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Salidroside Protects Human Erythrocytes against Hydrogen Peroxide-Induced Apoptosis

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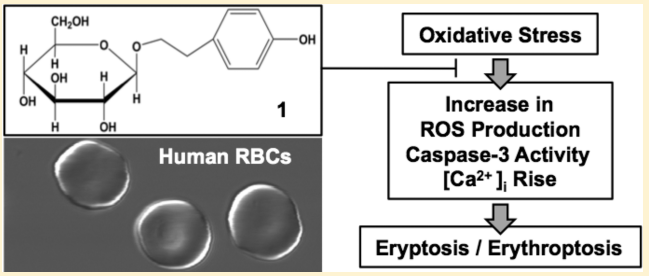
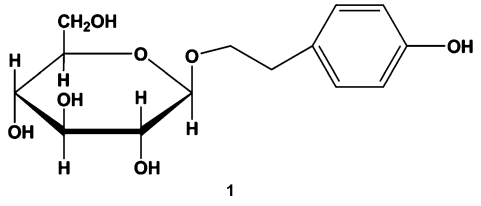
ABSTRACT: *Rhodiola rosea* is a commonly used folk medicine for the treatment of high altitude sickness, mountain malhypoxia, and anoxia. Its active ingredient, salidroside [2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside (**1**)], has been reported to have a broad spectrum of biological effects. However, the protective role of **1** in human erythrocytes remains unclear. This study therefore has investigated the effects of **1** on oxidative stress-induced apoptosis in human erythrocytes (also known as eryptosis or erythroptosis). Compound **1** increased cell survival significantly and prevented human erythrocytes from undergoing eryptosis/erythroptosis mediated by H_2O_2, as confirmed by the decreased expression of phosphatidylserine on the cell surface and reduced leakage of calcein through the damaged membrane. Mechanistically, **1** was found to exert its protective effects through its antioxidative activity and the inhibition of caspase-3 activation and stress-induced intracellular Ca^{2+} rise in a dose-dependent manner. Compound **1** is a protective agent in human erythrocytes against oxidative stress and may be a good adaptogen to enhance the body's resistance to stress and fatigue.



The plant *Rhodiola rosea* L., belonging to the family Crassulaceae, is used popularly as a folk medicine in Europe and mainland China. Previous studies have indicated that *R. rosea* is able to enhance both physical and mental performance.[1−4] Its active constituent, salidroside (**1**) [2-(4-hydroxyphenyl)ethyl β-D-glucopyranoside],[4] has been reported to have a broad spectrum of pharmacological properties, such as antiaging,[5] anti-inflammatory,[6] antihypoxia,[7,8] and antioxidative[9,10] effects.

R. rosea has long been used as a blood tonic and adaptogen to prevent high altitude sickness and to treat mountain malhypoxia and anoxia.[11,12] There are published studies showing that *R. rosea* and **1** can provide cardiovascular protection against ischemic/reperfusion injuries.[13,14] Also, **1** has been demonstrated to prevent oxygen-glucose deprivation-induced apoptosis in rat neonatal cardiomyocytes.[15] A commercial extract of *R. rosea* containing **1** (70%) extended the lifespan (24%) of *Drosophila melanogaster* by reducing the level of endogenous superoxide.[16] Recently, **1** was demonstrated to protect PC12 cells against H_2O_2-induced apoptotic cell death.[17] However, the protective effect of **1** in erythrocytes remains unclear. In light of this, it was hypothesized that **1** rescues human erythrocytes from apoptosis triggered by oxidative stress.

It has been known for some time that apoptosis is not limited to nucleated cells, since mature enucleated erythrocytes also share a program for self-destruction through apoptosis. Although the mechanism is poorly understood, mature red blood cells (RBCs) can undergo a type of apoptosis known as eryptosis or erythroptosis without the involvement of the mitochondria or the nucleus. Similar to apoptosis, eryptosis/erythroptosis is characterized by membrane blebbing, cell shrinkage, activation of proteases, externalization of phosphatidylserine (PS) on the cell surface, and loss of membrane integrity. During apoptosis, the PS-exposed RBCs are recognized and removed by macrophages through receptor-mediated phagocytosis. Apart from senescence, eryptosis/erythroptosis can be induced by oxidative stress. Activation of Ca^{2+}-permeable cation channels is one of the major mechanisms that triggers apoptosis in RBCs besides the activation of caspases.[18,19] In the present study, it is reported that **1** protects erythrocytes from H_2O_2-induced apoptosis through an antioxidative effect, suppression of the increase in the intracellular free Ca^{2+} ion concentration ($[Ca^{2+}]_i$), and inhibition of caspase-3 activation.



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RESULTS AND DISCUSSION

The potential cytotoxic effect of **1** on freshly isolated human erythrocytes was determined by hemolysis assay. As shown in

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Figure 1A, **1** did not exhibit any observable hemolytic effect at a concentration up to 800 μM in a 2-h incubation or 400 μM in a

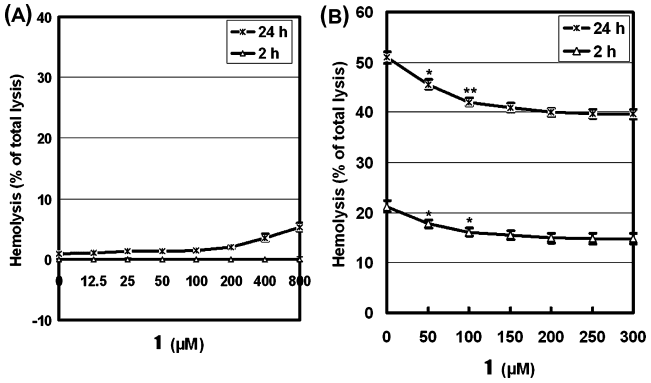

Figure 1. Effect of compound **1** on hemolysis in human erythrocytes. Erythrocytes were cultured with various concentrations of **1** in the absence (A) or presence (B) of H_2O_2 (1 mM) for 2 and 24 h at 37 °C, 5% CO_2. Hemolysis was then measured. Mean ± SEM, n = 5, *p < 0.05, **p < 0.001.

