

# Tetrahydroxystilbene Glucoside Exerts Cytoprotective Effect against Hydrogen Peroxide-induced Cell Death Involving ROS Production and Antioxidant Enzyme Activation

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Tian, *et al.*: Cytoprotective Effect of Tetrahydroxystilbene Glucoside

Epithelial injury caused by reactive oxygen species including hydrogen peroxide plays a critical role in the pathogenesis of gastric disorders. Tetrahydroxystilbene glucoside is an active component extracted from *Polygonum multiflorum*, a famous traditional Chinese herb. This study aimed to evaluate the protective effect of tetrahydroxystilbene glucoside on hydrogen peroxide-induced oxidative stress on non-malignant gastric epithelial cells and to see if these protective actions can be extended to gastric cancer cells. The results from MTT assay showed that incubating gastric epithelial cells and gastric cancer cells with 200  $\mu$ M hydrogen peroxide for 24 h significantly decreased cell viability, whereas pre-incubating cells with 10  $\mu$ M tetrahydroxystilbene glucoside for 24 h protected the cells against hydrogen peroxide cell damage, and more significantly in gastric epithelial cells. Using 2',7'-dichlorofluorescein diacetate, we found that tetrahydroxystilbene glucoside inhibited the production of intracellular reactive oxygen species in gastric epithelial cells and gastric cancer cells. In addition, it induced the expression of antioxidant enzymes hemeoxygenase-1 and NADPH quinone oxidoreductase-1. These results demonstrated that tetrahydroxystilbene glucoside exerted stronger cytoprotective effect against peroxide-induced cell death in gastric epithelial cells than gastric cancer cells and the cytoprotective mechanisms might be through decreasing oxidative stress and activating the expression of hemeoxygenase-1 and NADPH quinone oxidoreductase-1.

**Key words:** Tetrahydroxystilbene glucoside, GES-1 cells, SGC-7901 cells, H<sub>2</sub>O<sub>2</sub>

The impact of gastric diseases on human health, such as chronic gastritis, duodenal, gastric ulceration and adenocarcinoma, remains a big issue deserving worldwide attention. Oxidative stress is the major gastric pathologic feature and plays an essential role in the multiple progressions of gastric diseases<sup>[1]</sup>. The excessive generation of reactive oxygen species (ROS) is associated with cell death and generation of ROS is associated with cell death and keep cellular homeostasis, excessive ROS can cause damage to lipids and proteins as well as single stranded DNA breaks<sup>[2]</sup> and gastric epithelium is exposed to higher ROS level than other tissues<sup>[3,4]</sup>. Therefore, it is necessary for cells to effectively counteract ROS generation by triggering their own defensive mechanisms with the help of antioxidants. Hemeoxygenase-1 (HO-1) is a highly inducible, stress-responsive protein (also called heat

shock protein 32), which catalyzes the first and rate-limiting step in heme degradation, a potent oxidant<sup>[5]</sup>. Indeed, ample evidence currently supports the notion that HO-1 serves to provide potent cytoprotective effects in many *in vitro* and *in vivo* models of oxidant-induced cellular and tissue injury. The NADPH-quinone oxidoreductase 1 (NQO1) is a predominantly cytosolic enzyme which provides cells with multiple layers of protection against oxidative stress, including the direct detoxification of highly reactive quinones<sup>[6,7]</sup>.

