Revista Brasileira de Farmacognosia 28 (2018) 312–319

Brazilian Journal of Pharmacognosy
REVISTA BRASILEIRA DE FARMACOGNOSIA
www.elsevier.com/locate/bjp

Original Article

# Optimization for extraction of an oil recipe consisting of white pepper, long pepper, cinnamon, saffron, and myrrh by supercritical carbon dioxide and the protective effects against oxygen–glucose deprivation in PC12 cells

Jiaoning Li[a,1], Enshuang Guan[a,1], Li Chen[a], Xia Zhang[a], Lei Yin[a], Lin Dong[a], Qiong Pan[c], Xueyan Fu[a,b], Liming Zhang[a,b,*]

[a] *School of Pharmacy, Ningxia Medical University, Yinchuan, China*
[b] *Key Laboratory of Hui Ethnic Medicine Modernization, Ministry of Education, Ningxia Medical University, Yinchuan, China*
[c] *Second People's Hospital of Yinchuan, Yinchuan, China*

ARTICLE INFO

*Article history:*
Received 10 October 2017
Accepted 4 April 2018
Available online 1 May 2018

*Keywords:*
Hui medicine
Orthogonal design
Oxygen and glucose deprivation
Oxidative stress
PC12 cells

ABSTRACT

This study is to investigate the most efficient extractives of extracting oil recipe for stroke treatment and the protective effects on an oxygen and glucose deprivation model in PC12 cells. An orthogonal experimental design $L_9$ ($3^4$) was carried out for oil recipe's optimization with supercritical $CO_2$ fluid extraction. 2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide and enzyme-linked immunosorbent assay were conducted to evaluate cell activity and indexes in the cell lysate. The result showed that the optimum extraction condition was 30 Mpa, 50 °C, 100 min, the extracts were analyzed by gas chromatography–mass spectrometry and among forty detected compounds 27 were identified, representing 80.86% of the total oil content. *trans*-Cinnamaldehyde (14.14%), piperine (9.32%), β-amyrin (6.79%), lupenone (6.28%), longifolene (6.07%), β-caryophyllene (5.21%), α-bisabolol (4.11%), and β-bisabolene (2.56%) were high mass fraction. Oil recipe could significantly attenuate PC12 cell damage, the lactate dehydrogenase release and decreased the malondialdehyde levels, glutathione peroxidase and nicotinamide adenine dinucleotide phosphate oxidase activity, glutathione and nitric oxide content ($p < 0.01$) and increased the level of superoxide dismutase after oxygen and glucose deprivation. The protective mechanism may be related to oil recipe's antioxidant effect by scavenging free radicals.

© 2018 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

## Introduction

The country incorporated national characteristics into the medical system in China, including the traditional Chinese Hui medicine (TCHM) which is a representative folk medicine and an important branch of traditional Chinese medicine. The most characteristic of the TCHM is the aromatic drug mainly due to rich oil, and usually emits aromatic smell. Stroke is the third most frequent cause of adult death in most industrialized countries after cardiovascular disease and cancer and has been a serious threat to human health (Flynn et al., 2008). It is important to develop the effective treatment of neuroprotective drugs, due to few treatments for stroke and the imperative development of new therapeutics. Aromatic drugs in prescriptions for cerebral ischemia have been studied and found with remarkable treatment effects (Qi et al., 2016). *Trans*-cinnamaldehyde has been confirmed that played a major role in ameliorating cerebral ischemia-induced brain injury and protected myocardial ischemia damage (Zhao et al., 2015; Chen et al., 2016).

Our previous research show that treating cerebral stroke contains white pepper, long pepper, cinnamon, saffron, and myrrh as the main active components (equivalent to the monarch in the traditional Chinese medicine prescription) in TCHM (Li et al., 2013a,b). The main active components have many pharmacological effects, such as activating blood and dissolving stasis, anti platelet aggregation and prevention and treatment of atherosclerosis (Bai and Xu, 2000; Feng, 2004). Preliminary results show that oil recipe (OR) exert anti-inflammatory and analgesic effect (Zhang et al., 2014), which is key components of the mechanisms for treating ischemic

* Corresponding author.
*E-mail:* nyd322@126.com (L. Zhang).
[1] JiaoNing Li and EnShuang Guan equally contributed to this manuscript, all should be considered as first author.

https://doi.org/10.1016/j.bjp.2018.04.001
0102-695X/© 2018 Sociedade Brasileira de Farmacognosia. Published by Elsevier Editora Ltda. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

stroke and other forms of ischemic brain injury (Jin et al., 2013; Shichita et al., 2012). However, no direct evidence has confirmed that OR exerts a protective effect on cerebral ischemia. Rat pheochromocytoma PC12 cells, which are often adopted as *in vitro* cell culture model of ischemia, have been widely used as a cellular model for studying neuronal diseases (Ryu et al., 2014). Compared with the traditional extraction method, the application of supercritical $CO_2$ fluid extraction (SFE) has lots of advantages, such as fast extraction rate, avoiding heat deterioration and no residual solvent (Uquiche et al., 2015). In the present study, we verified the protective effect of OR against oxygen and glucose deprivation (OGD) in PC12 cells and thus provided scientific basis for the further development of drugs for stroke treatment.

## Materials and methods

White pepper (*Piper nigrum* L., Piperaceae), long pepper (*Piper longum* L., Piperaceae), myrrh (*Commiphora myrrha* (Ness.) Engl., Burceraceae), cinnamon (*Cinnamomum cassia* (L.) J.Presl, Lauraceae), and saffron (*Crocus sativus* L., Iridaceae) were purchased from Taiyuan Herbal Medicine Co., Ltd. (Anhui, China). All of these plants were classified by Lin Dong at the Pharmacognosy Department, College of Pharmacy, Ningxia Medical University, and voucher specimens were deposited in one unit (Herbarium number: 20130721, 20130723, 20130801, 20130811, 20130813).

The reagents, materials, and apparatuses used were as follows: *trans*-cinnamaldehyde reference substance (Chinese Food and Drug Inspection Institute, Voucher No.: 110710-201418, 99.4%); supercritical $CO_2$ extraction unit (US Applied Separations Inc.); 6890N Agilent gas chromatograph (American Agilent Company); gas chromatography–mass spectrometry (GC–MS) apparatus (American Agilent Company); anaerobic bag (Mitsubishi Ltd.); PC12 cell line (Shanghai Institute of Chinese Academy of Sciences); carbon dioxide ($CO_2$, 99.95%); high-glucose Dulbecco's modified Eagle's medium (DMEM; Hyclon); PBS (Hyclon); fetal bovine serum (FBS; PAN); nimodipine (Sigma); glucose-free culture medium (Gibco); commercial kits for lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), glutathione (GSH), glutathione peroxidase (GPX), nicotinamide adenine dinucleotide phosphate oxidase (NOX), and nitric oxide (NO) (Beijing Chenglin Bioengineering Institute). The remaining materials used were analytical reagents.