24 h treatment, indicating that the erythrotoxicity of **1** in human cells is minimal.

Next, it was examined as to whether **1** exerts any protective effect against hemolysis elicited by H_2O_2. When RBCs were incubated with H_2O_2 (1 mM) alone, about 20% and 50% of total hemolysis were obtained in the 2 and 24 h incubations, respectively (Figure 1B). When **1** was added, a dose-dependent prevention of H_2O_2-induced hemolysis was observed. In particular, hemolysis was reduced significantly when the concentration of **1** was 50 or 100 μM. Although the toxic effects from H_2O_2 could not be totally neutralized, the results obtained indicated that **1** can, at least in part, protect the RBCs from acute (2 h) and long-term (24 h) oxidative stress from H_2O_2.

To determine the effect of **1** on the H_2O_2-mediated apoptosis, a flow cytometric PE-annexin-V/calcein assay was employed to examine the protective effect. As shown in Figure 2A, most of the cells in the control group were found in the lower right quadrant (ca. 97%). These were healthy cells with normal membrane integrity and PS asymmetry (calcein$^+$ and annexin-V$^-$). To confirm the validity of the assay, Ca^{2+} ionomycin was used, which elicits apoptosis in erythrocytes as a positive control.[20] In the positive control group, Ca^{2+} ionomycin (1 μM) increased significantly the number of early apoptotic cells (calcein$^+$ and annexin-V$^+$) in the upper right quadrant (68.5% vs 2.6% in control), with a concomitant decrease in the number of healthy cells (0% vs 97% in control). Also, the number of late apoptotic cells (calcein$^-$ and annexin-V$^+$) in the upper left quadrant of the ionomycin-treated group increased slightly (25.2% vs 0% in control).

When cells were treated with H_2O_2 (1 mM) alone, few erythrocytes were found in the late apoptotic quadrant (<1%), with about 9% showing early apoptotic features, and 27.2% found in the lower left quadrant (calcein$^-$ and annexin-V$^-$) (Figure 2A). Findings from Bratosin et al. (2005) indicate that the inactivation of the enzyme esterase occurs earlier than that of the PS externalization during eryptosis/erythroptosis.[21] As a consequence of the inactivation of esterase, the calcein/AM could not be converted into calcein to generate fluorescence. Therefore, cells in the lower left quadrant are very early apoptotic cells. However, in the presence of **1**, the proportion of viable cells increased from 63% (0 μM **1**) to 70% (100 μM **1**) and 75% (300 μM **1**), while the very early apoptotic cells in the lower left quadrant decreased correspondingly from 27% to 17% and 14%. Experiments were repeated, and a similar response profile is demonstrated in Figure 2B.

Increase in $[Ca^{2+}]_i$ is an important apoptotic trigger in human RBCs.[18] Therefore the change in the $[Ca^{2+}]_i$ in RBCs was measured using fluo-4/AM. Without Ca^{2+} binding, fluo-4 is nonfluorescent, but the fluorescence increases in a concentration-dependent manner when bound with free Ca^{2+} ions. As expected, after calcium ionomycin treatment, the fluo-4 fluorescence signals moved to the upper right quadrant in the PE-annexin-V/Fluo-4 two-variant plot, indicating that ionomycin increased the $[Ca^{2+}]_i$ and at the same time promoted the PS externalization (Figure 3A). This finding agreed well with the previous observations that ionomycin is an apoptotic agent in erythrocytes.[18] When RBCs were challenged with H_2O_2 (1 mM) alone, the fluorescence signals moved to the upper right corner in the PE-annexin-V/Fluo-4 two-variant plot, indicating an increase in $[Ca^{2+}]_i$ and PS externalization. On the contrary, cells treated with **1** alone (100 and 300 μM) did not alter much in $[Ca^{2+}]_i$ and PS externalization when compared to that of the control group. When cells were co-treated with H_2O_2 (1 mM) and **1** (100 or 300 μM), more signals moved in a dose-dependent manner back to the lower left corner, indicating a reduction in the $[Ca^{2+}]_i$ and PS externalization (Figure 3A). Experiments were repeated, and the results are summarized in Figure 3B. As can be seen, the higher the dose of **1**, the stronger the protection. This observation again indicates that **1** protects the erythrocytes from H_2O_2-induced apoptosis through the inhibition of a rise in $[Ca^{2+}]_i$.

Activation of caspase-3 is an important step in apoptosis.[22] It also has been reported that caspase-3 activation was detected in the apoptosis of RBCs under certain conditions.[23,24] Accordingly, it was tested if caspase-3 is involved in the H_2O_2-mediated eryptosis/erythroptosis and whether **1** provides any protection in this aspect. To address these questions, the caspase-3 activity was studied using a fluorogenic caspase-3 assay. For the positive controls, erythrocytes were treated with staurosporine (STS), a potent apoptotic agent. As can be seen in Figure 4A, STS increased the caspase-3 activity (94.5% in the selected region) significantly when compared to that of control (0.3%) or **1** (300 μM) (0.2%). When RBCs were treated with H_2O_2 (1 mM) alone, the cell population moved to the right-hand side, indicating the activation of caspase-3 enzyme. However, the degree of activation was less than that of the STS-treated group (51.2% vs 94.5%). When cells were incubated with H_2O_2 (1 mM) together with **1**, the degree of caspase-3 activity was partially suppressed, also in a dose-dependent manner (Figure 4B).

