Identification of Anticoagulant Components in Korean Red Ginseng

Jae Joon Wee¹, Young Sook Kim¹, Jong Soo Kyung¹, Yong Bum Song¹, Jae Ho Do¹, Dong Chung Kim², and Sung Dong Lee³*

¹Ginseng Research Institute, Korea Ginseng Corporation, Daejeon 305-805, Korea
²Department of Human Nutrition and Food Science, Chungwoon University, Hongseong 350-701, Korea
³Department of Food and Nutrition, Korea University College of Health Science, Seoul 136-703, Korea

In this study, the anticoagulant compounds in Korean red ginseng (KRG) were investigated. KRG powder was extracted using hot methanol, and the methanol extract was fractionated into n-hexane, ethylacetate, n-butanol, and aqueous fractions by solvent partitioning. The remains from the methanol extraction were further extracted with water and then dialyzed to obtain low and high molecular weight fractions. The anticoagulant activities of the seven fractions were evaluated in terms of thrombin time, prothrombin time, and activated partial thromboplastin time. Among these fractions, the ethylacetate fraction showed the most potent anticoagulant activity. The active components in the ethylacetate fraction were identified as the phenolic compounds vanillic, caffeic, ferulic, and p-coumaric acid via TLC and HPLC. These findings suggest that the anticoagulant activities of phenolic compounds contribute to the cardiovascular effects of KRG.

Keywords: Korean red ginseng, Anticoagulant activity, Phenolic compounds

INTRODUCTION

Ginseng is one of the most highly valued herbal medicines in Asian countries, including Korea, China, and Japan. It has also become a leading herbal supplement in Europe and the USA in recent years. Numerous studies have demonstrated the possible curative and restorative properties of ginseng in the treatment of cancer, diabetes, and neurodegenerative disease. Furthermore, there is increasing evidence of a role of ginseng in the cardiovascular system. Clinical trials have demonstrated certain beneficial effects of ginseng in patients with hypertension, atherosclerosis, and cardiac disorders.

Korean red ginseng (KRG, processed Panax ginseng C. A. Meyer) has been shown to significantly reduce the 24-h mean systolic blood pressure and improve vascular endothelial dysfunction in patients with essential hypertension [1,2]. KRG increased PGI₂ formation in patients with atherosclerotic disorders and inhibited both ADP- and collagen-induced platelet aggregation in healthy volunteers [3,4]. Epidemiological studies have demonstrated that the long-term intake of ginseng significantly prolonged plasma clotting times [5,6]. Both in vitro and in vivo studies have indicated that ginsenosides, the active components in ginseng, have potential cardiovascular benefits. These effects have been shown to be due to vasorelaxation, effects on ion channels, decreased cardiac contraction, reduced platelet aggregation, fibrinolysis, improved lipid profiles, and regulation of the glycemic index.

Kim et al. [7] studied the effect of ginsenosides on the release of nitric oxide (NO) from endothelial cells and...
discovered that the ginsenoside $R_g_3$ was the most potent vasodilator among all of the ginsenosides examined. $R_g_2$-induced endothelium-dependent relaxation was markedly inhibited by tetraethylammonium, a non-selective K$^+$ channel blocker, suggesting that $R_g_2$ activates tetraethylammonium-sensitive K$^+$ channels in endothelial cells to promote Ca$^{2+}$ influx and the subsequent activation of endothelial NO synthase [8]. In contrast, Chen [9] examined the relaxation of pulmonary vessels in response to the ginsenosides $R_b_1$ and $R_g_3$, $R_b_2$, and $R_e$ decreased the contraction of adult rat ventricular myocytes [10], while ginsenosides $R_b_1$, $R_b_2$, and $R_b_3$ inhibited the contractility of normal myocardial cells [11]. Furthermore, $R_g_2$ exhibited potent anti-aggregatory activity in vitro when platelets were stimulated with collagen and arachidonic acid [12], and $R_g_3$ and its derivatives were shown to be potent antagonists of $[H]$-platelet activating factor [13]. Further, $R_b_2$ enhanced the fibrinolytic activity of bovine aortic endothelial cells [14]. In contrast, the hypolipidemic effects of ginseng saponins were examined in rats fed a high-fat diet in cyclophosphamide-induced hyperlipidemic rabbits [15,16]. Yokozawa et al. [17] demonstrated the hyperlipidemia-improving and hypoglycemic effects of $R_b_2$ on streptozotocin-induced diabetic rats.

