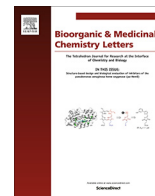




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7 α ,15-Dihydroxydehydroabietic acid from *Pinus koraiensis* inhibits the promotion of angiogenesis through downregulation of VEGF, p-Akt and p-ERK in HUVECs

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ABSTRACT

Pinus koraiensis pinecones are considered an undesired waste by-product of the processing of seeds. However, recent studies of the potential anti-tumor effects of the pinecones have led to increasing interest in their chemical constituents. The present study examined the potential antiangiogenic effects of the constituents of pinecones and further characterized their underlying mechanisms of action. Chemical investigation of a water extract of *P. koraiensis* pinecones led to the isolation and identification of the eight main components including five diterpenoids (**1–5**), two monoterpenes (**6,7**) and a phenolic acid (**8**). The structure of the compounds was determined by spectroscopic analysis of NMR spectra and LC/MS analysis. Of the isolated compounds, 7 α ,15-dihydroxydehydroabietic acid (**5**) significantly inhibited the promotion of angiogenesis in human umbilical vein endothelial cells (HUVECs). Compound **5** inhibited angiogenesis through downregulation of the VEGF, p-Akt and p-ERK signaling pathways. These results provide experimental evidence of a novel biological activity of 7 α ,15-dihydroxydehydroabietic acid (**5**) as a potential antiangiogenic substance. This study also suggests that compound **5** could potentially be a useful adjuvant therapeutic substance for cancer prevention and treatment.

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During angiogenesis, vascular endothelial cells in existing blood vessels divide and various proteins are secreted from these vascular endothelial cells and from the cells surrounding the blood vessels, resulting in new blood vessels that extend from the existing blood vessels.^{1,2} In addition to the growth of endothelial cells, a variety of complex processes, such as basement membrane infiltration, migration and differentiation, and capillary formation by endothelial cells, are required. Furthermore, activation of histolytic enzymes is necessary for the formation of angiogenesis, and this series of processes is very similar to the penetration process of cancer cells.^{3–5} Conventional anticancer drugs target cancer cells, while angiogenesis inhibitors target proliferating endothelial cells.

Therefore, angiogenesis inhibitors are expected to be less toxic than existing anticancer agents and to have fewer adverse effects.⁶

Pinus koraiensis Siebold & Zucc. (Pinaceae), commonly called the Korean pine, is widely distributed throughout the mountains of Korea, Russia, China and Japan. *P. koraiensis* is a large conifer famous for its seed crop, the pine nut, which is considered a nutritious food, rich in phenolic compounds with antioxidant activity.⁷ A recent study reported that procyanidins from *P. koraiensis* bark showed antitumor activity in mice bearing U14 cervical cancer.⁸ However, *P. koraiensis* pinecones, annual pine globular fruits without nutritious seeds inside, have been viewed as nothing more than hard woody shells, and treated as an unwanted by-product in the processing of seeds. While the Materia Medica Compendium has recorded that pinecones have been used in traditional Chinese medicine for the treatment of respiratory diseases, including excess production of phlegm and asthma,⁹ the pinecones are commonly burnt as firewood or discarded as waste. Nevertheless, several noteworthy studies of the pinecones have led to increasing interest,

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in the hopes of expanding the set of applications of *P. koraiensis* to include their use as a potential anti-tumor resource.^{10–12}

As part of our continuing efforts to discover bioactive compounds from Korean natural sources,^{13–18} our group recently explored the potential therapeutic effects of *P. koraiensis* pinecones against human lung adenocarcinoma cells, and further identified its underlying molecular mechanisms.¹⁹ In this study, a water extract of *P. koraiensis* pinecones exhibited cytotoxic activity in human lung cancer cells irrespective of their p53 status by inducing apoptotic cell death in a caspase-3-dependent manner. We also conducted a phytochemical investigation of a water extract of *P. koraiensis* pinecone, which led to the isolation and identification of the eight main components (**1–8**) of the pinecones. In this study, we examined the potential antiangiogenic effects of these eight main compounds (**1–8**) and further characterized their underlying mechanisms of action. To the best of our knowledge, this is the first study to show that there are potential antiangiogenic substances in *P. koraiensis* pinecones.

The dried pinecones were pulverized and extracted with water under reflux to obtain a water extract. Our recent study demonstrated that the water extract showed potent cytotoxic effects against human lung cancer cells and, further, that the anti-cancer effect is mediated, at least in part, by inducing apoptosis in a caspase-3-dependent fashion.¹⁹ To identify the compounds in the pinecones that induced the anti-cancer effects, the pinecone water extract was fractionated to yield soluble fractions of hexane, CH₂Cl₂, EtOAc, and *n*-BuOH. Chemical examination of the hexane-soluble fraction was performed by HPLC purification, which revealed the presence of a diterpenoid (**1**). The CH₂Cl₂-soluble fraction was further subjected to open column chromatography and HPLC purification, which yielded four additional diterpenoids (**2–5**) and two monoterpenes (**6,7**). Finally, the EtOAc-soluble fraction was further subjected to HPLC separation, which revealed the presence of a phenolic acid (**8**). The compounds were identified as dehydroabietic acid (**1**),²⁰ 15-hydroxy-7-oxodehydroabietic acid (**2**),²⁰ 7β,15-dihydroxydehydroabietic acid (**3**),²¹ β-D-glucopyranosyl labda-8(17),13-diene-(15,16)-lactone-19-oate (**4**),²² 7α,15-dihydroxydehydroabietic acid (**5**),²³ (1S,2S,4R)-(+)-limonene-1,2-diol (**6**),²⁴ sobrerol (**7**),²⁵ and 4-hydroxy-benzoic acid (**8**)²⁶ by comparing their spectroscopic data, including ¹H and ¹³C NMR, with previously reported values as well as LC/MS analysis (Fig. 1).

