was 1.0). The cells were treated with *cis*-mulberroside A at the indicated doses for 30 min and then left unstimulated (control) or stimulated with 1 μg/ml LPS. The levels of iNOS were determined using Western blot analysis 4 h after stimulated with LPS. The values represent the means ± S.E from three independent experiments; $^{**}P<0.01$; $^{***}P<0.001$, compared with the vehicle control.

NO production in LPS-stimulated RAW 264.7 macrophages. NO is an important mediator in the inflammatory process and is produced at inflamed sites by iNOS. High levels of NO have been reported in a variety of pathological processes including various forms of inflammation, circulatory shock, and carcinogenesis [18–20]. Therefore, an inhibitor of iNOS might be effective as a therapeutic agent for inflammatory diseases [21]. These results show that *cis*-mulberroside A inhibits LPS-induced NO production in a dose-dependent manner in RAW 264.7 macrophages. This suppression was correlated with the downregulation of the expression of iNOS protein in the cells.

In conclusion, the *cis*-mulberroside A from *R. mori* contains acute anti-inflammatory and analgesic activities. This compound also possesses an inhibitory activity on in vitro NO production in the stimulated mammalian cells. It is capable of diminishing expression of iNOS in LPS-stimulated RAW264.7 cells. Considering the use of *R. mori* in inflammatory processes in folk medicine, this compound may play a potent action.

Acknowledgments

This research was supported by grants from the National Natural Science Foundation of China (No. 30571406), and the Traditional Chinese Medicine Administration of Zhejiang Province (No. 2003KF005). We acknowledge all the staff for their valuable assistance in conducting this study.

References

[1] Wu YM, Zou YX, Liao ST, Yu YJ, Zhang YS, Yao XZ. Canye Kexue 2005;31:348–50.
[2] Wang R, Lu XC, Wang YW. J. Wuhan Botan Res 2002;20:467–9.
[3] Zhang ZF, Jin J, Shi LG. Environ Toxicol Pharmacol 2008;26:325–30.
[4] Kim BC, Mamura M, Choi KS, Calabretta B, Kim SJ. Mol Cell Biol 2002;22:1369–78.
[5] Santos ARS, Gadotti VM, Oliveira GL, Tibola D, Paszcuk AF, Neto A, et al. Neuropharmacology 2005;48:1021–34.
[6] Hunskaar S, Fasmer OB, Hole K. J. Neurosci Meth 1985;14:69–76.
[7] Dongmo AB, Nguelefack TB, Lacaille-Dubois MA. J Ethnopharmacol 2005;98:201–6.
[8] Yesilada E, Kupeli E. J Ethnopharmacol 2007;110:504–15.
[9] Sherman MP, Aeberhard EE, Wong VZ, Griscavage JM, Ignarro LJ. Biochem. Bioph Res Co 1993;191:1301–8.
[10] Dickenson A, Besson J. The pharmacology of pain. Berlin: Springer Verlag; 1997. p. 1–20.
[11] Kumazawa T, Mizumura K, Koda H, Fukusako H. J Neurophysiol 1996;75:2361–8.
[12] Hunskaar S, Hole K. Pain 1987;30:103–14.
[13] Santos ARS, Calixto JB. Neuropeptides 1997;31:381–9.
[14] Beirith A, Santos ARS, Calixto JB. Brain Res 2002;924:219–28.
[15] Sakurada T, Matsumura T, Moriyama T, Sakurada C, Ueno S, Sakurada S. Pharmacol Biochem Be 2003;75:115–21.
[16] Luiz AP, Moura JD, Meotti FC, Guginski G, Guimarães CL, Azevedo MS, et al. J Ethnopharmacol 2007;114:355–63.
[17] Perianayagam JB, Sharma SA, Pillai KK. J Ethnopharmacol 2006;104:410–4.
[18] Ohshima H, Bartsch H. Mutat Res 1994;305:253–64.
[19] Szabo C. New Horizontes 1995;3:2–32.
[20] MacMicking J, Xie QW, Nathan C. Annu Rev Immunol 1997;15:323–50.
[21] Koo TH, Lee JH, Park YJ, Hong YS, Kim KW, Lee JJ. Planta Med 2001;67:103.