As described above, the ginsenosides involved in cardiovascular pharmacology (e.g., vasorelaxation, anti-platelet aggregation, hypolipidemia, and hypoglycemia) have been studied extensively, whereas other components of KRG have not been fully explored. Many epidemiological studies have shown protective effects of plant-based diets on cardiovascular disease, leading to the discovery of various bioactive compounds, including phenolic compounds, phytosterogens, carotenoids, organosulfur compounds, and monoterpenes. Many phenolic compounds have antioxidative properties, and some studies have demonstrated favorable effects on thrombosis [18]. Thus, we searched for novel active compounds in KRG using an anticoagulation assay system. Through the screening of various fractions of KRG, we found that the ethylacetate (EtOAc) fraction possessed potent anticoagulant activity in vitro, and phenolic acids were identified as the active components in this fraction.

**MATERIALS AND METHODS**

**Materials**

KRG powder (Jungkwanjang) was provided by Korea Ginseng Corporation (Daejeon, Korea). Citrated human plasma was obtained from the Red Cross Blood Center (Daejeon, Korea). Thromboplastin, bovine thrombin, and authentic phenolic compounds were purchased from Sigma Chemical Co. (St. Louis, MO, USA). TLC plates were obtained from Merck (Darmstadt, Germany). HPLC columns and the packing material for reverse-phase column chromatography were purchased from YMC Co. (Kyoto, Japan). All other chemicals were of analytical grade.

**Extraction and fractionation**

KRG powder (100 g) was extracted with 500 mL of hot methanol (MeOH) four times. The MeOH extract was pooled and concentrated. Next, the MeOH extract was dissolved in distilled water and fractionated by solvent partitioning (Fig. 1) to produce n-hexane, ethylacetate (EtOAc), n-butanol (BuOH), and aqueous fractions; the yields were 0.43, 1.90, 10.63, and 22.32%, respectively. The residue remaining from the MeOH extraction was further extracted with water. The water extract was dialyzed against tap water for two days, and the resultant inner portion was precipitated with ethanol to obtain low and high molecular fractions (Fig. 1). The lipophilic fraction was dissolved in 10% dimethylsulfoxide; all others were dissolved in distilled water before use.

**Anticoagulation assay**

To screen the fractions for anticoagulant activity, the clotting times were measured using a blood coagulation analyzer (Behnk Elektronik, Norderstedt, Germany). To measure the thrombin time (TT), 50 μL of 0.02 M CaCl$_2$, 50 μL of thrombin, and 50 μL of each fraction were preincubated at 37°C for 3 minutes. The coagulation reaction was started by the addition of 100 μL of citrated human plasma. The prothrombin time (PT) was measured by the preincubation of 100 μL of human plasma with 50 μL of each fraction, followed by the addition of 100 μL of thromboplastin-D. To measure the activated partial thromboplastin time (aPTT), 100 μL of human plasma, 100 μL of aPTT-XL, and 50 μL of each fraction were preincubated at 37°C for 3 minutes, after which 100 μL of 0.02 M CaCl$_2$ were added to start the coagulation reaction.

**Column chromatography and preparative HPLC**

The anticoagulant-active EtOAc fraction was further fractionated by reverse-phase column chromatography. Briefly, the EtOAc fraction was passed through a C$_{18}$ glass column (Φ15×150 mm, 75 μm) to obtain subfractions. The column was eluted with 50% MeOH followed
Wee et al. Anticoagulant Components in Korean Red Ginseng

http://ginsengres.org

by 100% MeOH. The subfractions were assayed for anticoagulant activity. The 50% MeOH subfraction was further subjected to HPLC using a C<sub>18</sub> column (Φ20×250 mm, 10 μm) and CH<sub>3</sub>CN/H<sub>2</sub>O/phosphoric acid (20:80:0.2, v/v) mobile phase at a flow rate of 2 mL/min. The n-BuOH fraction was chromatographed on a silica gel (70-230 mesh) column with CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O as a developing solvent to separate the saponins protopanaxadiol (PPD) and protopanaxatriol (PPT).

