Electronic Journal of Biotechnology 47 (2020) 89–99

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

Research article

The protective ability and cellular mechanism of *Koelreuteria henryi* Dummer flower extract against hydrogen peroxide-induced cellular oxidative damage

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ARTICLE INFO

Article history:
Received 22 March 2020
Accepted 29 July 2020
Available online 4 August 2020

Keywords:
Antioxidant
Apoptosis
Flower extract
Heme oxygenase-1
Hydrogen peroxide
Koelreuteria henryi Dummer
Nuclear factor-erythroid 2-related factor
Oxidative damage
Taiwan

ABSTRACT

Background: Koelreuteria henryi Dummer is an indigenous plant in Taiwan. The species has been used in traditional folk medicine for the promotion of liver functions and for treating malaria and urethritis. The present study investigated the antioxidant activity of the flower extract of *Koelreuteria henryi* Dummer. The extraction conditions were optimized by the contents of total phenolic acids and total flavonoids, and antioxidant activity assays. Moreover, an *in vitro* study for investigating antioxidant activity of *K. henryi* flower extract was demonstrated by hydrogen peroxide (H_2O_2)-induced apoptosis.
Results: K. henryi flower extracted for 150 min showed high contents of total phenolic acids and total flavonoids. In an *in vitro* model, L929 cells were pretreated with *K. henryi* flower extract, and then treated with H_2O_2 to induce oxidative damage. Results demonstrated that H_2O_2-induced apoptosis was inhibited by the treatment of 200 μg/ml *K. henryi* flower extract through the mitochondria-mediated pathway and mitogen-activated protein kinase (MAPK) pathway. The caspase 8/9 activity and expression of p-p38 and pERK were repressed by *K. henryi* flower extract. In addition, the prevention of H_2O_2-induced apoptosis by *K. henryi* flower extract activated the nuclear factor-erythroid 2-related factor (Nrf2) stress response pathway to transcript heme oxygenase 1 (HO-1). Also, *K. henryi* flower extract prevented H_2O_2-induced apoptosis through HO-1 production, as evident by the use of HO-1 inhibitor.
Conclusions: The present study demonstrated that *K. henryi* flower extract could inhibit the H_2O_2-induced apoptosis in L929 cells through the activation of the Nrf2/HO-1 pathway.
How to cite: Tsai WC, Chang HC, Yin HY, et al. The protective ability and cellular mechanism of Koelreuteria henryi Dummer flower extract against hydrogen peroxide-induced cellular oxidative damage. Electron J Biotechnol 2020;47. https://doi.org/10.1016/j.ejbt.2020.07.006.

1. Introduction

Free radicals are reactive chemicals with unpaired electrons and can be generated through normal cellular metabolism or exposure to environmental oxidants. Moreover, free radicals can also be produced when people are exposed to extensive exercise or environmental pollutants/toxic compounds, such as cigarette smoke, alcohol, pesticides, ionizing and ultraviolet (UV) radiation, and ozone. Reactive oxygen species (ROS), including both free radical and non-radical oxygen-containing molecules, play an essential role in the induction of oxidative stress. Oxidative damage has been reported to be involved in more than 100 diseases, including cancer, atherosclerosis, rheumatoid arthritis, Alzheimer's disease, or Parkinson's disease [1]. As free radicals attack cells, they may directly fracture DNA chain, or indirectly break the DNA chain with a ROS oxidative product malondialdehyde, leading to interference in replication and transcription, and finally gene mutation [2]. When ROS attacks proteins, they can interrupt the cross-linking of peptides, alter protein folding, and thereby inactivate protein functionality [3]. Under severe oxidative stress, ROS induces extrinsic or intrinsic apoptotic signaling pathways by activating Jun N-terminal protein kinase (JNK). In the extrinsic pathway, JNK activates the

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Peer review under responsibility of Pontificia Universidad Católica de Valparaíso.

https://doi.org/10.1016/j.ejbt.2020.07.006

activation protein 1, which induces the expression of tumor necrosis factor-α (TNF-α) and Fas ligand for the activation of downstream caspase 8 to initiate apoptosis [4,5,6]. In the intrinsic pathway, JNK or ROS alter the permeability of mitochondria membrane resulting in a decline of mitochondria transmembrane potential ($\Delta\Psi_m$) and the release of cytochrome c, which activates caspase 9 and caspase 3 to trigger apoptosis [7,8].

Nowadays, people use antioxidants as supplements for scavenging free radicals or activating defensive protein systems. Redox balance is essential to maintain healthy cellular functions since it supports the optimal function of redox-sensitive signaling proteins [9]. HO-1, an oxidative stress response protein, catalyzes heme to produce equimolar amounts of biliverdin, iron, and carbon monoxide (CO) [10]. Previous studies have demonstrated that biliverdin protects from hydrogen peroxide-induced oxidative damage or enzymatically oxidative stress in different cell lines, such as vascular smooth muscle cells, endothelial cells, and cardiac myocytes [11,12,13]. Moreover, CO can induce the intracellular signaling process in anti-inflammatory and anti-apoptosis by modulating the MAPK family [14]. The expression of HO-1 is activated when Nrf2, a redox-sensitive transcriptional factor, binds to the genetic antioxidant response element and activates protective antioxidant genes [15]. Therefore, several studies have been carried out to find natural materials that can protect against cellular oxidative damages by activating Nrf2 and its downstream HO-1. For example, through the Nrf2/HO-1 pathway activation, epigallocatechin-3-gallate from green tea extract inhibited irradiation-induced pulmonary fibrosis [16]; *Ginkgo biloba* extract decreased high glucose-induced endothelial adhesiveness to monocytes for endothelial protection [17], and antroquinonol from mycelium of *Antrodia cinnamomea* protected HepG2 cells from oxidative stress [18].

K. henryi Dummer is an indigenous species in Taiwan, and it grows at altitudes below 1000 M. When in bloom, the whole tree is full of yellow flowers. Therefore, it is known as the 'Flame Golden Tree' in Taiwan. The roots, bark, twigs, leaves, and flowers of *K. henryi* have been used in traditional folk medicine for the promotion of liver functions and for treating malaria and urethritis [19,20]. Regarding anticancer effects of *K. henryi*, anthraquinone, stilbene, and flavonoids from crude extracts of *K. henryi* have shown a significant inhibitory activity to protein-tyrosine kinase, a potential target for controlling tumor cell growth [21]. Also, three cyclolignans (koelreuterin-1, austrobailignan-1, and austrobailignan-2) from the leaves of *K. henryi* have shown significant cytotoxicity to various human tumor cells, possibly through the prevention of tubulin polymerization [22]. Chow's group had found that astragalin from *K. henryi* has better anticancer ability in inhibiting the expression of dihydrodiol dehydrogenase [23]. Recently, Wu and co-workers demonstrated that austrobailignan-1 from *K. henryi* is a topoisomerase 1 inhibitor that can break DNA chains and subsequently trigger DNA damage response signaling for the cell cycle G2/M arrest and apoptosis in non-small cell lung cancer cells [24]. Besides anticancer effects, the leaves of *K. henryi* also have shown the antioxidant capability. Compared to the ethanol extracts from the leaves of 12 selected plants indigenous to Taiwan, the extract of *K henryi* contained abundant phenolic compounds, displayed good antioxidant and potent HO-1 induced activities [25]. Moreover, several flavonol glycosides isolated from the leaves of *K. henryi* exhibited comparatively higher potent radical-scavenging activity than that of trolox [25]. Among 27 cultivated plant species from Taiwan, only the acetone extract of the leaves of *K. henryi* showed strong inhibitory activities towards xanthine oxidase, tyrosinase, and lipoxygenase [26]. So far, there is no report on the evaluation of the antioxidant activity of flowers of *K. henryi* against H_2O_2-induced apoptosis by *in vitro* study. Therefore, in this study, the water extract from flowers of *K. henryi* was used to evaluate its protective effect on H_2O_2-induced oxidative damage in L929 cells, and its potential defense against H_2O_2-induced apoptosis. According to the results of this study, the authors illustrate the schematic representation of the cellular mechanism of inhibition of H_2O_2-induced apoptosis in L929 cells treated with *K. henryi* flower extract as shown in Fig. 1.