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*Polygonum multiflorum*, a traditional Chinese medicinal herb, has been widely used as a tonic and antiaging agent<sup>[8]</sup>. Tetrahydroxystilbene glucoside (TSG) is an active component extracted from *P. multiflorum*. Recently, TSG has received a great deal of attention owing to its biological properties, including antioxidative<sup>[9]</sup>, antiatherosclerotic<sup>[10]</sup> and antiinflammatory<sup>[11]</sup> activities. However, whether TSG has a protective activity against oxidative stress related injury to gastric epithelial cells remains unknown. TSG is the water-soluble active component. Therefore, TSG may well penetrate the cells with free diffusion. Some reports found that TSG was mainly absorbed in the stomach, but the absorption rate was reduced. So its absorption in the stomach may be by active transport. Lv *et al.*<sup>[12]</sup> discovered that TSG was rapidly absorbed into the body fluids and widely distributed throughout the body, with great efficiency of utility, followed by quick elimination. Sun *et al.*<sup>[13]</sup> preliminarily determined that the metabolites of TSG were glucuronic acid combination. Wang *et al.*<sup>[14]</sup> found that bile excretion might be the main drainage way of TSG and its metabolites.

Herein, in the present study, we investigated whether TSG protected ROS induced damage on human gastric epithelial cell line (GES-1) with oxidative stress mediated by hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) via the antioxidant enzymes HO-1 and NQO1. More importantly, we demonstrated whether TSG performed the same cytoprotective effect on gastric cancer cell line SGC-7901, thus weakening the antitumor effect of other drugs through decreasing ROS generation.

## MATERIALS AND METHODS

TSG (purity above 98%) was purchased from Putian Biological Technology Co., Ltd. (Beijing, China). 2',7'-dichlorofluorescein diacetate (DCFH-DA), MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) and H<sub>2</sub>O<sub>2</sub> were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium DMEM/F12 and trypsin ethylenediamine tetra acetic acid (EDTA) were purchased from Gibco Invitrogen (Carlsbad, CA, USA).

### Cell culture:

GES-1 and gastric cancer cell line SGC-7901 were purchased from Bogoo (Shanghai, China) and cultured in DMEM/F12 medium, supplemented with 10% heat inactivated fetal bovine serum (FBS, Gibco) and 1%

penicillin/streptomycin (Gibco). All the cells were incubated at 37° in humidified 5% CO<sub>2</sub>, 95% air atmosphere.

### Cell proliferation assay:

Inhibition of cell proliferation by TSG was measured by the MTT assay. GES-1 cells and SGC-7901 cells were plated in 96-well plates at a density of 4000 cells per well. After incubation with medium for 24 h, cells were incubated with indicated concentrations of TSG for 24 h. Then, MTT was added to cell cultures at a final concentration of 5 mg/ml for 4 h at 37°, after that the media were carefully removed. The adherent cells were solubilized with 100 µl of DMSO. Absorbance was measured at 570 nm using a microplate reader (BIO-RAD, California, USA).

### Measurement of ROS:

Generation of intracellular ROS was detected using the non-fluorescent probe DCFH-DA. DCFH-DA, a non-fluorescent substance, passively diffuses into cells and is deacetylated by esterases to form non-fluorescent DCFH. In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cell<sup>[12]</sup>. The cells were treated with H<sub>2</sub>O<sub>2</sub> for 4 h after being pretreated with or without TSG for 24 h. Then cells were harvested and incubated with 5 µM DCFH-DA at 37° for 30 min and analyzed by flow cytometry (Millipore, Bedford, USA).

### Quantitative real time polymerase chain reaction:

After treating with TSG, the total RNA was extracted with Trizol (Invitrogen, California, USA), using the standard method. cDNA synthesis was performed with 1 µg of total RNA, using the PrimeScript™ II 1st Strand cDNA Synthesis Kit. A quantitative real-time polymerase chain reaction (qRT-PCR) was performed on the CFX 96 Touch™ Real-Time PCR Detection System (BIO-RAD, California, USA), using the iTaq™ Universal SYBR® Green Supermix (Bio-Rad, Shanghai, China) according to the manufacturer's protocol. Glyceraldehyde-3 phosphate dehydrogenase (GAPDH) was used as the endogenous control. The gene-specific primers of human HO-1 and NQO1 were listed in Table 1. Data were presented as mean±SD. Statistical analysis was performed using one-way ANOVA with P<0.05 being considered to indicate statistical significance.

