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ORIGINAL PAPER

Lipid-Soluble Extracts as the Main Source of Anticancer Activity in Ginseng and Ginseng Marc

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Abstract The anticancer activity of ginseng originated mainly from lipid-soluble components. The hexane extract of ginseng marc (HEGM) showed a potent inhibitory activity on human hepatoma (HepG2, $GI_{50} = 41.7$ μg/ml) and breast (MCF-7, $GI_{50} = 54.4$ μg/ml) cancer cell proliferation in vitro in a concentration-dependent manner as did the hexane extract of ginseng (HEG), with GI_{50} values of 21.1 μg/ml in HepG2 and 41.2 μg/ml in MCF-7. The water extract of ginseng (WEG) possessed a low anticancer activity against both cancer cell lines, but the hexane-soluble fraction of WEG (HSF/WEG) showed a potent anticancer activity against HepG2 ($GI_{50} = 38.7$ μg/ml) and MCF-7 cells ($GI_{50} = 51.1$ μg/ml). The hexane extraction in ginseng was a very promising protocol for the maximum recovery of the anticancer active components in high concentrations. Also the adoption of hexane extraction after water extraction of ginseng was successful in the effective utilization of the residual lipid-soluble anticancer active components in ginseng marc.

Keywords Anticancer activity · Lipid-soluble components · Hexane extraction · Ginseng · Ginseng marc

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Introduction

Ginseng (*Panax ginseng* C.A. Meyer) roots have been traditionally considered to be one of the most important herbal medicines with restorative, tonic, and prophylactic properties. They contain various biologically active compounds such as ginsenosides, acidic polysaccharides, polyacetylenes, phenolic compounds, peptides, and essential oils [1]. Most studies on pharmacological activities of ginseng have employed water-soluble components such as saponins (ginsenosides) and acidic polysaccharides. Ginsenosides with various biological activities including anticancer, anti-allergy, anti-inflammatory, antifatigue and immunomodulatory have been regarded as the major pharmacological components in ginseng root [2, 3], and acidic polysaccharides have been reported to possess antitumor, immunomodulating, hypoglycemic, and anticomplementary activities [4]. In addition to the water-soluble components of ginseng root, lipid-soluble components have been shown to play an important role in pharmacological activities by

displaying anticancer activity in vitro and in vivo. Polyacetylenes such as panaxynol, panaxydol, and panaxytriol have been reported to be responsible for this anticancer effect [5, 6].

In spite of the great potential of the lipid-soluble components, industrial production and commercial use of ginseng have concentrated on water-soluble extracts consisting primarily of ginsenosides and carbohydrates. The aqueous extract of ginseng, which is generally called "ginseng tonic," is the most important product in the ginseng industry. In general it has been produced by a conventional method via direct extraction of dried or wet ginseng in hot water or mixed solvent of water and ethanol because most saponins and acidic polysaccharides are soluble in aqueous conditions [7]. Since the total yield of crude saponins extracted from ginseng is hardly affected by the ethanol concentration in mixed solvent (ethanol/water) extraction, hot water extraction has been widely adopted for the conventional production of water-soluble ginseng extracts [8]. The water-insoluble ginseng marc (WIGM), a by-product of the manufacturing process for the production of water-soluble ginseng extracts, is discarded as a waste even though it still contains both the residual polysaccharides and the lipid-soluble components with biological activities that cannot be recovered by the conventional water extraction process. In all previous studies, the ginseng marc was exploited in feed and culture media or only applied to the recovery of residual polysaccharides with an immunomodulating activity [9].

It is important to develop a new sequential extraction process for the recovery of lipid-soluble bioactive components in water-insoluble ginseng marc. A sequential extraction process (SEP) is based on different solubilities of target molecules in various solvents, allowing them to be recovered in a series of progressive extraction steps [10]. An SEP of ginseng root including a series of water extraction steps and *n*-hexane extraction steps can be applied to recover almost all bioactive components including water-soluble ginsenosides and acidic polysaccharides, and lipid-soluble polyacetylenes. The lipid-soluble components with anticancer activity recovered from water-insoluble ginseng marc by SEP can be used as a value-added co-product and be applied to produce a multi-bioactive ginseng extract containing both water-soluble and lipid-soluble components.

