A Structure-Function Relationship Exists for Ginsenosides in Reducing Cell Proliferation and Inducing Apoptosis in THP-1 Cells

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Abstract

Ginsenosides of 20(S)-protopanaxadiol and 20(S)-protopanaxatriol classification including the aglycones, PD, PT and ginsenosides Rh2, Rh1 were shown to posses characteristic effects on proliferation of THP-1 human leukaemia cells. A similar result was not apparent for ginsenoside Rg3 or dexamathasone. The concentration to inhibit 50% of cells (LC_{50}) for PD, Rh2, PT and Rh1 were 13 μg/mL, 15 μg/mL, 19 μg/mL and 210 μg/mL respectively. Cell cycle analysis showed apoptosis with PD and PT treatment of THP-1 cells resulting in a build up of sub-G1 cells after 24, 48 and 72 hours of treatment. Rh2, and dexamathasone treatments also increased apoptotic cells after 24 hours, where as Rh1 did not. After 48 and 72 hours Rh2, Rh1 and dexamathasone similarly increased apoptosis, but these effects were significantly ($P<0.05$) lower than observed for both PD and PT treatments. Furthermore, treatments that produced the largest build up of apoptotic cells were also found to have the largest release of lactate dehydrogenase (LDH). It can be concluded from these studies that the presence of sugars to PD and PT aglycone structure reduces the potency to induce apoptosis, and alternately alter membrane integrity. These cytotoxic effects to THP-1 cells were different from dexamethasone.

Introduction

Ginseng (*Panax ginseng*, C.A. Meyer) has a long history of traditional medicinal usage as a general tonic to promote health [1]. Ginseng is increasingly being employed as herbal supplement and for more contemporary neutraceutical or function food usages. It is generally believed that the major bioactive components of ginseng are the triterpene saponins, also referred to as ginsenosides [3]. Over thirty-one different ginsenosides have been isolated and structures determined [1], [5].

Ginsenosides share a similar basic structure, consisting of gonane steroid nucleus having 17

carbon atoms arranged in four rings. Differences in ginsenoside structure which include the type, position and number of sugar moieties attached by a glycosidic bond at positions C-3 and C-6 of the rings can characteristically influence biological response [6-8]. Based on these structural differences, two main categories of ginsenosides exist, the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol classifications. Specifically, from the 20(S)-protopanaxadiol group ginsenosides Rg3 and Rh2 have been reported to posses bioactivity in cell culture experiments and can alter cancer cell proliferation, induce apoptosis and perturb normal cell cycle events [8-11]. Many ginsenosides are thought, based on structural similarities to steroids to interact with plasma membranes [12], possess some steroid-like activity [13] and may be a functional ligand of the glucocorticoid receptor [14].

The purpose of this study was to test the hypothesis that the relative bioactivity of related ginsenosides derived from the 20(S)-protopanaxadiol and 20(S)-protopanaxatriol families will decrease as the glycosidic attachment of sugar moieties located on position C-3 and C-6 of the gonane structure increases. We examined a possible structure-function relationship for cell proliferation and apoptosis in cultured human (THP-1) cells.

Materials and methods

Test compounds and materials

Five ginsenoside standards were obtained from Bioherb (Changchun, China) and purity confirmed by HPLC. The 20(S)-protopanaxadiol and 20(S)-protopanaxatriol families. which includes the 20(S)-protopanaxadiol aglycone (PD), ginsenoside-Rh2 (Rh2), ginsenoside-Rg3 (Rg3) and the 20(S)-protopanaxatriol aglycone (PT) and ginsenoside-Rh1 (Rh1) are shown in Figure 1. Dexamethasone (Dex) was obtained from Sigma (St. Louis, MO). The test compounds were initially dissolved in 70% ethanol and subsequently diluted with culture medium to the final concentration prior to use. Unless stated, all test materials were obtained from Sigma (St. Louis, MO).

