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# Total flavones of *Dracocephalum moldavica* L. protect astrocytes against H<sub>2</sub>O<sub>2</sub>-induced apoptosis through a mitochondria-dependent pathway

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## Abstract

**Background:** The active components of *Dracocephalum moldavica* L. (TFDM) can inhibit myocardial ischemia by inhibiting oxidative stress. However, the effects of TFDM on astrocytes have not been investigated in vitro. The current study aimed to explore whether TFDM protects astrocytes against H<sub>2</sub>O<sub>2</sub>-induced apoptosis through a mitochondria-dependent pathway.

**Methods:** The human glioma cell line U87 was used to investigate the ability of TFDM to protect astrocytes against H<sub>2</sub>O<sub>2</sub>-induced apoptosis. The cell counting kit-8 assay and flow cytometry were used to detect cell viability, apoptosis, MMP, Ca<sup>2+</sup> influx and reactive oxygen species (ROS). Lactate dehydrogenase (LDH) and malonic dialdehyde (MDA) levels were measured by ELISA. In addition, protein and mRNA expression changes were detected by Western blotting and qRT-PCR.

**Results:** TFDM (0.78~200 µg/ml) had limited cytotoxic effects on the viability of U87 cells. Compared with the model group (treated with H<sub>2</sub>O<sub>2</sub> only), cells treated with medium- and high-dose TFDM exhibited reduced MDA concentrations ( $P < 0.05$ ) and ROS production ( $P < 0.05$ ) and decreased MMP ( $P < 0.05$ ) and reduced apoptosis ( $P < 0.05$ ). The percentage of annexin V-FITC-stained cells was markedly suppressed by TFDM, confirming its anti-apoptotic properties. WB results showed that protein expression of Bcl-2-associated X protein (BAX), Caspase-3, Caspase-9, Caspase-12, and B-cell leukemia/lymphoma 2 (Bcl2) was reduced in the TFDM group compared with that in the model group ( $P < 0.05$ ) and that expression of these proteins was normalized by TFDM treatment in a dose-dependent manner. According to RT-qPCR results, TFDM pretreatment resulted in reduced mRNA expression of BAX, Caspase-9, Caspase-12, p38MAPK, and CaMKII and increased mRNA expression of mTOR compared with the model group.

**Conclusions:** The current study revealed the protective effects of TFDM on U87 cells under oxidative stress conditions through the inhibition of a mitochondria-dependent pathway that is associated with the CaMKII/P38MAPK/ERK1/2 and PI3K/AKT/mTOR pathways.

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## Background

Stroke is the second most common cause of death worldwide, and effective treatment methods have not yet been developed [1]. *Dracocephalum moldavica* L., the dry aerial parts of which are widely used as a traditional Chinese medicine in Xinjiang, is a member of the family Lamiaceae [2]. The active components of *D. moldavica* L. (TFDM) have heart- and brain-tonifying functions, activate blood circulation and improve blood stasis [3–6]; therefore, it is mainly used for the treatment of cerebrovascular diseases. TFDM contains the main active components of *D. moldavica* L., accounting for 53.06% of the total activity. The main active ingredients of TFDM are tilianin (13.0 to 17.0%), acacetin-7-O- $\beta$ -D-glucuronide (10.0 to 13.5%), luteolin-7-O- $\beta$ -D-glucuronide (8.0 to 9.0%), diosmetin -7-O-  $\beta$ -D-glucuronide (7.0 to 8.6%) and apigenin-7-O- $\beta$ -D-glucuronide (2.0 to 3.0%). It has been reported that these flavonoids have anti-inflammatory and antioxidant stress effects [7–12]. In general, flavonoids are the most powerful and widely used bioactive compounds in plants and have cardioprotective and neuroprotective effects and have cardioprotective and neuroprotective effects [13–16]. Single-drug preparations of *D. moldavica* L., called Yixin Badiran Jibuya granules, have been used in the treatment of angina pectoris. Our previous studies found that TFDM can inhibit myocardial ischemia and stroke through the inhibition of oxygen free radical production [17–20], and recent evidence confirms that TFDM functions as an effective and stable free radical scavenger [21]. The purpose of this study was to investigate the role of TFDM in astrocytes, which are the most abundant cells in the central system and comprise approximately 20% of the total brain cells in mammals.

In the brain, neuronal cells are sensitive to oxidative and stress injury, and their survival depends on the protection of neighboring astrocytes against oxidation [22]. It has been reported that neuronal degeneration and astroglial scar destruction caused by astrocyte failure are associated with poststroke inflammatory diffusion and infarct volume in mice [23, 24]. Mitochondria are the main target of oxidative stress injury, and ROS result in an increased free  $\text{Ca}^{2+}$  concentration in mitochondria, which ultimately leads to apoptosis in astrocytes. Therefore, the survival of astrocytes under oxidative stress may be critical for reducing neuronal death [25]. Human glioma U87 cells have been used as model astrocytes in vitro [26].

Calmodulin kinase II (CaMKII) is an important target for cerebral ischemic nerve injury; phosphorylated CaMKII migrates from the cytoplasm to the cell membrane [27]. The effect is enhanced neuronal discharge and calcium inflow, aggravation of mitochondrial dysfunction and upregulation of the expression of downstream

proteins related to apoptosis. The current study explored whether TFDM protects astrocytes against oxidative stress-induced apoptosis by attenuating a CaMKII-dependent mitochondria pathway.

## Methods

### Reagents and antibodies

TFDM was prepared by Xinjiang Institute of Materia Medica (China).

DMEM high-glucose culture medium was obtained from HyClone, and fetal bovine serum was procured from Gibco (USA). Dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Sigma. A CCK-8 kit was obtained from Boster (China). We purchased all antibodies from Cell Signaling Technology (USA), Annexin V-FITC and Propidium Iodide Staining Solution were purchased from BD Company (USA), and Mitochondrial Membrane Potential Assay Kit with JC-1 was obtained from Solarbio (China).