In this study the anticancer activity of lipid-soluble extracts in ginseng marc as well as that of lipid-soluble extracts in an original ginseng root is demonstrated. We describe, herein, the production process for the maximum recovery of lipid-soluble components in a ginseng root, mainly focusing on SEP.

Experimental Procedures

Materials

Dried white ginseng roots (first grade, 6 years old) were purchased from Nonghyup Koreainsam (Jeungpyeong, Korea) and milled to a particle size of less than 400 μm. Human hepatoma (HepG2) and human breast cancer (MCF-7) cell lines were obtained from American Type Culture Collection (Manassa, VA, USA). RPMI-1640, fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were products of HyClone (Logan, UT, USA). Cell Counting Kit-8 was from Dojindo (Kumamoto, Japan). Other reagents were of analytical grade.

Aqueous Extraction of a Ginseng Root

Since it was reported that more than 94% of total saponins were recovered by carrying out four replicates of 8-h extractions at 80 °C [11], the aqueous extraction process with the same conditions was applied to extract the water-soluble components from a dried ginseng powder (Fig. 1a).

The first aqueous fraction was extracted by combining 100 g of ginseng with 900 ml of distilled water. This suspension was agitated at 80 °C for 8 h in a shaking waterbath and centrifuged at 2,890×*g* for 20 min. The first supernatant was collected, pellets were re-suspended in 800 ml of distilled water, and the extraction process was repeated. The second supernatant was collected through centrifugation, pellets were re-suspended in 500 ml of distilled water, and the extraction process was repeated. The third supernatant was collected through centrifugation, pellets were re-suspended in 500 ml of distilled water, and the extraction process was repeated. The forth supernatant was collected through centrifugation. The final pellet was the water-insoluble ginseng marc (WIGM). The WIGM was dried at 60 °C in a forced-air convection oven to 13 wt.% moisture before being extracted with hexane. The supernatants as a water extract of ginseng (WEG) were frozen at −70 °C and then lyophilized in a programmable freeze dryer (Ilshin Lab, Yangju, Korea) at −80 °C under a pressure of 8 mmTorr.

Hexane Extraction of Ginseng, Ginseng Marc, and Water-Soluble Extract of Ginseng

The experimental design for preparation of lipid-soluble fractions of ginseng is illustrated in Fig. 1. The hexane-soluble fraction in dried ginseng (HSF/DG) is identical to the hexane extract of ginseng (HEG). The hexane extract of ginseng marc (HEGM) represents the hexane-soluble fraction in WIGM (HSF/WIGM). WEG is divided into two

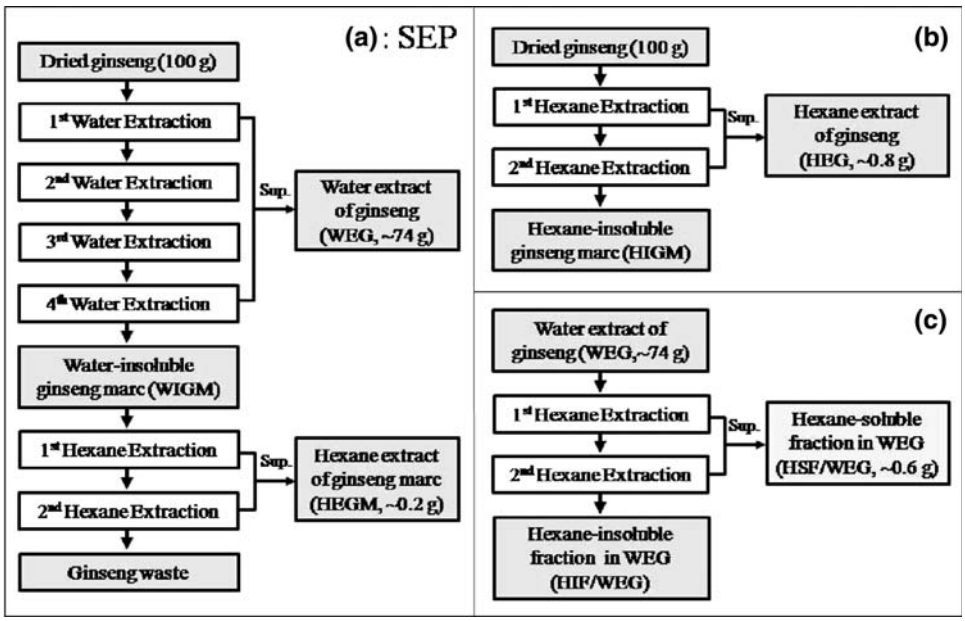

Fig. 1 Schematic diagrams depicting the recovery process for extraction of bioactive components in ginseng. **a** The sequential extraction process (SEP) including hexane extraction of the residual ginseng marc after water extraction of ginseng. **b** The hexane extraction process of ginseng. **c** The hexane extraction process of WEG obtained by water extraction of ginseng. Data are averages of duplicate measurements with range less than 10%