Cell culture

Human acute monocytic leukaemia suspension cell line (THP-1) was obtained from ATCC (Manassas, Virgina). Cells were maintained in RPMI 1640 medium supplemented with HEPES (10 mM), fetal bovine serum (10%) (Gibco, Grand Island, NY), 2-mercaptoethanol (0.05 mM), penicillin (100 U) and streptomycin (100 μg/mL) (Gibco, Grand Island, NY). Cells were subcul-

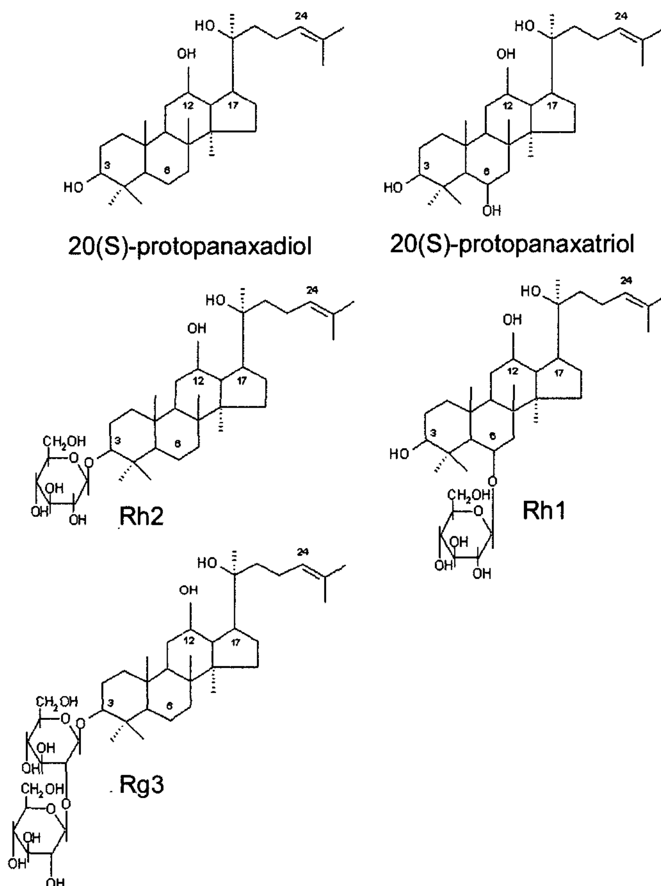

Fig. 1. The structure of test compounds 20 (S)-protopanaxadiol aglycone (PD), ginsenosides Rh_2, Rg_3 and 20 (S)-protopanaxatiol aglycone (PT) and Rh_1.

tured by total medium replacement using centrifugation every 5 days depending on cell number and incubated at 37°C in a 5% CO_2 humidified incubator. Viable cells and cell count were assessed by trypan blue (0.04%) exclusion dye using a hemocytometer. Viable cell numbers were assessed in quadruplicate.

Cell viability MTT assay dose response

A cell viability assay, based on MTT (3-(4,5-dimethylthiozol-2-yl)-2,5-diphenyl tetrazolium bromide) reduction by viable cells, was used in order to establish an LC_{50} (e.g. concentration to inhibit 50 % of cells). Fifty ml of RPMI 1640 medium was added to each well of a 96 microwell plate. Test

compounds (PD, Rh2, Rg3, PT, Rh1, Dex) were serial diluted by mixing 50 μl with 50 μl medium and in a 2-fold dilution transferred to adjacent wells. THP-1 cells were seeded to each well to a final concentration of 5×10^5 cells/mL. Controls contained test model cells, culture medium but no test compounds. Cells were incubated for 3 days before MTT was added to a concentration of 0.5 mg/mL and incubated in the dark for 4 hours as first described by Mosmann [15]. The optical density was read at 550 nm absorbance in a microplate reader (Biorad, Cambridge, MS).

Cell LDH activity

THP-1 cells were seeded at a concentration of 1×10^6 cell/mL in 24 well plates. The test compounds (PD, Rh2, PT, Rh1, and Dex) were added to the wells to stimulate THP-1 cells at LC_{50} determined previously from MTT assays; the concentrations used were 13, 15, 19, 210 and 13 μg/mL respectively. Untreated cells acted as control. Cells were incubated at 37°C in a 5% CO_2 humidified incubator for 24, 48 and 72 hours. Cell-free supernatant was obtained by centrifugation (400×g) for 10 minutes. Two millilitres of Tris-EDTA-NADH buffer and 50 μL of cell-free supernatant were mixed and incubated in a 37°C water bath for 10 min and 200 μL of pre-warmed (37°C) pyruvate solution was added as outline in [16]. The mixture was transferred to a 3 mL cuvet and the initial reaction velocity was recorded by continuous monitoring of the absorption at 340 hm at 37°C (Schimadzu Corp, UV-160 Spectrophometer, Kyto Japan).

