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DISSERTATION

Titel der Dissertation

Plants from ethnomedicines used against diabetes mellitus –
rationale and quality control of *Leonurus sibiricus* L.
(Lamiaceae) and *Juglans regia* L. (Juglandaceae)

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1 Introduction

1.1 Diabetes mellitus

The pathogenesis of diabetes mellitus (DM) is unequivocally linked with the hormone insulin. Therefore, before entering on the subject of DM, the role of insulin is discussed first.

1.1.1 Physiological background on insulin

Insulin is the most potent anabolic hormone in the human body. It promotes storage of carbohydrates, lipids and proteins, while inhibiting their degradation and release into circulation. Its main function is to keep the blood glucose concentration between 70 and 150 mg/dL. Concentrations above this range would be toxic. Even though other factors are also involved in the glucose-homeostasis, insulin's involvement is crucial (Skyler, 2012).

Insulin is produced in the β -cells of the islets of Langerhans in the pancreas. It is released from its secretory vesicles upon different stimuli, the most important of which is glucose. When glucose enters the β -cell via the insulin-independent glucose transporter GLUT2, it gets phosphorylated to glucose-6-phosphate (G-6-P) by the enzyme glucokinase. Further metabolism of G-6-P activates the mitochondrion and enhances thereby the conversion from ADP to ATP. The increasing ATP/ADP ratio leads to the closure of K_{ATP}^+ -channels, stopping the outflow of potassium. This causes the cell membrane to depolarize, which in return opens voltage-gated Ca^{++} -channels. The increased intracellular calcium concentration promotes the exocytosis of insulin-containing vesicles and its release into the blood stream (Newsholme et al., 2014).

Insulin release from the β -cells slows down or stops when blood glucose levels reach physiological values. Thus, there is a direct correlation between the blood glucose concentration and insulin release. If blood glucose level drops below the physiolog-

ical range, secretion of hyperglycemic hormones is initiated. The most prominent is glucagon, which forces the release of glucose into the blood from cellular stores, especially liver cell stores of glycogen. By increasing blood glucose concentrations, hyperglycemic hormones prevent life-threatening hypoglycemia. A balance between insulin and its counterpart glucagon is necessary for an efficient glucose metabolism and body function (Saltiel and Kahn, 2001).

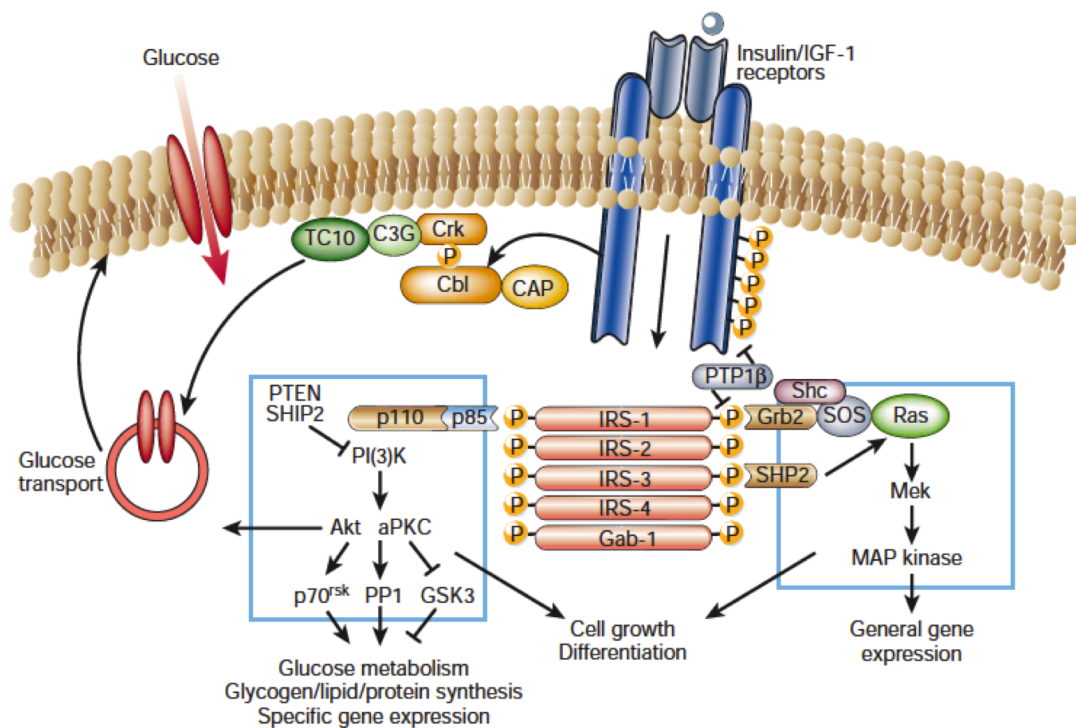


Figure 1.1: Insulin signal transduction
Source: Saltiel and Kahn (2001)

Once insulin reaches the blood stream, it can bind on insulin receptors (IR) of various cell types. Primary target tissues are the liver, the muscle and fat tissue. The processes of insulin action are illustrated in Figure 1.1 (page 2), according to Saltiel and Kahn (2001). The insulin receptor is a transmembrane receptor that is activated by insulin and insulin-like growth factor I (IGF-I). It belongs to the family of receptor tyrosine kinases, which generally mediate their activity by autophosphorylation. The binding of insulin to the extracellular α -domain of IR induces structural changes of the transmembrane β -subunits, which leads to autophosphorylation of tyrosine residues within the intracellular domain of the receptor. These changes facilitate a diverse series of sig-

naling pathways, eventually promoting the trafficking of glucose transporters to the cell membrane. These transporters take up glucose from the blood stream into the cell, where it is stored as glycogen (Skyler, 2012). If the action of insulin fails, diabetes mellitus will occur.

Two targets of the insulin signaling cascade are discussed in more detail, since they are of importance for further understanding. Protein tyrosine phosphatase 1B (PTP1B) is a negative regulator of IR signaling. It causes dephosphorylation of intracellular substrates of the insulin receptor kinase, resulting in a reduction of the insulin signal intensity. Therefore, PTP1B inhibitors would increase insulin sensitivity by blocking the PTP1B-mediated negative pathway (Panzhinskiy et al., 2013; Thareja et al., 2012). Peroxisome proliferator-activated receptor gamma (PPAR γ) is a nuclear receptor protein that functions as a transcription factor. The activation of PPAR γ promotes the expression of certain genes which modulate the glucose consumption of muscles and the hepatic gluconeogenesis (Consoli and Formoso, 2013).

1.1.2 Pathology

The term diabetes mellitus (DM) describes a severe metabolic disorder of the endocrine system. People suffering from this disease cannot use or produce insulin properly, which results in elevated blood glucose levels (hyperglycemia) and an imbalance in carbohydrate, fat and protein metabolism. The symptoms of diabetes are frequent urination (polyuria), excessive thirst (polydipsia), constant hunger, weight loss, fatigue and dry skin.

There are three types of diabetes: type 1 (previously known as insulin-dependent DM), type 2 (formerly known as non-insulin-dependent DM) and gestational diabetes. Type 1 DM occurs when the pancreatic β -cells fail to produce insulin. The exact causes remain unclear, but much research is being undertaken in this field. It most likely results from a combination of genetic susceptibility and exposure to environmental trigger (Van Belle et al., 2011). Gestational diabetes occurs during pregnancy and usually ceases after the child's birth.

Type 2 DM is a polygenetic disorder characterized by defects in the peripheral insulin action (insulin resistance). The most critical factor in the emergence of DM type 2 is

obesity. The majority of all patients with type 2 diabetes are or have been obese (Eckel et al., 2013). At the onset of DM type 2, insulin receptors become increasingly insensitive to insulin. Therefore, higher insulin concentrations are needed to maintain physiological blood glucose concentrations. Under normal conditions, the β -cells increase their insulin release sufficiently to overcome the reduced insulin efficacy. Only if the β -cells are unable to fully compensate the enhanced need for insulin, diabetes mellitus will develop. This β -cell dysfunction and insulin resistance may occur due to a yet unknown number of genetic polymorphisms. Together with obesity, these factors are likely to cause type 2 DM (Tsai et al., 2015).

1.1.3 Complications

If DM is not diagnosed, acute metabolic crises may occur. In normal conditions, insulin suppresses hepatic glucose production. In a state called diabetic ketoacidosis, the lack of insulin leads to glucose overproduction in the liver as well as high levels of counter-regulatory hormones (glucagon and catecholamines), which potentiate the effect by increasing glycogenolysis and gluconeogenesis. The patient suffers from potentially life threatening conditions, like dehydration, nausea, confusion, weakness and coma (Azevedo et al., 2014).

Aside from such an acute condition, DM leads to chronic long-term complications, which relate to the damage of small (microangiopathy) and big (macroangiopathy) blood vessels. The high blood glucose concentrations cause oxidative stress and glycation of endothelial plasma proteins and lipids, resulting in advanced glycation end-products (AGEs). Once AGEs are built, they induce cross-linking of other proteins such as collagen. This leads to alterations of the vascular structure (Llaurado et al., 2014; Beisswenger, 2012). Microangiopathies, like diabetes-specific retinopathy, neuropathy and nephropathy cause major morbidities, such as blindness, limb amputation and end-stage renal disease. Macroangiopathies are not diabetes-specific, but emerge faster and more severe than in non-diabetics. They considerably increase the risk for heart attack and stroke (Kampoli et al., 2009).

1.1.4 Epidemiology

According to the World Health Organization (2015a), 9% of the earth's adult population (age above 18) suffered from diabetes in the year 2014. The numbers are constantly increasing due to aging, population growth, unhealthy life-style (high fat diet, less physical action) and urbanization. These factors have a larger impact on low- and middle-income countries than on high-income countries. The number of diabetes patients is expected to increase by 55 % by 2035. However, estimations on how many people will be affected by diabetes in the future are regularly overthrown by newer studies (Guariguata et al., 2014) and projections worsen. DM is the seventh leading cause of death. In 2012, an estimated 1.5 million people died of the consequences of DM. 50% of people with DM die of cardiovascular disease. Approximately 1% of global blindness is ascribed to consequences of diabetes mellitus (World Health Organization, 2015a).

Not only is diabetes mellitus a threat to the human's health, it also imposes a big economical burden. The estimated global health expenditure on diabetes totals at least USD 376 billion or 12% of the total health expenditure in 2010. About USD 1330 are spent on each person with diabetes on average. Until the year 2030, the global health expenditures for diabetes are expected to increase by 30-34% (Zhang et al., 2010b).

1.1.5 Clinical treatment

The focus of diabetes therapy is to avoid acute metabolic crisis, such as diabetic ketoacidosis and to prolong or avoid the development of long-term complications (see section 1.1.3, page 4). This is achieved by keeping the blood glucose level in the physiological range of 70-150 mg/dL. The patients need to monitor their blood sugar levels regularly. As clinical marker serves glycated haemoglobin (HbA_{1c}). It provides information on the average plasma glucose concentration over a period of a few months. Glucose and haemoglobin can form a complex, which is stable until the red blood cell's death (~ 120 days). The amount of glycated haemoglobin reflects the amount of glucose to which the erythrocyte has been exposed and can therefore be used to assess the

effectiveness of anti-diabetic therapy (Abduhl-Ghani et al., 2011). High HbA_{1c} values are associated with an increased risk of all-cause mortality (Tako et al., 2014).

Since type 1 DM and type 2 DM have different causes, they need to be treated differently. Type 1 DM patients completely lack insulin and have therefore be treated with insulin or insulin analogues. A combinational therapy maintains a certain basal insulin level with a long lasting analog and increases insulin concentrations when needed (i.e. after food intake) with short acting insulins. This therapy concept allows adequate control of blood glucose levels and is used in the intensive conventional insulin therapy (CIT). All insulins are injected subcutaneously.

The treatment plan for type 2 DM consists of several stages. The first grade is a three month dietary- and exercise therapy. If, after that time, the HbA_{1c} value is > 7%, oral hypoglycemic agents are applied. First in mono-therapy, later in combinations. Only if these measures fail to lower the HbA_{1c} value below 7%, CIT is started as last initiative of the plan (American Diabetes Association, 2014).

Currently, there are five mechanistic classes of agents, besides insulin, which are used against type 2 DM (International Diabetes Federation Guideline Development Group, 2014):

- insulin secretagogues increase insulin excretion from the β -cells (sulfonylureas and analogues)
- insulin sensitizer enhance the peripheral responsiveness of insulin (biguanides and PPAR γ agonists)
- agents which delay the absorption of monosaccharides (α -glucosidase inhibitors)
- incretin potentiators
- amylin analogues

Detailed discussion of all hypoglycemic agents would surpass the scope of this introductory section. The most prominent therapies are listed in Table 1.1 (page 7), alongside their mechanisms and adverse side effects.

Irrespective of what type of diabetes is treated, the risk of hypoglycemia during diabetes-therapy must be kept as low as possible. The term hypoglycemia describes an abnormally low content of blood glucose and is the most frequent adverse effect of diabetic

medications (Snell-Bergeon and Wadwa, 2012). Symptoms and effects vary, but the most dangerous consequences arise from an inadequate supply of glucose to the brain and might in worst cases lead to permanent brain damage or death.

Treatment	Mechanism	Adverse side effects
Sulfonylureas	stimulate insulin secretion by K_{ATP}^+ -channel closure	weight gain, hypoglycemia
Biguanides	reduce glucose production by AMP increase	lactic acidosis
Glitazones	insulin-sensitization by gene regulation	hypoglycemia
α -Glucosidase inhibitors	reduce glucose absorption	GI-complications
Incretin potentiators	incretin mimetic, DPP-4 inhibitor	nausea sore throat, headache, diarrhea
Amylin analogues	lower postprandial blood glucose levels	nausea, hypoglycemia

Table 1.1.: Oral hypoglycemic therapy options for diabetes mellitus (left), mechanism of action (middle) and adverse side effects (right)

Although a large number of agents with different mechanistic properties already exist, only 37% of adults with diagnosed DM reach the goal HbA_{1c} value of < 7% (American Diabetes Association, 2014). Consequently there is still a pressing need for the discovery of new anti-diabetic agents as well as new biological targets.

1.1.6 Phytotherapy for DM

Prior to the discovery of insulin in 1921 and the rise of oral anti-diabetics in the 1950ies, people with diabetes relied on herbal preparations to ease their suffering (Meyer et al., 2002). These treatments were based on knowledge from different folk medicines. In Europe, for instance, the most often used plants for diabetic symptoms (diuresis, glucosuria) were *Syzygium cumini* (L.) Skeels., *Atropa belladonna* L. and its alkaloids, *Vaccinium myrtillus* L. and *Phaseolus sp.*. The drugs were used either as powders or decoctions (Helmstädter, 2007). In India, *Allium sativum* L., *Momordica charantia* L. and

Stevia rebaundia (Bertoni) Bertoni. are the most often traditionally used plants for diabetic disorders (Patel et al., 2012). For some of the mentioned plants, scientific studies could proof a beneficial effect on diabetes. Triterpenoids in *M. charantia*, for instance, stimulate AMP-activated protein kinase and increase fatty acid oxidation (Tan et al., 2008). Metformin, one of the routinely used oral anti-diabetics nowadays, was originally also derived from a plant. Its derivative isoamylene guanidine from *Galega officinalis* L. (French lilac) was the molecular lead on which metformin synthesis was built on (Witters, 2001). This example shows that traditionally used plants can constitute a valuable source for the discovery of new lead compounds.

1.2 Traditional Mongolian medicine (TMM)

The major part of this thesis deals with the microscopic and phytochemical investigation as well as the assessment of the *in vitro* activity on glucose metabolism of *Leonurus sibiricus* L. (*L. sibiricus*), a plant regularly used in the Traditional Mongolian Medicine (TMM). Since this medical system is rather unknown to the Western world, a review about the history and main characteristics of TMM is provided in section 1.2.2 (page 9 ff). *L. sibiricus* is one of the most often applied plants in Mongolia and ingredient in many traditional recipes (School of Pharmacy and Ministry of Health, 2007).

1.2.1 *Leonurus sibiricus* L.

L. sibiricus (Siberian Motherwort) belongs to the plant family of the Lamiaceae and is native to China, Mongolia, South Siberia, China, Korea, Japan and Southeast Asia (World Health Organization, 2015b; Hegi, 1906). Additionally it is naturalized in South, Central and North America (Global Biodiversity Information Facility, 2014). The herb is annual or biennial and grows to a height of 20 to 80 cm. The leaves are 3-palmatisect with narrowly oblong-rhombic lobes. The flowers are arranged in verticillasters with white or reddish to red-purple corollae. The upper lip of the flower is oblong and longer than the lower lip. After blooming between July and September, brown nutlet fruits are produced (Grubov, V.I., 2001; eFloras, 2008; Encyclopedia of Life, 2014).

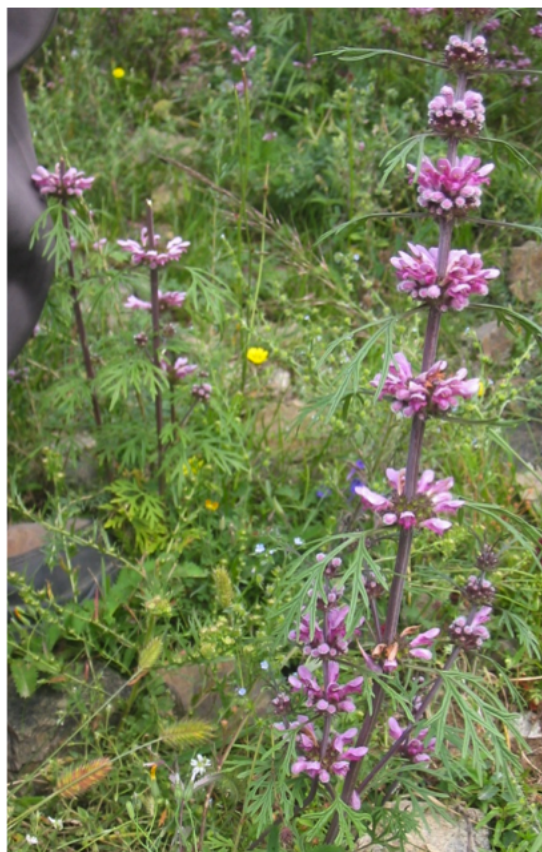


Figure 1.2.: *Leonurus sibiricus* L.
Source: Narantuya

Reported secondary metabolites of *L. sibiricus* include alkaloids (Luo et al., 1985), flavonoids (Pan et al., 2006) and iridoids (Hayashi et al., 2001). However, current scientific literature on secondary metabolites and bio-activities of *L. sibiricus* is hard to interpret, since it is often confused with another *Leonurus* species, *Leonurus japonicus* HOUTT. (*L. japonicus*). Indeed, the lack of microscopic anatomical characteristics of *L. sibiricus* makes a differentiation difficult. To shed light on this situation, *L. sibiricus* and *L. japonicus* have been investigated for characteristic anatomical features. This subject is discussed in more detail in section 4.3 (page 91 ff).

In TMM, *L. sibiricus* is traditionally used against high fever, poisoning and diarrhea (World Health Organization, 2015b) and, according to oral tradition, it is also applied against diabetic symptoms (Narantuya, 2011). However, effects of *L. sibiricus* on the glucose-homeostasis has not scientifically been shown yet. In this thesis, this need is approached from two points of view. First, the effects of *L. sibiricus* on the insulin's action site (glucose-uptake) are investigated in the publication of section 4.1 (page 47).

Secondly, *L. sibiricus*' potential to increase insulin release is assessed in the publication of appendix D (page 163). The latter project was mostly executed by cooperation partners at the Paracelsus Medizinische Privatuniversität Salzburg and is therefore added in the appendix. Qualitative and quantitative characterization of active extracts are given in sections 4.1 (page 47) and 4.2 (page 89).

1.2.2 Published review

The paper added in this section (page 11 ff) serves to introduce main characteristics of TMM. The more general part of the manuscript is followed by a discussion about five TMM plants, which are used against liver disorders. These five plants had been worked on previously on the Department of Pharmacognosy and were added to show an example of successful coalition of TMM with modern science. They do not relate to this thesis in any other way.

At this point the author wants to highlight a detail, which is mentioned in the paper (footnote on page 16), but should receive special explanation: The English names, synonyma, description, distribution, and habitat in the monographs of the review were adopted from a manuscript on Mongolian medicinal plants, prepared by S. Narantuya and S. Purevsuren in cooperation with WHO. When the review presented in this PhD thesis was submitted for final revision, the manuscript on Mongolian medicinal plants was close to publication as e-book. Finally, both review and e-book were published simultaneously by the end of 2013. S. Narantuya and S. Purevsuren are co-authors of the review and are the coordinators of the e-book published by WHO as mentioned in its Acknowledgements.

Traditional Mongolian Medicine: history and status quo

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Abstract Traditional Mongolian Medicine (TMM) plays an important role within the medical system of Mongolia nowadays. This medical system is rather unknown in the Western world, and detailed information can hardly be found in literature. In this article various aspects of TMM are highlighted. The eventful history of TMM is presented, and the centres which offer today's traditional medical care are introduced. Institutions which provide education in TMM are outlined, and the latest developments in the national standard are highlighted, according to which the different institutions have to develop their curricula. Furthermore, an overview is given about herbal medicines in Mongolia and the health situation in this

country. Finally, the international and Mongolian literature of *Achillea asiatica*, *Dianthus versicolor*, *Euphorbia pallasii*, *Lilium pumilum*, and *Saussurea amara*, which are all used in TMM to cure liver diseases, is reviewed.

Keywords Traditional Mongolian Medicine · Education · Herbal drugs · Liver diseases · *Achillea asiatica* · *Dianthus versicolor* · *Euphorbia pallasii* · *Lilium pumilum* · *Saussurea amara*

Introduction

Traditional medical systems, especially of Asian origin, gained more public attention in the Western countries during the last decades. Traditional Chinese Medicine, Ayurveda and Traditional Tibetan Medicine are popular and, in many European countries, medicinal preparations based on these traditions are often used for alternative or complementary medical treatments. The rather unknown Traditional Mongolian Medicine (TMM) developed from Mongolian folk medicine and was highly influenced by Ayurveda and Traditional Tibetan Medicine (see section History of TMM). The TMM went down under the Soviet influence in the first half of the twentieth century, but experienced a revival after the political changes in the Soviet Union. Nowadays, traditional medicine is officially recognized as its own Mongolian medical

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heritage (Kletter et al. 2008; Unkrig 2002). The revival of TMM during the 1990s initiated a new development. Education and training centres for TMM were established (see section Centres of Education for TMM; Natsagdorj 2002), and Mongolian academic institutions started to focus on investigations providing a scientific basis for TMM. In many cases cooperation with foreign academic institutions was sought. Today, various hospitals and clinics apply Western as well as traditional methods. Many Mongolians even prefer to consult primary doctors who are trained in both medical systems (Bernstein et al. 2002). By 2007, the percentage of Mongolians who received traditional medical health care reached 23.6 % of the total population (Munkhdelger and Tserenkhagva 2007; Unkrig 2002; Bolormaa et al. 2007). In this review we report on the history of TMM, the different institutions offering education in TMM and the production of herbal medicines in Mongolia. In the last section, the international and Mongolian literature of *Achillea asiatica*, *Dianthus versicolor*, *Euphorbia pallasii*, *Lilium pumilum*, and *Saussurea amara*, which are all used in TMM to cure liver diseases, is reviewed.

History of TMM

Mongols have been practising medical methods and traditions since ancient times (Shagdarsuren 1989). The traditional medicine was influenced by the Mongolians' lifestyle as nomads and herdsmen, their culture, and by the harsh climate conditions they had to overcome (Natsagdorj 2011a, b). Over the centuries other medical systems, such as Ayurveda and to a certain extent Chinese medicine, impacted the existing traditional medical knowledge. However, Mongolian medicine developed its own tradition and concepts, and it was taught in independent medical schools, which had been established in the thirteenth century (Shagdarsuren 1989). In the sixteenth century, when Lamaism became the leading religion in Mongolia, traditional Tibetan medicine strongly influenced the medical system (Kletter et al. 2008). Tibetan Buddhism largely influenced daily life, the religious practice of the Mongolians and the education of the Mongolian physicians. In the monasteries, the monks were educated in the teachings of the *rgyud bzhi*, the main medical treatise of Tibetan medicine, and they

played an important role in the health system of the past (Kletter et al. 2008). As a consequence, in the seventeenth century, the Tibetan language was introduced as the “medical language”. The physicians diagnosed the diseases by reading the pulses, examining the tongue, checking the urine by smell, colour and taste, and questioning the patients (Kletter et al. 2008; Kletter and Kriechbaum 2001). Recipe books were written in Tibetan language and later translated into Mongolian (Gerke 2004). Even though the recipes based on the same literature and the ingredients bore the same names in Tibetan and Mongolian medical texts, the medicinal drugs used for the medicines were different ones. The physicians substituted plants, exchanged plant parts or altered the formula of the recipe, depending on the individual experience of each physician (Gerke 2004). The traditional Mongolian physicians were highly approved in whole Central Asia and served even the Chinese court (Sartor 2007). Politically, the Qing Dynasty maintained control of Mongolia from the seventeenth century until 1911 (Baabar 1999; Wikipedia 2012). In 1921 it declared its independence, which was followed by the establishment of the Mongolian People's Republic with support from the former Soviet Union in 1924 (Wikipedia 2012). The communists of the neighbouring Soviet Union exerted great influence on Mongolian politics and, in 1936, the Mongolian republic entered into an alliance with the powerful neighbour (Kletter et al. 2008). This political development led to a modernization of Mongolian life. New buildings equipped with electricity, sanitation, a clean water supply and an effective centralised heating system were erected in Mongolian towns. The literacy rate increased to over 95 %, and a well structured and staffed (Western) health care system became accessible to most inhabitants of the country (Manaseki 1993). On the other hand, the Soviet communist influence in Mongolia caused dramatic changes in political and social structures. Lamaism, which had dominated the Mongolian life until the twentieth century, was banned officially. In 1937, monasteries were torn down and the monks persecuted. In consequence, the religious institutions and the traditional medical system collapsed (Kletter et al. 2008). The last Tibetan pharmacy was closed officially in 1937 (Munkhdelger and Tserenkhagva 2007). However, traditional medicine was still popular and used secretly, especially in the treatment of chronic diseases. In 1959, traditional

medicine was again promoted and research in traditionally used medicinal plants and traditional medical concepts started. After the political changes in the Soviet Union in 1989, all restrictions on Traditional Mongolian Medicine were completely lifted, and its revival has been ongoing right up to the present day (Kletter et al. 2008).

