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**TITLE:** Pharmacological and phytochemical insights on the pancreatic  $\beta$ -cell modulation by *Angelica* L. roots.

**RUNNING TITLE:** Angelica roots and insulin secretion

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**HIGHLIGHTS:**

- Monographed (Ph. Eur. 11th) *Angelica L.* roots were phytochemically profiled on LC-MS, showing distinct metabolite profiles.
- *Angelica* roots rich in coumarins modulate glucose-induced insulin secretion.
- Prenylated coumarins were found to be responsible for the insulinotropic effect of *angelica* root extracts.
- Imperatorin potentiated insulin secretion in a dose- and glucose-dependent manner.

**TITLE:** Pharmacological and phytochemical insights into pancreatic  $\beta$ -cell modulation by *Angelica* L. roots.

## **ABSTRACT**

### **ETHNOPHARMACOLOGICAL RELEVANCE**

Angelica roots are a significant source of traditional medicines for various cultures around the northern hemisphere, from indigenous communities in North America to Japan. Among its many applications, the roots are used to treat type 2 diabetes mellitus; however, this application is not mentioned often. Ethnopharmacological studies have reported the use of *A. japonica* var. *hirsutiflora*, *A. furcijuga*, *A. shikokiana*, and *A. keiskei* to treat diabetes symptoms, and further reports have demonstrated the three angelica roots, i.e., *A. japonica* var. *hirsutiflora*, *A. reflexa*, and *A. dahurica*, exhibit insulin secretagogue activity.

### **AIM OF THE STUDY**

This study aimed to phytochemically characterize and compare angelica roots monographed in the Ph. Eur. 11<sup>th</sup>, isolate major plant metabolites, and assess extracts and isolates' capability to modulate pancreatic  $\beta$ -cell function.

### **MATERIALS AND METHODS**

Root extracts of *Angelica archangelica*, *Angelica dahurica*, *Angelica biserrata*, and *Angelica sinensis* were phytochemically profiled using LC–MS. Based on this analysis, simple and furanocoumarins were isolated using chromatography techniques. Extracts and isolated compounds were studied for their ability to modulate insulin secretion in the INS-1 pancreatic  $\beta$ -cell model. Insulin was quantified by the homogeneous time-resolved fluorescence method.

### **RESULTS**

Forty-one secondary metabolites, mostly coumarins, were identified in angelica root extracts. *A. archangelica*, *A. dahurica*, and *A. biserrata* root extracts potentiated glucose-induced insulin secretion, which correlated with their high coumarin content. Subsequently, 23 coumarins were isolated from these roots and screened using the same protocol. Coumarins substituted with the isoprenyl group were found to be responsible for the extracts' insulinotropic effect.

### **CONCLUSIONS**

Insulinotropic effects of three pharmacopeial angelica roots were found, the metabolite profiles and pharmacological activities of the roots were correlated, and key structures responsible for the modulation of pancreatic  $\beta$ -cell function were identified. These findings may have implications for the traditional use of angelica roots in treating diabetes. Active plant metabolites may also become lead structures in the search for new antidiabetic treatments.

**KEYWORDS:** angelica; coumarins; pancreatic  $\beta$ -cell; insulin secretion.

## MAIN TEXT

### 1 Introduction

Type 2 diabetes mellitus (T2DM) is a disease that is associated with insulin resistance and pancreatic  $\beta$ -cell dysfunction. According to current knowledge on the pathophysiology of this disease, multiple antidiabetic therapies have been developed to improve glycemic control and slow disease progression.

Although numerous antidiabetic medications have been developed, the side effects accompanying the medications, including hypoglycemia, gastrointestinal symptoms, heart failure, weight gain, edema, impaired kidney function, pancreatitis, and genital infections, are disadvantageous (Irondi et al., 2015). The incidence rate of diabetes is higher in low- and middle-income countries, which causes another challenge, as modern therapies are lacking or unaffordable for the majority of diabetic patients in these countries. In this context, plant extracts and their active constituents are important sources for potential new antidiabetic treatments (Furman et al., 2020; International Diabetes Federation, 2021).

In traditional medicine systems, such as traditional Chinese medicine (TCM), Japanese Kampo, Indian Ayurveda, and African, Native American and European traditional medicine, numerous plants are used to treat diabetes. Support for the efficacy of these medicines is anecdotal, although some scientific evidence is present in the literature for more than 800 plants (Furman et al., 2020). In many cases, data on plant extracts and their metabolites are limited. The effect may be measured only in terms of blood glucose levels, and thus, the mechanism underlying the antidiabetic effect (when found) is unknown, and the active constituents responsible for the effect of the extract are often unknown.

For centuries, humans have utilized plants from the *Angelica* L. (*Apiaceae*) genus for culinary, ceremonial, and medicinal reasons. *Angelica* L. roots are widely used as a traditional and folk medicine worldwide and are mentioned in many pharmacopeias and medicinal systems, such as Traditional Chinese Medicine, Ayurveda, and Kampo (Figure 1).



Figure 1. Application of angelica roots worldwide in traditional medicine.

*Angelica archangelica* L. (syn. *Angelica officinalis* Hoffm.) is native to North and Eastern Europe and Western Asia and had been used for centuries as a spice and vegetable for culinary purposes in these areas (Kaur and Bhatti, 2021). In Western traditional medicine and the Ayurvedic medicinal system, *A. archangelica* L. roots are used as an appetite stimulant, an antispasmodic, a sedative, an anxiolytic, and a medication for gastrointestinal symptoms, such as bloating, poor digestion, eructation, and flatulence. Its main constituents are essential oil and simple and furanocoumarins. At present, it is recognized by the United States and European Pharmacopeias as well as the National Formulary of India (Kaur and Bhatti, 2021). *A. archangelica* has been naturalized in Eastern North America even though *Angelica atropurpurea* L, another representative of the *Angelica* genus with similar properties, naturally present in these regions. (Wszelaki et al., 2011). Like *A. archangelica*, *A. atropurpurea* L has been used as a vegetable and spice. Among the Native Cultures of North America, such as the Muscogee, Cherokee, Lenape, and Menominee, the plant was used for ceremonial, nutritional, and medicinal purposes to treat anxiety, back pain, and gastrointestinal disorders (Lewis and Jordan, 2002). Ceremonial and therapeutic uses of *Angelica dawsonii* S.Watson by the Blackfoot, *Angelica breweri* A.Gray by the Miwok, Paiute, Shoshoni, and Washo, *Angelica genuflexa* Nutt. By the Nuxalk and Gitxan, *Angelica lineariloba* A.Gray by the Paiute, *Angelica lucida* L. by the Aleut and Inuit, *Angelica pinnata* S.Watson by the Goshute, *Angelica tomentosa* S.Watson by the Pomo and Yana and *Angelica venenosa* (Greenway) Fernald by the Iroquois were also noted (Dexter et al., 2014; Moerman, 1998).

In parts of Europe, North America, and South Asia, *Angelica sylvestris* L. roots were used as an ethnomedicine. Among the Mi'kmaq of Eastern Canada, the roots were used to treat coughs, colds and sore throats (Moerman, 1998). In Estonia, it was used in folk medicine to treat cancer (Raal et al., 2022), while in Pakistan, it was used to treat rheumatism and muscle pain

(Malik et al., 2018). In the Pakistani and Indian Himalayan regions, another angelica root were used, i.e., that of *Angelica glauca* Edgew. Its roots are commonly used to treat gynecological disorders, such as dysmenorrhea, metrorrhagia, amenorrhea, polycystic ovary syndrome, and gastrointestinal disorders (Ikram et al., 2015; Kumar et al., 2022).

*Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav. Is widely distributed in Korea, Northeast China, Japan, Mongolia, and Siberia. Its root contains simple and furanocoumarins, which are probably responsible for its anti-inflammatory, analgesic, and antioxidative properties. It has been used in TCM, Kampo, and traditional Korean medicine to treat the common cold, rhinitis, headache, toothache, and some skin diseases (Zhao et al., 2022). The Korean Pharmacopoeia also mentions the root of *Angelica gigas* Nakai, which exhibits similar properties and is used to treat the common cold, headaches, neuralgia, arthralgia, and gynecological disorders (Jeong et al., 2015).

In China, the most known Angelica product is perhaps the root of *Angelica sinensis* (Oliv.) Diels, which is also traditionally used to treat gynecological disorders, such as amenorrhea and irregular periods. Its active constituents are phthalides, organic acids, and polysaccharides (Wei et al., 2016). *A. sinensis* was also used in Indonesian and Arabian traditional medicine, and the plant was probably transported to those countries *via* trade (Alqethami et al., 2020; Kusuma et al., 2022). *Angelica acutiloba* (Siebold & Zucc.) Kitag. Roots are sometimes used in TCM as a substitute for *A. sinensis* and in Kampo to treat menstrual disorders, amenorrhea, and premenstrual syndrome (Jeong et al., 2015). Chinese Pharmacopoeia also lists *Angelica anomala* Avé-Lall. As a treatment for gynecological disorders (He et al., 2012).

Apart from *A. dahurica* and *A. sinensis* roots, the Chinese Pharmacopoeia also lists *Angelica biserrata* (R.H.Shan & Yuan) C.Q.Yuan & R.H.Shan (syn. *Angelica pubescens* Maxim. f. *biserrata* R.H.Shan et C.Q.Yuan) roots, which are used in TCM to alleviate pain, fever, and rheumatism. Coumarins are their primary constituents and are probably responsible for their biological effects. The plant is widespread in Japan and present in mainland China and Taiwan at significantly lower levels but is not mentioned in the Japanese Pharmacopoeia (Ma et al., 2019; Wang et al., 2023). The common Latin name of the pharmacopoeial plant material *Angelicae pubescentis radix* may be misleading, as it applies to the roots of *A. biserrata*, which is sometimes considered an *A. pubescens* subspecies. *Angelica polymorpha* Maxim. Is mentioned in the Chinese Pharmacopoeia as a substitute for *A. pubescentis radix*.

Other species used in traditional oriental medicine include *Angelica decursiva* Franch. & Sav. (syn. *Peucedanum decursivum* Maxim.), which is also mentioned in the Japanese Pharmacopoeia for its anti-inflammatory effects (Zhao et al., 2012), and *Angelica tenuissima* Nakai, which is used to treat dental pain in Korean folk medicine (Kim and Song, 2011).

Angelica roots are sometimes used to treat T2DM. These include *Angelica furcijuga* Kitag., *Angelica shikokiana* Makino ex Y.Yabe, *Angelica keiskei* Koidz. and *Angelica japonica* var. *hirsutiflora* (Liu, Chao & Chuang) T.Yamaz. (Aulifa et al., 2022; Leu, Yann Lii et al., 2009; Mira

et al., 2013; Yoshikawa et al., 2006). In traditional medicines (Chinese or European) diabetes was described by the symptoms, which also depend on the stage of the disease, such as excessive thirst, hunger and urination. However, in the prediabetic state we also observed a chronic fatigue and weakness, a decrease in concentration ability, and an impairment in wound healing. In TCM the prediabetic stage is described as accompanied with nervousness, distention, and fullness in the chest and rib-side, while the middle stage principal symptoms are abdominal fullness and distention (Guo et al., 2014). Angelica species use in TCM, has been mentioned in treating some symptoms of diabetes: to promote blood circulation, in anti-diabetic prescriptions (*Angelica sinensis*), diabetes-induced skin ulcer (*Angelica dahurica*) (Li et al., 2004; Zao et al., 2022; ). Interestingly, the European *A. archangelica* has pleiotropic actions and is used as a general tonic and stimulant, in various GIT and biliary disorders (dyspepsia, flatulence, fullness) (Kaur and Bhatti 2021), however never described as strictly connected with diabetes symptoms.

Moreover, some *Angelica* L. root extracts were found to possess insulin secretagogue activity. (Leu, Y. L. et al., 2009) Osthole isolated from *Angelica pubescens* alleviated hyperglycemia in a mice model (Liang et al., 2009). In another study, Kim et al. discovered that Korean *Angelica reflexa* B.Y.Lee and some of its metabolites potentiate glucose-induced insulin secretion (Kim et al., 2023). Lastly, Park et al. screened over 1500 plant extracts for GPR119 agonistic actions, and *A. dahurica* root extract was the most active, and its effect on the modulation of insulin secretion was also established. The authors argued that coumarins may be responsible for this activity (Park et al., 2016).

