

# **Phytochemical, pharmacological and toxicological studies of alkaloid and sesquiterpene lactone- containing medicinal plants**

Summary of PhD. Thesis

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## 1. INTRODUCTION

The kingdoms of plants, animals and fungi are rich and important sources of natural products. For centuries, various diseases have been treated with living organisms in raw or processed form. In modern medicine, drugs and natural products are important raw materials for the pharmaceutical industry and serve as lead compounds in the research and development of medicines. Taking into consideration the high ratio of pharmaceuticals of natural origin to synthetic compounds, it is indeed the New Golden Age of natural product discovery.

Traditionally applied medicinal plants are of great importance. In case of effectiveness and safety, the authorisation often relies on long-term medicinal use. However, the pharmacovigilance data of medicinal herbs reveal a need for continuous re-evaluation. Till recently, the *per os* application of *Symphytum officinale* roots and *Chelidonium majus* were considered as safe. However, the application had to be revised, because of the pyrrolizidine alkaloid-content of *S. officinale* and the possible cardiac side effects of *Chelidonium majus* due to its hERG activity.

Modern approaches of phytochemistry, pharmacology and toxicology might lead to new breakthroughs in drug research. The better understanding of traditional application and processing of medicinal herbs resulted in the discovery of novel mechanisms of action and new active constituents of these plants. The traditionally applied *Aconitum* species are good examples how new results can open new ways in the research of traditionally used plants. Although neither the exact chemistry of these drugs, nor the pharmacological mechanisms have been thoroughly clarified, aconite drugs have been used as medicinal herbs more than two thousand years. The chemical analysis of raw plant materials and processed drugs made it possible to identify their biological active compounds and their change during processing. The studies identified diterpene alkaloids as biological active compounds. The least toxic compounds are diterpene alkaloids esterified with long-

chain fatty acids at position C-8 and C-3. These lipo-alkaloids are minor compounds in unprocessed drugs, their amount is increased during traditional process method, while the amount of tri- and diester alkaloids is significantly decreasing. The identification of lipo-alkaloids as biologically active constituents triggered experiments to semisynthesize new compounds and investigate their pharmacological effects. Even though the well-known *Aconitum* species were removed from the Western Pharmacopoeias, natural products of aconite origin are promising as anti-inflammatory or antiarrhythmic pharmacons.

In the recent years, medicinal plants are typically marketed as food supplements. Several products contain herbs that have not been extensively used before, and there are no pharmacological, toxicological and phytochemical data available to serve as basis for the assessment of their effectiveness, safety and quality. Plants that have not been consumed to a significant degree by humans in the European Union prior to 1997 are considered as novel food. One example for novel food (though unauthorised) is *Ambrosia artemisiifolia*, which has become quite popular as a medicinal plant in the recent years in Hungary. However, this plant has not been consumed or used as a medicinal herb before, hence its toxicological profile is unexplored and the risk related to its consumption are unknown.

## **2. AIMS OF THE STUDY**

In 2000, the research group of the Department of Pharmacognosy (University of Szeged) started a screening programme for isolation and identification of Ranunculaceae alkaloids in order to find new biologically active compounds and the rational explanations of the folk medicinal use of the toxic species. Later, the scope of the experiments was extended to the safety pharmacology, toxicology and chemistry of traditional and newly discovered medicinal plants.

According to this comprehensive approach, the aim of the present work was the chemical, pharmacological and toxicological investigation of diterpene alkaloid-containing traditional medicinal plants and one, only recently applied

species, to reveal the dangers of their use and to identify their potential role in modern medicine.

In order to achieve the aims, the main tasks were:

- Review the literature of the *Aconitum*, *Spiraea* genera and *Ambrosia artemisiifolia*, from aspects of the chemistry and pharmacological properties of the plants.
- Diterpene alkaloid extraction and identification from *Aconitum napellus* subsp. *firmum*.
- Investigation of the activity of Ranunculaceae diterpene alkaloids and lipo-alkaloids on GIRK and hERG channels.
- Evaluation of antiarrhythmic potential of Ranunculaceae diterpene alkaloids.
- *In vivo* toxicological evaluation of diterpene alkaloids using bdelloid rotifer assays.
- Extraction of *Spiraea* species with various alkaloid extraction methods for alkaloid-content screening, and investigation of the antibacterial and xanthine oxidase inhibitory activity.
- *In vivo* toxicological examination of common ragweed on rats.