### *Oil extraction*

After proper crushing and sieving in 40 mesh, the white pepper, long pepper, myrrh, cinnamon, and saffron were mixed at the conventional ratio 1:2:2:2:2 (w/w). Then, 45 g sample was loaded into the extraction cell. Carbon dioxide played a cooled and liquefied role which from a gas cylinder passed to refrigerator unit. In the high pressure pump the liquefied $CO_2$ was compressed, then passed into a surge tank and transferred to the main extraction column. The other conditions were maintained to be constant within the different experimental conditions was tested in triplicate. Percentage content of oil can be calculated on basis of the dried powder weight (Sodeifian et al., 2017).

$$\text{OR yield}\,(\%) = \frac{\text{OR weight}\,(\text{g})}{\text{material loaded}\,(\text{g})} \times 100\%$$

### *Determination of cinnamonaldehyde content*

GC was performed using an Agilent 6890N gas chromatograph, HP-5MS quartz capillary column (30.0 m × 320 μm × 0.25 μm), and carrier gas for high-purity nitrogen with 1.2 ml/min column flow. The heating program included a column temperature of 50 °C for 1 min; a temperature ramp of 8 °C/min to 150 °C, which was held for 3 min; a temperature ramp of 3 °C/min to 210 °C, which was held for 5 min; and finally, a temperature ramp of 5 °C/min to 240 °C, which was held for 15 min. The inlet temperature was 250 °C, the temperature of the detector was 280 °C, and the sample volume was 0.2 μl. The split ratio was 20:1.

### *Standard curve preparation for* trans*-cinnamaldehyde*

*trans*-Cinnamaldehyde reference substance (CRS) was dissolved in methanol to produce a solution containing 2.98 mg/ml of the substance as the reference solution. The control products above were diluted 5, 10, 20, 30, and 40 times, generating a series of standard solutions and triplicates for each sample. These solutions were then delivered separately into the gas chromatograph for analysis. The measured average peak area Y corresponds to the vertical coordinate, and the *trans*-cinnamaldehyde concentration X is in the horizontal coordinate. A standard curve of *trans*-cinnamaldehyde (Deng et al., 2014) content $y = 233.76x - 4.1337$, with $r = 0.9999$, was obtained. The linear relationship between 0.0745 and 2.98 mg/ml was favorable.

### *Single-factor experiment*

To determine a reasonable range for the orthogonal experiment, we performed single-factor tests for the factors affecting the OR yield, namely, extraction pressure, temperature, and time. When a certain factor was investigated, other factors became unchanged.

### *Orthogonal experiment*

Given the single-factor experimental results and the OR yield and *trans*-cinnamaldehyde content as indices, selected extraction temperature A (45 °C, 50 °C, and 55 °C), extraction pressure B (20, 25, and 30 Mpa), and extraction time C (80, 100, and 120 min) as three factors for the factor level. Using an $L_9$ ($3^4$) orthogonal experimental design, investigated the influence of the three factors on the OR extraction yield and the *trans*-cinnamaldehyde content obtained by SFE.

$$\textit{trans}\text{-Cinnamaldehyde}\,(\text{mg/g}) = \frac{\textit{trans}\text{-cinnamaldehyde}\,(\text{mg})}{\text{material loaded}\,(\text{g})}$$

In the composite-grade method, the weight assignment for the OR yield was 0.3, whereas that for *trans*-cinnamaldehyde was 0.7. The specific calculation method was applied in nine samples. The extraction yield of each sample or the content of the *trans*-cinnamaldehyde was divided by the extraction yield of nine samples or the maximum value of the content of *trans*-cinnamaldehyde, and then the weight coefficient was multiplied. The contribution value of the composite grade of extraction yield or the content of *trans*-cinnamaldehyde in the sample was obtained. The sum of the extraction yield and contribution value of the *trans*-cinnamaldehyde content in the same sample was regarded as the composite grade of the sample.

### *OR extraction and analysis*

The appropriate amount of oil at the optimal extraction process, obtained the total ion chromatogram through the automatic request of spectral graphs from GC–MS. Comparing the mass spectra with those of authentic compounds previously analyzed and stored in the database from the national institute of standards and technology, the components were identified individual (Sylvestre et al., 2006; Cheng et al., 2008; Argyropoulou and Skaltsa, 2012; Chen et al., 2013; Murugan and Mallavarapu, 2013; Singh

et al., 2013; Langhasova et al., 2014; Sereshti et al., 2014; Tian et al., 2014). All the chromatographic peaks were identified, and the relative content of each component was determined by the area normalization method. Individual components were identified by comparing the mass spectra with those of authentic compounds previously analyzed and stored in the data base from the national institute of standards and technology.

GC–MS was conducted using a 6890N Agilent gas chromatograph and MS Rxi-5sil capillary column (30 m × 0.25 μm, 0.25 μm). The inlet temperature was 250 °C, and the heating program included an initial temperature of 50 °C for 1 min; a temperature ramp of 7 °C/min–120 °C, which was held for 3 min; a temperature ramp of 3 °C/min–200 °C, which was held for 3 min; and a temperature ramp of 5 °C/min to 290 °C, which was held for 10 min. The carrier gas, volume flow rate, sample volume, and split ratio were 99.999% helium, 1.0 ml/min, 1 μl, and 25:1, respectively.

Meanwhile, MS was performed using an EI ionization source, an ion source temperature of 230 °C, interface temperature of 320 °C, and scanning quality range of 33–500 *m/z*.

### *PC12 cell culture and OGD establishment*

PC12 cells were cultured in a medium containing 10% FBS, 100 U $ml^{-1}$ penicillin, and 100 μg $ml^{-1}$ streptomycin in high-glucose DMEM. The cells were then incubated at 37 °C with a humidified atmosphere containing 5% $CO_2$ and 95% air, 0.25% trypsin digestion, and passage. Cells in the exponential growth phase were selected for the experiment.

PC12 cells were seeded into 96-well plastic plates at the density $2 \times 10^4$ cells/well, after 24 h from experiment initiation. The modeling method was based on literature (Zhao et al., 2009) with slightly modified experimental conditions and content. The experimental groups were divided into the control group, Nimo (positive control) group, model group, and OR (50, 12.5, and 3.13 μg/ml) group. The culture medium was replaced, and the control and model groups were added with high-glucose and serum-free medium and placed in an incubator for culture. The Nimo group was supplied with blank serum high-glucose DMEM medium with 10 μmol/l nimodipine (Rahbar-Roshandel et al., 2008). The OR group was added with blank serum high-glucose DMEM medium containing 50, 12.5, and 3.13 μg/ml OR. Except for the supernatant of the control group, supernatant fluids from the other groups were discarded after 24 h, and the culture medium was replaced with serum-free and no-glucose DMEM. In an anaerobic pocket of the incubator culture at 37 °C, when the indicator changed to pink, we began recording time until 8 h for the OGD.

