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Synthesis and synergetic effects of chrysin–organogermanium (IV) complex as potential anti-oxidant

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ABSTRACT

Organogermanium(IV) (Ge) is considered to play an important role in the anti-oxidative activities of some Chinese medicines. Here, a new chrysin–organogermanium (Chry–Ge) complex was synthesized and investigated for its potential biological activities. The radicals-sensitive Ge–O bond was introduced to Chry–Ge complex to enhance bioactivities of organic Ge or Chry. Results showed that Chry–Ge complex possessed great anti-oxidative activities, showing stronger hydroxyl scavenging effects than their corresponding ligands. We also demonstrated Chry–Ge complex inhibited ROS-dependent oxidative damage in cells. Moreover, the morphological and biophysical recoveries in oxidation-damaged cells induced by Chry–Ge complex were characterized by atomic force microscopy. All these results collectively suggested that Chry–Ge complex has synergetic effect for radicals scavenging and could be served as promising pharmacologically active agent against anti-oxidative treatment.

Germanium (Ge) is a valuable constituent of many Chinese medicines, such as ginseng, which has important pharmacological activity.[1,2] The compounds of Ge(IV) are classified into inorganic and organic forms. Studies showed that organic Ge has good bioactivities, such as low toxicity, inhibition of oxidative damage and anti-cancer.[3,4] Because organic Ge has a distinguishing chemical structure of Ge–C bond, electrons transfer between Ge and free radicals was relatively easy, which was beneficial to scavenge free radicals and then reduce oxidative damage.[5,6] Several potential Ge-based metallodrugs had been successfully synthesized and evaluated for their pharmacological and biological properties.[7,8] Among many organogermaniums known, bis-(carboxyethylgermanium) sesquioxide, which is generally referred to as triphenylgermanium bromide, has been studies as a promising anti-oxidative agent.[9]

Chrysin (Chry), one kind of flavonoid, possessed high anti-oxidative activity.[10] The biological activities of Chry are mainly attributed to hydroxyl and keto groups in its rings.[11,12] Metal–flavonoid complexes are reported to possess significantly high biological activity than those of flavonoids, showing potential applications in the treatment of disease.[13,14] Vanadium–flavonol complex,[15] iron–quercetin complex,[16] copper–naringenin complex[17,18] and copper–genistein complex[19,20] have been reported in recent years and showed attractive and promising anti-oxidative activities. Vitamin C–organogermanium complex showed good radical scavenging effects and cytoprotecting activities.[21] We have also proved that Ge (IV)–quercetin complex exhibited more potent anti-oxidative effects than free ligand or quercetin.[22] Study also showed that Ge–O bond are sensitive to scavenge free radicals.[9] Considering anti-oxidative activity of organic Ge or Chry, it is important to synthesize a new Chry–Ge complex to synergistically enhance their bioactivities. Therefore, it is still a challenge to synthesize Chry–Ge complex containing radical-sensitive Ge–O bond.

In order to introduce Ge–O bond in Chry–Ge complex, triphenylgermanium bromide was used as organic Ge, and chry sodium salt was pre-synthesized as the source of Chry. Chry–Ge complex was synthesized as indicated in Supplementary data. The synthesized complex was further purified by recrystallization. Figure 1A shows the proposed reaction formulas for the formation of Chry–Ge complex. To further verify the formation of Chry–Ge complex, ^{1}H NMR, FT-IR and ESI-MS were performed, respectively. In the spectrum of Ge (Fig. 1B), the peaks at 733.2 and 698.7 cm^{-1} were assigned to the bending vibration of C-H, while the peak at 459.9 cm^{-1} was corresponded to the stretching vibration of Ge-C.[23] This indicated that the bonding of Chry with organic Ge(IV). Importantly, the Ge–C bond of organic Ge was not destroyed in the synthesized process, which effectively protected the bioactivity of Ge. Moreover, the appearance of new peak at 859.4 cm^{-1} was observed, which was ascribed to the stretching vibration of Ge–O bond. This showed that successful formation of Ge–O bond in Chry–Ge(IV) complex. *M*/*Z* values of ESI-MS indicated that the stoichiometric numbers of Ge(IV) versus Chry, which reflected the coordination ratio of Ge(IV) and ligands, were considered to be 1:1. The molecular formula of Chry–Ge complex was therefore

