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Chapter

How Do Extraction Methods and Biotechnology Influence Our Understanding and Usages of Ginsenosides?: A Critical View and Perspectives

Christophe Hano, Duangjai Tungmunnithum, Samantha Drouet, Mohamed Addi, Saikat Gantait and Jen-Tsung Chen

Abstract

Ginseng saponins, aka ginsenosides, are bioactive phytochemicals from Panax species. Panax comes from the Greek word “panakos,” which means “cure-all.” Owing to their involvement in the creation of numerous medications and nutritional supplements, ginseng saponins play an essential part, especially in the pharmaceutical sector. The main ginsenosides (i.e., Rb1, Rb2, Rc, Rd and Rf) are extracted using a variety of extraction methods, although from a limited number of Panax species. However, more than ca 1000 unique ginsenosides and 18 Panax species have been reported so far, thus demonstrating our present challenge in better understanding of the potential medicinal uses of these compounds. Moreover, ginsenoside production and extraction methods are both time-consuming and inefficient, which has stimulated the development of several efficient extraction and biotechnological technologies to speed up these processes. In this chapter, we highlighted the need to expand the cutting-edge research approaches involving these unique ginsenosides to better understand their biological activities and discover new bioactive ginsenosides as well. The main objective of this chapter is to discuss the undiscovered aspects and limitations of the current biotechnological and extraction technologies, eventually to provide a platform for the production of these unique ginsenosides.

Keywords: biotechnology, extraction methods, ginsenosides, Panax, pharmaceutical applications

1. Introduction

The word “ginseng” refers to products that are derived from Panax species and relates to the “man-like” form of the root [1]. Panax ginseng C. A. Meyer (Araliaceae), sometimes known as Asian, Chinese, or Korean ginseng, and Panax
*Panax quinquefolius* L., often known as American or North American ginseng, are the two most well-known ginseng species [2], but a total of 18 plant species, including infraspecific taxa, have been already identified as *Panax* members worldwide [3]. *Panax* is derived from the Greek word “panakos,” which means “all-healing” or “cure-all,” which was first coined by Russian botanist Carl A. Meyer [4]. Thus, the herb ginseng has been used in various traditional medicinal remedies for over 5000 years [4]. Although, different species and parts of ginseng plants have distinct uses in traditional medicine preparations, the root is the most widely used medicinal component of the plant, and saponins are the principal active elements in most of them [5]. Preparations of ginseng dried roots are used to treat hyperglycemia, cardiovascular disease, cancer, and insomnia due to its beneficial biological properties [6–8]. Ginseng is also used as a tonic or adaptogenic supplement that helps to restore biological functions, improve physical performance, and boost tolerance to several stresses [1, 4].

The main bioactive ingredients of *P. ginseng* are a series of tetracyclic triterpenoid saponins also called ginsenosides. In recent years, many excellent reviews on ginsenosides have been published, focusing on structures or bioactivities [1, 8, 9], isolation and analysis [10–13], and metabolic regulation [14–18], thus evidencing our ever-increasing understanding of all these aspects of this thousand-year-old medicinal plant family. However, it is important to keep in mind that the ginsenoside contents greatly vary depending on the species, organs, growing season, and producing location, which implies that their pharmacological properties widely differ as well. Therefore, some important considerations should not be overlooked to continue to improve our understanding:

1. The majority of study relies on the use of *Panax* extracts, which provide less information regarding their ginsenosides compositions. However, it is important to keep in mind that the phytochemicals accumulated in greater abundance are not necessarily the most active ones.

2. Despite certain similarities, the quantities and the composition of ginsenosides widely differ depending on the *Panax* species, organs, growing season, and producing location. Different ecotypes/natural populations of the same *Panax* species may have substantially different phytochemical profiles.

3. The use of different extraction procedures may generate different types and quantities of ginsenosides (even from the same starting materials). Ginsenosides are categorized based on their polarity, although most studies look at only one kind of solvent, which leads to the production of extracts with very similar ginsenoside compositions. As a result, our ability to discover novel bioactive ginsenosides and/or biological activity is severely limited.

4. Traditional plant propagation takes around six years and is inconvenient for proper industrial production, which in turn has led to the involvement of biotechnological approaches, notably *in vitro* culture, to provide fast and continuous access to bioactive *Panax* extracts. However, the phytochemical profiles of these *in vitro* cultures, might significantly differ, both in terms of ginsenoside quantities and compositions, from those of the initial explants.

