Ferroptosis-Inhibitory Effect and Possible Mechanisms of Ellagitannin Geraniin

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The search for safe and effective ferroptosis-inhibitors has become an important topic. Geraniin, an ellagitannin bearing hexahydroxydiphenoyl (HHDP) and dehydrohexahydroxydiphenoyl (DHHDP) groups, was observed to inhibit erastin-induced ferroptosis in bone marrow-derived mesenchymal stem cells (bmMSCs). To determine the mechanism, geraniin was further analyzed using UV-vis spectra and several colorimetric assays, where its IC_{50} values were always much lower than that of the Trolox positive control. When interacted with several free radicals, geraniin gave no radical adduct formation (RAF) peak in the ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry. In conclusion, geraniin exhibits ferroptosis-inhibitory potential towards erastin-treated bmMSCs; such potential may mainly stem from its strong lipid peroxidation (LPO)-inhibition, Fe^{2+}-chelating, and antioxidant actions. Geraniin gives neither dimer nor radical adduct, owing to the bulky HHDP (or DHHDP) group; thus, it is considered as a safe and effective ferroptosis-inhibitor.

Ferroptosis is a newly identified form of cell apoptosis characterized by the accumulation of lipid peroxidation (LPO), Fe^{2+}, and reactive oxygen species (ROS).[1,2] Inhibition of ferroptosis has promise for clinical applications.[3] The synthetic ferroptosis inhibitor ferrostatin-1, however, has been recently to cause various rearrangement products. Thereby, there may be a metabolic risk.[4] Search for a safe and effective ferroptosis-inhibitor has become a hot issue nowadays.[1]

Owing to that plant-derived phenolics have been consumed by human beings for thousands of years and proven to play a safe and beneficial role, phenolics are thus postulated to be a library of safe ferroptosis inhibitors. In fact, some plant-derived phenolic flavonoids have been indicated as natural ferroptosis-inhibitors, such as galangin,[5] icariin,[6] and baicalein.[7] Recently, our team also found that quercetin and its Diels-Alder *anti*-dimer could also act as ferroptosis-inhibitors.[8] Compared with these flavonoids, ellagitannins however contain more phenolic –OHs, and are presumed to have higher ferroptosis-inhibitory potential.

According to the clue, geraniin, an ellagitannin existing in some edible plants (e.g., *Geranium thunbergii* Sieb. *et* Zucc.), was selected as a representative in the study. Structurally, geraniin bears a hexahydroxydiphenoyl (HHDP) group and a dehydrohexahydroxydiphenoyl (DHHDP) group (Figure 1 and Figures S1–2). The two groups enrich a lot of phenolic -OHs; Furthermore, the two are attached to a glucopyranosyl skeleton to twist its conformation (Figure S3).[9] The conformational twist is thought to enhance the reactivity of ROS-scavenging and promote the chelating effect of Fe^{2+} ion, a central role of LPO.[10–12]

To explore the effectiveness of geraniin, it was assayed using the 4,4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid (C11-BODIPY) assay,[13,14] cell counting kit-8 (CCK-8) assay, and flow cytometry assay. All these assays were based on bone marrow-derived mesenchymal stem cells (bmMSCs). The ferroptotic bmMSCs model however was created by erastin, a common inducer.[15]

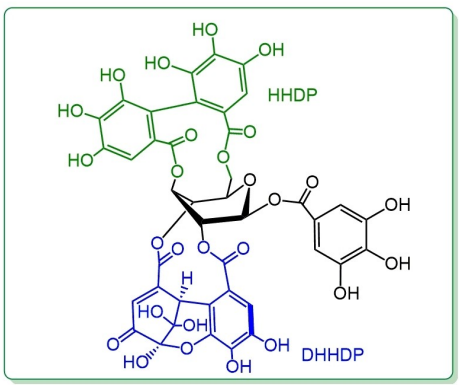

Figure 1. The structure of geraniin

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As seen in Figure 2A, the sample group (geraniin) showed a lighter green color compared with the model group (erastin) in C11-BODIPY assay, indicating an LPO-inhibition effect of geraniin. In CCK-8 assay and flow cytometric assay (Figures 1B–C), geraniin was also found to increase the cellular viability. This clearly indicates that, geraniin can effectively inhibit LPO and ferroptosis of MSCs. MSCs, however, are the most common stem cell type for transplantation engineering[16,17] and are usually limited by the low cellular survival derived from ferroptosis.[18] The identification of geraniin as a ferroptosis inhibitor will also provide positive information for MSC transplantation engineering in clinical applications.

Considering that Fe^{2+} ion plays a central role in the initiation of LPO, Fe^{2+}-chelating action of geraniin was further analyzed using UV-vis spectra.[11] As shown in Figures S4A–4C, when geraniin was mixed with Fe^{2+}, it produced an evident red-shift in the UV spectra, and the reaction solution gave a shoulder peak with a darker color in the visible spectra. In the Fe^{2+}-chelation quantitative evaluation, geraniin gave a good dose-dependent curve (Figure S5). These results mean that geraniin may experience Fe^{2+}-chelating pathway to exert its LPO-inhibition action and ferroptosis-inhibitory action.