From Figure 3A, it seems likely that **1** suppresses the H_2O_2-mediated increase in $[Ca^{2+}]_i$. As cellular Ca^{2+} ion is a crucial trigger for the induction of apoptosis in human RBCs, it was determined whether **1** suppresses a rise in $[Ca^{2+}]_i$ elicited by ionomycin. As shown in Figure 3C and D, **1** partially inhibited the ionomycin-induced $[Ca^{2+}]_i$ rise. This indicates that the protective effect is due, at least in part, to the Ca^{2+} efflux through the plasma membrane in the RBCs, in which organelles for taking up Ca^{2+} ions into internal stores are lacking. Likewise, **1** partially blocked the STS-mediated activation of caspase-3 (Figure 4C and D). Previous studies have shown that **1** is an antioxidant,[9,10] so the potential protection from **1** was

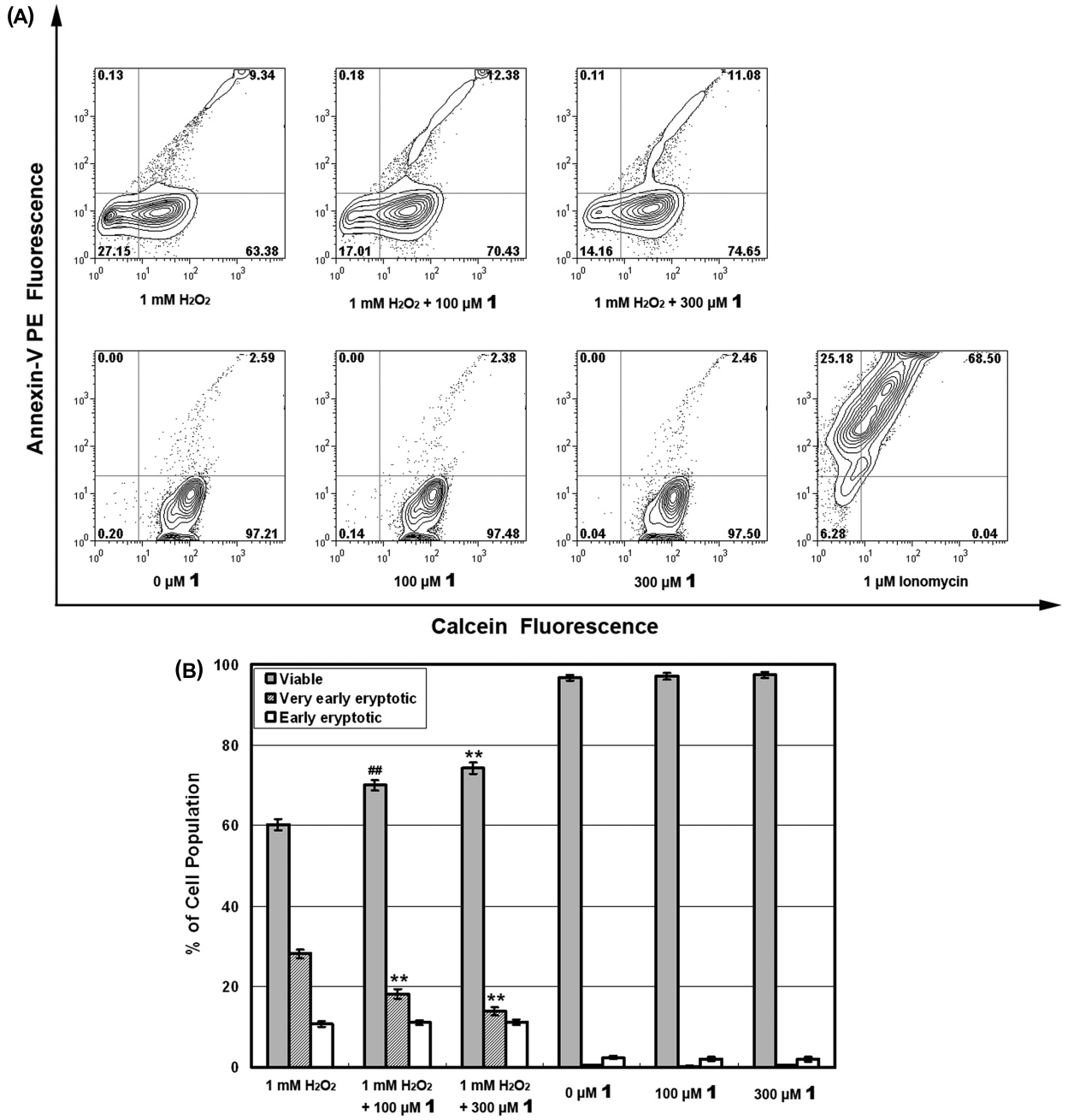

Figure 2. Effect of compound **1** on H_2O_2-induced apoptosis in human erythrocytes. Erythrocytes were treated with medium alone, **1** (100 or 300 μM), or ionomycin (1 μM) in the absence or presence of H_2O_2 (1 mM) as indicated at 37 °C, 5% CO_2 for 24 h. Externalization of PS and loss of membrane integrity were determined by flow cytometry. Figure represents the % of cell population in the quadrant (A). Mean ± SEM, $n = 4$ (B). $^{\#\#}p < 0.005$, $^{**}p < 0.001$, compared to the H_2O_2-treated group.