2. Materials and methods

2.1. Plant material and extract preparation

The flowers of *K. henryi* were collected from September to December in 2013 in the wild in Taichung City in Taiwan. Proper identification and authentication were done by Professor Hung-Chi Chang at Chaoyang University Technology (Taichung, Taiwan). First, flowers were cleaned and dried in an oven (25°C) and then ground into a fine powder that could pass a 60 mesh screen. Each 50 g of flower powder was extracted with 1 l distilled water with stirring for 150 min at 100°C. The extract was centrifuged at 8000 rpm for 10 min, and the supernatant was filtered using a Whatman No. 1 filter paper. The filtrate was lyophilized by a freeze-dryer (FTS, Stone Ridge, USA), and the lyophilized powder was packaged with a vacuum sealer and stored in an electronic dry cabinet until use.

2.2. Chemicals and reagents

Acetonitrile, 2,2′-azobis (2-methylpropionamidine) dihydrochloride (AAPH), methanol, dimethyl sulfoxide (DMSO), Folin-Ciocale reagent, sodium bicarbonate, Tris (hydroxymethy) aminomethane hydrochloride (Tri-HCl) and Tris (hydroxymethyl) aminomethane (Tris-Base), sodium dodecyl sulfate (SDS), and Tween 20 were obtained from Merck (Darmstadt, Germany). 2,2′-Azino-bis(3-ethyl benzothiazoline-6-sulfonic acid) diammonium salt (ABST), 6-hydroxy-2,5,7, 8-tetramethylchroman-2-carboxylic acid (Trolox), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 30% hydrogen peroxide were purchased for Sigma-Aldrich Co (MO, USA). 3-(4-,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's modified eagle medium powder (DMEM), non-essential amino acids (NEAA), L-glutamine (LG), and 0.4% trypan blue stain were purchased from Invitrogen (Carlsbad, CA, USA).

2.3. Antioxidant activities

2.3.1. DPPH free radical scavenging ability

DPPH free radicals have a maximum absorption at 515 nm, which decreases as radicals are reduced by antioxidant compounds in samples [27]. In this assay, 250 μl of 0.1 mM DPPH solution in methanol was mixed with 50 μl of sample and incubated in the dark for 10 min. The absorbance of the mixture was measured by a plate reader (infinite M200, TECAN, Swiss) at 517 nm. The DPPH free radical scavenging activity was calculated.

2.3.2. Reducing power assay

In this assay, potassium ferricyanide was first reduced by the sample and then reacted with Fe^{3+} to form Prussian blue, which could be detected at 700 nm [28]. Briefly, samples were mixed with 0.2 M phosphate buffer and 1% potassium ferricyanide, and the mixture was subsequently incubated at 50°C for 20 min. After cooling, 10% trichloroacetic acid was added to the mixture and then centrifuged at 1000 × g for 10 min. The supernatant was mixed with distilled water and 0.1% ferric chloride, and incubated at room temperature for 10 min. The absorbance at 700 nm was measured. The increase of the absorbance indicated the increase of the reducing power of the samples.

2.3.3. Oxygen radical absorbance capacity (ORAC) assay

AAPH, a free radical initiator, can produce peroxyl radicals to break down the fluorescent probe, β-phycoerythrin, leading to an increase of fluorescence intensity. Thus, in the ORAC assay, the antioxidant compounds in samples were used to protect the degradation of β-phycoerythrin from the attraction of the free radical [29]. First, the flower extract was mixed with β-phycoerythrin and pre-

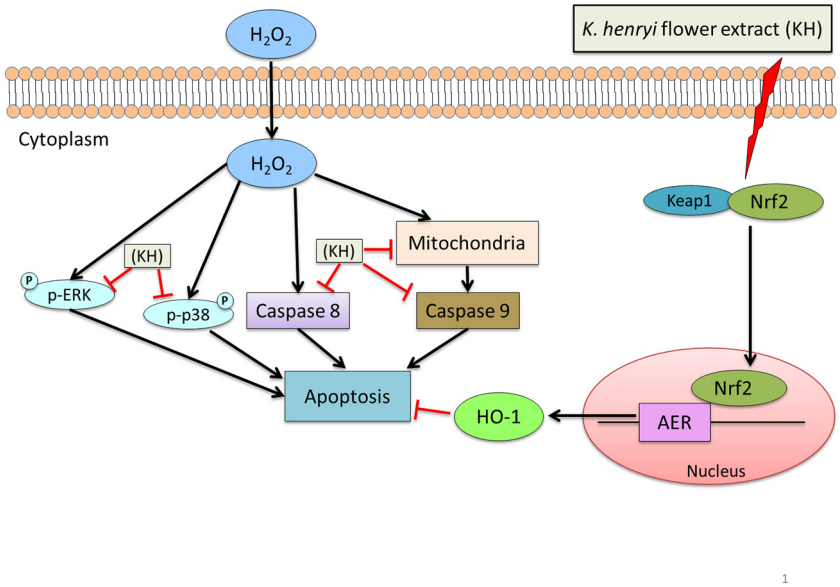

Fig. 1. Schematic representation of the cellular mechanism of inhibition of H_2O_2-induced apoptosis in L929 cells treated with *K. henryi* flower extract.

incubated at 37°C for 5 min. After adding the APPH solution with a final concentration as 12 mM, the fluorescence of reaction mixture was recorded for 60 min at excitation and emission wavelengths of 485 and 530 nm, respectively. A blank sample of phosphate buffer was used to replace the flower extract, and four concentrations of Trolox were used to establish a standard curve. To quantify the antioxidant activity of the sample, the area under the curve (AUC) was calculated by integrating the relative fluorescence curve. The net AUC of samples was calculated by deducting the AUC of a blank sample. Based on the standard curve with Troxol, the ORAC value of each sample was determined and expressed as mmol Trolox/g of plant extract.

2.3.4. Trolox equivalent antioxidant capacity (TEAC) assay

The TEAC assay was based on suppressing the absorbance of ABST•$^+$ radical by the antioxidant compound in the sample, as ABST incubates with a peroxidase and hydrogen peroxide. The ABST•$^+$ radical has a bluish-green color with maximum absorbance values at 734 nm. The solution containing 0.6 ml double distilled water, 0.1 ml ABST, 0.1 ml H_2O_2, 0.1 ml peroxidase, and 100 μl sample (or standard; Trolox) was mixed and incubated for 10 min, and subjected to a plate reader at 734 nm [30].

2.3.5. The total phenolic and flavonoids content

The total phenol content of samples was measured based on the Folin–Ciocalteu method with gallic acid as the standard [31]. Thirty microliters of the sample (or standard) was mixed with 150 μl of 1% Folin–Ciocalteu's reagent and 120 μl 7.5% sodium bicarbonate solution for 10 min. The absorbance of the mixture was measured by a plate reader at 765 nm, and the results were expressed as mg gallic acid/g of extract. Also, the total flavonoid content of samples was analyzed by the aluminum chloride colorimetric method with rutin as the standard. One hundred-microliter sample (or standard) was mixed with 100 μl of 2% aluminum and incubated for 10 min. The mixtures were detected by a plate reader at 420 nm, and the data were expressed as mg rutin/g of extract.

2.4. In vitro cell viability study

The L929 mouse fibroblast cell line (ATCC number CCL-1) was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). These cells were cultured in Eagle's minimum essential medium supplemented with 10% heat-inactivated new horse serum, 2 mM L-glutamate, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 1.0 mM sodium pyruvate. The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. Oxidative stress was induced by exposing the cells to hydrogen peroxide, and the MTT assay was conducted to evaluate the viability of L929 cells [32]. Briefly, the L929 cells (4×10^4 cells/well) were incubated in 24-well plates for 24 h and then washed with phosphate-buffered saline (PBS) before they were treated with the flower extracts, followed by incubation with 0.25 mM hydrogen peroxide for 3 h. After treatment, cells were washed with PBS, and MTT (5 mg/ml) was added to react for 4 h. Furthermore, the medium was removed, and 0.8 ml of DMSO was added to each well, and the absorbance at 570 nm of the dissolved solutions was measured by a plate reader. The survival rate of the L929 cells (%) to that of the control was calculated by (Absorption treated) / (Absorption control) × 100%.