fractions: the hexane-soluble fraction (HSF/WEG) and the hexane-insoluble fraction (HIF/WEG).

The lipid-soluble components were recovered by *n*-hexane extraction of ginseng, WIGM, and lyophilized WEG. Each hexane-soluble fraction was prepared by combining one part powder with ten parts *n*-hexane for 4 h at room temperature in a shaking waterbath. Each suspension was centrifuged at 2,890×*g* for 20 min, each supernatant was collected, and each pellet was then resuspended in *n*-hexane of the original volume used. The extraction process was repeated and the next supernatant was collected through centrifugation. Each pellet was dried at 30 °C in a forced-air convection oven, and each supernatant was filtered using a filter paper (Whatman No. 2). Hexane was eliminated from each lipid-soluble extract using a rotary vacuum evaporator (Eyela, Tokyo, Japan) at 50 °C.

Determination of Crude Saponin and Carbohydrate Contents

The crude saponin content was determined as described by Kwon et al. [12]. Ten grams of each sample was mixed with 50 ml of distilled water and then defatted twice with 50 ml of diethyl ether. The aqueous layer was extracted four times with 50 ml of water-saturated *n*-butanol. The butanol-soluble fraction was washed twice with 30 ml of distilled water to remove impurities and then dried using the rotary vacuum evaporator. The weight of residues corresponded to the crude saponin content of each sample. The carbohydrate content was determined by the phenol–sulfuric method with glucose as a standard as described by Dubious et al. [13].

Inhibitory Effects of Ginseng Extracts on Human Cancer Cell Proliferation

The effect of water-soluble and lipid-soluble ginseng extracts on proliferation of human cancer cells was assessed using a commercial assay kit, Cell Counting Kit-8 (CCK-8), following the supplier's instructions [14]. The lipid-soluble extracts were dissolved in ethanol and further diluted in cell culture media such that the final ethanol concentration did not exceed 0.1% (v/v). The water-soluble extracts were dissolved in the culture media. The prepared extracts were subjected to a sterile syringe filter (0.2 μm) prior to use. HepG2 and MCF-7 cancer cells were cultured in 96-well microplates at 4×10^4 cells/well in 200 μl of RPMI-1640 medium containing 5% FBS and penicillin–streptomycin for 24 h at 37 °C and 5% CO_2. After culturing, the media were changed with fresh RPMI-1640 containing lipid-soluble or water-soluble ginseng extracts (0–1,000 μg/ml). At the end of 24-h incubation, the media were eliminated by a suction pump. Ten microliters of CCK-8 solution and 190 μl of serum-free RPMI-1640 were then added to each well, and the absorbance of each well was read using a microplate reader (Molecular Devices, CA, USA) at 450 nm. These experiments were repeated three times, and the data were expressed as mean ± standard error (SE). SEs and *p*-values were calculated using Student's *t* test. The absorbance of ginseng extract-treated samples was compared to that of untreated control. The inhibition rate was calculated as follows:

$$\text{Inhibition rate } (\%) = \left(\text{OD}_{\text{Control}} - \text{OD}_{\text{Sample}}\right)/\text{OD}_{\text{Control}} \times 100$$

The GI_{50} values, which represent the test-sample concentration that causes 50% growth inhibition of cancer cells, were calculated as described by Nakatsu et al. [15].

Results and Discussion

Extraction Processes of Lipid-Soluble Components in Ginseng and Ginseng Marc

After water-soluble components in ginseng were recovered by hot water as a water extract of ginseng (WEG), the residual marc was treated using *n*-hexane for extraction of the lipid-soluble components. About 74 out of 100 g of ginseng was recovered as water-soluble components by four repetitions of hot water extraction (recovery yield $\approx$ 74 wt.%), and approximately 0.2 g of lipid-soluble components as a hexane extract of ginseng marc (HEGM) was then recovered in the residual ginseng marc by two repetitions of the *n*-hexane treatment (Fig. 1a). The use of SEP, including a series of water and *n*-hexane extractions, could successively recover water-soluble and lipid-soluble components in ginseng.