evaluated against oxidative stress from H_2O_2. As depicted in Figure 5A, H_2O_2 (1 mM) increased the fluorescence of 2′,7′-dichlorofluorescein (DCF), indicating the generation of reactive oxygen species (ROS) in the cytoplasm after treatment. When cells were co-treated with H_2O_2 and **1**, protection of RBCs was observed in a dose-dependent manner (Figure 5B). Taken together, the results suggest that **1** protects RBCs from H_2O_2-elicited eryptosis/erythroptosis through an antioxidative effect, as well as by suppression of Ca^{2+} rise and inhibition of caspase-3 activation.

R. rosea has long been used as a blood tonic and adaptogen to treat mountain malhypoxia. Salidroside (**1**) has been used in traditional Tibetan medicine as an adaptogen and has been documented as a strong antioxidant and protective agent against cell death in neuronal cells, possibly through an antioxidative effect.[25] However, the mechanism for the protection in human erythrocytes and erythroblasts is unclear. Our recent study has indicated that **1** promotes erythropoiesis in EPO-treated cells, and it also reduces the degree of cell death in erythroblasts after H_2O_2 treatment through the upregulation of the protective proteins thioredoxin-1 and glutathione peroxidase-1.[26] On the other hand, low alveolar oxygen tension (ca. 4% O_2 vs 16% O_2 at sea level) or hypoxia was shown to promote the production of ROS during an ascent to high altitude.[27] When oxidized hemoglobins accumulate, they induce phospholipid oxidation and disrupt the membrane. Under this oxidative imbalance condition, RBCs are committed to

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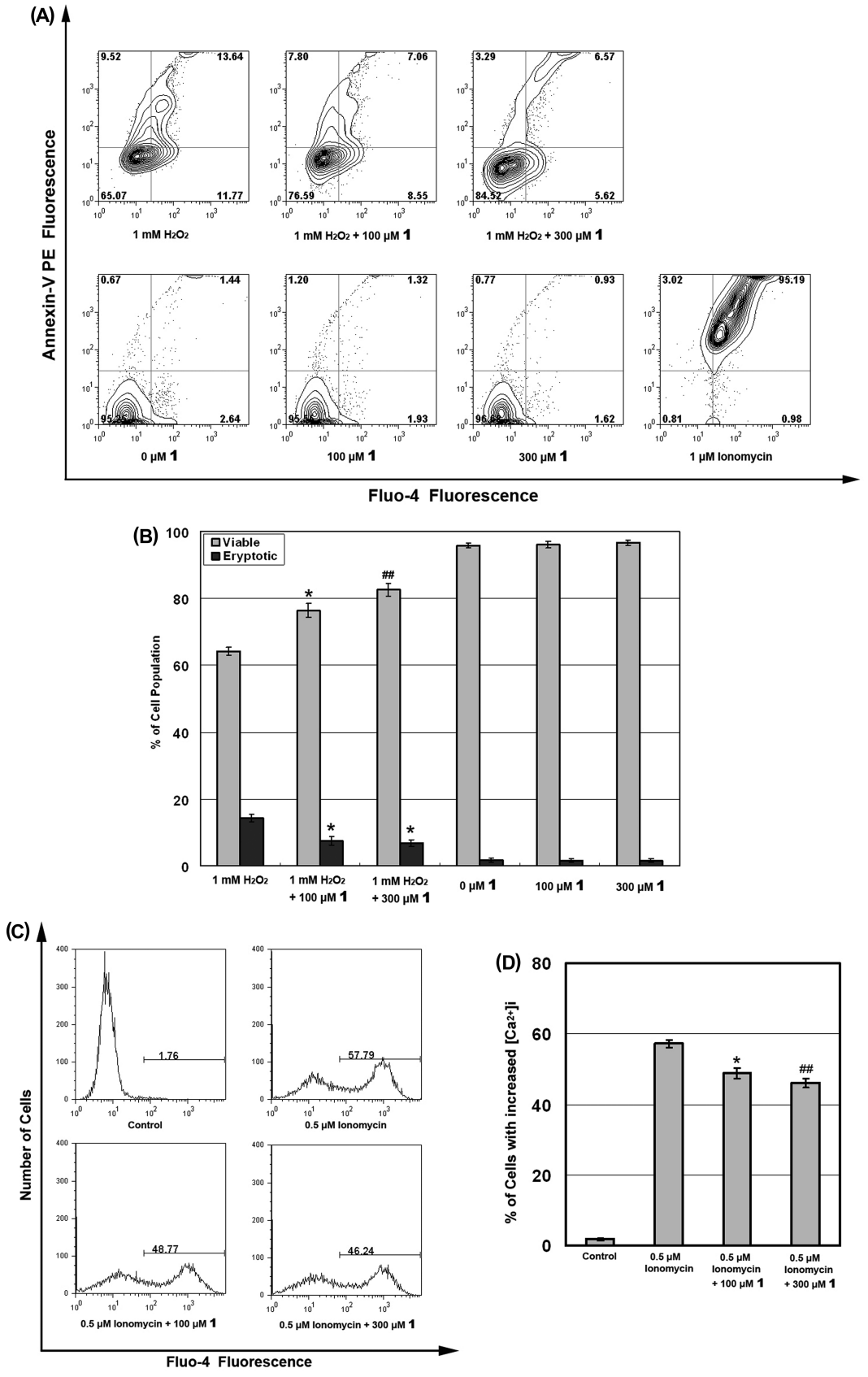