There have been numerous attempts at assessing coumarins' antidiabetic potential, although the researchers mostly target  $\alpha$ -glucosidase (Channa Basappa et al., 2020; Hu et al., 2019; Sepehri et al., 2020). Single studies were conducted in vivo and on  $\beta$ -cell models to evaluate the protective and insulin secretion-modulating effects of coumarins. Umbelliferone (7-hydroxycoumarin)  $\beta$ -D-galactopyranoside was found to increase insulin levels in streptozotocin (STZ)-induced diabetic rats at doses ranging from 10 mg/kg to 40 mg/kg (Kumar et al., 2013). In another study, its aglycone (umbelliferone) induced a similar effect in alloxan-induced diabetic rats at 100 mg/kg (Ramu et al., 2016). Daphnetin (7,8-dihydroxycoumarin) was found to protect INS-1 cells from STZ-induced  $\beta$ -cell death and dysfunction (Vinayagam and Xu, 2017). Ahmed et al. studied 13 coumarin sulfur analogs isolated from *Clutia lanceolata* Forssk on murine pancreatic islets and found that 7 increased glucose-induced insulin secretion, although the concentration used in this study was relatively high (200  $\mu$ mol/L) (Ahmed et al., 2020). Another coumarin, scopoletin (7-hydroxy-6-methoxycoumarin), increased glucose-induced insulin secretion through  $K_{ATP}$  and VGCC channel-dependent pathways in INS-1 cells at concentrations below 20  $\mu$ mol/L (Park et al., 2022). Imperatorin was found to increase plasma GLP-1 levels and increase insulin secretion in STZ-induced diabetic rats at 3-10 mg/kg and to modulate glucose-induced insulin secretion in INS-1 cells at 0.1 mg/mL (370  $\mu$ mol/L) (Adebajo et al., 2009; Wang et al., 2017). Unfortunately, data on the

effect of coumarins on  $\beta$ -cells are scarce and mostly limited to high doses beyond bioavailability.

Pancreatic  $\beta$ -cells play a central role in glucose homeostasis by secreting insulin, a hormone that maintains glycemia within the physiological range (mainly by promoting glucose uptake and utilization by peripheral tissues). Moreover, the progressive decline in  $\beta$ -cell capacity to secrete insulin in response to glucose is a cardinal feature of the pathogenesis and evolution of type 2 diabetes (Rorsman and Ashcroft, 2018). Therefore, considering that some angelica representatives are used to manage diabetes and that medicinal plants may exhibit unknown pharmacological activities, which may pose a risk of undesirable effects, this study aimed to evaluate the effect of the angelica roots monographed in the European Pharmacopeia 11<sup>th</sup> edition on insulin secretion by pancreatic  $\beta$ -cells and to isolate compounds responsible for this activity. Four plant materials derived from angelica species that are monographed in the Ph. Eur. 11<sup>th</sup> and may be sold in Europe were phytochemically profiled and evaluated for insulin secretion in the INS-1  $\beta$ -cell line.

## 2 Material and methods

### 2.1 Plant material

Dried and shredded roots of *Angelica archangelica* L. were purchased from Nanga (Złotów, Poland, batch 13/09/21). Roots of *Angelica dahurica* (Hoffm.) Benth. & Hook.f. ex Franch. & Sav., *Angelica biserrata* (R.H.Shan & Yuan) C.Q.Yuan & R.H.Shan and *Angelica sinensis* (Oliv.) Diels. were purchased from Bozhou Zhongzheng Nature (Bozhou, China, batch 210625, 210611 and ### respectively). The plant material was authenticated according to the European Pharmacopoeia 11th ed. by Prof. Anna Karolina Kiss. Plant material was milled to powder and freeze-dried. All plant material was stored at -20 °C until further use.

### 2.2 Phytochemical reagents

Acetic acid, methanol, chloroform, dichloromethane, DMSO, ethyl acetate, formic acid, n-hexane and sodium acetate were purchased from POCh (Gliwice, Poland). Acetonitrile was purchased from Merck (Darmstadt, Germany). Water for HPLC experiments was prepared using the Milli-Q Plus system (Millipore, Billerica, MA, USA) (18.2 M $\Omega$  cm). All solvents used for chromatography were of gradient grade. Esculetin, imperatorin, (Z)-ligustilide, osthole, and scopoletin were purchased from Extrasynthese (Lyon, France). Oxypeucedanin was purchased from Phytolab GmbH (Vestenbergsgreuth, Germany). TLC 60G F<sub>254</sub> silica gel plates (20 × 20 cm) and silica gel (60-200  $\mu$ m) were purchased from Merck (Darmstadt, Niemcy). Deuterated solvents (methanol-d<sub>4</sub> and CDCl<sub>3</sub>) for NMR analysis were purchased from Glazer (Basel, Switzerland).

### 2.3 Pharmacology reagents

RPMI-1640 w/o L-glutamine and HEPES 1 M were purchased from Capricorn Scientific (Ebsdorfergrund, Germany). Sodium pyruvate (100 mM) and 2-mercaptoethanol (50 mM)

were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Fetal calf serum (FCS) was purchased from Eurobio Scientific (Les Ulis, France). Bovine serum albumin (BSA), Dulbecco's phosphate-buffered saline (DPBS) w/o CaCl<sub>2</sub> and MgCl<sub>2</sub>, trypsin-EDTA HBSS, poly-L-lysine hydrobromide, L-glutamine-penicillin–streptomycin solution, glucose, TWEEN 20, DMSO (sterile) and DMSO (molecular biology) were purchased from Sigma–Aldrich Chemie GmbH (Steinheim, Germany). An HTRF insulin ultrasensitive dosing kit was purchased from PerkinElmer (Waltham, MA, USA). HEPES was purchased from Carl Roth GmbH (Karlsruhe, Germany). Sodium bicarbonate (NaHCO<sub>3</sub>), monopotassium phosphate (KH<sub>2</sub>PO<sub>4</sub>), sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl<sub>2</sub>), magnesium sulfate heptahydrate (MgSO<sub>4</sub>×7H<sub>2</sub>O) and disodium phosphate (Na<sub>2</sub>HPO<sub>4</sub>) were purchased from Chempur (Piekary Śląskie, Poland).

#### 2.4 Extract preparation

Dried and shredded roots of *Angelica archangelica* (500 g), *Angelica dahurica* (500 g), and *Angelica biserrata* (500 g) were milled to powder and extracted with 2 L of n-hexane in a Soxhlet apparatus for 15 hours. The extract was filtered through filter paper and evaporated to dryness on a rotary evaporator, generating a residue of 14.4 g, 9.3 g and 13.0 g. These extracts were used for phytochemical profiling and coumarin isolation.

Dried and shredded roots of *Angelica archangelica* (5.0 g), *Angelica dahurica* (5.0 g), *Angelica biserrata* (5.0 g) and *Angelica sinensis* (5.0 g) were milled to powder and extracted with 50 mL of 60% (v/v) aqueous ethanol under reflux for 2 h. The extracts were then filtered through filter paper, reduced in a rotary evaporator, and lyophilized, resulting in dried ethanolic extracts. These extracts were used for pharmacological studies.

#### 2.5 Thin layer chromatography (TLC)

TLC analysis was performed on glass plates coated with 60G F<sub>254</sub> silica gel (Merck, Darmstadt., Germany). The plates were developed in a vertical chamber previously saturated with mobile phase for at least 15 minutes. The mobile phase was a mixture of n-hexane and ethyl acetate (85:15, v/v) or anhydrous acetic acid, ethyl acetate and toluene (1:10:90, v/v/v). The chromatogram was visualized and evaluated under UV light at  $\lambda_1=254$  nm and  $\lambda_2=365$  nm and afterward treated with a 10% (v/v) solution of sulfuric acid in methanol, heated at 100 °C for 5 minutes and examined in daylight.

#### 2.6 Column chromatography (CC)

Fractionation of plant extracts was performed on glass chromatography columns of different lengths and widths, including 60 x 2 cm and 65 x 5 cm. The columns were filled with silica gel (0.063–0.100 mm; Merck, Darmstadt., Germany). No external pressure was used (liquid flow under gravity). Fraction collection was volume-based, using the Foxy Jr. Fraction Collector (Teledyne ISCO, Lincoln NE, USA). Step gradients of chloroform–methanol, n-hexane–ethyl acetate and toluene–ethyl acetate were used depending on the polarity of the fraction constituents. The fractions were monitored and pooled based on their TLC profiles.

## 2.7 Preparative chromatography (HPLC-PREP)

Preparative HPLC was performed with a Shimadzu LC20-AP instrument (Shimadzu, Japan) using a Kinetex XB-C<sub>18</sub> column (150 mm × 21.2 mm, 5 μm) (Phenomenex, Torrance, CA, USA) at a flow rate of 20.0 mL/min and detection at λ<sub>1</sub>=254 nm and λ<sub>2</sub>=325 nm. The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using the following gradient: 0-60 min, 15-100% B. The injection volume was 400 μL. Fraction collection was based on set retention times.

## 2.8 Isolation of pure compounds

*Angelica archangelica* root n-hexane extract was subjected to silica gel column chromatography (65 × 5 cm) and eluted with a chloroform/methanol gradient (100:0 → 90:10) of 11 steps, 500 mL each, to obtain 100 fractions of 55 mL, which were pooled into 8 main fractions (AAH1–AAH8) based on their TLC profiles. From fraction AAH4 (1880 mg), **psoralen** (5.5 mg; 15.25–16.60 min), **bergapten** (11.7 mg; 18.75–20.20 min), **imperatorin** (5.0 mg; 24.25–25.5 min), **osthole** (112.2 mg; 28.70–30.30 min) and **isoimperatorin** (9.4 mg; 30.50–31.60 min) were isolated using preparative HPLC. Using the same technique, **oxypeucedanin hydrate** (5.5 mg; 12.40–13.40 min) and **isopimpinellin** (9.9 mg; 18.80–20.00 min) were isolated from fraction AAH5 (2570 mg). Fraction AAH6 (4050 mg) was rechromatographed on a silica gel column (60 × 2 cm) with an n-hexane/ethyl acetate gradient (90:10 → 75:25) of 4 steps, 750 mL each. One hundred forty-four fractions of 20 mL each were collected and pooled into 5 main fractions (AAH6A–AAH6E) based on their TLC profiles. Using preparative chromatography **isoimperatorin** (3.2 mg; 30.50–31.60 min) was purified from fraction AAH6B (320 mg), **imperatorin** (0.6 mg; 24.25–25.50 min), **osthole** (9.0 mg; 28.70–30.30 min), **phellopterin** (0.8 mg; 30.35–31.10 min), **archangelicin** (110.4 mg; 38.60–40.00 min) and **2'-angeloyl-3'-isovaleryl viginate** (97.2 mg; 40.10–41.75 min) were isolated from fraction AAH6C (820 mg) and **xanthotoxin** (9.3 mg; 16.40–18.50 min), **bergapten** (2.2 mg; 18.75–20.20 min), **osthenol** (22.1 mg; 20.50–22.20 min), **vaginidiol-O-angelate** (16.5 mg; 23.30–24.1 min), **imperatorin** (12.4 mg; 24.25–25.5 min) and **isoedultin** (24.5 mg; 31.80–33.20 min) were isolated from fraction AAH6D (1630 mg). Fraction AAH7 (2620 mg) was rechromatographed on a silica gel column (60 × 2 cm) with an n-hexane/ethyl acetate gradient (90:10 → 75:30) of 5 steps, 750 mL each. Four hundred fractions of 10 mL were collected and pooled into 6 main fractions (AAH7A–AAH7F). Preparative chromatography was used to obtain **angelicin** (64.0 mg; 16.00–16.70 min) from fraction AAH7B (80 mg), **isoedultin** (64.0 mg; 31.80–33.20 min) from fraction AAH7D (160 mg), **heraclenol-2'-O-angelate** (68.2 mg; 21.40–22.40 min), **byakangelicin-2'-O-angelate** (102.2 mg; 22.50–23.50 min) and **byakangelicin-2'-O-isovalerate** (14.4 mg; 23.90–24.70 min) from fraction AAH7F (680 mg).

*Angelica dahurica* root n-hexane extract was subjected to silica gel column chromatography (65 × 5 cm) and eluted with a chloroform/methanol gradient (100:0 → 50:50) over 26 steps (250 mL each) to generate 100 fractions of 65 mL, which were pooled into 4 main fractions (ADH1–ADH4) based on their TLC profiles. Preparative chromatography was used to obtain

**psoralen** (0.3 mg; 15.25–16.60 min), **bergapten** (13.1 mg; 18.75–20.20 min) and **isoimperatorin** (135.9 mg; 30.50–31.60 min) from fraction ADH1 (1000 mg) and **xanthotoxin** (1.2 mg; 16.40–18.50 min), **bergapten** (13.5 mg; 18.75–20.20 min), **imperatorin** (206.7 mg; 24.25–25.5 min), **cnidilin** (3.3 mg; 28.80–30.40 min) and **isoimperatorin** (12.1 mg; 30.50–31.60 min) from fraction ADH2 (970 mg). Fraction ADH3 (3900 mg) was rechromatographed on a silica gel column (60 × 2 cm) with an n-hexane/ethyl acetate gradient (100:0 → 70:30) of 7 steps, 750 mL each. Three hundred forty-eight 15 mL fractions were collected and pooled into 8 main fractions (ADH3A–ADH3H). Preparative chromatography was used to purify and isolate **phellopterin** (6.3 mg; 30.35–31.10 min) from fraction ADH3B (360 mg), **imperatorin** (173.7 mg; 24.25–25.5 min) and **cnidilin** (221.2 mg; 28.80–30.40 min) from fraction ADH3C (830 mg), and a mixture (3:1) of **neobyakangelicol** and **pabulenol** (10.6 mg; 19.90–21.50 min) and **byakangelicin** (5.5 mg; 11.50–12.40 min) from fraction ADH3F (130 mg).

