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Pharmacodynamics of Ginsenosides: Antioxidant Activities, Activation of Nrf2 and Potential Synergistic Effects of Combinations

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Abstract

Ginseng has long been used in the Asian Countries for more than 2,000 years. Currently, in the "Western World or Western Medicines", many reports have indicated that they have used herbal medicines, and Ginseng is one of the most popular herbs. Several recent reports have indicated that the antioxidant / anti-oxidative stress activities of ginseng play a role in the benefits of ginseng, however the precise mechanism is lacking. The antioxidant response element (ARE) is a critical regulatory element for the expression of many anti-oxidant enzymes and phase II/III drug metabolizing/transporter genes, mediated by the transcription factor nuclear factor (erythroid-derived 2)-like 2 (Nrf2). The aim of this study was to examine the potential activation and synergism of Nrf2-ARE-mediated transcriptional activity between three common ginsenosides present in ginseng, ginsenoside Rb1 (Rb1), ginsenoside Rg1 (Rg1) and ginsenoside 20(S)-protopanaxytriol (20S). We tested whether these ginsenosides and their combinations, could induce Nrf2-ARE activities in HepG2-C8 cells with stably transfected ARE luciferase reporter gene. Cell proliferation, antioxidant and ARE activities, western blotting of Nrf2 protein and qPCR of mRNA of Nrf2 were conducted for Rb1, Rg1 and 20S as well as the combinations of 20S with Rb1 or Rg1. To determine the combination effects, the combination index (CI) was calculated. Rb1 and Rg1 are relatively non-toxic to the cells, while 20S at 50 μM or above significantly inhibited the cell proliferation. Rb1, Rg1 or 20S induced total antioxidant activity and ARE activity in a concentration-dependent manner. Furthermore, combinations of 20S with either

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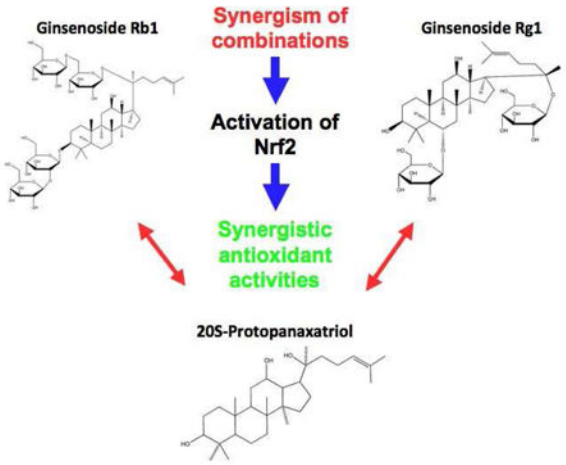
Rb1 or Rg1 induced total antioxidant and ARE activity synergistically. Induction of Nrf2 protein and mRNA were also found to be synergistic with the combination treatments. In summary, in this study, we show that ginsenosides Rb1, Rg1 and 20S possess antioxidant activity, transcriptionally activating ARE as well as potential of synergistic activities. The Nrf2-ARE-mediated antioxidant pathway could play a role for the overall anti-oxidative stress activities, which could be important for ginseng's health beneficial effects such as cancer chemopreventive activities.

Graphical abstract



Keywords

Antioxidant response element (ARE); nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2); ginsenoside Rb1; ginsenoside Rg1; ginsenoside 20(S)-protopanaxytriol (20S); combination; synergism; anti-oxidative stress

Introduction

The root of ***Panax Ginseng, C.A. Meyer,*** commonly known as **ginseng** has a long history in traditional herbal Medicine. Ginsenosides are a class of compounds found exclusively in the plant genus *Panax* (ginseng). Ginseng has been used in almost all kinds of illnesses including internal medicine, external medicine, gynecology and pediatric among others. It has been used to prevent diseases and delaying aging for more than 2,000 years in traditional Chinese medicine (TCM). Many ancient records and studies have suggested the value of health benefits of ginseng such as improving vitality and even today, ginseng is continuing to be widely used as health improving product.[1] It has been marketed successfully as a tonic to invigorate weak bodies and it is ranked as one of the most popular herbal products in the US including the internet market.[2] Its sales has been increasing steadily over the years and shows with $300 million USD in sales in 1997.[3]

Recent report shows that ginseng possesses preventive and therapeutics anti-cancer properties.[4] Oral administration of the ginsenosides-enriched **American Ginseng (*Panax quinquefolius*)** extracts to healthy volunteers significantly reduced the oxidative stress

biomarkers such as F2-isoprostane and 8-hydroxy-deoxyguanosine, implicating ginseng may possess anti-oxidative stress properties in human.[4] Since these studies did not measure specific anti-oxidant enzymes or other biomarkers, it is not clear whether the anti-oxidative stress responses are due to direct scavenging effects of ginseng or due to other indirect mechanisms such as the induction of anti-oxidative stress enzymes. Most recently, we have reviewed the literatures and found a possible involvement of the nuclear factor (erythroid-derived 2)-like 2 (NFE2L2 or Nrf2), implicating a role of Nrf2 in the anti-oxidative stress/ anti-inflammatory responses modulated by ginseng.[5] Nevertheless, the direct investigation of the role of Nrf2 in ginseng's mechanism of action is still lacking.

A recent report shows the protective effects of ginsenoside Rb1 (Rb1) against 6-hydroxydopamine-induced oxidative stress potentially via enhanced heme oxygenase 1 (HO-1) expression through Nrf2-dependant pathway.[6] Nrf2 is a key regulator of the antioxidant response element (ARE)-mediated gene expression and is a potential molecular target of cancer chemopreventive compounds.[7] ARE is a critical regulatory element found in the promoters of many Phase II protective/ detoxification and anti-oxidative stress enzymes mediated by Nrf2. Many naturally occurring dietary compounds, including herbal medicines and phyto-chemopreventive agents, possess potent cancer chemopreventive effects in numerous preclinical model systems and ongoing clinical trials are showing some promising results. Many of these compounds have been shown to activate the Nrf2-ARE pathway and many health benefits of ginseng have been reported such as anti-cancer activities.[8-11] Therefore, it appears that ginseng may be a potential cancer preventive agent.[5, 12] In this study, we hypothesized that ginsenosides would activate Nrf2-dependent anti-oxidative stress pathway and with appropriate combinations, ginsenoside Rb1 (Rb1), ginsenoside Rg1 (Rg1) and ginsenoside 20(S)-protopanaxytriol (20S) (Figure 1) could exert synergistic benefits in Nrf2 activation. We investigated whether these ginsenosides and their combinations, could also induce antioxidant activities using an *in vitro* system. To accomplish these goals, we utilized the human liver hepatoma cell line (HepG2-C8-ARE-luciferease cells, HepG2 cell stabilized with the ARE-luciferase reporter gene.[13] We have utilized this stably transfected HepG2-C8 cells to study the Nrf2-ARE signaling pathways mediated by many other dietary phytochemicals,[14-20] including synergistic effects of phytochemical indoles and isothiocyanates[21] among others. Our present results show that the ginsenosides alone can transcriptionally activate ARE, and more importantly, the combinations can also act synergistically in activating the ARE pathway. The expression of Nrf2 as well as the total antioxidant activities was also induced synergistically by the combination treatment.