### Cell culture and treatment

U87 cells were obtained from the Chinese Academy of Medical Sciences (CAT NO.TCHu38). The cells were incubated in DMEM high-glucose culture medium supplemented with 10% FBS at 37 °C in an atmosphere containing 5%  $\text{CO}_2$ .

We established a modified model of  $\text{H}_2\text{O}_2$ -induced injury in vitro to simulate pathological changes. U87 cells were incubated with 0.63 mM  $\text{H}_2\text{O}_2$  for 20 min to induce oxidative stress. The cells were divided into three groups: control,  $\text{H}_2\text{O}_2$ -induced injury and  $\text{H}_2\text{O}_2$ -induced injury pretreated with gradient concentrations of TFDM (100, 50, 25, 12.5, 6.25, 3.13, and 1.56  $\mu\text{g}/\text{ml}$ ) for 24 h.

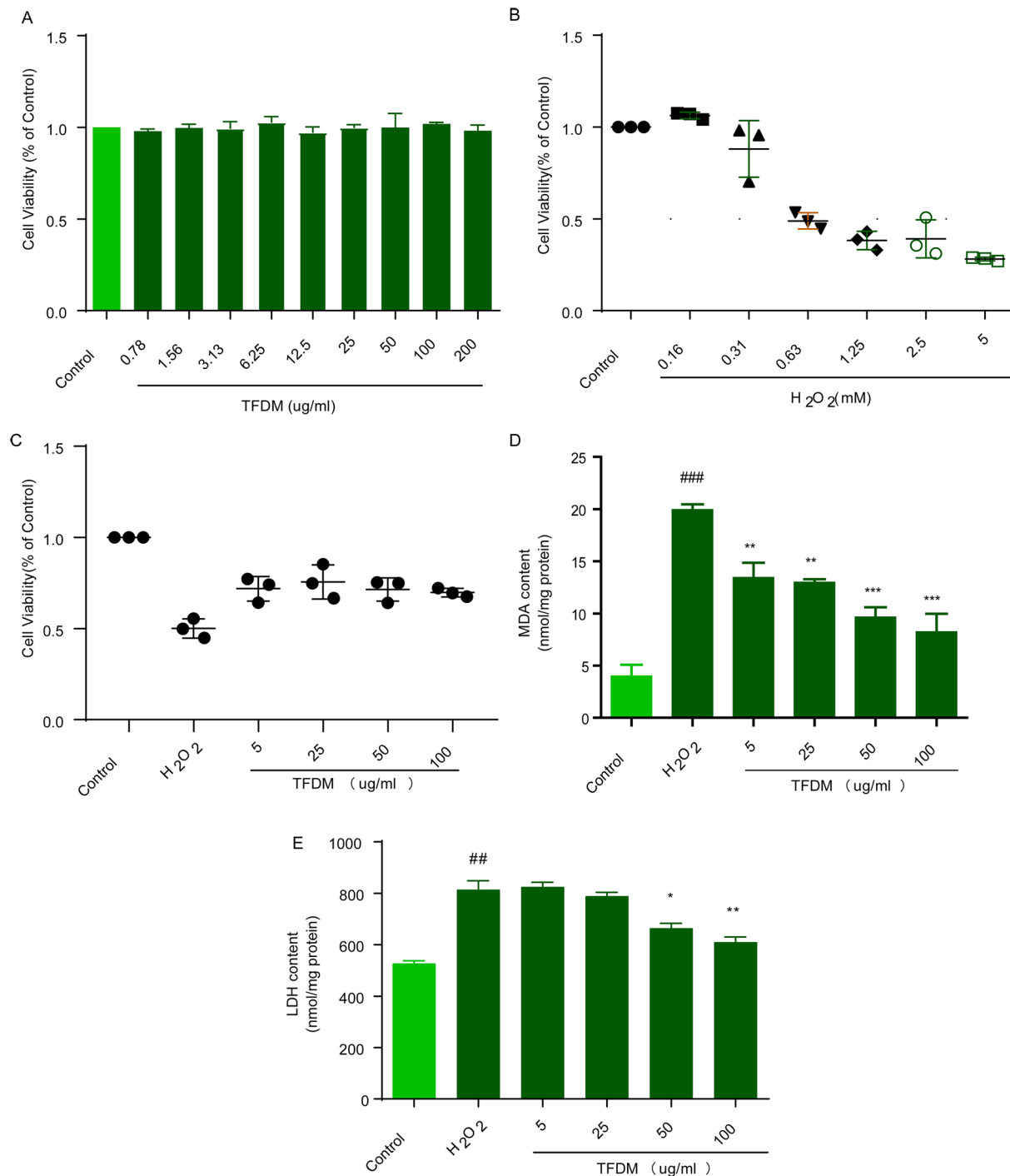
**Table 1** RT-qPCR primer sequences

| Primer mane | Primer sequence                | Primer mane  | Primer sequence               |
|-------------|--------------------------------|--------------|-------------------------------|
| CaMKII      | F:GCTGCTATTGTTGTTGGT<br>GTGGAG | GAPDH        | F:TGCACCACCAACTG<br>CTTAGC    |
|             | R:ACAGGTGAAGCTTGAG<br>AGGAAGTG |              | R:GGCATGGACTGTGG<br>TCATGAG   |
| mTOR        | F:GACCGTCCGCCTTC<br>ACAGATAC   | P38-<br>MAPK | F:CTTCCAGTCGGCGG<br>TCCATG    |
|             | R:GCCGAGTCCGTTT<br>CTTCTC      |              | R:TCAGATCGGCGTCC<br>ATCAAGTG  |
| CHOP        | F:AACAGAGGTCACAC<br>GCACATCC   | Bax          | F:CCGCACGTCCACGA<br>TCAGTC    |
|             | R:CGCTCGTTCTCCTG<br>CTCCTTC    |              | R:CATCACTGCCGCTG<br>CCTCTC    |
| Caspase-9   | F:AGAAGACCGGAGTG<br>CAATGGATG  | Caspase-12   | F:ACACGTCTGGCTCT<br>CATCATCTG |
|             | R:TGGCAGGTAGAGGA<br>CAGAGACAG  |              | R:TCTGAGGACTGGTG<br>CTCTGGAC  |