Figure 3. Effect of compound **1** on $[Ca^{2+}]_i$ in human erythrocytes after H_2O_2 treatment. Erythrocytes were treated with medium alone, **1** (100 or 300 μM), or ionomycin (1 or 0.5 μM) in the absence or presence of H_2O_2 (1 mM) as indicated at 37 °C, 5% CO_2 for 24 h. Level of PS externalization and increase in $[Ca^{2+}]_i$ were then determined. Figure represents the % of cell population in the quadrant. Mean ± SEM (bar chart). $^*p < 0.05$, $^{\#\#}p < 0.005$, compared to the H_2O_2- (A, B, $n = 4$) or ionomycin- (C, D, $n = 3$) treated group.

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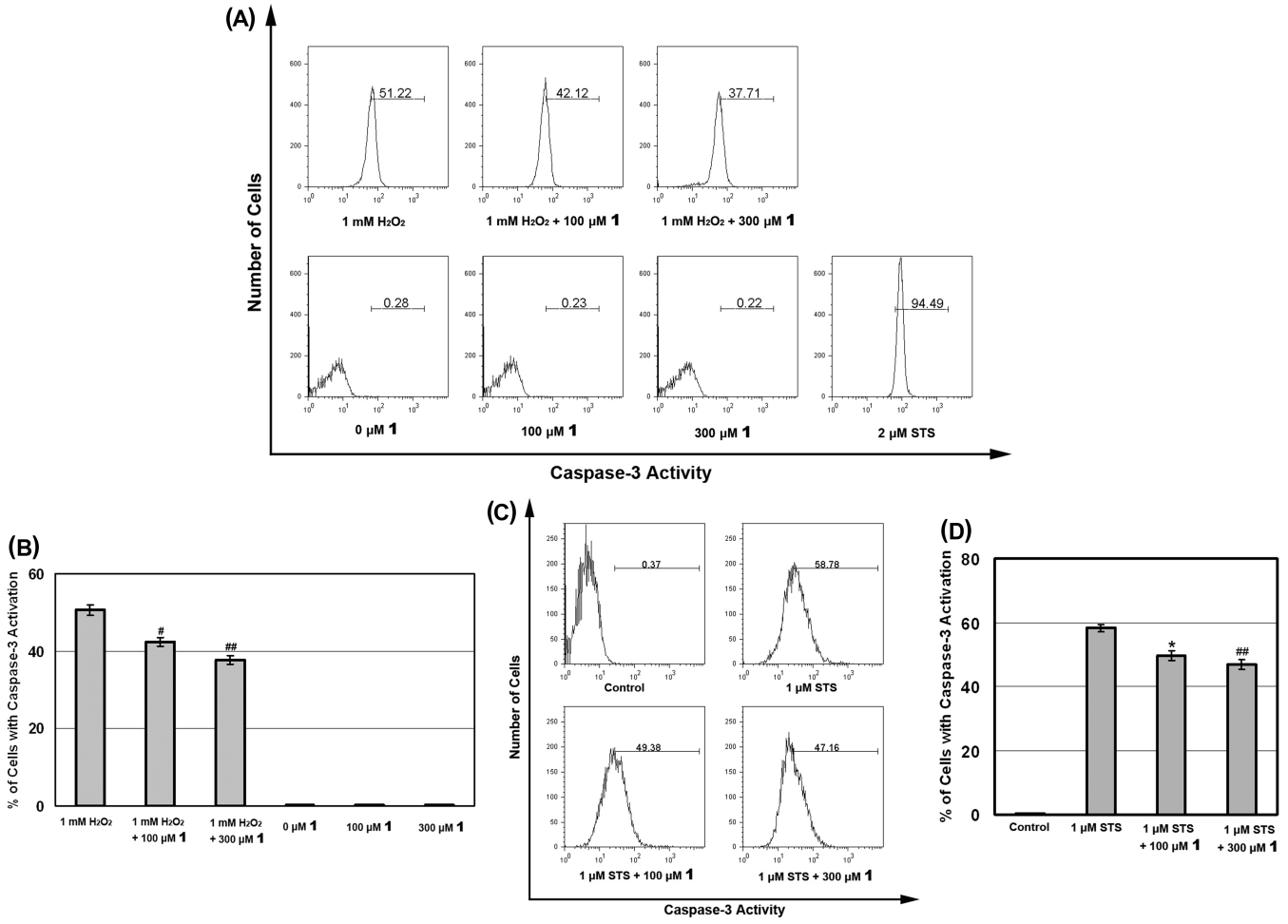

Figure 4. Effects of compound **1** on the level of caspase-3 activation in human erythrocytes after H_2O_2 treatment. Erythrocytes were treated with medium alone, **1** (100 or 300 μM), or staurosporine (STS) (1 or 2 μM) in the absence or presence of H_2O_2 (1 mM) as indicated at 37 °C, 5% CO_2 for 24 h. Level of caspase-3 activation was then determined. Figure represents the % of cell population in the selected region. Mean ± SEM, n = 3 (bar chart). $^{\#}p < 0.01$, $^{*}p < 0.05$, $^{\#\#}p < 0.005$, compared to the H_2O_2- (A, B) or STS- (C, D) treated group.