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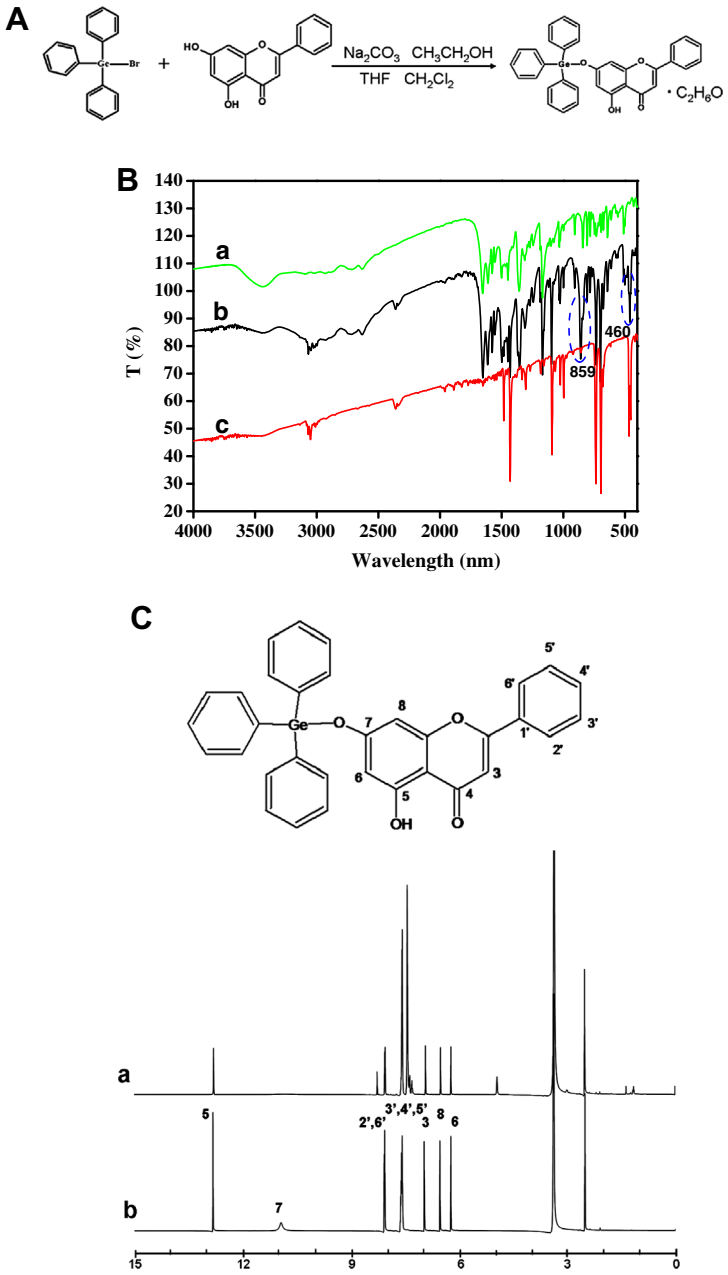

Figure 1. Chemical composition and structure characterization of Chry–Ge complex: (A) Chemical reaction and chemical structure of the Chry–Ge complex; (B) FT-IR spectra of Chry (a), Chry–Ge (b) and Ge (c); (C) ^{1}H NMR of Ge (a) and Chry–Ge (b).

$C_{33}H_{24}O_4Ge{\cdot}C_2H_6O$. ^{1}H NMR spectra of Chry showed the signals of 12.82 and 10.94 ppm (Fig. 1C), which were corresponded to 5-OH and 7-OH, respectively. In the ^{1}H NMR spectra of Chry–Ge complex, the signal of 10.94 ppm was disappeared, whereas the signal of 12.82 ppm was not obviously changed, which was due to the formation of Ge–O bond. Taken together, the changes in ESI-MS, ^{1}H NMR and FT-IR spectra validated new formation of Ge–O bond in Chry–Ge complex and maintained Ge–C bond in organic Ge, which successfully introduced the radicals-sensitive group and protected the anti-oxidative activity of organic Ge.