2.5. In vitro apoptosis study

2.5.1. Mitochondrial membrane potential

Mitochondrial membrane potential was measured by JC-1 Mitochondrial Membrane Potential Assay Kit. First, L929 cells were seeded in 6-well plates (2×10^5 cells/well) for 24 h. After treatment with flower extract for 2 h, cells were exposed to 0.25 mM H_2O_2 for 3 h. Cells were then trypsinized, and the cell pellets were suspended and incubated with JC-1 dye for 20 min at 37°C in the dark. After washing with PBS, cells were re-suspended in the assay buffer and analyzed using a fluorescent plate reader. In healthy cells, JC-1 forms J-aggregates, which exhibited red fluorescence, while in apoptotic cells, JC-1 was present as monomers, which exhibited green fluorescence. The increase in the ratio of red to green fluorescence indicates the increase of mitochondrial membrane potential, $\Delta\Psi m$.

2.5.2. Apoptosis assay with Annexin V-FITC and Propidium iodide (PI) double stain

Annexin V-FITC Apoptosis Detection kit (Biovision, America) was used to distinguish apoptosis and necrosis state of cells. L929 cells (2×10^5 cells/well) cultured in 6-well plate for 24 h at 37°C. After pretreatment with the flower extract, cells were treated with 1 mM H_2O_2 for 3 h and harvested by trypsinization and incubated with

Annexin V-FITC solution and PI solution for 15 min in the dark. The cells were analyzed by flow cytometry (CyFlow® space, Partec, Münster, Germany) to access the degree of apoptosis. A minimum of 10,000 cells were tested for each assay at a flow rate of <100 cells/s [33].

2.5.3. Caspase 8 and caspase 9 fluorometric assay

L929 cells (2.5×10^5 cells/well) were seeded in 6-well plates for 24 h and then collected at 10000 × g for 10 min at 4°C. Cells were re-suspended in 50 μl of chilled lysis buffer and incubated on ice for 10 min. The protein concentration of the supernatant was quantified and prepared as 50 μg/50 ml. Fifty microliters of 2× reaction buffer containing 10 mM dichloro-diphenyl-trichloroethane was added to each sample. Five microliters of the 1 mM IETD-AFC substrate (caspase 8) or 1 mM LEHD-AFC substrate (caspase 9) was added and incubated at 37°C for 2 h. Signals of samples were detected by a fluorescence plate reader with a 360 nm excitation and 460 nm emission.

2.6. Measurement of intracellular ROS

Intracellular ROS was measured with Dichloro-dihydro-fluorescein diacetate (DCFH-DA) cut by intracellular esterase to form DCFH. Subsequently, DCFH was oxidized by intracellular ROS to produce the highly fluorescent 2,7-dichlorofluorescein (DCF) [34]. Cells were seeded in 6-well plates (2.5×10^5 cells/well) and treated with 200 μg/ml of flower extract for different durations (30–360 min). After washing with PBS, the cells were treated with 10 μM DCFH-DA for 60 min in the dark, after that, washed with PBS and then collected by trypsinization. The cells were sonicated for 1 min and detected with a fluorescence microplate reader in excitation at 485 nm and emission at 530 nm.

2.7. Preparation of nuclear protein extract

L929 cells cultured in 10 cm dishes were collected, washed with PBS, and then re-suspended in 250 μl hypotonic buffer in a pre-chilled microcentrifuge tube. After incubation for 15 min on ice, 12.5 μl detergent was added, and samples were vortexed for 10 s at the maximum speed. After centrifugation for 30 s at 14000 × g at 4°C, the supernatant was removed, and the nuclear pellet was re-suspended in 25 μl complete lysis buffer. The supernatant of nuclear protein was obtained with 14,000 × g for 10 min in a centrifuge at 4°C after incubation for 30 min on ice.

2.8. Western blot

L929 cells were seeded in 6-well plates at the density of 8×10^5 cells/10 cm dish. After treatment, the protein samples extracted from treated cells were analyzed with SDS-PAGE and then transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA) with 100 mA for 1 h. The nonspecific binding site was blocked with 0.1% gelatin in Tris-buffered saline with 0.1% Tween 20 buffer. Furthermore, the membrane was incubated with the primary antibody for overnight at 4°C followed by incubation with the secondary antibody at room temperature for 1 h [35]. The band was visualized by electrochemiluminescence reagent (NEN, Boston, MA) in the Luminescence image system. Protein quantity was determined by using ImageJ software.

2.9. Statistical analysis

Statistical analysis was performed with SAS software, and the values were expressed as mean ± standard deviation. Differences among the groups were compared, and the results of the probability level lower than 5% ($p < 0.05$) were considered as significantly different.

3. Results and discussion

3.1. The antioxidant ability of K. henryi flower water extract

To optimize the extraction time for *K. henryi* flower water extract, the antioxidant ability of *K. henryi* flower was determined by different assays, including reducing power, DPPH, ORAC, and TEAC assay. *K. henryi* flower was extracted with water for 60, 90, 120, 150, 180, or 210 min, and the results of antioxidant ability obtained are shown in Table 1. DPPH has the free radical to react with antioxidants, which were followed by monitoring the decrease in its absorbance. The results of the DPPH free radical scavenging ability showed that there was no significant difference in each extraction time except 210 min. The EC_{50} of *K. henryi* flower extract was superior to other extracts at 210 min. The result of reducing power assay showed that the EC_{50} of *K. henryi* flower extract had a marginal decrease after 150-minute extraction. The EC_{50} was the highest at 90 min, but there was no significant difference at 60–120 min. In the ORAC assay, *K. henryi* flower extracted for 60 min was 1.83 M Trolox, and it had the lowest antioxidant capacity compared with other extraction periods. The value increased with the increase of extraction time from 60 to 150 min. The ORAC value of *K. henryi* flower extract was the highest at 150 min, and there was no significant difference when extracted at 120–180 min. However, the results of the TEAC assay showed that the value increased with the increase of extraction time. There was no difference in the TEAC results for *K. henryi* flower extracted at 120–210 min.

Polyphenols are a large group of natural antioxidants that can protect people against cancer and cardiovascular diseases. Polyphenols have two major groups, phenolic acids and flavonoids, and the content of total phenolic and total flavonoids were measured in this study to see the connection with antioxidant activity assay. The total phenolic acids content increased with an increase in extraction time from 60 to 150 min but decreased marginally from 180 to 210 min. The maximum content of total phenolic acids was recorded at 150 min (402.48 mg gallic acid/g) (Table 1). A similar trend was observed with total flavonoids with the maximum contents at 150 min (9.17 mg rutin/g). Moreover, the results of DPPH (EC_{50} 0.04 mg/ml), reducing power (EC_{50} 0.131 mg/ml), and ORAC (2.53 mmol Trolox/g) assays had better value in 150-minute extract than others due to the abundance of phenolic acids and flavonoids. Given these results, the extraction time of 150 min was selected.

Chen and co-workers found that there were more antioxidant components in the water extract of *K. henryi* flower (27.2%) than that in the ethanol extract (27.2%) and methanol extract (20.2%) [36]. The total phenolics content of the water extract of *K. henryi* flower in our study were higher than those of ethanol extract of *K. henryi* leaves in Lee's study [25], and the DPPH free radical scavenging ability of the ethanol extract of *K. henryi* leaves was better than the water extract of *K. henryi* flower. It may be the effect of the extraction solvent since antioxidant compound was extracted by ethanol. The antioxidant activity could be improved by isolation of the antioxidant compounds from abundant of antioxidant components in the water extract of *K. henryi* flower. Moreover, two compounds of 1,3,4,5-tetra-O-galloylquinic acid and kaempferol 3-O-(200,300-di-O-galloyl)-a-L-rhamnopyranoside isolated from *K. henryi* leaves had good antioxidant activity [19,37].

3.2. Determination of the treatment model for L929 cells

To investigate the concentration of *K. henryi* flower extract that did not have toxicity to cells, L929 cells were treated with various concentrations (100–500 μg/ml) of extract for 6 h (Fig. 2). The survival rate of L929 cells treated with *K. henryi* flower extract (100–250 μg/ml) was around 100% in contrast to survival rates between 93.1 and 72.2% with the treatment of higher concentrations (300–500 μg/ml). The concentrations of *K. henryi*

Table 1
The antioxidant ability and the contents of total phenolics and total flavonoids in *K. henryi* flower extract extracted with distilled water for 60, 90, 120, 150, 180 and 210 min.