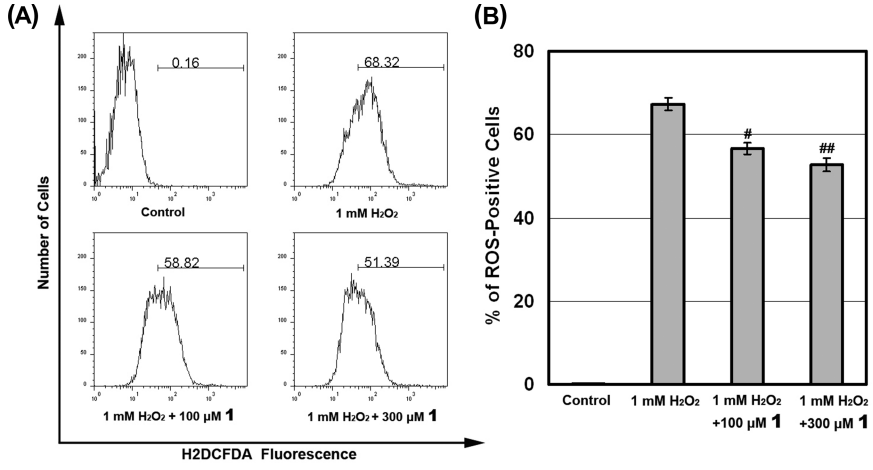

Figure 5. Effects of compound **1** on ROS levels in human erythrocytes after H_2O_2 treatment. Erythrocytes were treated with medium alone or H_2O_2 (1 mM) in the presence or absence of **1** (300 μM) as indicated at 37 °C, 5% CO_2 for 24 h. ROS levels were then determined using H_2DCFDA. The figure represents the % of cell population in the selected region (A). Mean ± SEM, n = 3 (B). $^{\#}p < 0.01$, $^{\#\#}p < 0.005$, compared to the H_2O_2-treated group.

senescence and to cell death.[28] As a result of this, the oxidative stress from ROS is regarded as one of the major triggers of apoptosis in RBCs.[29] In the present study, the apoptotic effect from oxidative stress was well supported from the results using H_2O_2 to elicit the reduction in esterase activity, the increase in PS externalization, and the loss of membrane integrity (Figure 2).

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These are the very early, early, and late apoptotic events during the cell death of RBCs. As these occur in cells devoid of mitochondria and their nuclei, eryptosis/erythroptosis represents a unique cell death system through the regulation at the cell membrane. In this study, evidence has been provided that **1** is able to protect the RBCs against H_2O_2-induced apoptosis.

It has been reported that **1** is able to lower the mitochondrial superoxide levels in *Drosophila melanogaster*.[16] As human erythrocytes lose their mitochondria upon maturation through the pro-apoptotic BH3-only like factor Nix,[30] it is of interest to identify the mechanism as to how **1** protects human RBCs from eryptosis/erythroptosis under oxidative stress. In human erythrocytes, oxidative stress activates the Ca^{2+}-permeable cation channels that stimulate Ca^{2+} entry.[31] It is believed that a high $[Ca^{2+}]_i$ value activates the Ca^{2+}-dependent cysteine protease μ-calpain, which degrades the cytoskeleton.[32] In the present study, the data showed that H_2O_2 increases the cytosolic Ca^{2+} levels. In fact, the rise in Ca^{2+} alone elicited by ionomycin was able to trigger PS externalization (Figure 3A). In this regard, **1** reduced both the H_2O_2- (Figure 3A and B) and ionomycin-mediated (Figure 3C and D) increase in the cellular level of Ca^{2+}, suggesting that this compound might have effects on membrane Ca^{2+} pumps to extrude Ca^{2+} ions out of the cytosol of RBCs. In fact, RBCs are equipped with powerful plasma membrane Ca^{2+} pumps to keep the $[Ca^{2+}]_i$ low.[33] A similar effect on the suppression of H_2O_2-induced $[Ca^{2+}]_i$ elevation was found in human cortical neurons and human hepatocytes HL-7702 after incubation with *R. rosea* extract or **1**.[34,35]

Although caspase-3 is more than a cysteinyl aspartate-specific protease in programmed cell death and elicits many non-apoptotic functions,[36] it is a common executor in the intrinsic and extrinsic apoptotic pathway in nucleated cells. In mature erythrocytes, caspase-3 is shown to be functional.[24] Although devoid of mitochondrial apoptotic regulators, the caspase-3 in human RBCs can be activated by oxidative stress.[23] This notion was confirmed in this study in that H_2O_2 is capable of activating caspase-3 in human RBCs, although the effect of H_2O_2 was less than that of staurosporine, which inhibits protein kinase C.[37] Compound **1** can block, at least in part, the H_2O_2- (Figure 4A and B) or STS-mediated (Figure 4C and D) activation of caspase-3. Previous studies have indicated a role of **1** in blocking oxidative harm from *tert*-butyl hydroperoxide and the poor growth conditions achieved during hypoglycemia and serum starvation in human U-937 macrophages.[9] Such observations are now endorsed by the present results.

EXPERIMENTAL SECTION

Materials. Salidroside (**1**), an active component of the medicinal plant *R. rosea*,[4] was determined to be 98% pure by HPLC and was purchased from International Laboratory (San Bruno, CA, USA). Fluo-4/AM, calcein/AM, H_2DCFDA, and phycoerythrin (PE) conjugated annexin-V were purchased from Molecular Probes and BD Transduction Laboratories. Calcium ionomycin was obtained from Sigma.

