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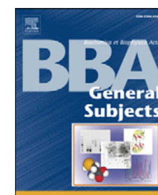

Geraniin exerts cytoprotective effect against cellular oxidative stress by upregulation of Nrf2-mediated antioxidant enzyme expression via PI3K/AKT and ERK1/2 pathway

Peng Wang [a,b,c,1], Xiao Peng [a,b,1], Zuo-Fu Wei [a,b], Fu-Yao Wei [a,b], Wei Wang [a,b], Wei-Dong Ma [a,b], Li-Ping Yao [a,b], Yu-Jie Fu [a,b,*], Yuan-Gang Zu [a,b,**]

[a] Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin 150040, PR China
[b] Engineering Research Center of Forest Bio-preparation, Ministry of Education, Northeast Forestry University, Harbin 150040, PR China
[c] Key Laboratory of Myocardial Ischemia, Harbin Medical University, Ministry of Education, Heilongjiang Province, PR China

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ABSTRACT

Background: Geraniin, an active compound with remarkable antioxidant activity, was isolated from *Geranium sibiricum*. The present study aimed to investigate whether geraniin has the ability to activate Nrf2, induce antioxidant enzyme expression and protect cells from oxidative damage.
Methods: The cells were pretreated with geraniin for 24 h and exposed to hydrogen peroxide (H_2O_2) for 4 h. Intracellular reactive oxygen species (ROS) levels, mitochondrial membrane potential and apoptosis were measured. We also investigated intracellular glutathione (GSH) levels and changes in nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated signaling cascade in cells treated with geraniin.
Results: We investigated the protective effects of geraniin against H_2O_2-induced apoptosis in HepG2 cells. Geraniin significantly reduced H_2O_2-induced oxidative damage in a dose dependent manner. Further, geraniin induced the expression of heme oxygenase-1 (HO-1), NAD(P)H quinone oxidoreductase-1 (NQO1) and level of glutathione (GSH) in a concentration- and time-dependent manner, and increased Nrf2 nuclear translocation. The Nrf2-related cytoprotective effects of geraniin were PI3K/AKT and extracellular signal-regulated protein kinase1/2 (ERK1/2) pathway-dependent. However, inhibitors of PI3K/AKT and ERK1/2 (LY294002 or U0126) not only suppressed geraniin-induced nuclear translocation of Nrf2 but also abolished the expression of HO-1, NQO1 and GSH.
Conclusions: These results demonstrated that geraniin induced Nrf2-mediated expression of antioxidant enzymes HO-1 and NQO1, presumably *via* PI3K/AKT and ERK1/2 signaling pathways, thereby protecting cells from H_2O_2-induced oxidative cell death.
General significance: Geraniin, at least in part, offers an antioxidant defense capacity to protect cells from the oxidative stress-related diseases.

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1. Introduction

The excessive generation of reactive oxygen species is associated with cell death and involved in various chronic diseases including atherosclerosis [1], cancer and aging [2]. If the generation and elimination of ROS fail to keep cellular homeostasis, excessive ROS can cause damage to the lipids, proteins and DNA [3]. Therefore, it is necessary for cells to effectively counteract ROS generation by triggering their own defensive mechanisms with the help of antioxidants.

Under normal conditions, nuclear factor-erythroid 2-related factor 2 (Nrf2), a member of the cap "n" collar subfamily of bZIP transcription factors [4], is sequestered in the cytoplasm binding with Kelch-like ECH-associated protein-1 (Keap1), which regulates proteasomal degradation of Nrf2 *via* the Cullin3-based E3-ligase ubiquitination complex [5]. In response to intracellular oxidative stress, activated Nrf2 is released from Keap1, and translocates to the nucleus where it forms a heterodimer with the small Maf protein. Then, it binds to antioxidant-related elements (ARE) in the DNA and activates the transcription of a series of phase II detoxifying antioxidant genes [6], such as hemeoxygenase 1 (HO-1), NAD(P)H: quinone oxidoreductase1 (NQO1) and γ-glutamate cysteine ligase (GCL). The latter one is the rate-limiting enzyme in GSH synthesis. These findings have suggested the importance of the Nrf2–ARE pathway