Although, all of these differences in ginsenoside contents and compositions may appear to be disadvantageous or anecdotal, we have decided to highlight them in this chapter to emphasize that they may, on the contrary, be an asset to our understanding of ginsenoside biological activity and discovery of new bioactive ginsenosides.
2. A Tour d’Horizon of the Ginsenosides chemical diversity

Initially, the term “ginsenosides” was used to design a series of tetracyclic triterpenoid saponins from *P. ginseng*. According to different aglycones, triterpenoid saponins may be classified into tetracyclic triterpene saponins (e.g., dammarane-type saponins, DAMS) and pentacyclic triterpene saponins (e.g., oleanolic-type (OT) and ocotillol-type (OA) saponins). The main aglycones of which are protopanaxadiol (PPD), protopanaxatriol (PPT), oleanolic acid, and ocotillol [1, 11]. A variety of saponins are biosynthesized with different types of glycosides groups and/or linkage orders. DAMS, such as PPTs and PPDs, generally contain 1 to 4 glycosyl groups linked with the aglycone structure. Sugar chains are usually linked to the C3 or C4 position of the aglycone in PPD type saponins, whereas regularly linked to the C6 or C20 position in PPT type saponins.

The ginsenoside chemical annotation is ‘Rx’, where ‘R’ stands for root and ‘x’ stands for chromatographic polarity in alphabetical order. To date, the Rb (protopanaxadiols) and the Rg groups (protopanaxatriols) are the most studied ones (Figure 1). With the availability of commercial standards, these ginsenosides Rb1, Rb2, Rc, and Rd from the Rb group (or PPD), and the ginsenosides Rg1, Rg2, Re, and Rf from the Rg group (or PPT) (Figure 1) were more readily analyzed during the extraction procedure, thus leading in greater available information on their biological activity.

Panax plant phytochemistry has been investigated since the mid-nineteenth century, mostly with *P. ginseng* or *P. quinquefolius* as starting materials. Samuel S. Garrigues isolated the first ginsenoside, “panaquilon,” from *P. quinquefolius* roots in 1854 [19]. Due to the renewed interest in natural compounds and traditional medicines, various unique *Panax* species, such as *P. vietnamensis* Ha et Grushv. and *P. sokpayensis*, have piqued the interest of many phytochemists since 1970s [20, 21]. Between 1970 and 2000, owing to the development and democratization in laboratory techniques such as two-dimensional nuclear magnetic resonance (2D NMR) or quadrupole time of flight mass spectrometry (Q-TOF-MS) that were employed to detect *Panax* chemical components and clarify stereo configurations, it was found that the structures of many saponin compounds mostly belonged to C17 side-chain that varied for both PPD- and PPT-type ginsenosides [22–26]. But since the 2000s, an impressive and growing number of new saponins have been studied. This phenomenon is greatly credited to the use of advanced analytical techniques and the renewed interest in natural compounds from many parts of the world. The annotation of ginsenosides at this time is much more diverse and complex than that used in the earlier era. This report aimed to provide a snapshot of the current state of the ginsenoside field, from the molecular level to the plant extracts, and to assist chemists and researchers interested in this area. The overview might expand the understanding of ginsenosides and stimulate discoveries in this field.

![Figure 1](image-url)

*Figure 1. Chemical structure of Panax species’ common protopanaxadiol (PPD or Rb) and protopanaxatriol (PPT or Rg) ginsenosides.*
discovered owing to the significant advances in chromatography, spectroscopy and mass spectrometry methods that allow rapid and efficient screening of natural Panax products. The work of Yao et al. [27] perfectly illustrated this impressive progress by resolving 945 ginsenosides from the leaves of *P. notoginseng* by using two-dimensional liquid chromatography (2D-LC) separation technology, based on high-performance liquid chromatography coupled with high-resolution mass spectrometry (HPLC-HRMS) platform, 662 of which were novel.