Human Erythrocytes. Two to three droplets of fresh human red blood cells was obtained from healthy donors following informed consent by puncturing fingertips with a sterile lancet with depth setting. Heparinized RBCs were washed with PBS, pH 7.4, for experiments. Some variations in viability were found in the erythrocytes taken from different donors or from the same donor at different dates. Experiments were repeated, and the response trend was similar. To avoid any bias potentially introduced by the use of different batches of erythrocytes, a comparison was always made within a given erythrocyte batch. Results shown in this paper represent typical responses from 3 to 5 trials.

Hemolysis Assay. For the hemolysis assay, RBCs (1×10^6/mL) were treated with **1** in the absence or presence of H_2O_2 at 37 °C, 5% CO_2 in HEPES physiological buffer ((in mM) 140 NaCl, 5 KCl, 10 HEPES, 2.5 $CaCl_2$, 10 glucose, and 0.1% (w/v) BSA, pH 7.4). After treatment, absorbance of supernatant was measured at 415 nm with an ELISA plate reader (Bio-Rad) for the leakage of hemoglobin. Triton X-100 [0.05% (v/v)] was used to lyse the RBCs for the 100% total cell lysis.

Flow Cytometric Assays. To evaluate apoptosis in RBCs, flow cytometric analysis using annexin-V and calcein/AM, similar to the annexin-V/propidium iodide assay for nucleated cells, was employed.[21,38] After each treatment, erythrocytes were loaded with PE-annexin-V and calcein/AM (1 μg/mL) for 20 min at room temperature. PE-annexin-V is able to label the PS externalized on the outer leaflet of the plasma membrane. Calcein/AM is a nonfluorescent dye. After diffusing into the cytoplasm across the plasma membrane, the ester linkage between calcein and the AM group is cleaved by the cytosolic esterases, so the fluorescent hydrophilic calcein cannot diffuse out. However, in cells with a damaged membrane, calcein molecules are leaked out and less calcein is kept in the cytosol, therefore leading to a reduction in calcein fluorescence in the cells.

For the determination of $[Ca^{2+}]_i$, erythrocytes were loaded with fluo-4/AM (10 μM) for 20 min at room temperature, and changes in the $[Ca^{2+}]_i$ after various treatments were measured semiquantitatively by flow cytometry with an excitation at 488 nm. Similar to calcein/AM, fluo-4/AM is a nonfluorescent dye. After diffusing into the cytoplasm across the cell membrane, the ester linkage between fluo-4 and the AM group is cleaved by the cytosolic esterases and the fluorescent hydrophilic fluo-4 cannot diffuse out. Ca^{2+} levels are reported in the cytosol in a semiquantitative way.

The nonfluorescent compound CM-H_2DCFDA, after diffusion into cells, will be oxidized to form the highly fluorescent DCF in the presence of ROS such as H_2O_2.[39] To study ROS production, erythrocytes after treatment were incubated with CM-H_2DCFDA (5 μM) for 30 min. After washing, cells were immediately analyzed by flow cytometry.

For the measurement of caspase-3 activity, cells (1×10^6/mL; 0.3 mL) were incubated with a fluorogenic caspase-3 FAM-DEVD-FMK assay (Apologix), according to the manufacturer's instructions (Cell Technology Inc., Mountain View, CA, USA). After washing, cells were analyzed.

Flow cytometric analysis was performed on a FACSCanto flow cytometer (BD Biosciences), using FlowJo software for data acquisition and analysis. Green and red fluorescence after excitation at 488 nm from a minimum of 10 000 cells were determined. In the two-variant plots, each dot stands for one single cell, and dot density is expressed by contour. The figure at each corner represents the percent of total cell population in the quadrant.