**Cell viability assay**

U87 cells ( $1 \times 10^4$ ) were plated in 96-well plates. Six parallel replicates were used for each group. CCK-8 working solution (Boster Biological Technology, USA)

was added to each well and incubated for 1.5h according to the instruction manual. Optical density at 450 nm was detected using a microplate reader (BioTek, USA). Cell viability calculation formula was



**Fig. 1** TFDM treatment protected U87 cells against H<sub>2</sub>O<sub>2</sub>-induced cytotoxicity. **a** The cytotoxic potential of TFDM. **b** The effects of different concentrations of H<sub>2</sub>O<sub>2</sub> on U87 cell viability. **c** TFDM increased cell viability, as evaluated by the MTS assay. **d** TFDM decreased MDA release after H<sub>2</sub>O<sub>2</sub>-induced injury. **e** TFDM decreased LDH release after H<sub>2</sub>O<sub>2</sub>-induced injury. The data are presented as the mean ± SD, n = 3. #p < 0.05 vs. control; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 vs. H<sub>2</sub>O<sub>2</sub>

as follows: [(experimental well-blank well) / (control well-blank well)] × 100%.

### Detection of LDH and MDA

According to the instructions of the LDH and MDA test kits, purchased from Nanjing Jiancheng Company (China), LDH released from cells was measured, and the content of malondialdehyde (MDA) in a cell homogenate was assessed.

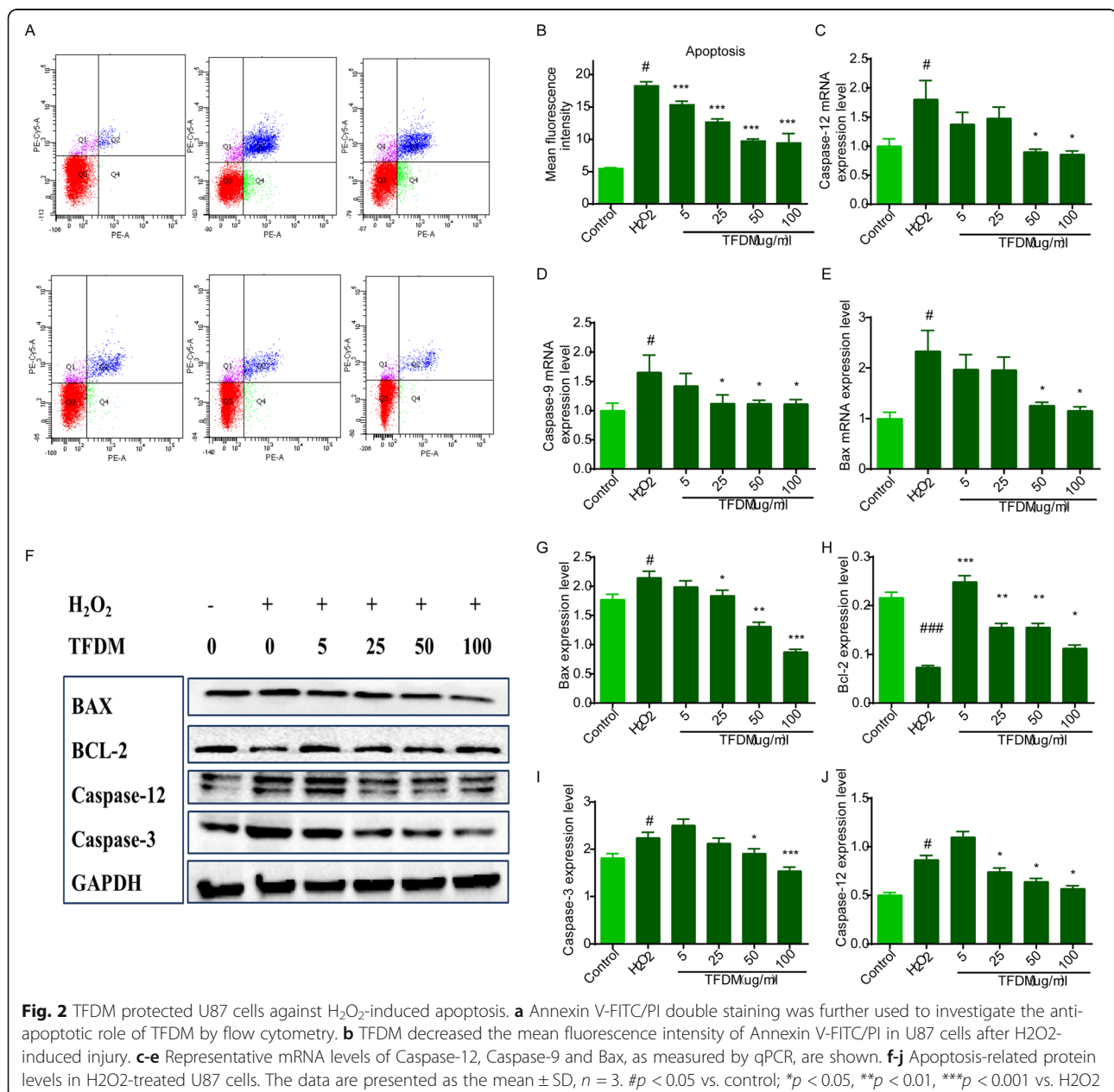
### Analysis of apoptosis

Cells were treated as described above; the medium was discarded, and the cells were washed twice with pre-chilled PBS. After trypsin (excluding EDTA) digestion, the

cells were collected and washed with pre-chilled PBS. Next, 100 µl of pre-configured 1× buffer was added to re-suspend the cells and mixed well; 5 µl of Annexin-V FITC reagent and PI reagent were each added, as was 400 µl of 1× buffer, and the plates were incubated in the dark. The samples were filtered through a 300-mesh cell sieve, and the apoptotic rate was detected by flow cytometry. The total apoptotic rate was calculated as annexin-V FITC positivity and annexin-V FITC/PI double positivity.

