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Synthesis of novel caffeic acid derivatives and their protective effect against hydrogen peroxide induced oxidative stress via Nrf2 pathway

Xiaoyu Peng[1,3], Gang Wu [2], Anran Zhao[4], Kelin Huang[3], Ling Chai[5], Bharathi Natarajan[6], Songxin Yang[2], Hailan Chen*[2], Cuiwu Lin*[1]

[1] School of Chemistry and Chemical Engineering, Guangxi University, Nanning, Guangxi, 530004, China

[2] College of Animal Science and Technology, Guangxi University, Nanning, Guangxi, 530005, China

[3] China Academy of Science and Technology Development Guangxi Branch, Nanning, Guangxi 530022, China

[4] First affiliated hospital of Guangxi Medical University, Nanning, Guangxi, 530021, China

[5] Guangxi Key Laboratory of Traditional Chinese Medicine Quality Standards, Guangxi Institute of Traditional Medical and Pharmaceutical Sciences, Nanning, Guangxi, 530022, China

[6] College of Medicine, Jiaxing University, Jiaxing, Zhejiang, 314001, China

Corresponding author:

Dr. Hailan Chen

Room 124, College of Animal Science and Technology, Guangxi University,

100 Daxue Road, Xixiangtang District, Nanning, Guangxi 530004, China

Fax: +86-771-3270149;

Tel: +86-771-3235635;

E-mail: hlchen319@163.com

Dr. Cuiwu Lin

Room 114, School of Chemistry and Chemical Engineering, Guangxi University,

100 Daxue Road, Xixiangtang District, Nanning, Guangxi 530004, China

Fax: +86-771-3275878;

Tel: +86-771-3233718;

E-mail: cuiwulin@163.com

Abstract

Aim: This study was aimed to synthesize novel caffeic acid derivatives and evaluate their potential applications for the treatment of oxidative stress associated disease.

Main methods: Caffeic acid sulfonamide derivatives were synthesized by coupling sulfonamides to the backbone of caffeic acid and fully characterized by melting points test, FT-IR, MS, NMR, UV-*vis* and n-octanol–water distribution assay. Their free radical scavenging ability was evaluated using DPPH assay and cytotoxicity against A549 cells were determined by MTT assay. The protective effect of these derivatives against hydrogen peroxide (H_2O_2) induced oxidative injury was assessed in A549 cells from cell viability, production of reactive oxygen species (ROS) and malondialdehyde (MDA), alternation of antioxidase activities, and expressions of Nrf2 and its target genes.

Key findings: Six novel caffeic acid sulfonamide derivatives were obtained. The derivatives showed better liphophilicity than the parent caffeic acid. CASMZ, CAST and CASQ exhibited similar DPPH scavenging capability as caffeic acid, while the protection of hydroxyl groups on the benzene ring with acetyl groups caused decrease in radical scavenging activity. No inhibitory effect on the proliferation of A549 cells were observed up to a concentration of 50 μM. Pretreatment of cells with these derivatives strongly inhibited H_2O_2 induced decrease of cell viability, reduced the production of ROS and MDA, promoted antioxidase activities, and further upregulated the expression of Nrf2 and its target genes.

Significance: Caffeic acid sulfonamide derivatives were synthesized with simple reactions under mild conditions. They might protect cells from H_2O_2-induced oxidative injury via Nrf2 pathway.

Key words: caffeic acid sulfonamide derivative, free radical, H_2O_2, oxidative stress, Nrf2

1 Introduction

Reactive oxygen species (ROS), including oxygen superoxide anion ($O_2\cdot^-$), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and nitric oxide (NO), are the middle products of normal metabolism in human or animal body playing important roles in signal transduction and regulation of cell functions [1, 2]. However, large amounts of ROS are produced under the conditions of bacterial and viral infection, heavy metal exposure, stress, radiation, chemotherapy drugs

administration, and other pathological conditions. When the balance between the production of ROS and their clearance by endogenous antioxidative system is destroyed, oxidative stress is created [3]. Excess ROS can damage cell proteins, lipids, DNA, and other cellular components, leading to cell death and tissue injury. Researchers believe that free radicals induced macromolecules destroy and cell damage is the basic cause of aging, cancer and other diseases [4]. Modern medicine and free radical science have also increasingly shown that free radicals are closely associated to many diseases, such as cancer, arteriosclerosis, myocardial ischemia reperfusion injury, cataracts, neurological disorders, hypertension, arthritis and rheumatoid arthritis [5, 6]. Many studies suggested that the use of exogenous antioxidants can eliminate excess ROS, alleviate oxidative stress induced cell and tissue injury, and is helpful for the treatment of oxidative stress-related diseases [7, 8].

Caffeic acid is a secondary metabolite of plants which is found in almost all plants. Antioxidative, anti-cancer and anti-virus activities have been frequently reported in studies on caffeic acid [9, 10]. However, the low solubility and compatibility of caffeic acid in common solvent systems, difficulties in penetrating biomembranes, and the limited availability of natural derivatives of caffeic acid, are limiting the widely application of caffeic acid in the biomedical field [11, 12]. In recent years, the chemical modification of caffeic acid produce various derivatives and these new compounds exhibit excellent scavenging capability on free radicals and strong inhibitory activity on lipid peroxidation [13]. For example, caffeic acid phenethyl ester (CAPE) could decrease the levels of malondialdehyde (MDA), myeloperoxidase (MPO) and catalase (CAT), and increase the activities of superoxide dismutase (SOD) and xanthine oxidase (XO) in the lung tissue of burned rats [14, 15]. Thus, the synthesis of caffeic acid derivatives not only maintain its biological activity, but also expand the scope of applications.

In our previous studies, several active phenolic acids, including caffeic acid, chlorogenic acid and gallic acid, have been isolated from *Blumea riparia* DC. Series phenolic acid derivatives were synthesized by modulating the chemical structure of these phenolic acids. Obtained compounds exhibited strong activities of antioxidation, thrombin inhibition, anticancer and chondrocytes proliferative promotion [16-18]. In this study, six novel caffeic acid sulfonamide derivatives were synthesized through simple reaction steps under mild conditions. Their physicochemical properties were fully characterized using melting points

(mp), mass spectra (MS), nuclear magnetic resonance (NMR), Fourier transfer-infrared spectrometry (FT-IR), and ultraviolet spectroscopy (UV). Their radical scavenging activity was evaluated by DPPH assay and cytotoxicity was determined via MTT assay. Their protective effect against oxidative stress associated damage was studied using H_2O_2-induced oxidative stress A549 cell models according to cell viability, ROS and MDA production, endogenous antioxidative enzymes activities and transcription of Nrf2 pathway associated genes.

2 Material and methods

2.1 Material

Caffeic acid with a purity of 99% was purchased from Zhuhai Jiaxinkang Pharmaceutical Technology Co. (Zhuhai, China). Sulfamethoxazole (SMZ), sulfathiazole sodium (ST) and 4-amino-N-(2-quinoxalinyl)benzenesulfonamide sodium (SQ-Na) were purchased from Shenzhen Remote Technology Development Co. 2,7-dichlorodi-hydrofluorescein diacetate (DCFH-DA) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazoliumbromide (MTT) were bought from Sigma–Aldrich, USA. High glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin sodium and streptomycin were purchased from Gibco, USA. Commercial kits for the determination of malondialdehyde **(**MDA) contents and activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). Other reagents in research grade were supplied by Xilong Scientific Co. and were used as received.

2.2 Synthesis of caffeic acid sulfonamide derivatives

Caffeic acid sulfonamide derivatives were prepared from caffeic acid and SMZ, ST or SQ-Na following the synthetic scheme in Fig.1. Firstly, 9.0 g caffeic acid was reacted with 80 mL acetic anhydride at room temperature for 24 h, followed by a concentration process using rotary evaporation. The obtained product was purified by recrystallizing in ethanol and then reacted with thionyl chloride (SOC_{l2}) to produce the intermediate produce of (E)-4-(3-chloro-3-oxoprop-1-en-1-yl)-1-2-phenlene diacetate (CPD). CPD was then dissolved in 45 mL tetrahydrofuran (THF) by stirring for 1 min and standing for 12 hours without stirring at room temperature. SMZ, ST or SQ-Na and 10 mL of pyridine was added sequentially to above solution. The mixture was stirred at 0 °C for 6 hours and at room temperature for another 24 hours. Upon completion, THF and pyridine was removed by vacuum evaporation. Obtained product was

washed by distilled water and purified by recrystallizing in a THF-methanol system. One kind of white powder and two kind of pale yellow power were obtained and named as (Z)-4-(3-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenylamino)-3-oxoprop-1-enyl)-1,2-phenylene diacetate (ACASMZ), (Z)-4-(3-oxo-3-(4-(N-thiazol-2-ylsulfamoyl)phenylamino)prop-1-enyl)-1,2-phenylene diacetate (ACAST) and (Z)-4-(3-oxo-3-(4-(N- quinoxalin-2-ylsulfamoyl) phenylamino)prop-1-enyl)-1,2-phenylene diacetate (ACASQ), respectively. Acetyl groups protecting hydroxyl groups on the benzene ring of ACASMZ, ACAST and ACASQ were removed by reacting with hydrochloric acid in THF at 55°C for 4 hours. After removing excess THF solvent and purifying by recrystallization, (Z)-3-(3,4-dihydroxyphenyl)-N-(4-(N-(5-methylisoxazol-3-yl)sulfamoyl)phenyl)acrylamide (CASMZ), (Z)-3-(3,4-dihydroxyphenyl)-N-(4-(N-thiazol-2-ylsulfamoyl)phenyl)acrylamide (CAST) and (Z)-4-(3-oxo-3-(4-(N-thiazol-2-ylsulfamoyl)phenylamino)prop-1-enyl)-1,2-phenylene diacetate (CASQ) were obtained.