### *2-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and LDH release assay*

The specific method was as follows. PC12 cells were seeded into 96-well plastic plates at a density of $2 \times 10^4$ cells/well. Each well was then provided with eight holes, and the cells were treated with OGD as described above. After treatment, 10 μl MTT was dissolved in full culture medium and added to each well. The plates were incubated for 4 h, and then the liquid was siphoned. Dimethylsulfoxide (150 μl) was added to each well to fully dissolve the purple crystals, and the absorbance of each well was detected at 495 nm on the enzyme detector. The experiment was repeated three times.

Cell injury was confirmed by measuring the amount of LDH released. Specific methods were performed for measurement in accordance with assay kit specifications at 450 nm.

### *Assessment of NO content and NOX activity in the PC12 cell supernatant*

After administration and OGD treatment, cell supernatant was collected by sterile tube and centrifuged for 20 min (400–900 × *g*). The supernatant was collected carefully in accordance with assay kit requirements and analyzed under a detection wavelength of 450 nm.

### *Assessment of MDA and GSH contents and SOD and GPx activities in the cell lysate*

After administration and OGD treatment, the cells were washed with ice-cold PBS (pH 7.2–7.4) twice and diluted to form a cell suspension, which was subsequently homogenized to about 1 million/ml. The suspension was subjected to repeated freezing and thawing to induce cell damage and component release. The cell lysate was then centrifuged at 400–900 × *g* for 20 min, and the supernatant was carefully collected in accordance with assay kit requirements to measure GSH and MDA contents as well as, GPx and SOD activities. The detection wavelength was 450 nm.

### *Flow cytometry analysis of cell death*

Apoptosis was determined using an apoptosis detection kit. Apoptosis was assayed by Annexin V-FITC and PI staining followed by analysis with fluorescence-activated cell sorting (BD FACSCalibur, USA). Briefly, cells were cultured in culture dish (300,000 cells per well) and collected after OGD by trypsinization and washed with cold PBS three times (500 rpm for 5 min). Then, the cells were resuspended in 400 μl binding buffer, the FITC-annexin V (5 μl) and PI (5 μl) were added and incubated for 15 min at 4 °C in the dark. After 20 min, cells were analyzed on the flow cytometer.

### *Statistical analysis*

The experimental results were expressed as means ± standard deviations (SDs). The results were analyzed using the statistical program SPSS Statistics, Version 19.0. The data were subjected to ANOVA, and p values of $^{*}p < 0.05$ and $^{**}p < 0.01$ were regarded as statistically significant.

## Results

### *Single-factor experiment*

Drug powder (45 g) was accurately weighed and extracted under different extraction temperature (40, 45, 50, and 55 °C), pressure (20, 25, 30 and 35 Mpa) and time (20, 40, 60, 80, 100, and 120 min). The result shows the OR yield was highest when the temperature was 50 °C, and the OR yield decreased with temperature increase. The OR yield was the highest at 25 Mpa, the curves approximated a straight line or even a downward trend more than 25 Mpa. And the OR yield was the highest at 100 min, the yield was stabilized more than 100 min (Fig. 1).

### *Orthogonal experiment*

According to the above results, employed OR yield and *trans*-cinnamaldehyde content as index, and adopted an orthogonal $L_9$ $(3)^4$ test design. Repeated three times each experiment in parallel, the orthogonal experimental design and results were shown in Table 1, and the variance analysis in Table 2. Intuitive analysis shows that the effects of various factors on the extraction process follows the order B > A > C; the effect of pressure on extraction efficiency was the greatest, followed by temperature and

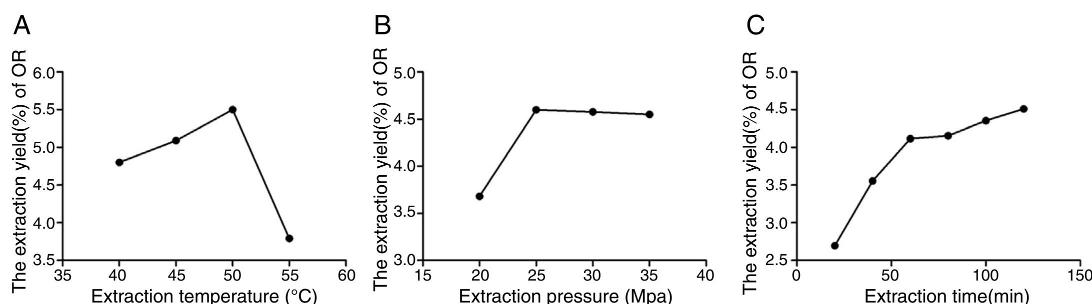


**Fig. 1.** Single-factor experiment. Effect of different extraction temperatures on extraction yield (A), effect of different extraction pressures on extraction yield (B), effect of different extraction times on extraction yield (C).

**Table 1**
The results of the orthogonal experiment analysis and the yield of *trans*-cinnamaldehyde for the oil recipe extraction.

| No | (A) Extraction temperature (°C) | (B) Extraction pressure (Mpa) | (C) Extraction time (min) | Error column | The yield of OR (%) | The content of *trans*-cinnamaldehyde (mg/g) | Colligation score |
|---|---|---|---|---|---|---|---|
| 1 | 45 | 20 | 80 | 1 | 1.11 | 0.53 | 0.24 |
| 2 | 45 | 25 | 100 | 2 | 3.99 | 1.05 | 0.59 |
| 3 | 45 | 30 | 120 | 3 | 4.72 | 1.37 | 0.75 |
| 4 | 50 | 20 | 100 | 3 | 1.40 | 0.52 | 0.26 |
| 5 | 50 | 25 | 120 | 1 | 1.56 | 0.73 | 0.34 |
| 6 | 50 | 30 | 80 | 2 | 1.96 | 0.97 | 0.44 |
| 7 | 55 | 20 | 120 | 2 | 1.41 | 0.51 | 0.26 |
| 8 | 55 | 25 | 80 | 3 | 1.43 | 1.23 | 0.49 |
| 9 | 55 | 30 | 100 | 1 | 2.79 | 2.14 | 0.88 |
| | $K_1$ 0.527 | 0.253 | 0.390 | 0.487 | | | |
| | $K_2$ 0.347 | 0.473 | 0.577 | 0.430 | | | |
| | $K_3$ 0.543 | 0.690 | 0.450 | 0.500 | | | |
| | $R$ 0.197 | 0.437 | 0.187 | 0.070 | | | |

**Table 2**
Analysis of variance (ANOVA) of the oil recipe extraction.

| Factor | SS | f | S | F | Significant |
|---|---|---|---|---|---|
| Extraction temperature | 0.071 | 2 | 0.036 | 8.61 | |
| Extraction pressure | 0.286 | 2 | 0.143 | 34.51 | [a] |
| Extraction time | 0.544 | 2 | 0.272 | 6.57 | |
| Error column | 0.008 | 2 | 0.004 | 1.00 | |

[a] Significant at 0.05 level.

then extraction time. Variance analysis reveals that the extraction pressure, but no temperature and time, significantly affected the experimental results. Along with the single-factor tests, determined that the best conditions for $A_2B_3C_2$ are as follows: extraction pressure of 30 Mpa at 50 °C for 100 min. Under such conditions, the OR yield was 4.8% (relative SD [RSD] = 0.02%, $n$ = 3), and the cinnamic aldehyde content was 2 mg/g (RSD = 1.8, $n$ = 3). These findings indicate that the process was reasonable and effective.