### 3. MATERIALS AND METHODS

*Aconitum napellus* subsp. *firmum* was collected in the Retezat Mountains (South Carpathians, Romania) in August 2007. *Spiraea crenata* and *S. salicifolia* were collected in Sepsibükszád (Romania) and Alsórákos (Hungary). *S. nipponica*, *S. x vanhouttei* and *S. x billardii* were collected in Botanical Garden of University of Szeged. *S. media* and *S. chamaedryfolia* were harvested in Daugavpils (Latvia). Ragweed puree was purchased online in 2015. The investigated diterpene alkaloids were isolated from investigated *Aconitum* species and other Ranunculaceae species previously studied by our group. The lipo-alkaloids were semisynthesised previously.

Diterpene alkaloids were isolated using multistep chromatographic methods [open column chromatography (CC), centrifugal planar chromatography (CPC), gel filtration chromatography (GFC), preparative layer chromatography (PLC) and thin layer chromatography (TLC)]. The alkaloid-content of fractions was monitored using SiO<sub>2</sub> TLC, the detection were performed by spraying with Dragendorff's reagent and 5% aqueous NaNO<sub>2</sub> solution. The isolated compounds were identified by means of LC-MS and NMR spectroscopy.

The inhibitory activity on GIRK and hERG potassium channels of diterpene alkaloids and lipo-alkaloids were measured on stable transfected HE239-GIRK (Kir3.1/3.4) and HEK293 cells using automated planar patch clamp technology. In hERG channel inhibitory assay amitriptyline and in GIRK channel inhibitory assay propafenone were used as positive controls. The toxicity of diterpene alkaloids were examined *in vivo* using bdelloid rotifer assays by evaluation of the animals viability parameters (toxicity and survival lifespan; body size index; mastax contraction frequency assay and CRC: cellular reduction capacity).

Among investigated species only *S. chamaedryfolia* contained alkaloid. The alkaloid-distribution was localised by within secondary root by microscopy, using alkaloid-specific staining method. The fractions of *S. chamaedryfolia* were tested using nine bacteria strains [*Streptococcus pneumoniae* (ATCC 49619), *S. agalactiae* (ATCC 13813), *S. pyogenes* (ATCC 19613) *Staphylococcus epidermidis* (ATCC 12228), *S. aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Klebsiella pneumoniae* (ATCC 700603), *Moraxella catarrhalis* (ATCC 25238), *Staphylococcus aureus* (MRSA) (ATCC 43300)]. The xanthine oxidase inhibitory activity of fractions was also tested using allopurinol as reference compound.

The sesquiterpene lactone-content of ragweed puree was confirmed by means of TLC and LC-MS methods. Repeated-dose toxicity was tested in healthy, male Wistar rats. During 28 days long experiment control animals received plain cookie dough without ragweed, for further groups the ragweed puree was administered in doughs, in low dose (500 mg/kg b.w.) and high dose (1000 mg/kg



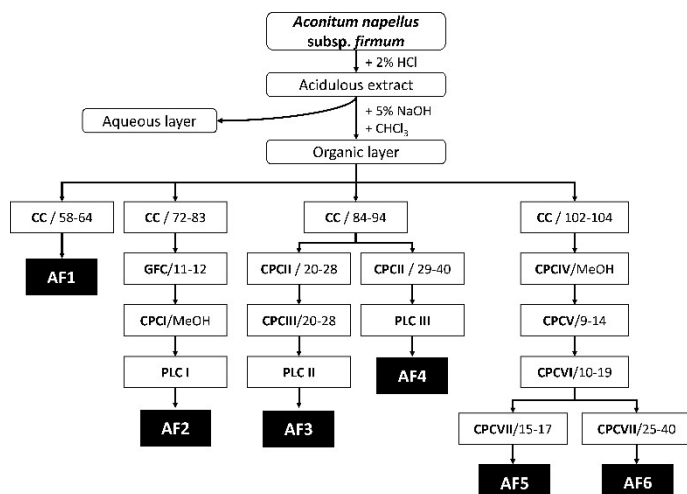
b.w.). The toxicity was evaluated by analysis of general clinical symptoms, blood serum parameters and the organ-to-body weight ratio.

Pharmacological investigations were performed in cooperation with the Rytmion Ltd. (Szeged, Hungary), Department of Public Health and Department of Psychiatry (University of Szeged, Faculty of Medicine).