From a separate experiment involving two repetitions of hexane extraction of ginseng without an aqueous extraction step (Fig. 1b), it was found that about 0.8 out of 100 g of ginseng was recovered as a hexane extract of ginseng (HEG). Moreover, when the lyophilized WEG was re-extracted by two repetitions of hexane extraction, about 0.6 out of 74 g of WEG was recovered as a hexane-soluble fraction (HSF/WEG) (Fig. 1c). From the experimentally determined extraction yield (0.8 wt.%, based on dried ginseng) for the process scheme described in Fig. 1b, the total hexane-soluble fraction in dried ginseng (HSF/DG) can be estimated as the amount of HEG (0.8 g) as follows:

$$\text{Mass(HSF/DG)} \approx \text{Mass(HEG)} \approx 0.8 \text{ g}$$

In Fig. 1c, the hexane-soluble fraction in water-insoluble ginseng marc (HSF/WIGM) can be calculated using the following equation:

$$\begin{aligned}\text{Mass(HSF/WIGM)} &\approx \text{Mass(HSF/DG)} \\ &\quad - \text{Mass(HSF/WEG)} \\ &= 0.8 - 0.6 = 0.2 \text{ g},\end{aligned}$$

which shows that the WIGM might contain about 0.2 g of hexane-soluble fraction. Interestingly, the amount (0.2 g) of HSF/WIGM is the same as that of HEGM obtained in the process scheme described in Fig. 1a, indicating that 0.2 g of the lipid-soluble components still remained in ginseng marc as HEGM after the aqueous extraction of ginseng (Fig. 1a). The results showed that 0.6 out of 0.8 g of the total hexane-soluble fraction (denoted as "HEG") could also be extracted by hot water through four repetitions of aqueous extraction in SEP of ginseng.

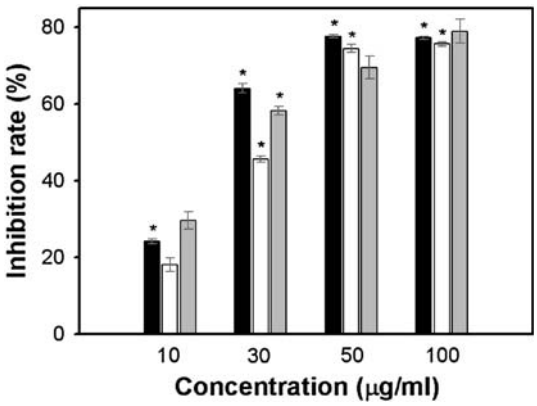

Fig. 2 Effects of HEG, HEGM, and HSF/WEG on human hepatoma cancer cell (HepG2) growth. *Black bars*, HEG; *white bars*, HEGM; *gray bars*, HSF/WEG. Data are means and SE of triplicate measurements. $^*P < 0.05$

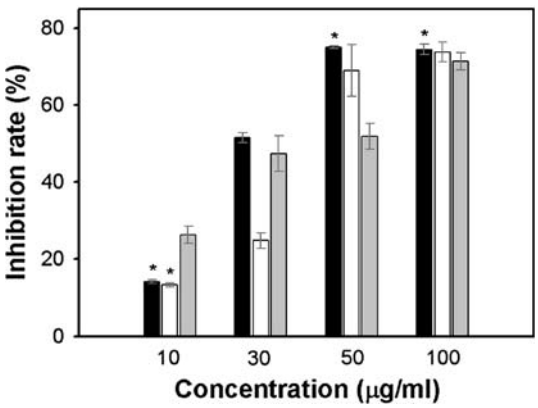

Fig. 3 Effect of HEG, HEGM and HSF/WEG on human breast cancer cell (MCF-7) growth. *Black bars*, HEG; *white bars*, HEGM; *gray bars*, HSF/WEG. Data are means and SE of triplicate measurements. $^*P < 0.05$

In Vitro Anticancer Activity of Ginseng Extracts on Human Cancer Cell Proliferation