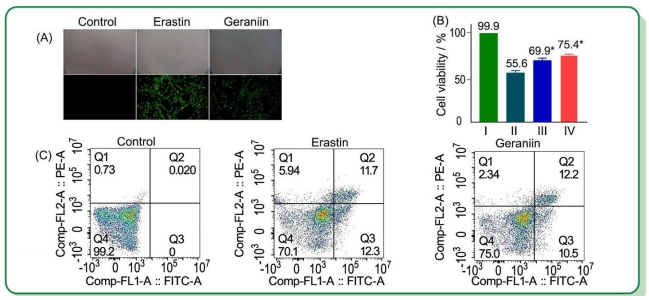

Figure 2. The inhibitory effect of geraniin on erastin-induced ferroptosis in bmMSCs: (A) C11-BODIPY assay. (B) CCK-8 assay; the control group (I) was cultured in medium only, while the ferroptosis model group (II) was treated with erastin. The sample group was damaged by erastin and then treated with 0.3 (III) and 3.1 μM (IV) geraniin. Each value is expressed as the mean ± SD, n = 3; *, $p < 0.05$, significant difference *vs* the model group. (C) Flow cytometry assay; the assay was conducted to distinguish live cells (Q4), necrotic cells (Q1), early apoptotic cells (Q3), and late apoptotic cells (Q2). The experiment was performed with three different batches of cells and each batch was tested in triplicate.

Besides Fe^{2+}-chelating, ROS-scavenging (e.g., $^{\bullet}O_2^-$-scavenging) is also a pathway used to inhibit LPO.[19] To quantitatively assess the ROS-scavenging level of geraniin, it was evaluated using an $^{\bullet}O_2^-$-scavenging assay.[20] In the assay, geraniin had an $^{\bullet}O_2^-$-scavenging level 5.73 times higher than Trolox (Table 1, Figure S6), implying that LPO inhibition may also be mediated by a ROS-scavenging pathway.

The ROS-scavenging pathway, however, can be fulfilled through electron transfer (ET) and proton transfer (PT) pathways. The Cu^{2+}-reducing, Fe^{3+}-reducing, Folin-Ciocalteu-reducing, and 4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide radical ($PTIO^{\bullet}$)-scavenging (pH 4.5) assays are ET-based antioxidant assays.[21] The evidence that geraniin could dose-dependently increase its percentages in the four antioxidant assays (Table 1, Figures S8–S11, and Table S1), suggested ET as one of the antioxidant pathways. To explore the PT possibility, the $PTIO^{\bullet}$-scavenging assay was repeated at pH 7.4 (Figure S12). As seen in Table 1, geraniin doubled its scavenging activity at pH 7.4, suggesting that the pH value affects scavenging activity, namely, H^+ (proton) is also involved in the geraniin ROS-scavenging activity.[21] In 1,1-diphenyl-2-picrylhydrazyl radical ($DPPH^{\bullet}$)-scavenging assay, ET is synergistically accompanied by PT to present the hydrogen-atom transfer (HAT) pathway.[22] The fact that geraniin could successfully scavenge the $DPPH^{\bullet}$ indicated that the above two basic pathways (ET and PT) might occur synergistically during the geraniin antioxidant process (Table 1 and Figure S13).

However, regardless of which pathway was mediated by, geraniin was much more active than Trolox. As shown in Table 1, geraniin had 5.4 times higher activity than Trolox in average. So high antioxidant level is postulated to be from the specific structure, particularly HHDP and DHHDP groups.

Our calculation based on MM2 revealed that, the twisted conformation caused by HHDP and DHHDP groups was actually $^{3,O}B$ skew-boat form rather than chair form (Figures S3B–3C). The total energy of $^{3,O}B$ skew-boat was 100.5 kcal/mol; while chair conformation (e.g., $^1C_4$ chair, Figure S14) was 106.5 kcal/

Table 1. IC_{50} values (μM) of geraniin in antioxidant colorimetric assays.