## *GC–MS analysis*

The total ion chromatogram of the compounds and the relative OR contents are shown in Table 3, respectively. By GC–MS analysis, forty compounds were detected, of which 27 compounds were identified, representing 80.86% of the total composition, *trans*-cinnamaldehyde (14.14%), piperine (9.32%), β-amyrin (6.79%), lupenone (6.28%), longifolene (6.07%), β-caryophyllene (5.21%), α-bisabolol (4.11%), and β-bisabolene (2.56%) were found to be the major constituents of oil (Ching, 2011; Lima et al., 2014; Chaskar et al., 2017). The content of *trans*-cinnamaldehyde in OR was the highest, and it was shown to have the effect of dilating blood vessels and lowering blood pressure (Zhou et al., 2015). Thus, selecting *trans*-cinnamaldehyde as index component for process optimization is a reasonable action. However, some high-content compounds have not been identified in the figure and require further study.

**Table 3**
Chemical composition and retention indices of the oil recipe.

| [a]*Rt* | [b]$RI^{calc}$ | [c]$RI^{lit}$ | *Compound* | [d]% |
|---|---|---|---|---|
| 5.64 | 940 | 928 | α-pinene | 1.07 |
| 10.01 | 1185 | 1188 | α-terpinenol | 1.24 |
| 12.45 | 1276 | 1271 | *trans*–cinnamaldehyde | 14.14 |
| 15.83 | 1380 | 1382 | α-cubebene | 1.71 |
| 17.37 | 1424 | 1426 | β-caryophyllene | 5.21 |
| 17.79 | 1436 | 1438 | α-guaiene | 1.32 |
| 19.42 | 1481 | 1482 | germacrene D | 0.74 |
| 19.52 | 1483 | 1460 | *allo*-aromadendrene | 1.82 |
| 19.64 | 1487 | 1489 | β-selinene | 0.67 |
| 19.81 | 1491 | 1492 | valencene isomer | 0.72 |
| 20.14 | 1501 | 1500 | β-bisabolene | 2.56 |
| 20.43 | 1508 | 1508 | α-farnesene | 0.91 |
| 20.84 | 1520 | 1519 | myristicin | 1.17 |
| 24.78 | 1628 | 1630 | apiol | 0.92 |
| 26.62 | 1679 | 1678 | α-santalol | 1.58 |
| 26.93 | 1687 | 1685 | α-bisabolol | 4.11 |
| 27.43 | 1701 | 1700 | *n*-heptadecane | 2.40 |
| 32.88 | 1859 | 1857 | elemol | 2.04 |
| 33.65 | 1882 | 1889 | nerolidol isobutyrate | 1.70 |
| 35.05 | 1952 | 1953 | 3-ethyl-3-hydroxyandrostan-17-one | 0.62 |
| 40.61 | 2246 | 2225 | sclareol | 1.64 |
| 46.95 | 2454 | 2499 | pentacosane | 2.36 |
| 51.69 | 2669 | – | longifolene | 6.07 |
| 54.52 | 2853 | – | tetracontane | 1.75 |
| 57.56 | 3055 | – | piperine | 9.32 |
| 64.6 | 3456 | – | β-amyrin | 6.79 |
| 65.79 | 3483 | – | lupenone | 6.28 |

[a] *Rt*-retention time (min) from a linear temperature program.
[b] $RI^{calc}$-retention index calculated for each compound.
[c] $RI^{lit}$-retention index obtain from published literature.
[d] %-relative abundances from peak area integration.

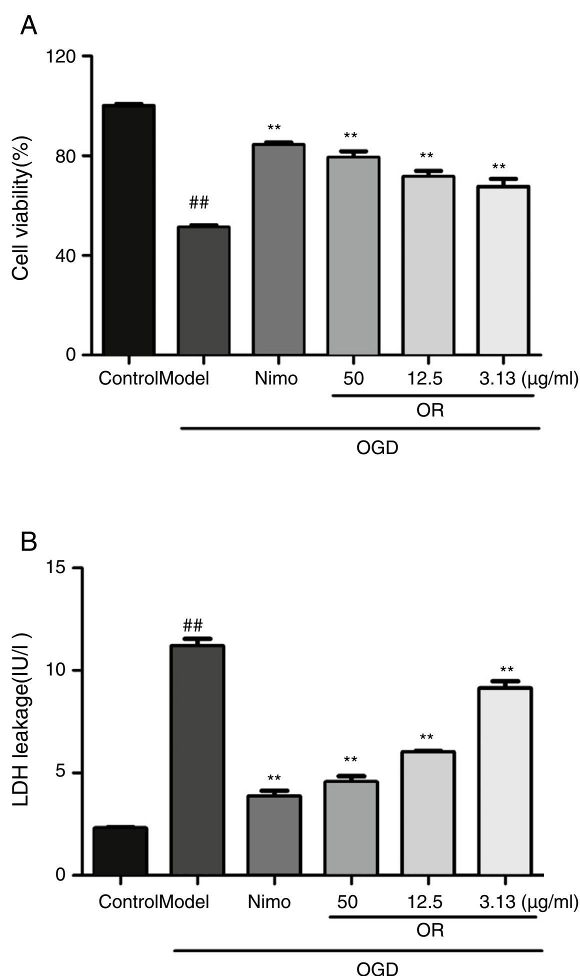


**Fig. 2.** Effects of OR on PC12 cell viability under OGD for 8 h as determined by MTT (A) and LDH release (B) assays, Histograms represent means ± SDs at $n = 8$. $^{**}p < 0.01$ OGD + model group vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. OGD + model group.

*Promotion of the viability of the OGD-injured PC12 cells by OR*

The effects of OR on cell viability were determined by MTT assay (Fig. 2A), and the oil at 50, 12.5, and 3.13 μg/ml concentrations significantly promoted PC12 cell viability. The cell viabilities were 79.27% ± 6.07%, 71.52% ± 6.05%, and 60.01% ± 6.01%, respectively, compared with that of the model group at 51.17% ± 2.31%, indicating significant increases.

