Chinese Journal of Integrative Medicine
Available online at link.springer.com/journal/11655
Journal homepage: www.cjim.cn/zxyjhen/zxyjhen/ch/index.aspx
E-mail: cjim_en@cjim.cn

Original Article

Genotoxicity and Embryotoxicity Study of Bicyclol Methyl Ether, Main Impurity in Bicyclol

ZHANG Qian-qian[1], LI Qiang[2], DONG Lin[3], LI Wan-fang[1], LI Chao[3], WANG Ai-ping[1,3], WEI Jin-feng[1,3], and JIN Hong-tao[1,3]

ABSTRACT **Objective**: To assess the genotoxicity and embryotoxicity of bicyclol methyl ether (BME), the main impurity in bicyclol. **Methods**: Five concentrations of BME (0.5, 5, 50, 500 and 5000 μg/plate) were used in the Ames test to detect gene mutation. In the chromosome aberration test, Chinese hamster lung cells were used to detect chromosomal aberration of BME (15, 30, 60, 120 μg/mL) with or without S9 mixture. Embryotoxicity test was also conducted to determine any embryotoxicity of BME (7.5, 22.5, 67.5 μg/L) using zebrafish embryos. **Results**: No significant differences were observed in the Ames test and the chromosome aberration test in the BME groups compared with the vehicle control group. The zebrafish embryos toxicity test also showed no embryo development toxicity of BME, including hatching rate, body length, pericardial area and yolk sac area. **Conclusions**: Bicyclol methyl ether has no genotoxicity *in vitro* and embryotoxicity in zebrafish embryos, and the impurity in bicyclol is qualified.

KEYWORDS bicyclol methyl ether, genotoxicity, embryotoxicity, Ames test, chromosome aberration test, zebrafish

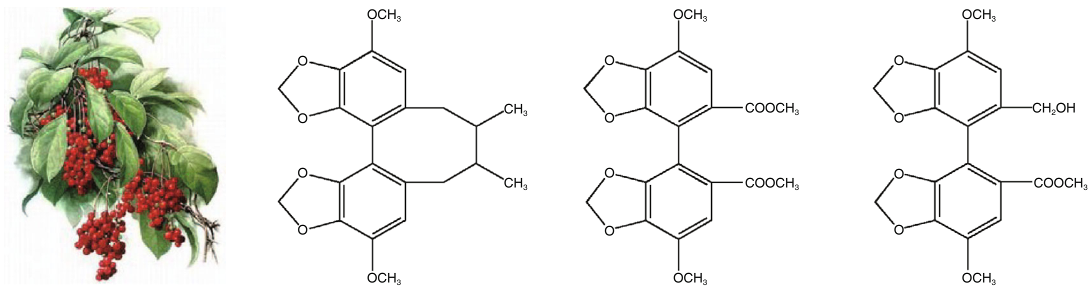
The Chinese herb *Fructus Schiznadrae* (Figure 1A) has been found to be effective in improving the liver function of patients with chronic viral hepatitis in the early 1970s. Based on this clinical practice, some dibenzo cycloocten lignans were isolated from *Fructus Schiznadrae* and their liver injury protect effects were screened to further determine the effective constituents by chemists in the Institute of Materia Medica, Chinese Academy of Medical Sciences. Of these lignans, schizandrin C (Figure 1B) was found to be most active in liver protection against chronic viral hepatitis. Because of the failure in total synthesis of schizandrin C, a number of analogues were synthesized(1,2) and their bioactive effects on liver protection were studied. Fortunately, biphenyl dimethyl dicarboxylate (4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene-dioxy-2, 2'-dicarboxylate biphenyl, DDB) (Figure 1C) was discovered to have remarkable effect on improvement of impaired liver function in the clinical trial with rare side effects.(2-5) However, the anti-viral activity and bioavailability of DDB were poor. Therefore, a number of DDB derivatives were synthesized and a novel substitute of DDB, bicyclol (4, 4'-dimethoxy-5, 6, 5', 6'-dimethylene-dioxy-2-hydroxymethyl-2'-carbonyl biphenyl, Figure 1D), was discovered to be more effective than DDB in liver protection and hepatitis virus replication inhabitation.(6,7)

Studies have shown that bicyclol has good hepatoprotective effect(6,8) and the mechanism of its effect may include eliminating free radicals and inhibiting the hepatocyte nuclear DNA damage and apoptosis.(7,9) Bicyclol has been widely used in treatment of chronic viral hepatitis in China since China Food and Drug Administration issued license to manufacture bicyclol in 2001.(10) It has been proved that bicyclol is effective for anti-hepatitis virus and liver protection in clinical practice these years. Bicyclol acquired imported drug license in Russia in 2015 and it has been registered in 9 countries so far.