Materials and Methods

Materials

All compounds were purchased from Sigma Aldrich Chemical Company (St Louis, USA): ginsenoside Rb1 (Rb1, catalog #00170580, 98.31%, Fig. 1A), ginsenoside Rg1 (Rg1, catalog #68317, 98.73%, Fig. 1B) and ginsenoside 20S-protopanaxatriol (20S, catalog #P0034, 99.4%, Fig. 1C). The ginsenosides were dissolved in dimethyl sulfoxide (DMSO),

and the final concentration of DMSO in the media was less than 0.1% DMSO. The vehicle control of 0.1% DMSO was used for all tests.

Cell culture

The Nrf2-mediated ARE in human hepatoma HepG2-C8-ARE luciferase cells, previously established in our laboratory using the pARE-TI-luciferase reporter gene was utilized in this experiment.[13] The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1.17 g/L sodium bicarbonate, and 100 unit/mL penicillin, 100 μg/ml streptomycin at 37 °C in a humidified incubator with 5% CO_2.

Cell proliferation MTS assay

The cytotoxicity of the ginsenosides was tested in HepG2-C8 cells using the CellTiter 96 aqueous non-radioactive cell proliferation assay MTS assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS] (Promega, Madison, WI). The cells were first cultured in 96-well plates for 24 h and then were treated with ginsenosides at various concentrations for 24 h using 1% FBS medium. The cells were then treated with MTS for 1 h at 37°C. Absorbance of the formazan product was read at 490 nm with μQuant Biomolecular Spectrophotometer from Bio-Tek Instruments Inc. (Winooski, VT). At least three independent studies were conducted and four samples were performed for each treatment each time.

Total antioxidant activity test

The antioxidant potential of ginsenosides in HepG2-C8 cells were tested using the Total Antioxidant Power kit (Oxford Biomedical Research, MI, USA; catalog # TA02) according to the manufacturer' protocol. Briefly, the cells were first cultured in 12-well plates for 24 h and then were treated with ginsenosides at various concentrations for 24 h using 1% FBS medium. After 24 h, the experiment was terminated by washing the cells with ice-cold phosphate buffered-saline (PBS, pH 7.4), the cells were homogenized with lysis buffer (Promega, WI, USA). The lysate was then subjected to centrifugation at 604 × *g* for 12 min at 4°C, supernatant was collected for the assays and protein concentrations were determined by BCA protein assay (Pierce, Rockford, USA). For normalization of the total antioxidant activity of the treatment, the activity is expressed as percentage compared with the control 0.1% DMSO.

The reduction potential of the samples or the standards which effectively converts Cu^{2+} to Cu^{1+}, thus changing the ion's absorption characteristics. This reduced form of Cu^{1+} would selectively form a stable 2:1 complex with the chromogenic reagent with an absorption maximum at 450 nm. A known concentration of Trolox was used to create a calibration curve, and the results were expressed as mM Trolox equivalents or in μM copper reducing equivalents and the activity is expressed as percentage compared with 0.1% DMSO. At least three independent studies were conducted in triplicates. All samples were diluted to fall within the standard curve.

ARE-luciferase assay

HepG2-C8 cells were cultured in 12-well plates and each well contained 100,000 cells in 1 ml of 1% FBS medium. The cells were treated with compounds for 24 h. The luciferase activity was determined as described previously[14-21] using a luciferase kit from Promega (Madison, USA) according to the manufacturer's instructions. Briefly, after treatments for 24 h, the cells were washed twice with ice-cold PBS and harvested in 1 × reporter lysis buffer and kept at -20°C overnight. After centrifugation at 4°C, 9,660 × *g* for 5 min, a 10 μl aliquot of the supernatant was assayed for luciferase activity with a SIRIUS luminometer (Berthold Detection System GmbH, Pforzheim, Germany). The luciferase activity was normalized against protein concentration determined by BCA protein assay (Pierce, Rockford, USA). The luciferase activity was expressed as percentage of induction of the samples over that of control vehicle-treated cells. At least three independent studies were conducted in triplicates.

Combination index calculation

To determine the synergistic effect between the combination of two different compounds, the combination index (CI) can be calculated with the following formula: $CI= d1/Dx,_1 + d2/Dx,_2$ where d1 and d2 are doses of drugs 1 and 2 in combination, which produces an effect x. $Dx,_1$ and $Dx,_2$ are the doses of drug 1 and drug 2 that produce the same effect x when given alone.[21, 22] When the CI is equal to, less than or greater than 1, the combination will be additive, synergistic or antagonistic, respectively, as we have described previously.[21, 22] This approach is based on the Loewe additivity model and although the exact mechanism of interaction may be unknown, this model is one of the most commonly used reference models for evaluating potential drug-drug interactions[23] and was first introduced by Chou TC el al.[24] Using this CI calculation for the ARE-luciferase activity and total antioxidant activity induced by the combinations of ginsenosides, it is possible to identify whether the combination of these ginsenosides at some concentrations would be synergistic, antagonistic or additive.