LDH was measured to further assess the protective effect of the oil against OGD on the cultured PC12 cells (Fig. 2B). The LDH contents were 4.70 ± 0.38, 6.1 ± 0.16, and 9.14 ± 0.94 IU/l in the 50, 12.5, and 3.13 μg/ml groups, respectively, compared with the model group (10.82 ± 0.45 IU/l). The OR group significantly reduced LDH release, and the effect of the high dose was similar to that in the Nimo group (4.11 ± 0.2 IU/l).

*Determination of NO content, NOX activity in the supernatant and MDA and GSH content, GPx and SOD activities in the cell lysate*

Oxidative stress induced by OGD is an important cause of cell death (Cao et al., 2010). After OGD, the MDA content increased from 1.59 ± 0.1 nmol/l (control group) to 6.3 ± 0.5 nmol/l (model group). OR (50, 12.5, and 3.13 μg/ml) significantly prevented the rise of MDA under OGD-induced injury (Fig. 3A). Meanwhile, SOD activity was decreased in the model group (Fig. 3B). Compared with control group, the SOD activity in the 50 and 12.5 μg/ml OR groups significantly increased, but the 3.13 μg/ml OR group did not significantly change. The GSH level and GPX activity decreased to 336.38 ± 51.17 ng/l and 19.97 ± 2.63 pmol/ml after OGD, however, OR (50, 12.5, and 3.13 mg/ml) can significantly inhibit the decline of GSH and GPX (Fig. 3C and D). The NOX activity and NO content show a decided increase over control group ($^{*}p < 0.05$), while OR (50, 12.5, and 3.13 μg/ml) markedly prevented the increase of NOX activity and NO content (Fig. 4A and B).

*OR prevented OGD – induced apoptosis in PC12 cells*

To analyze the possible anti-apoptotic capability of OR under OGD-condition by flow cytometry using FITC-Annexin V/PI double staining. Relative to the percentage of apoptotic cell in control group (6.58 ± 0.32%), cell apoptosis was 27.05 ± 1.89% in the model group. However, pretreat of the cells with OR (50, 12.5, and 3.13 μg/ml) and Nimo (10 μmol/l) were very effective for attenuating OGD-induced apoptosis cell death, reducing the apoptosis cell count to 16.63 ± 1.58%, 21.9 ± 1.28% and12.85 ± 0.87% (Fig. 5).

## Discussions

TCHM is an important branch of traditional Chinese Medicine that covers disease prevention and treatment, as well as other important health care aspects, in the Hui people. However, to date, TCHM research is in the initial stage. Hui medicine treatment and literature are poor and lacking systematic pharmacological research; hence, its clinical application lacks scientific support. This paper studied the extraction, component analysis, and pharmacological activity of component herbs in a TCHM to provide an experimental basis for the development and utilization of the corresponding TCHM prescription.

As shown in other researches, *trans*-cinnamaldehyde has neuroprotective effects against glutamate-induced oxidative stress and piperine exerts a neuroprotective effect on corticosterone-induced neurotoxicity in PC12 cells, as well as β-bisabolol decreased nervous excitability elicited caused by an irreversible blockade of voltage-dependent sodium channels (Alves et al., 2010; Lv et al., 2017; Mao et al., 2012). Therefore the major component of the oil may be protecting the nervous system in stroke, the development of this oil recipe research is imperative.

SFE has been widely used in extracting volatile oil in recent years with advantages of simple technology, convenient operation, high-purity oil yield, and retention of all components (Peng et al., 2012). *trans*-Cinnamaldehyde had the protective effect against glutamate-induced oxidative stress, and was the major component consisting of OR shown in result (14.14%), so it is reasonable as an index compound. In the experiment, an exceedingly high temperature produced powder adhesive in the paste mill chamber wall. Hence, we adjusted the crushing ambient temperature to around 25 °C and the mill power to a level not exceedingly high to prevent OR volatilization. A sealing method was also used to prevent the loss of crushed medicinal powder.

The alterations of LDH, MDA, SOD, GPX and GSH were caused by oxidative stress-induced cell injury. SOD was the important line of defense against cell injury in cytotoxic reactive oxygen species. Nox, a major source of brain reactive oxygen species, MDA, the end product of lipid peroxidation, expressed throughout the central nervous system are as reliable biomarkers for assessing the severity of oxidative stress. LDH released from the cytoplasm into the extracellular environment in response to cellular damage, due to endogenous cellular mechanisms or as a result of exogenously applied insults. The whole of these shown above is relevant to neuronal cell death, oxidative stress, glutamate neurotoxicity, and

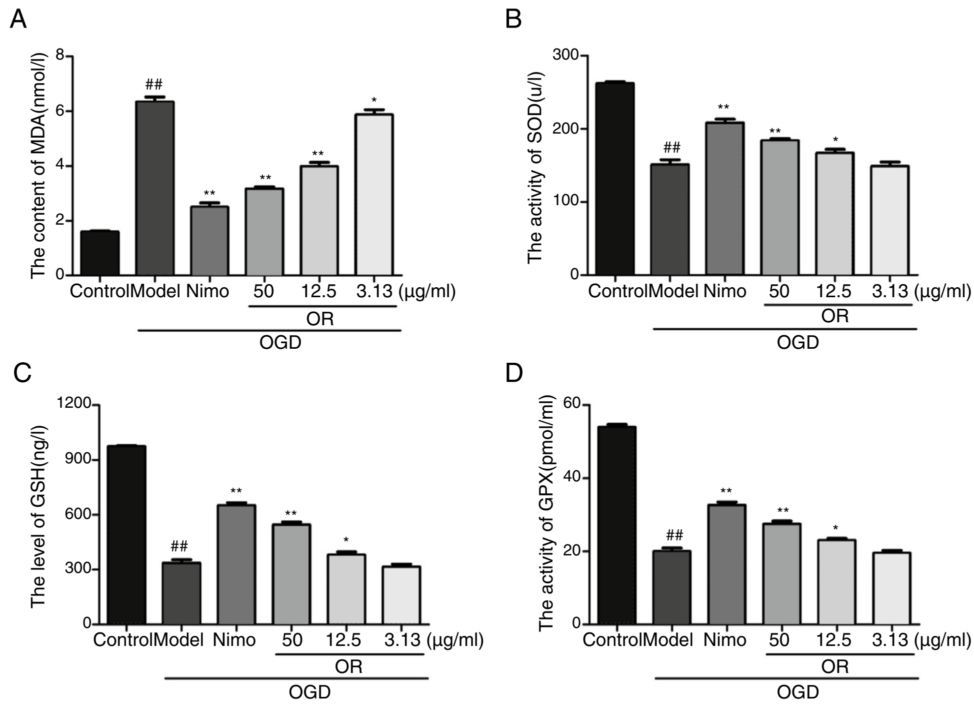


**Fig. 3.** Effects of OR on PC12 cell viability under OGD for 8 h as determined by MDA (A), SOD (B), GSH (C) and GPX (D) assays. Histograms represent means ± SDs at $n = 8$. $^{**}p < 0.01$ OGD + model group vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. OGD + model group.