With the wide application of bicyclol, its safety evaluation has become necessary and important. Previous study showed that bicyclol had no

1. New Drug Safety Evaluation Center, Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing (100050), China; 2. Beijing Union Pharmaceutical Factory, Beijing (100026), China; 3. Beijing Union-Genious Pharmaceutical Technology Ltd., Beijing (100176), China
Correspondence to: Prof. JIN Hong-tao, Tel: 86-10-67817730, E-mail: jinhongtao@imm.ac.cn
DOI: https://doi.org/10.1007/s11655-018-2553-x

Chin J Integr Med 2019 Oct;25(10):743-749



(A) *Fructus Schiznadrae* (B) Schizandrin C (C) Biphenyl dimethyl dicarboxylate (D) Bicyclol

Figure 1. Chemical Structure of Schizandrin C Derivatives and Discovery of Bicyclol

obvious chronic toxicity as well as mutagenicity and teratogenicity in animal models.[11] So the evaluation has been focused on the impurities in bicyclol.

Bicyclol methyl ether (BME) is an identified impurity in bicyclol. The limitation of BME in bicyclol is 0.3% according to Pharmacopoeia of the People's Republic of China,[12] which is higher than the qualification threshold of 0.15% for maximum daily dose < 2 g per day intake based on the ICH guideline (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use). Therefore, genotoxicity studies are necessary to determine any clinically relevant adverse effects according to the ICH guideline regarding to impurities in new drug substances.[13] On the other hand, because of their rapid development and sensitivity to toxic substances, zebrafish embryos have been utilized for assessing embryotoxicity these years and have become an attractive vertebrate animal model for *in vivo* toxicological study.[14]

Hence, we evaluated the genotoxicity of BME using Ames test and chromosome aberration test *in vitro*. In the meantime, embryotoxicity test *in vivo* was also conducted using zebrafish embryos. Together, this study aims to assess the safety of BME to determine the possible side effects of bicyclol in clinical use.

METHODS

Test Material

BME (Lot No. 20140906) was obtained from Beijing Union Pharmaceutical Factory and stored dry and sealed. For treatments in the Ames test and chromosome aberration test, BME was dissolved in dimethyl sulphoxide (DMSO, Sigma, USA), for treatment in embryo development toxicity test, BME was dissolved in ethanol.

Ames Test

Bacteria

Salmonella typhimurium tester strains TA 97, TA 98, TA 100, TA 102, TA 1535 originally obtained from Krishgen Biosystems (India) were used in this experiment. The tester strains were freshly prepared in nutrient broth (AOBOX, China) at 37 ℃ for 16 h with shaking at 100–120 r/min.

Chemicals

4-nitroquinolineoxide (4-NQO), sodium azide (NaN_3) and cyclophosphamide (CP) were purchased from Sigma Aldrich (USA), 2-aminofluorene (2-AF) was purchased from Merck (USA), mitomycin C (MMC) and nicotinamide adenine dinucleotide phosphate (NADP) were purchased from Solarbio Life Sciences (China). Other chemicals used in this test were obtained from Sinopharm Chemical Reagent Beijing Co., Ltd., (China).

S9 Preparation

Liver S9 homogenate was prepared from male rats induced with phenobarbital and β-naphthoflavone. The S9 mixture were prepared freshly before use and consisted of 0.1 mL S9, 8 μmol/L $MgCl_2$, 33 μmol/L KCl, 5 μmol/L glucose-6-phosphate, 4 μmol/L NADP and 0.5 mL phosphate buffer solution (PBS) per milliliter. The amount of S9 supernatant was 10 % v/v in the S9 mixture.