In order to compare the anticancer activity of HEG and HEGM, dose–response assays were used to assess the effects on proliferation of HepG2 and MCF-7. Cell growth was potently inhibited by the HEG and the HEGM in both cell lines in a concentration-dependent manner (Figs. 2 and 3). HepG2 cells were more sensitive to both extracts of HEG and HEGM. The inhibitory effect of HEG was potent in HepG2 (GI_{50} = 21.1 μg/ml; Fig. 2) and MCF-7 (GI_{50} = 41.2 μg/ml; Fig. 3) cells. The HEGM with GI_{50} values of

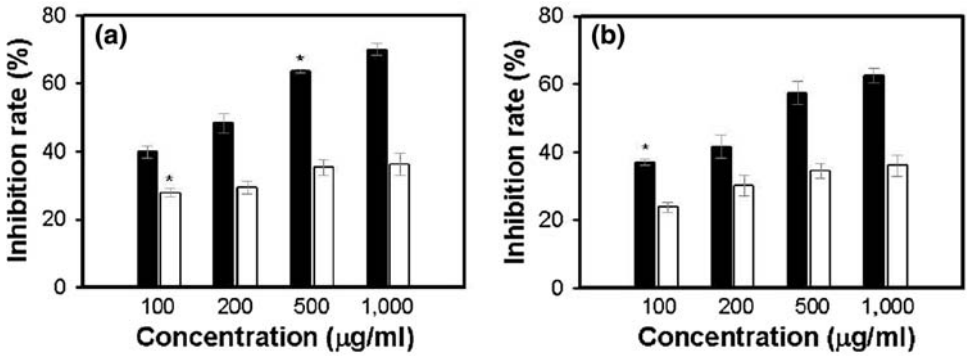

Fig. 4 Effect of WEG and residual HIF/WEG on human hepatoma (**a**) and human breast (**b**) cancer cell growth. *Black bars*, WEG; *white bars*, HIF/WEG. Data are means and SE of triplicate measurements. $^{*}P < 0.05$

41.7 μg/ml in HepG2 (Fig. 2) and 54.4 μg/ml in MCF-7 (Fig. 3) showed slightly less efficacy than HEG. The results show that ginseng marc as well as ginseng possesses anticancer active components. Thus, ginseng marc can be utilized for the production of bioactive lipid-soluble components with an anticancer activity. This strongly suggests that ginseng marc would be an industrially cost-effective raw material for valuable ginseng products.

WEG, HSF/WEG, and the residual hexane-insoluble fraction after hexane extraction of WEG (HIF/WEG) were prepared to demonstrate the anticancer activity of water-soluble and lipid-soluble components in ginseng (Fig. 1c). While WEG showed a low anticancer activity against HepG2 (GI_{50} = 268.5 μg/ml; Fig. 4a) and MCF-7 cells (GI_{50} = 466.6 μg/ml; Fig. 4b), HSF/WEG showed a potent anticancer activity against HepG2 (GI_{50} = 38.7 μg/ml, Fig. 2) and MCF-7 cells (GI_{50} = 51.1 μg/ml, Fig. 3) in a concentration-dependent manner similar to the activity of HEGM. Also the anticancer activity of residual HIF/WEG was greatly reduced showing GI_{50} values greater than 1,000 μg/ml in both HepG2 (Fig. 4a) and MCF-7 (Fig. 4b). These results indicate that the anticancer activity of WEG mainly originates from lipid-soluble components that could be extracted in both water and hexane. Although the water-soluble fraction in ginseng was the most abundant, its degree of dose-dependent anticancer activity was very low. On the other hand, the lipid-soluble fraction possessed a higher anticancer activity despite its small concentration in ginseng.

Assuming that lipid-soluble components in ginseng were fully recovered as HEG, the hexane extraction of ginseng could be an effective process (described in Fig. 1b) for the maximum recovery of lipid-soluble components with anticancer activity in high concentrations. The protocol developed in this study for SEP (described in Fig. 1a) of ginseng including a series of water extraction steps and hexane extraction steps could be effective in the recovery of all water-soluble and lipid-soluble anticancer active components in ginseng. Also the hexane extraction of waste ginseng marc in the ginseng industry can provide an attractive industrial process for the effective utilization of the residual lipid-soluble anticancer active components.