*Angelica biserrata* root (*Angelicae pubescentis radix*) n-hexane extract was subjected to silica gel column chromatography (65 × 5 cm) and eluted with a chloroform/methanol gradient (100:0 → 70:30) over 16 steps (400 mL each) to obtain 100 fractions of 65 mL, which were pooled into 5 main fractions (APH1–APH5) based on their TLC profiles. Fraction APH4 (6320 mg) was rechromatographed on a silica gel column (60 × 2 cm) with a toluene/ethyl acetate gradient (99:1 → 70:30) of 14 steps, 500 mL each. Seven hundred fractions of 10 mL were collected and pooled into 11 main fractions (APH4A–APH4K). Preparative chromatography was used to purify and isolate **columbianadin** (108.9 mg; 32.45–33.60 min) from fraction APH4D, **columbianetin acetate** (30.7 mg; 21.6–22.5 min) from fraction APH4G (470 mg), **bisabolangelone** (23.7 mg; 20.65–21.75 min) from fraction APH4I (590 mg), and **columbianetin** (14.2 mg; 12.10–12.90 min) from fraction APH4J (20 mg).

## 2.9 Nuclear magnetic resonance (NMR)

<sup>1</sup>H, <sup>13</sup>C, and 2D NMR spectra (ROESY, COSY, HSQC, HMBC) were obtained on a Bruker Avance III 500 NMR spectrometer (Bruker BioSpin, Rheinstetten, Germany) operating at 500 and 126 MHz, respectively, using standard pulse programs and 5 mm NMR tubes. All measurements were performed at 295 K. Spectra were recorded in methanol-d<sub>4</sub> or in CDCl<sub>3</sub>. In each case, spectra were calibrated at residual solvent resonances at 3.31 ppm for <sup>1</sup>H and 49.15 ppm for <sup>13</sup>C (methanol-d<sub>4</sub>) or 7.24 ppm for <sup>1</sup>H and 77.23 ppm for <sup>13</sup>C (CDCl<sub>3</sub>-d<sub>6</sub>). All isolated compounds were characterized using NMR techniques.

## 2.10 Phytochemical characterization by LC-DAD-ESI-MS/MS

Liquid chromatography with a diode array detector coupled with electrospray ionization tandem mass spectrometry (LC-DAD-ESI-MS/MS) analysis was performed on a UHPLC-3000 RS system (Dionex, Dreieich, Germany) outfitted with a diode array detector (Dionex, Dreieich, Germany) coupled with an AmaZon SL ion trap mass spectrometer with an ESI interface (Bruker Daltonik GmbH, Bremen, Germany). Separation was performed on a Kinetex XB-C<sub>18</sub> analytical column (150 mm × 2.1 mm, 1.9 μm) (Phenomenex, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) using

the following gradient: 0–60 min, 15–100% B, and then 10 min of equilibration. Samples for LC-DAD-ESI-MS/MS analysis were prepared by dissolving dried extracts in 0.1% formic acid in methanol at a concentration of 10 mg/mL. Reference solutions were prepared in the same way at a concentration of 1 mg/mL. The flow rate was 0.2 mL/min, the injection volume was 5  $\mu$ L, and the column temperature was set at 25 °C. The LC eluate was introduced into the ESI interface without splitting, and compounds were analyzed in both positive and negative ion mode with the following settings: nebulizer pressure of 40 psi, drying gas flow rate of 9 L/min, nitrogen gas temperature of 300 °C, and capillary voltage of 4.5 kV. The mass scan ranged from 100 to 2200 m/z.

UV spectra were recorded in the range of 190–400 nm, and the compounds were identified based on the UV–Vis spectra. Then, their molecular mass and fragmentation profile were determined and compared to reference compounds and literature data.

### 2.11 Quantitative liquid chromatography

Quantitative analysis was performed according to the methods described in Ph. Eur. 11<sup>th</sup>. Root ethanolic extracts were weighed (50 mg), dissolved in 10 mL of methanol, transferred to a volumetric flask, diluted to 50.0 mL with methanol, and filtered through a 0.45  $\mu$ m membrane.

A reference solution of imperatorin was prepared by dissolving 5 mg of imperatorin in methanol and diluting to 50.0 mL. One milliliter of this solution was transferred to a 10 mL volumetric flask, diluted to 10.0 mL with methanol and filtered through a 0.45  $\mu$ m membrane, generating a 10  $\mu$ g/mL solution.

A reference solution of osthole was prepared by dissolving 5 mg of osthole in methanol, diluted to 100.0 mL in a volumetric flask, and filtered through a 0.45  $\mu$ m membrane, generating a 50  $\mu$ g/mL solution.

The quantitative analysis was performed using an apparatus equipped with low-pressure gradient pumps LC-20AD, a sampler SIL-20A, a column oven CTO-10AS set at 25 °C, and a DAD detector SPD-M20A (Shimadzu, Kyoto, Japan). Separation was performed according to the validated Ph. Eur. 11 methods on a Luna RP-C18 column (250 mm  $\times$  4.6 mm, 5  $\mu$ m) (Phenomenex, Torrance, CA, USA) at a flow rate of 1.0 mL/min (imperatorin) or 0.23 mL/min (osthole) and detection at  $\lambda_1=210$  nm (imperatorin),  $\lambda_2=322$  nm (osthole). The mobile phase for imperatorin quantification consisted of water (A) and acetonitrile (B) using the following gradient: 0–15 min, 55% B; 15–33 min, 55–95% B; 33–35 min, 95% B. The mobile phase for osthole quantification was a mixture of water and acetonitrile (40:60, v/v). The injection volume was 20  $\mu$ L (imperatorin) or 10  $\mu$ L (osthole). The LabSolutions system (Shimadzu, Kyoto, Japan) was used for operating procedures and calculation management and to provide complete quantitative data.

The total percentage content of imperatorin was calculated from the following expression:

$$\frac{A_1 \times m_2 \times p}{A_2 \times m_1 \times 10}$$

$A_1$  = area of the peak due to imperatorin in the chromatogram obtained with the test solution,

$A_2$  = area of the peak due to imperatorin in the chromatogram obtained with reference solution,

$m_1$  = mass of extract to be examined, in g.

$m_2$  = mass of imperatorin used to prepare reference solution, in g.

$p$  = percentage content of imperatorin in the reference compound.

The total percentage content of osthole was calculated from the following expression:

$$\frac{A_1 \times m_2 \times p \times 0.5}{A_2 \times m_1}$$

$A_1$  = area of the peak due to osthole in the chromatogram obtained with the test solution,

$A_2$  = area of the peak due to osthole in the chromatogram obtained with reference solution,

$m_1$  = mass of extract to be examined, in g.

$m_2$  = mass of osthole used to prepare reference solution, in g.

$p$  = percentage content of osthole in reference compound.

## 2.12 Preparation of compound and extract solutions

Compounds and extracts stored for future pharmacological studies were dissolved in sterile DMSO, filtered through a 0.20  $\mu\text{m}$  membrane, aliquoted and stored at  $-20\text{ }^\circ\text{C}$  until the experiment. In general, extracts were prepared to obtain a concentration of 10 mg/mL, and for compounds, a concentration of 20 mmol/L.

## 2.13 INS-1 cell culture

INS-1 cells (rat radiation-induced insulinoma cell line) were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated (30 min,  $56\text{ }^\circ\text{C}$ ) fetal calf serum, 100 U/mL penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin, 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L<sup>-1</sup> sodium pyruvate and 50  $\mu\text{mol}/\text{L}$  2-mercaptoethanol in a humidified atmosphere (5%  $\text{CO}_2$ ,  $37\text{ }^\circ\text{C}$ ). The cells used for experiments had a passage number from 33 to 39.

### 2.14 Insulin secretion experiments on INS-1 cells

INS-1 cells were seeded in 24-well plates precoated with poly-L-lysine ( $4 \times 10^5$  cells/well) and cultured for 5 days before insulin secretion experiments to obtain a cell confluence of approximately 80%. The cells were washed twice with HEPES-balanced Krebs-Ringer bicarbonate buffer (123 mmol/L NaCl, 5.4 mmol/L KCl, 1.3 mmol/L  $\text{KH}_2\text{PO}_4$ , 1.4 mmol/L  $\text{MgSO}_4$ , 2.9 mmol/L  $\text{CaCl}_2$ , 5 mmol/L  $\text{NaHCO}_3$  and 20 mmol/L HEPES, adjusted to pH 7.4 with NaOH) saturated with carbon dioxide (15 min) and supplemented with 1 g/L bovine serum albumin (KRB). Then, the cells were preincubated for 1 h (5%  $\text{CO}_2$ , 37 °C) in 500  $\mu\text{L}$  of KRB buffer containing 1.4 mmol/L glucose. Afterward, the buffer was discarded, and the cells were incubated for 1 h (5%  $\text{CO}_2$ , 37 °C) in 500  $\mu\text{L}$  of KRB buffer containing tested compounds or extracts in basal glucose conditions (1.4 mmol/L glucose) or stimulating glucose conditions (8.3 mmol/L glucose). To limit the measurement variations, each condition was carried out in triplicate on the same plate. Control treatments were always performed using the same amount of vehicle (DMSO) in each well.

At the end of the 1 h incubation period, the supernatants were sampled and stored at -20 °C until the insulin assay by homogeneous time-resolved fluorescence technology (HTRF) according to the manufacturer's instructions (PerkinElmer). Two anti-insulin antibodies were used, one labeled with  $\text{Eu}^{3+}$ -Cryptate (fluorescence donor) and one labeled with XL665 (fluorescence acceptor), which recognize distinct epitopes. When these two fluorophores bind to insulin molecules, the two antibodies come into proximity, allowing fluorescence resonance energy transfer (FRET) to occur between  $\text{Eu}^{3+}$ -Cryptate and XL665. This FRET increases proportionally with the insulin concentration.

### 2.15 Insulin secretion experiments on INS-1 cells

Cell viability was determined using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. INS-1 cells were seeded in 96-well plates pre-coated with poly-L-lysine ( $1 \times 10^5$  cells/well) and cultured for 4 days before experiments. Then, the cells were preincubated with tested compounds for 48h (5%  $\text{CO}_2$ , 37 °C) in culture medium. At the end of the 48 h preincubation period, cells were washed twice with KRB buffer and KRB containing 5 mg/mL MTT was added to each well. Plates were then incubated for 3 h in the dark in a humidified atmosphere (5%  $\text{CO}_2$ , 37 °C). Cells were washed with DPBS, and precipitates were dissolved in 50  $\mu\text{L}$  DMSO. The absorbance of the reduced intracellular formazan product was read at 492 nm on a microtiter plate reader (Tecan, Lyon, France). Experiments were performed in quadruplicate.

### 2.16 Data and statistical analysis

The data and statistical analyses comply with the recommendations on experimental design and analysis in pharmacology (Curtis et al., 2022). All data were analyzed using GraphPad Prism v9 software (GraphPad Software, San Diego, USA). All averaged data are expressed as the mean  $\pm$  standard error of the mean (SEM). The group size (n) represents the number of

experimental independent repeats, each carried out with distinct biological preparations. Group sizes suitable for each set of experiments were estimated to ensure adequate power and to detect a prespecified effect based on the available literature, on pilot studies, and on previous reports (Bayle et al., 2019; Peraldi-Roux et al., 2022; Toubal et al., 2020). To ensure the reliability of single values, technical triplicates were used for insulin secretion experiments.

Statistical analyses were run only on group size of  $n$  independent samples  $\geq 3$ , as follows. First, each dataset was tested for normality (Shapiro–Wilk test,  $p > 0.05 =$  passed) and for variance homogeneity (Brown-Forsythe test,  $p > 0.05 =$  passed). Parametric statistical tests (ANOVAs) were used only if the data were normally distributed and no inhomogeneity of variance was observed. If only one dataset failed the Shapiro–Wilk test, nonparametric analyses were performed using Kruskal–Wallis tests. Comparisons between multiple sets of data were performed by one-way ANOVA or Kruskal–Wallis tests with no matching. Holm–Sidak or Dunn’s post hoc comparisons were conducted only if ANOVA or Kruskal–Wallis tests, respectively, achieved statistical significance ( $p < 0.05 =$  passed). The limit of statistical significance was set at  $p < 0.05$  and indicated by an asterisk \*.