## 4. RESULTS

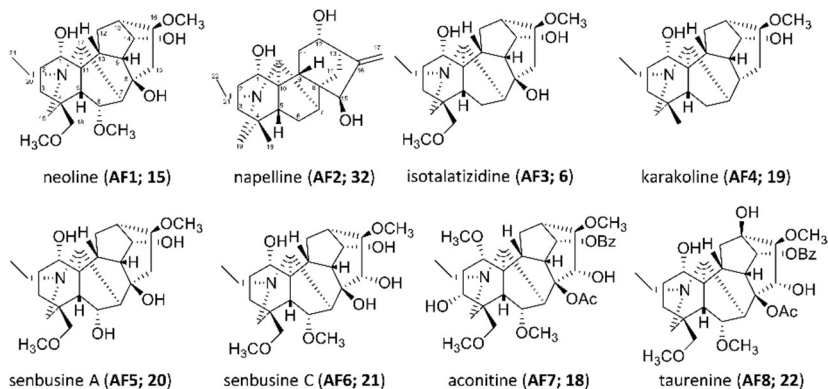
### 4.1. Alkaloids isolated from *A. napellus* subsp. *firmum*

The dried and crushed plant material was extracted with 2% aqueous hydrochloric acid. After the pH was adjusted to alkaline with 5% aqueous NaOH solution, alkaloids were extracted by solvent-solvent partitioning using  $\text{CHCl}_3$ . The organic phase was collected and evaporated. Six diterpene alkaloids [neoline (**AF1**), napelline (**AF2**), isotalatisidine (**AF3**), karakoline (**AF4**), senbusine A (**AF5**) and senbusine C (**AF6**)] were isolated from crude alkaloid-rich fraction using multistep chromatographic method (CC, CPC, GFC, PLC) (Fig 1).



**Figure 1.** Isolation of alkaloids from *A. napellus* subsp. *firmum*

The isolated compounds were identified by means of NMR spectroscopy. The isolated compounds were confirmed as genuine constituents by LC-MS, furthermore two compounds [aconitine (**AF7**) and taurenine (**AF8**)] were identified. The compounds **AF2-AF4** and **AF6** have been reported for the first time of *A. napellus* subsp. *firmum*.

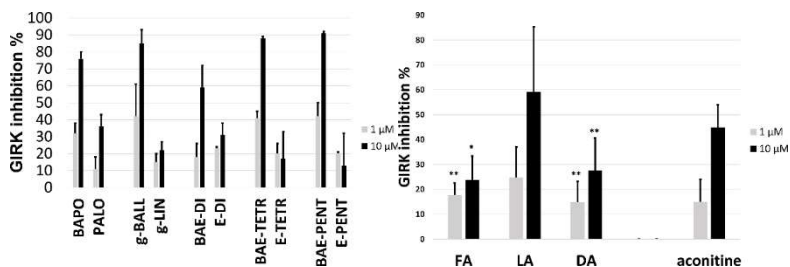


**Figure 2.** The isolated and two identified compounds from *A. napellus* subsp. *firmum*

#### 4.2. Activity on GIRK and hERG potassium channels

Moderate inhibitory activity on GIRK and hERG channels was exerted only by aconitine. The effect on GIRK channels exerted by aconitine have been reported by our research group for the first time. These results suggest a more complex cardiac action of aconitine with a multiple ion channel effect as it was earlier suspected.

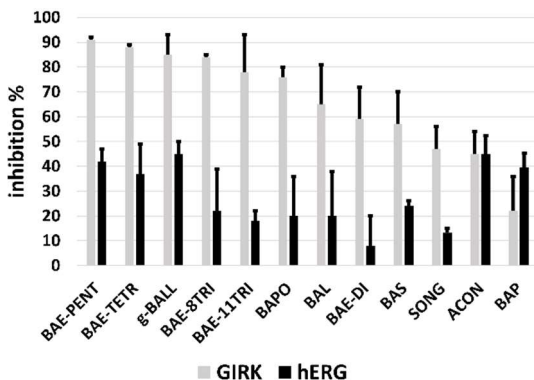
Lipo-alkaloids possess remarkable inhibitory activity on both GIRK and hERG channels. The analysis of activity allowed to perform structure-activity relationship. Significant difference between the activity of lipo-alkaloids, aconitine and free fatty acids indicates that the 14-BzA-part of the molecule is necessary for the ion channel inhibitory effect. Further structure-activity relationship was recognised concerning the saturation grade of the esterifying fatty acids. The unsaturation of the esterifying fatty acids is a crucial factor for GIRK inhibitory activity (Fig 3).