Flow cytometry cell cycle analysis

PD, Rh2, PT, Rh1 and Dex were added to THP-1 cells (1×10^6 cells/mL) at LC_{50}. Cells were incubated at 37°C in a 5% CO_2 humidified incubator for 24, 48, and 72 hours with the untreated cells acting as a control. After treatment, cells in suspension were centrifuged for 10 min (400× g). The supernatant was discarded and cells were further washed twice in PBS and re-centrifuged to remove the cell pellet. The pellet was vortexed vigorously and 1 mL of ice-cold 70% ethanol was added slowly and fixed overnight at 4°C. Ethanol was removed by centrifugation (10 min, 300×g) and gently vortexed followed by the addition of 1 mL of PBS containing propidium iodide (50 μg/mL) and RNase A (100 μg/mL). Samples were incubated at room temperature for 1 hour and analysed by FACscan flow cytometry (Becton-Dickinson, Mountain View, CA) as outlined in [18]. Flow cytometry data were analysed using WINMDI software package (La Jolla, California).

Statistical analysis

Oneway ANOVA (SPSS release 9.0) was used to analyse the experimental data at 24, 48 and 72 hour time periods. Significance was judged at $P< 0.05$ using Tukey post hoc multiple comparisons of observed means.

Results

Dose response LC_{50} determination

Figure 2 shows the different concentration response effects of test compounds PD, Rh2, Rg3 (bottom panel) and PT, Rh1 and Dex (top panel) on the proliferation of THP-1 cells. The 20(S)-protopanaxadiol family of compounds were determined to have an LC_{50} of 13 μg/mL for the PD

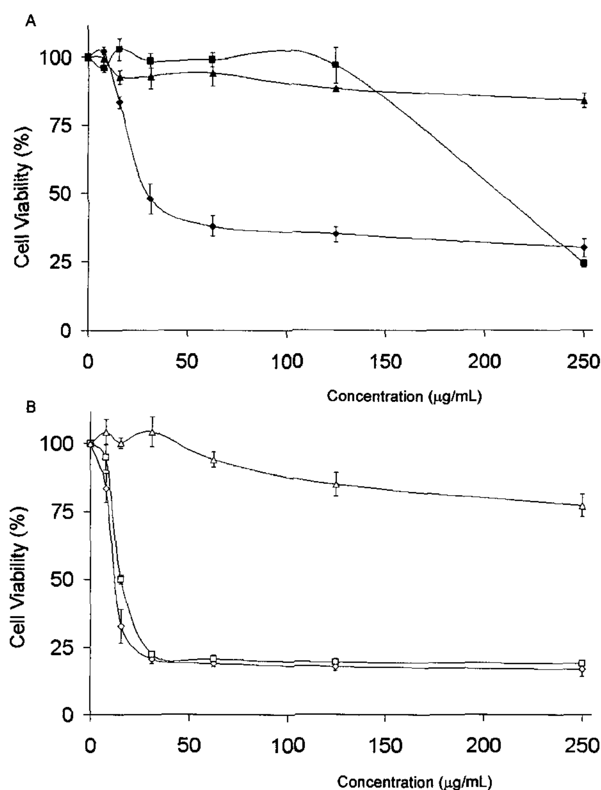

Fig. 2. Dose response relationship between test compounds (PT, Rh_1, Dex and PD, Rh_2, Rg_3), in THP-1 cells after three days measured in triplicate by MTT viability assay as outlined in the material and methods. Figure labels corresponding to the top panel (A) are represented by PT (◆), Rh_1 (■), and Dex (▲). The bottom panel (B) labels are represented by PD (◇), Rh_2 (□), Rg_3 (△). Values are expressed as a percentage of untreated cells (mean±SD).

aglycone, followed by 15 μg/mL for Rh2, and Rg3 had no inhibitory effect of proliferation of the cells at concentrations greater than 250 μg/mL. The 20(S)-protopanaxtriol classification of compounds were determined to have an LC_{50} of 19 μg/mL for the PT aglycone followed by Rh1 at 210 μg/mL. Dexamethasone (Dex) had no effect on proliferation of the THP-1 cells at concentrations greater than 250 μg/mL.