The in vitro cytotoxic effects of Chry–Ge complex and Chry were screened against normal breast epithelial cells by MTT assay. After incubation with 20 μg/ml of Chry–Ge complex and Chry, the cell survival rates have 73.33% and 80.17%, respectively. This result indicated that Chry–Ge complex has low cytotoxicity to normal cells. Free radicals were regarded as potent and dangerous metabolites, the ability of elimination of radicals was therefore taken as an important goal for anti-oxidant administration. Under the pH value for DPPH assay, synthesized Chry–Ge complex remained stable. As shown in Figure 2, DPPH assay showed anti-oxidative (Fig. 3) activities of Chry–Ge complex was a dose-dependent manner. When concentration of Chry–Ge complex was 70 μg/ml, scavenging rate of DPPH radicals reached 43.5%, which was significantly higher than that of 70 μg/ml Chry for 21.5%. Therefore, Chry–Ge complex showed higher suppression effect toward DPPH radicals than that of free Chry, indicating higher scavenging effect of Chry–Ge complex toward DPPH radical. The value of DPPH inhibition rate of the Chry–Ge complex was close to a known antioxidant,

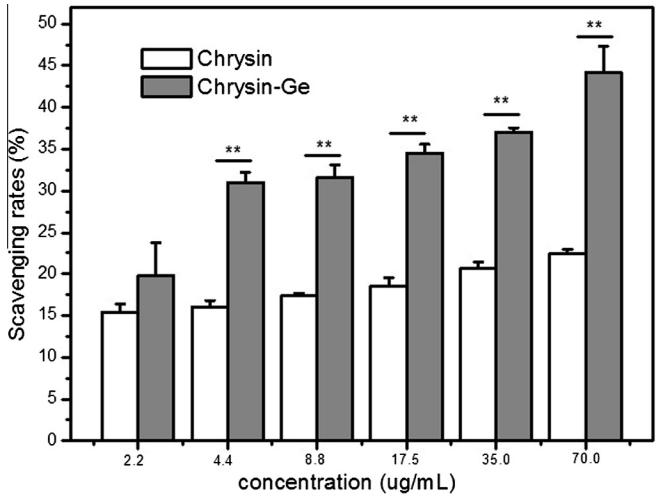

Figure 2. Antioxidant activities of Chry and Chry–Ge complex using the DPPH assay. Bars with different characters are statistically different at the P <0.01 level.

such as ascorbic acid,[24] which inhibited DPPH oxidation by 45% 50 μM. This are mainly attributed to synergetic effects of Chry–Ge complex.

It is known that the generation of intracellular reactive oxygen species (ROS) is a biomarker of oxidative stress in cellular level. Excess intracellular ROS can attack cellular membrane lipids, membrane proteins and DNA, inhibit their normal functions, and finally cause oxidative damage.[25] Hydroxyl radial (OH·) was regarded as potent and dangerous oxygen metabolite from H_2O_2. To further investigate anti-oxidative activity of Chry–Ge complex at cellular level, normal BRL cells were pretreated with H_2O_2 and its biological activity was investigated. Intracellular ROS was therefore detected by flow cytometry as using fluorescein-labeled dye of DCFH-DA. After 4 h pretreatment of BRL cells with Chry–Ge complex, anti-oxidative activity of Chry–Ge complex was characterized by observing changes in fluorescent intensity of DCFH-DA. Compared with controlled group, BRL cells treated with H_2O_2 showed a significant increase in fluorescent intensity of 2705 (Fig. 3), demonstrating that H_2O_2-induced oxidative damage led to over-expression of intracellular ROS. As shown in Fig. 3 the anti-oxidative activity of Chry–Ge complex against ROS were all concentrations related. The pretreatment of 7.5 μg/mL Chry–Ge complex reduced the fluorescent intensity to 998, while same concentration of Chry only reduced to 1112. This showed that anti-oxidative activities of Chry–Ge complex against intracellular ROS were significantly higher than that of their corresponding Chry. Results showed that Chry–Ge complex prevented H_2O_2-induced oxidative damage by inhibition ofROS-dependent pathway.