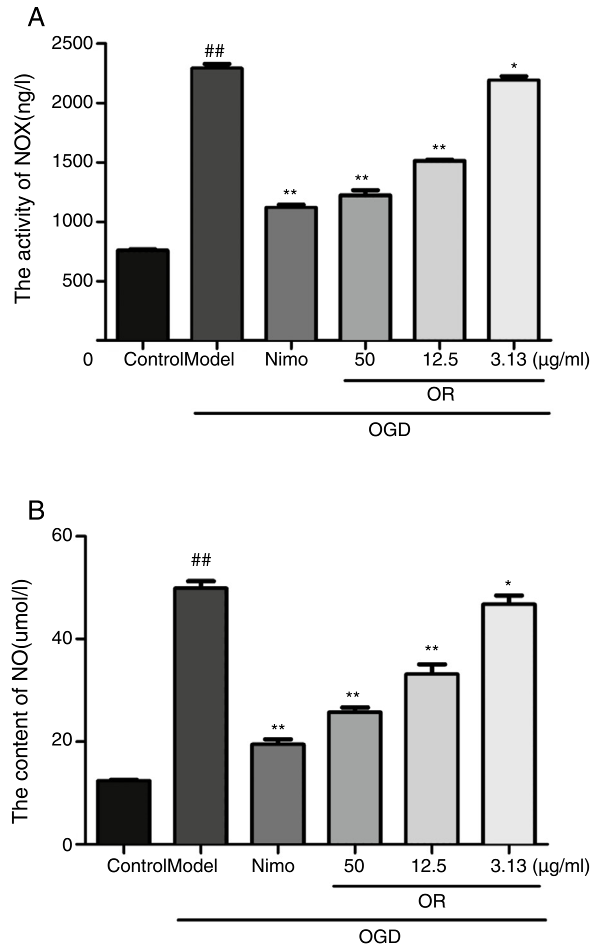


**Fig. 4.** Effects of OR on PC12 cell viability under OGD for 8 h as determined by NOX (A) and NO (B) assays. Histograms represent means ± SDs at $n = 8$. $^{**}p < 0.01$ OGD + model group vs. control group; $^{*}p < 0.05$ and $^{**}p < 0.01$ vs. OGD + model group.

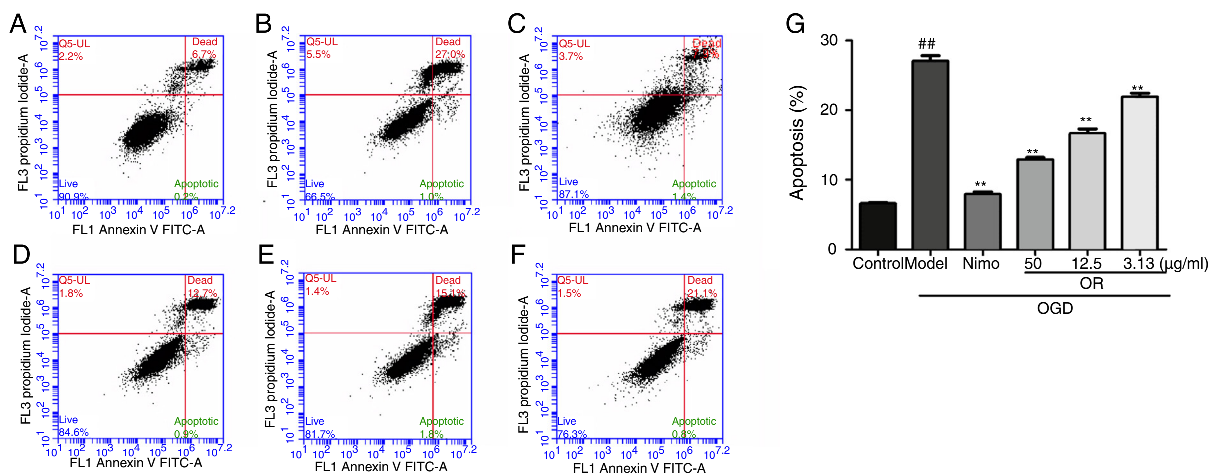


**Fig. 5.** The level of apoptotic in PC12 cell was measured by flow cytometer (four quadrants represent dead neurons, late apoptotic neurons, normal neurons, early apoptotic-neurons, respectively). (A)–(F) The results of apoptotic neurons in control group, model group, OR (50, 12.5, and 3.13 μg/ml) groups, and nimodipine respectively. (G) The apoptotic neurons of different groups. Data are expressed as mean ± SEM ($n = 6$). ##$p < 0.01$ vs control group; *$p < 0.05$, **$p < 0.01$ vs vehicle group.

neuroinflammation (Shu et al., 2016; Kaja et al., 2017; Liu et al., 2017).

This study established PC12 cells in an *in vitro* model of OGD, simulated *in vivo* hypoxic/ischemic conditions, and explored the protective effect of OR against OGD injury. Compared with the hypoxia model group, the OR group showed a significant increase in PC12 cell viability and reduction in LDH release with obvious dose dependence. The effect of the OR group (50 μg/ml) was similar to that of the Nimo group, suggesting that the oil can significantly ameliorate OGD injury in PC12 cells. The study confirmed (Lecht et al., 2012) that cell OGD injury is induced by a large amount of oxygen free radicals, and the free radicals easily cause lipid peroxidation of cell and mitochondrial membranes rich in phospholipids. Consequently, the cell and mitochondrial membranes were damaged, and neuronal injury and neurological functional defect ensued. The end product of lipid peroxidation is MDA, which is an important index for evaluating body oxidation reaction levels. Compared with the model group, the preventive treatment groups showed significant reductions in MDA content in the PC12 cells (*$p < 0.05$). OR can increase MDA formation by inhibiting the hypoxia and glucose deprivation in the cells. As a result, lipid peroxidation levels diminished, and the cells were protected. SOD is that the major enzyme involved in the *in vivo* metabolic activity against oxygen-centered radicals. Our study found that the SOD activities in the high- and medium-dose groups were higher than that in the model group (*$p < 0.05$). This result reveals the cellular antioxidant capacity enhancement and increased cell resistance against oxygen stress and peroxidation to avoid injury. As an important antioxidant, GSH can combine with free radicals and can hence protect normal cell metabolism (Kepekci et al., 2013). After giving drug treatment, GSH content increased significantly, suggesting that oil can enhance cell antioxidant capacity. GPX can effectively remove free radicals *in vivo* through the catalytic GSH reduction of hydrogen peroxide and thus protects cells from oxidative damage. Compared with the model groups, the OR groups showed different degrees of enhanced GPX activity. Excessive NO production can cause cell toxicity; NO and $O_2^-$ may react and generate $OONO^-$ (Raij, 2006). The NO content in the model group increased significantly (**$p < 0.01$) and that in the treatment groups reduced significantly (*$p < 0.05$) relative to that in the control. In the OGD injury of the brain, NOX can produce a substantial number of reactive oxygen species (ROS) after activation (Tang et al., 2010; Chen et al., 2011; Shen et al., 2011). ROS induces cell apoptosis from oxidative stress and even cell necrosis. Thus, high NOX activity indicates that the cells are damaged seriously. In the experiment, the NOX activity in the model group was significantly increased whereas those in the OR groups significantly decreased (*$p < 0.05$) with respect to that in the control group.