### Evaluation of mitochondrial membrane potential

According to the kit instructions, 1× JC-1 staining buffer was prepared, and 0.5 ml of 1× JC-1 staining buffer was



**Fig. 2** TFDM protected U87 cells against H<sub>2</sub>O<sub>2</sub>-induced apoptosis. **a** Annexin V-FITC/PI double staining was further used to investigate the anti-apoptotic role of TFDM by flow cytometry. **b** TFDM decreased the mean fluorescence intensity of Annexin V-FITC/PI in U87 cells after H<sub>2</sub>O<sub>2</sub>-induced injury. **c-e** Representative mRNA levels of Caspase-12, Caspase-9 and Bax, as measured by qPCR, are shown. **f-j** Apoptosis-related protein levels in H<sub>2</sub>O<sub>2</sub>-treated U87 cells. The data are presented as the mean ± SD, *n* = 3. <sup>#</sup>*p* < 0.05 vs. control; <sup>\*</sup>*p* < 0.05, <sup>\*\*</sup>*p* < 0.01, <sup>\*\*\*</sup>*p* < 0.001 vs. H<sub>2</sub>O<sub>2</sub>

added to each tube. After mixing, the cells were incubated at 37 °C for 30 min and centrifuged at 1000 r • min<sup>-1</sup> for 4 min. The cells were collected, and 1 ml of pre-cooled buffer was added and mixed well, followed by centrifugation at 1000 r • min<sup>-1</sup> for 3 min; the cells were washed 2–3 times, and 0.5 mL of 1× JC-1 buffer was added and mixed well. The samples were filtered through a 300-mesh cell sieve and examined by flow cytometry. The transition (ratio) of red fluorescence to green fluorescence indicates a decrease in cell membrane potential.

### [Ca<sup>2+</sup>] content assay

Ca<sup>2+</sup> in U87 cells was detected using Fluo-4/AM (a fluorescent calcium indicator) according to the manufacturer's instructions. U87 cells were trypsinized and collected according to the above method, and 5 μmol/Fluo-4/AM was added for 45 min at 37 °C. The cells were washed twice in pre-chilled PBS for 5 min each. The fluorescence intensity of the cells was measured by flow cytometry to represent the content of Ca<sup>2+</sup>.

### Western blot analysis

U87 cell protein was extracted on ice, and a protein lyase inhibitor was added. The protein concentration was determined by the BCA method. Protein loading buffer was added, and the proteins were denatured by boiling at 95 °C for 5 min in a metal bath. After SDS-polyacrylamide gel electrophoresis, the proteins were transferred to a PVDF membrane. After blocking with

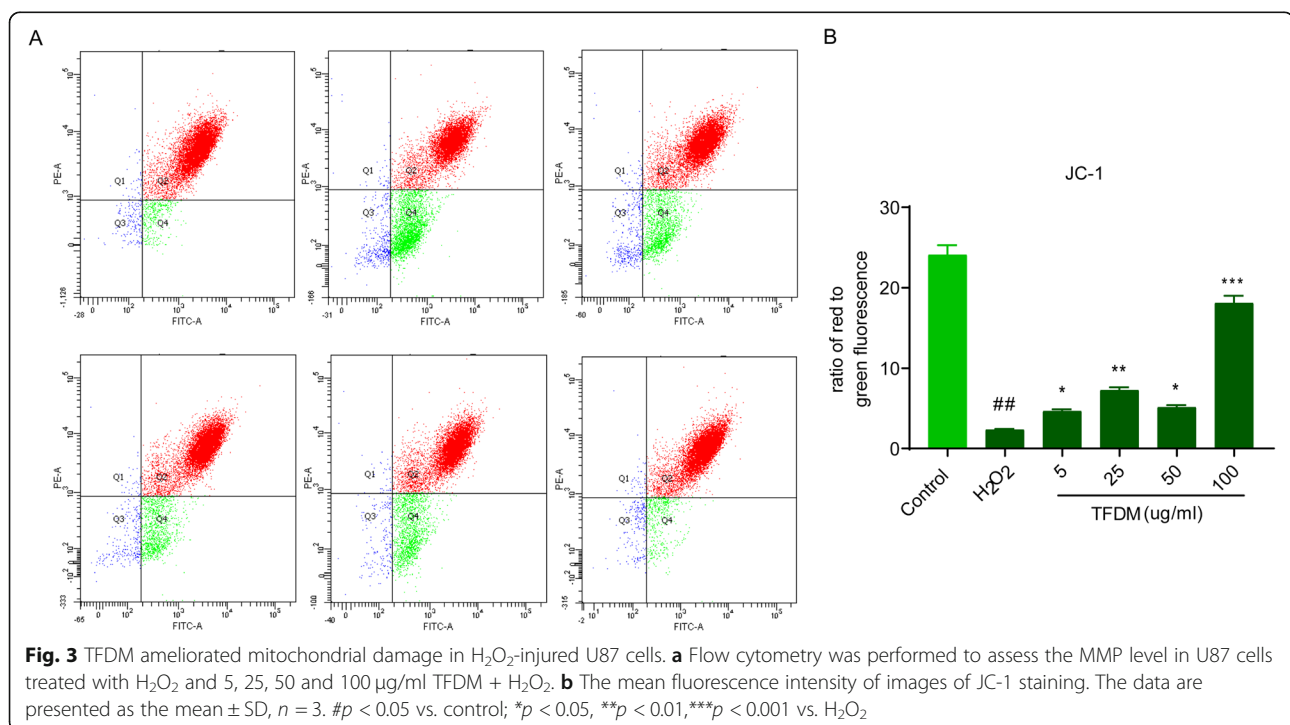
5% skimmed milk powder, the primary antibody was added and incubated overnight at 4 °C. Antibodies against GAPDH, phospho-CaMKII (Thr286), phospho-p38MAPK, phospho-p44/42MAPK, phospho-P13K, phospho-AKT, and downstream related proteins Bax, Bcl-2, Cleaved caspase-3 and Caspase-12 were used. After the blots were washed to remove excess antibody, they were incubated with the corresponding secondary antibody (1:2000) for 2 h. A photoluminescence solution (Millipore) was used to acquire images with a BioRad imaging system, and the image grayscale was analyzed with Image J (National Institutes of Health, USA).