From this brief historical background, it is indisputable that the emergence of more efficient analytical approaches has substantially improved our understanding of the chemical variety of ginsenosides. Here are some additional important observations on the chemical diversity to be taken into account for future development (beginning with the most well-known compounds):

1. There are 94 PPD-type ginsenosides known to date. Their sugar moieties are linked to the C3 and/or C20 position(s) (e.g., Rb1, Rb2, Rc, and Rd, Figure 1), while acylation, particularly of the 6-OH function of the C3 glucose, has been reported. It is now undeniable that acylation is a key source of new structures in PPDs, and it deserves more attention both from chemical and biological perspectives.

2. There are 93 PPT-type ginsenosides described. Typically, the sugar moieties are linked to the ring at the C6 position (e.g., Rg1, Re, and Rg5, Figure 1), and possibly at the C20 position. In addition, some other interesting substitutions have been reported as well. For instance, the direct substitution of malonyl or acetyl at the C-6' or C-3 positions appeared to boost antiproliferative activity [1], or the presence of an olefine acid ester group and acetyl at the C-6 position has been linked to significant inhibition of antimycin A-induced mitochondrial oxidative stress [28]. These two examples highlight the necessity of focusing on new structures and extended structure-function studies to increase our current understanding of PPT ginsenosides' pharmaceutical potential.

3. The 34 OA- and 23 OT-type ginsenosides forms a minor group of ginsenosides. The main OA-type ginsenoside Ro is thought to be biosynthesized from oleanolic acid, and was initially identified in trace amounts in *P. ginseng*. OT-type ginsenosides are tetracyclic triterpene saponins with a furan ring on the side chain as described in *P. pseudoginseng*, *P. quinquefolius*, *P. vietnamensis* and *P. japonicus* extracts. Further research on these chemicals is required to characterize their medicinal potentials. In addition, the chemotaxonomic and authentication potentials of these OA and OT compounds should be further explored.

4. Around 220 saponins with C-17 varied side chain (including lupane-triterpenes) and 53 others structural saponins (465–516) have been reported to date. Some lupane-triterpene compounds have demonstrated effective anti-inflammatory activity acting on various targets (inhibition of cyclooxygenase-2 (COX2), decrease in cellular NO synthase (iNOS) concentrations) [29]. This also emphasizes the importance of exploring even the “minor” (in accumulation concentrations) ginsenoside structures for future pharmaceutical product development.
3. A critical evaluation of the analytical procedures used in the extraction of ginsenosides

Over the last decades, different extraction procedures were studied and have yielded different kinds and quantities of ginsenosides. A recent review has comprehensively compiled the different results obtained [11]. So far, the bulk of studies to far have focused on two Panax species (P. ginseng and P. quinquefolius) and a small number of ginsenosides (mainly the Rb and Rg type ginsenosides). Here, we combined the various processes utilized to extract these main ginsenosides from the roots of P. ginseng and P. quinquefolius so that we could compare the results and draw conclusions as well as future directions (Table 1).

This review of the literature found a wide range of variations in the ginsenoside extraction yields [from 1.0 [51] to 79.5 mg/g DW [36], with no extraction approach appearing to be ideal. However, given our previous observations on the variability of these ginsenoside contents (which varied greatly depending on the species, organs, growing season, and production location), and the fact that the majority of these studies did not use different types of starting material to eliminate these variabilities associated with this material, it is difficult to draw firm conclusions from these data. Nevertheless, based on the critical analysis of Table 1, the following critical observations can be made:

1. Traditional extraction methods such as Soxhlet and heat reflux (Figure 2) yielded a wide range of ginsenoside content in P. ginseng and P. quinquefolius, depending on the solvent, extraction time, and sample preparation. High ginsenoside content can be achieved, but at the expense of a long extraction time and/or a high temperature, both of which are costly in terms of energy use.

2. The use of modern extraction methods (ultrasound, microwave, high pressure) (Figure 2) can be a good alternative to these traditional extraction procedures, since they are less time-consuming, need less solvent, are readily automated, and result in higher extraction yields. Extraction using high pressure or pressurized liquid (e.g., accelerated solvent extraction or pressurized fluid extraction) dramatically enhanced extraction yields and considerably reduced extraction time.

3. There were no consistent direct proportional connections found between ginsenoside extraction yield and extraction parameters (solvent concentration, extraction pressure, and extraction time).