Centres offering traditional medical care and/or education in TMM

Traditional Mongolian medical knowledge was passed on over generations through private practitioners, who got their medical knowledge from the elders, and through educational and faculty systems, which had been established in the 16th century. “Manba Datsan” is the general name for a Buddhistic monastic school of traditional medicine, mostly designed to educate physicians, but also to provide the possibility of scientific studies of various subjects for scholars (Natsagdorj 2002). Between 1585 and 1920, there were 222 Manba Datsans distributed all over Mongolia. 203 of them offered the general doctor’s training, 6 offered an advanced education, the maaramba training, and 13 Manba Datsans played a role in the teaching of religious chants related to medicine. The general education of the Manba Datsan’s training was divided into three main phases: preliminary phase, main education phase and high level phase. In the preliminary phase, children began to study at the age of 5–6 years. They became an apprentice of a highly educated teacher, learned letters and began to study some sutras. At first, they had to learn sutras by heart, and later on their teachers explained to them the principle of the sutras. In the main education phase, the pupils had to study for 12–13 years. Every year they had to take an exam, and after their successful passing they entered the next class. In the high level phase, the students had to be enrolled in the advanced training to become a doctor. This phase offered four different levels that could be reached. “Manba Duirava” entitled a person, who achieved the basic medical level of traditional medicine. This matches the qualification of today’s nurse. A “Manba Duirava” performed blood drawing, moxibustion and massage, he collected medicinal plants and prepared traditional remedies, but did not diagnose diseases. The doctors who graduated from the following two higher levels

bore the titles “Manba Gavj”, comparable to today’s physician, and “Manramba/Maaramba”, which corresponds to today’s PhD. Finally, at the highest educational level the graduate was awarded the title “Manba Bumramba” (Saruul-Oyun and Natsagdorj 2011) comparable to the Western “Doctor of Science”. There were only a few persons who ever reached the highest level (Natsagdorj 2001). However, during the Soviet supremacy TMM disappeared. None of the old Manba Datsans survived and, along with the breakdown of the above-mentioned system, the traditional medical education vanished. Only since the late 1990s, after the rehabilitation of TMM, traditional medical doctors have been again educated in Mongolian public and private institutions. In January 2013, the Center of Standardization and Measurement of Mongolia approved a national standard which obliged the institutions to develop their curricula (Center of Standardization and Measurement of Mongolia 2012). This standard defines obligatory subjects, fields of knowledge, and skills which have to be taught in TMM education centres.

There are several public institutions in Mongolia who teach TMM. One of them, the “School of Traditional Medicine” of the Health Sciences University of Mongolia (HSUM) was founded in 1989 originating from the Department of TMM of the former Medical University. In 2000, it was re-established as “School of Traditional Medicine” representing one of seven independent institutes of HSUM. It offers all degrees of education in traditional medicine and promotes research in this field. In 1993, the first traditional medical doctors graduated. Since then, 565 students have graduated as traditional doctors from this school (Batbaatar and Lkhagvasuren 2012).

In two other public institutions outside the capital Ulaanbaatar, in “Darkhan City’s Medical College” and “Govi-Altai’s Medical College”, assistants to traditional medical doctors and nurses are educated.

There are also private medical colleges offering education in TMM. The “Ach Medical College” provides both, education in Western medicine and TMM, whereas at the “New Medicine Traditional Medical College” exclusively TMM is taught. The latter was established in 2000 as one branch of the private “Monos Medical College”. Since 2000, the “New Medicine Traditional Medical College” has

graduated 362 traditional doctors. Only in 2012 it separated from the “Monos Medical College” and became independent.

The first public hospital which offered traditional medical care after 1989 was the “State Clinical Central Hospital”. In 1989, a TMM subdivision was founded including 50 beds and 8 supervision rooms. Ten years later, in 1999, this division expanded and, by decision of the Mongolian government, changed its name to “Traditional Medicinal Scientific Technology Corporation”. At present, this institution comprises three subdivisions, the “Research Center”, the “Traditional Medicine Clinic”, and the “Manufactory of Traditional Medicine”. The “Traditional Medicine Clinic” houses an in-patient hospital with 100 beds and an ambulatory division with 12 cabinets, which can serve 120–150 patients per day. Interestingly, the “Manufactory of Traditional Medicine” had already existed when Mongolia was still under Soviet influence. Since its foundation in 1973 it had been a subdivision of the “Traditional Hospital Institute” which belonged to the Mongolian Academy of Sciences. It produced exclusively products for experimental purpose developed by the “Traditional Hospital Institute”. Only later, the government decided to integrate this factory into the “Traditional Medicinal Scientific Technology Corporation” to which it still belongs. This factory produces 110 traditional medicinal products and serves the “Traditional Medicine Clinic”.

The only religious centre, and the most important one, is the new “Manba Datsan” which, nowadays, provides medical education in Mongolia. It was founded in 1990 as the first Mongolian private hospital of traditional medicine with the aim to restore TMM in the country. This highly recognized institution includes a hospital and a training centre (Natsagdorj 2002), and will be introduced in detail in the following section.

Manba Datsan Clinic and Training Center for Mongolian Traditional Medicine

After 70 years of communist regime, the political democratic movement in 1990 enabled the founding of Manba Datsan (“Clinic and Training Centre for Traditional Medicine”), by one of the most venerable lamas in Mongolia, Dr. Prof. Khamba Lama Natsagdorj D. The institution, located in the city of

Ulaanbaatar, aims to re-establish Mongolian Traditional Medicine, custom, treatment and medicine-production. This new Manba Datsan succeeded the historical Manba Datsan “Monastery for Medicine to Help Others”, which had been established in 1760 by the 3rd Bogd Ishdambiinyam. It had conducted its activities for a period of 178 years and was erased because of the political repression in the winter of 1938 (Natsagdorj 2001).

The new Manba Datsan “Clinic and Training Centre for Mongolian Traditional Medicine” offers various services, e.g. hospital, production of medicines, and religious activities. The monks who live in the monastery spend their monastic life by studies, meditation, services, and gatherings on special religious occasions. The lamas are also sent abroad, e. g. to Tibet, to study spiritual custom and tradition. The Manba Datsan is financially supported by the Ministry of Education and Science, the Ministry of Health and the Ministry of Justice and Internal Affairs. It embraces the following branches:

- “Otoch Manramba University of Traditional Mongolian Medicine”
- “Manba Datsan Monastery for Medicine to Help People”
- “Manba Datsan Hospital”
- “Manba Datsan Medicament Production”
- “Shinjeech Otoch Laboratory”
- “International Training, Treatment and Nursing Complex”
- “The Children of Manba Buddha Association”

The “*Manba Datsan Hospital*” houses an ambulatory and in-patient clinic with 60 beds, which was accredited by the National Centre of Health Development in 2002. More than 230,000 patients have been treated in the hospital since its foundation. Most of the expenses for operations are covered by the national health care system. The patient has to contribute a small amount of money to the costs of the operation. 25 specialized and well educated physicians, and 10 nurses are employed and ready to take care of the patients.

The “*Manba Datsan Medicament Production*”, a facility for the production of medicaments, was established in 1994. It was renewed and modernized according to ISO-9001 by the project “Peking Paris Ralley” supported by the Development Agency of Switzerland in 2007. More than 400 different types of

raw materials, from local and international providers are handled. The quality of the raw materials is checked in the institution's own laboratory and/or in the state laboratory. The raw materials are processed to more than 197 traditional herbal medicines following prescriptions of age-long experience, including pills, decoctions, oils, powders etc. Apart from Mamba Datsan hospital, the “Manba Datsan Medicament Production” serves more than 20 local traditional hospitals.

The “*Otoch Manramba University of Traditional Mongolian Medicine*” was founded in 1991. Within the new Manba Datsan, it represents the centre for education in TMM. It was the first institute in Mongolia, to offer education in TMM after its comedown in the 1930ies. As each other educational institution in Mongolia, it needs to be approved by the Ministry of Education and Science, which issues special training licenses. The institute achieved accreditation in 2000 by the “National Council for Education Accreditation”, and was re-accredited successfully for the period from 2006 to 2016. The following degrees are offered:

- Bachelor Degree “General Physician of Traditional Medicine” (6 years)
- Bachelor Degree “Nurse in Traditional Medicine” (4 years)
- Master Degree training (2 years)
- Qualification training and specialization after graduation

After graduation, the official licence for traditional medicine is issued by the “Center of Health Development of Mongolia” which authorizes alumni to treat humans.

Herbal medicines in Mongolia

The medical preparations are usually complex mixtures of a varying number of ingredients, mainly plants but also minerals and animal drugs of local and foreign origin. In the past, each traditional physician prepared the medicines for his patients himself according to the prescriptions of medical texts or his own experience. Also, the necessary plants, minerals, and animal products for certain medications were collected by the physician himself (Kletter et al. 2008; Munkhdelger and Tserenkhagva 2007).

Today, the mode of production and distribution of herbal medicines, traditional remedies, and non-traditional remedies has greatly changed. Herbal medicines are either regulated as prescription medicines, non-prescription medicines or traditional medicines, and can be sold with health claims in pharmacies or by licensed practitioners. Mongolia has a registration system for herbal medicines and is in process of establishing GMP for herbal medicinal products (WHO Regional Office for the Western Pacific 2012). Traditional medicines are dispensed to the public by traditional medicine pharmacies, while traditional hospitals have their own traditional medicine pharmacies. To date, 11 Mongolian traditional products have been registered. However, a large amount of traditional remedies which are not yet registered, is produced in the centres of traditional medicine and in Mongolian companies using modern production methods. As far as traditional herbal preparations are concerned, Mongolia has presently six manufacturing units which produce several hundred types of traditional medicines in large scale (14 tons annually) (Ministry of Health of Mongolia 2012a). Recipes mentioned in ancient books, transliterated sutras and more recent formula handbooks are used for the production of the preparations.

As far as quality control is concerned, steps towards GMP were taken only recently. Before 2011, an approved list of standardized medicinal raw materials and the inspection guidelines for traditional medicinal materials and formulas served as basis for the quality control. In 2011, the first Mongolian pharmacopoeia was published, which includes 76 herbal monographs. In case the Mongolian pharmacopoeia does not contain a certain monograph, the standards approved before 2011 and the pharmacopoeias of China and Russia are legally binding. The Mongolian State Central Inspection Laboratory and the State Inspection Agency are responsible for monitoring traditional preparations to ensure their quality. Mechanisms of regulation are included in the Mongolian “Law on Medicine and Medical Devices”, which was established in 1998 and amended several times since then.

Health situation in Mongolia

In 2011 a general classification of common diseases according to Traditional Mongolian Medicine was

approved by the Ministry of Health of Mongolia (Ministry of Health of Mongolia 2012b). It represents a guideline for traditional doctors defining various types of diseases, and is used for diagnosis and treatment. TMM has an excellent reputation among Mongolia's population. In specific diseases such as chronic diseases, head trauma, diseases of liver, stomach and kidneys, the Mongolians prefer to consult traditional practitioners (Natsagdorj and Odontsetseg 2001; Natsagdorj 2011b).

Both, traditional medical doctors, and Western medical doctors are concentrated in urban areas. In the private sector, usually a fee for the traditional medicine service is paid. In rural areas, “bagiin emch”, as called in Mongolian language, provides general Western medical care to the nomadic herdsman families and communities. They are trained in mid-level health care in one of the previously mentioned medical centres. They live and work in their own house, and provide basic medical services such as home visits, antenatal and postnatal care, health promotion and education or prescription of essential drugs (WHO Western Pacific Region Health Services Development 2012).

An epidemiologic monitoring showed an increasing prevalence of lifestyle-related chronic diseases since the beginning of the 1990s. According to the Health Indicators 2011, the leading causes for morbidity all over Mongolia in 2011 were diseases of the respiratory system, the digestive system, the genitourinary system, and the circulatory system (Health Indicators 2011). The incidence of those diseases was, in general, higher in rural than in urban areas. Compared to the figures of 2000, the number of inpatients with liver problems increased from 18.9 to 26.1 % (Health Indicators 2011). Therefore, a joint project between Mongolian and Austrian institutions was initiated, which focused on medicinal plants used in TMM to treat liver disorders. Traditional methods of diagnosis do not necessarily correlate with diseases as classified in Western medicine (Kletter et al. 2008). TMM classifies, in general, hot and cold disorders; regarding liver impairment, there are 13 hot disorders and 5 cold ones. However, some symptoms listed in the traditional texts relate to Western disease syndromes. For example, regarding liver disorders, the traditional texts mention fever, localisation of pain or yellow skin, symptoms which indicate a liver involvement (Kletter et al. 2008). Within the Mongolian-

Austrian project, several plants were investigated for their influence on bile flow in the isolated perfused rat liver, and for their phytochemical composition. In the following section we review the literature of five selected plants, which exerted remarkable effects on bile secretion.

Monographs¹

The Mongolian, Tibetan and English names of the five herbal drugs, their synonyma, the used plant parts, and the traditional prescriptions which contain the respective plants are listed in Table 1.

Achillea asiatica SERG. (Asteraceae)

Description

Perennial herb, with rhizome. Stem 20–50 cm tall, whitish due to long, slender, entangled hairs, erect, branched only at the inflorescence. Basal leaves 10–20 cm long, 1–2 cm wide, cauline leaves smaller, sessile, lanceolate, two to three times pinnatisected, linear and acute segments not more than 3 mm in width, closely arranged. Heads, with 2–5 mm long peduncles, forming a dense corymb. Ligulate flowers usually purple, sometimes white, ca. 3 mm long. Disk flowers are yellow.

Habitat

Sandy terraces on western and eastern slopes of mountains, forest fringes (Gubanov 1996; Malishev and Peshkova 1979; Sanchir et al. 2003; Ligaa et al. 2005).

Traditional use

The taste is bitter and hot and the potency is coarse and sharp. It is used for treating persistent fever.

¹ English names, synonyma, description, distribution, and habitat were adopted from a manuscript on Mongolian medicinal plants, prepared by S. Narantuya and S. Purevsuren in cooperation with WHO, which is close to publication.

Table 1 Names, synonymy, distribution, and used plant parts of *A. asiatica*, *D. versicolor*, *E. pallasii*, *L. pumilum* and *Saussurea amara*, and names of the traditional prescriptions in which these five plants are contained

	<i>Achillea asiatica</i>	<i>Dianthus versicolor</i>	<i>Euphorbia pallasii</i>	<i>Lilium pumilum</i>	<i>Saussurea amara</i>
Mongolian name	Aziin tologch ovs	Alag bashir	Pallasiin syyt ovs	Odoi saraana	Gashuun Banzdoo
Tibetan name	Bambo bam-po (bam-po)	Umodeujin b-S-k-dmr-po (ba sha ka dmar po)	Durjid, àon-bu (khron bu)	Aviha a-bi-Ê (a bi sha)	Gazriin khokh Lugzi dovo lug-rtsi-do-bo (lug-rtsi-do-bo)
English name	Asiatic Yarrow	Colour-changing Pink, Versicolor Pink	Pallas Milkwort	Low Lily	Meadow Saussurea
Synonym	<i>A. setacea</i> auct. non WALDST. et KIT. (Olziikhutag 1983)			<i>L. tenuifolium</i> FISCH. ex SCHRANK (Olziikhutag 1983)	<i>S. marginata</i> Borszca. ex Trautv., <i>S. glomerata</i> Poir., <i>S. amara</i> (L.) DC. var. <i>glomerata</i> (Poir.) Trautv., <i>S. glomerata</i> var. <i>chinensis</i> F.H. Chen, <i>S. japonica</i> var. <i>alata</i> (Chen.) Nakai et Kitag., <i>S. centauroides</i> Tausch, <i>S. macrocephala</i> Less., <i>S. scabra</i> Less., <i>S. gmelini</i> Hort. Dorp. ex Herder, <i>S. microcephala</i> C.A.Mey., <i>S. microcephala</i> var. <i>aptera</i> Nakai et Kitag., <i>S. microcephala</i> f. <i>leucocephala</i> Nakai et Kitag., <i>S. japonica</i> f. <i>leucocephala</i> (Nakai et Kitag.) Nakai et Kitag., <i>S. amara</i> Less. var. <i>microcephala</i> (Franch.) Lipsh., <i>S. tenuicaulis</i> Ling, <i>Serratula amara</i> L., <i>Theodorea amara</i> Cass., <i>T. glomerata</i> (Poir.) Soják (Olziikhutag 1983)
Distribution	Mongolia: Khangai, Khyangan, Khentii, Khovd, Khovsgol, Mongol-Dagur, Ikh nuur	Mongolia: Dagur, Dornod Dundad, Gobi-Altai, Ikh nuur, Khalkh, Khangai, Khentii, Khovd, Khovsgol, Mongol, Mongol-Altai, Mongol-Khyangan, Olon nuur China Kazakhstan Siberia Ukraine	Mongolia: Dornod, Mongol (Khalkhiin gol), Khyangan	Mongolia: Dundad Dornod, Mongol, Khalkh, Khyangan, Khovsgol, Khentii, Khangai, Mongol-Dagur China East Russia Eastern Siberia North Korea	Mongolia: Dornod Mongol, Dundad Khalkh (west north), Ikh nuur, Khangai, Khovsgol (Darkhad), Khyangan, Mongol Altai, Mongol-Dagur, Olon nuur, Zyngar
Parts used	Root, herb	Herb, flowers	Root	Flowers	Herb

Table 1 continued

	<i>Achillea asiatica</i>	<i>Dianthus versicolor</i>	<i>Euphorbia pallasii</i>	<i>Lilium pumilum</i>	<i>Saussurea amara</i>
Ingredient in following traditional prescriptions	Agar-11 Ar ur-7 Dilmanmar Dorijan Ganman-7 Gavar-9 Gurgum-8 Shinjyd-21 Tsarvan-15 (Ligaa et al. 2005; Yuthok, eighth–ninth century; Danzanpuntsag, eighteenth century, Boldsai Khan 2004)	Bashaga-7 Digda-4 Ruda-6 Zandan-18 (Ligaa et al. 2005; Yuthok, eighth–ninth century; Danzanpuntsag, eighteenth century; Boldsai Khan 2004)		Bushelz-7 Davsén-6 Luded-18 (Ligaa et al. 2005; Yuthok (eighth–ninth century); Danzanpuntsag, eighteenth century; Boldsai Khan 2004; Khurelchuluun et al. 2007)	Bavo-13, 14 Banzido-2, 6, 11, 12 Bontag-25 Bragshun-8, 25 Givan-7, 10, 20 Gurgum-7, 9 Dudzi-10 Seru-15 Tsarvon-4 Tanchin-10 Tuglogunsel (Ligaa et al. 2005; Yuthok, eighth–ninth century; Danzanpuntsag, eighteenth century; Boldsai Khan 2004; Khurelchuluun et al. 2007)

Chemical constituents

For the plant ubiquitous sugars (Kalinkina et al. 1989a) and organic acids (Kalinkina and Beresovskaya 1974) are described. Polysaccharides consisting of galacturonic acid, rhamnose, arabinose, xylose, mannose, glucose, galactose, and an unidentified sugar were obtained after extraction of the herb with hot water (Kalinkina et al. 1988). Coumarins, such as umbelliferone and scopoletin were detected (Kalinkina et al. 1989b). The flavonoids (Valant-Vetschera 1984; Narantuya et al. 1999) comprise kaempferol (Kalinkina et al. 1989b), vitexin, isovitexin, orientin, isoorientin (Valant-Vetschera 1984), apigenin, diosmetin, centaureidin, and apigenin-7-*O*-glucoside (Narantuya 1996). Moreover, essential oil is contained up to a content of 0.2–0.5 %, with α -pinene, β -pinene, sabinene, camphor, limonene, 1,8-cineole, *n*-cymol, camphor, borneol, and β -caryophyllene as the main constituents (Kalinkina and Beresovskaya 1974; Motl et al. 1990; Sokolov et al. 1993). Steam distillation of the drug provides a blue coloured oil which is predominated by hydrocarbons (58 %) and chamazulene (17.5 %) according to Motl et al. (1990), and Yusubov et al. (2000). Kalinkina and Beresovskaya (1975), detected changes in the essential oil and proazulene contents during the growth season. The highest proazulene contents were measured at full blooming in the inflorescences, whereas the level in the stalks were found to be low during the entire growth period. Therefore, the authors recommend to harvest inflorescences including leaves and upper parts of the stalks at the stage of full flowering. A number of sesquiterpene lactones, among them several proazulenes, have been isolated and structurally elucidated (see Table 2; Narantuya et al. 1999; Narantuya 1996; Gunbilig 2003; Glasl et al. 2001a, b). The proazulene pattern is very similar to the one of *A. collina*.

Bioactivities

An aqueous extract showed anti-inflammatory, haemostatic and bile-expelling activities (Myagmar 1992). In an other trial, the preparation “achigran”, isolated by extraction with ethanol-water from *A. asiatica*, was investigated for its acute and chronic antiulcer activity after direct intragastral administration via catheter in both rat and mouse (Slipchenko et al. 1994). Application of an aqueous *A. asiatica* extract in dogs one

month after implanting fistulas led to a reduced activity of proteolytic enzymes and to a decrease of the acidity in the gastric juice, whereas the mucus synthesis and excretory function of the stomach were activated (Vymiatnina and Gridneva 1997). The antiphlogistic systemic effect of a lipophilic extract from *A. asiatica*, enriched with sesquiterpenes, was tested in mice. The animals were treated orally for 5 days at a daily dose of 24.5 mg/kg. Inflammation was elicited by injection of oovalbumin in the leg of the experimental animal, and the volume of inflammation was measured at time points 0, 30, 60 and 120 min. The *A. asiatica* extract showed significant activity after 30 and 120 min which decreased at the end of the experimental period (Gunbilig et al. 2003). In vitro and in vivo experiments in rabbits exhibited an anthelmintic effect. *A. asiatica* was active against the larvae of trichostrongyle gastrointestinal nematodes (Nemeth and Bernath 2008).

Additional data

The gene structure of diploid and tetraploid *A. asiatica* and several other taxa has been investigated by AFLP analyses in order to explain the complex relationships and evolutionary processes within the polyploid *A. millefolium* aggregate (Guo et al. 2005, 2013).

Dianthus versicolor FISCH EX LINK.
(Caryophyllaceae)

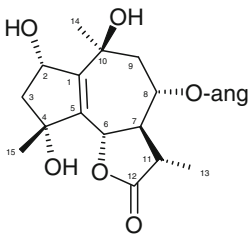
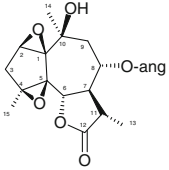
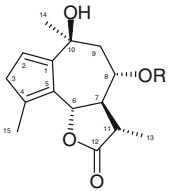
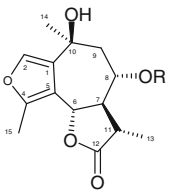
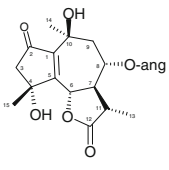
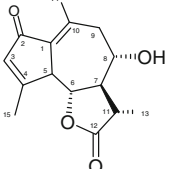
Description

Thick roots produce many flowering stems, but not vegetative shoots. Stem 10–35 cm tall, erect, branched, leaves with short, stiff, sparse hairs, or glabrous. Leaves 3–6 cm long, 2–7 mm wide, narrow linear, acute. Solitary, or two to three flowers at the tip of stems and branches. Bracts mostly four, ovate, tapering abruptly into long and lanceolate-linear tip. Uppermost bract very close to the flower. Sepals 13–18 mm long, tube-like, tapering to the apex. Petals 20–25 mm long, pink, their limbs 10–12 mm long, unevenly dentate at the tip.

Habitat

Slopes of mountains and hills in forest-steppe and steppe zone (Olziikhutag 1983; (Gubanov 1996; Malishev and Peshkova 1979; Sanchir et al. 2003; Ligaa et al. 2005).

Table 2 Guaianolides from the aerial parts of *Achillea asiatica*

	8α-angeloxy-2α,4α,10β-trihydroxy-6βH,7αH,11βH-1(5)-guaien-12,6α-olide	Proazulene ^a	Glasl et al. (2001a, b)
	8α-angeloxy-1β,2β:4β,5β-diepoxy-10β-hydroxy-6βH,7αH,11βH-guaien-2,6α-olide	Proazulene	Glasl et al. (2001a, b)
	8α-tigloxy-artabsin (R = tiglyl)	Proazulene	Narantuya et al. (1999)
	8α-angeloxy-artabsin (R = angelyl)	Proazulene	
	8α-acetoxy-artabsin (= achillicin, R = acetyl)	Proazulene	
	8α-tigloxy-3-oxa-artabsin (R = tiglyl)		Narantuya et al. (1999)
	8α-angeloxy-3-oxa-artabsin (R = angelyl)		
	8α-acetoxy-3-oxa-artabsin (= 3-oxa-achillicin, R = acetyl)		
	8α-angeloxy-4α,10β-dihydroxy-2-oxo-6βH,7αH,11βH-1(5)-guaien-12,6α-olide		Glasl et al. (2001a, b)
	8-desacetyl-matricarin		Glasl et al. (2001b)

^a Proazulene degrades to blue coloured derivatives of the chamazulene type under treatment with heat or acid

Traditional use

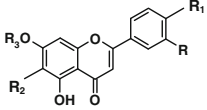
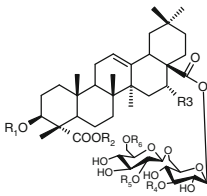
The taste is astringent and the potency is cool. It is used to treat the following diseases: pneumonia, typhoid, typhoid fever, and scurvy disease.

Chemical constituents

The plant contains ascorbic acid. Early papers report on the flowers to contain saponins (Fedorov et al.