### Reverse transcription and quantitative real-time PCR

Total RNA was extracted from cultured U87 cells using Trizol reagent. RNA was reverse transcribed into cDNA following the instructions of TIANScript cDNA First Strand Synthesis Kit. The Ct value was detected by quantitative real-time PCR in accordance with the instructions of QuantiNovaTMSYBER® Green PCR Kit and the BioRad CFX Manager detection system. GAPDH was used as an endogenous control. Primers were designed and synthesized by Biomed (Table 1).

### Measurement of ROS production

Cell plating and pretreatment methods were the same as above. DCFH-DA was diluted and incubated with cells according to the manufacturer's instructions. Levels of 2', 7'-dichlorofluorescein (DCF) in the cells were detected by flow cytometry at Ex = 485 nm and Em = 535 nm.



### Statistical analysis

SPSS 21.0 was used to analyze the data, and the results are all expressed as the mean  $\pm$  standard deviation. Three independent experiments were performed to ensure the reproducibility of the results. One-way ANOVA was employed for comparison between groups, and Bonferroni correction was applied. A  $p$  value  $< 0.05$  was considered statistically significant.

## Results

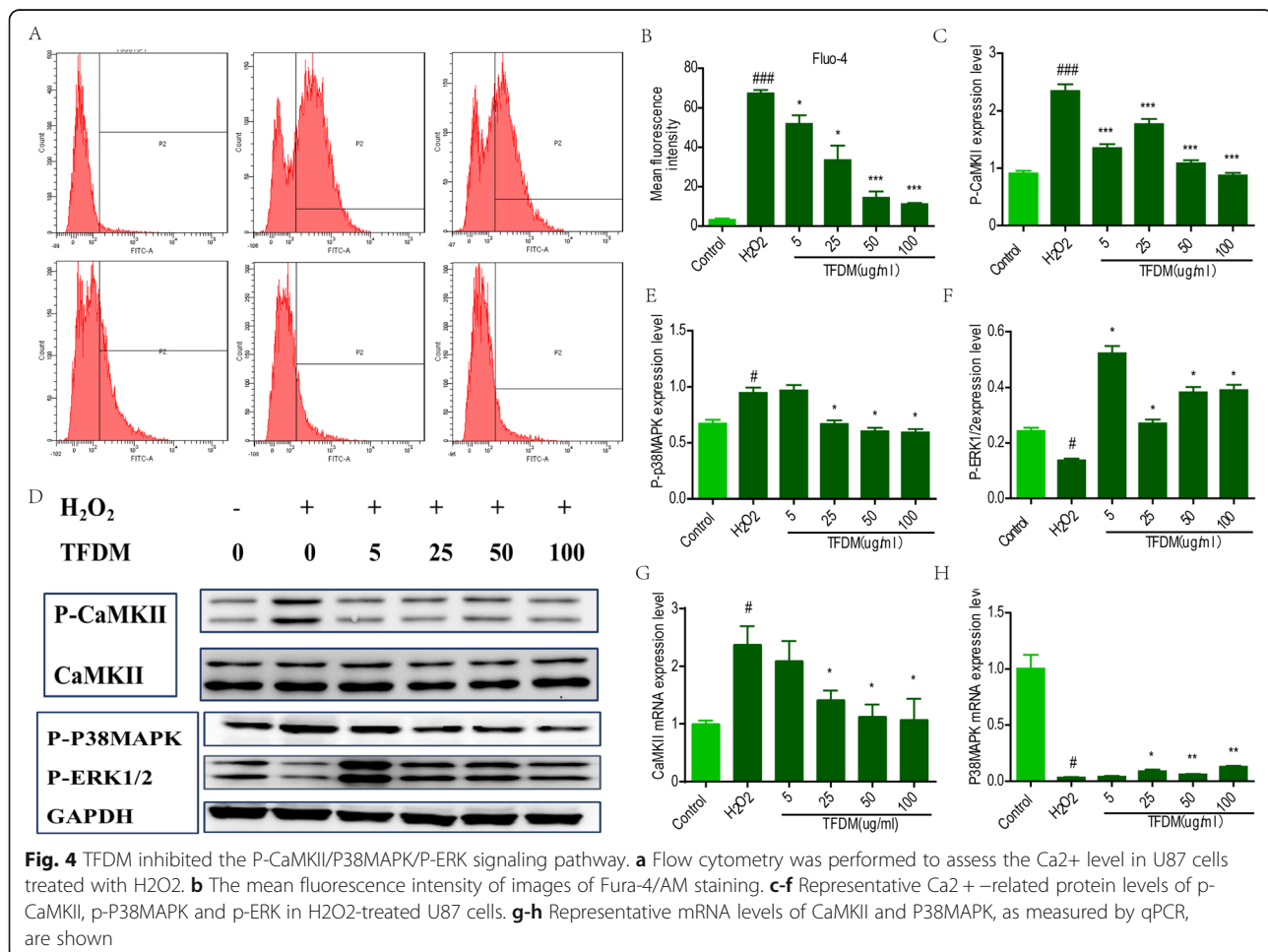
### TFDM protected U87 cells against H<sub>2</sub>O<sub>2</sub> injury

Based on CCK-8 and LDH kit results, TFDM treatment for 24 h had no significant effect on the viability of U87 cells (Fig. 1a). However, the viability of U87 cell incubated with H<sub>2</sub>O<sub>2</sub> (0.63–5 mM) for 20 min (Fig. 1b) was significantly decreased. Thus, 0.63 mM H<sub>2</sub>O<sub>2</sub> was chosen as the dose of H<sub>2</sub>O<sub>2</sub> for U87 cells. TFDM (5, 25, 50, and 100  $\mu$ g/ml) dose-dependently restored cell viability (Fig. 1c). Similarly, the level of LDH release and the level of MDA were much higher in the H<sub>2</sub>O<sub>2</sub> group than in the control group and

were dose-dependently reversed by TFDM (5, 25, 50, and 100  $\mu$ g/ml) (Fig. 1d-e).