This study verified that the OR can protect PC12 cells from OGD injury at the cellular level. The mechanisms may be related to antioxidant effects, free-radical scavenging, prevention of PC12 cell lipid peroxidation, and maintenance of the normal cell membrane structure.

## Authors' contributions

JL and EG collected the plant samples and conducted laboratory work. LD identified the medicinal materials, and EG analyzed the data. LC, QP, and XF critically proofread the manuscript. LZ designed the study, supervised the laboratory work, and contributed to the critical reading of the manuscript. All the authors read the final manuscript and approved its submission.

## Ethical disclosures

**Protection of human and animal subjects.** The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

**Confidentiality of data.** The authors declare that they have followed the protocols of their work center on the publication of patient data.

**Right to privacy and informed consent.** The authors declare that no patient data appear in this article.

## Conflicts of interest

The authors declare no conflicts of interest.

## Acknowledgments

This work was supported by grants from the National Science and Technology Major Project of China (Project no. 2013BAI11B07).

## References


Alves, A.D.M.H., Gonçalves, J.C.R., Cruz, J.S., Araújo, D.A.M., 2010. Evaluation of the sesquiterpene (−)-α-bisabolol as a novel peripheral nervous blocker. Neurosci. Lett. 472, 11–15.

Argyropoulou, C., Skaltsa, H., 2012. Identification of essential oil components of *Marrubium thessalum* Boiss & Heldr., growing wild in Greece. Nat. Prod. Res. 26, 593–599.

Bai, Y.F., Xu, H., 2000. Protective action of piperine against experimental gastriculer. Acta Pharmacol. Sin. 21, 357–359.

Cao, X., Xiao, H., Zhang, Y., Zou, L., Chu, Y., Chu, X., 2010. 1,5-Dicaffeoylquinic acid-mediated glutathione synthesis through activation of Nrf2 protects against OGD/reperfusion-induced oxidative stress in astrocytes. Brain Res. 1347, 142–148.

Chaskar, P.K., Doshi, G.M., Tank, S.H., 2017. Gas chromatography–mass spectroscopy studies on *Cestrum nocturnum* macerated methanolic extract. Asian J. Pharm. Clin. Res. 10, 259–263.

Chen, H., Kim, G.S., Okami, N., Narasimhan, P., Chan, P.H., 2011. NADPH oxidase is involved in post-ischemic brain inflammation. Neurobiol. Dis. 42, 341–348.

Chen, Y., Zhou, C., Ge, Z., Liu, Y., Liu, Y., Feng, W., Li, S., Chen, G., Wei, T., 2013. Composition and potential anticancer activities of essential oils obtained from myrrh and frankincense. Oncol. Lett. 6, 1140–1146.

Chen, Y.F., Wang, Y.W., Huang, W.S., Lee, M.M., Wood, W.G., Leung, Y.M., Tsai, H.Y., 2016. Trans-cinnamaldehyde, an essential oil in cinnamon powder ameliorates cerebral ischemia-induced brain injury via inhibition of neuroinflammation through attenuation of iNOS COX-2 expression and NFkappa-B signaling pathway. NeuroMol. Med. 18, 322–333.

Cheng, S.S., Liu, J.Y., Lin, C.Y., Hsui, Y.R., Lu, M.C., Wu, W.J., Chang, S.T., 2008. Terminating red imported fire ants using *Cinnamomum osmophloeum* leaf essential oil. Bioresour. Technol. 99, 889–893.

Ching, J., 2011. Chemical and pharmacological studies of *Ardisia elliptica*: antiplatelet, anticoagulant activities and multivariate data analysis for drug discovery, http://scholarbank.nus.edu.sg/handle/10635/30563 (accessed February 2012).

Deng, X., Liao, Q., Xu, X., Yao, M., Zhou, Y., Lin, M., Zhang, P., Xie, Z., 2014. Analysis of essential oils from Cassia bark and Cassia twig samples by GC–MS combined with multivariate data analysis. Food Anal. Method. 7, 1840–1847.

Feng, N.P., 2004. The distill technics and easing pain function of mastic and myrrh. Chin. Tradit. Pat. Med. 17, 75–78.

Flynn, R.W., MacWalter, R.S., Doney, A.S., 2008. The cost of cerebral ischaemia. Neuropharmacology 55, 250–256.

Jin, R., Liu, L., Zhang, S., Nanda, A., Li, G., 2013. Role of inflammation and its mediators in acute ischemic stroke. J. Cardiovasc. Transl. 6, 834–851.

Kaja, S., Payne, A.J., Naumchuk, Y., Koulen, P., 2017. Quantification of lactate dehydrogenase for cell viability testing using cell lines and primary cultured astrocytes. Curr. Protoc. Toxicol. 72, 2.26.1–2.26.10.

Kepekci, R.A., Polat, S., Celik, A., Bayat, N., Saygideger, S.D., 2013. Protective effect of *Spirulina platensis* enriched in phenolic compounds against hepatotoxicity induced by CCl4. Food Chem. 141, 1972–1979.

Langhasova, L., Hanusova, V., Rezek, J., Stohanslova, B., Ambroz, M., Kralova, V., Vanek, T., Lou, J.D., Yun, Z.L., Yang, J., Skalova, L., 2014. Essential oil from *Myrica rubra* leaves inhibits cancer cell proliferation and induces apoptosis in several human intestinal lines. Ind. Crops Prod. 59, 20–26.

Lecht, S., Rotfeld, E., Arien-Zakay, H., Tabakman, R., Matzner, H., Yaka, R., Lelkes, P.I., Lazarovici, P., 2012. Neuroprotective effects of nimodipine and nifedipine in the NGF-differentiated PC12 cells exposed to oxygen–glucose deprivation or trophic withdrawal. Int. J. Dev. Neurosci. 30, 465–469.