It has been reported that the detailed morphological and mechanical properties of cell membrane are important indicators of physiological and pathological processes for cells.[26] In this study, AFM was used to observe the changes of surface morphology, membrane ultrastructure and biophysical properties of normal BRL cells before or after treatment of Chry–Ge(IV) complex treatment. Cell samples were fixed by paraformaldehyde solution and detected in air. The results in Figure 4A1 indicated that BRL cells had ellipse shape and their cell membranes were relatively smooth and intact. The cell membrane architecture of cells was homogeneous and represented granular morphology with the surface particles (Fig. 4A2), which was related to protein molecules on cell membrane.[27] After treated with 100 μM H_2O_2, BRL cells were both significantly deformed to form collapsed cell morphology with shrunk cell tails (Fig. 4B1). For ultrastructure of cell membrane, heterogeneous and aggregation of large-sized particles on cell membrane were observed (Fig. 4B2). The changes of cell membrane morphology and membrane particles size implied that

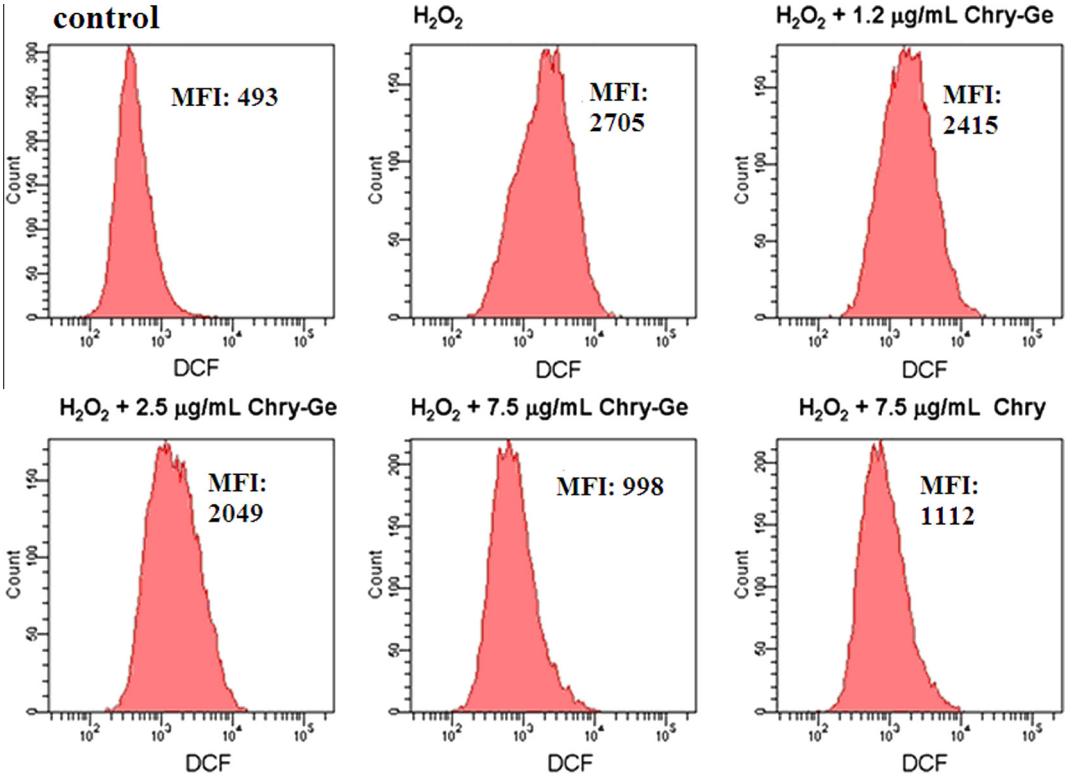

Figure 3. Protective effects of Chry and Chry–Ge complex on H_2O_2-induced ROS generation in BRL cells. Cells were pretreated with different concentrations (1.2, 2.5, 7.5 μg/mL) of Chry–Ge for 4 h and further treated with 100 μM H_2O_2 for 2 h only.