### 3 Results

#### 3.1 TLC analysis of angelica roots

*Angelicae archangelicae radix*, *Angelicae dahuricae radix*, *Angelicae pubescentis radix* and *Angelicae sinensis radix* are pharmacopeial plant materials coming from the roots of distinct angelica species (i.e., *A. archangelica*, *A. dahurica*, *A. biserrata* and *A. sinensis*, respectively). Although the metabolite content of these plant materials is somewhat varied, as they are standardized for the content of different secondary metabolites, Ph. Eur. 11<sup>th</sup> provides a common TLC method for their characterization based on the presence or absence of three compounds – osthole, imperatorin and (Z)-ligustilide. TLC analysis confirmed the presence of osthole and imperatorin in *A. archangelica* roots and excluded the presence of (Z)-ligustilide. Similar chromatogram, with some distinct zones (described in Ph. Eur. 11<sup>th</sup>) was observed for *A. biserrata* root, although the zone for imperatorin was hardly observable due to the low content of this coumarin in this plant material (its presence is not mandatory for confirmation of the origin of this root). For *A. dahurica* root, only the zone for imperatorin was observed, and for *A. sinensis* root, the presence of (Z)-ligustilide was confirmed. These findings were later confirmed by LC-DAD-ESI-MS/MS analysis.

#### 3.2 Phytochemical characterization of angelica root extracts

Furthermore, for a more comprehensive analysis of angelica roots from different species, LC-DAD-ESI-MS/MS analysis was performed, which was supported by the isolation and characterization of some compounds through NMR analysis. Through this analysis, we identified or partly identified 41 compounds. Apart from one phthalide and one sesquiterpene, most compounds were simple or furanocoumarins. The metabolite profiles of

the studied angelica roots are shown in Table 1, and chromatograms obtained at 254 nm are presented in Figure 2.

Table 1. Retention time, UV, and MS/MS data of the compounds identified in *Angelicae* roots.

Compounds	Tr [min]	UV <sub>max</sub> [nm]	Extracted ion [m/z]	Fragment ions [m/z]	Species	Ref.
1. Scopoletin*	9.5	225, 342	193 [M+H] <sup>+</sup> 215 [M+Na] <sup>+</sup>	137	AA	(Tian et al., 2022)
2. Unknown coumarin	12.4	220, 339	179 [M+H] <sup>+</sup> 201 [M+Na] <sup>+</sup> 379 [2M+Na] <sup>+</sup>	147	AA	(Tian et al., 2022)
3. Pabularinone	15.5	264, 306	287 [M+H] <sup>+</sup>		AD	(Shi et al., 2022)
4. Columbianetin**	16.8	207, 218sh, 260, 328	247 [M+H] <sup>+</sup> 269 [M+Na] <sup>+</sup> 493 [2M+H] <sup>+</sup> 515 [2M+Na] <sup>+</sup>	175	AB	(Tian et al., 2022; Wang et al., 2014)
5. Oxypeucedanin hydrate**	17.1	220, 266sh, 311	305 [M+H] <sup>+</sup> 327 [M+Na] <sup>+</sup> 631 [2M+Na] <sup>+</sup>	203 286, 267	AA, AD	(Shi et al., 2022; Wszelaki et al., 2011)
6. Byakangelicin**	17.8	219, 268, 314	335 [M+H] <sup>+</sup> 357 [M+Na] <sup>+</sup> 691 [2M+Na] <sup>+</sup>		AD	(Shi et al., 2022)
7. Psoralen**	19.9	217, 243, 294, 325sh	187 [M+H] <sup>+</sup> 209 [M+Na] <sup>+</sup> 395 [2M+Na] <sup>+</sup>		AA, AD, AB	(Shi et al., 2022; Tian et al., 2022)
8. Angelicin**	20.7	246, 300	187 [M+H] <sup>+</sup> 209 [M+Na] <sup>+</sup> 395 [2M+Na] <sup>+</sup>		AA	
9. Angelol A/Angelol D/Angelol G/Isoangelol A	20.9	204sh, 219, 328	377 [M+H] <sup>+</sup> 399 [M+Na] <sup>+</sup> 775 [2M+Na] <sup>+</sup>		AB	(Wang et al., 2014)
10. Xanthotoxin**	21.3	217, 245, 301	217 [M+H] <sup>+</sup> 239 [M+Na] <sup>+</sup> 433 [2M+H] <sup>+</sup> 455 [2M+Na] <sup>+</sup>		AA, AD, AB	(Shi et al., 2022; Tian et al., 2022; Wszelaki et al., 2011)
11. Angelol A/Angelol D/Angelol G/Isoangelol A	21.3	219, 247sh, 263sh, 302	377 [M+H] <sup>+</sup> 399 [M+Na] <sup>+</sup> 775 [2M+Na] <sup>+</sup>		AB	(Tian et al., 2022; Wang et al., 2014)
12. Angelol A/Angelol D/Angelol G/Isoangelol A	23.2	220, 326	377 [M+H] <sup>+</sup> 399 [M+Na] <sup>+</sup> 775 [2M+Na] <sup>+</sup>		AB	(Wang et al., 2014)
13. Angelol C	23.7	220, 268, 317	379 [M+H] <sup>+</sup> 401 [M+Na] <sup>+</sup>	361, 347, 278, 259, 219, 207	AB	(Wang et al., 2014)
14. Bergapten**	23.8	220, 249, 267, 312	217 [M+H] <sup>+</sup> 239 [M+Na] <sup>+</sup> 433 [2M+H] <sup>+</sup> 455 [2M+Na] <sup>+</sup>		AA, AD, AB	(Shi et al., 2022; Tian et al., 2022; Wszelaki et al., 2011)
15. Isopimpinellin**	24.0	221, 249, 268, 313	247 [M+H] <sup>+</sup> 269 [M+Na] <sup>+</sup> 493 [2M+H] <sup>+</sup> 515 [2M+Na] <sup>+</sup>		AA, AD	(Shi et al., 2022; Wszelaki et al., 2011)
16. Suberenol	24.9	217, 261, 325	261 [M+H] <sup>+</sup> 283 [M+Na] <sup>+</sup> 543 [2M+Na] <sup>+</sup>	243, 217, 189	AB	(Tian et al., 2022)

17. Pabulenol	25.1	220, 267, 313	287 [M+H] <sup>+</sup> 309 [M+Na] <sup>+</sup> 595 [2M+Na] <sup>+</sup>	224	AD	(Shi et al., 2022; Tian et al., 2022)
18. Neobyakangelicol**	25.1	220, 267, 313	317 [M+H] <sup>+</sup> 339 [M+Na] <sup>+</sup>	224	AD	(Shi et al., 2022)
19. Osthenol**	25.7	256, 325	231 [M+H] <sup>+</sup> 253 [M+Na] <sup>+</sup> 483 [2M+Na] <sup>+</sup>	201, 189, 175	AA	(Tian et al., 2022; Wszelaki et al., 2011)
20. Bisabolangelone**	26.1	247	271 [M+Na] <sup>+</sup> 519 [2M+Na] <sup>+</sup>		AB	
21. Byakangelicol	27.4	221, 268, 312	317 [M+H] <sup>+</sup> 339 [M+Na] <sup>+</sup> 633 [2M+H] <sup>+</sup> 655 [2M+Na] <sup>+</sup>	299, 233	AD	(Shi et al., 2022)
22. Oxypeucedanin*	27.6	220, 248, 308	287 [M+H] <sup>+</sup> 309 [M+Na] <sup>+</sup> 573 [2M+H] <sup>+</sup> 595 [2M+Na] <sup>+</sup>	203	AA, AD	(Shi et al., 2022; Wszelaki et al., 2011)
23. Columbianetin acetate**	27.8	202, 259, 326	289 [M+H] <sup>+</sup> 311 [M+Na] <sup>+</sup> 577 [2M+H] <sup>+</sup> 599 [2M+Na] <sup>+</sup>	187	AB	(Tian et al., 2022; Wang et al., 2014)
24. Heraclenol-2'-O-angelate**	28.9	217, 247, 301	387 [M+H] <sup>+</sup> 409 [M+Na] <sup>+</sup> 795 [2M+Na] <sup>+</sup>	369, 269, 203 342, 225, 207, 185	AA	(Wszelaki et al., 2011)
25. Byakangelicin-2'-O-angelate**	30.7	220, 246sh, 267, 309	417 [M+H] <sup>+</sup> 439 [M+Na] <sup>+</sup> 855 [2M+Na] <sup>+</sup>	399, 299, 231, 167	AA	(Wszelaki et al., 2011)
26. Vaginidiol-2'-O-angelate**	32.1	220, 267, 313	345 [M+H] <sup>+</sup> 367 [M+Na] <sup>+</sup>		AA	
27. Byakangelicin-2'-O-isovalerate**	32.6	221, 269, 313	419 [M+H] <sup>+</sup> 441 [M+Na] <sup>+</sup> 859 [2M+Na] <sup>+</sup>	383, 339, 255, 239, 209, 187	AA	(Wszelaki et al., 2011)
28. Imperatorin**	33.2	218, 247, 261sh, 301	271 [M+H] <sup>+</sup> 293 [M+Na] <sup>+</sup> 536 [2M+Na] <sup>+</sup>	203	AA, AD, AB	(Shi et al., 2022; Tian et al., 2022; Wszelaki et al., 2011)
29. (Z)-Ligustilide*	33.9	281, 324, 350	191 [M+H] <sup>+</sup> 381 [2M+H] <sup>+</sup>	173	AS	(Wei et al., 2016)
30. Osthole**	34.8	203, 221, 324	245 [M+H] <sup>+</sup> 267 [M+Na] <sup>+</sup> 489 [2M+H] <sup>+</sup> 511 [2M+Na] <sup>+</sup>	189	AA, AB	(Tian et al., 2022; Wszelaki et al., 2011)
31. Cnidilin**	34.9	222, 269, 313	301 [M+H] <sup>+</sup> 323 [M+Na] <sup>+</sup> 623 [2M+Na] <sup>+</sup>	233	AD	(Shi et al., 2022; Tian et al., 2022)
32. Phellopterin**	36.0	221, 268, 312	301 [M+H] <sup>+</sup> 323 [M+Na] <sup>+</sup> 623 [2M+Na] <sup>+</sup>	254	AA, AD	(Shi et al., 2022; Tian et al., 2022; Wszelaki et al., 2011)
33. Isoimperatorin**	36.4	221, 249sh, 269sh, 310	271 [M+H] <sup>+</sup> 293 [M+Na] <sup>+</sup> 563 [2M+Na] <sup>+</sup>	259, 224, 203	AA, AD, AB	(Shi et al., 2022; Wszelaki et al., 2011)
34. Suberosin	36.9	221, 330	245 [M+H] <sup>+</sup> 267 [M+Na] <sup>+</sup> 511 [2M+Na] <sup>+</sup>		AD	(Shi et al., 2022)
35. Columbianadin I	37.0	210, 259sh, 326	329 [M+H] <sup>+</sup> 351 [M+Na] <sup>+</sup>	229, 187	AB	(Tian et al., 2022; Wang et al., 2014)

			657 [2M+H] <sup>+</sup> 679 [2M+Na] <sup>+</sup>			
36. Columbianadin II**	37.3	210, 259sh, 326	329 [M+H] <sup>+</sup> 351 [M+Na] <sup>+</sup> 657 [2M+H] <sup>+</sup> 679 [2M+Na] <sup>+</sup>	229, 187	AB	(Tian et al., 2022; Wang et al., 2014)
37. Dihydrocolumbianadin I	37.8	219, 260sh, 326	331 [M+H] <sup>+</sup> 353 [M+Na] <sup>+</sup> 661 [2M+H] <sup>+</sup> 683 [2M+Na] <sup>+</sup>	229, 187	AB	(Wang et al., 2014)
38. Dihydrocolumbianadin II	38.1	219, 260sh, 326	331 [M+H] <sup>+</sup> 353 [M+Na] <sup>+</sup> 661 [2M+H] <sup>+</sup> 683 [2M+Na] <sup>+</sup>		AB	(Wang et al., 2014)
39. Isoedultin**	38.3	218, 321	409 [M+Na] <sup>+</sup> 795 [2M+Na] <sup>+</sup>		AA	
40. Archangelicin**	45.3	202, 217, 321	449 [M+Na] <sup>+</sup> 875 [2M+Na] <sup>+</sup>		AA	(Wszelaki et al., 2011)
41. 2'-Angeloyl-3'-isovaleryl vaginate**	47.1	202, 217, 321	451 [M+Na] <sup>+</sup> 879 [2M+Na] <sup>+</sup>		AA	(Wszelaki et al., 2011)

AA – *Angelica archangelica*; AD – *Angelica dahurica*; AB – *Angelica biserrata*; AS – *Angelica sinensis*. \* compared with the reference standard; \*\* isolated from angelica root and identified by NMR.

Compound **1** ( $t_r = 9.5$  min) exhibited pseudomolecular ions at  $m/z$  193 [M+H]<sup>+</sup> and  $m/z$  215 [M+Na]<sup>+</sup>. It was assigned as scopoletin based on comparison of UV, retention time and MS signals with reference compound. Compound **2** ( $t_r = 12.4$  min) exhibited pseudomolecular ions at  $m/z$  179 [M+H]<sup>+</sup>,  $m/z$  201 [M+Na]<sup>+</sup> and  $m/z$  379 [2M+Na]<sup>+</sup>. Its retention time was compared with that of a reference standard for esculetin, but the times did not match. Thus, compound **2** was assigned as an unknown coumarin.