4. However, it appeared that the ginsenoside extraction yield can be greatly influenced by solvent use. However, it was notable that only a restricted number of solvents with very similar polarity were evaluated. Given the vast range of variations that were observed for the various ginsenosides (especially for the less studied ones), more solvents should be investigated in the future. Natural deep eutectic solvents, for instance, may have a greater range of extraction capability and might be a good option to investigate a wider range of ginsenosides, as recently demonstrated by Liu et al. [53].

5. In this context, supercritical carbon dioxide extraction (Figure 2) emerged as an appealing approach for studying less polar ginsenosides. However, for compounds with higher polarity, the polarity of the fluid phase must be
### Ginseng - Modern Aspects of the Famed Traditional Medicine

<table>
<thead>
<tr>
<th>Species</th>
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<th>Duration (min)</th>
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<td></td>
<td></td>
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<td>70°C</td>
<td>26.1</td>
<td>[46]</td>
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P. ginseng and P. quinquefolius. The relative concentrations of the widely investigated Rb and Rg ginsenosides were shown by the colors (blue = low, red = high, orange = detected but not quantified), and the resulting total ginsenosides content was given in the column “Total.”

<table>
<thead>
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<th>Microwave</th>
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<td>70% ETOH</td>
<td>15</td>
<td></td>
<td>[34]</td>
</tr>
</tbody>
</table>

| Pressurized Liquid | Water | 30 | 110°C, 0.4Mpa | 11.2 | [47] |
|                   | 70% ETOH | 5 | 150°C, 6.7Mpa | 8.7 | [42] |
|                   | 50% MeOH | 25 | 120°C, 10Mpa | 26.0 | [46] |
|                   | 100% MeOH | 15 | 150°C, 10.3Mpa | 58.9 | [49] |
|                   | 100% MeOH | 15 | 150°C, 6.9Mpa | 62.2 | [50] |
|                   | 100% MeOH | 15 | 150°C, 6.9Mpa | 18.6 | [50] |
|                   | 100% MeOH | 20 | 140°C, 3Mpa | 12.9 | [37] |
|                   | 100% MeOH | 20 | 140°C, 3Mpa | 39.2 | [37] |

| Supercritical Fluid | 100% CO₂ | 240 |       | 1.0 | [51] |
|                     | 100% CO₂ | 240 | 45°C, 24Mpa | 23.2 | [41] |
|                     | 100% CO₂ | 240 | 40°C, 30Mpa | 3.2 | [34] |
|                     | 100% CO₂ | 1200 | 110°C, 48.3Mpa | 71.8 | [38] |

| High-Pressure/Microwave | 70% ETOH | 10 | 0.4Mpa | 43.3 | [36] |
| High-Pressure/Microwave | 70% ETOH | 11 | 0.5Mpa | 49.4 | [32] |

| Ultrahigh-Pressure | Water | 5 | 25°C, 300Mpa | 23.8 | [45] |
|                    | 50% ETOH | 2 | 500Mpa | 73.3 | [41] |
|                    | 50% ETOH | 2 | 25°C, 200Mpa | 11.9 | [43] |
|                    | 70% ETOH | 5 | 25°C, 200Mpa | 43.9 | [33] |

Table 1. Variations in ginsenoside content were obtained using different extraction procedures from the two most frequently investigated Panax species [30–53].
increased during extraction with supercritical carbon dioxide and a small addition of polar modifiers like methanol, ethanol or DMSO.

6. In the future, more *Panax* species and ginsenoside structures should be investigated, particularly utilizing modern tools and techniques. In this context, for those ginsenosides that accumulated in lower concentrations, cutting-edge techniques such as macroporous resins or, more interestingly, magnetic analogue-imprinted polymers with high capacity and selectivity have been highlighted [54], should be considered.

4. A critical look at biotechnological interventions to ginsenoside bioproduction

The conventional approaches for ginsenoside pruning from natural populations or production using the classical agricultural systems can be time-consuming and/or not feasible, and thus it has paved the way for the development of various biotechnological approaches, which would ameliorate the productivity of ginsenosides. Plant tissue culture proved to be an important tool for the continuous production of bioactive compounds that are specialized metabolites in most of the instances. However, the notion of productivity is essential here, and it is far more significant than production. Naturally, secondary metabolites (*aka* specialized metabolites) are produced from primary metabolites (such as carbohydrates, lipids, and amino acids) that are required for plant growth and development. The key concern is that if the primary metabolites are involved too actively for the biosynthesis of a specific class of specialized metabolite, plant growth and development may deteriorate.
eventually. As a result, high productivity collectively defined as “biomass x production yield of bioactive specialized metabolite” is significantly more desirable for efficient and continuous output of specialized metabolite at the industrial level.