## RESULTS AND DISCUSSION

First of all, the effects of TSG on GES-1 cells and

**TABLE 1: THE LIST OF GENE PRIMERS USED IN QRT-PCR ANALYSIS**

Genes	Forward (5'-3')	Reverse (3'-5')
GAPDH	AGAAGGCTGGGGCTCATTTG	AGGGGCCATCCACAGTCTTC
HO-1	ACAGGGTGACAGAAGAGGCTAA	CTGTGAGGGACTCTGGTCTTTG
NQO1	CAGCGGCTCCATGTACT	GACCTGGAAGCCACAGAAG

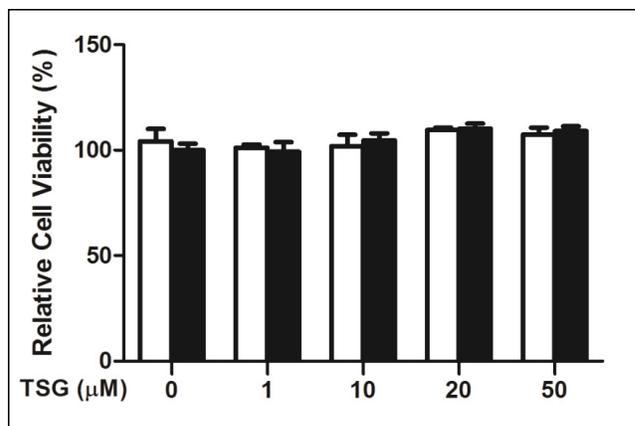
SGC-7901 cells proliferation were examined. Cells were treated with 0-50  $\mu\text{M}$  TSG for 24 h and cell proliferation was analyzed using the MTT assay. Compared with untreated control cells, we found that TSG did not inhibit either the proliferation of GES-1 cells or SGC-7901 cells (fig. 1).

To examine the cytotoxic potential of  $\text{H}_2\text{O}_2$ -induced cell damage, we selected the tumor cells (SGC-7901 cells) with high reproduction and easy accessibility. SGC-7901 cells were treated with increasing concentration of  $\text{H}_2\text{O}_2$  (0-800  $\mu\text{M}$ ) for 4 h, the cell viability was significantly decreased to  $42.2 \pm 0.62\%$ . The results shown in fig. 2A demonstrated that  $\text{IC}_{50}$  valued was  $510 \pm 4.95 \mu\text{M}$ . Therefore we selected the concentration of 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for the following experiment. As shown in fig. 2B, pretreatment with the indicated concentration of TSG reduced  $\text{H}_2\text{O}_2$ -induced cell damage.

After that, we examined the effect of TSG on ROS generation in GES-1 cells and SGC-7901 cells. Generation of ROS in cells was measured using fluorescence assay with DCFH-DA probe by flow cytometry. The results demonstrated that TSG could dose-dependently attenuate  $\text{H}_2\text{O}_2$ -induced ROS production compared with the  $\text{H}_2\text{O}_2$  treatment alone (figs. 3A and 3B).

To explore whether TSG leads to the expression of antioxidant enzymes, we examined the gene levels of HO-1 and NQO1. qRT-PCR analysis was conducted to confirm the induction of antioxidant gene. Treatment of cells with TSG (0, 5 and 10  $\mu\text{M}$ ) for 24 h showed that the compound induced the expression of NQO1 and HO-1 genes in a concentration-dependent manner (figs. 4A and 4B).