Compounds **3** ( $t_r = 15.5$  min), **17** ( $t_r = 25.1$  min) and **22** ( $t_r = 27.6$  min) exhibited pseudomolecular ions at  $m/z$  287 [M+H]<sup>+</sup>,  $m/z$  309 [M+Na]<sup>+</sup>,  $m/z$  573 [2M+H]<sup>+</sup> and  $m/z$  595 [2M+Na]<sup>+</sup>. Based on the elution order and comparison with reference standards, compound **3** was assigned as pabularinone, compound **17** as pabulenol and compound **22** as oxypeucedanin.

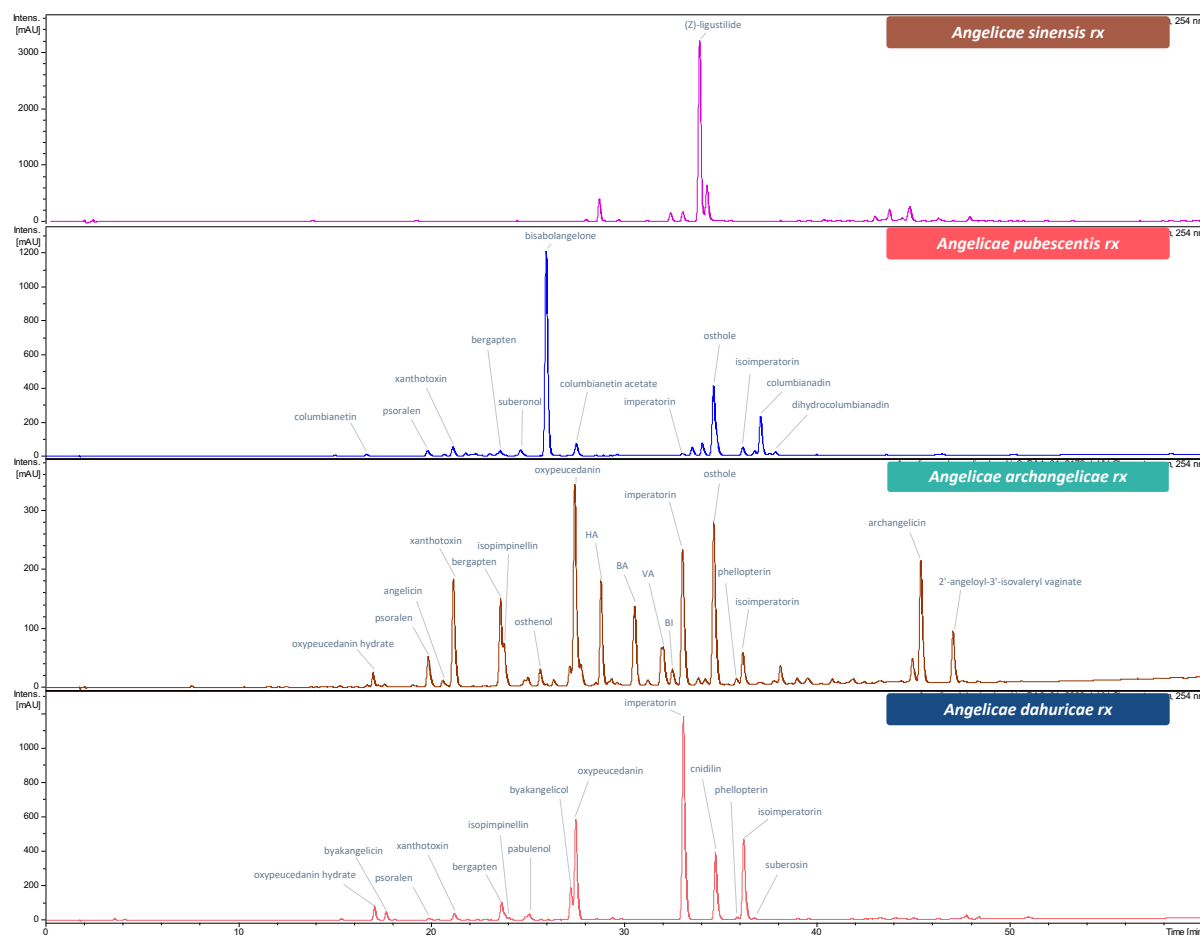


Figure 2. Chromatograms of angelica root n-hexane extracts obtained at 254 nm.

HA – heraclenol-2'-O-angelate; BA – byakangelicin-2'-O-angelate; VA – vaginidiol-O-angelate; BI – byakangelicin-2'-O-isovalerate.

Compounds **4** ( $t_r = 16.8$  min) and **15** ( $t_r = 24.0$  min) exhibited pseudomolecular ions at  $m/z$  247  $[M+H]^+$ ,  $m/z$  269  $[M+Na]^+$ ,  $m/z$  493  $[2M+H]^+$  and  $m/z$  515  $[2M+Na]^+$ . These compounds were compared to reference standards and identified; compound **4** was assigned as an angular furanocoumarin columbianetin, and compound **15** was assigned as a linear furanocoumarin isopimpinellin.

Compound **5** ( $t_r = 17.1$  min) exhibited pseudomolecular ions at  $m/z$  305  $[M+H]^+$ ,  $m/z$  327  $[M+Na]^+$  and  $m/z$  631  $[2M+Na]^+$  and was tentatively identified as oxypeucedanin hydrate. Similarly, compound **6** ( $t_r = 17.8$  min) exhibited its pseudomolecular ions at  $m/z$  335  $[M+H]^+$ ,  $m/z$  357  $[M+Na]^+$  and at  $m/z$  691  $[2M+Na]^+$ . It was tentatively assigned as byakangelicin.

Compounds **7** ( $t_r = 19.9$  min) and **8** ( $t_r = 20.7$  min) exhibited pseudomolecular ions at  $m/z$  287  $[M+H]^+$ ,  $m/z$  209  $[M+Na]^+$  and  $m/z$  396  $[2M+Na]^+$ . The two obvious candidates, linear furanocoumarin psoralen and angular furanocoumarin angelicin, could not be distinguished through the UV and MS spectra. Both compounds were assigned as psoralen and angelicin by comparison with reference standards.

Compounds **9** ( $t_r = 20.9$  min), **11** ( $t_r = 21.3$  min) and **12** ( $t_r = 23.2$  min) exhibited pseudomolecular ions at  $m/z$  377  $[M+H]^+$ ,  $m/z$  399  $[M+Na]^+$  and  $m/z$  775  $[2M+Na]^+$  and are 4 compounds previously described in *Angelica* species that could give these signals, namely, angelol A, angelol D, angelol G and isoangelol A. The three signals together were assigned as angelol A, angelol D, angelol G or isongelol A.

Compounds **10** ( $t_r = 21.3$  min) and **14** ( $t_r = 23.8$  min) exhibited pseudomolecular ions at  $m/z$  217  $[M+H]^+$ ,  $m/z$  239  $[M+Na]^+$ ,  $m/z$  433  $[2M+H]^+$  and  $m/z$  455  $[2M+Na]^+$ . The two obvious candidates, 5-*O*-methoxy- or 8-*O*-methoxy-substituted psoralen, could not be distinguished through the UV and MS spectra. Both compounds were assigned by comparison with reference standards as xanthotoxin (methoxalen) and bergapten.

Compound **13** ( $t_r = 23.7$ ) exhibited pseudomolecular ions at  $m/z$  379  $[M+H]^+$  and at  $m/z$  401  $[M+Na]^+$ . The  $[M+H]^+$  ion fragmented into ions at  $m/z$  361  $[M+H-18]^+$  from the loss of  $H_2O$  and a few minor ions at  $m/z$  347, 278, 259, 219 and 207. Through comparison with literature data, it was assigned as angelol C.

Compound **16** ( $t_r = 24.9$  min) exhibited pseudomolecular ions at  $m/z$  261  $[M+H]^+$ ,  $m/z$  283  $[M+Na]^+$  and  $m/z$  543  $[2M+Na]^+$ . The  $[M+H]^+$  ion fragmented into ions at  $m/z$  243  $[M+H-18]^+$  from the loss of  $H_2O$ ,  $m/z$  217  $[M+H-44]^+$  and at  $m/z$  189  $[M+H-72]^+$ . Based on literature data, it was assigned as suberenol.

Compounds **18** ( $t_r = 25.1$  min) and **21** ( $t_r = 27.4$  min) exhibited pseudomolecular ions at  $m/z$  317  $[M+H]^+$  and  $m/z$  339  $[M+Na]^+$ , with the latter also exhibiting ions at  $m/z$  633  $[2M+H]^+$  and  $m/z$  655  $[2M+Na]^+$ . The identity of these two compounds was later confirmed through isolation and NMR analysis. Compound **18** was identified as neobyakangelicol, while compound **21** rapidly degraded after isolation, giving signals and ions typical for byakangelicin (**6**). Considering that the cause of this degradation could be hydrolysis of the oxirane group in acidic eluent and judging from the product, compound **21** was assigned as byakangelicol.

Compound **19** ( $t_r = 25.7$  min) exhibited pseudomolecular ions at  $m/z$  231  $[M+H]^+$ , 253  $[M+Na]^+$  and  $m/z$  483  $[2M+Na]^+$  and was tentatively identified as osthénol.

Compound **20** ( $t_r = 26.1$  min) exhibited pseudomolecular ions at  $m/z$  271  $[M+Na]^+$  and  $m/z$  519  $[2M+Na]^+$ . It was isolated from *A. biserrata* roots and identified through NMR analysis as bisabolangelone, which was the only sesquiterpene found.

Compound **23** ( $t_r = 27.8$  min) exhibited pseudomolecular ions at  $m/z$  289  $[M+H]^+$ ,  $m/z$  311  $[M+Na]^+$ ,  $m/z$  577  $[2M+Na]^+$  and  $m/z$  599  $[2M+Na]^+$  and was tentatively assigned as columbianetin acetate.

Compounds **24** ( $t_r = 28.9$  min) and **39** ( $t_r = 38.3$  min) exhibited pseudomolecular ions at  $m/z$  409  $[M+Na]^+$  and  $m/z$  795  $[2M+Na]^+$ , with the former exhibiting an ion at  $m/z$  387  $[M+H]^+$ .

Both compounds were isolated from the roots of *A. archangelica* roots and identified through NMR analysis as heraclenol-2'-*O*-angelate (**24**) and isoedultin (**39**).

Compound **25** ( $t_r = 30.7$  min) exhibited its pseudomolecular ions at  $m/z$  417  $[M+H]^+$ ,  $m/z$  439  $[M+Na]^+$  and  $m/z$  855  $[2M+Na]^+$ . Based on literature data, it was tentatively identified as byakangelicin-2'-*O*-angelate.

Compound **26** ( $t_r = 32.1$  min) exhibited its pseudomolecular ions at  $m/z$  345  $[M+H]^+$  and at  $m/z$  367  $[M+Na]^+$ . The compound was isolated and assigned as vaginidiol-2'-*O*-angelate based on NMR analysis.

Compound **27** ( $t_r = 32.6$  min) exhibited its pseudomolecular ions at  $m/z$  429  $[M+H]^+$ ,  $m/z$  442  $[M+Na]^+$  and  $m/z$  859  $[2M+Na]^+$ . Based on literature data, the compound was tentatively identified as byakangelicin-2'-*O*-isovalerate.

Compounds **28** ( $t_r = 33.2$  min) and **33** ( $t_r = 36.4$  min) exhibited pseudomolecular ions at  $m/z$  271  $[M+H]^+$ ,  $m/z$  293  $[M+Na]^+$  and  $m/z$  536  $[2M+Na]^+$ , both producing fragment ions from  $[M+H]^+$  at  $m/z$  203. The peaks represented two major coumarins in *A. archangelica* and *A. dahurica* roots, with peak **28** being significantly more abundant. Based on the elution order and comparison with reference standards, compound **28** was identified as imperatorin (8-*O*-isoprenyloxypsoralen), and compound **33** was identified as isoimperatorin (5-*O*-isoprenyloxypsoralen).

Peak **29** ( $t_r = 33.9$  min), which was present only in *A. sinensis* root, showed UV spectra different from those of coumarins, with  $UV_{max}$  values at 281, 324 and 350 nm. The compound exhibited pseudomolecular ions at  $m/z$  191  $[M+H]^+$  and at  $m/z$  381  $[2M+H]^+$ . Based on literature data and comparison with the reference standard, it was assigned as (*Z*)-ligustilide.

Compounds **30** ( $t_r = 34.8$  min) and **34** ( $t_r = 36.9$  min) exhibited their pseudomolecular ions at  $m/z$  245  $[M+H]^+$ ,  $m/z$  267  $[M+Na]^+$  and at  $m/z$  511  $[2M+Na]^+$ , with the former additionally exhibiting a  $[2M+H]^+$  ion at  $m/z$  489. Based on elution order and comparison with reference standards, compound **30** was identified as osthole, and compound **34** was identified as suberosin. The characteristic absence of osthole in *A. dahurica* roots was also observed.