		Antioxidant assays			Total phenolics and flavonoids contents	
Time (min)	DPPH (EC_{50}, mg/ml)	Reducing power (EC_{50}, mg/ml)	ORAC (Trolox, mmol/g)	TEAC (Trolox, mmol/g)	Total phenolics (gallic acid, mg/g)	Total flavonoids (rutin, mg/g)
60	0.043 ± 0.001 ^{b}	0.141 ± 0.002 ^{a}	1.83 ± 0.07 ^{d}	6.86 ± 0.49 ^{c}	345.40 ± 2.71 ^{d}	7.07 ± 0.43 ^{d}
90	0.042 ± 0.002 ^{b}	0.142 ± 0.002 ^{a}	2.21 ± 0.09 ^{c}	8.24 ± 0.61 ^{bc}	365.99 ± 7.65 ^{c}	8.51 ± 0.31 ^{b}
120	0.041 ± 0.000 ^{b}	0.139 ± 0.001 ^{a}	2.38 ± 0.13 ^{abc}	9.32 ± 1.03 ^{ab}	374.88 ± 3.37 ^{bc}	8.78 ± 0.38 ^{ab}
150	0.040 ± 0.003 ^{b}	0.131 ± 0.002 ^{b}	2.53 ± 0.12 ^{a}	10.57 ± 0.85^{a}	402.48 ± 8.17 ^{a}	9.17 ± 0.09 ^{a}
180	0.043 ± 0.002 ^{b}	0.133 ± 0.003 ^{b}	2.48 ± 0.04 ^{ab}	10.71 ± 0.84 ^{a}	393.96 ± 2.32 ^{a}	8.69 ± 0.21 ^{ab}
210	0.049 ± 0.001 ^{a}	0.133 ± 0.003 ^{b}	2.32 ± 0.03 ^{bc}	11.08 ± 2.01 ^{a}	376.47 ± 3.11 ^{bc}	7.72 ± 0.22 ^{c}

The values are expressed as mean ± SD. The different letters within a row indicate a significant difference ($p < 0.05$).

flower extract lower than 250 μg/ml were selected for the investigation in the treatment model because the concentrations were not toxic to cells.

A prevention or therapy model was investigated to determine whether L929 cells should be treated with *K. henryi* flower extract before or after oxidative damage. For the prevention model, L929 cells were treated with *K. henryi* flower extract for 6 h, followed by 0.25 mM H_2O_2 treatment for 3 h. Inhibition of oxidative damage was observed by the treatment of *K. henryi* flower water extract in the prevention model, and it showed dose-dependent results (Fig. 3a). The survival rate of L929 cells treated with 25–150 μg/ml of *K. henryi* flower extract was 71.4–98.9%, while those treated with 200 μg/ml had a higher survival rate compared to others. In the therapy model, L929 cells were treated with 0.25 mM H_2O_2 for 3 h, followed by the treatment of *K. henryi* flower extract for 6 h. The results indicate that cells treated with *K. henryi* flower extract had survival rates in the range of 60–100% (Fig. 3b). In the therapy model, L929 cells showed an injury after the treatment of *K. henryi* flower extract, which might be due to the ROS produced by *K. henryi* flower extract. It was found that ROS was produced when L929 cells were treated with *K. henryi* flower extract for 60 min (Fig. 4). Thus, the intracellular H_2O_2 and *K. henryi*-induced ROS together severely damaged L929 cells and reduced its survival rates. Moreover, 200 μg/ml of *K. henryi* flower extract could repair the oxidative damage cells as normal cells in the prevention model. However, the high concentration of *K. henryi* flower extract (250 μg/ml) was needed to repair the oxidative damage cells as normal cells. Therefore, it revealed that the prevention model had higher survival rates than the therapy model. Hence, *K. henryi* flower extract concentration of 200 μg/ml in the prevention model was used in the following cell experiments.

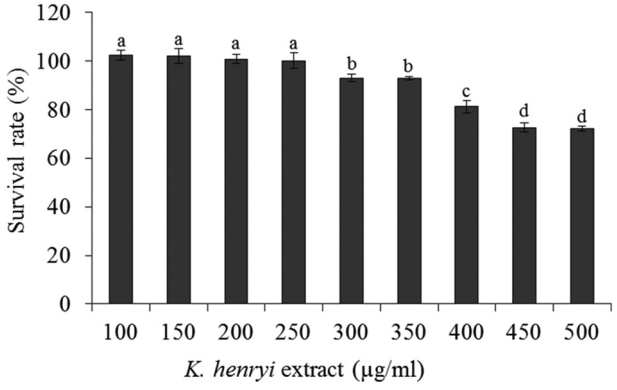

Fig. 2. The cytotoxicity of *K. henryi* flower extract in L929 cells. L929 cells were treated with different concentrations of *K. henryi* flower extract for 6 h, and then it was analyzed by MTT assay. The different letters indicate a significant difference ($p < 0.05$).

3.3. Mitochondrial membrane potential of L929 cells treated with K. henryi flower extract

When cells are attacked by ROS or stimulated by specific cytokines, they trigger the mitochondrial-mediated apoptosis. The dysfunction of mitochondria lets hydrogen ion lose from the intermembrane space, and the mitochondrial membrane potential decreases. Therefore, the mitochondrial membrane potential is one of the major keys to evaluate apoptosis. To study the effect of *K. henryi* flower extract on mitochondrial membrane potential, L929 was pretreated with 200 μg/ml *K. henryi* flower extract for different durations (30–240 min). The mitochondrial membrane potential decreased in H_2O_2-treated cells, and it indicated that —H2O2 induced the mitochondrial-mediated apoptosis. The mitochondrial membrane potential could not be repaired by the pretreatment of *K. henryi* flower extract for only 30 min. The mitochondrial membrane potential was repaired as normal cells when cells were pretreated with *K. henryi* flower extract for 60 and 120 min. However, it decreased when pretreated for 240 min. Hence, the optimal pretreatment duration for L929 cells with *K. henryi* flower extract was 120 min against the H_2O_2-induced apoptosis (Fig. 5a). Zhang and co-workers reported similar results on the inhibition of H_2O_2-induced apoptosis. The pretreatment of morroniside decreased mitochondrial membrane potential and suppressed H_2O_2-induced apoptosis in SK-N-SH human neuroblastoma cells through the mitochondria-mediated apoptosis [38]. Mitochondria mitochondria-mediated apoptosis belongs to intrinsic pathways, and this result indicated that the mitochondria-mediated apoptosis could be repressed by *K. henryi* flower extract.

3.4. Caspase 8 and 9 activity of L929 cells treated with K. henryi flower extract

Various caspases can activate apoptosis. For example, caspase 8 is an essential component of extrinsic cell death pathways initiated by TNF-α family [39]. In contrast, caspase 9 is a key component in the mitochondrial death pathway regulated by the Bcl-2 family on the surface of the mitochondrial membrane [40]. To understand the mechanism of *K. henryi* flower extract, the activities of caspase 8 and caspase 9 were investigated to know which one could be inhibited by *K. henryi* flower extract. Results show that caspase 8 activity gradually increased in H_2O_2-treated cells (Fig. 5b), indicating that H_2O_2 induced the extrinsic apoptosis pathway. The caspase 8 activity decreased on increasing the concentration of *K. henryi* flower extract from 100 to 200 μg/ml. According to an earlier study, the caspase 8 activity can be inhibited by compounds in the herbal extract. For example, it was found that luteolin inhibited caspase 8 activity triggered by H_2O_2-induced oxidative damage [41]. In the present study, the group only treated with 1 mM H_2O_2 showed an increase of caspase 9 activity due to activation of the mitochondria-mediated apoptosis (Fig. 5c). The caspase 9 activity of the L929 cells treated with 200 μg/ml extract showed a significant decrease compared to H_2O_2-treated L929 cells. These results indicate that