**Figure 3.** GIRK activities of lipo-alkaloids and their corresponding fatty acids.

(BAPO: 14-BzA-8-O-palmitoleate, PALO: palmitoleic acid, g-BALL: 14-BzA-8-O-γ-linolenate, g-LIN: γ-linolenic acid, BAE-DI: 14-BzA-8-O-eicosa-11Z,14Z-dienoate, E-DI: eicosa-11Z,14Z-dienoic acid, BAE-TETR: 14-BzA-8-O-eicosa-5Z,8Z,11Z,14Z-tetraenoate, E-TETR: eicosa-5Z,8Z,11Z,14Z-tetraenoic acid, BAE-PENT: 14-BzA-8-O-eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoate, E-PENT: eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoic acid)

Concerning promising pharmacons the high selectivity on GIRK/hERG inhibition is required (Fig 4). Two compounds, 14-BzA-8-O-eicosa-8Z,11Z,14Z-trienoate and 14-BzA-8-O-eicosa-11Z,14Z,17Z-trienoate possess low inhibitory activity on hERG and at the same time they are potent GIRK inhibitors, which renders them worthy of consideration for further pharmacological studies.



**Figure 4.** Selectivity of the most potent compounds on the GIRK and hERG channels

(BAE-PENT: 14-BzA-8-O-eicosa-5Z,8Z,11Z,14Z,17Z-pentaenoate, BAE-TETR: 14-BzA-8-O-eicosa-5Z,8Z,11Z,14Z-tetraenoate, g-BALL: 14-BzA-8-O-γ-linolenate, BAE-8TRI: 14-BzA-8-O-eicosa-8Z,11Z,14Z-trienoate, BAE-11TRI: 14-BzA-8-O-eicosa-11Z,14Z,17Z-trienoate, BAPO: 14-BzA-8-O-palmitoleate, BAL: 14-BzA-8-O-laurate, BAE-DI: 14-BzA-8-O-eicosa-11Z,14Z-dienoate, BAS: 14-BzA-8-O-stearate, SONG: songorine, ACON: aconitine, BAP: 14-BzA-8-O-palmitate).

### 4.3. Toxicity of diterpene alkaloids on bdelloid rotifers

The investigated 10 diterpene alkaloids were non-toxic in these assays, moreover significant elevation of these parameters could be observed, except napelline, that reduced all measured parameters (Fig 5). Bdelloid rotifers are possess wide tolerance range for the tested alkaloids, even for aconitine, which is highly toxic in mammals. This experiment supports low toxicity of songorine, reported by literature.

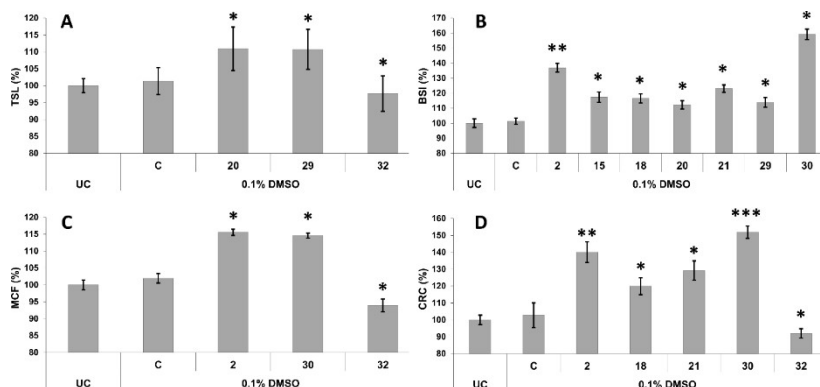


Figure 5. Viability assays on bdelloid rotifers

### 4.5. Pharmacological and phytochemical screening of *Spiraea* species

Phytochemical screening revealed alkaloid content in *S. chamaedryfolia* roots, nevertheless the other six *Spiraea* species were alkaloid-free. The alkaloid distribution of alkaloids are distributed in secondary cortex and secondary xylem. Phytochemical investigation in preparative scale of *S. chamaedryfolia* afforded alkaloid rich  $\text{CHCl}_3$  and EtOAc fractions (Fig 6).

Fractions of *S. chamaedryfolia* exerted antibacterial activity against *Staphylococcus aureus* (ATCC 29213), *Bacillus subtilis* (ATCC 6633), *Streptococcus pneumoniae* (ATCC 49619), *Moraxella catarrhalis* (ATCC 25238) and one fraction exerted antibacterial activity against methicillin-resistant *Staphylococcus aureus*

(MRSA) (ATCC 43300). The EtOAc, CHCl<sub>3</sub> and MeOH fractions exerted noteworthy xanthine oxidase inhibitory activity (>80%).