* Correspondence to: Y.-J. Fu, Key Laboratory of Forest Plant Ecology, Ministry of Education, Northeast Forestry University, Harbin 150040, PR China. Tel./fax: +86 451 82190535.
** Correspondence to: Y.-G. Zu, Engineering Research Center of Forest Bio-preparation, Ministry of Education, Northeast Forestry University, Harbin 150040, PR China.
E-mail address: yujie_fu2002@yahoo.com (Y.-J. Fu).
[1] These authors contributed equally to the work.

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for enhancing antioxidative defenses and survival of cells from oxidative damage. Moreover, some studies have identified a possible link between both mitogen-activated protein kinases (MAPKs), phosphoinositole 3-kinase (PI3K/AKT) and Nrf2 activation [7,8]. Indeed, MAPKs and PI3K/AKT play an important role in regulating cell proliferation, survival and apoptosis associated with Nrf2 activation.

So far, many natural resources have been proposed as therapeutic agents to counteract the oxidative stress. It is known that polyphenolic compounds possess a multitude of biological activities, such as antioxidant [9], antihypertensive [10] and antibacterial [11] properties. In human's everyday life, regular consumption of vegetables and fruits can effectively reduce the risk of illness, because they are rich in polyphenols, which are strong antioxidants for free radicals scavenging in the body [12]. *Geranium sibiricum* is commonly consumed as additive in distilled spirit for drink and for foods [13]. Among the polyphenolic compounds in *G. sibiricum*, geraniin is a major polyphenolic compound with strong antioxidant activity due to 2,4-hexahydroxydiphenoyl (HHDP)-bearing glucopyranose within 3,6-bridges. The human hepatoma cell line HepG2 represents a suitable model to study xenobiotic metabolism and antioxidant activity, because it is similar with normal hepatocytes and keeps many liver-specific characteristics, especially the activity of phase I, phase II and antioxidant enzymes [14]. Therefore, we have chosen HepG2 cells for our experiments.

In our previous study, geraniin showed efficient radical scavenging activities, including superoxide radical scavenging activity, DPPH radical scavenging activity and the reducing power [15]. To the best of our knowledge, the molecular mechanisms of geraniin-mediated antioxidant effects have not been elucidated yet. Therefore, the present study aimed to investigate whether geraniin conferred an antioxidant defense capacity *via* activation of Nrf2 and induction of its downstream target genes in hepatocytes. Nrf2 activation by geraniin was found to be due to the phosphorylations of PI3K/AKT and MAPK signaling pathways. Furthermore, geraniin had a cytoprotective effect against oxidative damage in hepatocytes.