Plate Incorporation Preincubation Methodology

Five concentrations of BME (0.5, 5, 50, 500 and 5000 μg/plate) dispersed in DMSO were used in this assay. The positive mutagens employed for the assay were 4-NQO (2.0 μg/plate) for TA 97 and TA 98, NaN_3 (1.5 μg/plate) for TA 100 and TA 1535, MMC (1.0 μg/plate) for TA 102 without the S9 mixture and 2-AF (60 μg/plate) for TA 97, TA 98, TA 100, TA 102, CP (200 μg/plate) for TA 1535 with the

S9 mixture. For preincubation treatment, 0.1 mL of the test solution or vehicle control (purified water and DMSO), 0.1 mL of bacterial suspension and 0.5 mL of PBS (for the test without S9 mixture) or the S9 mixture (for the test with the S9 mixture) were incubated at 37 ℃ for 20 min with shaking at 120 r/min before adding 2 mL top agar (kept in a 45 ℃ water bath) and pouring into the minimal bottom agar plates. The plates were incubated for 48 h at 37 ℃. Revertant colonies on each plate were counted manually after incubation.

Each concentration of test material was test in triplicate, with and without S9. Genetic toxicity were determined positive if revertant colonies in the BME groups (with or without S9 mixture) were more than twice of the vehicle control and dose dependent with the prerequisite of well-grown bacterial background lawn.

Chromosome Aberration Test

Materials

Chinese hamster lung (CHL) cells were obtained from Kunming Institute of Zoology, Chinese Academy of Science. Cells were cultured in DMEM medium (Hyclone, USA) and were incubated at 37 ℃ in a constant temperature incubator with 5% CO_2.

Preparation of the S9 mixture was the same as described previously in the Ames test. MMC and CP were used as positive control in this test.

Dose Range Selection

A cytotoxicity test was conducted to determine the highest concentration for the study. Eight concentrations of the test material (16.125, 31.25, 62.5, 125, 250, 500, 1,000, 2,000 μg/mL) were used. Triplicate cultures with 0.05 mL test material or vehicle control were incubated at 37 ℃ for 48 h and cell growth inhibiting rate was calculated. According to the result, the half maximal inhibitory concentration (IC_{50}) of BME was 119.03 μg/mL. Therefore, 15, 30, 60, 120 μg/mL of BME were used in the chromosome aberration test.

Treatment and Scoring

Cell cultures were treated with the solvent, the positive control, or with each test material concentration. The tests without S9 mixture were treated continuously for 24 h or 48 h, the tests with S9 mixture were treated for 6 h, and then the cells were washed 3 times with PBS buffer and cultured with fresh DMEM medium for 18 h. To arrest dividing cells in metaphase, colchicine was added prior to harvest, and then cells were digested, centrifuged and resuspended in KCl solution. After 10 min at room temperature, the cells were fixed by 5 mL fresh, ice-cold methanol/glacial acetic acid (3:1, v/v). After incubation for 10 min, the fixative was changed by centrifugation and resuspending several times until the supernatants were clear. Three slides were prepared from each concentration. The slides were air-dried, stained with 3% Giemsa solution, and then washed in tap water and distilled water, dried, and mounted for chromosome aberration scoring. Two-hundred metaphases from each slide were analyzed for chromosome aberrations. A result was defined as positive if the aberration rate was more than 10% and there was significant difference compared with the vehicle control.

Embryo Toxicity Test in Zebrafish

Preparation of Test Solution

The test material was dissolved in ethanol and then diluted in E3 solution (5 mmol/L NaCl, 0.17 mmol/L KCl, 0.33 mmol/L $CaCl_2$, 0.33 mmol/L $MgSO_4$), the egg medium, to the test concentrations. Three BME concentrations were used based on our previous pharmacokinetic study on bicyclol in beagle dogs (data not published): 7.5 μg/L (with 0.0025% ethanol), 22.5 μg/L (with 0.0075% ethanol), and 67.5 μg/L (with 0.0225% ethanol). The dosages used in this test were much higher than the calculated plasma concentration in beagle dogs. ethanol solution (0.0225%) was used as vehicle control and E3 solution was used as blank control.

Stability Analysis of the Test Solution

The zebrafish embryos were exposed to the test solution for 3–96 hours post fertilization (hpf). Therefore, stability of the solution should be analyzed. The stability analysis was performed on the Agilent 1200 HPLC system (Agilent Technologies, USA). Chromatographic separation was carried out at 40 ℃ on a Waters Symmetry C18 analytical column (250 mm × 4.6 mm, 5 μm). The mobile phase comprised acetonitrile and water (55:45, v/v) with 0.01% acetic acid at a flow rate of 0.5 mL/min. Three concentrations of the test solution (7.5 μg/L, 22.5 μg/L, 67.5 μg/L) were kept at the temperature of 28 ℃ and 200 μL of the solution was injected into the HPLC system for analysis at 0, 48 and 96 h, respectively.