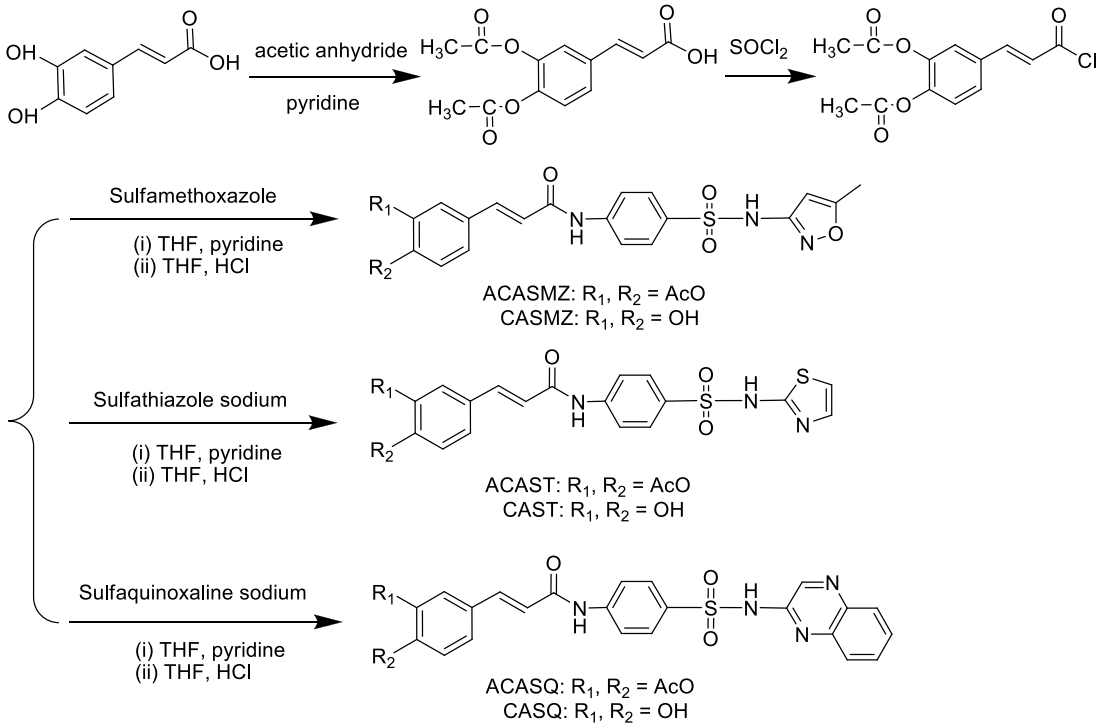

Fig. 1 Synthesis route of caffeic acid sulfonamide derivatives.

2.3 Characterization of caffeic acid sulfonamide derivatives

The mp of obtained caffeic acid sulfonamide derivatives were measured using an XT-4 micro melting apparatus (Beijing Tech Instruments, China). Electrospray ionization mass spectra (EIS-MS) was recorded by Shimadzu LC-MS 2010A (Shimadzu，Japan). ^{1}H-NMR and ^{13}C-NMR spectra scanning were carried out at 25°C using a Bruker Advance III 300 (Bruker, Swiss). The

frequency for ^{1}H-NMR and ^{13}C-NMR were set as 400 Hz and 125 Hz, respectively. For the description of the spin multiplicities, "s" represented "singlet" and d denoted "duplet". FT-IR spectra were measured on a Nicolet 380 spectrophotometer (Thermo Fisher Scientific, USA). The UV absorption spectra was determined using an UV-2600 (Shimadzu, Japan).

ACASMZ: $C_{23}H_{21}N_3O_8S$, white powder, ESI-MS: 498[M-H]$^-$ mp: 244-246 ℃; ^{1}H-NMR (300 MHz, DMSO-d_6): δ11.25 (s, 1H, SO_2-NH), 10.57 (s, 1H, CO-NH), 7.76 (d, *J* =9.1 Hz, 4H, 4×Ar-H), 7.52 (d, J =8.2 Hz, 2H, Ar-H), 7.49 (s, 1H, Ar-H), 7.25 (d, *J* = 15.1 Hz, 1H, CO-CH), 6.7 (d, *J* =15.7 Hz, 1H, Ar-CH), 6.03 (s, 1H, C-CH), 2.30 (d, *J* =0.6 Hz, 9H, C-CH_3). ^{13}C-NMR (75 MHz, DMSO): δ 168.77, 164.26, 150.07, 145.58, 143.60, 142.82, 139.89, 134.90, 133.92, 128.94, 126.67, 125.71, 125.21, 119.25, 20.89, 12.68.

CASMZ: $C_{19}H_{17}N_3O_6S$, light yellow powder, ESI-MS: 414[M-H]$^-$; mp: 258-260℃; ^{1}H-NMR (300 MHz, DMSO-d_6): 11.23 (s, 1H, SO_2-NH), 10.39 (s, 1H, CO-NH), 9.55-9.24 (s, 2H, Ar-*O*H), 7.90-7.76 (d, *J* =9.08 Hz, 4H, 4×Ar-H), 7.26 (d, *J* = 15.55 Hz, 1H, CO-CH), 6.96(d, *J* =8.2 Hz,, 2H, Ar-H), 6.69 (s, 1H, Ar-H), 6.45 (d, *J* =15.6 Hz, 1H, Ar-CH), 6.08(s, 1H, C-CH), 2.30(d, *J* =0.6 Hz, 3H, C-CH_3). ^{13}C-NMR (75 MHz, DMSO) δ170.74, 168.77, 165.02, 158.29, 151.55, 150.09, 145.11, 143.35, 143.83, 142.83, 139.90, 134.94, 129.15, 124.71, 123.18, 119.25, 106.05, 12.68.

ACAST: $C_{22}H_{19}N_3O_7S_2$, yellow powder, ESI-MS:500[M-H]$^-$; mp: 142-145℃; ^{1}H-NMR(300 MHz, DMSO-d_6): δ 12.59 (s, 1H, SO_2-NH), 10.51 (s, 1H, CO-NH), 7.83-7.58 (d, *J* =8.89 Hz, 4H, 4×Ar-H), 7.50 (d, *J* = 15.1 Hz, 1H, CO-CH), 7.41 (d, *J* = 4.6 Hz, 1H, S-CH), 7.37 (d, *J* =1.9 Hz, 2H, Ar-H), 7.20 (d, *J* = 4.6 Hz, 1H, N-CH), 7.09 (d, *J* = 8.3 Hz, 1H, Ar-H), 6.69 (d, *J* =15.7 Hz, 1H, Ar-CH), 2.14(d, *J* =4.6 Hz, 6H, C-CH_3). ^{13}C-NMR (75 MHz, DMSO) δ169.27, 168.73, 164.07, 143.54, 142.81, 139.88, 136.87, 133.66, 127.61, 126.69, 124.89, 123.14, 119.30, 118.74, 108.77, 20.88.

CAST: $C_{18}H_{15}N_3O_5S_2$, yellow powder, ESI-MS:416[M-H]$^-$; mp:176-179℃; ^{1}H-NMR(300 MHz, DMSO-d_6): 10.94 (s, 1H, SO_2-NH), 10.53 (s, 1H, CO-NH), 7.87-7.76 (d, *J* =8.89 Hz, 4H, 4×Ar-H), 7.49 (d, *J* = 15.1 Hz, 1H, CO-CH), 7.19 (d, *J* = 4.6 Hz, 1H, S-CH), 7.02(d, *J* =1.9 Hz, 2H, Ar-H), 6.82 (d, *J* = 4.6 Hz, 1H, N-CH), 6.79 (d, *J* = 8.3 Hz, 1H, Ar-H), 6.63 (d, *J* =15.6 Hz, 1H, Ar-CH). ^{13}C-NMR (75 MHz, DMSO) δ169.25, 165.05, 148.31, 146.06, 143.14, 142.34, 136.66, 127.46, 126.42, 124.95, 121.64, 119.19, 118.46, 116.23, 114.74.