Compounds **31** ( $t_r = 34.9$  min) and **32** ( $t_r = 36.0$  min) exhibited pseudomolecular ions at  $m/z$  301  $[M+H]^+$ ,  $m/z$  323  $[M+Na]^+$  and  $m/z$  623  $[2M+Na]^+$ . Their retention times and UV spectra were similar. Compound **31** was identified as cnidilin (5-*O*-isoprenyloxy-8-*O*-methoxypsoralen), and compound **32** was identified as phellopterin (8-*O*-isoprenyloxy-5-*O*-methoxypsoralen).

Compounds **35** ( $t_r = 37.0$  min) and **36** ( $t_r = 37.3$  min) exhibited pseudomolecular ions at  $m/z$  329  $[M+H]^+$ ,  $m/z$  351  $[M+Na]^+$ ,  $m/z$  661  $[2M+H]^+$ , and  $m/z$  683  $[2M+Na]^+$ . Their retention times and UV spectra were similar. They were tentatively assigned as enantiomers of columbianadin. Similarly, compounds **37** ( $t_r = 37.8$  min) and **38** ( $t_r = 38.1$  min) exhibited ions 2

Da higher, which could be explained by the presence of an isovaleryl moiety instead of an angeloyl moiety. Thus, the compounds were tentatively assigned as enantiomers of dihydrocolumbianadin.

Compound **40** ( $t_r = 45.3$  min) exhibited its pseudomolecular ions at  $m/z$  449  $[M+Na]^+$  and at  $m/z$  875  $[2M+Na]^+$ . It was tentatively assigned as archangelicin.

Compound **41** ( $t_r = 47.1$  min) exhibited its pseudomolecular ions at  $m/z$  451  $[M+Na]^+$  and at  $m/z$  879  $[2M+Na]^+$ . It differed only by 2 Da from compound **40**, presenting similar UV spectra and retention times. The compound was tentatively assigned as 2'-angeloyl-3'-isovaleryl vaginate, which is an archangelicin with an angeloyl moiety at 3' replaced with an isovaleryl moiety. This was later confirmed by NMR analysis of an isolate from *A. archangelica*.

### 3.3 Qualitative analysis of imperatorin and osthole content

Ethanollic root extracts of *A. archangelica*, *A. dahurica*, *A. sinensis* and *A. biserrata* were subjected to HPLC-UV chromatography to quantify their prenylated coumarin content according to Ph. Eur. 11<sup>th</sup> (Table 2).

Table 2. Imperatorin and osthole content in Angelica roots.

Root source	Imperatorin content [% of extract]	Osthole content [% of extract]
<i>Angelica archangelica</i>	3.2 ± 0.68	2.4 ± 1.2
<i>Angelica dahurica</i>	2.2 ± 0.12	n/d
<i>Angelica biserrata</i>	n/d	0.73 ± 0.020
<i>Angelica sinensis</i>	n/d	n/d

The values are represented as the mean ± SEM, n = 3; n/d – peak not detected in extract.

As expected imperatorin and osthole were not detected in the ethanolic extract from *A. sinensis* roots. *A. archangelica* root extract was the richest in imperatorin (3.2 ± 0.68%) and osthole (2.4 ± 1.2%). *A. dahurica* extract contained significant amounts of imperatorin (2.2 ± 0.12%), a compound used for the standardization of this plant material. Consistent with TLC and LC–MS analysis, no osthole peak was observed, which is another characteristic property used to identify Dahurian angelica root. The osthole content was quantified for *A. biserrata* roots, reaching 0.73 ± 0.020%. Although traces of imperatorin were detected in *A. biserrata* root in TLC and LC–MS analysis, its peak was not observed in this analysis.

### 3.4 Angelica root extracts modulate insulin secretion

Ethanollic root extracts of four pharmacopeial plants from the genus *Angelica* L., namely, *A. archangelica*, *A. dahurica*, *A. sinensis* and *A. biserrata*, were studied for their potential to modulate insulin secretion in vitro on the INS-1  $\beta$ -cell line. To this aim, INS-1 cells were exposed to these extracts (concentrations ranging from 1.6 to 50.0  $\mu\text{g/mL}$ ) for 1 h under glucose at a stimulating concentration (8.3 mmol/L), and then insulin secretion was quantified. This intermediate stimulating concentration of glucose was chosen because it prevents the system from becoming saturated, which could conceal a pharmacological activity [41].

The stimulating effect of glucose was observed relative to the basal condition (1.4 mmol/L glucose) by a 2.5-fold increase in insulin secretion. After one hour of incubation, the insulin concentration in the cell supernatant treated with 1.4 mmol/L glucose was  $22.43 \pm 1.061$  ng/mL, while in the supernatants treated with 8.3 mmol/L glucose, the concentration was  $55.75 \pm 2.432$  ng/mL (Figure 3).

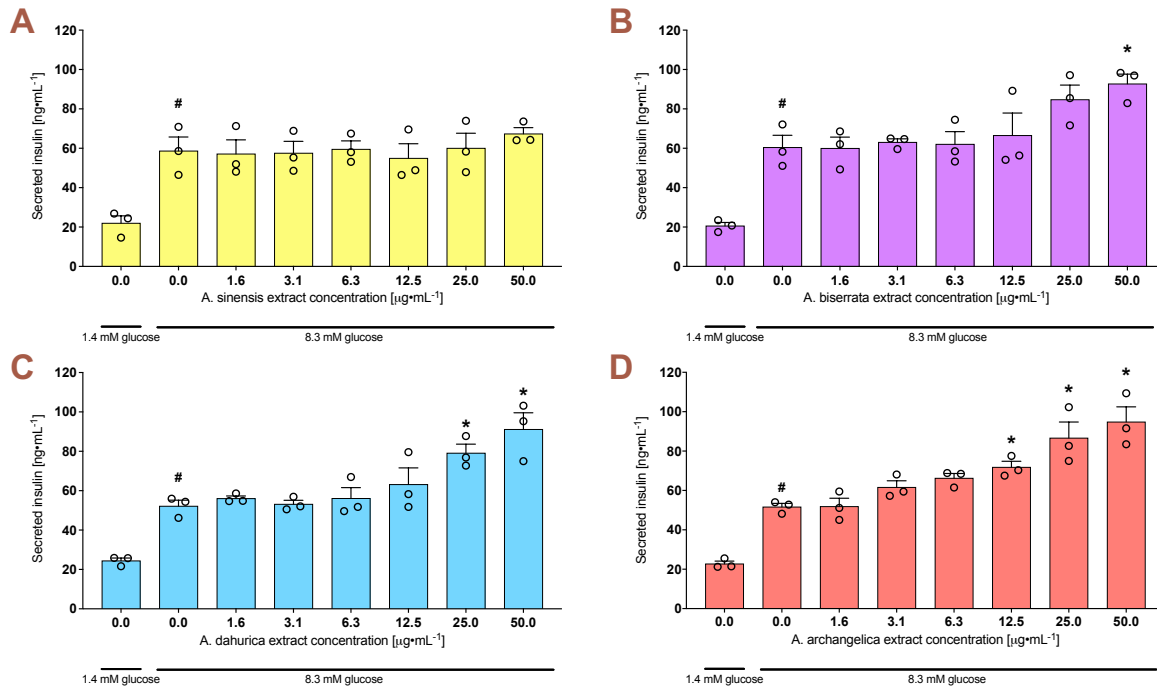


Figure 3. Modulation of insulin secretion in glucose-stimulating conditions after a 1 h incubation with angelica root extracts.

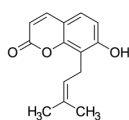
The bar graphs depict the mean  $\pm$  SEM of insulin secretion in ng/mL; (A) – *A. sinensis* root extract; (B) – *A. biserrata* root extract; (C) – *A. dahurica* root extract; (D) – *A. archangelica* root extract; \* $p < 0.05$  different from 0.0  $\mu\text{g/mL}$  of extract in 8.3 mmol/L glucose (#) with one-way ANOVA followed by the Holm–Sidak test,  $n = 3$ .

As expected, 8.3 mmol/L glucose significantly increased insulin secretion. *A. sinensis* root extract could not modulate glucose-induced insulin secretion at any concentration (Figure 3A), while an increase in insulin secretion for certain concentrations of the three other extracts was observed (Figure 3B–D). A significant increase in 8.3 mmol/L glucose-induced insulin secretion was observed for 50.0  $\mu\text{g/mL}$  of *A. biserrata* extract (secreted insulin of  $92.74 \pm 4.915$  vs.  $60.47 \pm 6.162$  ng/mL in the presence and absence of extract, respectively) (Figure 3B), for 25.0 and 50.0  $\mu\text{g/mL}$  of *A. dahurica* extract (secreted insulin of  $79.14 \pm 4.480$  and  $91.16 \pm 8.384$  vs.  $52.16 \pm 3.017$  ng/mL in the presence of 25.0 and 50.0  $\mu\text{g/mL}$  extract and in its absence, respectively) (Figure 3C), and for 12.5, 25.0 and 50.0  $\mu\text{g/mL}$  of *A. archangelica* extract ( $71.81 \pm 2.992$ ,  $86.60 \pm 8.126$  and  $94.80 \pm 7.657$  vs.  $51.68 \pm 1.781$  ng/mL in the presence of 12.5, 25.0 and 50.0  $\mu\text{g/mL}$  extract and in its absence, respectively) (Figure 3D). The insulinotropic effect was observed to follow a concentration dependency. As only extracts containing coumarins could modulate glucose-induced insulin secretion, we decided to study the effects of pure coumarins present in the studied species.

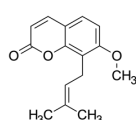
### 3.5 Coumarin modulation of insulin secretion

Twenty-three coumarins (Figure 4) isolated from the roots of *A. archangelica*, *A. dahurica* and *A. biserrata* were screened for their insulinotropic effect in 8.3 mmol/L glucose-stimulating conditions. Coumarins were tested at a concentration of 20  $\mu\text{mol/L}$ , a concentration often used in screening tests by various teams, including ours (Bardy et al., 2013; Youl et al., 2010).

**Simple coumarins:**

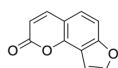


osthenol

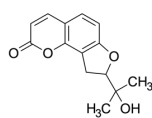


osthol

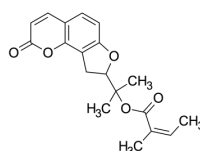
**Angular furanocoumarins:**



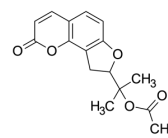
angelicin



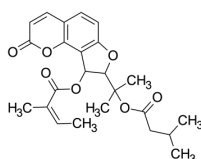
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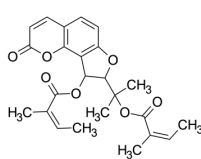
columbianadin



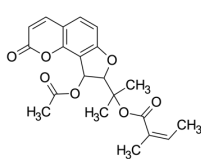
columbianetin acetate



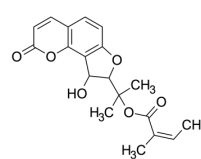
2'-angeloyl-3'-isovaleryl vaginate



archangelicin

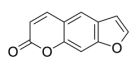


isoedultin

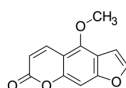


vaginidiol-O-angelate

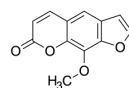
**Linear furanocoumarins:**



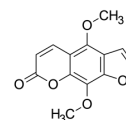
psoralen



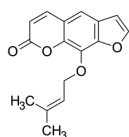
bergapten



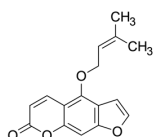
xanthotoxin



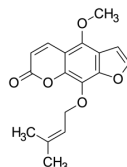
isopimpinellin



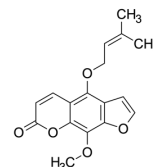
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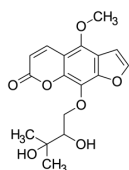
isoioperatorin



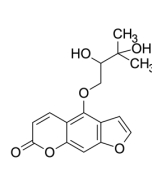
phellopterin



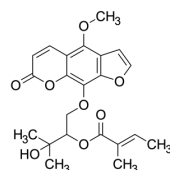
onidilin



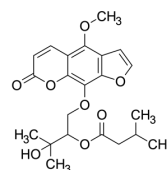
byakangelicin



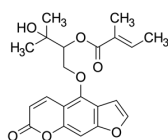
oxypeucedanin hydrate



byakangelicin-2'-O-angelate



byakangelicin-2'-O-isovalerate



heraclenol-2'-O-angelate

Figure 4. Structures of the isolated coumarins.

Quercetin, a natural compound with a confirmed insulinotropic effect, was used as a positive control. As expected, quercetin potentiated 8.3 mmol/L glucose-induced insulin secretion ( $220.8 \pm 12.31\%$ ). Among the twenty-three coumarins tested, our exploratory data suggested that six could increase glucose-induced insulin secretion when used at 20  $\mu\text{mol/L}$  (Figure 5).