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Zebrafish Husbandry and Exposure to BME

Zebrafish of AB strain (wild type) were maintained in a circulation system at standard laboratory conditions of 28 ℃ with a photoperiod of 14 h light/10 h dark cycle. The fish were fed twice a day with brine shrimp (hatched from eggs in salt water). For experiments, fertilized eggs were washed with E3 solution and healthy embryos were chosen under a stereomicroscope (Olympus, Japan) within 3 hpf and then transferred to 24-well culture plates. Thirty embryos were used in each group (5 embryos in 2 mL solution/well) and the experiment was replicated 3 times. The embryos were treated with the test sample for 3–96 hpf in an incubator with the temperature controlled at 28 ± 0.5 ℃ and a 14 h light/10 h dark photoperiod. Dead embryos were removed from the 24-well plates every 24 h.

Toxicological Endpoints

At 72 hpf, the hatching rate, which is a ratio of hatching embryos to the living embryos in each group, was evaluated. At 96 hpf, the embryonic mortality and notochord malformation of the embryo were also recorded. The photographs of embryos were captured and body length, pericardial area and yolk sac area were measured as diagrammed in Figure 2 using the Image J software to determine whether the test material could affect the growth of the embryos or cause pericardial and yolk sac edema.

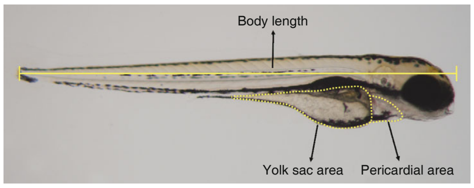

Figure 2. Diagram of Measurement of Body Length, Pericardial and Yolk Sac Area of Zebrafish Larvae

Statistical Analysis

The data of body length, pericardial area and yolk sac area were expressed as mean ± standard deviation ($\bar{x} \pm s$) and analyzed using one-way analysis of variance (ANOVA). The data of mortality, hatching rate and notochord malformation were analyzed using χ^2 test. All statistical analyses were conducted using SPSS software and differences were considered significant at P<0.05.

RESULTS

Ames Test

Revertant colonies induced by all the 5 positive mutagens 4-NQO (TA 97 and TA 98), NaN3 (TA 100 and TA 1535), MMC (TA 102), 2-AF (TA 97, TA 98, TA 100 and TA 102), and CP (TA 1535) were more than twice of the vehicle control. However, revertant colonies of the bicyclol methyl ether groups were comparative with that of the vehicle control group (Table 1).

Chromosome Aberration Test

Chromosome aberration induced by the positive control MMC and CP were significantly higher than that of the vehicle control and the aberration rates were higher than 10%.

There were no significant increases in the chromosome aberration rate at 6 h, 24 h or 48 h with or without S9 mixture in the BME groups compared with the vehicle control group (Appendix 1).

Embryo Toxicity Test in Zebrafish

Stability Analysis of the Test Solution

The results of the stability of BME solution were shown in Table 2. It was demonstrated that the samples were stable during the embryo toxicity test with the relative standard deviation (RSD) within general acceptable limit.

Toxic Effects in Zebrafish Embryos

No significant differences were observed in any parameters in the vehicle control group compared with the blank control, indicating that the 0.0225% ethanol did not induce any abnormal toxicity effects in zebrafish embryos.

Treatment of BME did not affect hatching rate and mortality of the embryos significantly (Table 3). Besides, there was notochord malformation in all the groups, but the incidence was comparative with the blank control, suggesting that the malformation was spontaneous and irrelevant to the treatment of BME. In addition, there were no significant differences in the body length, pericardium area and yolk sac area in the BME groups except for the pericardial area in the BME 7.5 μg/L group (P<0.05, Figure 3).