ACASQ: $C_{27}H_{22}N_4O_7S$, white powder, ESI-MS:545[M-H]$^-$; mp: 242-244℃; ^{1}H-NMR(300

MHz, DMSO-d_6): δ 12.01 (s, 1H, SO_2-NH), 10.59 (s, 1H, CO-NH), 8.59 (s, 1H, N-CH), 7.95 (d, *J* =8.8 Hz, 4H, 4×Ar-H), 7.83 (d, *J* = 15.7 Hz, 1H, CO-CH), 7.70 (d, *J* = 8.5 Hz, 1H, Ar-H), 7.56 (d, *J* = 10.30 Hz, 2H, C-CH), 7.51 (d, *J* = 10.30 Hz, 2H, CH-CH), 7.49 (s, 1H, Ar-H), 7.27 (d, *J* = 8.5 Hz, Ar-H), 6.73 (d, *J* = 15.7 Hz, 1H, Ar-CH), 2.21 (d, *J* = 2.29 Hz, 6H, CO-CH_3). ^{13}C-NMR (75 MHz, DMSO) δ168.74, 164.26, 146.63, 143.58, 143.34, 142.81, 139.95, 133.80, 131.39, 129.21, 128.78, 128.35, 127.88, 127.03, 126.68, 124.73, 123.18, 119.16, 118.63, 118.52, 118.06, 20.81.

CASQ: $C_{23}H_{18}N_4O_5S$, yellow powder, ESI-MS:461[M-H]$^-$; mp:255-258℃; ^{1}H-NMR(300 MHz, DMSO-d_6): 11.79 (s, 1H, SO_2-NH), 10.47 (s, 1H, CO-NH), 9.44 (s, 1H, Ar-*O*H), 9.18 (s, 1H, Ar-*O*H), 8.54 (s, 1H, N-CH), 7.90 (d, *J* =8.3 Hz, 4H, 4×Ar-H), 7.60 (d, *J* = 7.3 Hz, 2H, C-CH), 7.50 (d, *J* = 7.5 Hz, 2H, CH-CH), 7.35 (d, *J* = 15.5 Hz, 1H, CO-CH), 6.87 (dd, *J* = 1.9 Hz, 2H, Ar-CH), 6.69 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.43 (d, *J* = 15.6 Hz, 1H, Ar-CH). ^{13}C-NMR (75 MHz, DMSO) δ165.53, 165.03, 148.57, 146.65, 146.10, 144.11, 142.62, 131.40, 128.93, 127.95, 126.42, 121.65, 118.45, 118.26, 116.30, 114.49.

2.4 Lipophilicity determination

N-octanol–water distribution coefficient was used to determine the lipophilicity of caffeic acid sulfonamide derivatives. Firstly, series concentrations of caffeic acid sulfonamide derivatives in PBS saturated n-octanol were prepared and absorbance at respective peak wavelength was measured to plot the standard curves. To determine the lipophilicity of these caffeic acid derivatives, 40 μL of the derivatives (5 μM) was added into 10 mL of PBS saturated n-octanol and thoroughly mixed with a vortex. The obtained emulsion was then centrifuged at 4000 rpm for 5 min to separate the aqueous and organic phases. The UV absorption of caffeic acid derivatives in the organic phase was measured and their concentrations were determined according to respective standard curves. Log P was defined as the logarithm of the ratio between the compound concentration in the organic and that in the aqueous phases, and calculated as $Log[C_{(organic)}]/[C_{(aqueous)}]$ [19]. $C_{(organic)}$ and $C_{(aqueous)}$ represent the concentration of caffeic acid sulfonamide derivatives in the organic and aqueous phase, respectively.

2.5 DPPH Scavenging capacity evaluation

DPPH assay was used to evaluate the radical scavenging capacity of synthesized caffeic acid sulfonamide derivatives. Briefly, 3.94 mg DPPH was dissolved in 100 ml of anhydrous ethanol and 2 mL of this solution was mixed with series concentrations of derivatives in ethanol. The

mixture was kept at room temperature in dark for 30 min before measuring the absorbance at 519 nm using an UVmini-1285 (Shimadzu, Japan). The DPPH scavenging activity was calculated as percentage of $(A_{(control)} - A_{(sample)})/A_{(control)}$. $A_{(control)}$ and $A_{(sample)}$ represent the absorbance of control sample and samples with caffeic acid sulfonamide derivatives, respectively.

2.6 *In vitro* cytotoxicity assay

Human lung carcinoma A549 cells, purchased from the cell bank of the Chinese Academy of Science (Shanghai, China), were cultured in completed DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 U/mL streptomycin. They were maintained in humidified atmosphere at 37°C with 5% CO_2. The cytotoxicity of synthesized compounds against A549 cells was evaluated to access their potential safety issues using MTT assay. Briefly, cells were seeded in 96-well plates with a concentration of 5×10^4 cells/mL and 100 μL per well. After 12 h, the supernatant was replaced with new medium containing series concentrations of caffeic acid sulfonamide derivatives and cells were cultured for another 48 h later. The medium was discarded and 100 μL of MTT (0.5 mg/mL in DMEM) was added into each well. After incubating at 37°C for another 4 h, the supernatant was carefully removed and the purple MTT crystals were dissolved in DMSO. The absorbance at 490 nm were measured using a multi-functional microplate reader (TECAN, Switzerland) and cell viability was calculated as percentage of $(OD_{(test)} - OD_{(blank)})/(OD_{(control)} - OD_{(blank)})$. $OD_{(control)}$ and $OD_{(test)}$ represent the optical density of cells without and with caffeic acid sulfonamide derivatives pre-treatment, respectively. $OD_{(blank)}$ denote the optical density of wells without cells and drugs.

2.7 Effects of caffeic acid sulfonamide derivatives on H_2O_2 induce oxidative stress

2.7.1 Determination of H_2O_2 concentration

A549 cells were cultured in 96-well plates with 5000 cells per well for 36 h and then incubated in series concentrations of H_2O_2 diluted with serum-free culture medium for 4 h. Cell viability was measured according to the method described in 2.6. The concentration of H_2O_2 causing around 50% decrease in cell viability was selected for following experiments and the concentration was determined to be 800 μM.

2.7.2 Cell viability

A549 cells were seeded into 96-well plates with a concentration of $5x10^4$ cell/mL and 100 μL per well. After overnight cultured, cells were treated with series concentration of

caffeic acid sulfonamide derivatives for 24 h, followed by administration with 800 μM of H_2O_2 for 4 h. Cell viability was measured following the procedure described in 2.6.

2.7.3 ROS generation

To analyze the ROS production, cells cultured in 12-well and 48-well plates were treated with caffeic acid sulfonamide derivatives (12.5 μM) for 24 h and then administrated with H_2O_2 (800 μM) for 4 h. Cells in 24-well plates were collected using trypsin and incubated with 10 μM of DCFH-DA in serum free DMEM at 37°C for 30 min in dark, followed by washing twice with serum-free DMEM. Fluorescence intensity was measured using a flow cytometry (BD, USA) and data was analyzed using FlowJo software. Cells cultured in 48-well plates were labeled with DCFH-DA and imaged on an inverted microscope (Nikion, Japan).

2.7.4 MDA production

Cells were cultured in 6-well plates with a concentration of $5x10^4$ cell/mL and 2 mL per well. After treating with 12.5 μM of caffeic acid sulfonamide derivatives for 24 h, cells were then administrated with 800 μM of H_2O_2 for 4 h. Cells were harvested using 0.25% trypsin followed and collected by centrifugation at 1500 rpm for 5 min. These cells were disrupted by ultrosonication on ice, followed by centrifugation at 3000 rpm for 10 min at 4°C. MDA levels in the supernatant were measured using commercial assay kits following the manufacturer's instructions.

2.7.5 Intracellular antioxidative enzyme activities analysis

The activities of SOD, GSH-Px and CAT in the supernatant prepared in 2.6.5 were analyzed using commercial assay kits according to the manufacturer's instructions.

2.7.6 mRNA levels of Nrf2 pathway associated genes

Cells in control, model group and caffeic acid sulfonamide derivatives treated groups were lysed using Trizol and total mRNA was extracted using Total RNA Extraction Reagent (Vazyme, China). mRNA was reverse transcribed into cDNA using HiScript III RT SuperMix for qPCR kit (Vazyme, China). SYBR-Green I PCR kit (Vazyme, China) was used to amplify cDNA using the following conditions: initial denaturation at 95°C for 30 sec, followed by 35 amplification cycles at 95°C for 10 sec, 55°C for 10 sec, 72°C for 15 sec, and a final extension at 72°C for 10 min. β-actin was used as an internal reference gene. Relative values for mRNA levels were calculated using $2^{-\Delta\Delta Cq}$ methods and presented as fold change of internal reference gene β-actin. Primers used

for Nrf2, NQO1, HO-1 and TXNRD1 are listed in table 1.