TLC
Silica gel TLC was performed to identify the active components. The plate was developed with CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (65:35:10, v/v), 0.5% FeCl<sub>3</sub> in 0.1 N HCl was sprayed to detect phenolic compounds.

Analytical HPLC
The EtOAc fraction and its subfractions were analyzed by HPLC (Waters Corp., Milford, MA, USA) using a C<sub>18</sub> column (Φ4.6×250 mm, 5 μm) to detect ginsenosides or phenolic compounds. The mobile phase was a CH<sub>3</sub>CN/H<sub>2</sub>O gradient for ginsenosides, and CH<sub>3</sub>CN/H<sub>2</sub>O/phosphoric acid (8:92:0.2, v/v) for phenolic compounds, respectively. The detection wavelength was 203 nm for ginsenosides and 310 nm for phenolic compounds, respectively.

RESULTS AND DISCUSSION

Effect of the Korean red ginseng extract fractions on blood coagulation
Seven fractions obtained by the fractionation of a KRG MeOH extract were evaluated for their anticoagulant activity. Among them, only the EtOAc fraction prolonged clotting time markedly, as measured by the TT (Table 1). Based on this result, the EtOAc fraction was used to isolate anticoagulant-active components. The EtOAc fraction was further fractionated by reverse-phase column chromatography to yield subfractions (50% and 100% MeOH). The 50% MeOH subfraction showed remarkably potent inhibitory activity against blood coagulation compared to the 100% MeOH subfraction (Table 2). Generally, the EtOAc fraction is used to isolate less polar ginsenosides, including Rh<sub>1</sub>, Rh<sub>2</sub>, Rg<sub>1</sub>, and Rg<sub>3</sub>. Therefore, we analyzed the 50% MeOH subfraction by HPLC to examine whether these ginsen-
osides were present. The HPLC traces revealed that Rg1, Rf, Rh1, and Rg3, which appeared in the EtOAc fraction, were weakly detected in the anticoagulant-active 50% MeOH subfraction (Fig. 2). This result indicates that saponins do not contribute to the anticoagulation activity of the EtOAc fraction. To confirm this, we prepared PPD and PPT saponin fractions, and examined their anticoagulation activities (Table 3). As shown in Table 3, the PPD and PPT fractions did not show anticoagulation activity. The 50% MeOH subfraction was further separated using preparative HPLC to give fractions 1-4, in order of elution. Fractions 2 and 3 showed potent anticoagulation activity (data not shown). The anticoagulant-active components in fraction 2 and 3 were studied chromatographically.

### Table 1. Effect of various fractions prepared from Korean red ginseng methanol extract on thrombin time

<table>
<thead>
<tr>
<th>Fractions</th>
<th>Thrombin time (s)</th>
<th>Final concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0</td>
<td>2.5</td>
</tr>
<tr>
<td>n-Hexane</td>
<td>18.1</td>
<td>-</td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>&gt;200</td>
<td>&gt;200</td>
</tr>
<tr>
<td>n-Butanol</td>
<td>23.7</td>
<td>-</td>
</tr>
<tr>
<td>Aqueous</td>
<td>27.4</td>
<td>-</td>
</tr>
<tr>
<td>Outer</td>
<td>32.6</td>
<td>-</td>
</tr>
<tr>
<td>Inner-supernatant</td>
<td>27.2</td>
<td>-</td>
</tr>
<tr>
<td>Inner-precipitate</td>
<td>22.1</td>
<td>-</td>
</tr>
</tbody>
</table>

Each value represents the average of duplicate experiments. Each fraction was dissolved in 10% dimethylsulfoxide or distilled water at a concentration of 25 mg/mL.

### Table 2. Effect of subfractions derived from the ethylacetate fraction on plasma clotting times

<table>
<thead>
<tr>
<th>Subfractions</th>
<th>Clotting time (s)</th>
<th>Thrombin time</th>
<th>Prothrombin time</th>
<th>Activated partial thromboplastin time</th>
</tr>
</thead>
<tbody>
<tr>
<td>50% methanol</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>76.8</td>
<td></td>
</tr>
<tr>
<td>100% methanol</td>
<td>22.4</td>
<td>35.5</td>
<td>26.2</td>
<td></td>
</tr>
</tbody>
</table>

Each value represents the average of duplicate experiments.