DISCUSSION

Bicyclol is a derivative of schizandrin C, isolated from *Fructus Schiznadrae*. Preclinical study showed that bicyclol had hepatoprotective effect in animal models(6-9) and anti-hepatitis virus activity in

Table 1. Ames Test of BME with and without S9 Mixture

Conditions	Test article	Concentration (μg/plate)	Numver of revertant colonies ($\bar{x} \pm s$)				
			TA97	TA98	TA100	TA102	TA1535
Without S9	Purified water	0	117.7±12.4	25.3±3.1	101.3±8.5	278.0±17.1	13.3±3.1
	DMSO	0	119.2±14.0	25.7±3.0	100.3±7.7	273.2±17.1	13.3±1.8
	BME	0.5	115.8±13.2	25.0±3.0	100.2±9.4	276.5±17.2	12.5±2.3
	BME	5.0	116.5±11.7	27.5±4.1	99.0±10.0	275.5±16.7	14.0±1.9
	BME	50.0	118.3±14.6	24.2±2.5	99.5±8.4	273.5±17.7	12.7±2.2
	BME	500.0	119.5±14.3	23.8±2.1	100.5±9.2	276.8±18.2	13.2±3.2
	BME	5000.0	115.2±10.6	23.3±1.9	98.3±6.3	270.7±11.6	12.7±2.8
	4-NQO	2.0	>1000	>1000	–	–	–
	NaN3	1.5	–	–	>1000	–	>500
	MMC	1.0	–	–	–	>1000	–
With S9	Purified water	0	138.8±11.5	36.2±3.5	115.5±6.0	295.5±5.1	23.7±1.9
	DMSO	0	136.5±14.0	35.0±4.0	114.7±7.9	292.5±5.4	22.8±3.0
	BME	0.5	138.3±15.6	35.5±4.9	115.7±6.9	293.3±5.3	23.0±2.7
	BME	5.0	139.0±14.1	36.8±4.8	115.2±7.9	291.0±6.7	24.7±2.1
	BME	50.0	136.7±15.7	34.8±4.4	116.5±6.0	292.7±7.6	22.7±4.4
	BME	500.0	139.0±14.7	35.2±5.7	116.5±7.3	295.2±6.7	22.5±1.4
	BME	5000.0	132.7±16.3	33.7±5.5	114.8±6.1	287.5±4.4	21.0±3.7
	2-AF	60.0	>1000	>1000	>1000	>1000	–
	CP	200.0	–	–	–	–	>500

Note: "–" stands for not suitable for the test strain.

Table 2. Stability Analysis for BME

Nominal concentration (μg/L)	Measured concentration (μg/L)	Peak area			
		0 h	48 h	96 h	Precision (RSD, %)
7.5	7.6	17.61	17.16	17.34	1.31
22.5	22.8	49.64	48.29	48.55	1.47
67.5	68.4	147.10	146.96	145.39	0.65

cell model and duck model.(10) Bicyclol has few side effect according to the clinical study and has been an effective drug mainly for treating chronic hepatitis B and C ever since its approval for clinical use.(15) Preclinical toxicity study of bicyclol showed no obvious chronic toxicity as well as mutagenicity and teratogenicity in animal models.(11)

As a main impurity existing in bicyclol, BME is a process-related organic impurity. Because the level of BME in bicyclol is greater than the qualification threshold, genotoxicity studies and general toxicity studies are recommended to conduct to detect point mutation, chromosomal aberration or other toxic effects according to the ICH guideline regarding to impurities in new drug substances.(13) In the previous studies, we assessed the acute toxicity and sub-chronic toxicity of BME in rats, and no obvious toxic effects were observed. Therefore, in the present study, we performed a bacterial reverse mutation test using the *Salmonella typhimurium* (Ames test) and chromosome aberrations in CHL cells to investigate the potential genotoxicity of BME as well as an

Table 3. Hatching Rate, Mortality and Notochord Malformation of the Zebrafish Embryos

Test material	Concentration (μg/L)	Concentration of ethanol (%, v/v)	Observing parameters		
			Hatching rate (%)	Mortality (%)	Notochord malformation rate (%)
E3 solution	0	0	85.6	1.1	2.3
Ethanol solution	0	0.0225	81.1	2.2	3.3
BME	7.5	0.0025	92.2	1.1	2.3
BME	22.5	0.0075	92.2	3.3	2.3
BME	67.5	0.0225	92.3	0	6.6