Table 1 Sequence of the primers for qRT-PCR.

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
Nrf2	GAGACAGGTGAATTTCTCCCAAT	GGGAATGTGGGCAACCTGGG
NQO1	AAGAAAGGATGGGAGGTGGT	GCTTCTTTTGTTCAGCCACA
TXNRD1	GGAACTAGATGGGGTCTCGG	TCTTGCAGGGCTTGTCCTAA
HO-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCG
β-actin	GATCATTGCTCCTCCTGAGC	ACTCCGCTTGCTGATCCAC

2.8 Statistical analysis

All assays were repeated at least thrice. Data were presented as means ± SD. For all statistical comparison, one way analysis of variance (ANOVA) followed by LSD post hoc test was used. *P* value < 0.05 was considered as statistically significant.

3 Results

3.1 Characterization of caffeic acid sulfonamide derivatives

The six compounds were characterized by melting points test and it was found that ACAST exhibited the lowest mp of 142-145℃, and CASMZ showed the highest mp of 258-260℃. From the ESI-MS results, the molecular ion peaks of m/z for these caffeic acid sulfonamide derivatives range from 414 to 545 (Fig .S1 - S6). The NMR spectra showed that the proton of the SO_2-SH group were highly reactive and the proton peak corresponding to this proton disappeared after exchange with heavy water. The proton peaks denoting the –OH group of CASMZ, CAST and CASQ were found at δ 9.55 - 9.18 (Fig. S13 - S18), while the proton peaks for the carbon of the $–CH_3$ group in ACASMZ, ACAST and ACASQ were appeared at δ 20.81 - 20.80 (Fig. S19 - S24). In the IR spectra, the absorption peaks of the O-H group in CASMZ, CAST and CASQ were observed between 3374.61-3525.53 cm^{-1}, whereas the C=O group of ester group in ACASMZ, ACAST and ACASQ were found between 1765.69-1772.16 cm^{-1}. The absorption peaks of the two -NH groups were ranged from 3028.85-3263.80 cm^{-1}, and the peaks of C=O group adjacent to the amide group of sulfonamide were found between 1664.57-1682.34 cm^{-1}.

3.2 Lipophilicity of caffeic acid sulfonamide derivatives

The UV spectra of caffeic acid sulfonamide derivatives in water, ethanol, PBS saturated

n-octanol and n-octanol saturated PBS between 250~600 nm was obtained on a UV-2600. There is a sharp absorption peak at ~316 nm and a broad absorption peak at ~340 nm in the spectrum of CASMZ, CAST and CASQ (Fig. 2A). The protection of hydroxyl groups on the benzene ring of caffeic acid backbone resulted in the disappearance of the broad peak at ~340 nm and thus only one sharp peak at ~316 nm was observed at the spectrum of ACASMZ, ACAST and ACASQ. Similar spectrum was obtained when different solvents were used and absorption peaks were appeared at the same wavelength. The maximum peak wavelengths for ACASMZ, CASMZ, ACAST, CAST, ACASQ, CASQ and caffeic acid were determined to be 316, 342, 316, 338, 316, 342 and 324 nm, respectively.

Standard curves exhibited good linear relationship between the UV absorbance and the concentrations of caffeic acid derivatives in the PBS-saturated n-octanol with R^2 of 0.999 (Fig. 2B). The linear range for caffeic acid was 0.312~5.0 μM and those for all caffeic acid sulfonamide derivatives were 1.25~20.0 μM. According to standard curves, concentrations of caffeic acid sulfonamide derivatives in the PBS-saturated n-octanol and that in the n-octanol saturated PBS were obtained according to their absorbance at peak wavelength. Their oil-water distribution coefficient (Log P) was calculated according to the concentration of derivatives in these two phase and results were shown in table 2. The conjugation of sulfonamides to the backbone of caffeic acid significantly increased its oil-water distribution coefficient. ACAST exhibited the strongest lipophilicity with an Log P of 1.6414, followed by CAST, ACASQ, CASQ, ACASMZ, and CASMZ with Log P of 1.4738, 1.1748, 0.9864, 0.6577, 0.3482 and 0.3482, respectively. Protection of hydroxyl groups in the benzene ring of caffeic acid increases the oil-water distribution coefficient. However, caffeic acid exhibited a negative Log P of -1.4171.

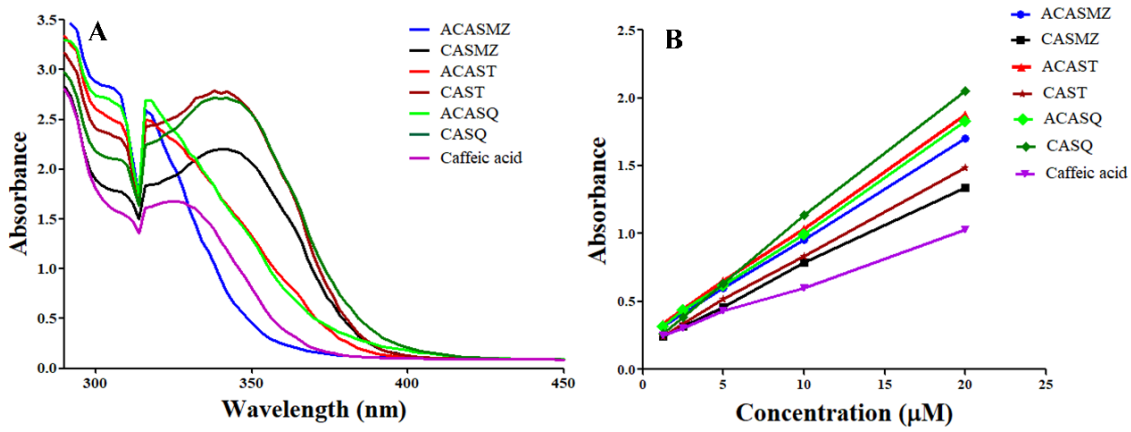

Fig. 2 UV spectrum of caffeic acid sulfonamide derivatives (A) and standard curves prepared by measuring the absorbance at peak wavelength of series concentration of these derivatives in n-octanol saturated PBS (B).

Table 2 Standard curves and Log P of caffeic acid sulfonamide derivatives.

Compound name	slope	Y-intercept	R^2	Log P
ACASMZ	0.07376	0.2235	0.9999	0.6577
CASMZ	0.05879	0.1735	0.9990	0.3482
ACAST	0.08156	0.2377	0.9998	1.6414
CAST	0.06593	0.1724	0.9994	1.4738
ACASQ	0.08018	0.2171	0.9993	1.1748
CASQ	0.0955	0.1543	0.9996	0.9864
Caffeic acid	0.03432	0.1773	0.9994	-1.4171

3.3 DPPH scavenging capacity

From Fig. 3, the radical scavenging rate increased as the concentration of caffeic acid derivatives increased, exhibiting a dose-dependent manner (Fig. 3). Table 3 showed that the IC_{50} is between 40~50 μM for derivatives with two active hydroxyl groups on the benzene ring, and these derivatives included CASMZ, CAST and CASQ. Protection of hydroxyl groups with acetyl groups resulted in almost five times higher IC_{50}, which were 182.7, 185.7 and 189.8 μM for ACASMZ, ACAST and ACASQ, respectively.

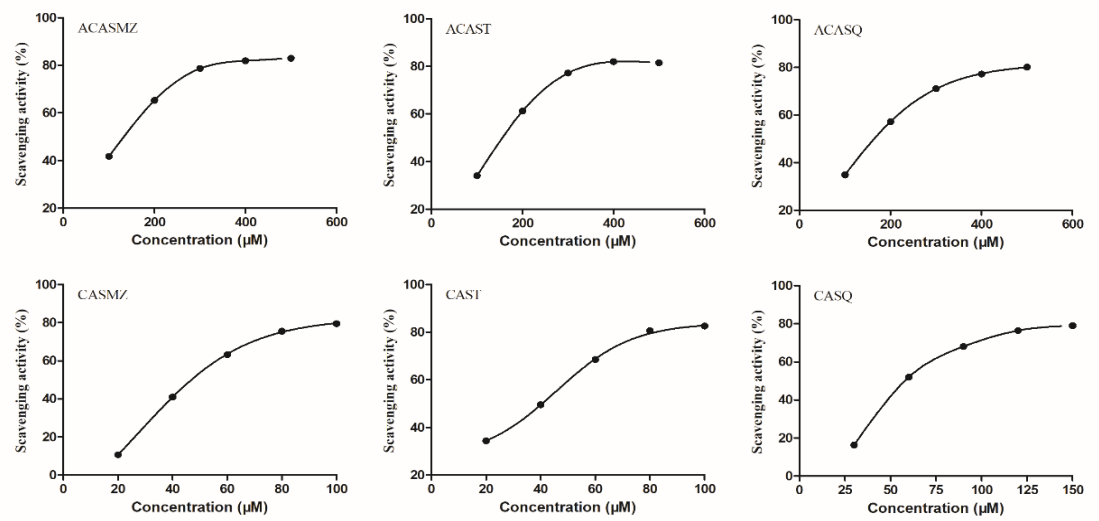

Fig. 3 Radical scavenging capacity of caffeic acid sulfonamide derivatives.