Western blotting

The cells were treated similarly as the MTS, and ARE-luciferase assays described above using 1% FBS medium. HepG2-C8 cells were treated with the compound for 48 h. Cells were harvested in 100 μl of Cell Culture Lysis Reagent (Promega E153A, Madison, WI) after washed with ice-cold PBS (pH 7.4) and kept at -20°C overnight. The next day, the frozen samples were thawed and collected as the homogenate. Then, the homogenate was centrifuged at 4°C, 9,660 × *g* for 5 min. The supernatants were collected and 15 μg of total protein were mixed with 5 μl Laemmli's SDS-Sample Buffer (Boston Bioproducts, Ashland, MA, USA) and denatured at 95°C for 5 minutes. The samples and the protein standard (Bio-Rad, Hercules, CA, USA) were then loaded onto a polyacrylamide gel (Criterion Tris–HCl gel, Bio-Rad Lab, Hercules, CA, USA) and gel electrophoresis was ran at 130 mA for 60 min. Proteins were transferred onto a polyvinylidene difluoride (PVDF) membranes (Immobilon-P, Millipore, Bedford, MA, USA) over 2 h using a semi-dry transfer system (BioRad, Hercules, CA, USA). The membranes were blocked with 5% BSA solution for 1 h at room temperature and incubated with the primary antibody (1:1000, in 3% BSA in Tris-buffered-saline and Tween 20, TBST) overnight at 4°C. Antibody against Actin (catalog no.

sc-1616) was purchased from Santa Cruz (Santa Cruz Biotechnology, Inc., CA, USA). Antibody against Nrf2 (catalog no. 2178-1) was purchased from Epitomics (Burlingame, CA, USA). After hybridization with primary antibody, membranes were washed with TBST four times. The immunoreactions were continued with the respective secondary antibodies (1:5000, in 3% BSA in TBST) purchased from Santa Cruz Biotechnology, Inc., CA, USA, for 1 h at room temperature. After washing four times with TBST, the immunocomplexes were determined using the enhanced chemiluminescent system to detect the horseradish peroxidase on the immunoblots (Thermo Scientific, Rockford, IL, USA) and the bands were visualized and captured by BioRad ChemiDoc XRS system (Hercules, CA, USA). The protein bands were quantified by densitometry using Image J (Version 1.44, National Institute of Health, USA).

RNA isolation and quantitative real-time polymerase chain reaction (qPCR) in HepG2-C8 and murine prostate cancer TRAMP C1 cell lines

The HepG2-C8 cells were treated similarly as the MTS and ARE-luciferase assays described above using 1% FBS medium. The incubation of the compounds with the cells was terminated at 6 h later. On the other hand, TRAMP C1 cells at a density of 1×10^4 cells/dish were cultured in 3 cm dishes, and the cells were treated with 20S (12.5 μM) after 24 h. The treatment medium was changed after 2 days, and total RNA was extracted from the cells using Trizol reagent (Invitrogen, Carlsbad, CA) after 3 days treatment. SuperScript III First-Strand Synthesis System from Invitrogen (Grand Island, NY) was further used to synthesize first-strand cDNA from total RNA according to the manufacturer's instructions. The first-strand cDNA was used as the template for the determination of mRNA expression levels by quantitative real time-PCR (qPCR) using Power SYBR Green PCR Master Mix (Applied Biosystem, Carlsbads, CA) in ABI7900HT system. The primer pairs were designed using Primer Quest Oligo Design and Analysis Tool by Integrated DNA Technologies Inc. (Coralville, IA, USA) and the sequences are listed in Table 1. At least 3 independent treatments were performed and duplicate samples were carried out for each treatment.

Statistical analysis

The results are presented as means ± standard error of the mean (SEM). The data were analyzed using one-way ANOVA with a post hoc multiple comparison analysis by Tukey's (PASW Statistics, version 18, SPSS Inc., Chicago, Illinois) or Student's *t*-test. *P* value of less than 0.05 was considered to be statistically significance. At least three independent experiments of each assay with three samples were conducted for each experiment.

Results

Ginsenosides and their combinations are relatively non-toxic

MTS assay was performed to determine the effects of Rb1, Rg1 and 20S and their combinations of 12.5 μM of 20S with 12.5 μM of Rb1 or Rg1 (designated as 20S Rb1 12.5 and 20S Rg1 12.5 respectively). Rb1 and Rg1 are relatively non-toxic as compared to the control (0.1% DMSO) even up to 100 μM (Figure 2), while 20S decreased cell proliferation in a dose-dependent manner at 50 μM and above 50 μM caused significant cell death (ANOVA, $p < 0.01$ for 50 and 100 μM compared with 0.1 % DMSO; Figure 2). All

subsequent experiments were conducted below 50 μM for compound 20S. Interestingly, the combinations of 20S Rb1 12.5 and 20S Rg1 12.5 did not increased the toxicities.

Induction of total antioxidant activity by ginsenosides and combination of ginsenosides show potential synergistic effect

To determine the antioxidant activity of Rb1, Rg1 and 20S and the combinations 20S Rb1 12.5 and 20S Rg1 12.5, the antioxidant activity was examined. The induction of the total antioxidant activity was increased by treatment of Rb1, Rg1 and 20S alone dose-dependently (Figure 3). Figure 3 shows the combinations 20S Rb1 12.5 and 20S Rg1 12.5 significantly induced total antioxidant activity (ANOVA, $p < 0.01$ compared with 0.1 % DMSO), significantly greater than the individual 12.5 μM Rb1 or Rg1 alone (both $p < 0.05$, data not shown). Table 2 shows the CI of the combinations resulted in CI < 1 significantly (Table 2), indicating synergistic effects when combining 12.5 μM 20S with 12.5 μM of Rb1 or Rg1.

Induction of ARE-luciferase activity by ginsenosides and their combinations show potential synergistic induction of ARE-luciferase activity

To test the transcriptional activation of ARE by ginsenosides, ARE-luciferase reporter assay was performed.[14-21] Figure 4 shows that the three ginsenosides alone or in combinations induced ARE-luciferase activity in HepG2-C8 cells with different potency. 20S at 25 μM strongly induced ARE-luciferase activity as compared to any other treatments ($p<0.01$). Combination of 12.5 μM 20S with 12.5 μM Rb1 or Rg1 synergistically induced the ARE luciferase activity with CI < 1 (Table 2), similar to the total antioxidant activity above (Figure 3).