Subfractions were obtained by reverse-phase column chromatography of the ethylacetate fraction and dissolved in 10% dimethylsulfoxide at a concentration of 25 mg/mL.

Fig. 2. HPLC chromatograms of the ethylacetate (EtOAc) fraction and its subfraction. (A) Korean red ginseng EtOAc fraction. (B) 50% methanol subfraction. Column, C18 (Φ4.8×250 mm, 5 μm); mobile phase, CH3CN/H2O gradient; flow rate, 1 mL/min; detection wavelength, 203 nm; AU, absorption unit.
Identification of phenolic components and their anticoagulant activity

Maltol was identified in fr. 2 by TLC based on the R<sub>f</sub> value and FeCl<sub>3</sub> color reaction (Fig. 3); this was confirmed by HPLC (Fig. 4). Additionally, protocatechuic acid was identified in fr. 2, while p-hydroxybenzoic, vanillic, caffeic, p-coumaric, and ferulic acid were identified in fr. 3 by HPLC (Fig. 4). p-Coumaric, ferulic, caffeic, vanillic, and protocatechuic acid, but not maltol, had potent inhibitory effects on blood coagulation, as shown by the TT (Table 4).

Blood coagulation and platelet aggregation are crucial events in thrombosis, which is a major cause of human mortality. When a blood vessel is injured, both the intrinsic and extrinsic coagulation pathways become activated, leading to the formation of blood clots to minimize blood loss. Problems with this process can result in the formation of an excessive number of platelet/fibrin-rich thrombi, which obstruct blood flow in the circulatory system. In a recent report, Jin et al. [19] showed that the administration of KRG extract to rats prevented carotid arterial thrombosis <i>in vivo</i>, whereas it failed to prolong coagulation times <i>ex vivo</i>. They concluded that the antithrombotic effect of KRG extract might not be due to its anticoagulation effect, but rather to antiplatelet aggregation activity. Nonetheless, Matsuda and Kubo [20] reported that a 70% MeOH extract of KRG prevented the disruption of the intravascular coagulative system.

### Table 3. Effects of saponin fractions on thrombin time

<table>
<thead>
<tr>
<th>Saponin fractions&lt;sup&gt;1,2&lt;/sup&gt;</th>
<th>Thrombin time (s) Final concentration (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0</td>
</tr>
<tr>
<td>Protopanaxadiol saponin fraction</td>
<td>16.9</td>
</tr>
<tr>
<td>Protopanaxatriol saponin fraction</td>
<td>16.6</td>
</tr>
<tr>
<td>Aspirin</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

Each value represents the average of duplicate experiments.

<sup>1</sup>Protopanaxadiol and protopanaxatriol saponin fractions were prepared from the n-butanol fraction by silica gel column chromatography.

![Fig. 3](image1.png)  
<sup>Fig. 3</sup>. TLC chromatogram of fraction 2. Lane 1, fraction 2; lane 2, maltol standard; plate, silica gel 60 pre-coated aluminum sheet, layer thickness 0.2 mm; solvent system, CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O (65:35:10, v/v); detection, FeCl<sub>3</sub> spray.

![Fig. 4](image2.png)  
<sup>Fig. 4</sup>. HPLC chromatograms of fractions 2 and 3. (A) fraction 2. (B) fraction 3. Column, μ-Bondapak C<sub>18</sub> (Φ3.9×300 mm, 5 μm); mobile phase, CH<sub>3</sub>CN/H<sub>2</sub>O/phosphoric acid (8:92:0.2, v/v); flow rate, 1 mL/min; detection wavelength, 310 nm; AU, absorption unit.
induced by endotoxin and thrombin in rats. Also, KRG significantly prolonged the aPTT and PT in a blood stasis rat model [21]. Furthermore, an antithrombin-active polysaccharide with an inhibitory effect on blood coagulation has been isolated from KRG [22].