Table 3 Radical scavenging capacity of caffeic acid sulfonamide derivatives.

Compounds	**IC_{50} (μM）**	**R^2**
ACASMZ	182.7	0.9951
CASMZ	42.2	0.9986
ACAST	185.7	0.9980
CAST	49.5	0.9626
ACASQ	189.8	0.9970
CASQ	45.5	0.9959
Caffeic acid	40.9	0.9955

3.4 *In vitro* cytotoxicity of caffeic acid sulfonamide derivatives against A549 cells

Safety issue is the most important concern in drug develpment. Hence, cytotoxicity of caffeic acid sulfonamide derivatives was firstly evluated using A549 cells. From Fig. 4, the viability of A549 cells was higher than 80% of control for all tested derivatives at a concentration ≤200 μM. Except 200 μM of ACAST ($P<0.05$), 200 μM of CAST ($P<0.01$), 100 μM ($P<0.05$) and 200 μM ($P<0.01$) of ACASQ, no significance on cell viability was observed between derivatives treated cells and control cells.

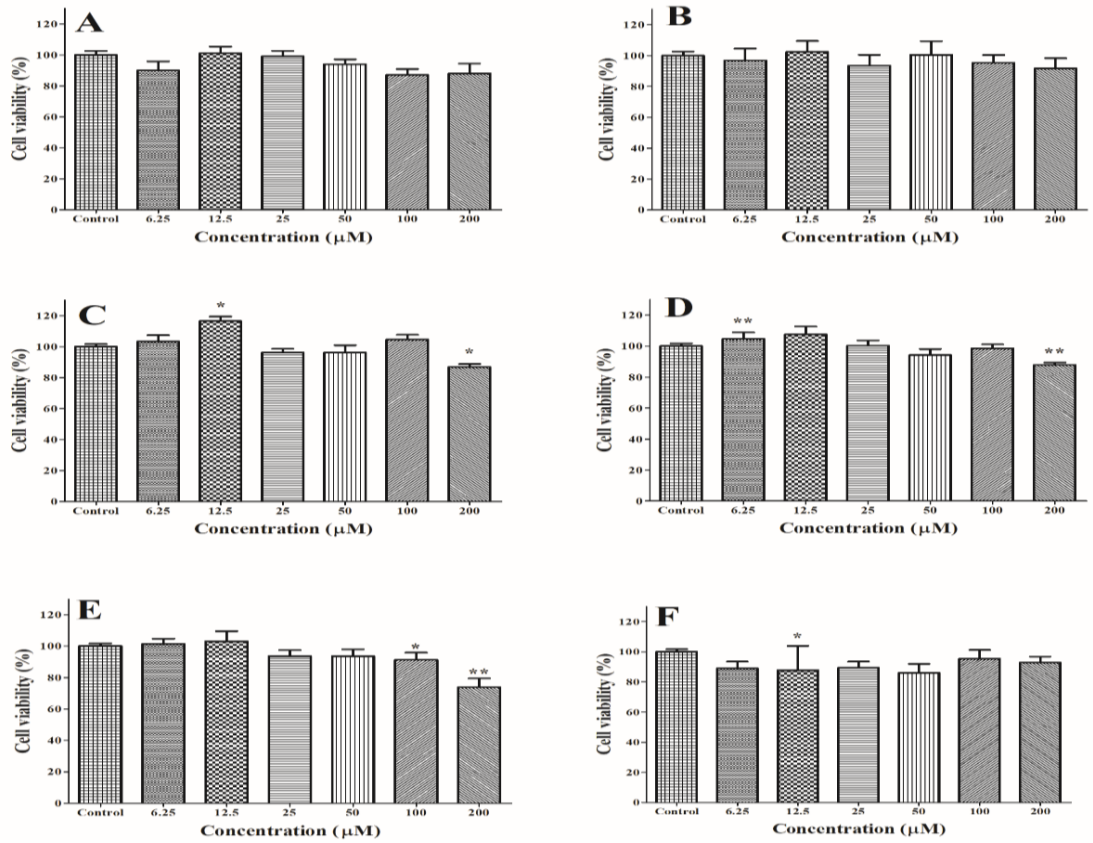

Fig. 4 Cytotoxicity of caffeic acid sulfonamide derivatives against A549 cells. A: ACASMZ, B: CASMZ, C: ACAST, D: CAST, E: ACASQ, F: CASQ. *, ** and *** indicate statistical difference from control in significant levels of $P<0.05$, $P<0.01$, $P<0.001$. (n=4)

3.5 Effect of caffeic acid sulfonamide derivatives on H_2O_2 induced oxidative stress in A549 cells

3.5.1 Determination of H_2O_2 concentration

Viability of A549 cells decrease as the concentration of H_2O_2 increase. Significantly lower viability was observed in cells treated with H_2O_2 in concentrations higher than 50 μM ($P < 0.05$) and the cell viability decreased to 61.61% and 50% when cells were administrated with 800 μM and 1000 μM H_2O_2, respectively (Fig. 5). To maintain sufficient number of cells to study other parameters, 800 μM of H_2O_2 was chosen for following experiments.

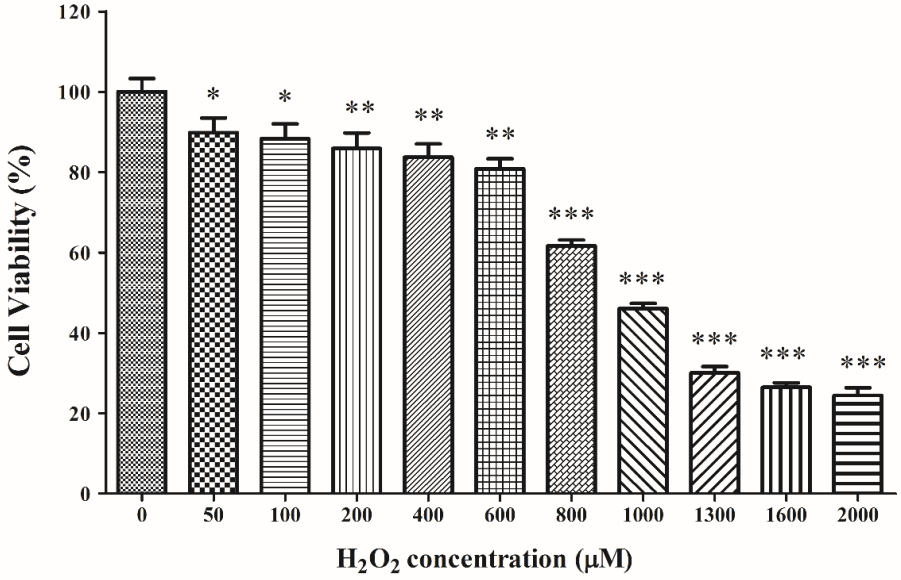

Fig. 5 Viability of A549 cells administrated with series concentrations of H_2O_2 for 4 h. *, ** and *** indicate statistical difference from control in significant levels of $P<0.05$, $P<0.01$, $P<0.001$; #, ## and ### indicate statistical difference from model group in significant levels of $P<0.05$, $P<0.01$, $P<0.001$. (n=4)

3.5.2 Caffeic acid sulfonamide derivatives reduced H_2O_2 induced cell death

From Fig. 6, 800 μM H_2O_2 treatment resulted in ~40% drop in the viability of A549 cells comparing to control. However, pretreatment of cells with series concentration of caffeic acid sulfonamide derivatives for 24 h significantly inhibited H_2O_2 induced decrease of cell viability. Over tested concentrations, the best preventive effect for most of these derivatives was obtained when a concentration of 12.5 μM was used and thus 12.5 μM was chosen for the following experiments.