Ginseonisdes induce Nrf2 protein expression

Figure 5 shows the protein expression of Nrf2 after the cells were treated with the ginsenosides individually or in combinations. The Nrf2 protein was not significantly induced at low doses of 12.5 μM by the three ginsenosides. However, the combinations of low doses of ginsenosides were able to significantly induce higher Nrf2 protein level as compared to the individual agent (Figures 5A & 5B). This result correlates with the synergistic effects (CI < 1) shown above in the total antioxidant activity (Figure 3) and ARE-luciferase assay (Figure 4).

Ginseonisdes induce mRNA expression of Nrf2 and Nrf2-target genes

To confirm that ginseonisdes can also induce endogenous Nrf2 and Nrf2 downstream target genes in cells, we conducted qPCR to quantify the mRNA expression (Figure 6). Values higher than one were considered positive in comparison to cells treated with control 0.1% DMSO. Figure 6A shows the results for the induction of Nrf2 and HO-1, Nrf2-dependent ARE-driven endogenous gene in HepG2-C8 cells (Figure 6A). When ginsenosides were tested alone at 12.5 μM, they showed two-fold induction of Nrf2 mRNA. Similar trend of mRNA induction of HO-1 was also shown (Figure 6A). Additionally, the mRNA induction of endogenous Nrf2 and Nrf2-mediated antioxidant and detoxifying enzymes such as HO-1, NQO1 and UGT1A1 in murine prostate cancer TRAMP C1 cells were also quantified using

qPCR after treatments with 20S for 3 days (Figure 6B). The results show that 20S (12.5 μM) significantly increased Nrf2 and UGT1A1 mRNA expressions compared to the controls ($p < 0.05$). 20S (12.5 μM) also slightly induced HO-1 and NQO1 mRNA expressions compared to the controls although not statistically significance. These results suggest that ginsenosides, especially 20S, have the potential of increasing endogenous mRNA expression of Nrf2 and Nrf2-mediated antioxidant and detoxifying enzymes in cells.

Discussion

The overall health benefits including prevention of diseases such as cancer conceivably can be achieved with increased consumption of fruits, vegetables and botanical dietary supplements containing rich sources of many phytochemicals.[7, 25] The exact mechanisms by which phytochemicals, including ginsenosides could prevent diseases such as cancer are not clear, but potentially appear to involve the Nrf2-ARE-mediated anti-oxidative stress pathway.[7, 25] In addition, ginseng is often consumed as a mixture comprising various ginsenosides. The preparation process of ginseng root such as steaming will cause a change of ginsenoside profile in ginseng products.[26] In the current study, we investigated the transcriptional activation of Nrf2-ARE, as well as the potential synergistic effects of the ginsenosides. Our results suggest that ginseng crude extract containing a combination of ginsenosides could potentially be more potent and beneficial than single ginsenoside in some settings. The concentrations used in our current study appear to be higher than that found in human study,[4, 27] which are commonly seen with many phytochemicals using in vitro cell lines. Nevertheless, as a proof of concept, the synergistic effect may be possible, which could be a reflection of consumption of natural ginsengs in human. Additional in vivo studies will be needed to correlate the in vitro cell culture model with human studies.

It has been reported recently that ginsenosides have positive effects against hydrogen peroxides or radicals,[28-30] but their total antioxidant activity has not been demonstrated in these previous studies. Negative finding has also been reported on the endogenous antioxidant activities when these ginsenosides were tested[31] However, the effectiveness of oral consumption of ginseng in human appears to suggest its anti-oxidative action.[4] Hence, in our current study we focus on the endogenous total antioxidant activities potentially link to the mechanism of action of ginseng.

Among the three ginsenosides tested (Rb1, Rg1 and 20S), it appears that 20S possesses more potent cell killing activity (Figure 2), total antioxidant activity (Figure 3), ARE-luciferase activity (Figure 4) and induction of mRNA of Nrf2-target genes (Figure 6). Interestingly, the combinations of 20S with Rb1 or Rg1 would exert synergistic induction if ARE luciferase activities (Figure 4, Table 2) and synergistically induced the total antioxidant activities (Figure 3, Table 2). The combinations also appear to be potentially synergistic induction of Nrf2 protein (Figure 5) and Nrf2 mRNA (Figure 6). Combination of gensenosides could also regulate Keap1 expression as previously reports with other chemopreventive compounds such as curcumin and quinones.[32] Future in vivo studies would be needed to examine whether the combination ginsenosides would induce Nrf2 target genes and the in vivo cancer chemopreventive effects.

In summary, the results of our current study suggest that ginsenosides activate the Nrf2-mediated anti-oxidant/oxidative stress pathway, which is the key regulatory pathway against oxidative stress. There is potential synergistic anti-oxidative effects of combinations of different ginsenosides which could be important for ginseng's overall health beneficial effects such as cancer chemoprevention.

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Abbreviations

ARE	Antioxidant response element
Nrf2	nuclear factor (erythroid-derived 2)-like 2
Rb1	ginsenoside Rb1
Rg1	ginsenoside Rg1
20S	ginsenoside 20(S)-protopanaxytriol