Li, T.T., Wang, X., Ma, X.Q., Chen, J., Fu, X.Y., 2013a. Prescription compatibility law research for stroke treatment in Hui medicine. Chin. J. Exp. Tradit. Med. 19, 307–311.

Li, T.T., Dong, L., Chen, G.T., Chen, J., Fu, X.Y., 2013b. Summary of Hui prescriptions for stroke. China J. Chin. Mater. Med. 38, 2412–2415.

Lima, E.M., Nascimento, A.M., Lenz, D., Scherer, R., Meyrelles, S.S., Boëchat, G.A.P., Andrade, T.U., 2014. Triterpenes from the *Protium heptaphyllum*, resin-chemical composition and cytotoxicity. Rev. Bras. Farmacogn. 24, 399–407.

Liu, Y.J., Wang, D.Y., Yang, Y.J., Lei, W.F., 2017. Effects and mechanism of dexmedetomidine on neuronal cell injury induced by hypoxia-ischemia. BMC Anesthesiol. 17, 0413–414.

Lv, C., Yuan, X., Zeng, H.W., Liu, R.H., Zhang, W.D., 2017. Protective effect of cinnamaldehyde against glutamate-induced oxidative stress and apoptosis in PC12 cells. Eur. J. Pharmacol. 815, 487–494.

Mao, Q.Q., Huang, Z., Ip, S.P., Xian, Y.F., Che, C.T., 2012. Protective effects of piperine against corticosterone-induced neurotoxicity in PC12 cells. Cell. Mol. Neurobiol. 32, 531–537.

Murugan, R., Mallavarapu, G.R., 2013. α-Bisabolol, the main constituent of the essential oil of *Pogostemon speciosus*. Ind. Crops Prod. 49, 237–239.

Peng, S., Li, Z., Zhou, D., Wang, Y., 2012. Advances in applications of supercritical carbon dioxide technology. ShanXi Chem. Ind. 32, 31–35.

Qi, X., Zhou, R., Liu, Y., Wang, J., Zhang, W., Tan, H., Niu, Y., Sun, T., Li, Y., Yu, J., 2016. Trans-cinnamaldehyde protected PC12 cells against oxygen and glucose deprivation/reperfusion (OGD/R)-induced injury via anti-apoptosis and anti-oxidative stress. Mol. Cell. Biochem. 421, 67–74.

Rahbar-Roshandel, N., Razavi, L., Tavakoli-Far, B., Mahmoudian, M., 2008. Mebudipine and dibudipine protect PC12 cells against oxygen–glucose deprivation and glutamate-induced cell death. Pathophysiology 15, 227–231.

Raij, L., 2006. Nitric oxide in the pathogenesis of cardiac disease. J. Clin. Hypertens. 8, 30–39.

Ryu, S., Seol, G.H., Park, H., Choi, I.Y., 2014. Trans-anethole protects cortical neuronal cells against oxygen–glucose deprivation/reoxygenation. Neurol. Sci. 35, 1541–1547.

Sereshti, H., Heidari, R., Samadi, S., 2014. Determination of volatile components of saffron by optimised ultrasound-assisted extraction in tandem with dispersive liquid–liquid microextraction followed by gas chromatography–mass spectrometry. Food Chem. 143, 499–505.

Shen, J., Bai, X., Qin, Y., Jin, W., Zhou, J., Zhou, J., Yan, Y., Wang, Q., Bruce, I.C., Chen, J., Xia, Q., 2011. Interrupted reperfusion reduces the activation of NADPH oxidase after cerebral I/R injury. Free Radic. Biol. Med. 50, 1780–1786.

Shichita, T., Ago, T., Kamouchi, M., Kitazono, T., Yoshimura, A., Ooboshi, H., 2012. Novel therapeutic strategies targeting innate immune responses and early inflammation after stroke. J. Neurochem. 123 (Suppl. 2), 29–38.

Shu, Y., Yang, Y., Zhang, P., 2016. Neuroprotective effects of penehyclidine hydrochloride against cerebral ischemia/reperfusion injury in mice. Brain Res. Bull. 121, 115–123.

Singh, S., Kapoor, I.P.S., Singh, G., Schuff, C., De Lampasona, M.P., Catalan, C.A.N., 2013. Chemistry antioxidant and antimicrobial potentials of white pepper (*Piper nigrum* L.) essential oil and oleoresins. Proc. Natl. Acad. Sci. India. B 83, 357–366.

Sodeifian, G., Sajadian, S.A., Saadati Ardestani, N., 2017. Experimental optimization and mathematical modeling of the supercritical fluid extraction of essential oil from *Eryngium billardieri*: application of simulated annealing (SA) algorithm. J. Supercrit. Fluids 127, 146–157.

Sylvestre, M., Pichette, A., Longtin, A., Nagau, F., Legault, J., 2006. Essential oil analysis and anticancer activity of leaf essential oil of *Croton flavens* L. from Guadeloupe. J. Ethnopharmacol. 103, 99–102.

Tang, X., Liu, K.J., Ramu, J., Chen, Q., Li, T., Liu, W., 2010. Inhibition of gp91(phox) contributes towards normobaric hyperoxia afforded neuroprotection in focal cerebral ischemia. Brain Res. 1348, 174–180.

Tian, J., Zeng, X., Zhang, S., Wang, Y., Zhang, P., Lü, A., Peng, X., 2014. Regional variation in components and antioxidant and antifungal activities of *Perilla frutescens* essential oils in China. Ind. Crops Prod. 59, 69–79.

Uquiche, E., Cirano, N., Millao, S., 2015. Supercritical fluid extraction of essential oil from *Leptocarpha rivularis* using $CO_2$. Ind. Crops Prod. 77, 307–314.

Zhang, Y., Wang, X., Ma, L., Dong, L., Zhang, X., Chen, J., Fu, X., 2014. Anti-inflammatory, antinociceptive activity of an essential oil recipe consisting of the supercritical fluid $CO_2$ extract of white pepper, long pepper, cinnamon, saffron and myrrh in vivo. J. Oleo Sci. 63, 1251–1260.

Zhao, J., Li, L., Ling, C., Li, J., Pang, J., Lin, Y., Liu, J., Huang, R., Wang, G., Pei, Z., Zeng, J., 2009. Marine compound xyloketal B protects PC12 cells against OGD-induced cell damage. Brain Res. 1302, 240–247.

Zhao, J., Zhang, X., Dong, L., Wen, Y., Zheng, X., Zhang, C., Chen, R., Zhang, Y., Li, Y., He, T., Zhu, X., Li, L., 2015. Cinnamaldehyde inhibits inflammation and brain damage in a mouse model of permanent cerebral ischaemia. Br. J. Pharmacol. 172, 5009–5023.

Zhou, M., Chen, Z.Y., Shen, S.T., 2015. Recent advances on cinnamaldehyde. J. Econ. Anim. 19, 1–5.