1985) and flavonoids (Fedorov et al. 1985; Boguslavskaya et al. 1983). Ma et al. isolated the pentacyclic triterpenoid saponins dianversicosides A-G from the herb (see Table 3; Ma et al. 2009). Various flavonoids were recently described from the herb, such as C- and O-glycosides of apigenin, luteolin, chrysoeriol and 3'-or 4' methylfluteolin (see Table 3; Obmann et al. 2011b). HPLC methods for the quantification of the flavonoids were developed by which the total flavonoid content in the crude drug was determined to

Table 3 Flavonoids and triterpene saponins from the aerial parts of *Dianthus versicolor*

	R	R ₁	R ₂	R ₃				
	OH	OH	Glc	rha(1 → 6)glc	Isoorientin-7-O-rutinoside		Obmann et al. (2011b)	
	OH	OH	Glc	rha(1 → 6)gal	Isoorientin-7-O-rhamnosyl-galactoside			
	H	OH	Glc	rha(1 → 6)glc	Isovitexin-7-O-rutinoside			
	H	OH	Glc	rha(1 → 6)gal	Isovitexin-7-O-rhamnosyl-galactoside			
	OMe	OH	Glc	rha(1 → 6)glc	Isoscoparin-7-O-rutinoside			
	OMe	OH	Glc	rha(1 → 6)gal	Isoscoparin-7-O-rhamnosyl-galactoside			
	OMe	OH	Glc	gal	Isoscoparin-7-O-galactoside			
	OH	OH	Glc	gal	Isoorientin-7-O-galactoside			
	H	OH	Glc	glc	Isovitexin-7-O-glucoside (saponarin)			
	H	OH	rha(1 → 2)glc	H	Isovitexin-2''-O-rhamnoside			
	H	OH	Glc	H	Apigenin-6-glucoside (isovitexin)			
	OH	OH	H	Glc	Luteolin-7-O-glucoside			
	H	OH	H	Glc	Apigenin-7-O-glucoside			
	OH	OH	H	H	Luteolin			
	H	OH	H	H	Apigenin			
	OMe	OH	H	H	Chrysoeriol			
	OH	OMe	H	H	Diosmetin			
	H	OMe	H	H	Acacetin			
	R ₁	R ₂	R ₃	R ₄	R ₅	R ₆		
	H	Glc	OH	H	Glc	HMG	Dianversicoside A	Ma et al. (2009)
	H	H	OH	Glc	Glc	HMG	Dianversicoside B	
	H	Glc	H	Glc	Glc	HMG	Dianversicoside C	
	H	H	H	Glc	Glc	HMG	Dianversicoside D	
	H	Glc	OH	Glc	Glc	H	Dianversicoside E	
	H	Glc	OH	H	Glc	H	Dianversicoside F	
	Ara	H	H	Glc	Glc	H	Dianversicoside G	

HMG 3-hydroxyl-3-methylglutaryl

vary between 0.07 and 0.57 % (Obmann et al. 2011a, 2012).

Bioactivities

The extract shows antihypertensive activity, the decoction hemostatic and uterine-stimulating activities (Fedorov et al. 1985). The extraction of the aerial parts with 80 % ethanol yielded dianversicosides A–G, triterpene saponins of the oleanane type. These compounds exhibited more potent activities against HFL-I, EVC-304, and BGC-803 cells (IC₅₀ values between 2 and 10 μM) than against MCF-7 and Hep G2 cells (Ma et al. 2009). Methanolic and aqueous extracts of the herb stimulate the bile flow in the model of the isolated perfused rat liver (Obmann et al. 2010).

Euphorbia pallasii TURCZ. ex LEDEB.
(Euphorbiaceae)

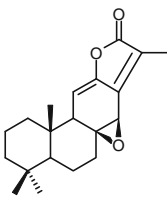
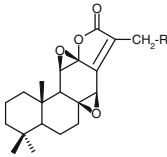
Description

Cauline leaves: lowermost squamiform, the others verticillate (lower ones by 3, upper ones by 5), oblong-elliptic, coriaceous, with revolute margins. Umbel rays strongly divaricate. Plant green (Grubov 2008).

Habitat

Steppe stony and debris slopes and rocks (Grubov 2008)

Table 4 Diterpene lactones in the roots of *Euphorbia pallasii*

	Pseudojolkinolide A	Sirchina et al. (1985)
	Pseudojolkinolide B (R = H) 16-hydroxy-pseudojolkinolide B (R = OH)	

Traditional use

The taste is sweet. The power is warm, coarse and sharp. It is used to treat pyic wounds, vomiting phlegm, and acts as a laxative for all disorders (Boldsai Khan 2004).

Chemical constituents

The root contains the triterpenoids sitosterin, lupeol, and cycloartenol acetate (Tarmaeva and Belova 1980), O-acetyl-N-(N'-benzoyl-L-phenylalanyl)-L-phenylalanine (Uemura et al. 1975), flavonoids and anthringlycosides (Sokolov et al. 1986, no chemical structures available). The acetone soluble fraction of a chloroform extract from the roots yielded three diterpene lactones of the pseudojolkinolide type (see Table 4; Sirchina et al. 1985).

Bioactivities

The acetone and hexane extracts show activity against hypoxia (Sokolov et al. 1986). The pseudojolkinolide containing chloroform extract exhibited antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Sirchina et al. 1985).

Lilium pumilum DELILE (Liliaceae)

Description

Perennial herbs. Bulbs 3–4 cm long, white, ovate, covered by grey scales. Stem thin, smooth, 18–80 cm

tall. Leaves sessile, narrow, linear, 3–10 cm long, 1–3 mm wide, a clear vein on lower surface, margins slightly curved down. Flowers drooping, tepals oblong lanceolate, their tips curved out, bright red. Raceme 1.5–3 cm long, consisting of 2–6 flowers.

Habitat

Mountain slopes, meadow slopes, stony slopes, pine forests (Gubanov 1996; Malishev and Peshkova 1979; Sanchir et al. 2003; Ligaa et al. 2005).

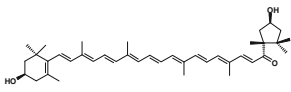
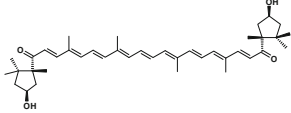
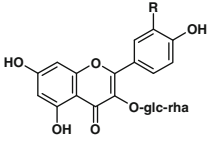
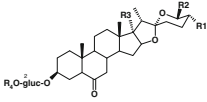
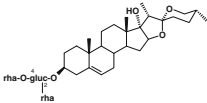
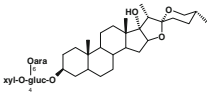
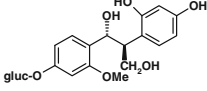
Traditional use

The taste is sweet and the potency is cool. It is used to increase urination, to decrease edema and to promote phlegm. The flowers are used to treat hemostatis, to dry out lymph, to treat wounds and menorrhagia.

Chemical constituents

Early publications report on alkaloids in the aerial parts of the plant, but do not give any chemical formulae (Antsupova 1975, 1976). The flowers contain the carotenoids capsorubin and capsanthin (see Table 5; Partali et al. 1987). Zhou et al. isolated several steroidal saponins and a phenolic glycoside (see Table 5; Zhou et al. 2012) from the bulbs. The belowground organs also contain gallic acid, p-coumaric acid, rutoside, catechin, epicatechin, myricetin, rutin, quercetin, and kaempferol (Jin et al. 2012). According to Huang et al. the bulbs contain 0.006 %

Table 5 Carotenoids, flavonoids, steroids saponins and a phenolic glycoside from *Lilium pumilum*

	Capsorubin	Flowers	Partali et al. (1987)																				
	Capsanthin																						
	Kaempferol-3-O-rutinoside (R = H) Rutoside (R = OH) Isorhamnetin-3-O-rutinoside (R = OMe)	Aerial parts	Obmann et al. (2010)																				
	<table border="1"> <thead> <tr> <th>R₁</th><th>R₂</th><th>R₃</th><th>R₄</th></tr> </thead> <tbody> <tr> <td>CH₂OH</td><td>H</td><td>OH</td><td>rha</td></tr> <tr> <td>Me</td><td>H</td><td>OH</td><td>rha</td></tr> <tr> <td>Me</td><td>H</td><td>H</td><td>rha</td></tr> <tr> <td>Me</td><td>H</td><td>H</td><td>ara</td></tr> </tbody> </table>	R ₁	R ₂	R ₃	R ₄	CH ₂ OH	H	OH	rha	Me	H	OH	rha	Me	H	H	rha	Me	H	H	ara	Bulbs	Zhou et al. (2012)
R ₁	R ₂	R ₃	R ₄																				
CH ₂ OH	H	OH	rha																				
Me	H	OH	rha																				
Me	H	H	rha																				
Me	H	H	ara																				
	Dioscin																						
	(25R)-5 α -spirostan-3 β ,17 α -diol-3-O- β -D-xylopyranosyl-(1 \rightarrow 4)-[α -L-arabino-pyranosyl-(1 \rightarrow 6)]- β -D-glucopyranoside																						
	Threo-1-(4'-hydroxy-2'-methoxyphenyl)-2-(2'', 4''-dihydroxyphenyl)-1, 3-propanediol-4'-O- β -D-glucopyranoside																						

dioscin (Huang et al. 2011). Obmann et al. investigated the aerial parts and identified the flavonoids rutoside, kaempferol-3-O-rutinoside, and isorhamnetin-3-O-rutinoside (see Table 5; Obmann et al. 2010).

Bioactivities

The water extract of the flowers acts as an anti-inflammatory, spasmolytic and liver protective agent in rats (Tseng-Ayush 2001). The acidified methanol extract of the bulb has moderate antioxidative activity in DPPH assay, ABTS assay, Cupric ion Reducing Antioxidant Capacity assay and Hydroxyl Radical Scavenging Activities assay (Jin et al. 2012). The methanolic extract of the bulbs inhibit Na⁺/K⁺ + ATPase (Zhou et al. 2012). Aqueous and methanolic extracts stimulate the bile flow in the perfused rat liver model (Obmann et al. 2010).

Saussurea amara (L.) DC. (Asteraceae)

Description

Perennial herb with 7–60 cm tall, erect, strong, glabrous or scabrous stems, branched in upper part, sometimes simple. Radical lower leaves petiolate, 3–15 cm long, 1–4 cm wide, oblong-ovate, oblong-lanceolate, with big teeth or irregular dentate, sometimes almost entire, both surfaces green, scabrous, with small glands. Loose corymbose heads form terminal corymbiform panicle. Involucres 10–15 mm long, 6–10 mm wide, layered on each other, with short tomentum. Flowers pink, with glands.

Habitat

Alkaline sandy and rocky riverbanks, waterside alkaline waters, nomad camps, agricultural fields, flooded

places (Gubanov 1996; Malishev and Peshkova 1979; Sanchir et al. 2003; Ligaa et al. 2005).

Traditional use

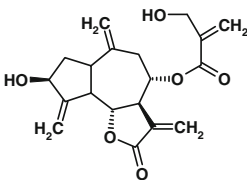
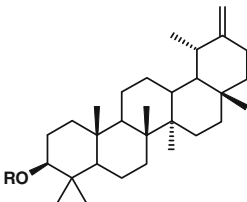
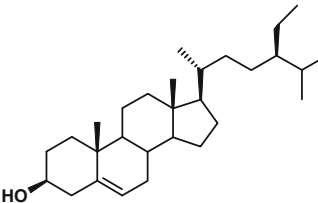
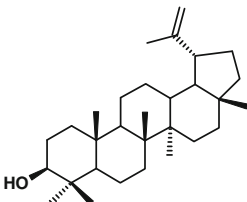
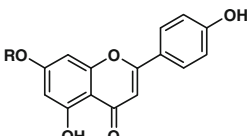
The taste is bitter and the potency is cool. It is used to treat infectious diseases, inflammation, bile disorders, and acts as antibacterial agent.

Chemical constituents

The fatty oil of the seeds amounts up to 27 %. It consists mainly of γ -linolenic acid and several other unsaturated fatty acids (Tsevegsuren et al. 1997;

Daariimaa 2006). Sokolov et al. identified sugars, coumarins, cardenolides, anthraquinone glycosides, alkaloids (0.1 %), and tannins (0.7 %)(Sokolov et al. 1993). The sesquiterpene cynaropicrin (see Table 6) was isolated from the aerial parts (Konovalova et al. 1979; Glasl et al. 2007). An HPLC method was developed for its quantification, the cynaropicrin content in the crude drug was determined to be 1.15 % (Glasl et al. 2007). Besides the sterols, taraxasterol, 3-O-acetyltaraxasterol, β -sitosterol, and lupeol, flavonoids were described, namely apigenin, apigenin-7-O-glycoside, and genkwanine (see Table 6; Daariimaa 2006). The total content of flavonoids, calculated as hyperosid, was determined spectrophotometrically and amounted to 0.054 % (Glasl et al. 2007).

Table 6 Terpenoids from the aerial parts of *Saussurea amara*

	Cynaropicrin	Konovalova et al. (1979) and Glasl et al. (2007)
	Taraxasterol (R = H) 3-O-acetyl-taraxasterol (R = acetyl)	Daariimaa (2006)
	β -sitosterol	
	Lupeol	
	Apigenin (R = H) Apigenin-7-O-glucoside (R = gluc) Genkwanin (R = Me)	Daariimaa (2006)

Bioactivities

The extracts show hemostatic and anti-neoplastic activities, as well as antibacterial properties against *S. aureus*, *E. coli*, and *P. aeruginosa* (Sokolov et al. 1993; Modonova et al. 1986). Cynaropicrin, apigenin, and apigenin-7-O-glycoside showed a choleric effect (Daariimaa 2006). Extracts of different polarity, obtained by extraction with ethylacetate, methanol and water, increased the bile flow in the isolated perfused rat liver at low concentrations. Higher concentrations partly caused a decrease in the bile flow (Glasl et al. 2007). Cynaropicrin provoked an increase of the bile flow in the isolated perfused rat liver at 5 and 10 mg/L, but caused a continuously increasing perfusion pressure at concentrations beyond (Glasl et al. 2007).

Additional data

The regulation of proline accumulation in seedlings and the activities of the key enzymes involved in the proline metabolism were studied at increasing salt concentrations (Wang et al. 2011).

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1.3 Austrian folk medicine

1.3.1 Volksmed databank

At the beginning of the 1980ies started a project at the Department of Pharmacognosy, University of Vienna under the leadership of Prof. Wolfgang Kubelka and Prof. Johannes Saukel with the aim to preserve knowledge from Austrian folk medicine. For that purpose, diploma students were sent out into different parts of Austria, South Tyrol and Bavaria, preferably into more rural regions, to interview "knowledgeable people", doctors and pharmacists about natural treatments for different diseases. Between 1983 and 1995, 42 diploma theses were written and the data compiled in the Volksmed databank. Information can be scanned by indication, application, plant family, plant species and used plant parts (Saukel and Kubelka, 1993, 1994).

When the term "diabetes mellitus" is entered into the Volksmed databank, a list of 58 traditionally used plants is provided alongside the used plant part and mode of application. This list served as basis for the investigation of traditionally used plants against diabetes mellitus in Austria. Leaves of *Juglans regia* L. are one of these herbs and have, after a thorough selection procedure (see chapter 3.1.2, page 34), been chosen to be investigated with respect to insulin-sensitizing properties and chemical composition.

1.3.2 *Juglans regia* L.

Walnut (*Juglans regia* L.) is a species belonging to the plant family of Juglandaceae and is distributed in Europe, North Africa, North America and East Asia.

The tree is about 10-25 m high, its leaves are alternately arranged, about 25 cm long and odd-pinnate with 7-9 elliptical leaflets. In young years, the bark is olive-brown, turning silvery-grey on older branches. The male flowers are dropping in long, green catkins and the female flowers are in clusters of two to three at the branch's terminal. The fruits of brown nuts are ripening in autumn and are covered with a green, fleshy husk (Wichtl et al., 2009).

Leaves of *J. regia* are blueish green in color and pubescent in young years, whereas adult leaves are dark green and almost glabrous, with only a few trichomes growing



Figure 1.3.: *Juglans regia* L.
Source: Wikipedia (2015)

on the vascular bundle. The venation on the leaf bottom shows a typical pattern with the secondary vein exiting from the main vein at about 45° and the tertiary vein being orthogonal to the secondary vein. Anatomical characteristics include druse in the spongy parenchyma, anomocytic stomata and short, thick glandular trichomes with a unicellular stalk and a multicellular head on both sides of the leaf. Phytochemical constituents are mainly of polyphenolic character: about 10% tannins, 3.4% flavonoids and phenol carbonic acids. Fresh leaves contain the naphthoquinone juglone, which polymerizes upon drying and is therefore hardly detected in dried material (Wichtl et al., 2009).

Standard literature states the use of walnut leaves (*Folium juglandis*, DAC) mainly due to its astringent nature - against massive perspiration and minor skin inflammations (Bundesvereinigung deutscher Apothekerverbände, 2012). In addition, recent studies report about antioxidative (Almeida et al., 2008), antibacterial (Sharafati-Chaleshtori et al., 2011), antifungal (Tomaszkiewicz-Potepa et al., 2005) and sedative (Girzu et al., 1998) effects. According to the Volksmed databank, *J. regia* leaves are traditionally used in Austrian folk medicine against disorders of the gastrointestinal tract and against diabetic disorders (Gerlach, 2007). Moreover, a positive effect on blood glucose levels and lipid profiles of walnut leaf extracts has been scientifically proven in mouse models

(Asgary et al., 2008) and in a human clinical trial (Hosseini et al., 2014). The mechanism of action, however, remained elusive. The publication provided in section 4.4.2 (page 124) elucidates a possible explanation for these hypoglycemic effects.

2 Objectives

Juglans regia L. and *Leonurus sibiricus* L. are applied against diabetic disorders in Austrian folk medicine and Traditional Mongolian Medicine, respectively. In this thesis, three goals are pursued: first, the plants' traditional use is probed by testing its effect on *in vitro* models related to insulin-sensitization. Secondly, effect-determining compounds are identified by bioactivity-guided fractionation of the active extracts, paralleled by their chemical characterization. In third instance, qualitative and quantitative chromatographic as well as microscopic methods are established to provide analytical means for quality control of the used drug.

As activity screening method serves a functional glucose-uptake assay in C2C12 myocytes. If applied extracts increase the glucose-uptake, the involvement of a prominent downstream target is evaluated: protein tyrosine phosphatase 1B.

Fractionation of active extracts is achieved by a series of chromatographic methods. After each fractionation step, the obtained fractions are tested in the bio-assays and the most potent fraction is used for further separation. This way, the most potent compounds of the plant can be narrowed down. Active fractions are chemically characterized by LC-MS dereplication, using authentic reference compounds and literature data for comparison. If dereplication is not possible due to lack of data, single compounds are isolated and their structure elucidated by NMR.

To define parameters for quality control (if such are not yet in existence), selective anatomical and phytochemical properties are evaluated, in accordance to modern monographs in pharmacopoeias. A quick qualitative assessment is achieved by HPTLC, detailed chemical analysis, including identification and quantitation of marker compounds, is realized by HPLC. Anatomical characteristics are determined by microscopic analysis of several plant samples and reference specimens of varying origin.

3 Material and methods

Published methods are described only shortly and can be found in more detail in the experimental section of the corresponding publication.

3.1 Plant material

3.1.1 *L. sibiricus*

The aerial parts of *L. sibiricus* were collected in August 2009 (a), August 2010 (b) and August 2014 (c) in Archangai province near Tstetserleg, Mongolia (Figure 3.1, page 33).



Figure 3.1.: Map of Mongolia. Region of collection of *L. sibiricus* is highlighted in green
Source: modified according to www.camamongolia.org

E. Ganbold, Head of Department of Biology, Ulaanbaatar University identified the plant material. A reference specimen of batch **a** is kept at the herbarium of the School of Pharmacy, Health Sciences University of Mongolia, Ulaanbaatar (specimen number 10080805). Reference specimens for batch **b** and **c** are kept at the Department of Pharmacognosy, University of Vienna, Austria (herbarium numbers HERB.LS.01/10/mon

and HERB_LS_01/14/mon, respectively). The drug was air-dried in Mongolia, all further processing was done in Vienna.

3.1.2 Austrian plants

The Volksmed databank (chapter 1.3.1, page 28) provided a list of 58 plants, which are traditionally used against diabetes mellitus in Austria. Six plants of this list were chosen to be screened for *in vitro* glucose lowering effects. The most potent of the plants was to be further investigated.

Plant (abb.)	Herb	Batch number (company)	DER of dry MeOH extract
<i>Arctium lappa</i> (AL)	Radix Bardanae	n.a. (Kottas)	7-8
<i>Equisetum arvense</i> (EA)	Equiseti Herba	KL-427804/07 (Kottas)	9-10
<i>Juglans regia</i> (JR)	Folium Juglandis	10240212 (Kottas)	4-5
<i>Plantago lanceolata</i> (PL)	Plantaginis Folium	KLA 70898 (Kottas)	5-6
<i>Ribes nigrum</i> (RN)	Fructus Ribis	1642511/05 (Richter)	10-11
<i>Sambucus nigra</i> (SN)	Sambuci Fructus	KLA 70086 (Kottas)	14-15

Table 3.1.: Extracted plants from Austrian folk medicine (left), used part of the plant (center-left), charge number and company (center-right), drug-to-extract ratio of the dry methanol extract (DER, right). n.a.=not available

To be considered as one of the selected candidates, the current literature had to provide information about possible *in vivo* hypoglycemic activities, and, in order to cover a broad spectrum of phytochemical ingredients, the plants had to belong to different families. The chosen plants were: *Arctium lappa* L. (AL, Asteraceae), *Equisetum arvense* L. (EA, Equisetaceae), *Juglans regia* L. (JR, Juglandaceae), *Plantago lanceolata* L. (PL, Plantaginaceae), *Ribes nigrum* L. (RN, Grossulariaceae) and *Sambucus nigra* L. (SN, Sambucaceae). The plant material was obtained either from Kottas Pharma GmbH (Vienna, Austria) or Richter Pharma AD (Wels, Austria). The plant name, used part of the plant and the corresponding batch number and company are summarized in Table 3.1 (page 34). There was no batch number available for *Arctium lappa*. Radix-, folium- and herba- drugs were cut, fructus drugs were *in toto*.

3.2 Extraction and purification

All herbs, except for fructus-drugs, were pulverized with a basic electric grinder before extraction. Ribis fructus and Sambuci fructus were not ground because of their high content of fats. All prepared powders were stored at room temperature and protected from sunlight before use.

3.2.1 *L. sibiricus*

The extraction of *L. sibiricus* was carried out by accelerated solvent extraction using an ASE®200 system from Dionex (Sunnyvale, CA, USA). 80 g of the powdered material were equally divided and filled into eight stainless steel extraction vessels. Each cell was extracted three times with 240 mL methanol, intermitted by a tube-cleaning step with acetone between every new cycle.

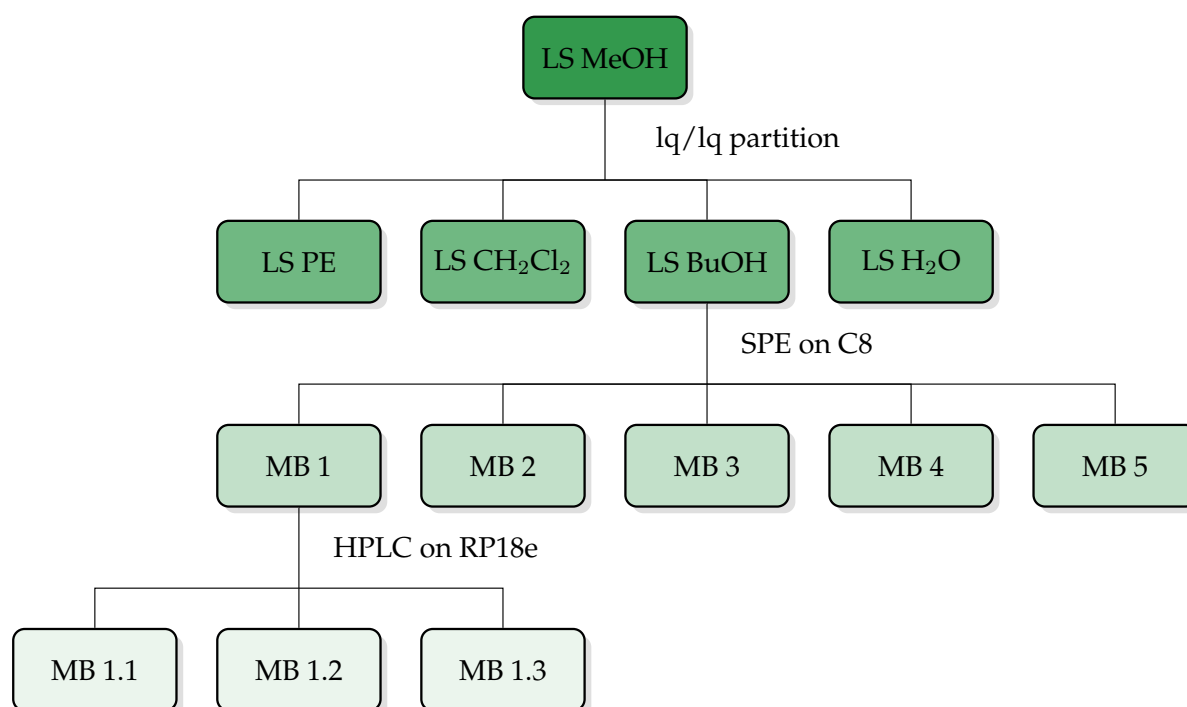


Figure 3.2.: Scheme of fractionation procedure of the *L. sibiricus* methanol (LS MeOH) extract. PE=light petroleum, CH₂Cl₂=dichloromethane, BuOH=butanol

Extractions were carried out at 40°C and under a pressure of 10 MPa. The resulting liquid extracts were combined and evaporated to dryness under reduced pressure at

40°C, yielding 13 g of dry extract (DER=6-7:1). The dried crude extract was stored in the dark at 4°C before usage.

For extract purification, 10 g of the dry methanol extract were re-dissolved in 500 mL methanol and partitioned by liquid-liquid extraction using three solvents of different polarity consecutively. The extraction with light petroleum was performed to deplete chlorophyll. The remaining methanolic layer was diluted with the same volume of water and partitioned against dichloromethane, followed by water-saturated butanol. The whole procedure resulted in four extracts after evaporation of the solvents under reduced pressure: the light petroleum extract (LS PE, yield=3.54 g), the dichloromethane extract (LS CH₂Cl₂, yield=2.12 g), the butanol extract (LS BuOH, yield=1.65 g) and the aqueous layer which remained after the partition with water saturated butanol (LS H₂O, yield=4.75 g; see Figure 3.2, page 35).

LS BuOH showed good activity in the used test system (see section 3.8, page 45) and was therefore further fractionated by a series of reversed phase chromatography methods. 50 mg of LS BuOH were subjected to a solid phase extraction on C 8 material. Five fractions were obtained by elution with methanol-water mixtures ranging from 10% (v/v) to pure methanol. The first of these fractions was further separated by semi-preparative HPLC on RP18e, yielding three sub-fractions. The material and methods section of the corresponding publication (page 54) describes the fractionation procedure in detail, Figure 3.2 (page 35) gives a graphic representation.