LDH activity

To assess the effect of PD, Rh2, PT, Rh1 and Dex on membrane integrity, lactate dehydrogenase (LDH) activity was measured in cultured THP-1 supernatant after exposure for 24, 48 and 72 hours (Figure 3). After 24 hours, the percentage of untreated cell LDH activity for PT treatment was significantly (P<0.05) higher (194±3% of untreated cells) compared to other test samples. Rh2 was found to be 113±8 percent, followed by PD (112±21%), Rh1 (107±3%) and Dex (100±5%). After 48 hours of treatment, PT was significantly (P<0.05) higher (206±18%) than all test samples followed by PD (143±7%) which was inturn significantly (P<0.05) higher than Dex (111±9%) and Rh2 (106±3%) respectively, but not Rh1 (121±9%). After 72 hours, both PT (196±7%) and PD (199±31%) treatment were significantly (P<0.05) higher than Rh1 (131±12%), Dex (126±13%) and Rh2 (115±8%) treatments.

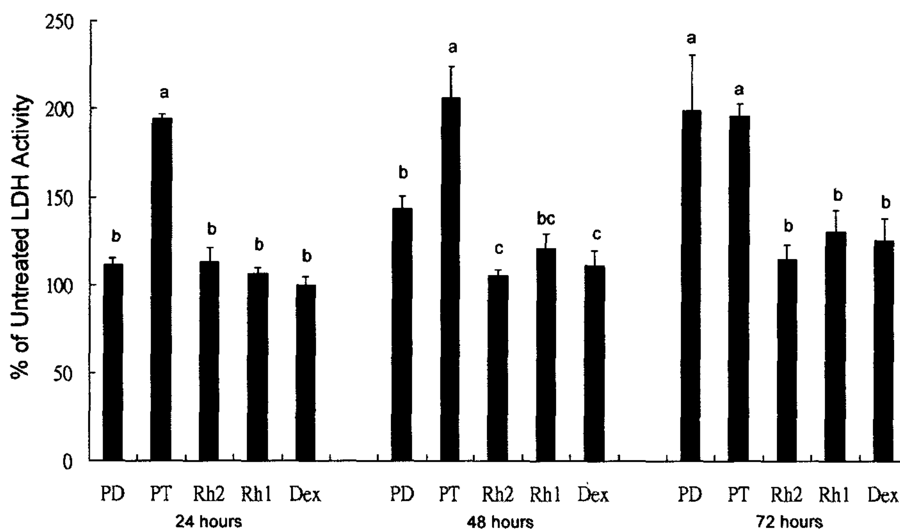

Fig. 3. Lactate dehydrogenase (LDH) activity was measured in THP-1 cells after 24, 48 and 72 hours of treatmentat at LC_{50} determined by MTT assay. LDH activity was determined in cell-free supernatant as outlined in the material and methods. Data is expressed as percentage of untreated samples (mean ± SD) of 3 separate experiments performed in triplicate. Bars within the same time period possessing different letters are significantly different (P< 0.05).