For this purpose, in vitro systems (Figure 3a) for various plant species have been developed over the last decades, including undifferentiated cell cultures like a callus and cell suspension, as well as differentiated organ cultures like adventitious root and hairy root, with widely disparate results in terms of biomass production and/or ginsenoside accumulation, as recently reviewed by Gantait et al. [18].

The most notable results of these various plant biotechnology techniques are critically reviewed below, along with some perspectives:

1. During a critical evaluation of the analytical procedures developed for the extraction of ginsenosides, we observed that only a limited number of Panax species, as well as a small number of different ginsenosides, have been thoroughly investigated with the aid of biotechnological methods to date.

Figure 3.

A. Flowchart depicting the main biotechnology approaches that have been developed using the various plant in vitro culture systems; b. Comparison of the total ginsenoside production (GS) obtained using various plant in vitro culture systems (callus, cell suspension, adventitious root and hairy root compared to naturally grown rhizome). *production done in a bioreactor; DW: dry weight. Pg: P. ginseng; Pq: P. quinquefolius; Pn: P. notoginseng; Pv: P. vietnamensis [55–82].
2. The total ginsenoside contents greatly varied from more than 2 orders of magnitude (from 0.4 mg/g DW in P. notoginseng cell suspension culture [58] to 59.9 mg/g DW for P. ginseng adventitious root culture [67] as a function of the considered Panax species, but also the type of in vitro system and growing conditions (Figure 3b).

3. When comparing in vitro cell cultures (callus and/or cell suspension) to naturally-cultivated ginseng root, it can be shown that in vitro cultures (callus and/or cell suspension) yielded 6-times less ginsenosides [55]. However, in vitro cell cultures can product massive and continuous biomass. Cell suspensions are more promising option than callus cultures for this purpose owing to their possibility to be scaled-up in a bioreactor. In addition, cell suspensions are obtained from a single cell or a small number of more genetically homogenous cells, hence they are commonly more stable in terms of growth and metabolite production capacities than callus cultures. On the other hand, callus cultures are usually a chimera of cells with very contrasting genetic profiles, resulting in contrasting and unstable growth and/or production profiles. For all of these reasons, cell suspensions are preferred for production, although calli are subjects of interest from a fundamental viewpoint to explore a wide range of developmental phases and thereby uncovering unusual ginsenoside accumulation profiles, both quantitatively and qualitatively.

4. Differentiated in vitro root-derived cultures, particularly in the case of hairy root, are promising in vitro production systems both in terms of biomass and ginsenoside production yields. This might be because ginsenoside is produced naturally in the root and rhizome. Furthermore, both adventitious and hairy roots may be scaled up in a bioreactor [69–71, 80, 82].

5. The selection of fast-growing and high-producing lines is an essential preliminary step before considering large-scale stable production since these parameters are highly variable [75]. Screening a large number of both wild-type [56, 75] or mutant [77] lines verified this interest. It has been demonstrated to contribute in the identification of certain lines capable of producing a single ginsenoside at significant levels [75]. It should be underlined that starting from different genotypes, cultivars or populations for the initiation of in vitro cultures may be especially significant for this purpose and have been largely unexplored.

6. The composition of plant culture medium is a significant factor in both the growth and the production of specialized metabolites. Therefore, the cultural conditions must be adjusted. For example, the production of various ginsenosides has been demonstrated to be dependent on the growing culture phase, as evidenced by several PPD derivatives [59, 78]. This is especially true for bioreactor production for which oxygenation is essential [71].

7. Elicitation strategies can have a significant impact on total ginsenoside yields. In particular, jasmonates (jasmonate, methyl-jasmonate, and 2-hydroxyethyl-jasmonate) have been extensively studied and found to enhance total ginsenoside production [57, 58, 76, 77, 80]. But, more interestingly, various jasmonate derivatives have been shown to preferentially stimulate PPD-type ginsenosides over PPT-type ginsenosides, indicating that it might be an
appealing option for redirecting ginsenoside biosynthesis [77]. It should be emphasized that elicitation has usually resulted in a reduction in growth [76].