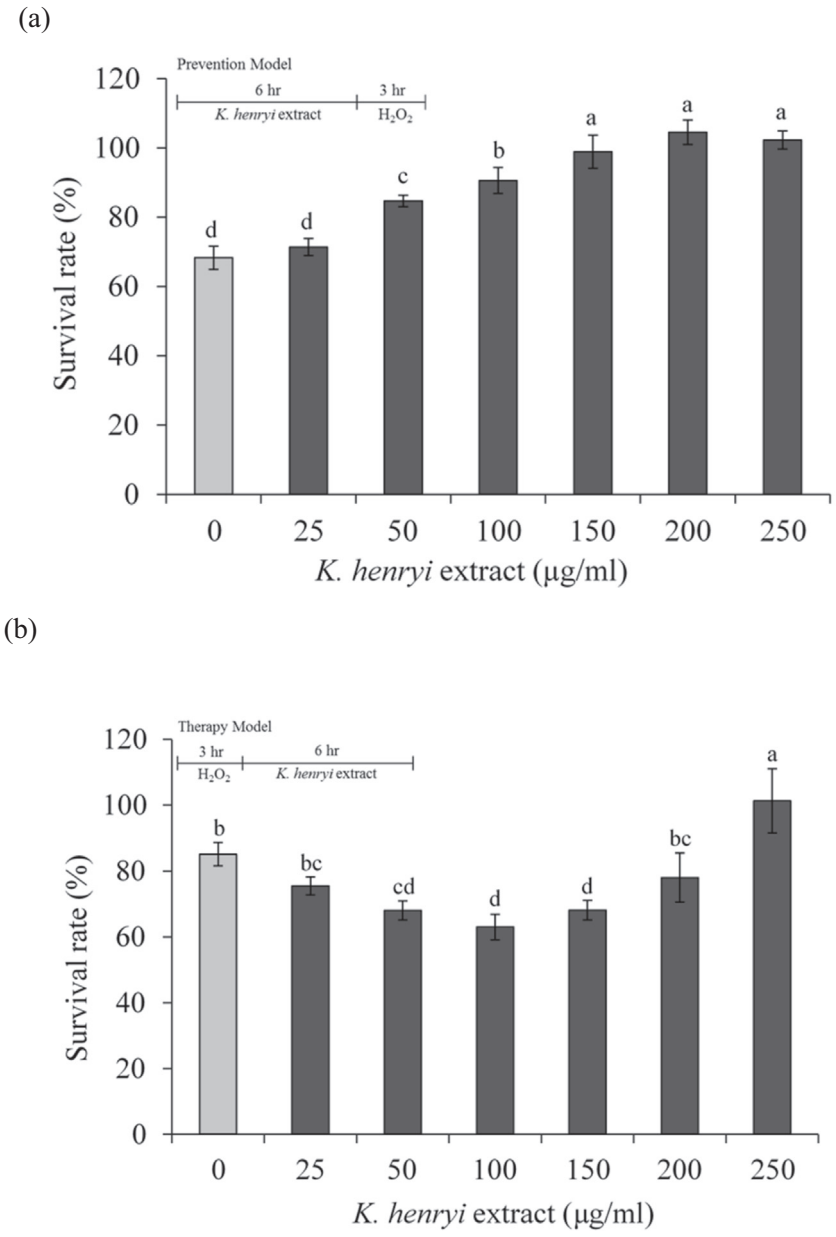

Fig. 3. Treatment models for *K. henryi* flower extract (a) Prevention model: L929 cells treated with various concentrations of *K. henryi* flower extract for 6 h and then treated with 0.25 mM H_2O_2 for 3 h. (b) Therapy model: L929 cells treated with 0.25 mM H_2O_2 for 3 h and then treated with various concentrations of *K. henryi* flower extract for 6 h. "0" represents that cells were treated with sterile deionized water and 0.25 mM H_2O_2, and were not treated with *K. henryi* flower extract. The different letters indicate significant difference ($p < 0.05$).

K. henryi can prevent oxidative stress damage or other apoptosis factors. However, the caspase 9 activity in the pretreatment of 100 μg/ml *K. henryi* group had no significant difference compared to the H_2O_2 group indicating that a concentration of 100 μg/ml *K. henryi* flower extract was not enough to prevent the caspase 9 activity. It has been reported that the apoptosis of Hela cell and mouse embryonic fibroblast was induced by H_2O_2 through the mitochondrial death pathway [42]. In conformity to this, results in the present study demonstrated that H_2O_2 could induce apoptosis by both extrinsic and intrinsic pathways involving the activation of caspases 8 and 9, respectively. The pretreatment of L929 cells with 200 μg/ml of *K. henryi* flower extract could effectively inhibit the signaling transduction in both apoptosis pathways. Moreover, mitochondrial-dependent intrinsic apoptosis lets cytochrome c release and activation of the caspase 9. Caspase 9 was downregulated by *K. henryi* flower extract, and it proved that mitochondria-dependent intrinsic apoptosis was inhibited by *K. henryi* flower extract.

3.5. The anti-apoptosis ability of K. henryi flower extract on L929 cells

According to the results, *K. henryi* flower extract inhibited the activity of caspase 8 and caspase 9 in L929 cells. The apoptosis triggered by oxidant damage was investigated by Annexin V-FITC & PI assay in L929 cells treated with *K. henryi* flower extract. The result of apoptosis is shown in Fig. 6, and there are four parts, including Q1 (necrosis cells), Q2 (late apoptosis cells), Q3 (normal cells), and Q4 (early apoptosis cells). Fig. 6a shows that the apoptosis results in the normal group were 0.34% for early apoptosis, 2.25% for late apoptosis, and 6.94% for necrosis. In the H_2O_2-treated group, the apoptosis results of L929 cells treated with 1 mM H_2O_2 for 3 h were 24.60% for healthy cells (Q3), 0.7% for early apoptosis (Q4), 58.27% for late apoptosis (Q2), and 16.43% for necrosis (Q1). A significant difference was noted in the late apoptosis and necrosis compared with the normal group. The L929

cells treated with 100 μg/ml extract for 2 h showed lower values in early apoptosis (0.44%), late apoptosis (16.55%), and necrosis (18.97%) compared with the normal group. On the other hand, cells pretreated with 200 μg/ml extract for 2 h could prevent apoptosis and had no significant difference to the normal cells. The total apoptosis ratios were a combination of early and late apoptosis, and the ratios of normal, H_2O_2-treated, two groups treated with *K. henryi* flower extract have been shown in Fig. 6b. The results indicate that *K. henryi* flower extract decreased oxidative-induced apoptosis in L929 cells, and the apoptosis ratio decreased with the increase of *K. henryi* flower extract concentrations from 100 μg/ml to 200 μg/ml. The apoptosis ratios of high concentration groups had no significant difference with the normal cells; H_2O_2-damaged cells could be repaired as normal cells. The apoptosis was inhibited due to the repression of extrinsic (caspase 8) and intrinsic (mitochondria and caspase 9) apoptosis. Prevention of H_2O_2-induced apoptosis in L929 cells by *K. henryi* flower extract may also be affected by the reduction in intracellular ROS level or inhibition of ROS generation. Previous studies have found that the inhibition of ROS production suppressed apoptosis. In one study, N-acetylctylcystein, an antioxidant compound, completely inhibited the cadmium-induced apoptosis pathway and blocked ROS generation in HepaG2 cells via upregulation of catalase [43]. In another report, quercetin, a major dietary flavonoid in foods, prevented H_2O_2-induced cell damage through its antioxidant activity [44]. Therefore, inhibition of H_2O_2-induced apoptosis in cells by *K. henryi* flower extract may be due to its antioxidant activity.

3.6. Phospho-p38 (pp38) and phospho-extracellular signal-regulated kinase (ERK) expression in L929 cells treated with K. henryi flower extract

In the study, H_2O_2-induced apoptosis was mediated by mitochondria and results shown in Fig. 4, Fig. 5, and Fig. 6 indicate that apoptosis triggered by 1 mM H_2O_2 was inhibited by *K. henryi* flower extract. Moreover, according to a previous report, p38 and ERK MAPK play an important role in inflammation, proliferation, differentiation, and apoptosis [45]. Therefore, the p38 and ERK MAPK pathways were investigated to determine which pathway was inhibited by *K. henryi* flower extract. Fig. 7a shows that the p-p38 expression increased when cells were treated with H_2O_2. L929 cells pretreated with 200 μg/ml of *K. henryi* flower extract significantly decreased the expression of p-p38, which may be the reason for the reduction in the activity of downstream caspase 8. Fig. 7b shows that

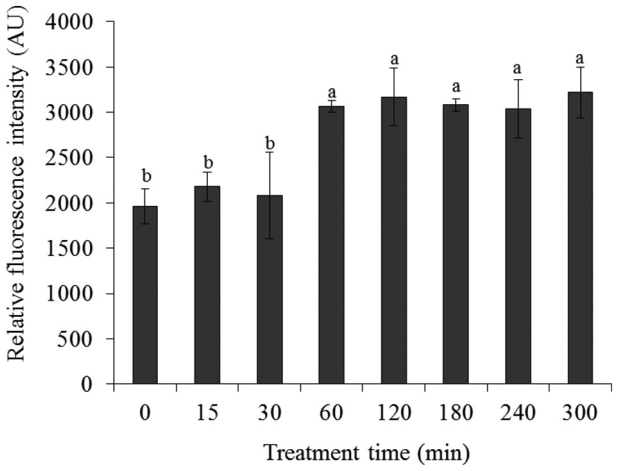

Fig. 4. Measurement of intracellular ROS in L929 cells treated with 200 μg/ml *K. henryi* flower extract for different time durations. The different letters indicate a significant difference ($p < 0.05$).