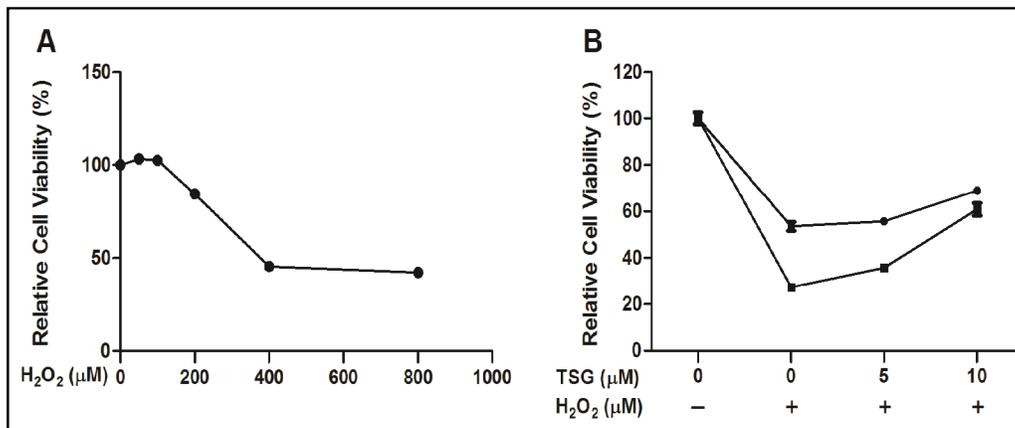
We examined the gene levels of HO-1 and NQO1 after the negative control  $\text{H}_2\text{O}_2$  treatment. The cells treated with 500  $\mu\text{M}$   $\text{H}_2\text{O}_2$  for 4 h showed that the expression of NQO1 and HO-1 genes were reduced. The elevated ROS is an essential factor in the onset and development of gastric epithelial injury and various gastric disorders. Cells possess a plenty of cytoprotective enzymes, which protect living cells from oxidative stress-induced damage. However, exogenous administration



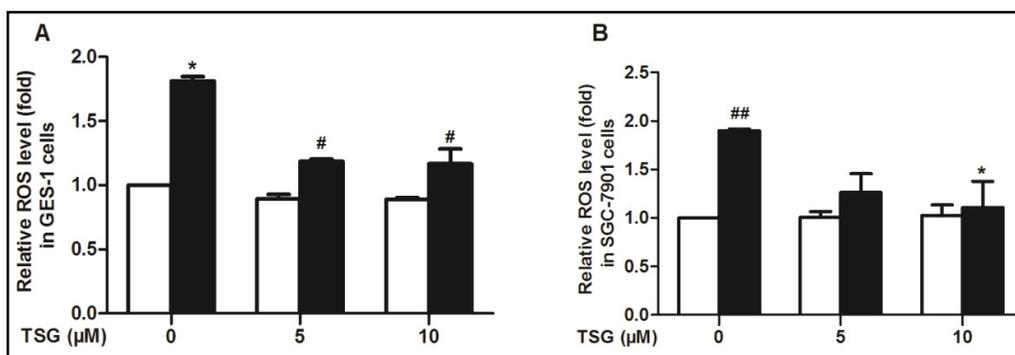
**Fig. 1: Effects of tetrahydroxystilbene glucoside on the proliferation of GES-1 cells and SGC-7901 cells. GES-1 cells ( $\square$ ) and SGC-7901 cells ( $\blacksquare$ ) were incubated with indicated concentrations of TSG. Then the cell proliferation was estimated by MTT assay. Data represent means  $\pm$  SD.**

of antioxidants is also an important strategy to protect cells against oxidative damage. TSG has been reported to counteract oxidative stress in different cell lines<sup>[9,15,16]</sup>. In this study, we first demonstrated that TSG could protect gastric epithelial GES-1 cells from oxidative damage. It is further found that TSG protected oxidative damages through inhibition of oxidative stress and up-regulation the expression of antioxidant enzymes HO-1 and NQO1.