### TFDM protected U87 cells against H<sub>2</sub>O<sub>2</sub>-induced apoptosis

H<sub>2</sub>O<sub>2</sub> treatment enhanced apoptosis from 2.1 to 29.1% (Fig. 2a), whereas TFDM dose-dependently reduced the apoptotic rate (Fig. 2b). Western blotting and qPCR were used to detect apoptotic signal transduction after cell injury, and activation of the mitochondria-related apoptosis proteins Bcl-2, Caspase-3, Caspase-9, and Caspase-12 was observed. Compared with the control group, protein expression of Bax, Caspase-3, and Caspase-12 in the oxidative stress model group increased significantly, and this increase was accompanied by a decrease in Bcl-2. This abnormal expression was reversed by treatment with 5–100  $\mu$ M TFDM (Fig. 2f-j). qPCR confirmed that the mRNA levels of Bax, Caspase-9 and Caspase-12 were significantly increased and that TFDM pretreatment reduced the level of apoptosis in a dose-dependent manner (Fig. 2c-e). These results were consistent with





the Western blotting results. Taken together, the results suggest that TFDM can reverse mitochondrial dysfunction and protect U87 cells against oxidative stress induced by H<sub>2</sub>O<sub>2</sub>.

**TFDM ameliorated mitochondrial damage in H<sub>2</sub>O<sub>2</sub>-injured U87 cells**

Depolarization of the mitochondrial membrane has crucial roles in stroke and is involved in the mitochondria-mediated apoptosis pathway. It was observed that JC-1 fluorescence at 596 nm (red) and 534 nm (green) increased and that H<sub>2</sub>O<sub>2</sub> significantly increased MMP in U87 cells (Fig. 3a). However, a dose-dependent recovery in MMP was observed in H<sub>2</sub>O<sub>2</sub>-treated U87 cells treated with gradient concentrations of TFDM (Fig. 3b).

**TFDM inhibited cytosolic Ca<sup>2+</sup> influx in U87 cells exposed to H<sub>2</sub>O<sub>2</sub>-induced injury**

It was observed that Fura-4/AM fluorescence was enhanced at 488 nm and 525 nm in the H<sub>2</sub>O<sub>2</sub> injury group; H<sub>2</sub>O<sub>2</sub> also promoted Ca<sup>2+</sup> influx into U87 cells (Fig. 4a). Conversely, treatment with gradient concentrations of

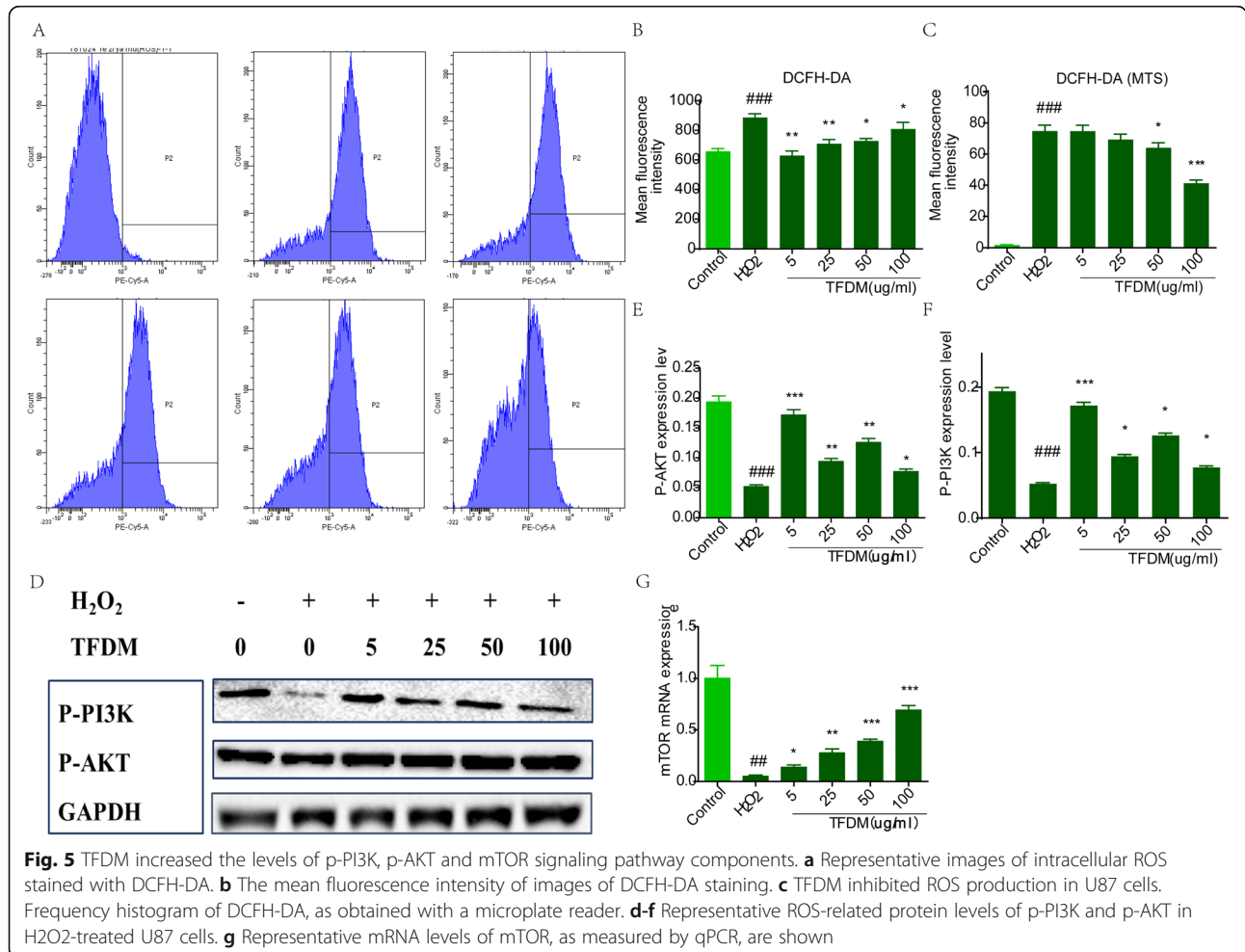
TFDM caused a dose-dependent recovery of Ca<sup>2+</sup> influx in H<sub>2</sub>O<sub>2</sub>-treated U87 cells (Fig. 4b).