ORIGINAL WATER EXTRACT (OWE)

In addition to the methanol extract, an aqueous *L. sibiricus* extract (original water extract - LS OWE) was produced, whose preparation takes the traditional way of application into account. In course of the extraction procedure, the digestion in the stomach is imitated by constant movement and low pH. Distilled water adjusted to pH 2 with trifluoroacetic acid served as extraction solvent. 10 g pulverized material were extracted for one hour at 40°C with 250 mL acidified water under constant movement on a rotary evaporator. Upon completion of extraction, the trifluoroacetic acid was evaporated and the remaining water extract lyophilized, yielding 3 g LS OWE (DER=3-4:1).

3.2.2 Austrian plants

The above-mentioned plants used in Austrian folk medicine, are applied as liquors. They are prepared either with schnaps or red wine. Taking this procedure into account, the dried plant material was extracted with methanol, which shows similar polarity to ethanol. The extraction of the six Austrian plants was carried out by accelerated solvent extraction, as described in section 3.2.1 (page 35). The drug-to-extract ratios (DER) are given in Table 3.1, page 34.

CHLOROPHYLL SEPARATION

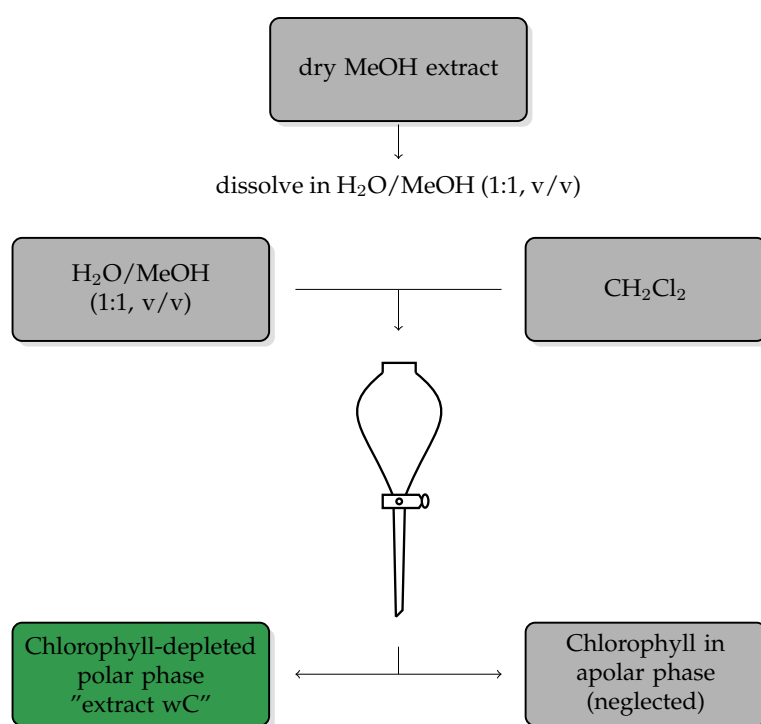


Figure 3.3.: Scheme of chlorophyll depletion

All folium- and herba drugs listed in Table 3.1 (see page 34) were separated from chlorophyll. 6 g of each crude methanol extract were re-dissolved in 50 mL H₂O/MeOH (1:1, v/v) and partitioned with equal volumes of CH₂Cl₂. The upper layer was separated and evaporated to dryness to yield the chlorophyll-depleted extract, while the lower layer was neglected. A scheme of this procedure is depicted in Figure 3.3 (page

37). Three chlorophyll depleted extracts ("wC" – without chlorophyll) were produced this way: EAwC (yield: 5.4 g), JRwC (yield: 4.5 mg) and PLwC (yield: 5.2 mg). The absence of chlorophyll was checked by thin layer chromatography, using derivatization method 3 (see Table 3.2, page 39).

TANNIN SEPARATION

Separation of tannins was achieved by the technique described by Wall et al. (1996) and performed with *J. regia*. 240 mg of the chlorophyll depleted, dried JRwC extract (see Figure 3.3) were re-dissolved in 10 mL H₂O/MeOH (1:9, v/v) and defatted with the same volume of hexane by liquid/liquid partition. The apolar phase was discarded and the polar phase, after dilution with water to H₂O/MeOH (1:1, v/v), extracted with CHCl₃. In the next step, the apolar phase was washed with 1% NaCl and the residue evaporated to dryness under reduced pressure, yielding 5 mg of a tannin-degraded extract (JRwCT – without chlorophyll and tannins). The absence of tannins was checked by thin layer chromatography, using derivatization method 2 (see Table 3.2, page 39).

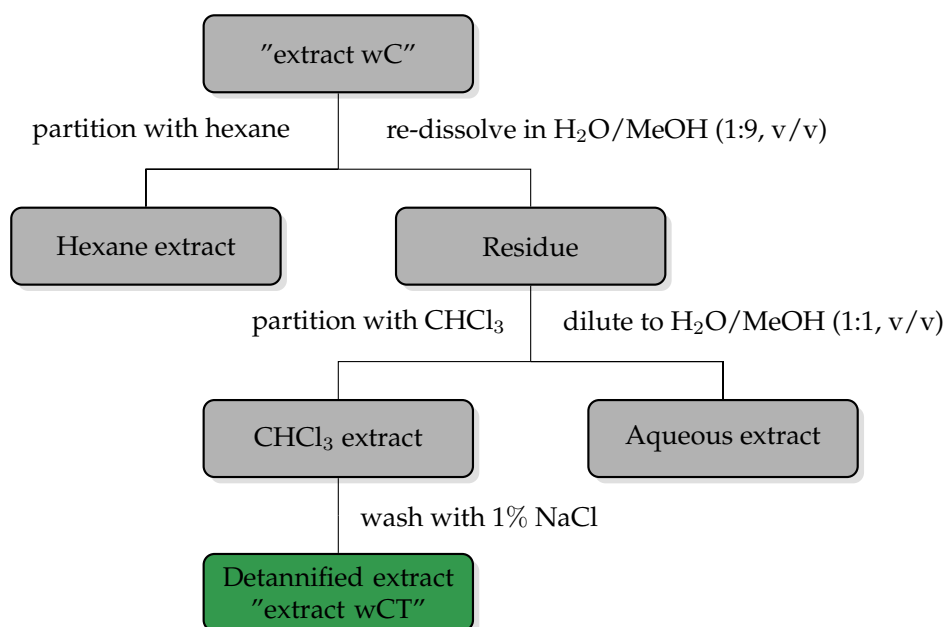


Figure 3.4.: Scheme of tannin separation, according to Wall et al. (1996)

3.3 HPTLC

High performance thin layer chromatography (HPTLC) was used for quick comparative analyses of different extracts and fractions as well as for verification of separation procedures (e.g. chlorophyll depletion). Parameters of HPTLC are summarized in Table 3.2 (page 39). As stationary phase served HPTLC silica gel 60 F₂₅₄ glass plates (Merck, Darmstadt, Germany).

Module	Parameter	
Application CAMAG ATS4	stationary phase	HPTLC silica gel 60 F ₂₅₄
	sample concentration	extracts 30 mg/mL pure compounds 10 mg/mL
	volume	5 μ L-15 μ L
	band length	10 mm
	distance from lower edge	130 mm
Development CAMAG ADC2	mobile phase	EtOAc, HCOOH, CH ₃ COOH, H ₂ O (100:11:11:26, v/v/v/v)
	humidity control	K ₂ CO ₃ (43% RH)
	saturation	20 min with saturation pad
	temperature	24°C
	total step time	59 min
Derivatization CAMAG immersion device III	reagent	detection
	(1) modified dragendorff	daylight
	(2) anisaldehyde-sulfuric acid	heat plate 7 min to 100°C; daylight, UV 366 nm
	(3) natural product reagent A	UV 366 nm
CAMAG visualizer	85% exposure	

Table 3.2.: Parameter of HPTLC for CAMAG operation system

As mobile phase served EtOAc, HCOOH, CH₃COOH, H₂O (100:11:11:26, v/v/v/v), a system suitable for the analysis of flavonoids and phenol-carboxylic acids. Extracts

and fractions were used at concentrations of 30 mg/mL, pure reference compounds at a concentration of 10 mg/mL. To improve solubility, sample solutions containing betaines were acidified with concentrated formic acid (0.2 μ L/mL). Samples were applied, developed and derivatized using automated modules of CAMAG (Berlin, Germany).

Plates were visualized at daylight, 245 nm and 366 nm before derivatization. Derivatization was performed by dipping the plate in the respective reagent, using CAMAG chromatogram immersion device III. Detection after derivatization varied, depending on the used reagent (see Table 3.2, page 39). For detection of flavonoids and phenolic acids served a 1% methanolic solution of diphenylboric acid- β -aminoethylester complex (natural product reagent A) followed by a 5% ethanol solution of polyethyleneglycol (PEG) 400. Anisaldehyde-sulfuric acid reagent (anisaldehyde, glacial acetic acid, methanol, sulfuric acid 0.5:10:85:5, v/v/v/v) was used to detect tannins and saccharides. For the detection of betaines and other alkaloids served a modified version of the Dragendorff reagent (see Table 3.3, page 40).

modified Dragendorff reagent	
Bi ₅ (OH) ₉ (NO ₃) ₄ O	0.085 g
H ₂ O _{dest}	25.00 mL
H ₂ SO _{4conc}	1.75 mL
CH ₃ COOH _{conc}	1.00 mL
KI solution, 40%	5.00 mL
H ₂ O _{dest}	ad 50.00 mL

Table 3.3.: Protocol for modified Dragendorff reagent

3.4 HPLC and detectors

High performance liquid chromatography (HPLC) was used for qualitative and quantitative assessments as well as semi-preparative isolation of pure compounds. Depending on the purpose, two different HPLC devices, described below, have been used.

HPLC and MS parameters (stationary & mobile phase, elution sequence etc.) varied and are described in more detail in the "material and method" section of the corresponding publication. For unpublished results, HPLC settings are summarized in Table 3.4, page 41.

The sample concentration of 30 mg/mL was used for analytical purposes. For semi-preparative isolation, the concentration was increased up to 80 mg/mL, depending on the capacity of the column.

	Method 1	Method 2	Method 3
Stationary phase	Luna phenyl hexyl 5 μ m, 250x2.0 mm	Luna phenyl hexyl 5 μ m, 250x2.0 mm	Aquasil C18 5 μ m, 250x4.6 mm
Elution sequence	Minute ACN [%] 0-10 5 \rightarrow 10 10-46 10 \rightarrow 23	Minute ACN [%] 0-70 5 \rightarrow 40	Minute MeOH [%] 0-45 10 \rightarrow 100
Flow	0.55 mL/min	0.20 mL/min	1.00 mL/min
Temperature	18°C	25°C	25°C
Detection	DAD, CAD	UV-254 nm, ELSD	UV-300 nm, ELSD
Sample	30-80 mg/mL 10 μ L	30-80 mg/mL 10 μ L	30-80 mg/mL 10 μ L

Table 3.4.: Parameter of used HPLC methods

3.4.1 UV-DAD-ELSD

HPLC for qualitative analyses and isolation of pure compounds was conducted on a Prominence LC-20AD coupled to a Prominence SPD-M20 Diode Array Detector and an evaporative light scattering detector (ELSD) (Shimadzu, Kyoto, Japan). Data analysis was carried out using LabSolutions version 1.25, LC-Postrun (Shimadzu). Details on the hardware can be found in Table 3.5, page 42.

Module	Description
Degaser	Shimadzu DGU-20As
Liquid chromatograph	Shimadzu LC-20AD
Auto sampler	Shimadzu SIL-20ACHT
Column oven	Shimadzu CTO-20AC
Communications bus module	Shimadzu CMB-20A
Diode array detector (DAD)	Shimadzu SPD-M20A
Evaporative light scattering detector (ELSD)	Shimadzu ELSD-LT

Table 3.5.: Parameters of Shimadzu HPLC

3.4.2 LC-CAD-MSⁿ

HPLC-CAD-ESI-MSⁿ analyses were performed on an UltiMate 3000 RSLC-series system coupled to a charged aerosol detector (CAD, Thermo, Waltham, MA, USA) and a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). CAD evaporation temperature was set to 25°C. The same device was used for quantification of main compounds in *L. sibiricus* and the subsequent method validation. Data analysis was carried out using Chromeleon version 7.1.1 (Dionex). Details on the hardware are summarized in Table 3.6, page 43.

3.5 Determination of sugar moieties

This chapter describes the procedure used to identify the glycosylation units of flavonoids isolated from *J. regia* and the determination of their configuration as described by Reznicek and Susman (1993).

Fischer projection differentiates between L and D saccharides. When condensation reaction with a racemic alcohol occurs, four enantiomers will emerge (DS, DR, LS, LR) (see Figure 3.5 a), which have the same physical and chemical properties. Thus, the pairs of enantiomers cannot be separated by chromatography. If, however, saccharides are chemically converted with a chiral reagent, two chromatographically separable di-

Module	Description
Degaser	Solvent Racks SRD-3x00
Pump	RS-pump series
Auto sampler	WPS-3000SL and WPS-3000RS
Column oven	TCC-3000SL and TCC-3000RS
UV detector	VWD-3x00
Diode array detector (DAD)	
Charged aerosol detector (CAD)	Corona Ultra RS, Thermo

Table 3.6.: Parameters of Dionex HPLC

astereomers will be generated (see Figure 3.5 b).

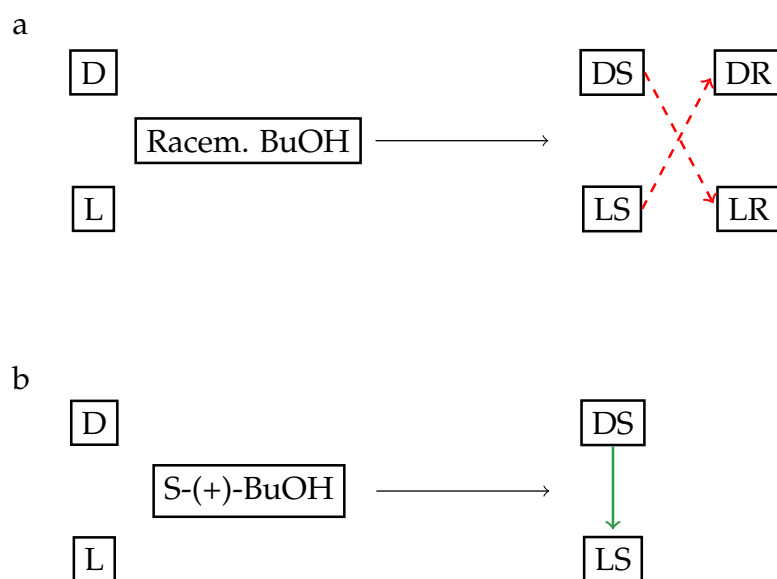


Figure 3.5.: Scheme of chemical reaction of saccharides with racemic butanol (a) and chiral butanol (b). Red, dashed arrows indicate enantiomers, green arrow indicates diastereomers

3.5.1 Acidic hydrolysis and derivatization

Isolated flavonoids were hydrolyzed for two hours with Kiliani reagent (5.5 mL H₂O, 3.5 mL CH₃COOH_{conc}, 1 mL HCl_{conc}) at 100°C to break the glycosylation bonds. The sample solution was partitioned three times with ethyl acetate. The apolar phase was

neglected, whereas the aqueous phase, containing the saccharides, was evaporated to dryness. The residue was treated with 0.45 mL S-(+)-butanol and 0.1 mL HCl_{conc} and heated to 100°C for 15 hours in a sealed reaction vial. This treatment resulted in the formation of the saccharides' corresponding diastereomeric butyl glycosides. Consequently, the sample solution was evaporated to dryness, re-dissolved in pyridin and derivatized with 5 μL hexamethyldisilacane and 5 μL trimethylchlorsilane, to form volatile derivatives. Before injecting to the GC-MS, the sample solution was kept at 80°C for 10 minutes in a tightly sealed reaction vial.

3.5.2 GC-MS

GC-MS analyses were performed on a QP2010 GC-MS (Shimadzu, Kyoto, Japan) using the following parameters:

GC: Phenomenex ZB-5 capillary column (65 m x 0.25 mm, thickness 0.25 μm); carrier gas: He 5.0; flow rate: 2.17 mL/min; split ratio 1:10; temperature gradient: 100-270°C at a rate of 3°C/min; injector and interface temperature: 270°C;

MS: ion source temperature 250°C; electron-impact ionization at 70 eV, scan range: 40 - 500 m/z.

The hydrolyzed monosaccharides were identified by comparison with authentic reference substances.

3.6 Microscopy

The settings for light microscopy and subsequent data analysis are detailed in the respective publication (page 98 f).

3.7 Cell culture of C2C12 myocytes

Conditions for the cultivation of C2C12 myocytes are outlined in the respective publication (page 78).

3.8 Assessment of glucose-uptake rate

The procedure for the assessment of the glucose-uptake rate is outlined in the respective publication (page 78 f).

3.9 *In vitro* PTP1B enzyme assay

The protocol for the *in vitro* PTP1B enzyme assay is outlined in the respective publication (page 79).

4 Results

4.1 *L. sibiricus*: *in vitro* activity and quantitation of main metabolites

To scientifically undermine the traditional use of *L. sibiricus* against DM in Mongolia, a bioactivity guided fractionation was performed. Two *in vitro* models served to monitor potential insulin-sensitizing effects: a functional glucose-uptake assessment in C2C12 myocytes and a target-oriented protein tyrosine phosphatase 1B (PTP1B) inhibition assay.

A methanol extract of herba leonuri sibirici (LS MeOH) proved to be active in both models. In course of the following fractionation, activity disintegrated in different fractions. However, an iridoid enriched fraction showed the highest activity among all sub-fractions. Thus, these glycosylated iridoids were quantified in different extracts of *L. sibiricus*, alongside phenylpropanoids, which constitute important marker substances of *L. sibiricus*. The paper summarizing the results is under revision in "Phytochemical analysis", given below (47 ff).

The wording of methods described in the following paper may resemble publications of co-authors, where similar methods were applied.

4.1.1 Paper under revision

Quantification of phenylpropanoids and iridoids in insulin-sensitizing extracts of *Leonurus sibiricus* L. (Lamiaceae)

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Keywords: *Leonurus sibiricus* L.; charged aerosol detector; quantification; validation; iridoids; phenylpropanoids; PTP1B inhibition

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Abstract

Introduction – *Leonurus sibiricus* L. (LS) is regularly used in the Traditional Mongolian Medicine (TMM). Respective Mongolian literature suggests the use of LS to treat symptoms of diabetes mellitus.

Objectives – This study was designed to provide a validated quantification method for the quality control of LS and to prove *in vitro* insulin-sensitization supporting the traditional use of LS.

Methodology – Pulverized LS material was either extracted with methanol or methanol-water_{dd} (25:75, v/v). HPLC separations were performed on a Luna phenyl-hexyl column with water and acetonitrile (both modified with 0.1% formic acid) as mobile phase. Gradient elution was employed using theophylline as internal standard. Tentative peak identification was facilitated by LC-MS. Validation was carried out according to ICH (International Conference on Harmonization) guidelines. Potential insulin sensitization of accordant extracts was assessed in glucose uptake experiments in C2C12 myocytes and protein tyrosine phosphatase 1B (PTP1B) enzyme assays.

Results – The established HPLC-CAD method allows sensitive quantification of the main secondary metabolites (glycosylated iridoids and phenylpropanoids) of LS, thereby providing a reliable tool for its quality control. Validation showed good accuracy, intermediate precision and robustness. The methanol extract of LS led to a 1.5 fold increase in insulin-stimulated cellular glucose-uptake and inhibition of PTP1B by 40 % at a concentration of 10 µg/mL.

Conclusion – This study describes a powerful chromatographic method to quantify the main secondary metabolites of LS with an internal standard and provides a rationale for LS's traditional use by showing its *in vitro* insulin-sensitizing activity in C2C12 myocytes.

Introduction

In the last decades, traditional medical systems like Traditional Chinese Medicine have gained global attention. Evermore patients turn to this age-long knowledge for disease prevention or as an alternative when conventional clinical therapy has failed (Patwardhan and Mashelkar, 2009). The rather unknown Traditional Mongolian Medicine (TMM) was strongly influenced by Ayurveda and Tibetan Medicine and is still the main medical care provider in Mongolia (Pitschmann *et al.*, 2013). Like in most traditional medical systems, TMM largely relies on herbal recipes. Today, officials in Mongolia are striving to scientifically prove the effectiveness of TMM preparations. In view of this development, thorough phytochemical characterization of the used plants and up-to-date standardized methods for their quality control are mandatory.

One of the most often used plants in Mongolia is *Leonurus sibiricus* L. (*L. sibiricus*, LS) (HSUM and MoH, 2007). The aerial parts are either consumed as aqueous/alcoholic preparation or swallowed as dry powder. The species belongs to the plant family of Lamiaceae and is spread in Mongolia, Russia and China. The herb is annual or biennial, reaches a height of 20-80 cm and has strigose anatomical elements. Flowers are arranged in verticillasters with white or reddish to purple-red corollae (eFloras, 2008; Grubov, 2001). LS is traditionally used in Mongolia to treat poisoning, diarrhea as well as high fever (WHO, 2013), and according to oral tradition it is also applied against diabetic symptoms (Schmidt *et al.*, 2013). Main secondary metabolites of LS include alkaloids (Luo *et al.*, 1985), flavonoids (Pan *et al.*, 2006), iridoids (Hayashi *et al.*, 2001), diterpenes (Wu *et al.*, 2011) and phenylpropanoids (Schmidt *et al.*, 2013).

The phytochemical classes of phenylpropanoids and iridoids are common in the plant kingdom and have been subject to an array of pharmacological studies (Galvez *et al.*, 2006; Tundis *et al.*, 2008), also revealing anti-diabetic actions. Observed effects ranged from decreasing advanced glycation end-products (AGEs) over improvement of insulin sensitivity to targeting glycogen phosphorylase- α , which is proposed to elicit anti-diabetic effects (Liu *et al.*, 2013; Vaidya *et al.*, 2013). Acetylharpagide, an iridoid glycoside present in LS, decreased the blood glucose levels of alloxan-induced diabetic mice by 29% after 2h of treatment (Ahmed *et al.*, 2003). Verbascoside, a major representative of the phytochemical group of phenylpropanoids in LS, has not been

tested as pure substance for a potential *in vivo* anti-diabetic activity yet. However, an extract of *Cistanche tubulosa* containing high amounts of verbascoside decreased fasting blood glucose levels in *db/db* mice (Xiong *et al.*, 2013). These reports strengthen the hypothesis of LS positively influencing glucose homeostasis.

In previous studies we showed that aqueous and methanolic extracts of LS increase insulin secretion in rat INS-1E insulinoma cells (Schmidt *et al.*, 2013). The current study extends the perspective of LS's influence on the glucose homeostasis by investigating *in vitro* effects of different extracts on the glucose uptake in C2C12 myocytes and on protein tyrosine phosphatase 1B (PTP1B) - a negative regulator of insulin signaling. Those extracts which show activity in our models are chemically characterized by chromatographic methods and mass spectrometry. This includes an LS methanol extract and its active butanol sub-fraction (enriched butanol fraction). Both are analyzed for the quantitative composition of selected secondary metabolites, which required the establishment of an HPLC method and its validation according to ICH (International Conference on Harmonization) guidelines. The same method is used to determine the quantity of selected marker compounds in the crude LS drug by using theophylline as internal standard. As to date there is no evidence in literature about quantitative data for LS. This serves, in view of LS's extensive prescription in traditional Mongolian recipes, as a reliable tool for the quality control of different LS batches and extracts thereof.

Experimental

Chemicals

Verbascoside (batch nr. 0082-05-80, purity: 86.31%) was purchased from Sigma Aldrich (St. Louis, MO, USA). 86.31% purity of verbascoside was taken into account for the preparation of standard solutions. Acetylharpagide (batch nr. NP-001679, purity: 99%) was obtained from AnalytiCon Discovery (Potsdam, Germany). Solvents used for extraction were of analytical grade; those used for HPLC were of gradient grade and obtained from VWR (West Chester, PA, USA).

Plant material

Aerial parts of three *Leonurus sibiricus* L. batches (LS **a-c**) were harvested at Tsetserleg in the Mongolian province Archangai (latitude 47° 28' 8.22"; longitude 101° 26' 32.14") in the summers of 2009 (**a**), 2010 (**b**) and 2014 (**c**). The plant material was identified by E. Ganbold, Mongolia. A reference specimen of batch **a** is kept at the herbarium of the School of Pharmacy, Health Science University of Mongolia, Ulaanbaatar, Mongolia (herbarium number 10080805). Reference specimens for batch **b** and **c** are kept at the Department of Pharmacognosy, University of Vienna, Austria (herbarium numbers HERB_LS_01/10/mon and HERB_LS_01/14/mon, respectively). The dried material was pulverized with a basic electric grinder and stored at room temperature, protected from sunlight.

Optimization of the extraction

To define the most effective extraction procedure, three different methods were compared with regard to their efficiency and impact on secondary metabolites in the resulting extract. For these preparatory tests, dried and pulverized LS material from batch **a** was used.

Extraction method 1. 2 g of dried and pulverized LS material were extracted three times with 20 mL MeOH each using an ASE 200 system from Dionex (Sunnyvale, CA, USA). Extractions were carried out at 40°C under a pressure of 10 MPa with an acetone tube cleaning step between every cycle. Resulting liquid extracts were combined and

evaporated to dryness under reduced pressure, yielding 455 mg of dry extract (drug to extract ratio (DER) = 4-5:1).

Extraction method 2. 2 g of LS material were extracted twice for one hour with 20 mL MeOH each under reflux at 60°C. The extracts were combined and evaporated to dryness under reduced pressure, to yield 412 mg (DER = 5:1) of dry material.

Extraction method 3. 2 g of LS material were extracted with 20 mL MeOH by stirring for 3 h at room temperature. The resulting extract was evaporated to dryness, yielding 344 mg (DER = 6:1).

Standard solutions for HPLC

Stock solutions for HPLC analysis of theophylline, acetylharpagide and verbascoside were prepared using MeOH-H₂O_{dd} (25:75, v/v) as solvent. Theophylline stock solutions were used at concentrations of 2 and 10 mg/mL, acetylharpagide and verbascoside stock solutions at 0.5 mg/mL.