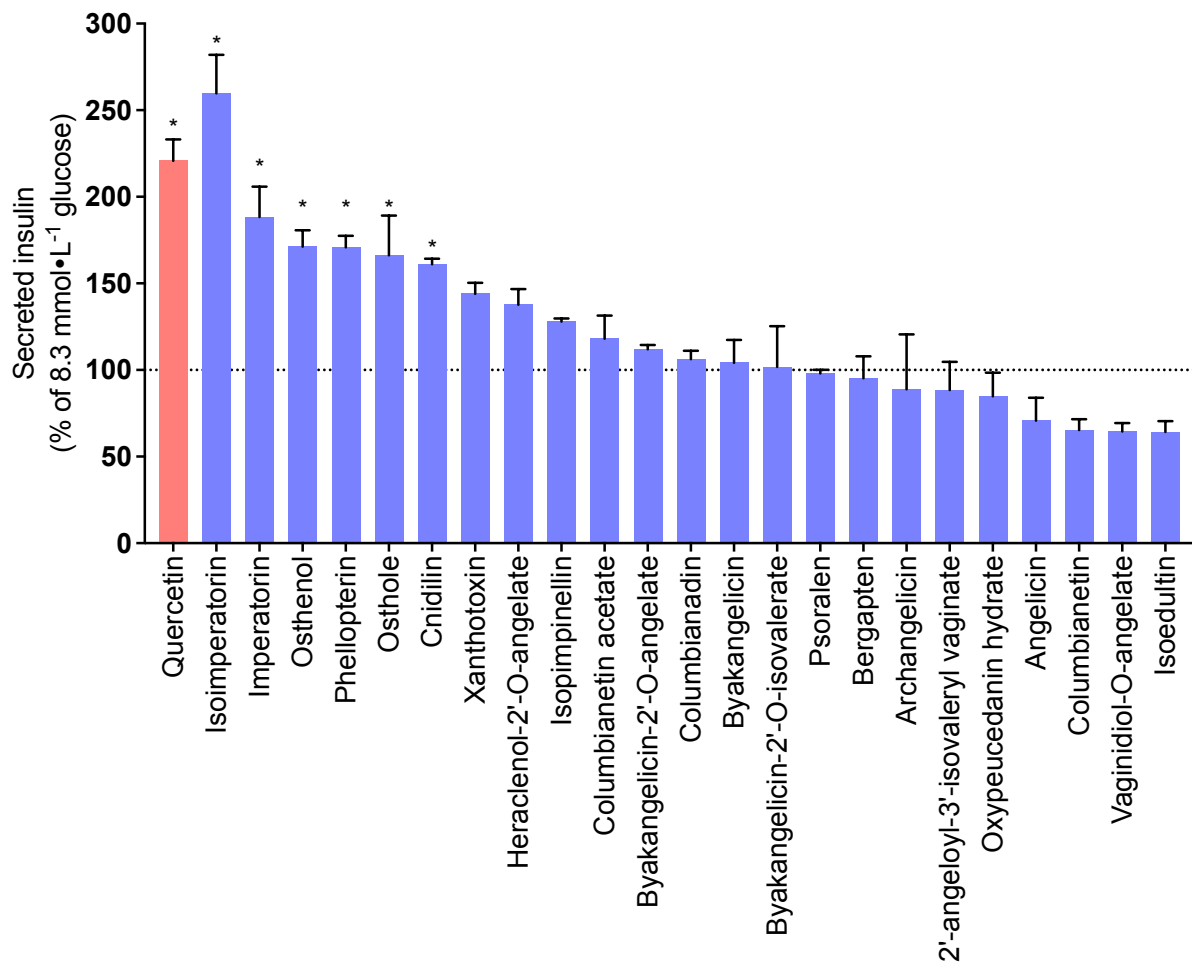


Figure 5. Exploratory data reporting the effect of a set of coumarins on glucose-induced insulin secretion in INS-1 cells.

The bar graph depicts the mean ± SEM of insulin secretion as a percentage of 8.3 mmol/L glucose-induced insulin secretion after 1 h incubation of INS-1 cells with 20 µmol/L coumarins; \*p < 0.05 different from 0 µmol/L of coumarins with one-way ANOVA followed by Holm-Sidak test, n = 3.

The compounds able to significantly amplify glucose-induced insulin secretion vs. the control were isoimperatorin (259.7 ± 22.29%), imperatorin (188.3 ± 17.64%), ostheno (171.3 ± 9.371%), phellopterin (170.8 ± 6.718), osthole (166.2 ± 13.27%) and cnidilin (161.1 ± 3.237%).

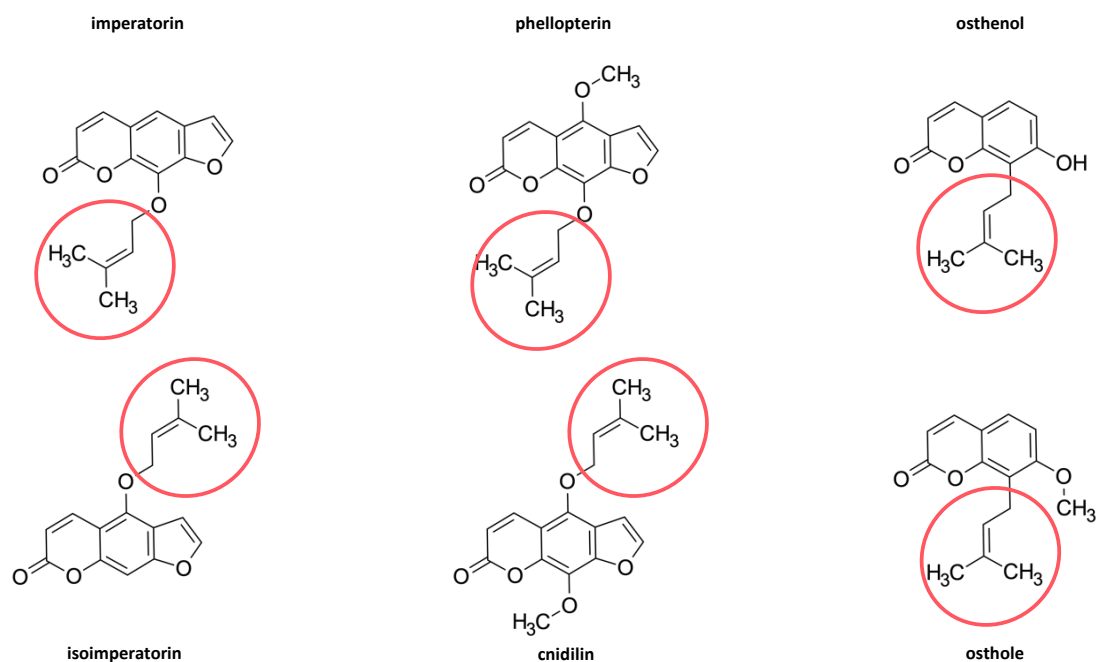


Figure 6. Structures of active coumarins with isoprenyl sidechains marked.

Based on these results, the structure-activity relationship was analyzed. It seems that insulinotropic activity is determined by an isoprenyl sidechain attached to the coumarin (Figure 6), while the nature of the heterocyclic coumarin ring may vary (both simple and furanocoumarins). Neither the position of substitution (both C5 and C8) nor its character (*C*-prenylation and *O*-prenylation) seem to be important for this activity. However, any substitution or prolongation of the isoprenyl sidechain leads to loss of function (Figure 7).

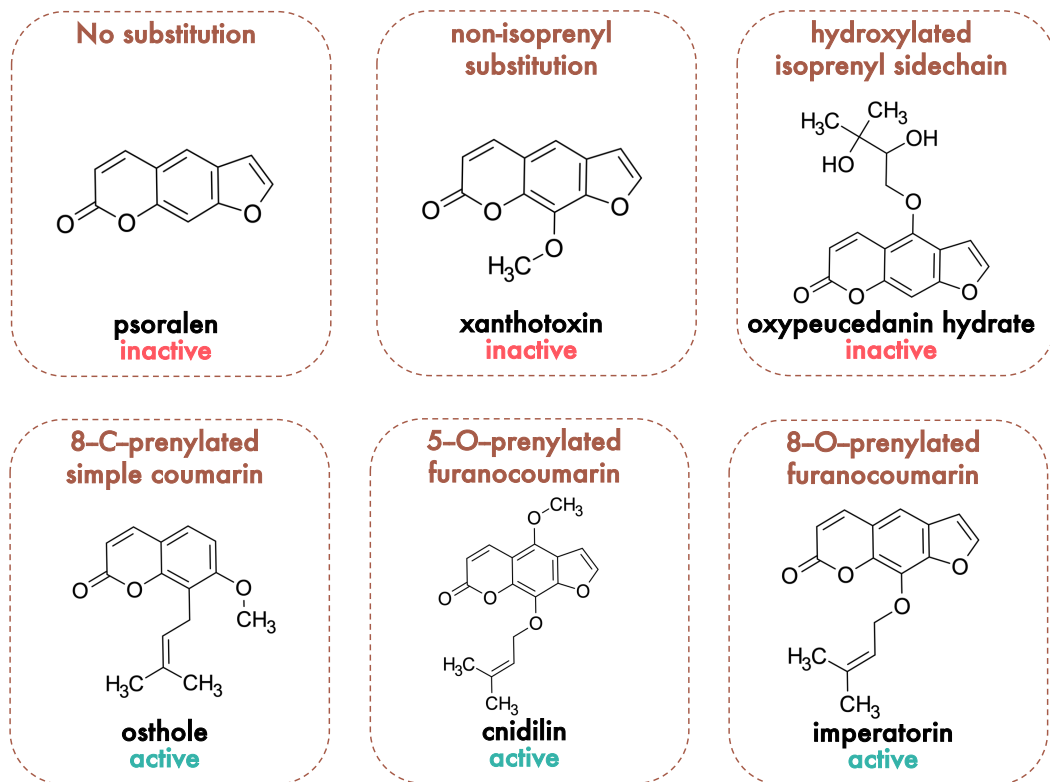


Figure 7. Structures of active coumarins compared to inactive structures.

It was decided that imperatorin, would be further evaluated, together with an inactive compound closely resembling its structure, i.e., psoralen, to use as a negative control. These compounds were tested in rising concentrations (from 5 to 40  $\mu\text{mol/L}$ ) in both basal (1.4 mmol/L) and glucose-stimulating (8.3 mmol/L) conditions. As expected, compared to 1.4 mmol/L glucose, 8.3 mmol/L glucose induced a significant insulin secretion ( $24.2 \pm 1.2$  ng/mL and  $50.7 \pm 1.4$  ng/mL respectively).

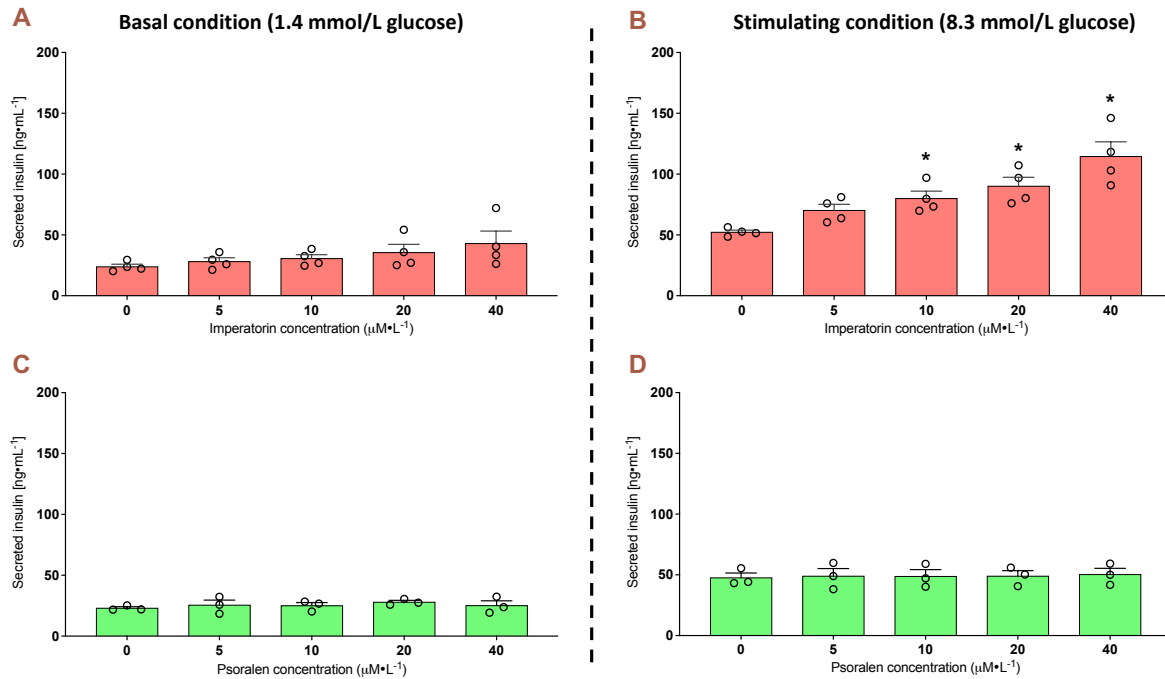


Figure 8. Effects of imperatorin and psoralen on basal (A and C) and glucose-induced (B and D) insulin secretion.