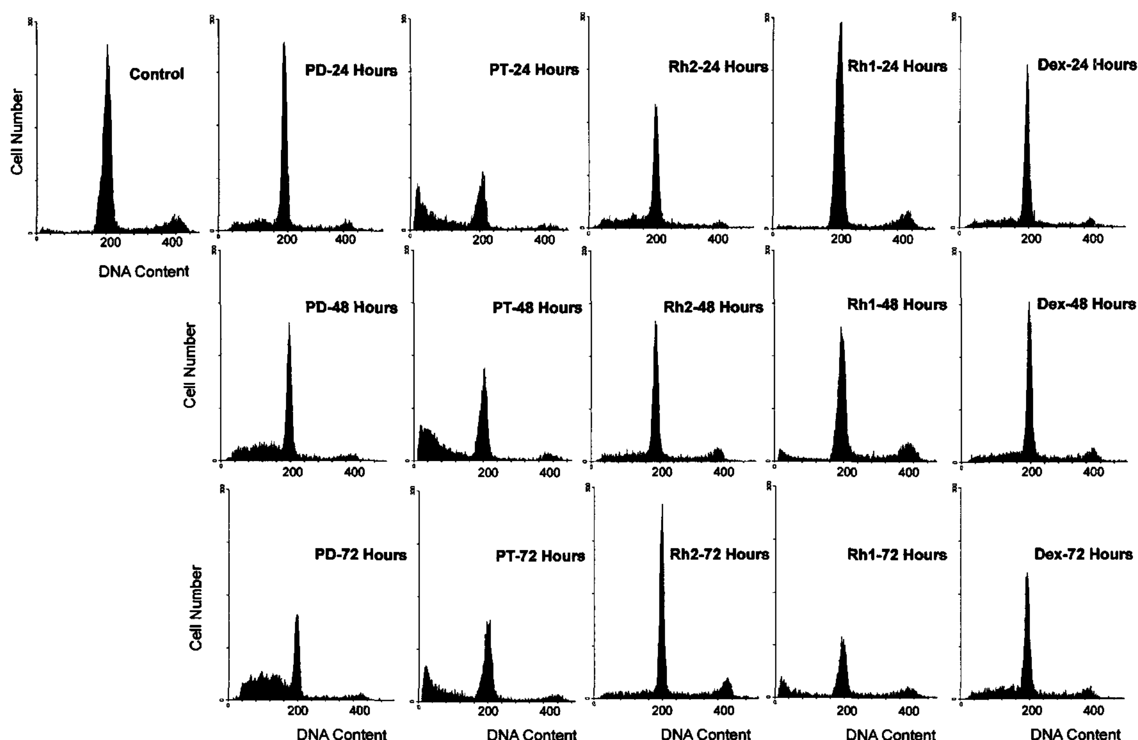

Fig. 4. DNA cell cycle histograms of untreated, PD, PT, Rh_2, Rh_1 and Dex treated cells for 24, 48 and 72 hours. Cells were fixed in ethanol and stained with PI as described in the material and methods. DNA histograms shown are representative histograms of three separate experiments.

Cell cycle analysis

The effect of PD, Rh2, PT, Rh1 and Dex on THP-1 cell cycle events measured using flow cytometry showed that these test compounds produced different effects represented by DNA histograms (Figure 4). After 24 hours, PT treatment produced a significantly ($P<0.05$) higher production of apoptotic cells (sub-G1) (42±3%), compared to untreated cells (2±0.1%) and other treatments at respective LC_{50s} (Figure 5). PD (20±2%), Rh2 (22±5%) and Dex (15±5%) treatments were also significantly ($P<0.05$) higher than untreated cells but Rh1 (4±1) treatment had no effect. After 48 hours, both PT (40±2%) and PD (34±3%) treatments were significantly ($P<0.05$) higher than untreated cells, followed by Rh2 (20±1%), Dex (18±2%) and Rh1 (10±3%). After 72 hours, PD (44±8%) was found to have the highest build up of apoptotic cells followed by PT (30±2%), Dex (22±2%), Rh2 (18±4%) and Rh1 (15±1%), all of which were significantly ($P<0.05$) higher than untreated cells.

Discussion

In this study we have shown that the concentrations of ginsenosides that inhibit proliferation of cultured THP-1 cells varies. For the 20(S)-protopanaxadiol family of ginsenosides, PD, the aglycone, was found to have the strongest effect relative to Rh2 whereas, Rg3 had no effect on cell proliferation. A similar finding was found for the agylcone PT relative to the effect on proliferation compared to Rh1. These results indicate that specific differences in ginsenoside chemical structure will influence the cytotoxic properties and proliferation of THP-1 cells. The THP-1 cell line was chosen as a model cell system with a range of similar properties to human monocytes and macrophages [19-22].

Ginsenosides are characterised according to the number and position of sugar moieties on the sterol chemical structure (Figure 1). Rh2 of the 20(S)-protopanaxadiol classification differs from the structure of the PD by the addition of one glucose moiety at position C-3 and Rg3 has the addition of two glucose moieties at the same position. Rh1 of the 20-protopanaxatriol family differs from PT by the addition of one glucose moiety at C-6 (Figure 1). Rh2 has been shown to suppress proliferation in a number of human cancer cells including, breast, prostate, hepatic, intestinal and animal cell lines [10-11], [22-23]. Rh2 and other ginsenosides have also been reported to act on specific membrane proteins or penetrate the plasma membrane and initiate genomic effects in addition to behaving as steroid hormones, and potent signalling molecules [12]. Much less is known about the 20(S)- protopanaxtriol family of ginsenosides. Rh1 has been reported to inhibit proliferation of NIH 3T3 mouse fibroblast cell line [6], but did not influence growth of B16 melanoma cells [7]. Our results indicate that PD, Rh2, PT and Rh1 had a substantially stronger effect at inhibiting proliferation of cancer cells than Dexamethasone (Dex). Dex, a synthetic glucocorticoid was chosen in this study to compare the effects of ginsenosides to steroids.