Sample solutions

Crude drug. 0.5 g pulverized LS drug material were spiked with 100 µL of theophylline 10 mg/mL standard solution and extracted three times with 6 mL of MeOH-H₂O_{dd} (25:75, v/v) each using extraction method 1. Resulting liquid extracts were combined and evaporated to dryness under reduced pressure at 40°C (LS MeOH/water). DER was 3-4:1. For HPLC-CAD analysis, the whole dry extract was dissolved in 10.00 mL MeOH-H₂O_{dd} (25:75, v/v) and centrifuged at 1300 g for 2 min. 10 µL of this solution were subjected to HPLC-CAD analysis.

Methanol extract. 80 g pulverized LS were extracted three times with 240 mL MeOH each using extraction method 1, as described above. The liquid extracts were combined and evaporated to dryness under reduced pressure at 40°C (LS MeOH). For HPLC analysis, 4 mg of this extract were re-dissolved in 950 µL MeOH-H₂O_{dd} (25:75, v/v) followed by the addition of 50 µL theophylline (2 mg/mL) standard solution. 10 µL of this sample solution were subjected to HPLC-CAD analysis.

Enriched butanol fraction. To yield the enriched fraction (LS BuOH), 10 g of LS MeOH were re-dissolved in methanol and purified by liquid/liquid partition using three solvents

of increasing polarity consecutively. The partition with light petroleum was performed to deplete chlorophyll. The remaining methanol layer was partitioned against dichlormethane followed by water-saturated butanol. The whole procedure resulted in four fractions after evaporation of the solvents under reduced pressure: the light petroleum fraction (DER 23:1), the dichlormethane fraction (DER 38:1) and the aqueous layer which remained after the partition with water-saturated butanol (DER 17:1), were not further investigated. 2 mg of the butanol fraction (LS BuOH; DER 48:1) were dissolved in 900 μL MeOH-H₂O_{dd} (25:75, v/v) followed by the addition of 100 μL theophylline (2 mg/mL) standard solution. 10 μL of this solution were subjected to HPLC-CAD analysis.

Iridoid and phenylpropanoid fraction. The LS BuOH sample was re-dissolved in methanol (50 mg in 200 μL) and applied to four SPE columns (BondElute RP 8, 6 cm³) each conditioned with 30 mL MeOH-H₂O (10:90, v/v). Elution with MeOH-H₂O mixtures ranging from 10% (v/v) to pure MeOH (36 mL each) yielded five fractions (fraction 1: 23.1 mg, 1.27% w/w; fraction 2: 8.8 mg, 0.49% w/w; fraction 3: 1.6 mg, 0.09% w/w; fraction 4: 4.6 mg, 0.25% w/w; fraction 5: 1.1 mg, 0.06% w/w;). Fraction 1 was further separated by semi-preparative HPLC on RP18e to give three sub-fractions: sub-fraction 3 (retention time: 32-53 min) was particularly enriched with phenylpropanoids (=phenylpropanoid fraction, 0.2 mg, 0.01% w/w), and sub-fraction 2 (retention time: 20-32 min) was particularly enriched with iridoids (=iridoid fraction, 0.6 mg, 0.04% w/w). Sub-fraction 1 (retention time: 0-20 min) remained disregarded.

HPLC-CAD and HPLC-DAD-MSⁿ analyses

Analyses were performed on an UltiMate 3000 RSLC-series system (Dionex) coupled to a Corona ultra RS charged aerosol detector (CAD, Dionex) and an HCT 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (Bruker Daltonics, Bremen, Germany). The CAD was used to assess relative and absolute quantities of LS constituents. The nebulizer temperature for HPLC-CAD analyses was set to 35°C. HPLC separation was carried out on a Luna Phenyl-Hexyl column (250x2 mm, 5 μm ; Phenomenex, Torrance, CA) with doubly distilled water (A) and acetonitrile (B), both modified with 0.1% formic acid, as mobile phase. Gradient elution started at 5% B,

increased in 10 min to 10% B and in further 36 min to 23% B. The flow rate was 0.55 mL/min and the temperature was set to 18°C.

Tentative identification of the main constituents was facilitated by HPLC-DAD-MS. The eluent flow was split 1:4 before the ESI ion source, which was operated as follows: capillary voltage: +3.5/-3.7 kV, nebulizer: 26 psi (N₂), dry gas flow: 9 L/min (N₂), and dry temperature: 340°C. Positive and negative ion mode multistage mass spectra up to MS⁴ were obtained in automated data-dependent acquisition (DDA) mode using helium as collision gas, an isolation window of 4 Th, and a fragmentation amplitude of 1.0 V. The aglycone part of all mentioned flavonoids was identified by spectrum matching to an in-house library containing the MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics).

Quantification of main constituents

Selected phenylpropanoids and iridoids were quantified in active LS extracts by HPLC-CAD using theophylline as internal standard. To establish the response factors of the marker compounds acetylharpagide (iridoid) and verbascoside (phenylpropanoid), varying amounts of either substance were added to a consistent concentration of theophylline and analyzed by HPLC-CAD. Individual response factors for each concentration were determined from the according peak areas using the formula $Rf = (AUC_{IS} \cdot C_A) / (C_{IS} \cdot AU_{CA})$. All values obtained over the whole concentration range were averaged and this number used as final response factor for acetylharpagide and verbascoside. These experimentally determined response factors were adopted for the calculation of the structurally related derivatives ajugoside (iridoid) and lavandulifolioside (phenylpropanoid). This approach was based on the assumption that the CAD response is largely independent of the analyte's chemical structure. Although it is known that peak areas increase with raising amounts of the organic solvent in the mobile phase (Vehovec and Obreza, 2010), these effects were neglected due to a very flat gradient and small differences in retention times (see Fig. 5: $\Delta t = 2$ min for acetylharpagide (**9**) / ajugoside (**11**); $\Delta t = 1$ min for lavandulifolioside (**28**) / verbascoside (**29**)). Other derivatives with significantly different retention times were not included in the quantification.

To determine the amount of iridoids and phenylpropanoids in LS crude drug and LS MeOH, theophylline was added in a concentration of 0.1 mg/mL. For LS BuOH, a theophylline concentration of 0.2 mg/mL was used to keep peak heights of theophylline and lavandulifolioside in a similar range.

HPLC-CAD method validation

Validation of the employed HPLC-CAD method was carried out with the enriched BuOH fraction according to ICH (International Conference on Harmonization) guidelines. During the whole validation process, peak areas and retention times were evaluated using Chromeleon 7.1.1 software (Dionex).

Linearity. Calibration curves for acetylharpagide and verbascoside were assessed in triplicate with six different concentrations, ranging between 8.6 µg/mL and 172.6 µg/mL. Linearity was determined within this range by calculation of a regression line by the method of least squares. The statistical significance of linearity was checked by F test.

Specificity. The specificity of the method was demonstrated by showing constant retention times and peak areas of the internal standard theophylline and the marker substances acetylharpagide and verbascoside in LS BuOH. To verify the identification and correct peak assignment of these two analytes in the extract, chromatograms of authentic reference substances were recorded. A possible carry-over of theophylline, acetylharpagide and verbascoside was excluded by regular monitoring of blank injections.

Detection and quantitation limits. Limits of detection (LOD) and quantitation (LOQ) were determined at a signal-to-noise ratio of 3.3 and 10, respectively. Signal-to-noise ratio was assessed by comparing peak heights obtained for low concentrations of marker substances with those of blank samples.

Precision. Repeatability was studied by analyzing the content of acetylharpagide, ajugoside, lavandulifolioside and verbascoside in six independently prepared LS BuOH sample solutions (2 mg/mL). Each sample was injected in triplicate. Intermediate precision was calculated over three days. The AUCs were assessed and the relative standard deviation computed.

Accuracy. The accuracy of the method was investigated by measuring the recovery of the marker substances in the enriched butanol fraction. 200 µg LS BuOH were spiked with three different concentrations of acetylharpagide and verbascoside and their recovery expressed in percentage of spiked LS BuOH. Analyses were carried out in triplicates.

Robustness. The method's robustness was studied by changing following chromatographic parameters: flow rate, pH of the aqueous part of the mobile phase and temperature.

Cell culture, assessment of glucose uptake rate and PTP1B inhibition assay

Settings were used as previously in Heiss *et al.* (2012) and can also be found in the supplementary information. For *in vitro* experiments, extracts were dissolved in MeOH-H₂O_{dd} (25:75, v/v) at a concentration of 10 mg/mL (stock solutions) and pure compounds at a concentration of 20 mM. Overall assay concentrations lay between 10 µg/mL and 25 µg/mL extract in buffer or cell culture media.

Statistical methods and data analysis

All values are expressed as the means ± SD. Statistical analyses were performed with GraphPad Prism software version 4.03. In glucose uptake experiments, the glucose uptake rate obtained without insulin under each treatment was set as 1 and is denominated with “no INS” in the respective graph. One-way ANOVA with Bonferroni's or Dunnett's post hoc test was used to determine statistical significance for PTP1B- and glucose uptake assays. Differences between data groups with $p < 0.05$ were considered as statistically significant.

Results and discussion

Extraction optimization

Three different extraction methods were compared with regard to their effectiveness and impact on the secondary metabolites' profile: Accelerated Solvent Extraction (ASE), reflux under 60°C, and stirring at room temperature. Double extraction under reflux resulted in a DER 4-5:1 in our study, but the relatively high temperatures bear the risk of sensitive compounds to degrade. Using stirring at room temperature avoids this risk, but is also less effective in terms of yield. The DER was only 5-6:1 in this case. When using ASE, the DER could be further increased to 4-5:1, presumably due to the application of pressure during the extraction process. Respective yields of 2 g LS crude drug are given in Figure 1.

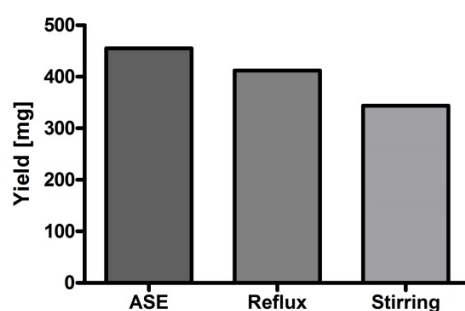


Figure 1. Effectiveness of different extraction methods in terms of yield in mg obtained from 2 g LS crude drug (n=1). ASE=Accelerated Solvent Extraction

TLC fingerprints of the extracts did not differ qualitatively from each other, but showed deviating intensities of selected bands, indicating variances in quantity (see Fig. S1). For further studies, ASE was chosen as extraction method, since it provided the highest yields and soft extraction conditions.

In vitro insulin-sensitizing activity of *L. sibiricus*

Striving to scientifically corroborate the traditional use of LS preparations against diabetes, we performed a series of *in vitro* assays in the course of a bio-activity guided fractionation. For this purpose, models related to insulin sensitivity were used. Protein tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signaling, was

examined for inhibition, while C2C12 myocytes were monitored for increased insulin-stimulated glucose-uptake when treated with LS extracts.

Initial tests with LS MeOH extract showed promising results in both models. At a concentration of 10 $\mu\text{g/mL}$, insulin-stimulated glucose-uptake of C2C12 myocytes could be increased to 1.5 fold of control cells and PTP1B was inhibited by about 40% (see Figure 2).

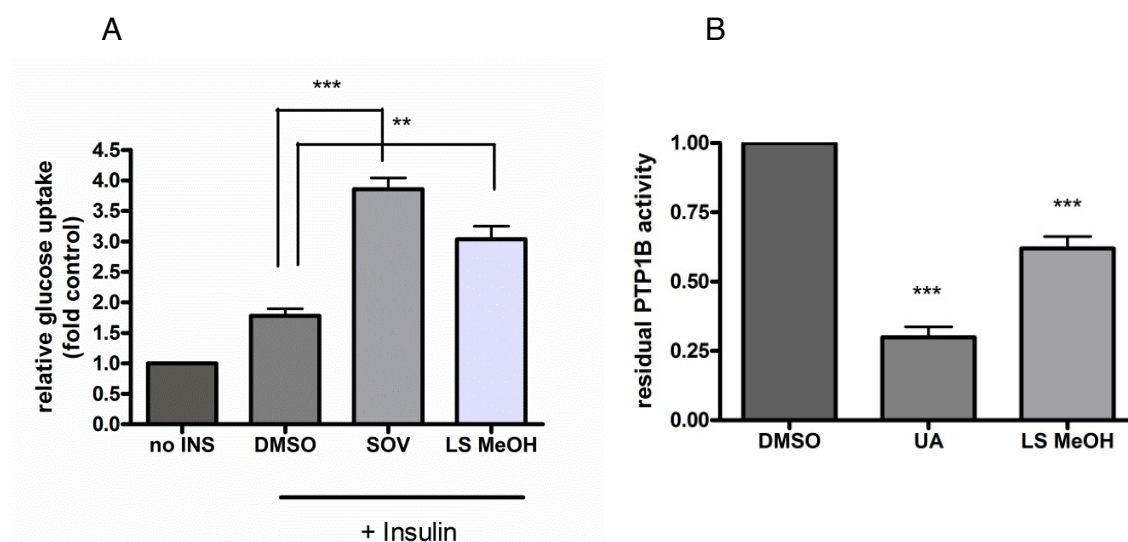


Figure 2. (A) Effect of LS MeOH 10 $\mu\text{g/mL}$ on the insulin-stimulated glucose-uptake of C2C12 myocytes. The glucose uptake rate without insulin under each treatment was set as 1 (no INS). SOV=sodium orthovanadate 10 μM , positive control, DMSO=negative control. (B) PTP1B inhibition by LS MeOH 10 $\mu\text{g/mL}$. UA=ursolic acid 30 μM , positive control; DMSO=negative control.. ** $P < 0.01$; *** $p < 0.001$ (ANOVA/Bonferroni)

In the course of the subsequent fractionation by liquid/liquid partition, solid phase extraction and semi-preparative HPLC, the primal activity could be narrowed down to a fraction mainly composed of iridoid glycosides (iridoid fraction). Ajugoside and acetylharpagide were found to be the main constituents in a relative ratio of 2:1 (see Fig. S2). At a concentration of 25 $\mu\text{g/mL}$, this iridoid fraction inhibited PTP1B by 55% and increased insulin-stimulated glucose-uptake by 1.5 fold (see Figure 3). When ajugoside and acetylharpagide were tested separately as pure compounds at concentrations of up to 100 μM , the observed activity vanished (data not shown). The insulin-sensitizing activity can therefore not be explained solely by the presence of these two main iridoid glycosides. Synergism with other minor compounds of the active fraction is conceivable. Phenylpropanoids, the other main secondary metabolites in LS,

however, can be ruled out to be of major significance for the observed insulin-sensitizing effect of LS in our models. A fraction (phenylpropanoid fraction) mainly composed of lavandulifolioside and verbascoside did not show activity (see Figure 3), nor did those compounds when tested separately at concentrations of up to 100 μ M (data not shown).

LS's *in vitro* insulin-sensitizing effect must be seen as interplay of several constituents. Glycosylated iridoids present in LS seem to contribute most to this activity. The previous finding of Cui *et al.* (2011), who reported PTP1B inhibition by five iridoids different from those in LS and isolated from *Plantago asiatica* underlines PTP1B as major target of iridoids in the insulin signaling pathway. At this place it should be noted that phenylpropanoids, although inactive in our models, may well contribute to the improved glucose homeostasis in diabetes, e.g. by inhibition of hepatic gluconeogenesis or impaired intestinal glucose absorption.

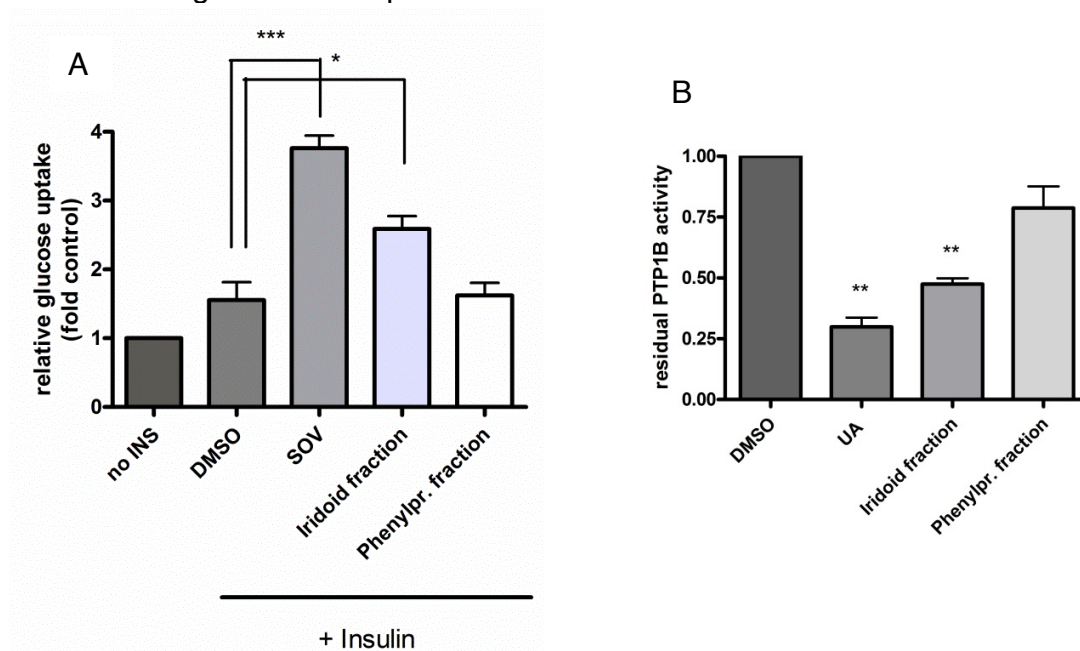


Figure 3. *In vitro* effects of LS MeOH-subfractions (iridoid and phenylpropanoid fraction) at concentrations of 25 μ g/mL. (A) Insulin-stimulated glucose-uptake of C2C12 myocytes. The glucose uptake rate without insulin under each treatment was set as 1 (no INS). SOV=sodium orthovanadate 10 μ M, positive control, DMSO=negative control. (B) Inhibition of PTP1B. UA=ursolic acid 30 μ M, positive control; DMSO=negative control. * $P < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (ANOVA/Dunnett)

High performance liquid chromatography analyses

Method optimization

For the development of a suitable HPLC quantification method, chromatographic conditions of a previous study (Schmidt *et al.*, 2013) were used as starting point and optimized in order to achieve better separation of critical peak pairs. Initial analyses with a methanol/water gradient on C18 material did not show satisfactory peak resolution for quantification purposes. Changing the mobile phase to an acidified acetonitrile/water-based gradient resulted in improved peak shape and resolution, but led at the same time to partly overlapping peaks. This obstacle was tackled by changing the stationary phase. After trying different materials, best separation was finally achieved with a phenyl hexyl column. The influence of the column temperature was studied between 18°C and 32°C. While the total run-time increased with lower temperatures, separations of the iridoid glycosides improved. The operating temperature was therefore set to 18°C. The flow rate showed to be best at 0.55 mL/min.

Fingerprint of L. sibiricus extracts

The phytochemical composition of three different LS extracts was analyzed by LC-MS: a methanol extract of the crude drug (LS MeOH), an enriched butanol fraction thereof (LS BuOH) and a methanol/water (25:75, v/v) extract (LS MeOH/water) of the crude drug.

The three extracts showed qualitatively similar patterns, dominated by the occurrence of glycosylated phenylpropanoids, iridoids, flavonoids and phenolic acids. Glycosylated phenylpropanoids, however, were less abundant in LS MeOH/water. Iridoid glycosides included acetylharpagide (**9**), ajugoside (**11**) and five further derivatives (**1-3**, **12**, **13**). Though MS data indicate the presence of iridoid glycosides, the identity of (**1-3**, **12**, **13**) could not be established with certainty.

Several phenylpropanoids structurally related to verbascoside (**29**) were detected: lavandulifolioside and isomers (**28**, **31**, **32**), pentosyl- β -hydroxy-verbascoside isomers (**15**, **17**), β -hydroxy-verbascoside isomer (**18**), pentosyl- β -methoxy-verbascoside isomers (**22**, **24**), β -methoxy-verbascoside (**27**), caffeoyl-hexosyl-verbascoside (**35**), leonoside A (**33**), leonoside B (**36**) and leucosceptoside A (**34**).

Molecular formulae of the main constituents are given in Figure 4, the chromatographic comparison of the three discussed extracts detected by CAD in Figure 5. Tentative peak

identification was facilitated by LC-MS (see Table 1 and Table S1). The presence of rutin (**21**), hyperoside (**23**), isoquercitrine (**26**), chlorogenic acid (**8**), caffeic acid (**10**), acetylharpagide (**9**), lavandulifolioside (**28**) and verbascoside (**29**) was verified by comparison with authentic reference substances. Their UV maxima are given in Table 1. Ajugoside (**11**) was isolated by semi-preparative HPLC and its structure confirmed by NMR (see Fig. S3) and comparison with literature data (Tatlı *et al.*, 2007).

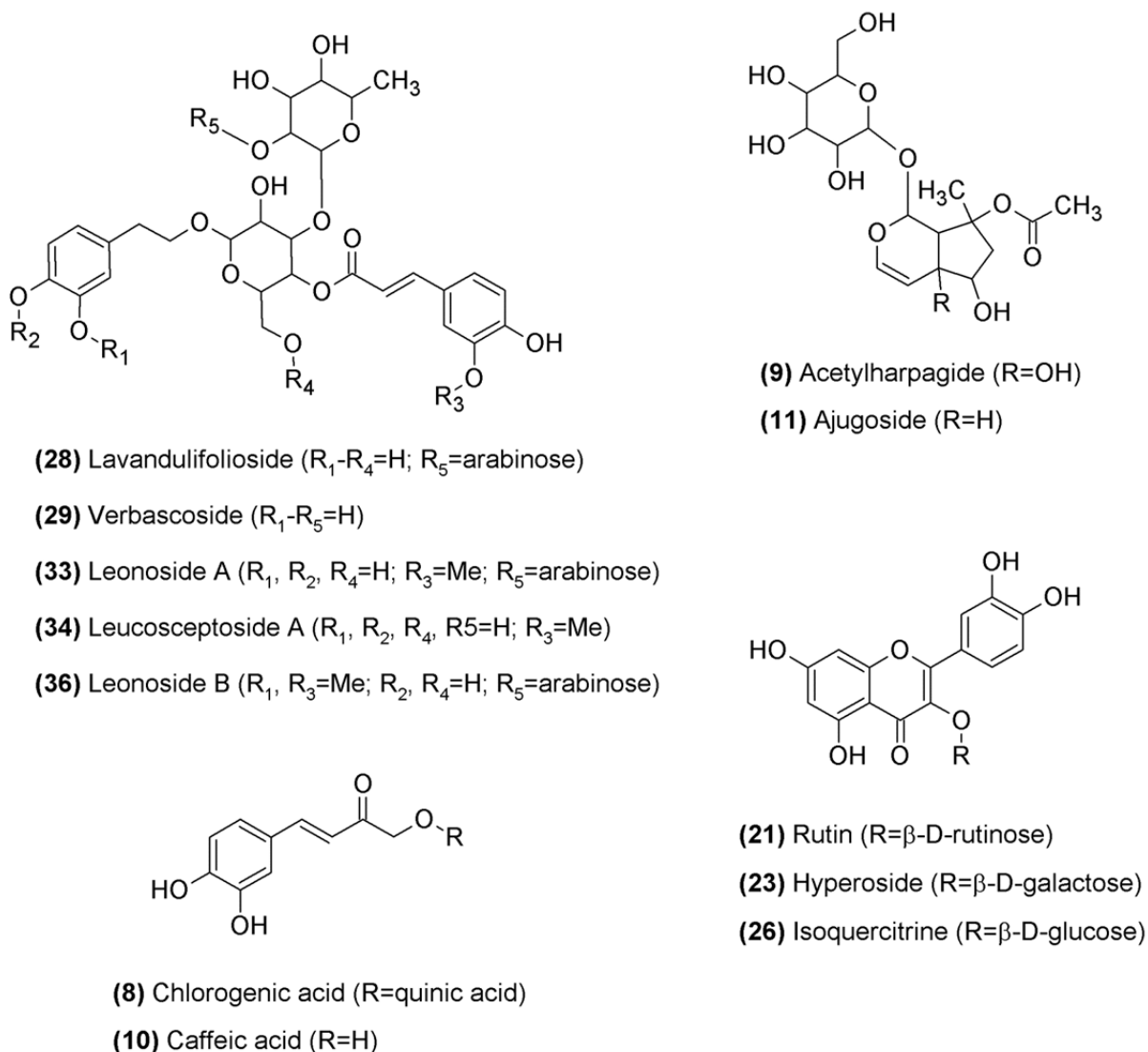


Figure 4. Molecular formulae of the main constituents in LS.