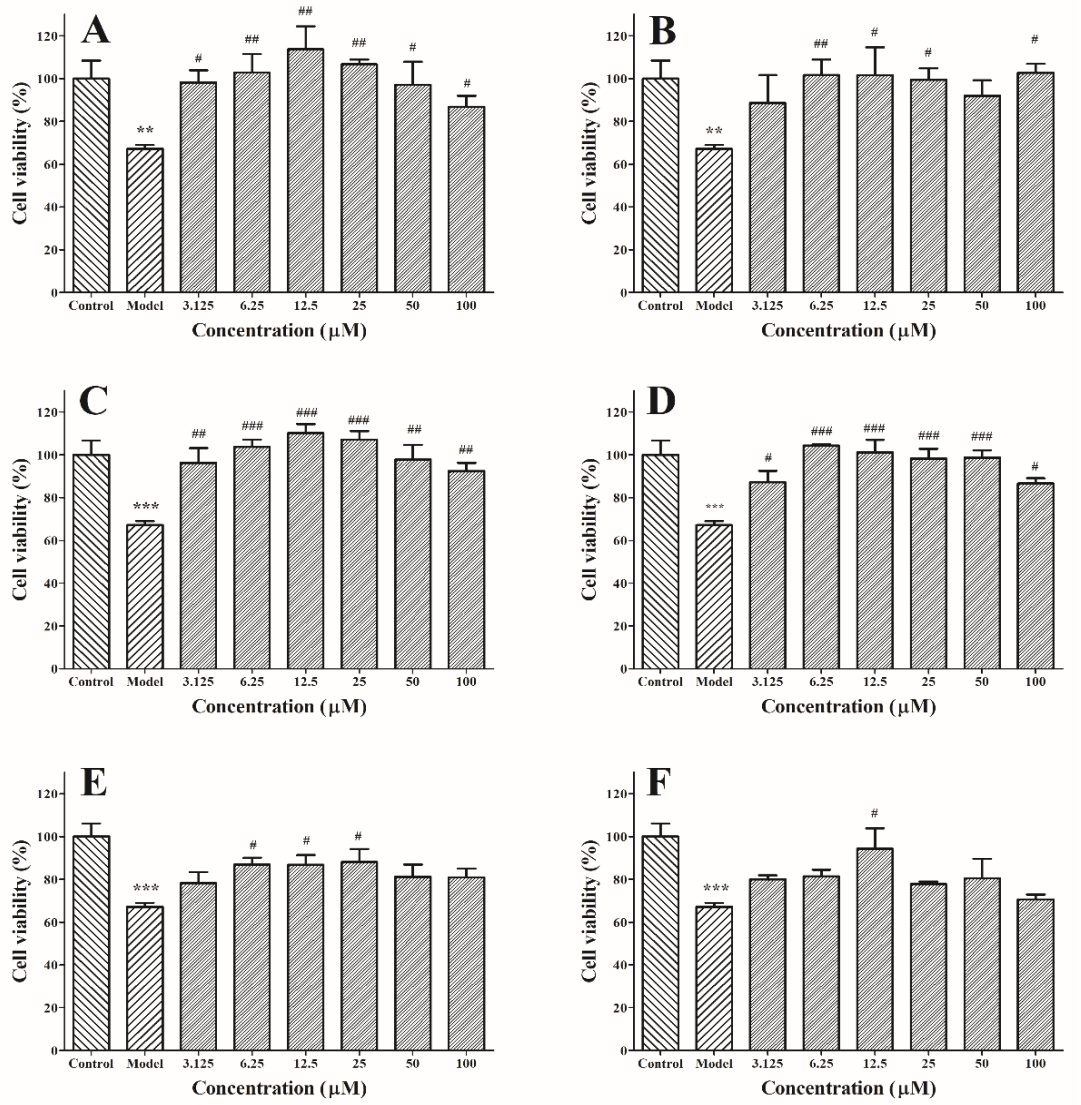

Fig. 6 Viability of A549 cells pretreated with series concentrations of caffeic acid sulfonamide derivatives for 24 h and then administrated with 800 μM of H_2O_2 for 4 h. A: ACASMZ, B: CASMZ, C: ACAST, D: CAST, E: ACASQ, F: CASQ. *, ** and *** indicate statistical difference from control in significant levels of P<0.05, P<0.01, P<0.001; #, ## and ### indicate statistical difference from model group in significant levels of P<0.05, P<0.01, P<0.001. (n=4)

3.5.3 Caffeic acid sulfonamide derivatives inhibited ROS generation

The content of ROS generation was analysed by DCFH-DA fluorescent probes using both flow cytometry and fluorescent microscope. From Fig. 7, brighter green fluorescence and larger number of green cells were observed in H_2O_2 group, comparing to control. Pre-treatment of cells with 12.5 μM of caffeic acid sulfonamide derivatives significantly reduced the green fluorescent intensity and the number of cells showing bright green color. From the optical microscopic images, the number of cells in H_2O_2 group was much smaller than that of control

and those of derivatives treated groups, which was in consistent with cell viability results. From the flow cytometry results, significantly higher level of ROS content was observed in cells stimulated with 800 μM H_2O_2, while derivatives pre-treatment strongly inhibited ROS generation upon H_2O_2 stimulation (Fig. 8A). Among these derivatives, CASQ exhibited the strongest inhibitory effect on ROS generation in H_2O_2 stimulated cells.

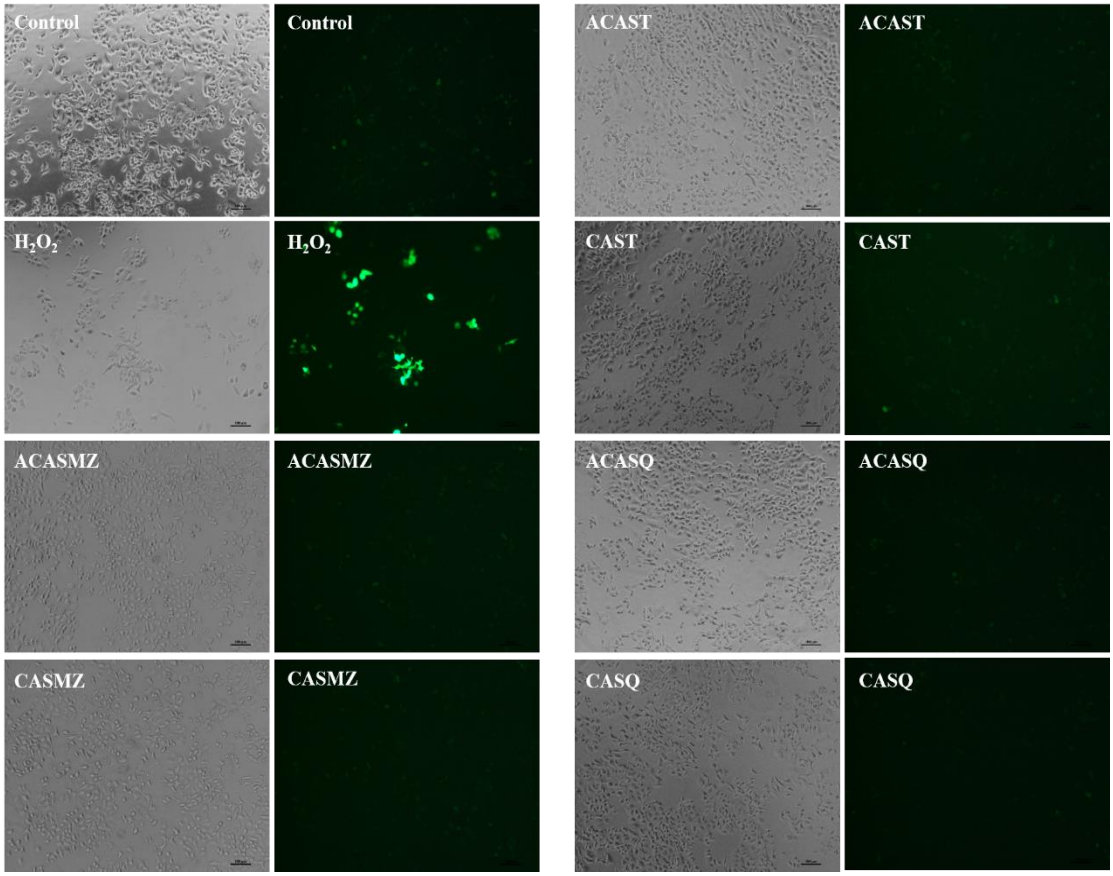

Fig. 7 Optical and fluorescent microscopic images of A549 cells pretreated with 12.5 μM of caffeic acid sulfonamide derivatives for 24 h and then administrated with 800 μM of H_2O_2 for 4 h.