8. Too infrequently, detailed metabolic investigation of the ginsenoside accumulation patterns has been investigated. The majority of investigations focused on total ginsenoside content or a limited number of ginsenosides. The study by Ha et al. [83] on the hairy roots of *P. vietnamensis* nicely demonstrated the benefits of in-depth LC-MS characterization for the discovery of unique accumulation patterns.

The current understanding of ginsenoside biosynthesis and regulation paves the way for metabolic engineering strategies to be developed [17]. For this purpose, in addition to the plant *in vitro* cultures, the microbial biosynthesis of ginsenosides from renewable resources may be a viable alternative technique for meeting the ever-increasing demand for ginsenosides in recent years [16]. Microbes have several benefits over plant cells, including the need for less area for growth, the ability to grow quickly with high cell density culture, the ability to regulate and describe genetics, and the ability to manipulate genetics. Yeasts, particularly *Saccharomyces cerevisiae*, are well-known as eukaryotic model organisms for the creation of high-value compounds with complex structures. In recent years, alternative approaches for ginsenoside production have been developed using the model yeast *Saccharomyces cerevisiae* and non-conventional yeasts such as *Yarrowia lipolytica* and *Pichia pastoris* [16].

5. Conclusions

“What you see is what you extract” remarked Y.H. Choi and R. Verpoorte [84]. This is especially true for ginsenosides. Most extraction methods continue to focus only on the major bioactive ginsenosides, although more holistic approaches to extraction-based research would substantially increase our understanding of the biological activities of this family of natural products. As critically discussed in the present chapter, ginsenosides may not have provided their full potential as medicinal resources due to a global lack of effective technologies for ginsenoside extraction and/or production.

The majority of the extraction procedures involve the most common bioactive components only (i.e., PPD-type ginsenosides: Rg3, Rb1, Rb2, Rc, and Rd; and PPT-type ginsenosides: Rg1, Re, and Rg5) from a limited number of *Panax* species (*P. ginseng* and *P. quinquefolius* mainly). On the contrary, some species, like *P. sokpayensis* and *P. stipuleanatus*, have received little attention. Additional bioactive components may be found using bioactivity-oriented separation methods. Further research will be needed to understand the molecular and cellular processes, toxicity using cellular and animal models, and clinical applications of less-studied ginsenosides. This would allow for more in-depth research of the structure-activity relationships of ginsenosides, which would provide important insights into the development of a *Panax* quality control method, based on faster and more accurate analytical procedures. In addition, the development of more effective holistic strategies vis-a-vis more specific targeted extraction procedures would go a long way toward ensuring that the *Panax* species continues to reveal new secrets. It is feasible to generate richer extracts through a more precise extraction strategy (for example, using NaDES combined with ultrasonic or high-pressure extraction) and then fractionate this extract with much more specific extraction methods for certain classes of ginsenosides (e.g., with bio-imprinted polymers).
Biotechnological production of different ginsenosides using in vitro cultures has not been thoroughly investigated to date, nor have quantitative analyses of less common ginsenosides been undertaken. Although, there have been several publications on cell suspension cultures and bioreactors, the use of elicitors has to be investigated more often, using omics technologies (metabolomics and transcriptomics) to provide full insight, since these compounds may have a substantial influence on ginsenoside biosynthesis. Recently, the microbial cell factory has been proposed as a source of the production of main ginsenosides, for which biosynthetic genes have been isolated. In this sense, plant and microbial biotechnology approaches are complementary: plant can reveal new structures, in particular, using elicitation coupled to omics studies and allow the identification of new genes that can then be used in both plant metabolic engineering or microbial synthetic biology approaches.

Panax species have been widely employed in traditional medicine and are known to have pharmaceutical uses. Ginsenosides have only recently been studied, owing to advances in analytical methods since the first comprehensive phytochemical descriptions in the 1970s. The current surge in the application of advanced technologies, such as HR-MS, has enabled the discovery of an increasing number of ginsenoside structures. These unique structures have not yet been explored due to their most recent discovery, and a lack of availability of adequate quantity.

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Conflict of interest

The authors declare no conflict of interest.
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