In the present study characteristic effects of specific ginsenosides on cell proliferation was achieved by examining the affinity of test compound to induce apoptotic. Apoptosis was effectively measured by cell cycle analysis using flow cytometry. In cell cycle analysis, the sub-G1 build up of cells is characteristic of apoptosis [24]. There are many reports linking the induction of apoptosis by Rh2. For example, Rh2 has been reported to activate caspase-3 protease, a major pro-enzyme involved in apoptosis [11]. Rh2-induced apoptosis has also been described to active cyclin A-associated-cyclin-dependent kinase 2 (cyclin A-Cdk2) by $p21^{WAF1/CIP1}$ in SK-HEP-1 cells (hepatoma cells) [26] and is important in the G1/S phase DNA-damage checkpoint control machinery.

In MCF-7 human breast cancer cells, Rh2 inhibits growth in an irreversible, concentration dependant manner, while also inducing a G1 arrest in the cell cycle, and an up-regulation of the expression of Cdk inhibitor $p21^{WAF1/CIP1}$ that results in reduced protein levels of cyclin D [23].

Both PD and PT treatments were found to have varied effects on apoptosis measured by flow cytometry. This procedure enabled us to determine that PT treatment produced the largest increase in the percentage of cells in the sub-G1 phase of the cell cycle after 24 hours, whereas, PD treatment induced the largest build-up after 72 hours. Cell-cycle analysis indicated a build-up of sub-G1 cells for Rh2 and Dex after 24 hours, and Rh2, Rh1 and Dex after 48 and 72 hours, respectively. The synthetic glucocorticoid Dex, which is known to induce apoptosis [25], was found to have a smaller increased of cells in sub-G1 phase of the cell cycle, compared to PD after 48 and 72 hours and PT after 24 and 48 hours, respectively. Both PD and PT affected cell cycle and induce apoptosis to a greater extent than dexamethasone. This finding would suggest that PD and PT bioactivity occurs independent of steroid activity, a popular suggestion that exists in the literature [13-14].

It is noteworthy that PD significantly increased the percentage of untreated cell LDH activity, when compared to Rh2 after 48 and 72 hours of treatment. In contrast, PT treatment resulted in greater increase in THP-1 LDH activity compared to Rh1 for all treatment periods. LDH, a stable enzyme present in all cells and found only in the cytoplasm [28], is released when the cell membrane is damage and a useful marker of membrane integrity. The fact that aglycones (PD and PT) affect cell membrane function in possible different ways compared to corresponding ginsenoside compounds Rh2 and Rh1, strongly indicates that these bioactivities are specifically related to an effect on apoptosis. This suggestion was confirmed here in with findings that showed treatments that produced the largest release of LDH activity, also exhibited the greatest build-up of apoptotic cells. Ginsenosides are known to pass directly into the nucleus or alternatively embed into the cell membrane [7], [29]. Our findings therefore suggest the possibility of an increased permeability of THP-1 cell membranes by PD and PT treatment in a manner reported for phytosterols [30]. For example, the increase in cell permeability may be explained, in part, by the different hydrophobic properties of individual test ginsenosides and thus preferential uptake in cell membrane in place of endogenous sterol.

To our knowledge, these findings are the first to show that the PD agylcone of Rh2 and Rg3 and the PT aglycone of Rh1 have a relatively stronger affinity to induce apoptosis than other structurally related ginsenosides. These differences cannot be compared to the known effects of

dexamethasone. Moreover, differences between specific ginsenosides reported herein are suggested to be related to the structural conformation and presence of sugars at position C-3 and C-6 which contribute to unique differences and will influence the hydrophobic character of the compounds required to interact with cell membrane function. Future studies are needed to determine if the relative ginsenoside composition of different varieties of ginseng constitute sufficient advantage for selection of use in the development of novel neutraceutical products.

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