The bar graphs depict the mean ± SEM of basal (left) and glucose-induced (right) insulin secretion in ng/mL; \*p < 0.05 different from 0 μmol/L of coumarins with one-way ANOVA followed by Holm–Sidak test, n = 4 (for psoralen n = 3).

In basal glucose condition none of the compounds at any concentration were able to significantly increase insulin secretion (Figure 8 A and C). In glucose-stimulating condition imperatorin significantly increased glucose-induced insulin secretion in a concentration-dependent manner (80.00 ± 6.028, 90.11 ± 7.275 and 114.6 ± 11.94 vs. 52.28 ± 1.611 ng/mL for 10, 20 and 40 μmol/L respectively) (Figure 8B). Psoralen was inactive regardless of concentrations (Figure 8D).

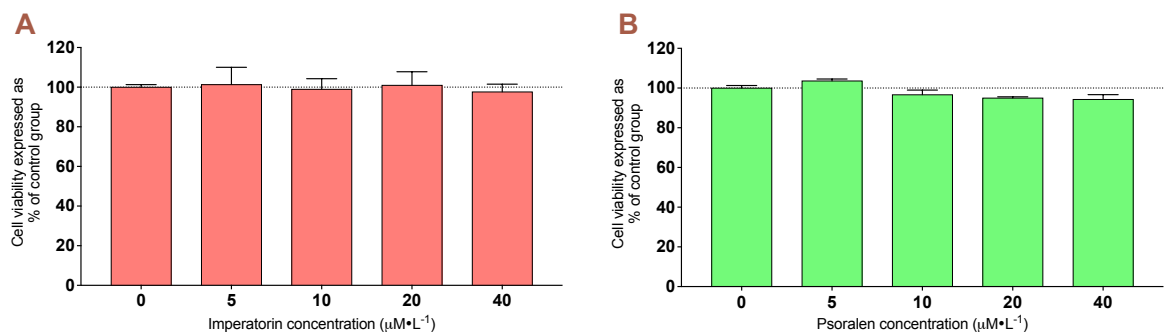


Figure 9. Effects of imperatorin (A) and psoralen (B) on cell viability.

To exclude any potential cytotoxic effect of studied coumarins on used cell model, i.e., INS-1, cell viability after 48 h of incubation with various concentrations of tested compounds was assessed using MTT assay (Figure 9). Studied compounds did not induce any cytotoxicity at the studied concentration range (5-40 μmol/L).

#### 4 Discussion

Angelica root extracts constitute a significant source of various traditional medicines for various cultures around the northern hemisphere, spanning from the indigenous cultures of North America to Japan. Among the many uses of the root, T2DM treatments is not often mentioned. Ethnopharmacological studies have reported the use of *A. japonica var. hirsutiflora*, *A. furcijuga*, *A. shikokiana*, and *A. keiskei* to treat diabetic symptoms (Kim et al., 2014; Leu, Y. L. et al., 2009; Mira et al., 2013; Oh et al., 2019). Further reports have demonstrated the insulin secretagogue activity of three angelica roots, i.e., *A. japonica var. hirsutiflora*, *A. reflexa*, and *A. dahurica*, belonging to the species spread in East Asia (China, Korea, Japan, and Taiwan) (Kim et al., 2023; Leu, Y. L. et al., 2009; Park et al., 2016). The metabolite profile of all angelica roots has not been studied thoroughly. There are many reports on the phytochemical composition of *A. dahurica* roots (Li et al., 2014; Shi et al., 2022; Tian et al., 2017; Zhao et al., 2022), but only a single report on *A. reflexa* (Kim et al., 2023). Only a few compounds have been isolated from *A. japonica var. hirsutiflora* and its composition has not been comprehensively studied (Pan et al., 2014; Wang et al., 2000). However, these limited data show that all these root extracts are rich in coumarins.

For this study, angelica roots monographed in the European Pharmacopeia (Ph. Eur. 11<sup>th</sup>) were chosen, i.e., *A. archangelica*, *A. dahurica*, *A. biserrata*, and *A. sinensis*. Through phytochemical and pharmacological data, we correlated the metabolite profile with the biological activity of the extracts on insulin secretion. The obtained results confirmed that *A. dahurica* roots modulate glucose-induced insulin secretion, and this effect was described for *A. biserrata* and *A. archangelica* roots for the first time, with the latter being active in the lowest concentrations of the three extracts (12.5 µg/mL). The fourth studied extract, from *A. sinensis* roots, did not show any activity at the studied concentrations (1.6 to 50.0 µg/mL). Based on the data obtained from the phytochemical examination of these extracts, only angelica roots rich in coumarins could modulate insulin secretion. Our results on insulin secretion are consistent with the conclusions from previous studies on *A. dahurica*, *A. reflexa*, and *A. japonica var. hirsutiflora* roots. Therefore, this study offers data on a new activity of traditionally used angelica roots in Europe.

Roots that were rich in coumarins in phytochemical examinations, i.e., those of *A. archangelica*, *A. dahurica* and *A. biserrata*, were subsequently used to isolate pure compounds, from 0.3 to 380.4 mg. The largest variety of coumarins was isolated from *A. archangelica* root. The plant contained simple C-prenylated coumarins, including osthole (121.2 mg) and osthenol (22.1 mg); linear furanocoumarins, including psoralen (5.5 mg), xanthotoxin (9.3 mg), bergapten (19.6 mg) and isopimpinellin (9.9 mg); O-prenylated linear furanocoumarins, including oxypeucedanin hydrate (5.5 mg), imperatorin (18.0 mg), isoimperatorin (12.6 mg), phellopterin (0.8 mg), heraclenol-2'-O-angelate (68.2 mg), byakangelicin-2'-O-angelate (102.2 mg) and byakangelicin-2'-O-isovalerate (14.4 mg); and angular furanocoumarins, including angelicin (64 mg), isoedultin (88.5 mg), vaginidiol-O-angelate (16.5 mg), 2'-angeloyl-3'-isovaleryl vaginate (97.2 mg) and archangelicin (110.4 mg).

The coumarin profile of dahurian angelica root had a much less diverse coumarin profile, but the plant contained large quantities of imperatorin (380.4 mg), isoimperatorin (148.0 mg) and cnidilin (224.5 mg) and several other linear furanocoumarins, including psoralen (0.3 mg), bergapten (26.6 mg), xanthotoxin (1.2 mg), phellopterin (6.3 mg), and byakangelicin (5.5 mg). Only 3 compounds were isolated from *A. biserrata* root. This was, however, not due to its poor coumarin content. As many compounds were already isolated from the first two roots, we attempted to only isolate compounds that were not present in the previous species. Thus, the following angular furanocoumarins were isolated: columbianetin (14.2 mg), columbianetin acetate (30.7 mg) and columbianadin (108.9 mg), and one sesquiterpene, bisabolangelone (23.7 mg).

The purity of all coumarins obtained during the course of this study was above 95%, which was deemed sufficient for pharmacological studies.

Furthermore, through phytochemical profiling of the active extracts and isolating major (and minor) coumarin compounds, we could explore which constituents could modulate  $\beta$ -cell function.

Out of the twenty-three coumarins isolated from *A. archangelica*, *A. dahurica*, and *A. biserrata* roots, six significantly increased glucose-induced insulin secretion, i.e., isoimperatorin, imperatorin, osthenol, phellopterin, osthole, and cnidilin. Therefore, imperatorin, isoimperatorin, osthole, phellopterin, and osthenol are implicated in the activity of *A. archangelica*; imperatorin, isoimperatorin, cnidilin, and phellopterin are implicated in the activity of *A. dahurica*; and osthole, imperatorin, and isoimperatorin are implicated in the insulinotropic activity of *A. biserrata*. These results could also confirm previous assumptions that coumarins are responsible for this activity in other angelica roots (Kim et al., 2023; Leu, Y. L. et al., 2009; Park et al., 2016).

Moreover, the quantitative content of prenylated coumarins in the studied extracts, especially imperatorin and osthole, correlates with the effect of extracts on insulin secretion. *A. archangelica* extract, which contained significant amounts of imperatorin and osthole, was the most active extract. This is an interesting observation because traditionally, this European representative of angelica was mainly used for gastric disorders. Our findings may have revealed a potentially new pharmacologic use of this plant material but also underline the new side effects, especially in patients with diabetes. This result also corresponds with data showing that *A. keiskei* root, which contains many isoprenylated coumarins, including imperatorin, isoimperatorin and osthenol, is used to treat diabetic symptoms (such as polyuria) (Kil et al., 2017; Kim et al., 2014).

Although the active compounds belonged to different coumarin subtypes (i.e., simple and linear furanocoumarins), they shared an isoprenyl sidechain attached to the coumarin in the C5 or C8 position. Insulinotropic activity may be determined by this isoprenyl sidechain, as its lack or any modification led to the loss of studied function. However, this must be confronted

with a recent report on scopoletin (7-hydroxy-6-methoxycoumarin), which does not possess an isoprenyl sidechain but is said to increase glucose-induced insulin secretion through  $K_{ATP}$  and VGCC channel-dependent pathways in INS-1 cells at concentrations below 20  $\mu\text{mol/L}$  (Park et al., 2022). We did not examine scopoletin in our study; thus, it cannot be excluded that this and other coumarins not studied here might fall out of the isoprenyl sidechain rule.

We found that imperatorin induced insulin secretion in a concentration dependent manner; 10 micromolar concentration being necessary to generate a significant pharmacological action on insulin secretion. This compound presents the same potencies that have been shown for flavonoids and are lower than those that were described for imperatorin in previous studies. Adebajo *et al.* have found that imperatorin modulated glucose-induced insulin secretion in INS-1 cells at 0.1 mg/mL (which is about 370  $\mu\text{mol/L}$ ) but did not show any effect at 0.01 mg/mL (equivalent to 37  $\mu\text{mol/L}$ ) (Adebajo et al., 2009). Park *et al.* observed phellopterin and imperatorin's ability to modulate glucose-induced insulin secretion in INS-1 cells at 100  $\mu\text{mol/L}$  but not at 10  $\mu\text{mol/L}$  (Park et al., 2016). Contrary to flavonoids (Fusi et al., 2020; Williamson et al., 2018), micromolar concentrations are much more likely to be reached *in vivo*, as coumarins have much better bioavailability (Deng et al., 2020).

Another important observation is that the insulinotropic activity of imperatorin is only evoked upon exposure of  $\beta$ -cells to a stimulating concentration of glucose, meaning that it follows a glucose-dependent pathway. This pharmacological feature of studied coumarins is particularly interesting, because it should minimize the likelihood of hypoglycemia, a common side-effect of anti-diabetic drugs present on the market (sulfonylureas) (Perreault et al., 2021).

Finally, we show that imperatorin is devoid of toxic effects, even over a long period of 48 hours, reinforcing the potential interest of this molecule in a diabetes treatment application.

## 5 Conclusions

Insulinotropic effects were found for the three pharmacopeial angelica roots, and their metabolite profiles were correlated with pharmacological activity; in addition, key structures responsible for the modulation of pancreatic  $\beta$ -cell function were identified. The nature of this effect should be further evaluated in mechanistic studies. These findings may have implications for the traditional use of angelica roots in treating diabetes. Active plant metabolites may also become lead structures in the search for new hypoglycemic treatments. Therefore, this study offers new perspectives for the development of novel antidiabetic therapies.

## 6 Glossary

ACh	acetylcholine
ATP	adenosine triphosphate
BSA	bovine serum albumin

CC	column chromatography
COSY	correlation spectroscopy
DAD	diode array detector
DMSO	dimethyl sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
FCS	fetal calf serum
FRET	fluorescence resonance energy transfer
GLP-1	glucagon-like peptide-1
HBSS	Hanks' balanced salt solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC or LC	high-pressure liquid chromatography
HSQC	heteronuclear single quantum coherence spectroscopy
HTRF	homogenous time-resolved fluorescence
K <sub>ATP</sub>	ATP-sensitive potassium channel
KRB	Krebs-Ringer bicarbonate buffer
MS	mass spectrometry
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NMR	nuclear magnetic resonance
Ph. Eur.	European Pharmacopeia
ROESY	rotating-frame nuclear Overhauser effect spectroscopy
RP	reversed-phase
RPMI 1640	Roswell Park Memorial Institute medium
STZ	streptozotocin

T2DM	type 2 diabetes mellitus
TCM	Traditional Chinese Medicine
TLC	thin layer chromatography
Tr	retention time
UHPLC	ultrahigh-performance liquid chromatography
UV	ultraviolet
VGCC	voltage-gated calcium channels

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