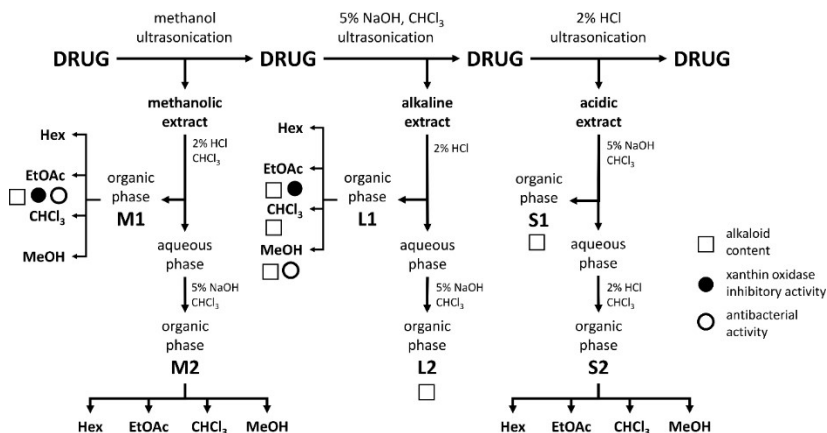
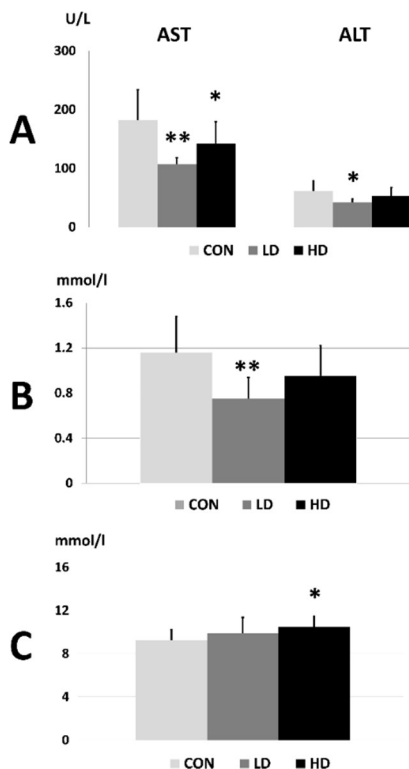


Figure 6. Alkaloid content and biological activities of *S. chamaedryfolia*

#### 4.6. Toxicology of ragweed puree

The sesquiterpene lactone-content of ragweed puree was confirmed by TLC and LC-MS methods.

General toxic symptoms could not be detected on animals fed with ragweed. However, the blood serum parameters significantly altered between low-, high dose and control group. The level of liver enzymes (AST, ALT), carbamide and tryglyceride were significantly reduced (Fig 6). A relative organ-to-body ratio of kidneys and brain was significantly reduced in animals fed with ragweed puree.



**Figure 7.** Levels of AST and ALT (A), tryglyceride (B) and carbamide (C) in blood serum of the control and treated rats.

## SUMMARY

The aim of this study was the phytochemical, pharmacological and toxicological evaluation of diterpene alkaloid-containing species (*Aconitum napellus* subsp. *firmum* and six *Spiraea* species) and the sesquiterpene lactone-containing *Ambrosia artemisiifolia*.

Six diterpene alkaloids were isolated and further two alkaloids were detected from *A. napellus* subsp. *firmum*, four of them were reported for the first time. The screening of seven *Spiraea* species resulted in the identification of alkaloids presence in *S. chamaedryfolia*.

Aconite alkaloids were screened for their inhibitory activity on cardiac potassium channel. The GIRK activity of aconitine is reported for the first time. 14-BzA-8-*O*-eicosa-8Z,11Z,14Z-trienoate and 14-BzA-8-*O*-eicosa-11Z,14Z,17Z-trienoate were identified as potent GIRK inhibitors with high GIRK/hERG ion channel selectivity.

The alkaloid-content of *Spiraea chamaedryfolia* is reported for the first time. Fractions of *S. chamaedryfolia* exerted noteworthy xanthine oxidase inhibitory activity (>70%) and moderate antibacterial activity.

Toxicity of 10 diterpene alkaloids was evaluated on bdelloid rotifer assay. Toxicological data are provided for senbusine A, senbusine C, septentriodine and hetisinone.