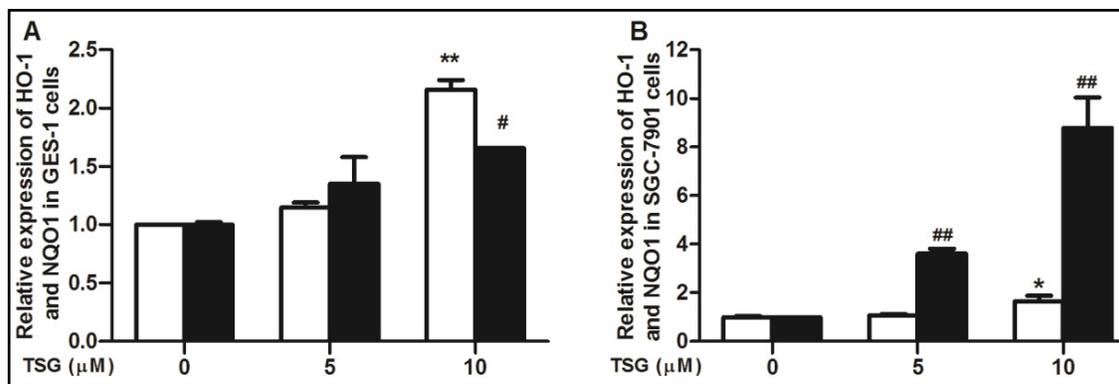
Different from endogenous  $\text{H}_2\text{O}_2$ , exogenously added  $\text{H}_2\text{O}_2$  is less effective at leading to a signaling response<sup>[17]</sup>, whereas it can rapidly diffuse across membranes and immediately increase ROS level, resulting in cell damage that ultimately decreases cellular function<sup>[18,19]</sup>. Our results demonstrated that GES-1 cells showed a dose-dependent viability loss after exposed to  $\text{H}_2\text{O}_2$  and ROS levels elevated immediately. Meanwhile, pretreated with different concentration of TSG significantly decreased cell viability loss, reduced the ROS generation and activating antioxidant enzymes of HO-1 and NQO1. As shown in fig. 4, pretreated TSG activated antioxidant enzymes of HO-1 and NQO1 in GES-1 cells and SGC-7901 cells. In GES-1 cells, HO-1 was increased about 2.16 folds compared with that of control group and NQO1 was increased about 1.66 folds. In SGC-7901 cells, HO-1 was increased



**Fig. 2:** Effects of tetrahydroxystilbene glucoside on the H<sub>2</sub>O<sub>2</sub>-induced cell cytotoxicity in GES-1 cells and SGC-7901 cells. A SGC-7901 cells (●—●) were incubated with indicated concentrations of H<sub>2</sub>O<sub>2</sub> for 4 h, and cell proliferation was estimated by MTT assay. B SGC-7901 cells (■—■) and GES 1 cells (●—●) were pretreated with the indicated concentration of TSG (0, 5 and 10 μM) for 24 h or not, and followed by H<sub>2</sub>O<sub>2</sub> (500 μM) administration. The cells were further cultured for 4 h and cell viability was tested. Data represent means±SD.



**Fig. 3:** Effects of tetrahydroxystilbene glucoside on the H<sub>2</sub>O<sub>2</sub>-induced ROS production in GES-1 cells and SGC-7901 cells. Cells were pretreated with the indicated concentrations of TSG (0, 5 and 10 μM) for 24 h or not, and followed by H<sub>2</sub>O<sub>2</sub> (500 μM) administration. A and B Intracellular ROS was evaluated and the data were quantified. Data represent means±SD. \*P<0.05 vs. Control (0 μM); #P<0.05 compared to H<sub>2</sub>O<sub>2</sub> treatment group. □ Control; ■ H<sub>2</sub>O<sub>2</sub>.