In the present study, we employed a systematic fractionation method to search for the anticoagulant-active components in KRG. Among seven fractions obtained from a MeOH extract of KRG powder, only the EtOAc fraction showed potent anticoagulant activity, whereas the other fractions showed almost no such activity (Table 1). The fact that the n-hexane and n-BuOH fractions showed no activity implies that non-polar fat-soluble compounds such as polyacetylenes and ginsenosides do not possess anticoagulant activity. In addition, the inner precipitate portion of the dialysis (a polysaccharide fraction), showed no anticoagulant activity, suggesting that this fraction did not contain the antithrombin-active polysaccharide isolated by Kim et al. [22]. Further fractionation of the EtOAc fraction by reverse-phase column chromatography afforded a remarkably potent anticoagulative subfraction (50% MeOH subfraction) (Table 2). The HPLC trace of the 50% MeOH subfraction indicated no ginsenosides, revealing that ginsenosides are excluded from these active components (Fig. 2). Finally, the phenolic acids p-coumaric, ferulic, caffeic, vanillic, and protocatechuic acid were identified in the active subfractions of fractions 2 and 3 by HPLC, implying that phenolic acids are anticoagulant-active compounds (Fig. 4). In fact, the phenolic acids in the EtOAc fraction showed strong anticoagulant activity in vitro, suggesting that phenolic acids contribute to the cardiovascular effects of KRG (Table 4). The chemical structures of these phenolic acids are illustrated in Fig. 5. As shown, hydroxybenzoic acid is based on a C6-C1 skeleton. Cinnamic acids are a series of trans-phenyl-3-propenoic acids with C6-C3 structures that differ in their ring substitutions. Caffeic acid, its esters, and ferulic acid are the most frequently encountered phenolic acids in plant foods. Phenolic acids constitute one of several categories of plant-derived phenolic compounds, including flavones, flavonols, isoflavones, anthocyanidins, fla-

Table 4. Anticoagulant activity of phenolic compounds identified in the ethylacetate fraction prepared from Korean red ginseng methanol extract

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th>Thrombin time (s)</th>
<th>Final concentration (mg/mL)</th>
<th>Remark</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.0</td>
<td>2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>27.0</td>
</tr>
<tr>
<td>Ferulic acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>24.3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>26.9</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>26.5</td>
</tr>
<tr>
<td>Protocatechuic acid</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>21.6</td>
</tr>
<tr>
<td>Maltol</td>
<td>34.7</td>
<td>23.5</td>
<td>17.4</td>
</tr>
</tbody>
</table>

Each value represents the average of duplicate experiments. Each phenolic compound was dissolved in methanol.

Anticoagulant activity of phenolic compounds identified in the ethylacetate fraction prepared from Korean red ginseng methanol extract

Fig. 5. Chemical structures of the phenolic acids in Korean red ginseng.
vanols, flavanones, tannins, stilbenes, and lignans. Phenolic compounds have received an increasing amount of attention because of their antioxidative properties, which may help prevent cardiovascular disease. For example, green tea catechin has antiinflammatory activity in rats, while p-coumaric acid has antiplatelet activity in rabbits [23,24].

Many natural products have potential benefits in the prevention and treatment of cardiovascular disorders, including citrus fruits, tea, coffee, ginkgo, tomato, grape, red wine, olive oil, fish oil, and soy. The bioactive compounds in these products include flavonoids, lycopene, resveratrol, omega-3 fatty acids, and isoflavone, as well as natural antioxidative nutrients such as ascorbate (vitamin C), tocopherols (vitamin E), and carotenoids. Epidemiological and clinical studies have shown that these natural products can reduce cardiovascular risk factors, including blood pressure, plasma lipids, blood coagulation, and platelet aggregation. Panax ginseng has also been studied extensively for its cardiovascular effects, and many experimental studies have demonstrated that ginsenosides, particularly Rg3, are active in vasorelaxation. However, in this study, we found that ginsenosides were inactive against blood coagulation, whereas phenolic acids were active, suggesting that they exert their cardiovascular effects through different mechanisms. On the other hand, it is unknown whether this effect occurs at the concentrations found in blood following the ingestion of ginseng products at the recommended doses, particularly considering that the content of total phenolic acids is around 0.01%, which is far less than that of ginsenosides [25].

In conclusion, the phenolic compounds in KRG have potent anticoagulant activity, whereas the saponin fractions, which were previously shown to possess antiplatelet aggregation activity, do not. Taken together, these results suggest that both saponins and phenolic compounds contribute to the cardiovascular effects of KRG through their antiplatelet aggregation and anticoagulant activities, respectively. Additional in vivo studies of the anticoagulant activities of phenolic compounds will be useful to better understand the pharmacology of these compounds.

REFERENCES