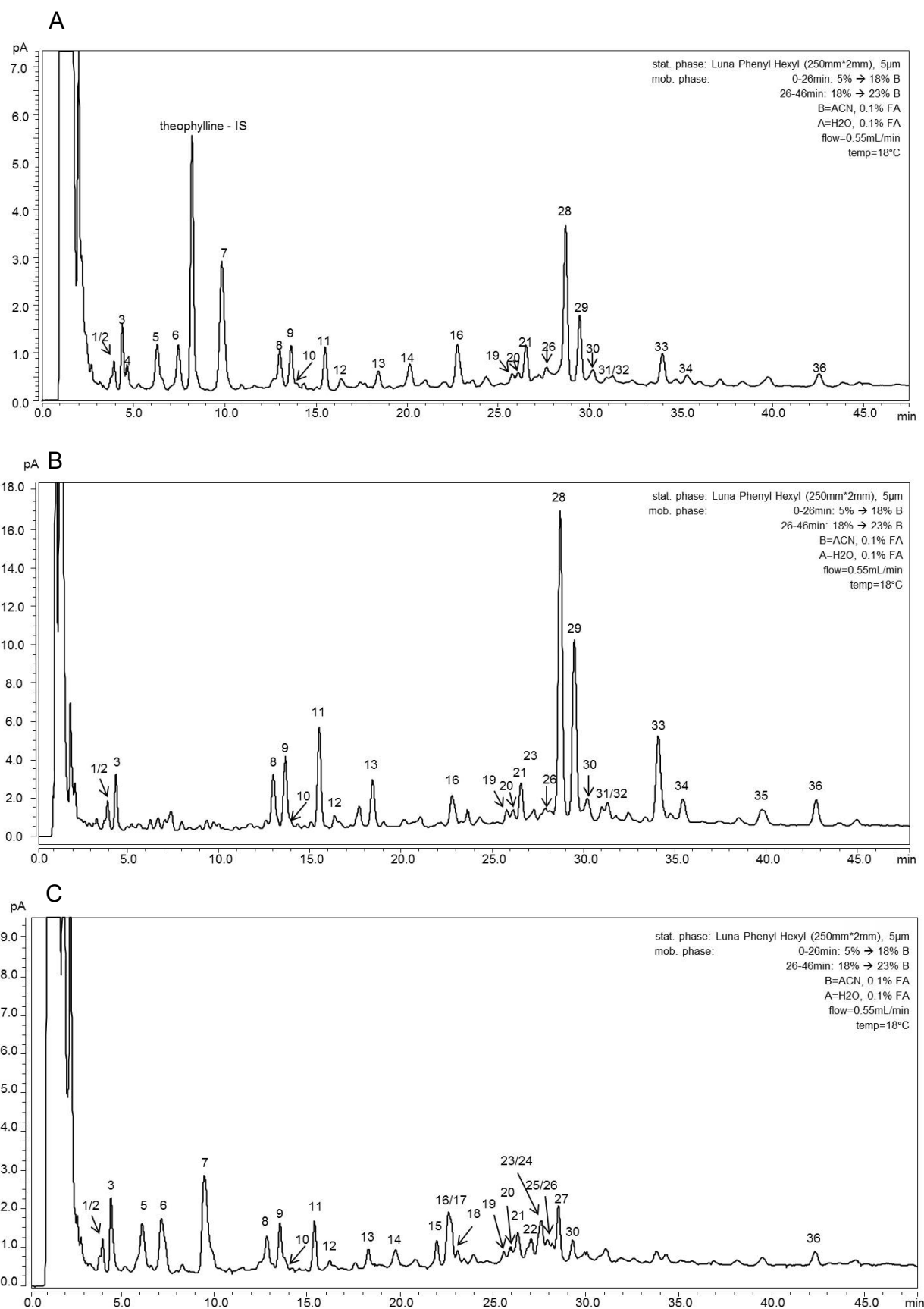


Figure 5. HPLC-CAD analyses of *L. sibiricus* extracts. Main secondary metabolites are numbered. (A) LS MeOH spiked with the internal standard (IS) theophylline, (B) LS BuOH and (C) LS MeOH/water.

Validation

To prove the validity of the developed method, Q2(R1) guidelines of the International Conference on Harmonization (ICH) were followed. First, the concentration range of acetylharpagide and verbascoside for linear behavior was established. Acetylharpagide showed linearity between 9-90 $\mu\text{g/mL}$ ($r=0.9983$) and verbascoside from 8.6-172.6 $\mu\text{g/mL}$ ($r=0.9998$). Corresponding regression equations are given in Table 2.

Limits of detection and quantification (LOD and LOQ) were 10 ng and 30 ng, respectively, for acetylharpagide and 20 ng and 60 ng, respectively, for verbascoside (see Table 2). Specificity was shown by constant retention times of acetylharpagide and verbascoside with RSD below 0.1% and by the absence of signals due to carry-over when pure solvent was injected. For precision analysis, repeatability and inter-day repeatability of acetylharpagide, ajugoside, lavandulifolioside and verbascoside in LS BuOH were determined. Relative standard deviations ranged from 2.75 % to 4.83 % (see Table 3). Accuracy was studied by spiking LS BuOH with low, medium and high concentrations of acetylharpagide and verbascoside. Recoveries lay between 91.73 % and 97.86 % (Table 4). Robustness was investigated by altering chromatographic conditions. The system tolerated flow rate shifts from 0.5 – 0.6 mL/min without considerable loss of resolution and pH alterations in the aqueous phase in a range of 2.3 – 3.1. Furthermore, changes in temperature from 16-19°C did not influence the chromatographic separation (see Fig. S4-S6).

Quantification

Having established LS's insulin-sensitizing potential *in vitro*, thorough phytochemical characterization of the active extracts was of interest. As mentioned above, the main compounds of LS showed none, or only faint PTP1B inhibition when tested individually. However, quantification of acetylharpagide, ajugoside, lavandulifolioside and verbascoside as the major marker substances for the two compound classes of glycosylated phenylpropanoids and iridoids constitutes an important part for the quality control of LS.

The unequal UV absorption maxima of phenylpropanoids and iridoids make a simultaneous UV detection-based quantification of both classes prone to bias. Therefore, a universal, absorption independent detector was needed. Previous studies showed that among the available evaporative/aerosol based detectors, the charged aerosol detector (CAD) proved to be most powerful (Almeling *et al.*, 2012) and it was successfully applied in natural product analysis (Jia *et al.*, 2013). Thus, CAD was the instrument of choice for our study. To compensate for errors during sample preparation or sample loading, we developed a quantification method using an internal standard. In our case, the general requirements for internal standards with respect to chemical properties and availability were best met by the xanthine theophylline. It is easily commercially available and its retention time of 8.2 minutes in the used HPLC system did not interfere with any other analyte of the extract.

From the four compounds that were chosen to be quantified in the LS extract, only the iridoid acetylharpagide and the phenylpropanoid verbascoside were commercially available. Response factors \pm SD were thus only assessed with these two marker compounds (see Table 5) The two most abundant other members of these compound classes, ajugoside and lavandulifolioside, which eluted very close to the respective marker compounds were also quantified using the determined response factor for the neighboring marker compound. Therefore, the chosen compounds for quantification were acetylharpagide, ajugoside, verbascoside and lavandulifolioside (see experimental, section quantification of main constituents).

Once response factors were established for the constituents of interest, their contents \pm SD in the extract were determined. For quantification in LS BuOH and LS MeOH,

theophylline was added to the respective liquid extract, while for the crude drug, it was added to the dried material before extraction. The latter procedure served to compensate for matrix effects or any errors inherent to the extraction process. Beforehand, the stability of theophylline against extraction conditions was ensured.

The quantity of the four glycosylated phenylpropanoids and iridoids were compared in three different extracts of LS (batch **a**). Highest contents of both substance classes were found in the enriched LS BuOH fraction with 2.4% combined iridoid content (acetylharpagide and ajugoside) and 13.0% combined phenylpropanoid content (lavandulifolioside and verbascoside). Concentrations in its mother fraction, LS MeOH, were smaller by about 82% and 88%, respectively. For quantification in the crude drug, MeOH-H₂O_{dd} (25:75, v/v) was used as extraction solvent. Interestingly, hardly any phenylpropanoids got extracted, despite their hydrophilic nature. The amount of iridoids in the crude drug was about one third to one fourth of the content in LS MeOH extract. The collected phenylpropanoid and iridoid glycosides quantities in LS crude drug, LS MeOH and LS BuOH are given in Table 5.

To get an idea of the biological variation of the iridoid pattern in the crude LS drug, three different LS batches were investigated in total. The material was harvested in the years 2009 (batch **a**), 2010 (batch **b**) and 2014 (batch **c**) from sites in geographical proximity to one another. Both iridoids were present in all three samples. The combined iridoid content of acetylharpagide and ajugoside in the crude drug shows some variation during these years, lying between 0.11% and 0.18% (see Figure 6). This may be attributed to the influence of exogenous factors, like fluctuations in climate or nutrient supply.

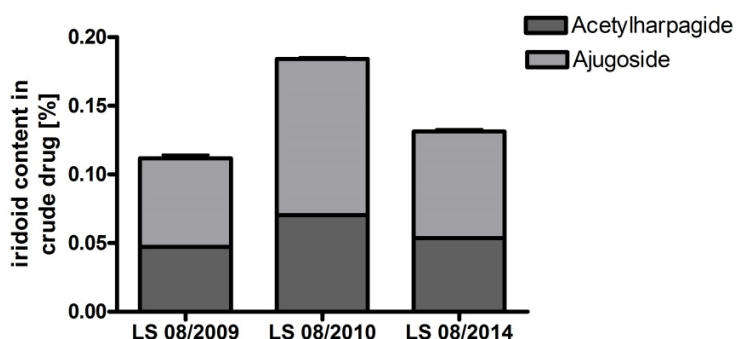


Figure 6. Comparison of iridoid content in LS batches from three different years

The presence of these two iridoids and the phenylpropanoids verbascoside and lavandulifolioside constitutes an important characteristic of LS and can therefore be applied in the quality control of this regularly used traditional plant. As far as the *in vitro* insulin-sensitizing activity of LS is concerned, our results show that the effect is linked to some extent to glycosylated iridoids. Even though the main iridoids acetylharpagide and ajugoside did not inhibit PTP1B when tested separately, a fraction highly enriched in them showed good activity. Highest quantities of these iridoids were recorded in a purified methanol extract. Finally, our newly developed method might also enable better quality control of species related to LS. Concurrent presence of glycosylated iridoids and phenylpropanoids is often observed in the Lamiales order (Akbay *et al.*, 2003; Harput *et al.*, 2004). Based on our results, investigations towards an anti-diabetic activity of related species should therefore be a promising strategy for further research.

Acknowledgement

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Appendix A. Supplementary materials

Supplementary data associated with this article can be found in the online version.

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Table 1. Proposed structure of the secondary plant metabolites identified in LS.

Compound	RT [min]	MW [Da]	Proposed structure	λ_{\max} [nm]
1	3.8	362	Iridoid glycoside	
2	4.0	364	Iridoid glycoside	
3	4.4	348	Iridoid glycoside	
4	4.7	372	Caffeoyl-hexaric acid isomer	
5	6.3	372	Caffeoyl-hexaric acid isomer	
6	7.5	372	Caffeoyl-hexaric acid isomer	
7	9.8	372	Caffeoyl-hexaric acid isomer	
8	13.0	354	Chlorogenic acid ^a	216.6, 236.5, 325.0
9	13.7	406	8-O-Acetylharpagide ^a	193.2
10	14.0	180	Caffeic acid ^a	216.5, 235.3, 322.0
11	15.6	390	Ajugoside ^b	
12	16.4	388	Iridoid glycoside	
13	18.3	358	Iridoid glycoside (acidic)	
14	20.1	296	Caffeoyl-malate	
15	22.2	772	Pentosyl- β -hydroxy-verbascoside isomer	
16	22.9	626	Quercetin-O-hexosyl hexoside ^c	
17	23.0	772	Pentosyl- β -hydroxy-verbascoside isomer	
18	23.3	640	β -Hydroxy-verbascoside isomer	
19	25.8	610	Kaempferol-O-hexosyl hexoside ^c	
20	26.1	610	Quercetin-3-O-robinoside ^c	
21	26.6	610	Rutin ^a	203.5, 256.0, 353.8
22	27.3	786	Pentosyl- β -methoxy-verbascoside isomer	
23	27.7	464	Hyperoside ^a	203.4, 255.7, 353.7
24	27.8	786	Pentosyl- β -methoxy-verbascoside isomer	
25	28.1	654	β -Methoxy-verbascoside isomer	
26	28.2	464	Isoquercitrine ^a	203.5, 255.9, 353.2
27	28.5	654	β -Methoxy-verbascoside isomer	
28	28.8	756	Lavandulifolioside ^a	195.5, 329.4, 217.0
29	29.6	624	Verbascoside ^a	195.5, 329.0
30	30.1	594	Kaempferol-3-O-rutinoside ^c	
31	30.8	756	Lavandulifolioside isomer	
32	31.1	756	Lavandulifolioside isomer	
33	34.0	770	Leonoside A	
34	35.4	638	Leucosceptoside A	
35	39.5	948	Caffeoyl-hexosyl-verbascoside isomer	
36	42.6	784	Leonoside B	

^a These constituents were confirmed by comparison of retention times and UV-spectra with reference compounds

^b The structure of this compound was confirmed by NMR and comparison with literature data

^c The aglycone part of the flavonoids was identified by spectrum matching to an in-house library containing MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics, Bremen, Germany)

Table 2. Calibration parameters, LOD and LOQ of acetylharpagide and verbascoside (n=3).

Parameters	Results	
	Acetylharpagide	Verbascoside
Linear range [$\mu\text{g/mL}$]	9.0-90.0	8.6-172.6
Regression equation ^a	$y = 0.0157x + 0.0567$	$y = 0.0145x + 0.0211$
Correlation coefficient r	0.9983	0.9998
Significance F	$1.1 \cdot 10^{-18}$	$5.2 \cdot 10^{-28}$
Limit of detection [ng]	11	22
Limit of quantitation [ng]	33	67

^a where x is the concentration in [$\mu\text{g/mL}$]

Table 3. Precision validation of acetylharpagide, ajugoside, lavandulifolioside and verbascoside in LS BuOH (batch **a**) at a concentration of 2 mg/mL

	Content [$\mu\text{g/mL} \pm \text{SD}$]	RSD [%]	Retention time [min]
<i>Repeatability n=9</i>			
Acetylharpagide	18.0 ± 0.5	2.75	13.75 ± 0.01
Ajugoside	29.2 ± 1.4	4.83	15.62 ± 0.02
Lavandulifolioside	169.1 ± 6.4	3.77	28.82 ± 0.02
Verbascoside	91.1 ± 3.7	4.09	29.59 ± 0.01

Table 4. Validation of the accuracy for acetylharpagide and verbascoside concentration in LS BuOH (batch **a**). n=12

	Theoretical concentration [µg/mL]	Found concentration [µg/mL, ± SD]	Recovery [% , ± SD]
Acetylharpagide			
low	29.46	29.14 ± 0.25	95.07 ± 1.10
medium	41.46	41.19 ± 0.52	97.86 ± 4.81
high	47.46	45.81 ± 0.69	95.05 ± 2.25
Verbascoside			
low	104.43	104.70 ± 2.25	95.29 ± 6.66
medium	113.45	110.82 ± 0.66	92.06 ± 9.70
high	130.43	125.35 ± 1.65	91.73 ± 4.12

Table 5. Response factors and contents of main secondary metabolites in *L. sibiricus* (batch **a**)

Compound	Response factor [mean \pm SD]	Content		
		Crude drug (n=9) [mean %, \pm SD] ^a	Methanol extract (n=9) [mean % \pm SD] ^b	Enriched butanol fraction (n=27) [mean %, \pm SD] ^b
Acetylharpagide	0.50 \pm 0.03	0.047 \pm 0.004	0.200 \pm 0.008	0.894 \pm 0.025
Ajugoside	0.50 \pm 0.03	0.065 \pm 0.008	0.223 \pm 0.009	1.519 \pm 0.060
Lavandulifolioside	0.67 \pm 0.02	n.a.	1.085 \pm 0.041	8.454 \pm 0.319
Verbascoside	0.67 \pm 0.02	n.a.	0.493 \pm 0.018	4.552 \pm 0.186
Combined iridoid content		0.112 \pm 0.013	0.422 \pm 0.015	2.413 \pm 0.066
Combined phenylpropanoid content		n.a.	1.578 \pm 0.056	13.006 \pm 0.481

^a w/w in relation to the crude drug^b w/w in relation to the dried extract

Quantification of phenylpropanoids and iridoids in insulin-sensitizing extracts of *Leonurus sibiricus* L. (Lamiaceae)

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Supplementary information

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Files in this data supplement

Experimental: Cell culture, assessment of glucose uptake rate, *in vitro* enzyme assay

Table S1

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Figure S6

Experimental

Cell culture

C2C12 myoblasts (ATCC) were maintained under sub-confluent conditions in DMEM medium supplemented with 10% FBS, glutamine, and penicillin/streptomycin under 5% CO₂ environment at 37 °C. For differentiation into myotubes and subsequent glucose uptake assays, cells (passage number <15) were seeded in 24-well plates and grown until confluency. Differentiation was initiated by changing to DMEM supplemented with 2% horse serum. After 96 hours, cells were considered fully differentiated and used for experiments within 2 days.

Assessment of the Glucose Uptake Rate

C2C12 myotubes were treated with extracts for 48 hours before they were serum starved for 4 hours and stimulated with 100 nM insulin as indicated for 30 minutes in standard Krebs-Ringer HEPES buffer, pH 7.4. Then, glucose uptake was assessed in the presence of 0.1 mM 2-deoxyglucose (DOG) spiked with 1 µCi/mL tritium-labelled ³H-DOG (NEN, Austria) for 10 minutes at room temperature. Then, cells were rinsed with ice cold PBS and lysed with 0.05 N NaOH. Aliquots of the lysates were analysed in a Perkin Elmer TriCarb 2100 liquid

scintillation counter and used for determination of the protein content (Bradford assay). The glucose uptake rate for each sample was determined as the amount of incorporated radiolabelled glucose per minute uptake time, normalized to the cellular protein content.

In vitro Enzyme Assay

PTP1B activity (human recombinant enzyme from R&D) was determined using 2 mM para-nitrophenylphosphate (pNPP) in 50 mM MOPS, pH 6.5, as a substrate. The known PTP inhibitor ursolic acid (UA, 30 μ M) was used as positive control. Fractions were first dissolved in DMSO and diluted to a final concentration of 1% DMSO in 100 μ L overall assay volume. The inhibitory action towards PTP1B was measured in 96-well format in the presence of 1 mM dithiothreitol (DTT). The reactions were subjected to kinetic absorbance readings at 405 nm for 30 min in a Tecan/SUNRISETM photometer. Subsequently, the reaction was stopped with 10 M NaOH and the absorbance again measured at 405 nm.

Table S1. Proposed structure and MS data of the secondary plant metabolites identified in the *Leonurus sibiricus* L. extracts.

#	Proposed Structure	[M-H] ⁻	Main Fragment Ions (>10% Rel. Int.)	References
1	Iridoid glycoside	361.1	MS ² [407.1]: 361.0 (100), 180.9 (22), 178.8 (19) MS ³ [361.0]: 324.9 (23), 282.9 (27), 253.0 (32), 222.8 (14), 194.8 (40), 182.0 (27), 180.9 (100), 178.9 (66), 177.0 (20), 160.8 (43), 159.5 (48), 155.9 (47), 154.9 (30), 142.9 (80), 137.8 (15)	-
2	Iridoid glycoside	363.1	MS ² [409.1]: 363.0 (100) MS ³ [363.0]: 200.9 (100), 182.9 (58), 178.8 (35), 164.8 (48), 156.9 (14), 118.9 (13)	Wu et al. (2010)
3	Iridoid glycoside	347.0	MS ² [393.0]: 347.0 (100), 166.9 (22), 148.9 (35) MS ³ [347.0]: 166.9 (77), 160.8 (19), 148.9 (100), 120.9 (26)	Sugaya et al. (1998)
4	Caffeoyl-hexaric acid isomer	371.0	MS ² [371.0]: 208.8 (100), 190.8 (32) MS ³ [208.8]: 190.8 (100), 132.8 (15), 84.9 (15)	Lorenz et al. (2012)
5	Caffeoyl-hexaric acid isomer	371.0	MS ² [371.0]: 208.8 (100), 190.9 (31) MS ³ [208.8]: 190.9 (100), 84.9 (24)	Lorenz et al. (2012)
6	Caffeoyl-hexaric acid isomer	371.0	MS ² [371.0]: 208.9 (100), 190.8 (30) MS ³ [208.9]: 190.9 (100), 84.9 (17)	Lorenz et al. (2012)
7	Caffeoyl-hexaric acid isomer	371.0	MS ² [371.0]: 352.9 (13), 208.8 (100), 190.8 (33) MS ³ [208.8]: 190.9 (100), 84.9 (19)	Lorenz et al. (2012)
8	Chlorogenic acid ^a	353.0	MS ² [353.0]: 190.9 (100)	Ritter et al. (2010)
9	8-O-Acetylharpagide ^a	405.1	MS ² [405.1]: 345.0 (100), 178.9 (27), 164.9 (29) MS ³ [345.0]: 178.8 (33), 164.8 (100), 160.8 (15),	Hayashi et al. (2001)

			124.9 (11), 118.8 (18), 112.9 (17)	
10	Caffeic acid ^a	178.9	MS ² [178.9]: 134.9 (100)	Ritter et al. (2010)
11	Ajugoside ^b	389.0	MS ² [435.0]: 389.0 (100), 329.0 (31), 231.0 (17), 166.9 (25)	Hayashi et al. (2001)
12	Iridoid glycoside	387.1	MS ² [387.1]: 369.0 (18), 206.9 (100), 162.9 (73) MS ³ [206.9]: 163.0 (100)	Han et al. (2011)
13	Iridoid glycoside (acidic)	357.0	MS ² [357.0]: 194.9 (100), 150.9 (47), 132.9 (12), 106.9 (69) MS ³ [194.9]: 150.8 (91), 132.8 (12), 106.9 (100)	Tasdemir et al. (1999)
14	Caffeoyl-malate	294.9	MS ² [590.9]: 294.9 (100), 178.9 (16) MS ³ [294.9]: 178.8 (100), 132.9 (86)	Hayashi et al. (2001)
15	Pentosyl- β -hydroxy-verbascoside isomer	771.2	MS ² [771.2]: 753.2 (100), 591.1 (25) MS ³ [753.2]: 621.1 (17), 601.0 (15), 591.0 (100), 459.1 (17), 306.9 (13)	Shi et al. (2013)
16	Quercetin-O-hexosyl hexoside	625.1	MS ² [625.1]: 445.0 (12), 300.9 (77), 299.9 (100), 270.9 (27), 254.9 (14) MS ³ [299.9]: 270.9 (100), 254.9 (51), 178.8 (24), 150.8 (26)	Schmidt et al. (2013)
17	Pentosyl- β -hydroxy-verbascoside isomer	771.2	MS ² [771.2]: 753.2 (100), 661.1 (10), 591.1 (18) MS ³ [753.2]: 621.0 (26), 591.0 (100), 469.1 (11)	Shi et al. (2013)
18	β -Hydroxy-verbascoside isomer	639.2	MS ² [639.2]: 621.1 (100), 459.0 (35), 178.8 (11)	Shi et al. (2013)
19	Kaempferol-O-hexosyl hexoside ^c	609.1	MS ² [609.1]: 429.0 (13), 286.0 (17), 284.9 (100), 283.9 (60), 256.9 (13), 254.9 (24)	-
20	Quercetin-3-O-robinoside ^c	609.1	MS ² [609.1]: 300.9 (100), 300.0 (69), 270.9 (20), 254.9 (17) MS ³ [300.9]: 271.8 (33), 270.9 (100), 254.9 (74), 212.9 (16), 211.2 (15), 179.7 (15), 178.8 (41), 150.8 (67)	Rolim de Almeida et al. (2008)
21	Rutin ^a	609.1	MS ² [609.1]: 300.9 (100), 300.0 (43), 270.9 (14) MS ³ [300.9]: 272.9 (15), 270.8 (100), 256.9 (22), 255.8 (12), 254.8 (72), 178.8 (69), 150.8 (76), 106.9 (11)	Rolim de Almeida et al. (2008)
22	Pentosyl- β -methoxy-verbascoside isomer	785.2	MS ² [785.2]: 767.2 (24), 753.3 (100), 591.2 (18) MS ³ [753.3]: 621.1 (11), 619.9 (18), 601.1 (21), 591.1 (100), 460.1 (19), 459.2 (10), 324.0 (10), 258.9 (11)	Shi et al. (2013)
23	Hyperoside ^a	463.0	MS ² [463.0]: 300.9 (100), 300.0 (35) MS ³ [300.9]: 270.8 (52), 255.0 (51), 242.8 (17), 239.0 (27), 229.8 (16), 228.8 (21), 178.8 (100), 152.9 (18), 121.0 (42)	Rolim de Almeida et al. (2008)
24	Pentosyl- β -methoxy-verbascoside isomer	785.2	MS ² [785.2]: 767.2 (19), 753.2 (100) MS ³ [753.2]: 621.1 (13), 601.0 (13), 591.1 (100), 307.0 (11)	Shi et al. (2013)
25	β -Methoxy-verbascoside isomer	653.2	MS ² [653.2]: 621.1 (100), 459.1 (33) MS ³ [621.1]: 459.0 (100), 306.0 (11), 202.9 (19), 178.8 (37)	Shi et al. (2013)
26	Isoquercitrine ^a	463.0	MS ² [463.0]: 300.9 (100), 300.0 (31) MS ³ [300.9]: 272.8 (18), 271.9 (32), 270.9 (44), 257.9 (14), 254.9 (55), 178.7 (57), 162.7 (21), 150.8 (100), 106.9 (13), 93.0 (18)	Rolim de Almeida et al. (2008)

27	β -Methoxy-verbascoside isomer	653.2	MS ² [653.2]: 636.0 (11), 635.0 (11), 621.1 (100), 459.1 (17) MS ³ [621.1]: 474.8 (12), 459.0 (100), 307.0 (12)	Shi et al. (2013)
28	Lavandulifolioside ^a	755.2	MS ² [755.2]: 593.1 (100), 461.0 (59) MS ³ [593.1]: 461.0 (100), 315.0 (17) MS ⁴ [461.0]: 315.0 (42), 134.9 (100)	Ritter et al. (2010)
29	Verbascoside ^a	623.1	MS ² [623.1]: 461.0 (100) MS ³ [461.0]: 315.0 (53), 160.9 (14), 142.8 (12), 134.9 (100)	Ritter et al. (2010)
30	Kaempferol-3-O-rutinoside ^c	593.1	MS ² [593.1]: 284.9 (100) MS ³ [284.9]: 266.9 (14), 258.0 (19), 256.9 (100), 255.9 (11), 254.9 (65), 242.9 (18), 240.8 (29), 238.9 (43), 229.9 (22), 229.0 (23), 214.8 (13), 212.9 (19), 210.9 (28), 199.0 (38), 194.8 (23), 186.9 (11), 168.9 (16), 162.8 (23), 93.9 (23)	Hayashi et al. (2001)
31	Lavandulifolioside isomer	755.3	MS ² [755.3]: 623.1 (12), 593.1 (100), 461.1 (62) MS ³ [593.1]: 461.0 (100) MS ⁴ [461.0]: 134.8 (100)	Schmidt et al. (2013)
32	Lavandulifolioside isomer	755.2	MS ² [755.2]: 593.1 (100), 591.2 (11), 461.1 (41) MS ³ [593.1]: 461.0 (100), 315.0 (15) MS ⁴ [461.0]: 314.9 (19), 297.0 (49), 134.9 (100)	Schmidt et al. (2013)
33	Leonoside A	769.2	MS ² [769.2]: 637.2 (65), 607.1 (12), 593.1 (100), 461.1 (43), 315.0 (10) MS ³ [593.1]: 461.0 (100) MS ⁴ [461.0]: 316.1 (16), 314.9 (16), 297.9 (10), 134.9 (100)	Calis et al. (1992)
34	Leucosceptoside A	637.2	MS ² [637.2]: 461.0 (100) MS ³ [461.0]: 413.5 (18), 409.1 (25), 316.0 (26), 314.9 (69), 160.9 (25), 134.9 (100)	Tasdemir et al. (1999)
35	Caffeoyl-hexosyl-verbascoside	947.3	MS ² [947.3]: 785.3 (100), 623.2 (41) MS ³ [785.3]: 623.1 (100) MS ⁴ [623.1]: 478.0 (100), 477.1 (54), 462.0 (24), 461.0 (25), 458.9 (15)	-
36	Leonoside B	783.3	MS ² [783.3]: 651.1 (45), 607.1 (100), 475.1 (18) MS ³ [607.1]: 475.0 (100), 329.1 (47)	Calis et al. (1992)

^a These constituents were confirmed by comparison of retention times and UV-spectra (if applicable) with reference compounds.