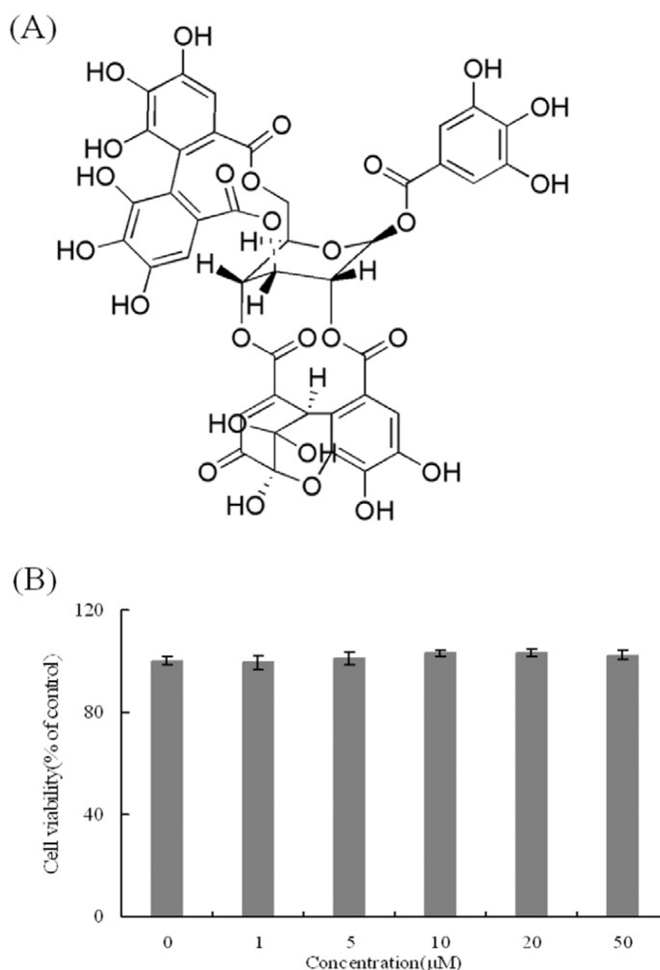

Fig. 1. Chemical structure of geraniin and effects of geraniin on cell viability. (A) Chemical structure of geraniin. (B) HepG2 cells were treated with indicated concentrations of geraniin for 24 h, and the viability was measured by MTT assay. The values for each geraniin concentration tested represent the average (mean ± S.D.) from eight replicate wells and are representative of three separate experiments.

2. Materials and method

2.1. Reagents and chemicals

Geraniin (purity ≥ 98%) was purchased from Delta Co. Ltd. (Anhui province, China). A 10 mM stock solution of geraniin was prepared in dimethyl sulfoxide (DMSO) and stored at −80 °C.

MTT (3-[4,5-dimehyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide) and dimethylsulfoximine (DMSO) were purchased from Sigma (St. Louis, MO). Antibodies against ERK, phospho-ERK1/2 (Thr158 + Tyr187 + Thr202 + Tyr204), Akt, phospho-Akt (Ser473), phospho-p38MAPK, p38 MAPK, JNK, phospho-JNK (Thr183/Tyr185), Bcl-2, Bax, caspase-3, β-actin and inhibitor compounds U0126 (a ERK inhibitor) and LY294002 (a PI3K inhibitor) were purchased from Beyotime Institute of Biotechnology (Beijing, China), anti-Nrf2, anti-HO-1, anti-NQO1 were purchased from Biosynthesis Biotechnology Company (Beijing, China). Other reagents and chemicals were purchased from Beijing Chemical Reagents Co. (Beijing, China). Secondary antibodies were obtained from Biosynthesis Biotechnology Company (Beijing, China). Deionized water was purified by a Milli Q Water Purification system from Millipore (Millipore Corp., Bedford, MA). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Millipore Corp., Bedford, MA).

2.2. Cell culture

The human hepatocarcinoma cell line HepG2 was purchased from Harbin Medical University, China, and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin (Beyotime Institute of Biotechnology, Beijing, China). The cells were kept at 37 °C in a humid atmosphere containing 5% CO_2.

2.3. Cell proliferation assay and morphology examination

Inhibition of cell proliferation by geraniin was measured by the MTT assay. HepG2 cells were plated in 96-well plates at a density of 1×10^4 cells per well. After incubation with medium for 24 h, cells were incubated with indicated concentrations of geraniin for 24 h. Then, MTT was added to cell cultures at a final concentration of 5 mg/mL 4 h at 37 °C, after that the media were carefully removed. The adherent cells were solubilized with 100 μL of DMSO. Absorbance was measured at 570 nm using an ELISA reader. The morphological change was observed under a microscope, and photomicrographs were taken with an Olympus digital camera.

2.4. Measurement of ROS

Formation of intracellular ROS was detected using the non-fluorescent probe DCFH-DA. DCFH-DA, a non-fluorescent substance, passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′,7′-dichlorofluorescein (DCFH). In the presence of ROS, DCFH reacts with ROS to form the fluorescent product DCF, which is trapped inside the cells. The cells were treated with H_2O_2 for 4 h after being pretreated with or without geraniin for 24 h. Then, the

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