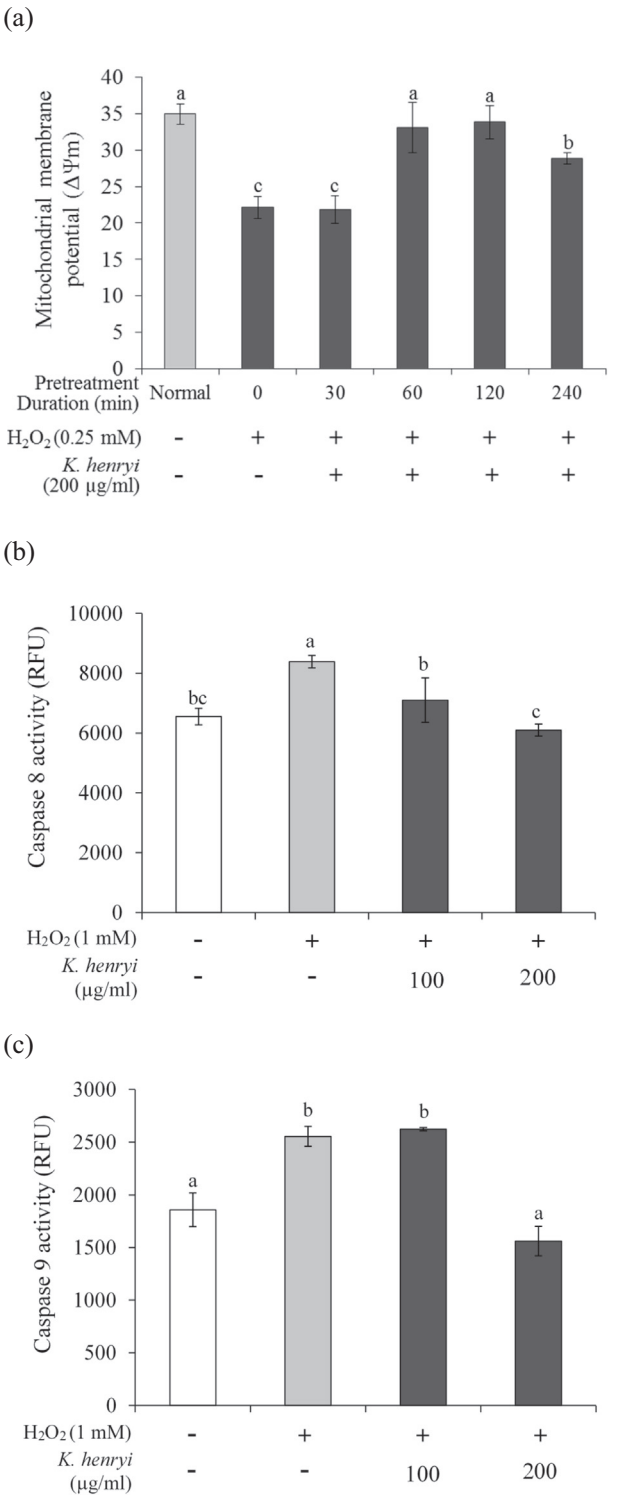

Fig. 5. Mitochondrial membrane potential and caspase activity in L929 cells. (a) Measurement of mitochondrial membrane potential in cells. Cells were treated with 200 μg/ml *K. henryi* flower extract for various time durations and then treated with 1 mM H_2O_2 for 3 h. (b) The activity of caspase 8 and (c) caspase 9 in cells. Cells were treated with 100 or 200 μg/ml *K. henryi* flower extract for 2 h and then treated with H_2O_2 for 3 h. "0" represents cells treated with sterile deionized water. The different letters indicate significant difference ($p < 0.05$).

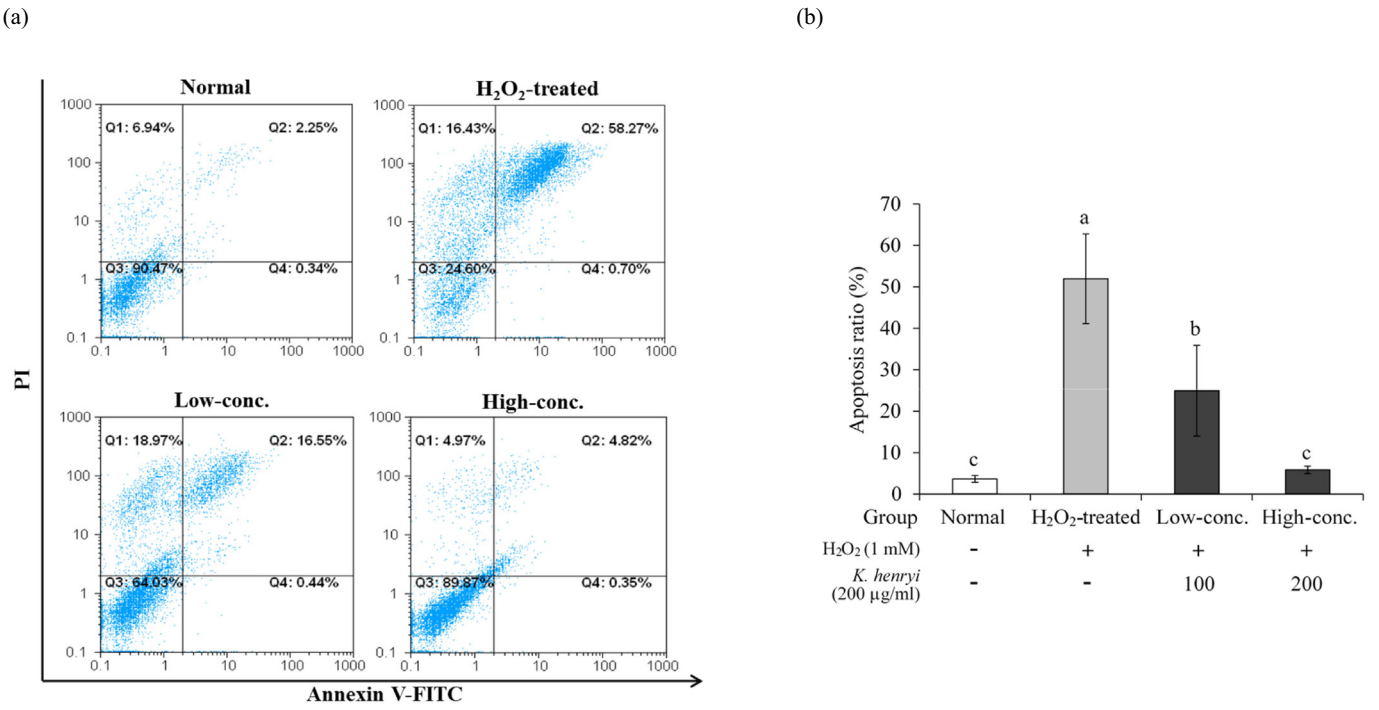

Fig. 6. Investigation on apoptosis in H_2O_2-treated L929 cells by Annexin V-FITC & PI assay. (a) Normal group: L929 cells were not treated with *K. henryi* flower extract and H_2O_2; H_2O_2-treated group: L929 cells were treated with 1 mM H_2O_2 for 3 h. Low-concentration group: L929 cells treated with 100 μg/ml *K. henryi* flower extract for 2 h and then treated with1 mM H_2O_2 for 3 h. High-concentration group: L929 cells treated with 200 μg/ml *K. henryi* flower extract for 2 h and then treated with 1 mM H_2O_2 for 3 h. (b) Apoptosis results in H_2O_2-treated L929 cells. The different letters indicate significant difference ($p < 0.05$).

the expression of pERK in L929 cells was suppressed when pretreated with 100 and 200 μg/ml of *K. henryi* flower extract. Therefore, it can be inferred that the oxidation-induced apoptosis in L929 cells treated by *K. henryi* flower extract was through the suppression of p-p38 and pERK expression in the MAPK pathway. There are reports in conformity with the present findings. For example, morroniside from *Cornus officinalis* and compounds from Sheng-mai san, Chinese medicines, inhibited H_2O_2-induced apoptosis by suppressing MAPK pathway, and decreasing pERK and p-p38 expression [46,47]. The H_2O_2-induced apoptosis was mediated not only through the mitochondrial pathway but also through p38 and ERK MAPK pathways, and these pathways inhibited by *K. henryi* flower extract lead to repression of apoptosis.