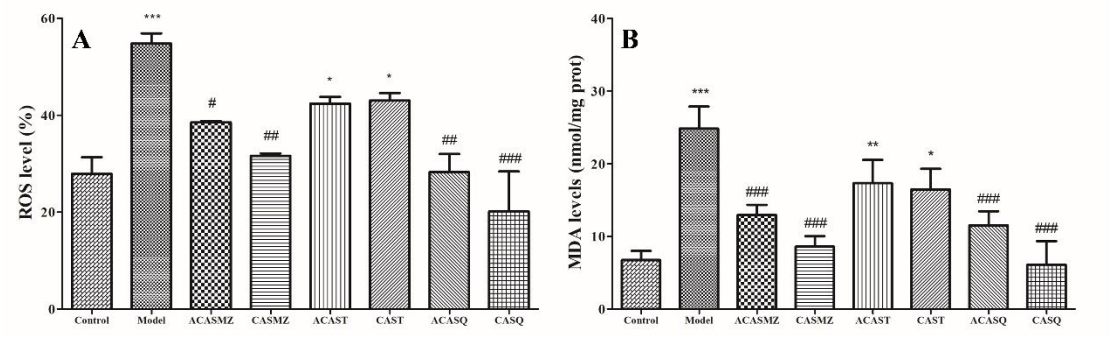

Fig. 8 Effect of caffeic acid sulfonamide derivatives on the production of ROS and MDA in A549 cells pretreated with 12.5 μM of caffeic acid sulfonamide derivatives for 24 h and then administrated with 800 μM of H_2O_2 for 4 h. *, ** and *** indicate statistical difference from

control in significant levels of $P<0.05$, $P<0.01$, $P<0.001$; #, ## and ### indicate statistical difference from model group in significant levels of $P<0.05$, $P<0.01$, $P<0.001$. (n=4)

3.5.4 Caffeic acid sulfonamide derivatives reduced MDA production

From Fig. 8B, H_2O_2 stimulation induced the generation of MDA, showing significantly higher MDA level compared to control cells ($P<0.001$). Pre-treatment of cells with ACASMZ, CASMZ, ACASQ and CASQ before H_2O_2 treatment strongly inhibited the production of MDA, showing significantly lower MDA levels compared to that of model group ($P<0.001$). Among these derivatives, CASMZ and CASQ exhibited the strongest protective effect against H_2O_2 induced lip peroxidation.

3.5.5 Caffeic acid sulfonamide derivatives increased activities of antioxidative enzymes

Fig. 9 shows the activities of SOD, CAT and GSH-Px, three important endogenous antioxidative enzymes, after H_2O_2 stimulation. 800 μM H_2O_2 treatment significantly reduced the enzyme activities ($P<0.001$), while pre-treatment with caffeic acid derivatives strongly attenuated H_2O_2 induced reduction of enzyme levels in significant level of 0.05 or 0.001. Among test derivatives, CASMZ and CASQ exhibited the strongest preventive effect on endogenous antioxidative enzymes against H_2O_2 exposure.

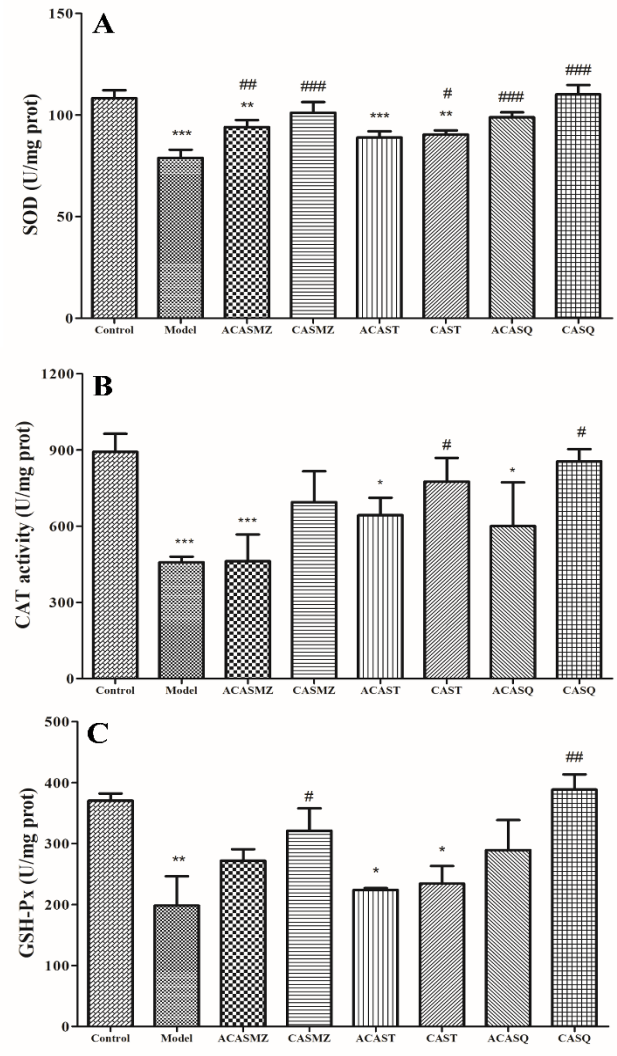

Fig. 9 Effect of caffeic acid sulfonamide derivatives on the activities of endogenous antioxidative enzymes in A549 cells pretreated with 12.5 μM of caffeic acid sulfonamide derivatives for 24 h and then administrated with 800 μM of H_2O_2 for 4 h. A: SOD, B: CAT and C: GSH-Px. *, ** and *** indicate statistical difference from control in significant levels of $P<0.05$, $P<0.01$, $P<0.001$; #, ## and ### indicate statistical difference from model group in significant levels of $P<0.05$, $P<0.01$, $P<0.001$. (n=4)

3.5.6 Caffeic acid sulfonamide derivatives upregulated critical gene expressions

qRT-PCR was used to analyze the mRNA expression of Nrf2 and Nrf2 target genes upon H_2O_2 exposure and relative mRNA expression levels were shown in Fig. 10. It was found that H_2O_2 administration stimulate the transcription of Nrf2, HO-1, NQO1 and TXNRD1. Pre-treatment of cells with caffeic acid sulfonamide derivatives further upregulated the mRNA levels of these genes. Among the tested derivatives, ACASQ and CASMZ exhibited the strongest

promoted effect on Nrf2 expression, while CASQ and CASMZ presented stronger effect on the transcription of HO-1, NQO1 and TXNRD1.

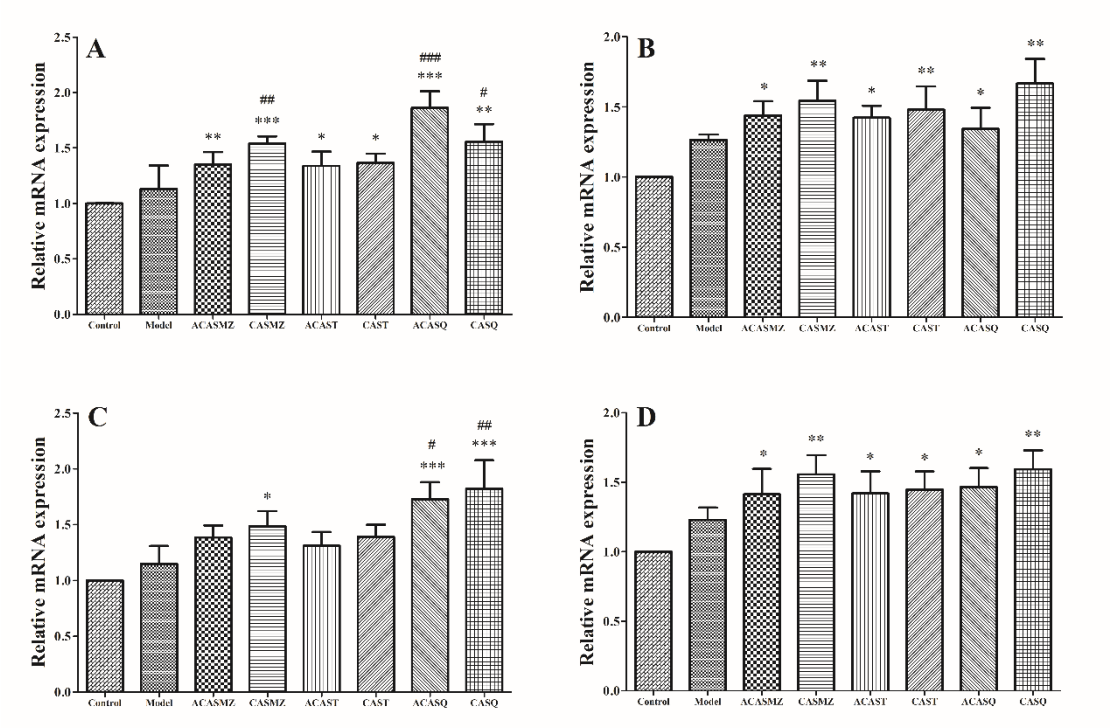

Fig. 10 Relative mRNA expression levels of Nrf2 (A), HO-1 (B), NQO1 (C) and TXNRD1 (D) in A549 cells pretreated with 12.5 μM of caffeic acid sulfonamide derivatives for 24 h and then administrated with 800 μM of H_2O_2 for 4 h. *, ** and *** indicate statistical difference from control in significant levels of $P<0.05$, $P<0.01$, $P<0.001$; #, ## and ### indicate statistical difference from model group in significant levels of $P<0.05$, $P<0.01$, $P<0.001$. (n=4)

4 Discussion

Based on the free radical theory of aging, Harmna firstly proposed the concept of oxidative stress in 1956. This theory holds that aging and malignant disease such as cancer are caused by free radical attack of biological macromolecules induced cell and tissue damages [20]. Studies have shown that oxidative damage is also directly related to cardiovascular disease, neurodegeneration, cancer, metabolic syndrome, inflammation and other important diseases [21, 22]. In recent years, researchers have screened and verified many traditional Chinese medicines, extracts from plants and algae, or well-structured monomers, which exhibit definite activities of anti-oxidative stress. They are helpful in the treatment of oxidative stress associated disease by scavenging excess free radicals and enhancing the activity of antioxidative enzymes [23, 24]. Caffeic acid and its derivatives have strong antioxidative activity, which come from the

combination of free radical elimination and metal ion chelation. In this study, six novel caffeic acid sulfonamides derivatives were synthesized by simple reaction steps under mild conditions. In particular, the purification procedure is extremely simple, without using complicated separation technology such as column chromatography. Final product with high purity was obtained via simple recrystallization based on the difference in physicochemical property between the final product and the raw materials, making it suitable for industrial production.