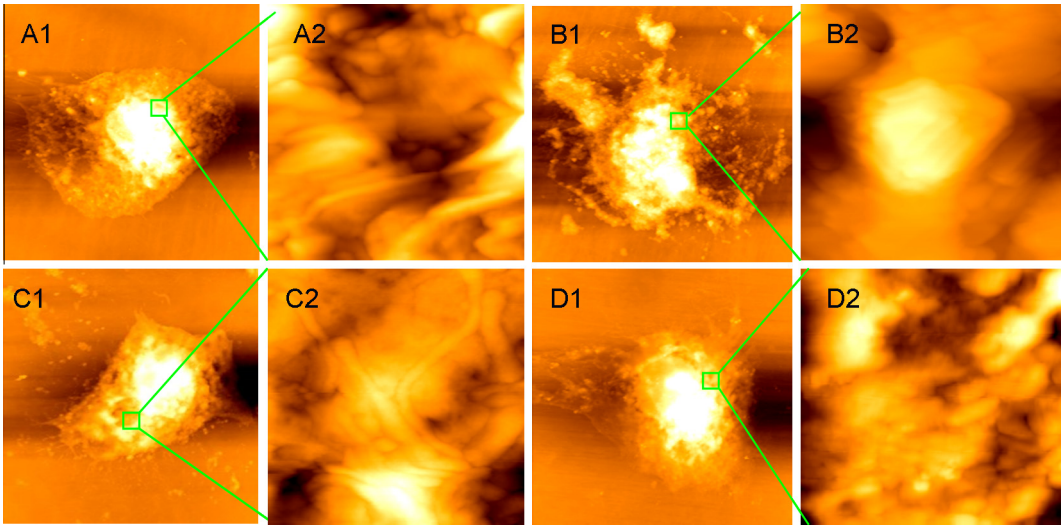

Figure 4. AFM images of single BRL cell. AFM images of normal BRL (A). AFM images of BRL cells were treated with 100 μM H_2O_2 for 2 h only (B). Cells were treated with 2.5 μg/mL Chry for 4 h in the presence of pre-treated with 100 μM H_2O_2 for 2 h (C). Cells were treated with 2.5 μg/mL Chry–Ge complex for 4 h in the presence of pre-treated with 100 μM H_2O_2 for 2 h (D).

some physiological changes had occurred in membrane proteins, indicating that radicals disrupted outer membrane proteins on cell membrane. Compared with Chry (Fig. 4C1-C2), pretreatment of cells with 2.5 μg/mL Chry–Ge complex in the presence of H_2O_2 alleviated oxidative damage of cell membrane, and ultrastructure of membrane proteins reversed to somewhat uniform (Fig. 4D1–D2). This showed that Chry–Ge complex effectively blocked oxidative stress induced by H_2O_2, and then recovered the proteins' functions of cells.

Studies showed that changes in cell membrane were related with the differences in nanomechanical properties of cells.[28] AFM-based force spectroscopy was used to probe nanomechanical properties to extract Young's modulus of control and complex-treated groups, respectively. Cell nucleus was chosen as nucleus points because it was easy to align the cantilever sphere over it, allowing data collection on the same spot over the experiment and then avoiding drifting or changes due to cell movements, such as contraction.[25] The statistical analysis of Force-distance consisting of arrays of 16 × 16 force curves were recorded in parallel with topographic images. Figure 5 shows elasticity histograms recorded on the top of cells. Most curves were well-described by the Hertz model, allowing us to obtain Young's modulus values. Elasticity analysis obtained at the control group showed average Young's modulus was 2.17 ± 0.76 kPa (Fig. 5A). When cells were treated with 100 μM H_2O_2, average Young's modulus of cell was significantly decreased to 1.05 ± 0.52 kPa (Fig. 5B). The elasticity properties of oxidation-damaged cells showed 50% softer compared to the control group, demonstrating that biological properties of cell membrane was destroyed under H_2O_2 treatment. Under the treatment of 2.5 μg/mL Chry–Ge complex, average Young's modulus of cell increased to 2.16 ± 0.71 kPa (Fig. 5D), which was higher than that of 1.86 ± 0.42 kPa for same concentrations of Chry pretreatment (Fig. 5C). Results showed that anti-oxidative ability of Chry–Ge complex was involved in the recovery of biophysical properties from oxidation-damaged cells. AFM images and force measurements showed Chry–Ge complex inhibited oxidative damage in H_2O_2-treated cells through recovery of ultrastructure of cell membrane and their biophysical properties.