3.7. HO-1 expression and Nrf2 pathway activated in L929 cells treated with K. henryi flower extract

H_2O_2, one of the ROS, would induce exogenous oxidative stress-induced apoptosis. Moreover, oxidative stress leads to the activation of many transcription factors like Nrf2. The role of Nrf2 is to regulate cellular redox balance and the suppression of oxidative stress [48]. To study whether the Nrf2 pathway was activated by *K. henryi* flower extract, the Nrf2 expression in L929 cells treated with *K. henry* extract for 30, 60, 120, and 180 min was analyzed in the cell nucleus. The results shown in Fig. 8a demonstrate that the maximum expression of Nrf2 was obtained when L929 cells were treated with *K. henryi* flower extract for 120 min. In an earlier study, activation of Nrf2 led to the production of antioxidant enzymes such as heme oxygenases [49]. Therefore, as a next step, an investigation was carried out on the HO-1 protein expression in L929 cells treated with *K. henryi* flower extract. It was observed that the expression of HO-1 increased when cells were treated with *K. henryi* flower extract (Fig. 8b). The highest expression of HO-1 protein was recorded at 120 min, like the results of Nrf2. The activation of Nrf2 in cells treated with *K. henryi* flower extract led to the upregulation of the HO-1, and protection of cells against the H_2O_2-induced apoptosis. Earlier, Lee et al. found that the ethanol extract of *K. henryi* leaves also could induce the expression of HO-1 in smooth muscle cells transfected with HO-1 gene [25]. Both leave and flower of *K. henryi* could induce the HO-1, and protect cells from oxidative damage. Moreover, the previous study has demonstrated the induction of HO-1 mediated adaptive cytoprotective response to oxidative stress in human fibroblasts [50]. Also, it has been reported that L929 cells treated with HO-1 can inhibit TNF-α induced apoptosis [51]. In another report, flavones in *Patrinia villosa* showed anti-apoptosis ability and increased the HO-1 expression through the mir-144-3p/Nrf2 pathway to protect Caco2 cells from H_2O_2-induced oxidative damage [52]. Therefore, the Nrf2 pathway induced by *K. henryi* flower extract plays a major anti-apoptotic role in H_2O_2-treated L929 cells.

3.8. The protective effect of K. henryi flower extract on L929 cells treated by H_2O_2

To confirm that the H_2O_2-induced apoptosis was inhibited by *K. henryi* flower extract through the expression of HO-1, Zinc protoporphyrin-9 (ZnPP), a competitive inhibitor was used to inhibit HO-1 activity. Results shown in Fig. 9a reveal that the late apoptosis (Q2) and necrosis (Q1) in the H_2O_2-treated group increased when L929 cells were treated with H_2O_2. The late apoptosis and necrosis decreased in the group treated with the extract. It indicated that L929 cells were protected from H_2O_2-damage when it was treated with *K. henryi* flower extract. However, in the extract/ZnPP group, many cells were located at late apoptosis (Q2) and necrosis (Q1). It revealed that ZnPP inhibited the expression of HO-1 to abrogate the protection of *K. henryi* flower extract. Fig. 9b shows the combined ratios of early and late apoptosis. In the extract group, the apoptosis triggered by oxidative damage in L929

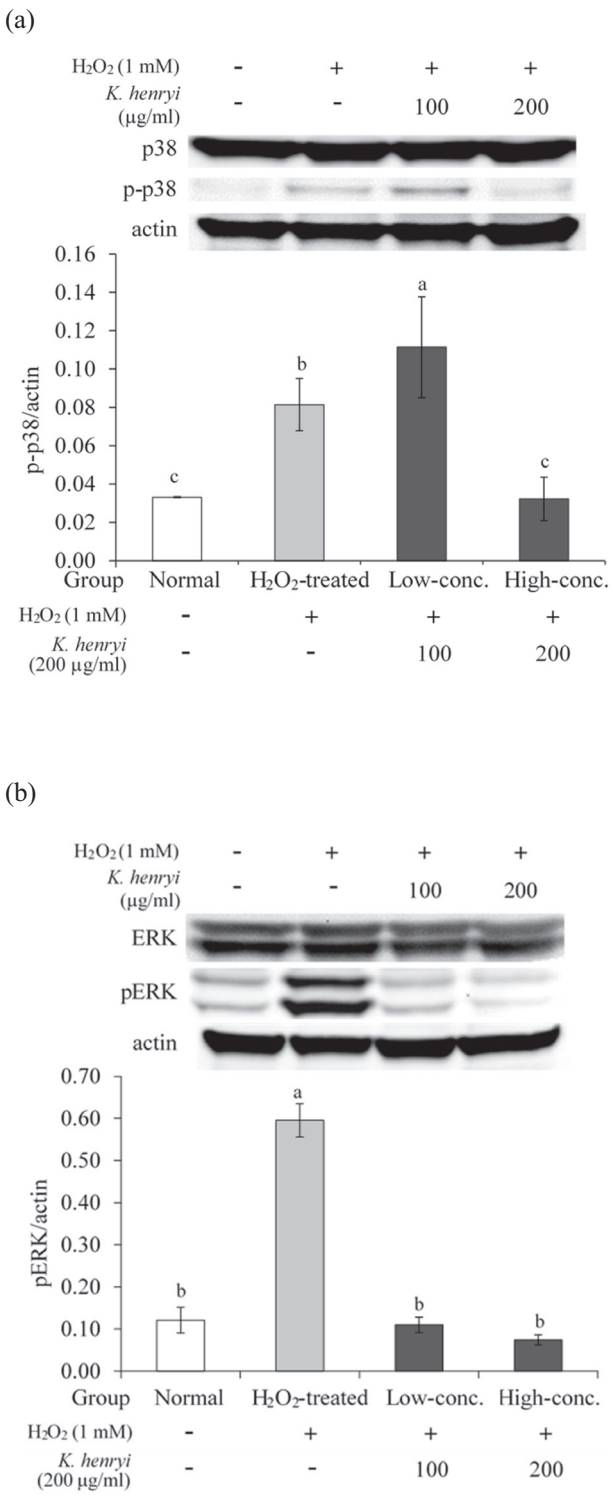

Fig. 7. The results of apoptosis through the MAPK pathway. Western blot analysis of (a) pp38 expression and (b) pERK expression. L929 cells were treated with 100 or 200 μg/ml *K. henryi* flower extract for 2 h and then treated with 1 mM H_2O_2 for 3 h. The different letters indicate significant difference ($p < 0.05$).