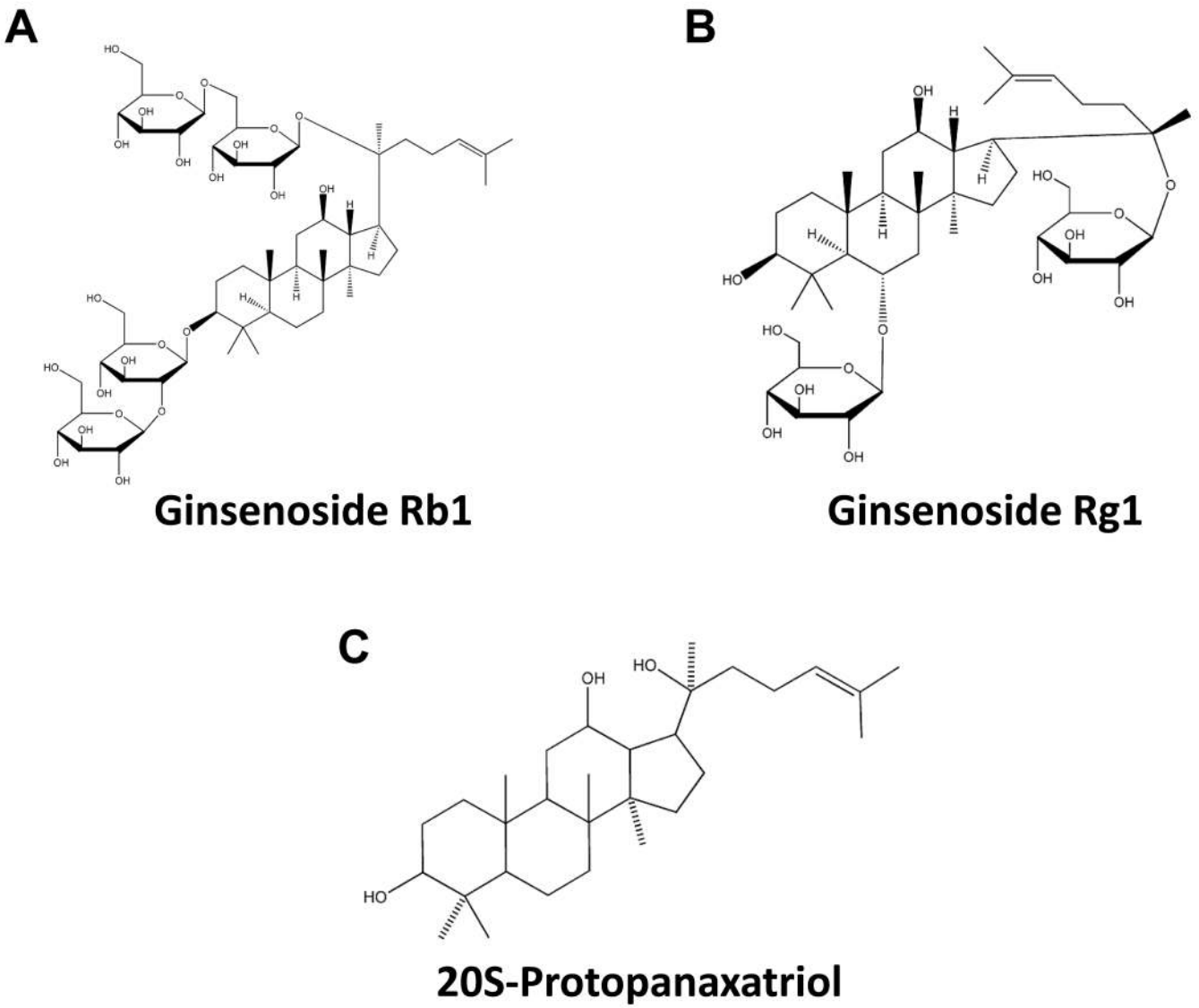

Figure 1. Chemical structures of gensenosides used in the current study

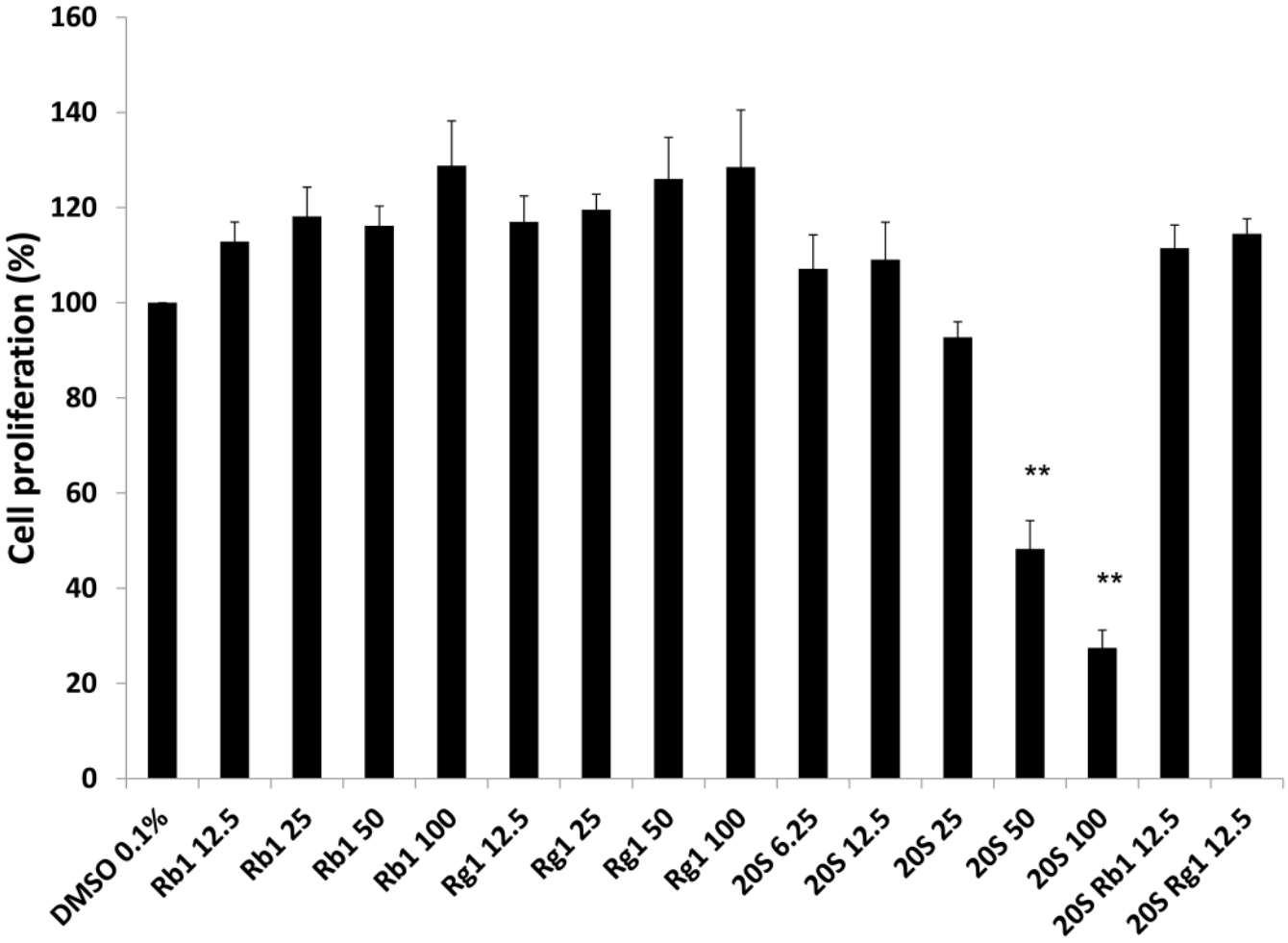

Figure 2.