The anti-oxidative activities of Chry–Ge(IV) complex could be attributed to its synergetic effects for three main reasons: first, the arrangements of outer electrons of Ge atom were $4S^24P^2$, which could make unpaired electrons be easily trapped by Ge atoms, and further made it possible for Ge to scavenge free radicals.[29] Second, Chry was reported to be excellent anti-oxidants.[10] The coordination of Chry with organic Ge gave Chry–Ge(IV) complex excellent radical scavenging activities. Third, the introduction of radical-sensitive Ge–O bond effectively enhanced the anti-oxidative activity of Chry–Ge complex. As shown in Figure 6, free radicals (R·) firstly attacked Ge–O bond in Chry–Ge complex, and then produced an oxide anion (RGeO), which effectively scavenged free radicals and reduced intracellular ROS generation. The anti-oxidative radical activity of Chry–Ge complex was closely related to the steric hindrance effect of phenols.[30] The radical scavenging activity of Chry–Ge complex was therefore most possible due to its synergetic effect, which resulted in synergetic effects of organic Ge, Chry–Ge complex, and Chry to coordinately catch free radicals.

In summary, we firstly synthesized and characterized novel Chry–Ge(IV) complex. The radical-sensitive Ge–O bond was introduced to Chry–Ge complex to synergistically enhance bioactivities of organic Ge or Chry. The biological analysis results showed Chry–Ge complex was with excellent anti-oxidative properties, which inhibited ROS-dependent oxidative damage in cells. The anti-oxidation assay showed that Chry–Ge complex possessed higher radical scavenging activity against hydroxyl and DPPH radicals, while Chry–Ge complex possessed higher radical scavenging activity than that of Chry. The flow cytometric analysis provided clear evidence that Chry–Ge(IV) complex were able to scavenge intracellular ROS. AFM morphological and biophysical data showed that complex not only recovered the ultrastructure of cell membrane particles, but also significantly increased the biophysical properties of cell membrane. The enhancement of the antioxidant activities of Chry–Ge complex could be possibly, at least partly, due to the introduction of Ge–O bond into Chry–Ge complex in the form of synergetic effect. This indicates that radical-sensitive Ge–O bond is an important chemical bond for enhancing its anti-oxidative activities. Further works are needed to reveal total antioxidant status of Chry–Ge complex in the cells. Collectively, all these results of this study provided further information for the design of organic Ge(IV)–flavonoid complex with predominant bioactivities and potential applications as promising anti-oxidative agents.