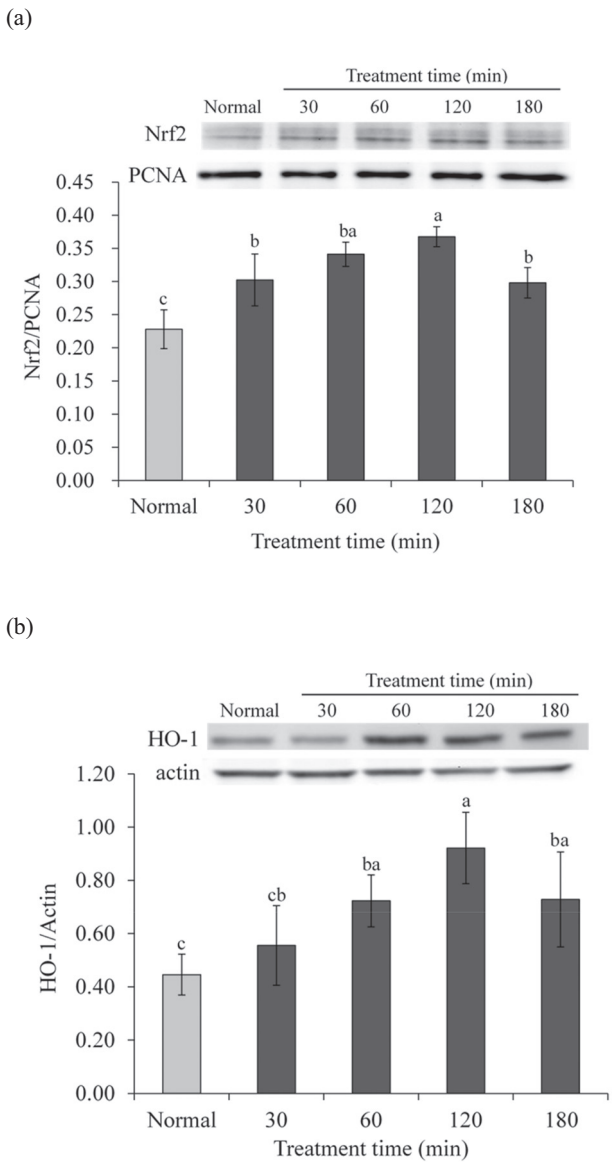

Fig. 8. Inhibition of apoptosis through the Nrf2 pathway. Western blot analysis of (a) The results of Nrf2 expression and (b) HO-1 expression. L929 cells were treated with 200 μg/ml *K. henryi* flower extract, and then treated with 1 mM H_2O_2 for 3 h. The normal group was not treated with *K. henryi* flower extract. The different letters indicate significant difference ($p < 0.05$).

cells was restored after cells were treated with *K. henryi* flower extract. There is no significant difference between the normal and the extract group. In the extract/ZnPP group, the apoptosis ratio was similar to H_2O_2-treated group since the expression of HO-1 was blocked by ZnPP. The result proved that HO-1 inducted by *K. henryi* played a major role against apoptosis triggered by oxidative stress through the Nrf2 pathway. Similar to these results, four components from *Gentianella acuta* inhibited H_2O_2-induced apoptosis in H9c2 cells by activating the Nrf2 pathway to express HO-1. The apoptosis was inhibited in the group without ZnPP; however, the group with ZnPP did not inhibit apoptosis [53]. In a nutshell, results obtained in the present study conclusively demonstrate that the treatment *K. henryi* flower extract activates the Nrf2 pathway to express HO-1 for the prevention of H_2O_2-induced apoptosis in L929 cells.

4. Conclusions

The antioxidant activity of *K. henryi* flower extract was investigated through antioxidant activity assays and *in vitro* study. First, *K. henryi* flower extracted for 150 min had high contents of total phenolic acids and total flavonoids, and good performance in antioxidant activity assays. *In vitro* study for investigating antioxidant activity of *K. henryi* flower extract was demonstrated by H_2O_2-induced apoptosis, and

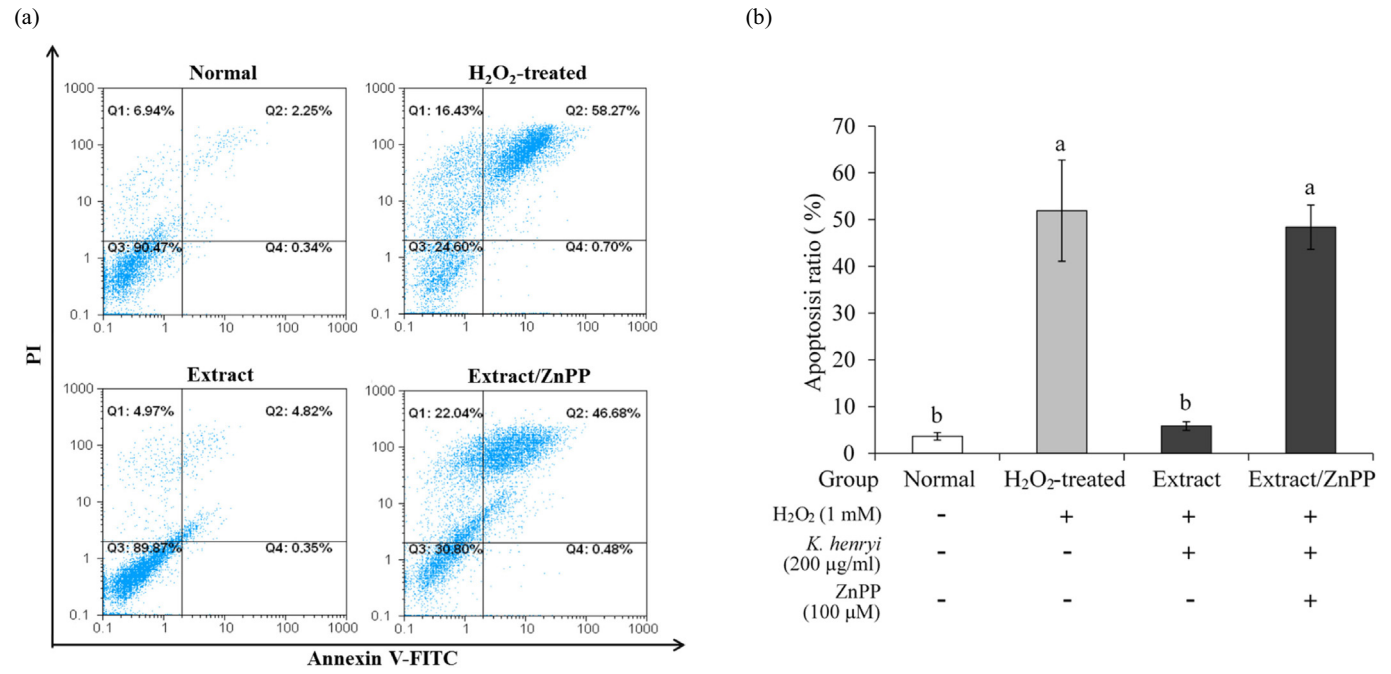

Fig. 9. Investigation on apoptosis in H_2O_2-treated L929 cells by Annexin V-FITC & PI assay. (a) Normal group: L929 cells were not treated with *K. henryi* flower extract and H_2O_2; H_2O_2-treated group: L929 cells treated with 1 mM H_2O_2 for 3 h. Extract group: L929 cells treated with *K. henryi* flower extract for 2 h and then treated with 1 mM H_2O_2 for 3 h. Extract/ZnPP group: L929 cells co-treated with *K. henryi* flower extract and ZnPP for 2 h and then treated with 1 mM H_2O_2 for 3 h. (b) Apoptosis results in H_2O_2-treated L929 cells. The different letters indicate significant difference ($p < 0.05$).

results showed that H_2O_2-induced apoptosis was inhibited through extrinsic and intrinsic apoptotic signaling pathways. The intrinsic part was the downregulation of caspase 9 in the mitochondrial-mediated pathway, and extrinsic part was the downregulation of caspase 8. Moreover, the MAPK pathway was also suppressed by *K. henryi* flower, leading to inhibition of H_2O_2-induced apoptosis. The expression of p-p38 and pERK were repressed in the MAPK pathway. Finally, it was found that *K. henryi* flower extract activated the Nrf2 pathway to induce the expression of HO-1 in L929 cells, and the HO-1 inhibited the H_2O_2-induced apoptosis. In conclusion, to the best of our knowledge, the present study is the first to demonstrate that *K. henryi* flower extract protects cells from H_2O_2 oxidative stress-induced apoptosis by activation of the Nrf2 pathway and the expression of HO-1. Therefore, *K. henryi* flowers have potential for the repression of oxidative stress.

Ethical statement

The authors declare that this article does not contain any studies with human participants and animals performed by any of the authors.

Conflict of interests

All authors declare that there is no conflict of interest of any kind among them.

Financial support

The authors would like to thank the Biotechnology Center of National Chung Hsing University, Taiwan, for financially supporting this research.

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