**TFDM inhibited the p-CaMKII/P38MAPK/p-ERK1/2 signaling pathway**

To further explore the protective mechanism by which TFDM protects against apoptosis, the possible involvement of CaMKII/P38MAPK/ERK1/2 in astrocytes was examined. We found that phosphorylation of CaMKII, P38MAPK, and ERK1/2 increased dramatically in U87 cells incubated with H<sub>2</sub>O<sub>2</sub> (Fig. 4c-g). After TFDM exposure, levels of p-CaMKII/p-P38MAPK/p-ERK were decreased. qPCR confirmed that the mRNA expression of CaMKII and P38MAPK was significantly elevated after oxidative damage and that TFDM significantly inhibited mRNA expression of these factors (Fig. 4h-i).

**TFDM ameliorated ROS production in U87 cells exposed to H<sub>2</sub>O<sub>2</sub>-induced injury**

Production of ROS in H<sub>2</sub>O<sub>2</sub>-treated U87 cells was evaluated by DCF with flow cytometry and a microplate reader (Fig. 5a). The results of fluorescence intensity



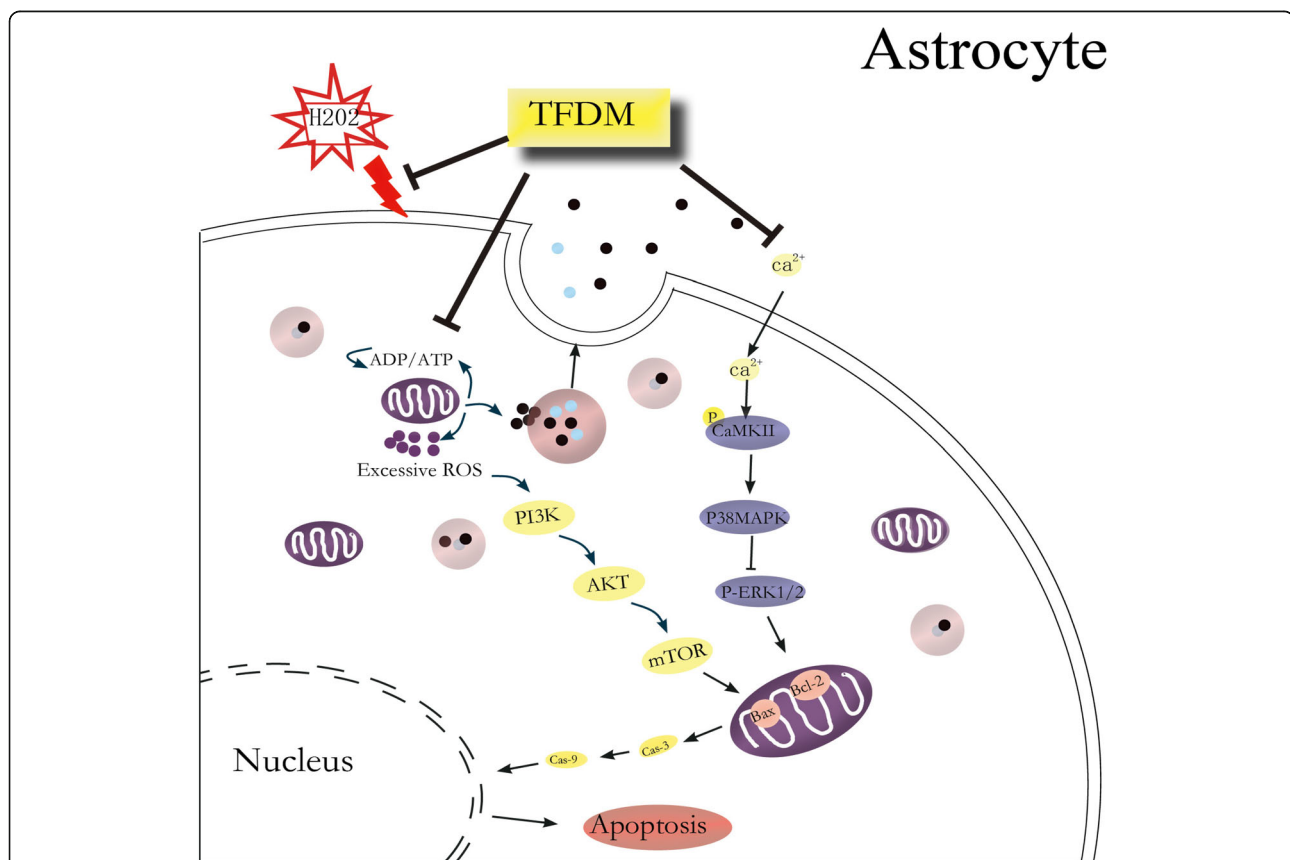
indicated that  $H_2O_2$  increased ROS production in U87 cells. After treatment with gradient concentrations of TFDM, ROS levels decreased in a dose-dependent manner (Fig. 5b), which was consistent with the results obtained using the microplate reader (Fig. 5c). As shown in Fig. 5d-f, TFDM reduced levels of p-PI3K and p-AKT in a dose-dependent manner. In addition, TFDM apparently blocked mRNA expression of mTOR in response to  $H_2O_2$  (Fig. 5g).