Statistical Analysis. Results of all experiments were expressed as mean ± SEM of three to five determinations. Data were compared using the Student's *t*-test; *p*-values less than 0.05 were considered statistically significant.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Shevtsov, V. A.; Zholus, B. I.; Shervarly, V. I.; Vol'skij, V. B.; Korovin, Y. P.; Khristich, M. P.; Roslyakova, N. A.; Wikman, G. *Phytomedicine* **2003**, *10*, 95−105.
(2) Spasov, A. A.; Wikman, G. K.; Mandrikov, V. B.; Mironova, I. A.; Neumoin, V. V. *Phytomedicine* **2000**, *7*, 85−89.
(3) Panossian, A.; Wikman, G. *Curr. Clin. Pharmacol.* **2009**, *4*, 198−219.
(4) Panossian, A.; Wikman, G.; Sarris, J. *Phytomedicine* **2010**, *17*, 481−493.
(5) Hu, X.; Lin, S.; Yu, D.; Qiu, S.; Zhang, X.; Mei, R. *Cell Biol. Toxicol.* **2010**, *26*, 499−507.
(6) Skopinska-Rozewska, E.; Malinowski, M.; Wasiutynski, A.; Sommer, E.; Furmanowa, M.; Mazurkiewicz, M.; Siwicki, A. K. *Pol. J. Vet. Sci.* **2008**, *11*, 97−104.
(7) Ye, Y. C.; Chen, Q. M.; Jin, K. P.; Zhou, S. X.; Chai, F. L.; Hai, P. *Zhongguo Yao Li Xue Bao* **1993**, *14*, 424−426.
(8) Yu, S.; Liu, M.; Gu, X.; Ding, F. *Cell. Mol. Neurobiol.* **2008**, *28*, 1067−1078.
(9) Kanupriya; Prasad, D.; Sai Ram, M.; Kumar, R.; Sawhney, R. C.; Sharma, S. K.; Ilavazhagan, G.; Kumar, D.; Banerjee, P. K. *Mol. Cell. Biochem.* **2005**, *275*, 1−6.
(10) Yu, P.; Hu, C.; Meehan, E. J.; Chen, L. *Chem. Biodiversity* **2007**, *4*, 508−513.
(11) Kelly, G. S. *Altern. Med. Rev.* **2001**, *6*, 293−302.
(12) Wu, Y. L.; Piao, D. M.; Han, X. H.; Nan, J. X. *Biol. Pharm. Bull.* **2008**, *31*, 1523−1529.
(13) Wu, T.; Zhou, H.; Jin, Z.; Bi, S.; Yang, X.; Yi, D.; Liu, W. *Eur. J. Pharmacol.* **2009**, *613*, 93−99.
(14) Zhang, J.; Liu, A.; Hou, R.; Jia, X.; Jiang, W.; Chen, J. *Eur. J. Pharmacol.* **2009**, *607*, 6−14.
(15) Zhong, H.; Xin, H.; Wu, L. X.; Zhu, Y. Z. *J. Pharmacol. Sci.* **2010**, *114*, 399−408.
(16) Schriner, S. E.; Abrahamyan, A.; Avanessian, A.; Bussel, I.; Maler, S.; Gazarian, M.; Holmbeck, M. A.; Jafari, M. *Free Radical Res.* **2009**, *43*, 836−843.
(17) Yu, S.; Shen, Y.; Liu, J.; Ding, F. *J. Mol. Neurosci.* **2010**, *40*, 321−331.
(18) Föller, M.; Huber, S. M.; Lang, F. *IUBMB Life* **2008**, *60*, 661−668.
(19) Lang, F.; Lang, K. S.; Lang, P. A.; Huber, S. M.; Wieder, T. *Antioxid. Redox Signaling* **2006**, *8*, 1183−1192.
(20) Bratosin, D.; Estaquier, J.; Petit, F.; Arnoult, D.; Quatannens, B.; Tissier, J. P.; Slomianny, C.; Sartiaux, C.; Alonso, C.; Huart, J. J.; Montreuil, J.; Ameisen, J. C. *Cell Death Differ.* **2001**, *8*, 1143−1156.
(21) Bratosin, D.; Mitrofan, L.; Palii, C.; Estaquier, J.; Montreuil, J. *Cytometry, Part A* **2005**, *66*, 78−84.
(22) Green, D. R.; Kroemer, G. *Science* **2004**, *305*, 626−629.
(23) Matarrese, P.; Straface, E.; Pietraforte, D.; Gambardella, L.; Vona, R.; Maccaglia, A.; Minetti, M.; Malorni, W. *FASEB J.* **2005**, *19*, 416−418.
(24) Mandal, D.; Mazumder, A.; Das, P.; Kundu, M.; Basu, J. *J. Biol. Chem.* **2005**, *280*, 39460−39467.
(25) Shi, T. Y.; Feng, S. F.; Xing, J. H.; Wu, Y. M.; Li, X. Q.; Zhang, N.; Tian, Z.; Liu, S. B.; Zhao, M. G. *Neurotox. Res.* **2012**, in press.
(26) Qian, E. W.; Ge, D. T.; Kong, S. K. *J. Ethnopharmacol.* **2011**, *133*, 308−314.
(27) Dada, L. A.; Chandel, N. S.; Ridge, K. M.; Pedemonte, C.; Bertorello, A. M.; Sznajder, J. I. *J. Clin. Invest.* **2003**, *111*, 1057−1064.
(28) Kiefer, C. R.; Snyder, L. M. *Curr. Opin. Hematol.* **2000**, *7*, 113−116.
(29) Barvitenko, N. N.; Adragna, N. C.; Weber, R. E. *Cell. Physiol. Biochem.* **2005**, *15*, 1−18.
(30) Schweers, R. L.; Zhang, J.; Randall, M. S.; Loyd, M. R.; Li, W.; Dorsey, F. C.; Kundu, M.; Opferman, J. T.; Cleveland, J. L.; Miller, J. L.; Ney, P. A. *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 19500−19505.
(31) Lang, K. S.; Duranton, C.; Poehlmann, H.; Myssina, S.; Bauer, C.; Lang, F.; Wieder, T.; Huber, S. M. *Cell Death Differ.* **2003**, *10*, 249−256.
(32) Lui, J. C.; Wong, J. W.; Suen, Y. K.; Kwok, T. T.; Fung, K. P.; Kong, S. K. *Arch. Toxicol.* **2007**, *81*, 859−865.
(33) Schatzmann, H. J. *Annu. Rev. Physiol.* **1983**, *45*, 303−312.
(34) Palumbo, D. R.; Occhiuto, F.; Spadaro, F.; Circosta, C. *Phytother. Res.* **2012**, in press.
(35) Guan, S.; Wang, W.; Lu, J.; Qian, W.; Huang, G.; Deng, X.; Wang, X. *Molecules* **2011**, *16*, 3371−3379.
(36) D'Amelio, M.; Cavallucci, V.; Cecconi, F. *Cell Death Differ.* **2010**, *17*, 1104−1114.
(37) Sopjani, M.; Foller, M.; Haendeler, J.; Gotz, F.; Lang, F. *J. Appl. Toxicol.* **2009**, *29*, 531−536.
(38) Vermes, I.; Haanen, C.; Steffens-Nakken, H.; Reutelingsperger, C. *J. Immunol. Methods* **1995**, *184*, 39−51.
(39) Sundaresan, M.; Yu, Z. X.; Ferrans, V. J.; Irani, K.; Finkel, T. *Science* **1995**, *270*, 296−299.