Effect of the compounds tested on the cell proliferation determined by MTS assay. Results are expressed as the mean ± SEM. **, $p < 0.01$, compared with corresponding value for the control 0.1% DMSO-treated cells.

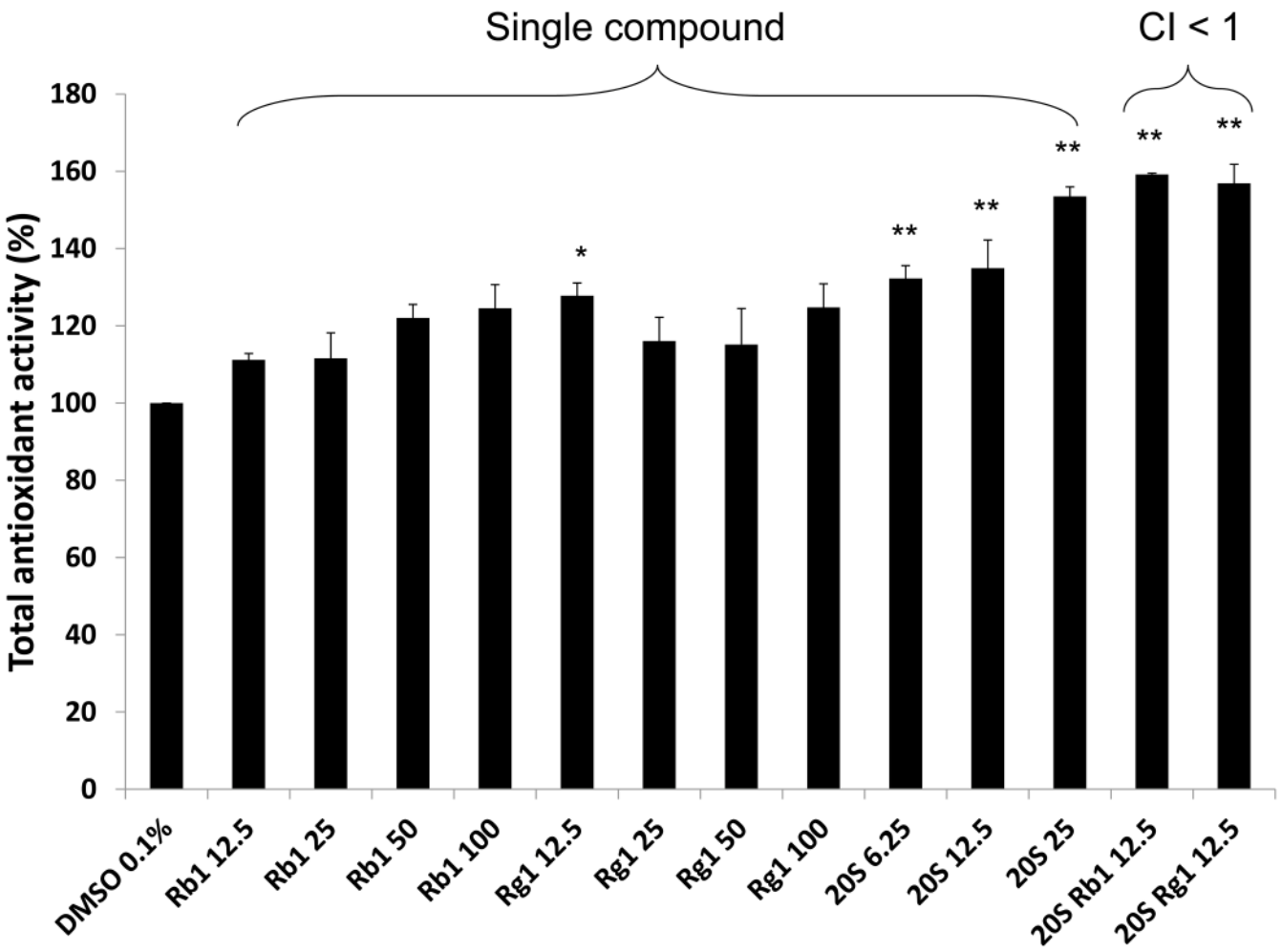

Figure 3.

Total antioxidant activity in HepG2-C8 cells. Cells were treated with Rb1, Rg1, 20S and the combinations for 24 h. Results are expressed as the mean ± SEM. *, $p < 0.05$; **, $p < 0.01$; compared with corresponding value for the control 0.1% DMSO-treated cells. The calculation of the combination index (CI) shows that the combinations of 20S with Rb1 or Rg1 at 12.5 μM are less than 1 indicates significant synergistic combination effects (see also Table 2).