The antioxidative capacity of chemical compounds usually depends on the number of hydroxyl groups, the presence of adjacent hydroxyl groups in the benzene ring, and the number of hydrogen donor groups (-NH, -SH) [25, 26]. In emulsion systems such as biomenbrane, the antioxidative activity is also associated to the oil-water Partition Coefficient (Log p) and its hydrophobicity [26, 27]. In this study, the lipophilicity of the derivatives obtained by conjugating different sulfonamides to the backbone of caffeic acid was significantly increased, which might be helpful for their diffusion in the lipid bilayer of cell membranes to exhibit antioxidative effect inside target cells [28, 29]. The positions and number of hydroxyl groups in the benzene rings of CASMZ, CAST and CASQ were the same as those of caffeic acid, thus similar IC_{50} values was obtained for these derivatives in the DPPH scavenging assays. The protection of hydroxyl groups on the benzene ring resulted in significant decrease of their radical scavenging ability, showing much higher IC_{50} values in the DPPH radical scavenging assays. However, besides chemical structure characteristics, the antioxidative activity of the chemicals in living cells or organisms also affected by their absorption and distribution, and a Log p between -1 and 2 is usually favorable for the chemical to pass through intestinal epithelial cells [28, 29].

Safety issue is a primary concern in drug development, and *in vitro* cell cytotoxicity assay is often used to verify the biological safety of chemicals in the very early period of drug development. The cytotoxicity of caffeic acid sulfonamide derivatives on A549 cells was assessed by MTT assay in this study. It was found that these derivatives did not show inhibitory effect on cell growth and proliferation at concentrations below 50 μM, suggesting that these derivatives is safe to the cells *in vitro* at concentrations below 50 μM.

H_2O_2 is a stable reactive oxygen species that can freely crossover cell membrane to enter cells. In the presence of transition metals, H_2O_2 convert to highly reactive hydroxyl groups, which can cause DNA damage and mitochondrial dysfunction, even lead to cell death [30, 31]. Thus,

H_2O_2 is often used to establish oxidative stress models for the study of oxidative stress-associated physiological and pathological changes in the organism, and for the evaluation of antioxidative properties of traditional Chinese medicines, extracts from plants, or well-structured monomers [32]. In this study, the antioxidative activity of caffeic acid sulfanilamide derivatives was evaluated by H_2O_2 induced oxidative stress model in A549 cells, and the functional mechanism was preliminary studied based on the expression level of Nrf2 and Nrf2 target genes.

The concentration of H_2O_2 for cell treatment was firstly determined from the viability of A549 cells administrated with series amount of H_2O_2. 800 μM of H_2O_2 caused 40% decrease of cell viability and it was chosen for the following experiments, because this concentration not only clearly distinguished the difference between model group and control group, but also reserved enough cells for the following analysis of oxidative stress products, endogenous antioxidative enzymes activity and the genes expressions. It is worth to mention that this dose of H_2O_2 is quite high comparing to the concentration used in the literature for other kind of cells. It might be associated with different tolerance of various kind of cells against oxidative stress. A549 is a cancerous lung epithelial cell that can use oxidative stress to form neo-antigens to escape the host immune surveillance. Thus, they are more resistant to certain degree of oxidative stress from serious cell damage comparing to other kind of cells, such as neural cells and primary cells [6]. However, 800 μM of H_2O_2 can cause 50% drop of cell viability when pig Alveolar macrophage was used in studies carried out by other group members.

After administrated with 800 μM of H_2O_2 for 4 h, ROS and MDA levels in A549 cells significantly increased, indicating the formation of oxidative stress and lipid peroxidation. The excess ROS not only attacked biomacromolecules but also depleted endogenous antioxidative enzymes. That is why the activities of SOD, CAT and GSP-Px significantly decreased upon H_2O_2 stimulation. In order to resist oxidative stress caused damages, Nrf2 signal pathway was activated and the expression of its target genes was upregulated [33, 34]. These genes include HO-1, NQO1 and TXNRD that encoding antioxidative and detoxification enzymes[35]. Antioxidant treatments not only directly scavenge ROS, block lipid peroxidation, inhibit ROS production, but also regulate Nrf2 signaling pathways to strengthen the endogenous antioxidative system [4]. The high lipophilicity of caffeic acid sulfonamide derivatives enable them to cross over the cell membrane and enter the cell. The derivatives then reduced the H_2O_2 induced cell damage by eliminating ROS

and inhibiting its production, and thus decreased ROS generation and reduced MDA production were observed. The less ROS consumed less antioxidative enzymes and thus higher activities of SOD, CAT and GSH-Px were found in cells pretreated with caffeic acid derivatives. Besides, the mRNA levels of Nrf2 and its target antioxidative and detoxification enzymes, including HO-1, NQO1 and TXNRD1 were further upregulated in derivatives pretreated cells to enhance their resist to oxidative damage. These results were in consistent with other studies on oxidative stress [36-38].

Conclusion

In this study, six novel caffeic acid sulfonamides derivatives were synthesized through simple chemical reactions under mild conditions. The products were easy to purify and the yield is high. They were fully characterized and it was found that they exhibited higher lipophilicity than the parent caffeic acid. The caffeic acid sulfonamide derivatives exhibited good DPPH radical scavenging activity and strong ability to protect A549 cells against damage caused by H_2O_2-induced oxidative stress. They inhibited H_2O_2-induced decline in cell activity, ROS generation, MDA production, and consumption of endogenous antioxidative enzymes. The regulation of Nrf2 signaling pathway may be a mechanism by which they enhance the ability of cells to resist oxidative stress.

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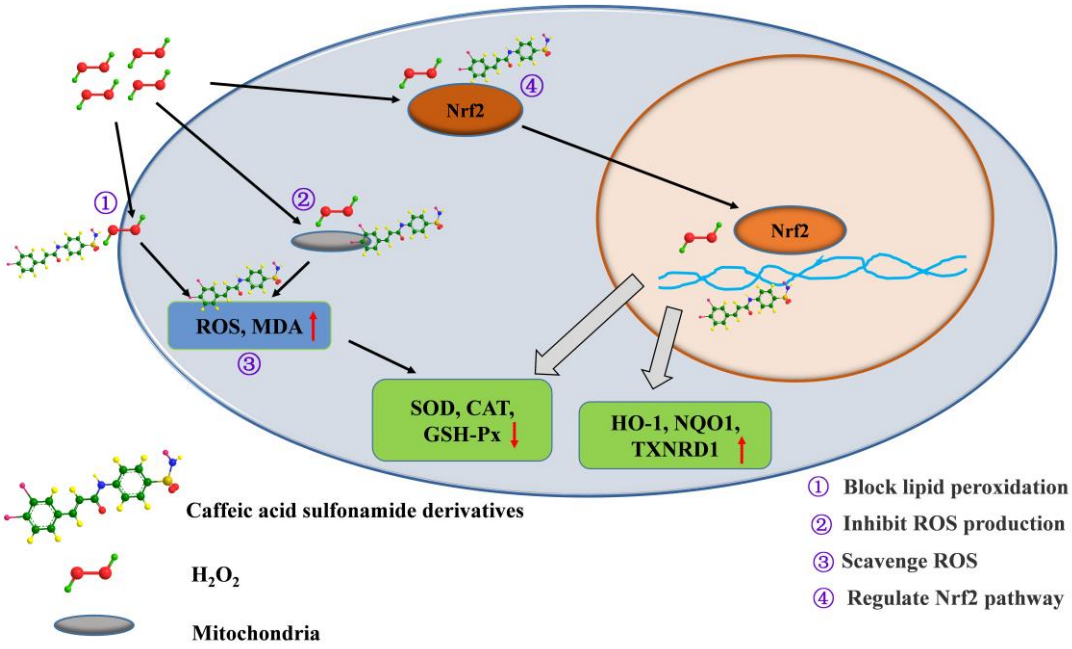

Graphical abstract

Highlights

1. Caffeic acid derivatives were obtained with simple reactions and high yield.
2. Sulfonamide conjugation increase lipophilicity of caffeic acid.
3. Obtained derivatives exhibit strong radical scavenging abilities.
4. Derivatives protect cells from H_2O_2 induced cell damage via Nrf2 pathway.