Crude Saponin and Carbohydrate Contents in Ginseng Extracts

Table 1 presents crude saponin and carbohydrate contents in WEG, HEG, and HEGM. Because most saponins and carbohydrates were recovered by water extraction, the components of the lipid-soluble fraction of ginseng, such as HEG and HEGM, contained a very small amount of saponins and no carbohydrates. It was reported that lipid-soluble components extracted by petroleum ether have a potent anticancer activity that mainly originates from polyacetylenes containing panaxynol, panaxidol, and panaxytriol [5, 6]. A panaxynol, which is a dominant compound of lipid-soluble polyacetylenes, was identified by GC-MS analysis in both HEG and HEGM. Polyacetylenes were reported to be quite unstable [16], but the panaxynol did not decompose during the storage of HEG and HEGM under nitrogen gas at 4 °C for ~3 months (data not shown). The ginseng extract obtained by organic solvent fractionation was reported to possess potent anti-oxidant compounds such as phenolic compounds, which can prevent oxidation/decomposition of polyacetylenes during storage [17]. The saponins in both HEG and HEGM (Table 1) are considered to be lipid-soluble saponins such as aglycon ginsenosides. It was reported that aglycon ginsenosides, which are present in natural ginseng in very small amounts, are extracted by chloroform and possess a

Table 1 Contents of crude saponin and carbohydrate in WEG, HEG, and HEGM

Extracts	Water extract of ginseng (WEG)	Hexane extract of ginseng (HEG)	Hexane extract of ginseng marc (HEGM)
Crude saponin (wt.%)	4.84	0.09	0.01
Carbohydrate (wt.%)	81.0	Not detected	Not detected

potent anticancer activity [18, 19]. While the lipid-soluble saponin content of HEG was 0.09 wt.% (Table 1), the polyacetylene content of HEG is considered to be 2.5–9.1 wt.% because 0.8 wt.% of dried ginseng was recovered as HEG (Fig. 1b), and the polyacetylene content of dried ginseng was reported to be 0.02–0.073 wt.% [16]. Accordingly it is assumed that the anticancer activity of HEG and HEGM originates mainly from polyacetylenes. The anticancer effect of lipid-soluble extracts was reported to be derived from the arrest of G1 phase in cell cycle progression [20]. Because water-soluble saponins and carbohydrates are not extracted by hexane, HIGM (Fig. 1b) including water-soluble components can be utilized as an industrially cost-effective raw material for the recovery of valuable water-soluble extracts.

The most significant finding of this study is that the anticancer activity of ginseng originates mainly from lipid-soluble components. The lipid-soluble fraction in ginseng marc and ginseng contained potent anticancer active compounds, and 25 wt.% of the lipid-soluble components still remained in ginseng marc after water extraction in ginseng. The WEG containing both water-soluble and lipid-soluble components showed anticancer activity. It was reported that the water-soluble or diluted ethanol-soluble extract in ginseng has anticancer activity [21–23]. From the results regarding the anticancer effect of various ginseng extracts, the anticancer activity of WEG is suggested to stem from the lipid-soluble compounds in combination with the water-soluble compounds such as saponins and acidic polysaccharides [4, 24, 25]. HSF/WEG (GI_{50} against HepG2 = 38.7 μg/ml) also possessed a potent anticancer activity, but the activity of residual HIF/WEG (GI_{50} against HepG2 >1,000 μg/ml), which contained only water-soluble compounds, was very low due to the elimination of anticancer active lipid-soluble compounds.

Conclusion

This work addressed the potent anticancer activity of the lipid-soluble components in ginseng and ginseng marc on human hepatoma and breast cancer cell proliferation in vitro. The cancer cell growth was strongly inhibited by HEGM as well as HEG in a concentration-dependent manner. While WEG possessed a low anticancer activity, HSF/WEG showed a potent anticancer activity against both cancer cell lines similar to the activity of HEGM. These results indicate that the anticancer activity of ginseng originates mainly from lipid-soluble components. During current SEP, a substantial amount of the anticancer active components was recovered during the water extraction steps, and the residual lipid-soluble anticancer active components in ginseng marc were then recovered by the hexane extraction steps. The current SEP can provide an efficient protocol for the utilization of ginseng marc, which is discarded as a by-product in the ginseng industry. Also the adoption of hexane extraction in ginseng is considered to be a very promising step for achieving maximum recovery of anticancer active components in high concentration.

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