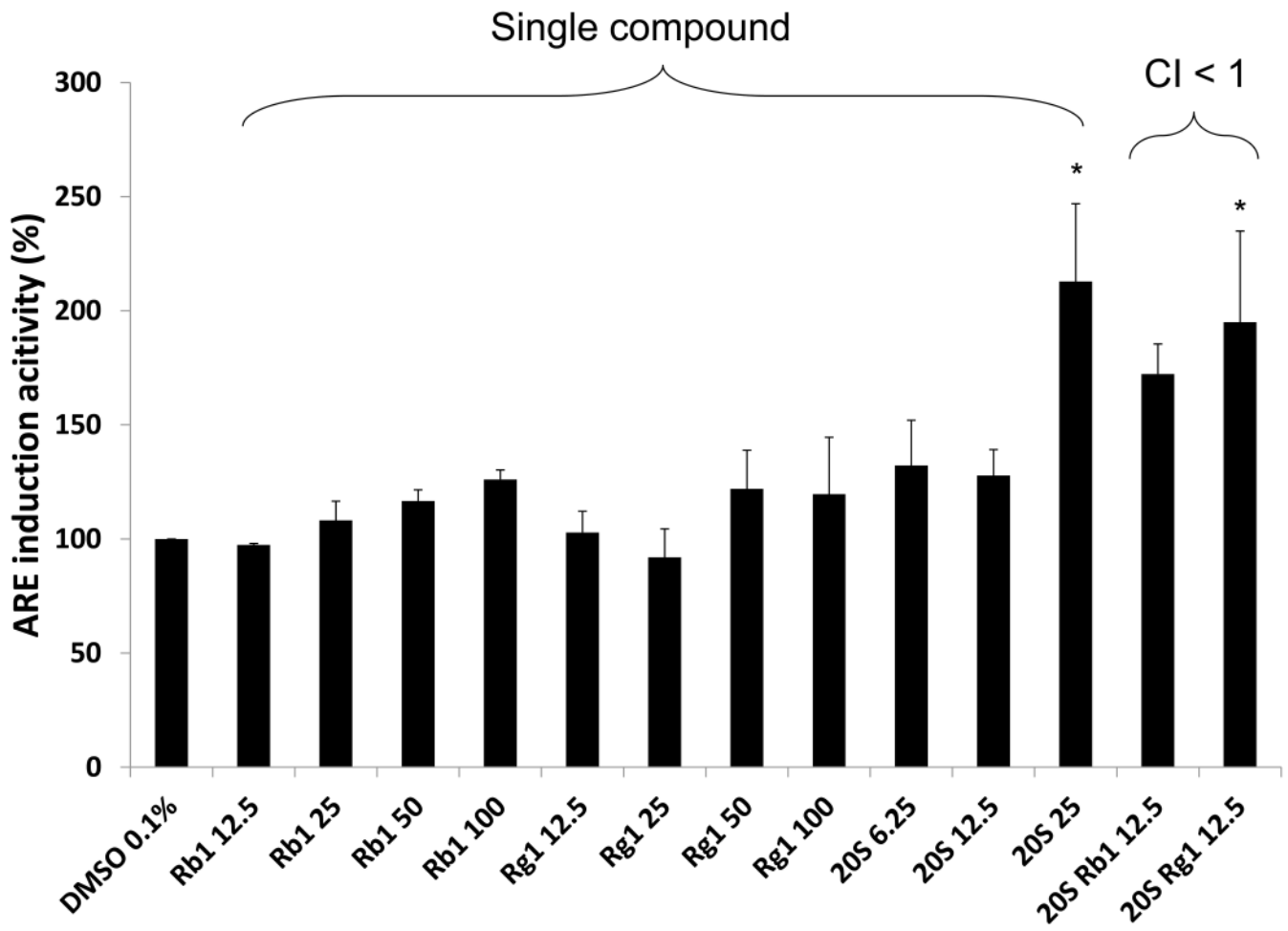

Figure 4.

ARE-luciferase activity in HepG2-C8 cells. Cells were treated with Rb1, Rg1, 20S or the combinations for 24 h. Results are expressed as the mean ± SEM. *, $p < 0.05$; compared with the corresponding value for the control 0.1% DMSO-treated cells. The CI < 1 for the combination of 20S with Rb1 or Rg1 at 12.5 μM indicates synergistic effects.

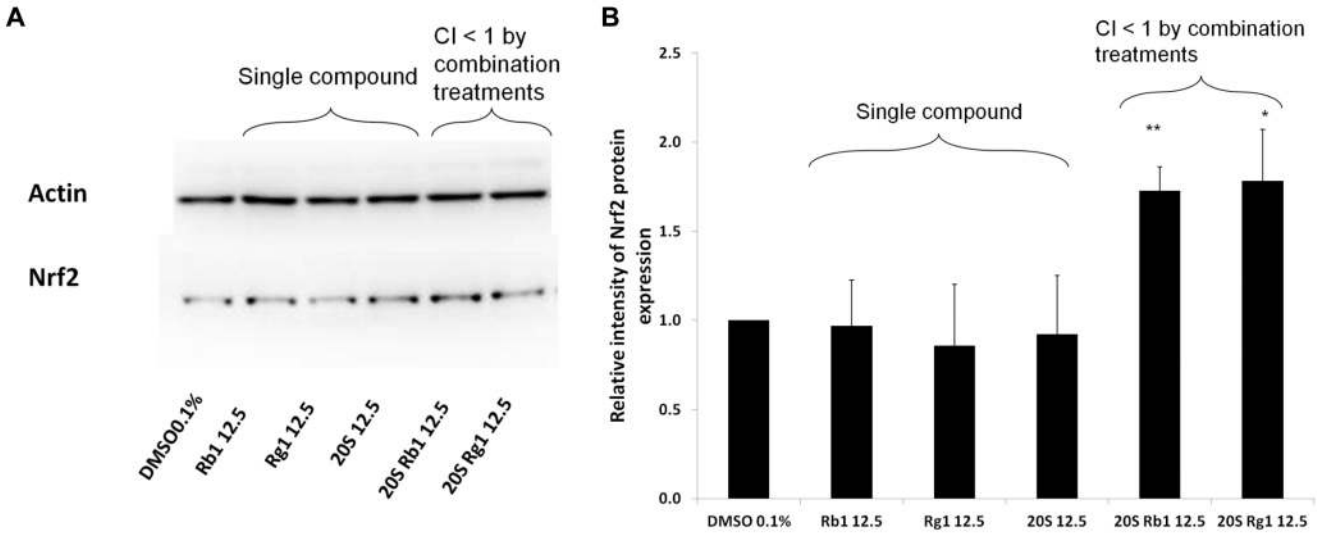

Figure 5.

Effects of Rb1, Rg1, 20S and their combinations on Nrf2 protein expression in HepG2-C8 cells by Western blotting using β-actin as housekeeping protein. The combinations of low doses of 20S with Rb1 or Rg1 were able to induce protein expression of Nrf2 synergistically. The tested concentrations were in μM. (A) Representative images are shown on the basis of similar results from three independent experiments. (B) Results of quantification are expressed as mean ± SEM. *, $p < 0.05$, compared with the control 0.1% DMSO-treated control cells.