Assays	Geraniin [μM]	Positive controls Trolox [μM]	BHA [μM]	Ratio
LPO-inhibition	$53.8 \pm 6.5^{[b]}$	$173.7 \pm 22.4^{[c]}$	$6.2 \pm 0.1^{[a]}$	3.23
Fe^{2+}-chelating	$60.1 \pm 6.8^{[a]}$	$131.5 \pm 17.9^{[b],*}$	No applicable	2.19
$^{\bullet}O_2^-$-scavenging	$1308.4 \pm 307.3^{[a]}$	$7503.5 \pm 1793.8^{[b]}$	$24808.4 \pm 5513.7^{[c]}$	5.73
Cu^{2+}-reducing	$13.0 \pm 0.4^{[a]}$	$130.2 \pm 5.2^{[b]}$	$137.8 \pm 15.6^{[b]}$	10.02
Fe^{3+}-reducing	$20.2 \pm 0.4^{[a]}$	$76.4 \pm 1.6^{[b]}$	$337.8 \pm 2.0^{[c]}$	3.78
Folin-Ciocalteu-reducing	$14.8 \pm 1.0^{[a]}$	$108.8 \pm 3.1^{[b]}$	$95.9 \pm 1.3^{[b]}$	7.35
$PTIO^{\bullet}$-scavenging (pH 4.5)	$113.1 \pm 1.8^{[a]}$	$288.6 \pm 26.7^{[b]}$	$283.4 \pm 71.1^{[b],**}$	2.55
$PTIO^{\bullet}$-scavenging (pH 7.4)	$67.1 \pm 9.1^{[a]}$	$444.7 \pm 34.8^{[c]}$	$255.8 \pm 54.6^{[b],**}$	6.63
$DPPH^{\bullet}$-scavenging	$4.4 \pm 0.4^{[a]}$	$19.4 \pm 1.0^{[b]}$	$32.0 \pm 4.0^{[c]}$	4.41
Average				**5.10**

The IC_{50} value (in μM) is defined as the final concentration of 50% radical inhibition or relative reducing/chelating power, calculated by linear regression analysis, and expressed as the mean ± SD (n = 3) (Table S1). Linear regression was analyzed by Origin 2017 professional software. The IC_{50} values with different superscripts ([a], [b], or [c]) in the same row, are significantly different ($p < 0.05$). Trolox is the positive control (*, the positive control is sodium citrate instead of Trolox; **, the positive control is vitamin C instead of butylated hydroxyanisole (BHA)). The dose-response curves are listed in Figures S5–S14. Abbreviations: LPO: lipid peroxidation; PTIO: 2-phenyl-4,4,5,5,-tetramethylimidazoline-1-oxyl 3-oxide; DPPH: 1,1-diphenyl-2-picrylhydrazyl. Ratio is defined as $IC_{50,\ Trolox}/IC_{50,\ geraniin}$.

mol. Now given that the HHDP and DHHDP groups break the three bridges to give rise to all depside geraniin (Figure S15), the molecular total energy was calculated as only 6.6 kcal/mol (Table S2). Thus, the HHDP and DHHDP groups remarkably improve the molecular energy to evaluate its antioxidant reactivity.

On the other hand, the HHDP and DHHDP groups may hinder the radical adduct formation (RAF) reaction.[23–26] To provide more evidence, three ellagitannins chebulagic acid, chebulinic acid, and punicalagin were also introduced in the study (Figures S16–21). As mentioned above, the three are ellagitannins bearing HHDP or DHHDP group. Similarly, after respectively interacting with four free radicals, including 16-DOXYL-stearic acid free radical, DPPH$^{\bullet}$, PTIO$^{\bullet}$, and 2,2,6,6-tetramethylpiperidine-1-oxyl free radical (TEMPO$^{\bullet}$), neither tannin-radical adduct nor tannin-tannin dimer was found in the ultra-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (UHPLC-ESI–Q-TOF-MS/MS) analysis (Figures S22–25). The experimental fact obviously supported the presumption that, the RAF inactivity was associated with steric hindrance from the HHDP or DHHDP groups. To some extent, the steric hindrance can hamper ellagitannin to pass through the cell membrane; Nevertheless, similar ellagitannins have been successfully metabolized in cells, such as strictinin[27] and pomegranate extract.[28] This means that, the bulky HHDP or DHHDP groups can hardly affect their bioavailability.

It should be noted that, of the above four free radicals, 16-DOXYL-stearic acid free radical is a mimic of lipid peroxidation radical of ferroptosis. The RAF inactivity with 16-DOXYL-stearic acid radical implies that, similar ellagitannins will give neither dimer nor radical-adduct when used as cellular ferroptosis-inhibitor. After all, the dimer and radical-adduct are newly generated species and may cause a toxic effect. If these ellagitannins link to biomolecular radicals (e.g., DNA and protein radicals), carcinogenesis may be caused in cells.[29–31] The RAF inactivity has actually released a positive sign, namely, ellagitannins bearing HHDP or DHHDP group cannot cause toxic or carcinogenic effects, and are regarded as safe ferroptosis-inhibitor. This can be regarded as an advantage of geraniin over the aforementioned ferrostatin-1.

The findings based on geraniin will also provide novel evidence on how other ellagitannins protect MSCs from erastin-induced ferroptosis. The findings will help to understand cellular mechanisms the ellagitannins bearing HHDP or DHHDP groups, e.g., chebulagic acid, chebulinic acid, punicalagin, granatin A, granatin B, helioscopinin A, carpinusin, phyllanthusiin D, and euphorscopin (Figure S26).

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: ferroptosis · lipid peroxidation reactions · ellagitannin · geraniin · mesenchymal stem cells

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