The antiangiogenic potential of the compounds was assessed using human umbilical vein endothelial cells (HUVECs). Endothelial cell proliferation is a complex multistep process involved in

angiogenesis.²⁷ First, the ability of the compounds to inhibit the proliferation of endothelial cells was examined using an MTT assay.^{28–31} We conducted dose-response studies on the proliferation of HUVECs *in vitro* to determine the effects of compounds **1–8** on endothelial cell cytotoxicity. The percent viability of HUVECs after treatment with compounds **1–8** is shown in Fig. 2, which demonstrates that compounds **2**, **3**, **7** and **8** had no effect on HUVECs at doses of up to 100 μM (Fig. 2B, C, G and H). In contrast, treatment with compounds **1**, **4**, **5** and **6** significantly decreased HUVEC cell viability (Fig. 2A, D, E and F).

The effects of a nontoxic dose of the compounds on HUVEC tube formation are shown in Fig. 3. Most compounds had no effect on tube formation in HUVECs. In contrast, compound **5** inhibited tube formation in HUVECs (Fig. 3B). Compared with the control, a 12.09% decrease in tube formation was achieved with 12.5 μM compound **1** and a 8.95% decrease in tube formation was achieved with 100 μM compound **3**, while a 20.53% decrease in tube formation was seen with 6.25 μM compound **5**. Among the tested compounds, compound **5** exerted the highest effect on the inhibition of tube formation.

Vascular endothelial growth factor (VEGF), which plays a central role in tumor angiogenesis, is a pro-angiogenic factor. Therefore, it is a promising target for therapeutic intervention.³² Proliferation, migration and tube formation of cultured endothelial cells are the typical characteristics of *in vitro* angiogenesis measured through the assay. These cellular events are usually stimulated by intracellular signal cascades, such as the MEK/ERK pathway for endothelial cell proliferation, and the Akt/eNOS axis, which promotes cell survival. Moreover, all of these signals contribute to endothelial differentiation and tube-like structure formation. Therefore, endothelial cell activation is an initial step in the angiogenic process.^{33,34} As shown in Fig. 4, our Western blot analysis showed that the expression of VEGF (0.89 ± 0.01 and 0.69 ± 0.02-fold at 3.125 and 6.25 μM, respectively), p-Akt (1.10 ± 0.01 and 0.68 ± 0.02-fold at 3.125 and 6.25 μM, respectively) and p-ERK (0.98 ± 0.01 and 0.82 ± 0.02-fold at 3.125 and 6.25 μM, respectively) were decreased in cells treated with compound **5**, compared to levels detected in control cells. Therefore, compound **5** inhibited the promotion of angiogenesis in HUVECs via downregulation of the VEGF, Akt and ERK signaling pathways. To the best of our knowledge, this is the first report on the beneficial effects of active compounds isolated from *P. koraiensis* on angiogenesis.

The most active compound **5** (7α,15-dihydroxydehydroabietic acid) is an abietane-type diterpenoid, and, to the best of our knowl-

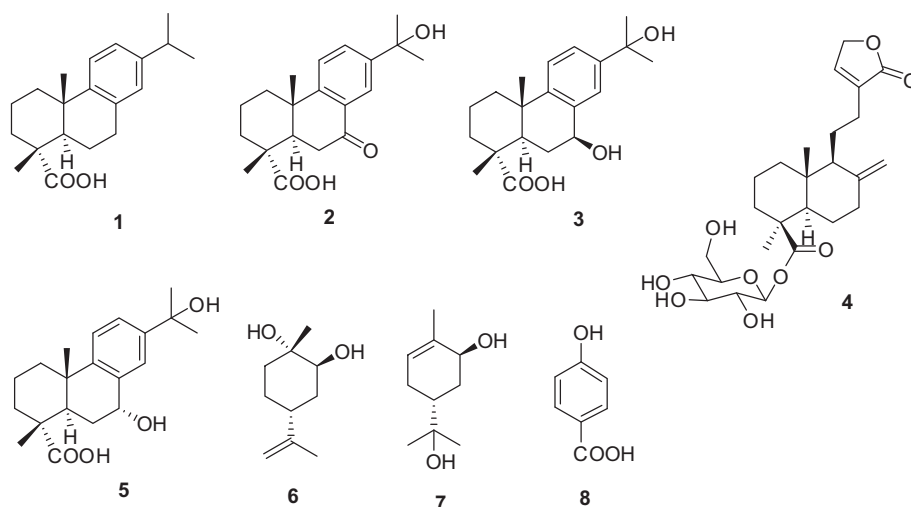


Fig. 1. Chemical structures of compounds **1–8**.

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