## Discussion

For decades, research on cerebral ischemia has mainly focused on neuronal cells. However, the effect is not obvious [28]. Astrocytes are important for the survival of neurons [21]. One study even suggested that it is more important to maintain and protect the function of astrocytes than neurons in cerebral ischemia [29]. Previous studies on TFDM have mainly focused on myocardial ischemia-reperfusion injury, though effects of TFDM on glial neurons in vitro have not been reported. Therefore,

the protective effects of TFDM on astrocytes and the associated mechanisms were investigated in the current study.

After stroke, astrocytes tend to rupture, leading to an imbalance in glutamate absorption and release and an increase in pro-apoptotic factors, which may ultimately accelerate the death of peripheral neurons [30, 31]. Oxidative stress is the basis of the pathogenesis of stroke. Moreover, the survival of astrocytes under oxidative stress conditions may be critical for reducing neuronal death and limiting infarct size [32]. In our study, incubation with  $H_2O_2$  caused cellular injury and apoptosis in astrocytes. It has been reported that the active fraction of TFDM, called thistle glycoside, has effective antioxidant activity. In the present study, TFDM increased survival rates and decreased apoptosis rates of astrocytes injured by  $H_2O_2$ . We first discovered that TFDM had protective effects on glial neurons by inhibiting oxidative stress. The protective effect of TFDM on astrocytes is related to the inhibition of apoptosis. Some antioxidants



**Fig. 6** Schematic diagram summarizing the protective effects of TFDM against  $H_2O_2$ -induced astrocyte apoptosis through a mitochondria-dependent pathway. Proposed protective mechanisms of TFDM against  $H_2O_2$  injury via  $CaMKII$ -dependent mitochondria-mediated apoptosis and  $PI3K/AKT/mTOR$  signaling pathways in astrocytes.  $H_2O_2$  induces an increase in  $[Ca^{2+}]$ , and  $H_2O_2$  exposure induces an increase in ROS levels, which might contribute to an elevation of the resting  $[Ca^{2+}]$ , possibly leading to neurotoxicity in the hippocampus. TFDM relieves  $CaMKII$ -dependent mitochondria-mediated apoptosis, including the expression of Bax, Bcl-2, and caspase-3/9 and the activation of p38MAPK and ERK1/2, following cellular  $H_2O_2$  injury



have been used to treat diseases of the human nervous system [17], which may facilitate the development of TFDM for clinical applications for ischemic injury.

Mitochondria are the source of cellular energy; however, mitochondria are damaged by oxidative stress, resulting in an insufficient ATP supply and excessive accumulation of  $\text{Ca}^{2+}$  in mitochondria [33, 34] and triggered MPTP opening [29, 35]. Mitochondrial dysfunction leads to the excessive release of mitochondrial apoptosis-related proteins, all of which promote Caspase-dependent neuronal apoptosis [17]. TFDM pretreatment contributes to a significant decrease in MMP in astrocytes. CaMKII was originally identified in the brain, and it is considered a prospective target for cerebral ischemia [36]. Furthermore, phosphorylation of CaMKII activates MAPK family proteins and ERK1/2. We demonstrate that TFDM pretreatment may reduce mitochondrial-mediated apoptosis and levels of p-CaMKII, its downstream MAPKs and p-ERK1/2 in astrocytes.

After mitochondrial injury, the MPTP opens and releases excessive ROS to induce lipid peroxidation and cytotoxicity, in turn contributing to mitochondrial dysfunction. The present results show that oxidative damage leads to a rapid increase in ROS, phosphorylation of PI3K, AKT and mTOR, and activation of the PI3K/AKT/mTOR pathway, which ultimately promotes apoptosis in astrocytes (Fig. 6).

## Conclusions

In summary, we conducted the first study to show that TFDM protects astrocytes against oxidative stress injury through a mitochondria-dependent pathway. We demonstrate that  $\text{H}_2\text{O}_2$  induces MMP dissipation and consequently leads to mitochondria-dependent apoptosis. TFDM can protect astrocytes against  $\text{H}_2\text{O}_2$ -induced apoptosis through a mitochondria-dependent pathway that is associated with inhibition of the p-CaMKII/P38MAPK/p-ERK1/2 signaling pathway and the PI3K/AKT/mTOR pathway.

## Abbreviations

BAX: Bcl-2-associated X protein; Bcl2: B-cell leukemia/lymphoma 2; CaMKII: Calcium/calmodulin-dependent protein kinase II; DCFH-DA: 2,7-dichlorodihydrofluorescein diacetate; LDH: Lactate dehydrogenase; MDA: Malonic dialdehyde; MMP: Mitochondrial membrane potential; qRT-PCR: Quantitative real-time polymerase chain reaction; ROS: Reactive oxygen species; TFDM: Total flavonoid extract of *Dracocephalum moldavica* L.

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## Authors' contributions

RFZ and YWD performed most of the experiments in this study and contributed to writing the manuscript. RFZ designed this study, CZ provided guidance, MX participated in some of the experiments and provided technical guidance, JGX provided funding support and provided guidance,

and HFW participated in some of the experiments. All authors read and approved the final manuscript.

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## Availability of data and materials

The datasets used and analyzed in the current study are available from the corresponding author on reasonable request.

## Ethics approval and consent to participate

Not applicable.

## Consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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