^b The structure of this compound was confirmed by NMR and comparison with literature data.

^c The aglycone part of the flavonoids was identified by spectrum matching to an in-house library containing MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics, Bremen, Germany).

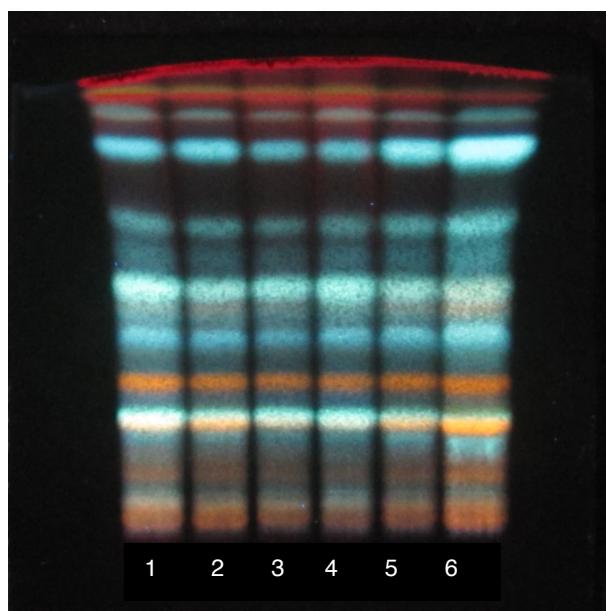


Figure S1. TLC comparison of LS MeOH extracts produced with different extraction methods. 1=reflux 1; 2=reflux 2; 3=stirring; 4=ASE 1; 5=ASE 2; 6=ASE3

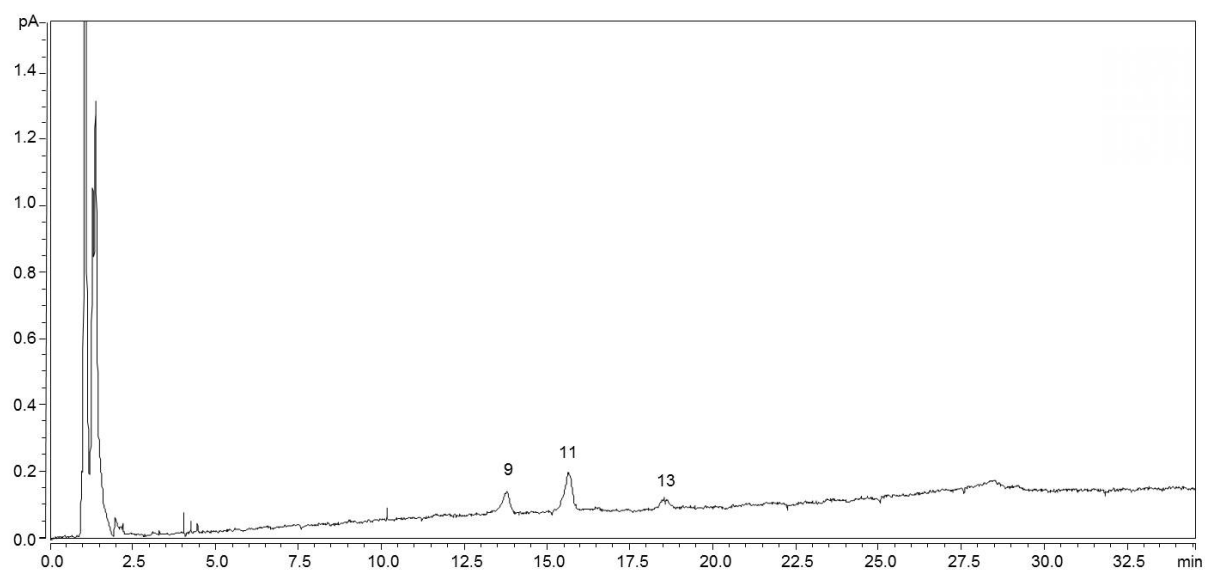


Figure S2. HPLC-CAD analysis of the LS iridoid fraction.

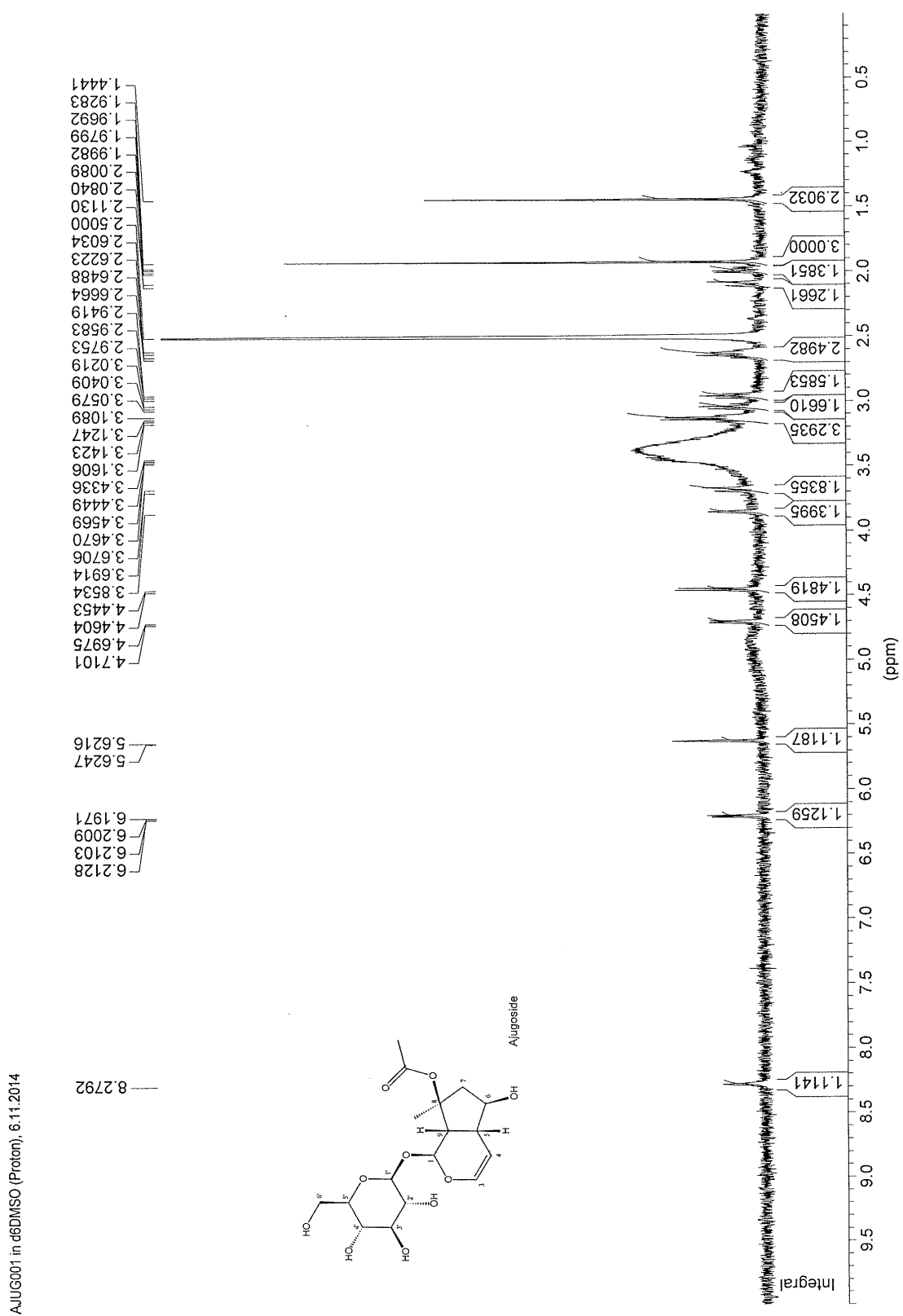


Figure S3. ^1H -NMR of ajugoside in DMSO-d_6

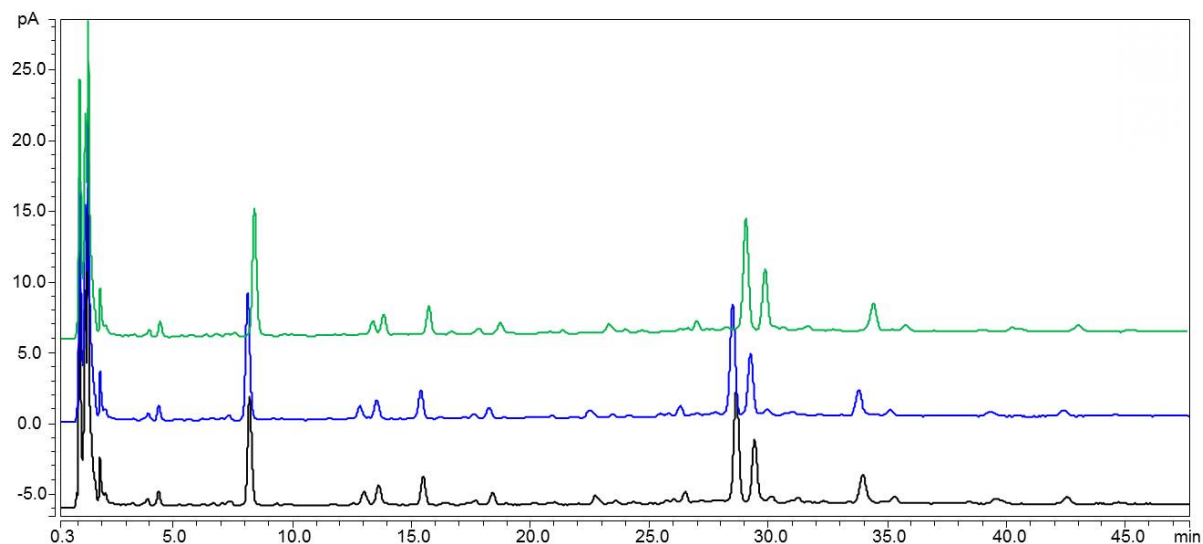


Figure S4. Validation of robustness: chromatograms of LS BuOH at different temperatures: 18°C (black), 19°C (blue) and 16°C (green)

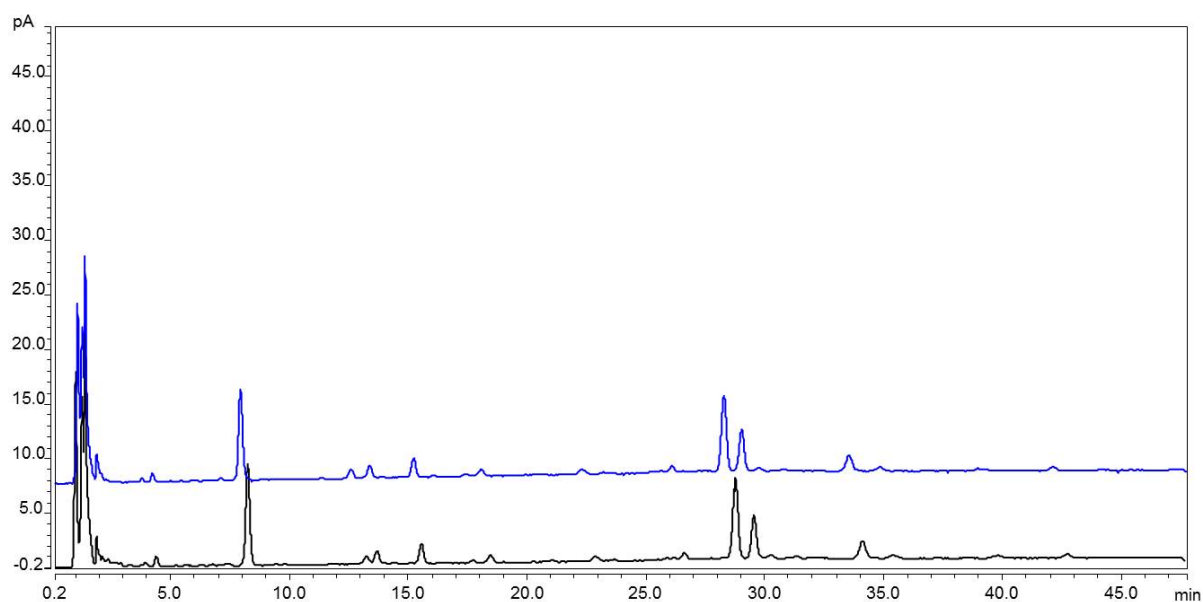


Figure S5. Validation of robustness: chromatograms of LS BuOH at pH 3.11 (black) and pH 2.3 (blue)

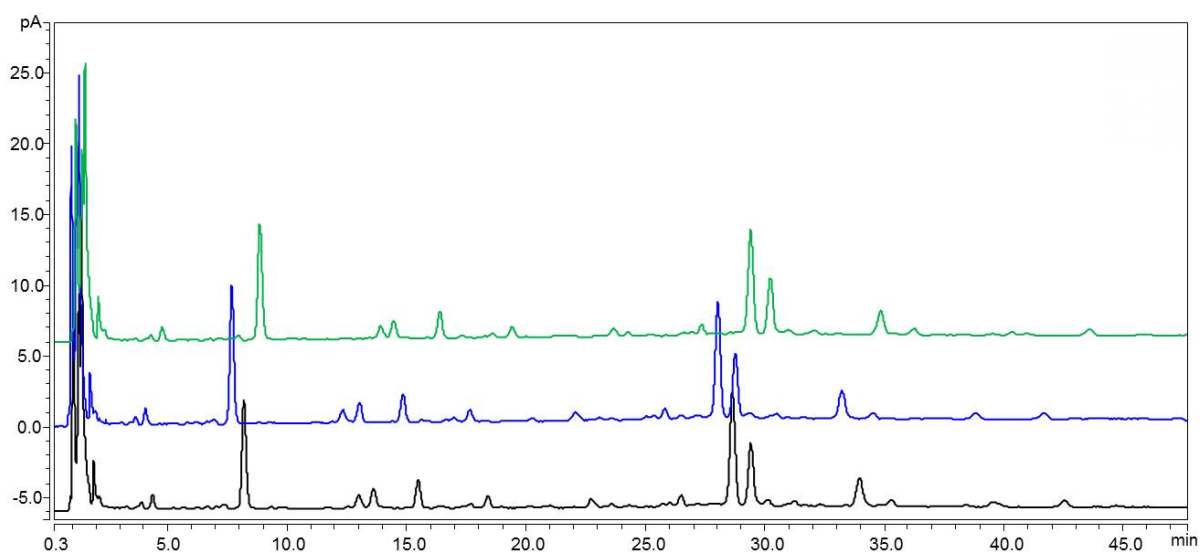


Figure S6. Validation of robustness: chromatograms of LS BuOH with different flow rates: 0.55 mL/min (black), 0.50 mL/min (blue) and 0.60 mL/min (green)

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Ritter, M., Melichar, K., Strahler, S., Kuchta, K., Schulte, J., Sartiani, L., Cerbai, E., Mugelli, A., Mohr, F.-W., Rauwald, H.W., Dhein, S., 2010. Cardiac and electrophysiological effects of primary and refined extracts from *Leonurus cardiaca* L. (Ph.Eur.). *Planta Med.* 76, 572-582.

Rolim de Almeida, L.F., Delachieve, M.E., Sannomiya, M., Vilegas, W., Campaner dos Santos, L., Mancini, E., de Feo, V., 2008. In vitro allelopathic potential of *Leonurus sibiricus* L. leaves. *J. Plant Interact.* 3, 39-48.

Schmidt, S., Jakab, M., Jav, S., Streif, D., Pitschmann, A., Zehl, M., Purevsuren, S., Glasl, S., Ritter, M., 2013. Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells. *J Ethnopharmacol.* 150, 85-94.

Shi, Y., Wu, C., Chen, Y., Liu, W., Feng, F., Xie, N., 2013. Comparative analysis of three *Callicarpa* herbs using high performance liquid chromatography with diode array detector and electrospray ionization-trap mass spectrometry method. *Journal of Pharmaceutical and Biomedical Analysis*. 75, 239-247.

Sugaya, K., Hashimoto, F., Ono, M., Ito, Y., Masuoka, C., Nohara, T., 1998. Antioxidative constituents from *Leonurii Herba* (*Leonurus japonicus*). *Food Sci. Technol. Int., Tokyo*. 4, 278-281.

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4.1.2 Additional data

For the sake of completeness, in this section those aspects are explained in greater detail, which were discussed only briefly in the publication. This concerns the identification of several compounds in *L. sibiricus* by comparative chromatography of authentic reference substances, the structure elucidation of verbascoside derivatives based on their MS/MS fragmentation patterns and PTP1B inhibition experiments of the iridoids acetylharpagide and ajugoside. The part about the identification of verbascoside derivatives by MS/MS is paid special attention to, since it facilitates a quick assessment of these compounds in *L. sibiricus*.

Figure 4.1 (page 86) shows a chromatographic comparison of LS BuOH (black) with caffeic acid (orange), chlorogenic acid (blue), rutin (pink), hyperoside (brown) and isoquercetrine (green) by HPLC method 1 (see Table 3.4, page 41). Retention times and UV spectra of the reference compounds were compared to the corresponding peaks in the LS BuOH extract. Caffeic acid, hyperoside and isoquercetrine are minor compounds in LS BuOH and are visible only by a small peak-shoulder in the chromatogram.

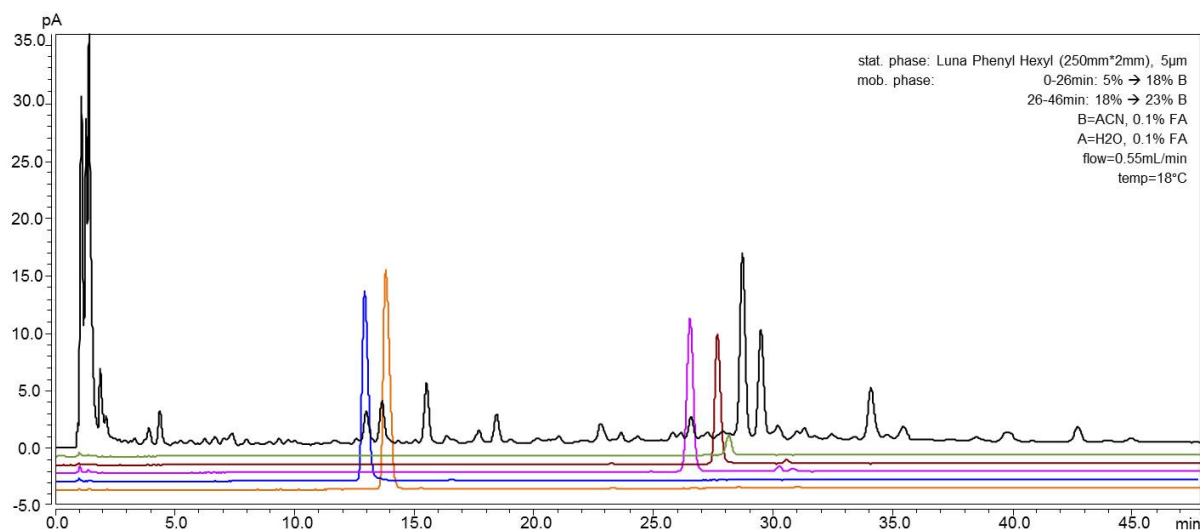


Figure 4.1.: HPLC-CAD analyses of caffeic acid (orange), chlorogenic acid (blue), rutin (pink), hyperoside (brown), isoquercetrine (green) and LS BuOH (black) by HPLC method 1 (see Table 3.4, page 41)

The *L. sibiricus* extracts discussed in the publication were rich in verbascoside and its derivatives. The MS/MS fractionation pattern of these structures showed characteristic properties, which are described in the following on the example of lavandulifo-

lioside (=pentosyl-verbascoside) (Figure 4.2, page 87 and Figure 4.3, page 88). The mentioned compounds showed better response in the negative ion mode. The $[M-H]^-$ ion of lavandulifolioside (m/z 755.2) lost either a pentosyl, caffeoyl or both residues at once to produce fragment ions of m/z 623.0, m/z 593.1 or m/z 461.0, respectively. Further loss of the deoxyhexosyl rest resulted in a fragment ion of m/z 315.0. The remaining molecule of m/z 134.9 was produced by the loss of the last sugar residue, a hexosyl rest. Similar fragmentation patterns of related compounds have been reported in literature (Wang et al., 2009).

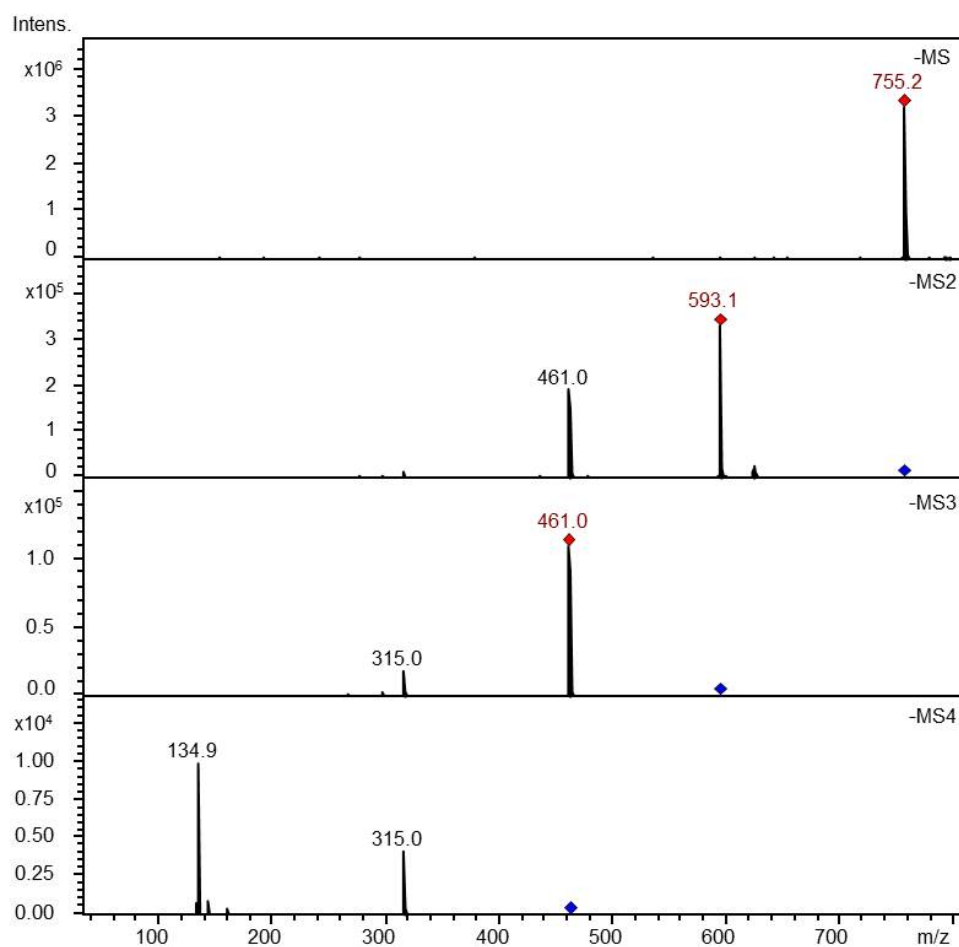


Figure 4.2.: MS⁴ spectra of lavandulifolioside in negative ESI ion mode

β -Hydroxy-verbascoside derivatives, like compound **18** in *L. sibiricus* (see Table S1, page 80), show similar fragmentation patterns, but are additionally characterized by the loss of water before the caffeoyl-residue is split off (Shi et al., 2013).