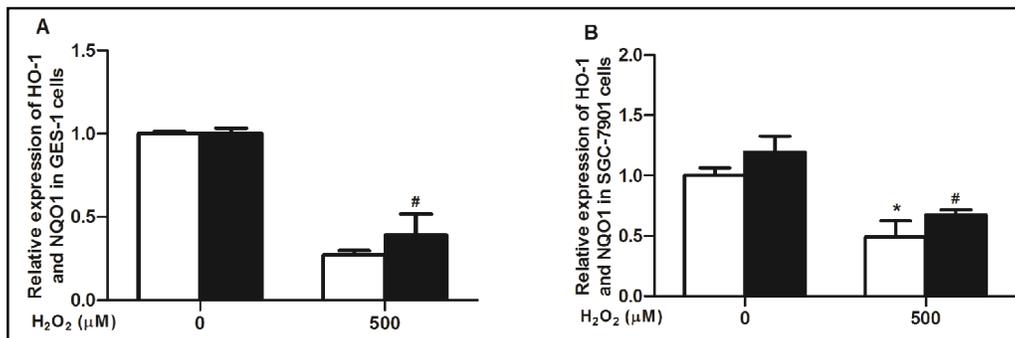


**Fig. 4:** Effect of tetrahydroxystilbene glucoside on the antioxidant enzyme expression in GES-1 cells and SGC-7901 cells. Cells were pretreated with the indicated concentrations of TSG (0, 5 and 10 μM) for 24 h or not. A and B Expression of NQO1 (■) and HO-1 (□) were evaluated and the data were quantified. Data represent means±SD. \*P<0.05 vs. Control (HO-1); #P<0.05 vs. Control (NQO1).

about 1.66 folds compared with that of control group and NQO1 was increased about 8.79 folds. These results indicated that HO-1 might be, at least in part, mainly involved in this process in GES-1 cells, and NQO1 might play a leading role in SGC-7901 cells,

though further investigation is required. These results suggested that TSG might exert gastro protective effect through an antioxidant pathway, which was in agreement with other reports<sup>[4,20]</sup>.

We also found that TSG exerted cytoprotective effect



**Fig. 5: Effect of H<sub>2</sub>O<sub>2</sub> on the antioxidant enzyme expression in GES-1 cells and SGC-7901 cells.**

Cells were pretreated with 500 μM H<sub>2</sub>O<sub>2</sub> for 4 h or not. A and B expression of NQO1 (■) and HO-1 (□) were evaluated and the data were quantified. Data represent means±SD. \*P<0.05 vs. Control (HO-1); #P<0.05 vs. Control (NQO1).

against H<sub>2</sub>O<sub>2</sub>-induced cell death in GES-1 cells, and also played a protective role in gastric cancer cells (SGC-7901), although the protection on SGC-7901 cells was not strong (fig. 5). These results indicated that TSG protected gastric epithelial cells from oxidative damage and at the same time, might reduce the effects of other antitumor drugs.

In summary, TSG presents cytoprotective effect against oxidative damage and may be further development as a therapeutic agent against gastric diseases. It should be mentioned that the detail molecular mechanisms for antioxidant effect of TSG are important aspects to study in the future. Furthermore, this study aimed to make available information on the prevention of TSG against hydrogen peroxide-induced cell death. It would be interest to test the treatment of TSG. We considered performing these experiments in our future studies.

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### Conflict of interest:

The authors have declared that there is no conflict of interest.

### REFERENCES

1. Bhattacharyya A, Chattopadhyay R, Mitra S, Crowe SE.

Oxidative stress: an essential factor in the pathogenesis of gastrointestinal mucosal diseases. *Physiological reviews* 2014;94:329-54.