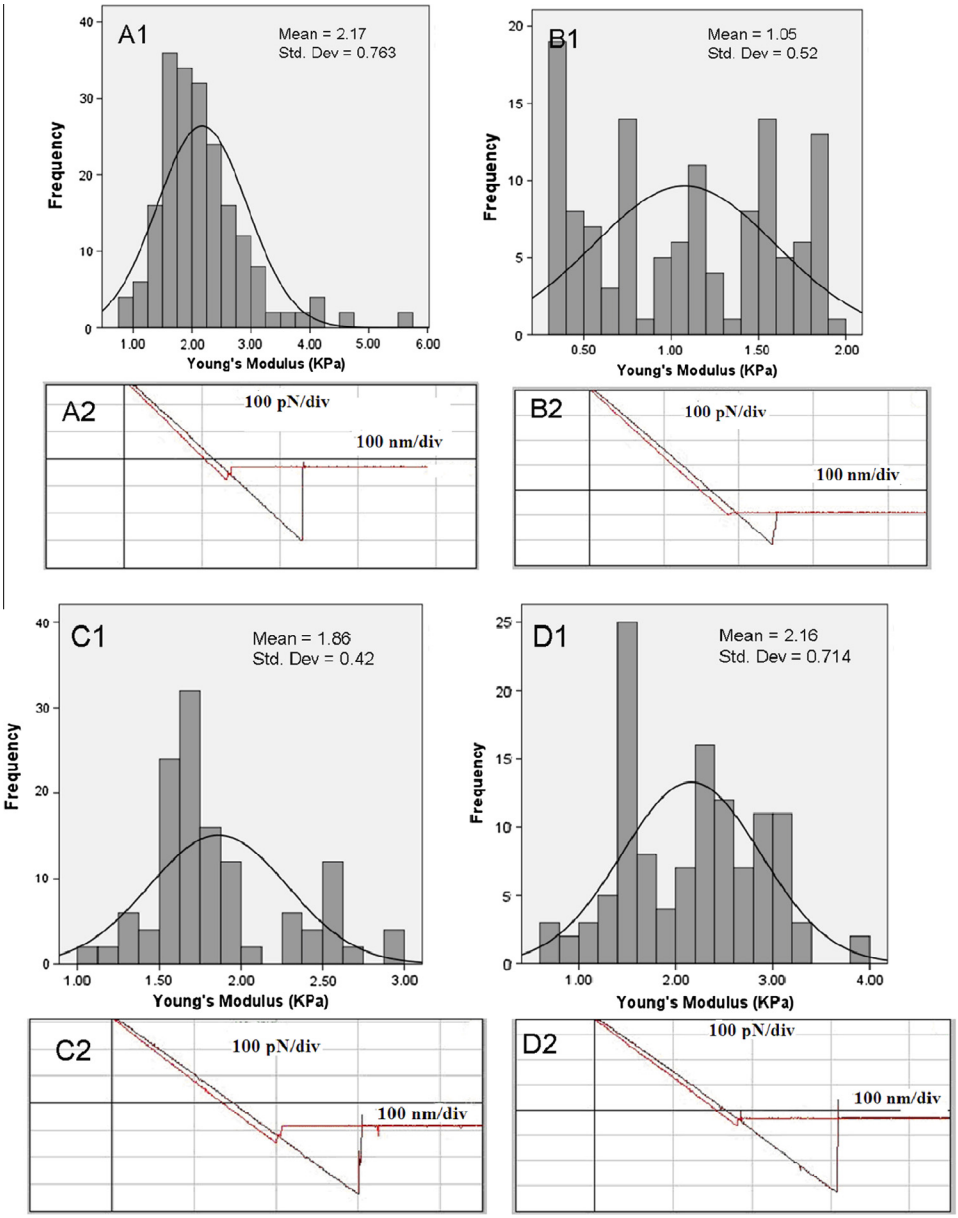

Figure 5. Force histograms with its Gaussian fits recorded by using SPSS 13.0 to gain the Gaussian distribution histograms of BRL cells (A1), cells in the presence of H_2O_2 (B1), cells in the presence of H_2O_2 with 2.5 μg/mL Chry (C1), cells in the presence of H_2O_2 with 2.5 μg/mL Chry–Ge complex (D1). (A2–D2) are the typical force-distance curves. Each group detected 10 cells. The result of each group is the average value of all the cells detected and presented as mean ± standard deviation. Size: 1 × 1 μm.

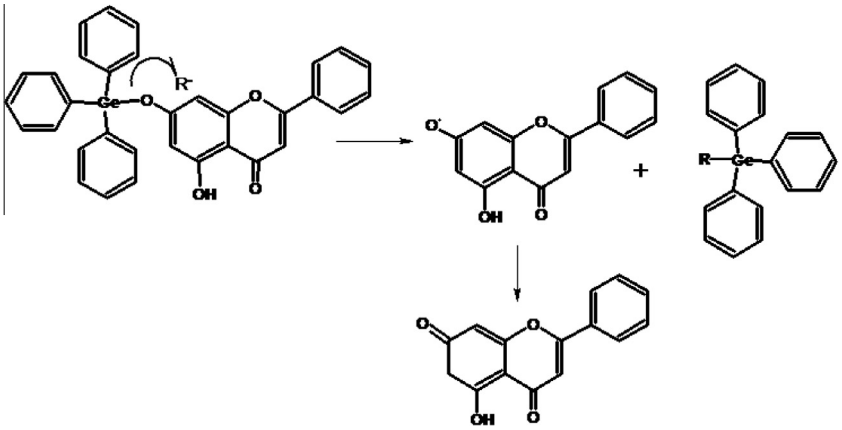

Figure 6. Proposed mechanism for scavenging of free radicals by Chry–Ge complex.

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Supplementary data

Supplementary data (experimental data) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2013.07.073.

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