The *Ambrosia artemisiifolia*-containing puree was analyzed *in vivo* in rats in a repeated-dose toxicity study. The study ended without any visible clinical symptoms, however the relative liver- and brain-weight was significantly reduced and blood parameters (AST, ALT liver enzymes, triglyceride and carbamide level) were significantly changed. Common ragweed-containing puree, as a novel food, showed to be toxic in rats, thus long-term human use is supposed to be not safe.

Our results demonstrate that modern phytochemical and pharmacological studies may contribute substantially to the deeper recognition of traditional medicinal herbs, and new results can open new ways in pharmaceutical research. Toxicological investigations of traditional and novel medicinal plants are unavoidable and useful tools in clarification of safety issues.

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**The thesis is based on the following publications:**

**1. Kiss T;** Orvos P; Bánsághi Sz; Forgó P; Jedlinszki N; Tálosi L; Hohmann J; Csupor D.  
Identification of diterpene alkaloids from *Aconitum napellus* subsp. *firmum* and  
GIRK channel activities of some Aconitum alkaloids

*FITOTERAPIA* 2013; **90**: 85-93. doi: 10.1016/j.fitote.2013.07.010

IF: 2.216

**2. Kiss T;** Szabó A; Oszánci G; Lukács A; Tímár Z; Tiszlavicz L; Csupor D.  
Repeated-dose toxicity of common ragweed on rats

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IF: 3.54\*

**3. Kiss T;** Borcsa B; Orvos P; Tálosi L; Hohmann J; Csupor D.

Diterpene lipo-alkaloids with selective activities on cardiac K<sup>+</sup> channels

*PLANTA MEDICA* 2017; accepted doi: 10.1055/s-0043-109556

IF: 1.99\*

**4. Kiss T;** Mácsai L; Csupor D; Datki Zs.

*In vivo* screening of diterpene alkaloids using bdelloid rotifer assays

*Acta Biologica Hungarica* 2017; accepted

IF: 0.616\*

**5. Kiss T;** Cank K; Orbán-Gyapai O; Liktör-Busa E; Rutkovska S; Zomborszki Z; Pučka  
I; Németh A; Csupor D.

Phytochemical and pharmacological investigation of *Spiraea chamaedryfolia* – A  
contribution to the chemotaxonomy of *Spiraea* genus

*BMC Research Notes* 2017; submitted

**Other publications:**

**1. Agapouda A;** Booker A; **Kiss T;** Hohmann J; Heinrich M; Csupor D.

Quality control of *Hypericum perforatum* L. analytical challenges and recent progress

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IF: 2.363\*

\* The impact factor for the year 2016 is given.

**Presentation held in the theme of the thesis:**

**1. Kiss T.**

Allergia a (növény)családban – Parlagfű és társai

XIII. Magyar Gyógynövény Konferencia (Kecskemét, 2013.09.27-28.)

**2. Kiss T; Csupor D; Orvos P; Tálosi L; Hohmann J**

Isolation and investigation of potassium channel activity of *Aconitum* diterpene alkaloids.

Phytokongress 2013 (Leipzig, Germany, 2013.03.10.)

ZEITSCHRIFT FÜR PHYTOTHERAPIE 2013; **34**: V25 doi: 10.1055/s-0033-1338200

**3. Kiss T; Borcsa B; Csupor D; Hohmann J**

Növényi nyersanyagokból izolált diterpén-alkaloidok szerkezet-meghatározása és farmakológiai aktivitásának vizsgálata.

Fiatal Gyógynövénykutatók Fórum (Budakalász, 2014.02.14.)

**4. Csupor D; Kiss T; Bauer R; Hohmann J**

Phytochemical and pharmacological research on Chinese and European *Aconitum* species

The 3rd Annual Meeting of GP-TCM RA (Nainjing, China, 2014.07.18-20.)

EUR J OF INT MED 2014; **6**: 695. doi: 10.1016/j.eujim.2014.09.024

**5. Kiss T; Cank K; Orbán-Gyapai O; Liktör-Busa E; Rutkovska S; Pučka I; Hohmann J; Csupor D.**

Screening for diterpene alkaloids in the *Spiraea* genus

63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Budapest, 2015.08.23-27.)

PLANTA MEDICA 2014; **81**: PM\_19. doi: 10.1055/s-0035-1565396

**6. Borcsa B; Kiss T; Csupor D; Orvos P; Tálosi L; Hohmann J**

The effect of diterpene alkaloids on the GIRK channels

63rd International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (Budapest, 2015.08.23-27.)

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