- Gal R, Halmosi R. The role of oxidative stress in heart failure. *Orvosi hetilap* 2015;156:1916-20.
- Halliwell B, Clement MV, Ramalingam J, Long LH. Hydrogen peroxide. Ubiquitous in cell culture and *in vivo*? *IUBMB life* 2000;50:251-57.
- Hu XT, Ding C, Zhou N, Xu C. Quercetin protects gastric epithelial cell from oxidative damage *in vitro* and *in vivo*. *Eur J Pharmacol* 2015;754:115-24.
- Otterbein LE, Choi AM. Heme oxygenase: colors of defense against cellular stress. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1029-37.
- Cui X, Li L, Yan G, Meng K, Lin Z, Nan Y, *et al.* High expression of NQO1 is associated with poor prognosis in serous ovarian carcinoma. *BMC Cancer* 2015;15:244.
- Rougee LR, Riches Z, Berman JM, Collier AC. The ontogeny and population variability of human hepatic NADPH dehydrogenase quinone oxido-reductase-1 (NQO1). *Drug Metab Dispos* 2016;44:967-74.
- Lin L, Ni B, Lin H, Zhang M, Li X, Yin X, *et al.* Traditional usages, botany, phytochemistry, pharmacology and toxicology of *Polygonum multiflorum* Thunb.: A review. *J Ethnopharmacol* 2015;159:158-83.
- Zhang JK, Yang L, Meng GL, Fan J, Chen JZ, He QZ, *et al.* Protective effect of tetrahydroxystilbene glucoside against hydrogen peroxide-induced dysfunction and oxidative stress in osteoblastic MC3T3-E1 cells. *Eur J Pharmacol* 2012;689:31-7.
- Yao W, Fan W, Huang C, Zhong H, Chen X, Zhang W. Proteomic analysis for antiatherosclerotic effect of tetrahydroxystilbene glucoside in rats. *Biomed Pharmacother* 2013;67:140-5.
- Huang C, Wang Y, Wang J, Yao W, Chen X, Zhang W. TSG (2,3,4',5-tetrahydroxystilbene 2-O-beta-D-glucoside) suppresses induction of pro-inflammatory factors by attenuating the binding activity of nuclear factor-κB in microglia. *J Neuroinflammation* 2013;10:129.
- Lv G, Lou Z, Chen S, Gu H, Shan L. Pharmacokinetics and tissue distribution of 2,3,5,4 tetrahydroxystilbene-2-O-β-D-glucoside from traditional Chinese medicine *Polygonum multiflorum* following oral administration to rats. *J Ethnopharmacol* 2011;137:449-56.
- Sun JH, Yuan ZF, Wang CY, Xu HJ, Zhang LT. Pharmacokinetics of stilbene glycoside from *Polygonum multiflorum* in rats *in vivo*. *Chinese Traditional and Herbal Drugs* 2005;36:405-8.

14. Wang CY, Yuan ZF, Wang Q, Xie FL, Yang W, Zhang LT. HPLC simultaneous determination of stilbene glycoside and its metabolite in rat bile liquid. *Chin J Pharm Anal* 2009;29:53-57.
  15. Zhang W, Chen XF, Huang YJ, Chen QQ, Bao YJ, Zhu W. 2,3,4',5-Tetrahydroxystilbene-2-O- $\beta$ -D-glucoside inhibits angiotensin II-induced cardiac fibroblast proliferation via suppression of the reactive oxygen species-extracellular signal-regulated kinase 1/2 pathway. *Clin Exp Pharmacol Physiol* 2012;39:429-37.
  16. Tao L, Li X, Zhang L, Tian J, Li X, Sun X, *et al.* Protective effect of tetrahydroxystilbene glucoside on 6-OHDA-induced apoptosis in PC12 cells through the ROS-NO pathway. *PLoS one* 2011;6:e26055.
  17. Choi MH, Lee IK, Kim GW, Kim BU, Han YH, Yu DY, *et al.* Regulation of PDGF signalling and vascular remodelling by peroxiredoxin II. *Nature* 2005;435:347-53.
  18. Ferreira IL, Resende R, Ferreiro E, Rego AC, Pereira CF. Multiple defects in energy metabolism in Alzheimer's disease. *Curr Drug Targets* 2010;11:1193-206.
  19. Jarrett SG, Lewin AS, Boulton ME. The importance of mitochondria in age-related and inherited eye disorders. *Ophthalmic Res* 2010;44:179-90.
  20. Harada S, Nakagawa T, Yokoe S, Edogawa S, Takeuchi T, Inoue T, *et al.* Autophagy deficiency diminishes indomethacin-induced intestinal epithelial cell damage through activation of the ERK/Nrf2/HO-1 Pathway. *J Pharmacol Exp Ther* 2015;355:353-61.
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