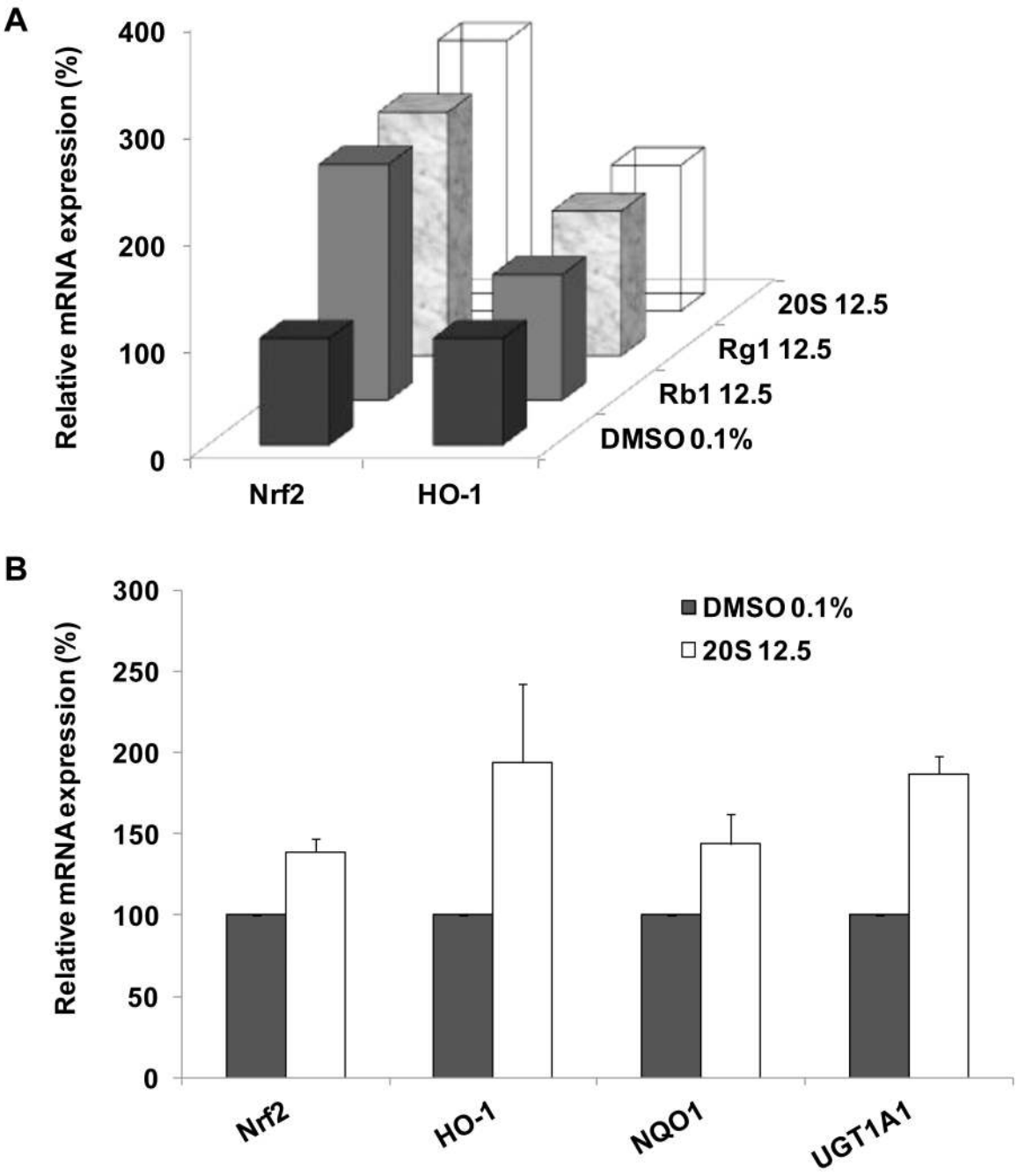

Figure 6.

Effect of gensenosides on the relative mRNA expressions of Nrf2 and Nrf2 related enzymes in HepG2-C8 (A) and TRAMP C1 (B) cells using quantitative real-time PCR (qPCR). (A) HepG2-C8 cells were treated with 12.5 μM of Rb1, Rg1 or 20S for 6 h. (B) TRAMP C1 cells were incubated with 12.5 μM of 20S for 3 days. The RNAs were extracted from three independent experiments. Data are expressed as mean ± SEM while GAPDH and β-actin were used as endogenous housekeeping gene in HepG2-C8 and TRAMP C1 cells,

respectively. * $p < 0.05$, significant increase in relative mRNA expression compared to control (DMSO 0.1%).

Table 1
Human and mouse oligonucleotide primers used for qPCR

Gene	Forward (5′) primer	Reverse (3′) primer
Human glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5′-TCG ACA GTC AGC CGC ATC TTC TTT-3′	5′-ACC AAA TCC GTT GAC TCC GAC CTT-3′
Human nuclear factor (erythroid-derived 2)-like 2 (Nrf2)	5′-TGC TTT ATA GCG TGC AAA CCT CGC-3′	5′-ATC CAT GTC CCT TGA CAG CAC AGA-3′
Human hemeoxygenase-1 (HO-1)	5′- ACG CGT TGT AAT TAA GCC TCG CAC-3′	5′-TTC CGC TGG TCA TTA AGG CTG AGT-3′
Mouse β-actin	5′-CGT TCA ATA CCC CAG CCA TG-3′	5′-GAC CCC GTC ACC AGA GTC C-3′
Mouse Nrf2	5′-AGC AGG ACT GGA GAA GTT-3′	5′-TTC TTT TTC CAG CGA GGA GA-3′
Mouse HO-1	5′-CCT CAC TGG CAG GAA ATC ATC-3′	5′-CCT CGT GGA GAC GCT TTA CAT A-3′
Mouse NAD(P)H dehydrogenase, quinone 1 (NQO1)	5′-AGC CCA GAT ATT GTG GCC G-3′	5′-CCT TTC AGA ATG GCT GGC AC-3′
Mouse UDP-glucuronosyltransferases 1A1 (UGT1A1)	5′-GAA ATT GCT GAG GCT TTG GGC AGA-3′	5′-ATG GGA GCC AGA GTG TGT GAT GAA-3′

Table 2

Combination index (CI) calculated for the combination treatments after 24 h

Final concentration (μM)	CI for total antioxidant activity	CI for ARE activity
20S Rb1 12.5	0.49 ± 0.08 **	0.72 ± 0.1 *
20S Rg1 12.5	0.46 ± 0.10 **	0.58 ± 0.13 *

CI calculated as described in the Materials and Methods. For the combination treatment, CI < 1, indicates synergistic; CI = 1, indicates additive; CI > 1 indicates antagonistic.

* $p < 0.05$;

** $p < 0.01$ compared to 1.