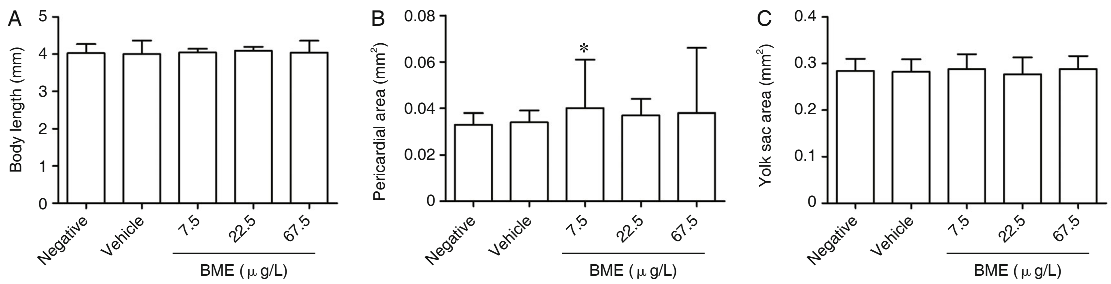

Figure 3. Effects of Bicyclol Methyl Ether on Body Length, Pericardial and Yolk Sac Area of Zebrafish Larvae

Notes: A: Body length of the zebrafish embryos, B: Pericardium area of the zebrafish embryos, C: Yolk sac area of the embryos. *P<0.05 *vs*. negative control.

embryotoxicity test using the zebrafish embryos to determine any embryo development toxicity.

The Ames test is a reliable and sensitive bacterial assay using amino acid-requiring strains of *Salmonella typhimurium*. It is extensively used to determine the potential of substances that can induce gene mutations or other genetic damage.(16,17) In our study, revertant colonies induced by all the 5 positive mutagens 4-nitroquinolineoxide (TA 97 and TA 98), NaN3 (TA 100 and TA 1535), mitomycin C (TA 102), 2-AF (TA 97, TA 98, TA 100 and TA 102), and CP (TA 1535) were more than twice of the vehicle control, indicating that the design of this test were reasonable. No positive mutagenic responses were observed in any of the tester strains compared with the vehicle control group both with and without application of the S9 mixture, suggesting that BME is negative in the Ames test.

The chromosome aberration test(18) is a classical and valuable technique to identify substances that can cause structural chromosome aberrations in cultured mammalian cells such as CHL cells, peripheral blood lymphocytes, etc.(19,20) In the present study, CHL cells were selected as the test system and no significant increases in the chromosome aberration rate at 6 h, 24 h or 48 h with or without S9 mixture in the BME groups compared with the vehicle control group, which is consistent with the results of the Ames test. Together, the results demonstrated that BME did not cause genotoxicity *in vitro*.

The zebrafish has been widely used as a vertebrate animal model for assessing toxicity and safety of chemicals.(21,22) The zebrafish embryos are transparent and develop outside the mother, which makes the observing of toxic effects easily. In addition, the zebrafish embryos are sensitive to compounds that exhibit teratogenicity in mammals and the developmental processes in the zebrafish are highly conserved, which means their response to toxic substances can be highly predictive in mammals.(14) In our embryo development toxicity test, samples were stable during the embryo toxicity test and no treatment-related toxicity was observed. The significantly larger pericardial area in the BME 7.5 μg/L group may be caused by the severe spontaneous pericardial edema of one larva in that group, which can be commonly observed in larvae. Besides, there is no dose–response relationship, so it can not be determined that BME induced toxic effect.

In conclusion, we conducted Ames test, chromosome aberration test and embryotoxicity test in zebrafish to investigate the genotoxicity and embryo development toxicity of BME. From the result above, it can be concluded that BME, main impurity in bicyclol, does not have genotoxicity *in vitro* and embryo development toxicity in zebrafish embryos, and the impurity in bicyclol is qualified. Furthermore, by combining *in vitro* genotoxicity tests with *in vivo* embryotoxicity tests, our study presented a valuable reference for assessment of other similar compounds.

Conflict of Interest

The authors declare there are no conflicts of interest.

Author Contributions

Zhang QQ, Dong L carried out the experiments and wrote the paper; Li Q, Li WF help sampling the test article and detection of HPLC; Li C, Wang AP, Wei JF supervised and directed the project; Jin HT as the corresponding author reviewed and revised the entire manuscript.

Electronic Supplementary Material: Supplementary material (Appendix) is available in the online version of this article at https://doi.org/10.1007/s11655-018-2553-x.

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(Accepted January 29, 2017; First Online September 22, 2018)
Edited by ZHANG Wen