In the paper, the PTP1B inhibition of an iridoid enriched fraction of *L. sibiricus* is discussed (see page 59). This fraction is mainly composed of acetylharpagide and ajugo-

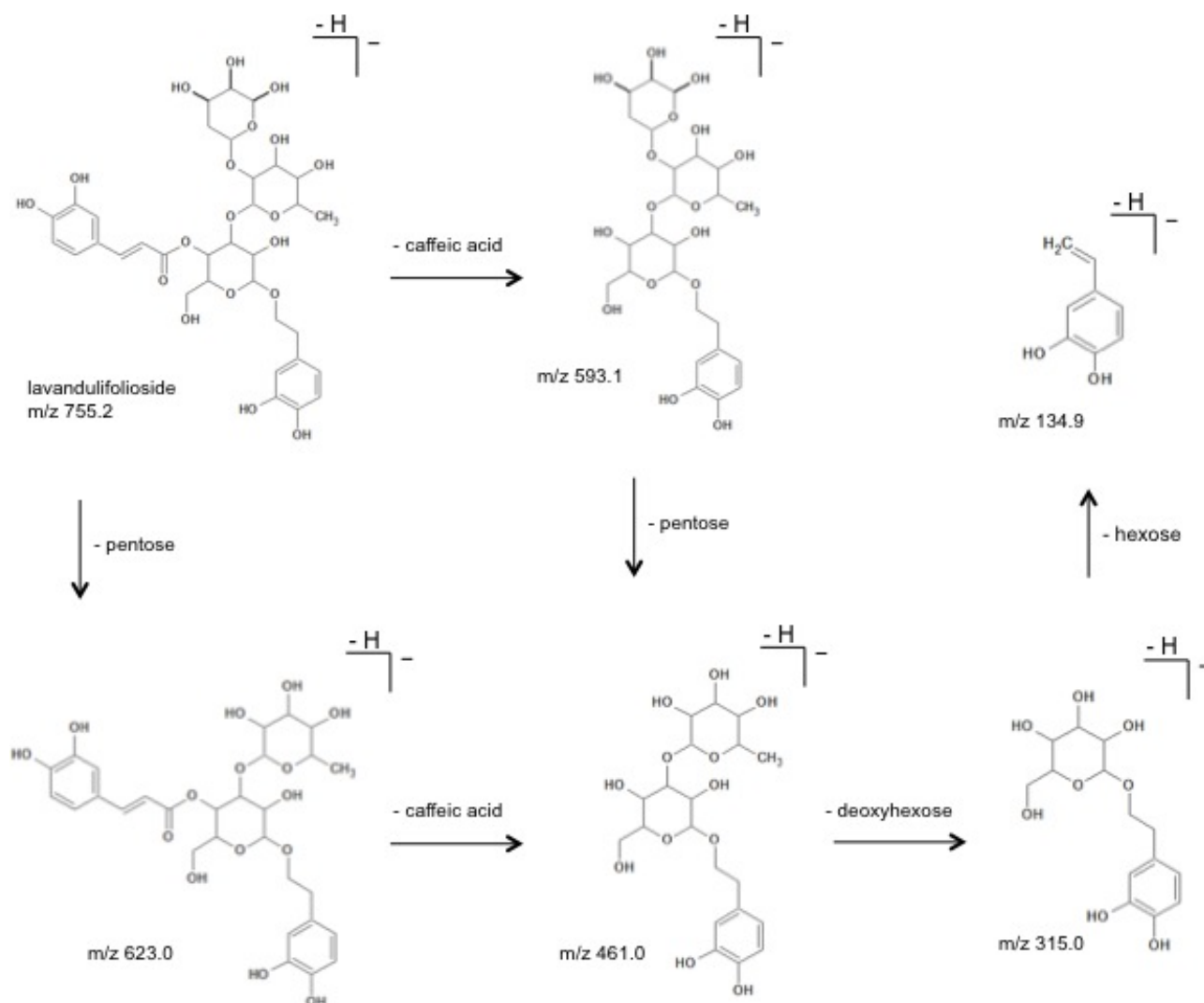


Figure 4.3.: Proposed fragmentation pathway of lavandulifolioside in the negative ESI ion mode

side, although another compound (compound 13) was detected in minor concentrations (see Fig S2, page 82). The injection peak at the beginning of the chromatogram was neglected. When acetylharpagide and ajugoside were tested separately at concentrations of up to 100 μM , the PTP1B inhibitory effect vanished. To examine whether a combination of both iridoids could elicit the same activity as the whole fraction, they were mixed in the same ratio as they occur in the fraction (ajugoside 40 μM , acetylharpagide 20 μM). Surprisingly, this iridoid mixture also failed to evoke PTP1B inhibition (see Figure 4.4, page 89). The activity of the iridoid fraction might therefore be explained by a synergistic effect of acetylharpagide, ajugoside and compound 13 or by the sole activity of compound 13. The identity of compound 13 could not be

determined with certainty, but MS data suggest it to be an acidic iridoid glycoside.

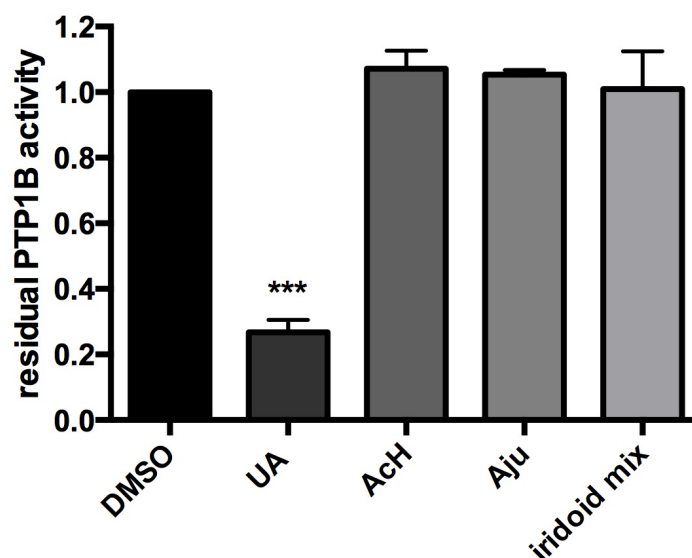


Figure 4.4.: PTP1B inhibition (see page 79) of acetylharpagide 100 μM (Ach), ajugoside 100 μM (Aju), a mixture of Ach 20 μM and Aju 40 μM (Iridoid mix). Negative control=DMSO, positive control=ursolic acid 30 μM (UA). *** $p < 0.001$ (ANOVA/Bonferroni)

4.2 Phytochemical characterization of LS OWE

An aqueous extract of *L. sibiricus* (LS OWE) was produced using special extraction conditions, which mimic the traditional way of intake (see section 3.2.1, page 36). When tested in the glucose-uptake assay (see page 78 f), LS OWE did not show activity. However, in an *in vitro* model using insulinoma INS-1E cells, this extract enhanced the insulin-secretion. In cooperation with partners at the Paracelsus Medizinische Privatuniversität, the effects of LS OWE on INS-1E cells were published in the "Journal of Ethnopharmacology" (see appendix D, page 163). The phytochemical characterization of LS OWE was subject of two diploma theses under the supervision of Prof. Glasl-Tazreiter at the Department of Pharmacognosy, University of Vienna (Burgstaller, 2014; Schallauer, 2014). This section summarizes their major findings.

To ensure that saccharides, which are known insulin secretors, do not mask the activity of other constituents in LS OWE, they had to be removed from the extract. This was one of the tasks in Gudrun Schallauer's diploma thesis. Different stationary phases

4. Results

for the separation of the sugars were tried and best results achieved by using solid phase extraction on C 8 material. Furthermore, several compounds of the HPLC fingerprint were identified by comparison with authentic reference substances or LC-MS. Figure 4.5 (page 90) shows an HPLC chromatogram of LS OWE and its main polyphenolic secondary metabolites detected by ELSD and UV=254 nm (Schallauer, 2014). Polyphenols detected in LS OWE also appear in LS MeOH (the extract, which showed activity in the glucose-uptake experiments), but with differences in quantity. These are namely several quercetin -and kaempferol derivatives, caffeoylquinic acids and phenylpropanoids in minor concentration.

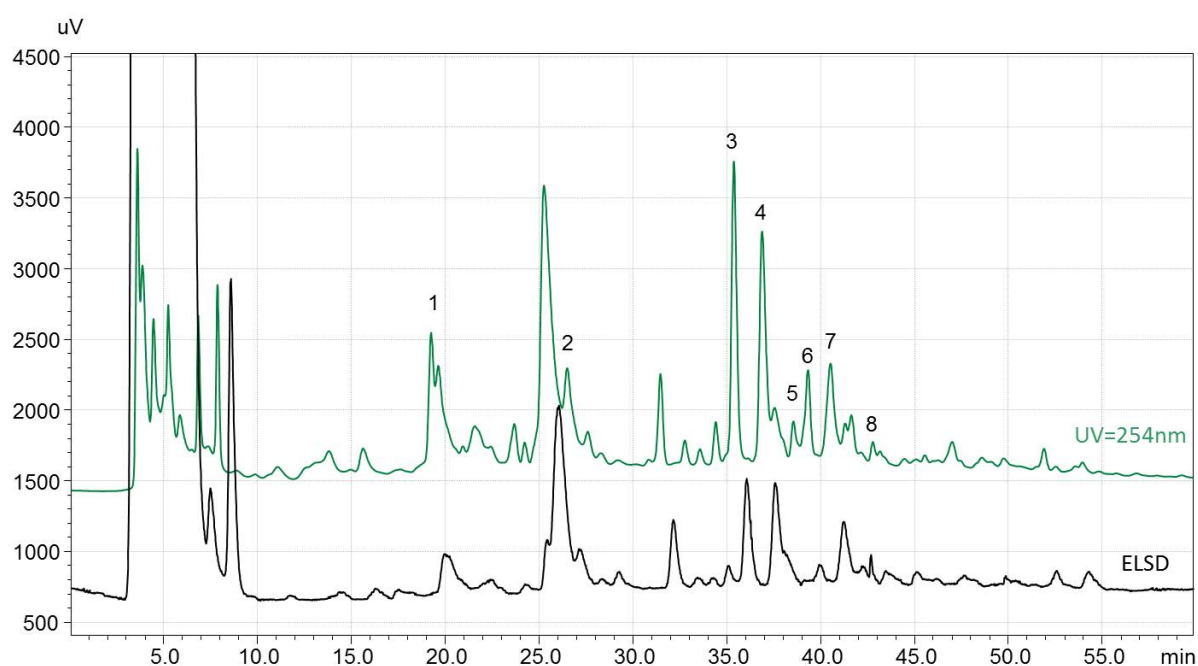


Figure 4.5.: HPLC analysis of LS OWE by HPLC method 2 (Table 3.4, page 41). 1=quercetin-trihexoside, 2=neochlorogenic acid, 3=quercetin-dihexoside, 4=cryptochlorogenic acid, 5=kaempferol-dihexoside, 6=rutin, 7=verbascoside, 8=lavandulifolioside. Source: Schallauer (2014)

Carina Burgstaller was able to proof the presence of stachydrine in LS OWE. Due to stachydrine's zwitterion character, the development of a suitable HPLC method was a time-consuming task. Good results were finally achieved by using a HILIC column with a 25 mM ammonium formate buffer as mobile phase. Representative amounts of stachydrine in *L. sibiricus* were proven by co-injection of the authentic reference substance and its MS data. As main secondary metabolite of LS OWE, it was of interest to examine whether stachydrine could elicit similar effects on the insulin secretion of

INS-1E insulinoma cells, as LS OWE. This, however, was not the case (see appendix D, page 163). Still, stachydrine's biological potency is a vivid field of investigation, especially in regard to cardiac protection (Shang et al., 2014).

4.3 Microscopic characteristics of *L. sibiricus* - paper in preparation

When reviewing available literature on *L. sibiricus*, confusions with another *Leonurus* species, *Leonurus japonicus* HOUTT. became apparent. In some cases, they were even used in synonymy. Clarification using literature data was not possible due to *L. sibiricus*' missing microscopic description. To compile criteria for distinction, anatomical and phytochemical characteristics of both species were assessed, according to monographs in pharmacopeias. Since *L. japonicus* is about to become adopted in the European Pharmacopeia (Ph.Eur.), *Leonurus cardiaca* L., the other *Leonurus* species monographed in Ph.Eur., was additionally included in the study. The data were summarized in the manuscript provided in the following pages (see page 92 ff), and prepared for submission to the "Journal of Ethnopharmacology".

The wording of methods described in the following paper may resemble publications of co-authors, where similar methods were applied.

Untangling the *Leonurus* confusion – microscopic and phytochemical differentiation of *L. sibiricus* L., *L. japonicus* HOUTT. and *L. cardiaca* L.

Anna Pitschmann¹, Christine Waschulin¹, Christina Sykora¹, Sodnomtseren Purevsuren², Sabine Glasl^{1,*}

Abstract

ETHNOPHARMACOLOGICAL RELEVANCE: Several *Leonurus* species are regularly applied in ethnomedicines: *L. sibiricus* L. in the Traditional Mongolian Medicine against high fever and diarrhoea, *L. japonicus* HOUTT. in TCM against gynaecological disorders and *L. cardiaca* L. against nervous heart complaints. *L. sibiricus* and *L. japonicus* are often used in synonymy implying that several studies may be built on improperly identified plant material, rendering conclusions questionable. To avoid further entanglement, we assessed species-specificity of anatomical, morphological and phytochemical characters.

MATERIAL AND METHODS: *L. cardiaca*, *L. sibiricus* and *L. japonicus* were compared according to their anatomical characteristics and their chemical HPTLC fingerprint. Using light microscopy, anatomical properties were established of reference specimens and confirmed by investigations of wild collected or commercially obtained plant material. For HPTLC analyses, methanol extracts of the three species were produced.

RESULTS: The three species are clearly distinguishable based on anatomy, morphology as well as phytochemistry. Anatomical characters involve presence, density and dimension of trichomes on different organs. Morphologically, species differed in the shape and length ratio of the upper and lower lip of the corolla. Chemical markers include leonurine, verbascoside and ajugoside on whose presence an identification can be based on. The absence of leonurine in *L. sibiricus* is described for the first time.

CONCLUSIONS: The three assembled character sets allowed for fast and reliable identification of *L. cardiaca*, *L. sibiricus* and *L. japonicus*. *L. japonicus*'s and *L. sibiricus*'s leaves and corollae are anatomically described for the first time and phytochemical fingerprints show type-specific patterns. In view of *L. japonicus*'s upcoming adaption in the European pharmacopoeia, proper distinction characteristics to *L. cardiaca*, the other *Leonurus* species monographed in Ph.Eur., meet a definite need.

Untangling the *Leonurus* confusion – microscopic and phytochemical differentiation of *L. sibiricus* L., *L. japonicus* HOUTT. and *L. cardiaca* L.

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Keywords: *Leonurus sibiricus* L.; *Leonurus japonicus* HOUTT.; *Leonurus cardiaca* L.; light microscopy; HPTLC fingerprint; leonurine

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1. Introduction

Leonurus, commonly known as motherwort, is a genus of the Lamiaceae family and comprises 25 species (The Plant List). Selected taxa are regularly applied in different traditional medicines: *Leonurus sibiricus* L. (LS) in the Traditional Mongolian Medicine (TMM) against high fever and diarrhea (WHO, 2013), *Leonurus japonicus* HOUTT. (LJ) in the Traditional Chinese Medicine (TCM) against gynaecological disorders (Stöger and Friedl, 2012) and *Leonurus cardiaca* L. (LC) against nervous heart complaints (Wichtl et al., 2009). LS and LJ are native to China, Korea and Japan. Additionally, LS is distributed in Russia and Mongolia and LJ became naturalized in Africa, Europe and South and North America (GBIF, 2014; WHO, 2013). The morphology is generally similar among all *Leonurus* species (Krestovskaja, 1992). The three mentioned types have white to red corolla and the flowers are arranged in verticillasters. Cauline leaves are dissected or 3- to 7-lobed (eFloras, 2008). LC is monographed in the European Pharmacopoeia (2014), LJ in the Chinese (Stöger and Friedl, 2012) and Japanese Pharmacopoeia (The Ministry of Health, 2011). In the respective monograph, anatomical characterization of the corresponding drug is offered. In case of LJ, the anatomical descriptions in the Chinese Pharmacopoeia only entail unspecific characteristics of fruits and stems. LS is not part of any Pharmacopoeia and has not been anatomically characterized. Reported secondary metabolites of LS, LJ and LC include flavonoids, phenylpropanoids, terpenoids and alkaloids (Hayashi et al., 2001; Kuchta et al., 2012; Luo et al., 1985; Schmidt et al., 2013; Shang et al., 2014).

When reviewing the available literature on these three species, it becomes apparent that LS and LJ have often been confused, sometimes used as synonyms (Harley and Paton, 2001; Hayashi et al., 2001; Kuchta et al., 2012). This circumstance is discussed in the work by Harley and Paton (2001). They emphasize the inequality of the common tropical weed LJ from LS by referring to lectotype specimens and further Siberian material from the Kew Herbarium. In their investigations, all specimens of LS shared a common characteristic: the calyces' deflexed lower lip. LJ's calyx however, has erect teeth and, in addition, is described to be smaller than that of LS. This distinction criterion was previously described by Krestovskaja (1992) in a systematic study of the genus *Leonurus*. There, the author not only shows differences in the calyx' shape of LS and LJ, but also of related genera and other *Leonurus* species. Additionally, the shape and size of different corolla parts are described to be deterministic for species assignment, although specific examples were not offered. Pollen grain examination could not be used for identification due to their great similarity between

the various species. The declaration of LS and LJ to be distinct species is accepted by reliable databases, like www.gbif.org and www.theplantlist.org.

Despite these taxonomic publications, LS and LJ are still interchangeably used in current literature. Hayashi et al. (2001), for example, declare: “*Leonurus sibiricus* (= *L. japonicus*, Labiatae) ...”. Kuchta et al. (2012) even cite above mentioned articles, but state nevertheless: “...the source plant [...] is *Leonurus japonicus* Houtt., including its taxonomic synonyms *Leonurus sibiricus* L., ...”. In this case, a misinterpretation of Krestowskaja’s and Harley and Paton’s works may be assumed. Shang et al. (2014) stress that various names for LJ exist and that this confusion slows research, but use *Leonurus sibiricus* at the same time as synonym for LJ in several tables. Further entangling is caused by miscitations. Moon et al. (2010), for instance, mention in their introduction biological effects and secondary metabolites of LS whilst citing articles, which in actuality deal with *L. persicus*, *L. glaucescens* and LJ. The same is the case in the article of Wu et al. (2011). They ascribe phytochemical properties to LS, which were originally reported for a different *Leonurus* species. Additionally, authors who follow the guidelines of the Japanese Pharmacopoeia on the monograph of Leonuri Herba may use LJ as well as LS for their investigations. Both species are listed as plant origin for Leonuri Herba in this Pharmacopoeia without giving any type-specific description of the plant material (The Ministry of Health, 2011).

Many studies dealing with LJ are based on plant material, which was obtained from a commercial provider, presumably in cut condition. Micromorphological details, like the shape of the calyx, may not be identifiable anymore once the plant material is cut. The previously reported distinction criterion between LS and LJ of the differently shaped calyx may thus not be applicable in these cases. Giving the varying use in the respective traditional medicine and the geographical closeness of LS and LJ, the importance of precise differentiation features becomes evident. To avoid mistakes in plant identification during collection as well as in the laboratory, it is of necessity to provide anatomical and phytochemical distinction characteristics. Furthermore, LJ is about to become adopted in the European Pharmacopoeia (Monschein et al., 2014), making a clear discrimination to LC, the other *Leonurus* species monographed in the Ph. Eur., necessary. For these reasons, anatomical and phytochemical differentiation characteristics of LC, LJ and LS are presented in this work.

2. Material and Methods

2.1 Chemicals

Solvents used for extraction and HPTLC analyses were of analytical grade. Reference substances verbascoside (batch nr. 0082-05-80, purity 86.3%) and leonurine (batch nr. SML0670, purity > 98%) were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetylharpagide (batch nr. NP-001679, purity 99%) was obtained from AnalytiCon Discovery (Potsdam, Germany). Ajugoside was isolated from *Leonurus sibiricus* L. and its structure confirmed by NMR in previous studies (Pitschmann et al., 2015). Glycerol gelatin was prepared by soaking 300 g gelatina alba in 1200 mL water followed by addition of 750 mL glycerol and conservation with 0.2% paraben.

2.2 Plant material

Five LC, two LJ and four LS reference specimens from different sources were investigated to establish morphological and anatomical characteristics of the respective species (see Table 1). For phytochemical analysis, commercially obtained or collected plant material was used to save the valuable specimens. Prior to extraction, these samples were subjected to an intensive microscopic screening, where the characteristics, which were previously established using the specimens, were verified. Respective specimen or lot numbers of these additional samples are given in Table 2.

2.2.1 *Leonurus cardiaca* L.

Reference specimens were provided by the Department of Pharmacognosy, University of Vienna, Austria (see Table 1). Four batches of herba leonuri cardiaceae were obtained from either Kottas Pharma GmbH (Vienna, Austria), Caesar & Loretz GmbH (Hilden, Germany) or MB-Holding GmbH & Co. KG (Vestenbergsgreuth, Germany) (see Table 2).

2.2.2 *Leonurus japonicus* HOUTT.

Reference specimens were provided from the Museum of Natural History, Vienna (see Table 1). Five different Herba Leonuri japonici samples were supplied by the Bayrische Landesanstalt für Landwirtschaft-Institut für Pflanzenbau und Pflanzenzüchtung (Freising-Weihenstephan, Germany) (see Table 2). According to the manufacturer's information, the plant material was characterized by DNA analyses.

2.2.3 *Leonurus sibiricus* L.

Reference specimens were provided by the Department of Pharmacognosy, University of Vienna, Austria and the Museum of Natural History, Vienna, Austria (see Table 1). Aerial parts of three further *Leonurus sibiricus* L. batches were harvested at Tsetserleg in the

Mongolian province Archangai (latitude 47° 28' 8.22''; longitude 101° 26' 32.14'') in the summers of 2009 (1), 2010 (2) and 2014 (3) (see Table 2). The plant material was identified by E. Ganbold, Mongolia. A reference specimen of batch 1 is kept at the herbarium of the School of Pharmacy, Health Science University of Mongolia, Ulaanbaatar, Mongolia (herbarium number 10080805). Reference specimens for batches 2 and 3 are kept at the Department of Pharmacognosy, University of Vienna, Austria (herbarium numbers HERB_LS_01/10/mon and HERB_LS_01/14/mon, respectively).

Species	Source	Specimen
<i>Leonurus cardiaca</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus cardiaca L. Rewnitz 871 Droge
<i>Leonurus cardiaca</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus cardiaca L. Gem. Herzgespann
<i>Leonurus cardiaca</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus cardiaca Leonurus bolus caulinis lanceolatis trilobis Didynamia gymnospermia
<i>Leonurus cardiaca</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus cardiaca Lin. Tirlemont Août. 1869
<i>Leonurus cardiaca</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP 667 Leonurus cardiaca L. Neilr. Flor. p. 342 Wien. In ruderatis, ad sepes in pagis. Jul., J.v.Kováts
<i>Leonurus japonicus</i> HOUTT.	Museum of Natural History Vienna, Austria	Herbarium W 2008-03539
<i>Leonurus japonicus</i> HOUTT.	Museum of Natural History Vienna, Austria	Herbarium W 2010-11477
<i>Leonurus sibiricus</i> L.	Museum of Natural History Vienna, Austria	Acqu. 1933 No.1210
<i>Leonurus sibiricus</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus sibiricus_Mon_1
<i>Leonurus sibiricus</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus sibiricus_Mon_2
<i>Leonurus sibiricus</i> L.	Dept. of Pharmacognosy, University of Vienna, Austria	WUP Leonurus sibiricus_Mon_3

Table 1 Source and specimen number of the investigated reference specimens of *Leonurus cardiaca* L., *Leonurus japonicus* HOUTT. and *Leonurus sibiricus* L.

Declaration	Sample name	Company/Identifier	Batch/specimen Number	DER of dry MeOH extract
<i>Leonurus cardiaca</i> L.	LC1	MB-Holding GmbH & Co. KG	16738/08 90435	13:1
<i>Leonurus cardiaca</i> L.	LC2	Kottas Pharma GmbH	W11201654	7:1
<i>Leonurus cardiaca</i> L.	LC3	Caesar & Loretz GmbH	72835188	7:1
<i>Leonurus cardiaca</i> L.	LC4	Kottas Pharma GmbH	W11202091	12:1
<i>Leonurus japonicus</i> HOUTT.	LJ1	LfL	Horizon herbs/2013	4:1
<i>Leonurus japonicus</i> HOUTT.	LJ2	LfL	B.T. world Seeds/2008	4:1
<i>Leonurus japonicus</i> HOUTT.	LJ3	LfL	Richters/2008	4:1
<i>Leonurus japonicus</i> HOUTT.	LJ4	LfL	JPK/2013	5:1
<i>Leonurus japonicus</i> HOUTT.	LJ5	LfL	Zhang/2011	4:1
<i>Leonurus sibiricus</i> L.	LS1	Dr. Ganbold	10080805 (Mongolia)	4:1
<i>Leonurus sibiricus</i> L.	LS2	Dr. Ganbold	HERB_LS_01/10/mon (Austria)	4:1
<i>Leonurus sibiricus</i> L.	LS3	Dr. Ganbold	HERB_LS_01/14/mon (Austria)	3:1

Table 2 Used drug samples (left), sample name (middle left), supplier of LC1-LJ5 and identifier of LS1-LS3 (middle), batch number of LC1-LJ5 and specimen number of LS1-LS3 (middle right) and DER (=drug to extract ratio) of the dry methanol extract (right). LfL=Bayrische Landesanstalt für Landwirtschaft-Institut für Pflanzenbau und Pflanzenzüchtung

2.4 Light microscopy

The anatomy of leaves and flowers of LC, LS and LJ was assessed by means of light microscopy. Preparation was conducted using a Nikon SMZ-U Zoom 1:10 stereo microscope. Leaves were rehydrated in water to soften the tissue, before cross sections were cut. Surface leaf preparations and preparations of the flowers were directly embedded in a few drops of chloral hydrate solution (60% in water), gently boiled and then examined using a Nikon Labophot-2 light microscope. Long term preparations were made using glycerol gelatin (see section 2.1). Lengths of trichomes were determined using a micrometer. The density of different trichomes types was evaluated in a field of 10 cm² using a Nikon Drawing tube. Pictures were taken with a Canon PowerShot S95 digital compact camera.

2.5 Data analysis and statistical methods

For the establishment of microscopical characters, one flower and one cauline leaf of each reference specimen (see Table 1) was used. Analyses of samples listed in Table 2 were conducted using five organs of each sample to ensure a representative data set. All

microscopic analyses were performed in quintuplicate. 30 leaf characters and 26 corolla characters were assessed in total. Table 3 enlists all the established characters for each species and the number of all measurements (data points). These numbers vary depending on the availability of the respective character, e.g. some organs were not present in all cases or certain structures were not recognizable due to thick cuticula.

Character	<i>L. cardiaca</i> – data points [Nr.]	<i>L. sibiricus</i> – data points [Nr.]	<i>L. japonicus</i> – data points [Nr.]
Cauline leaf			
US: trichome type 1 length	75	125	90
US: trichome type 1 width	70	125	90
US: trichome type 1 cell Nr.	75	125	90
US: trichome type 1 basal cells Nr.	75	125	85
US: trichome type 1 density	70	123	90
US: glandular trichome diameter	75	125	90
US: glandular trichome density	75	125	85
US: epidermis cells length	75	125	90
LS: stomata length	75	120	90
LS: stomata width	75	125	90
LS: stomata density	75	120	75
LS: trichome type 2 length	68	119	90
LS: trichome type 2 width	68	124	90
LS: trichome type 2 cell Nr.	68	124	90
LS: trichome type 2 basal cells Nr.	68	124	76
LS: trichome type 2 density	68	124	90
LS: glandular trichome 2 diameter	75	124	90
LS: glandular trichome 2 density	70	125	80
LS: glandular trichome 3 diameter	75	125	90
LS: glandular trichome 3 density	70	125	80
LS: epidermis cells length	75	125	85
Crystals length	75	120	90
Crystals width	75	121	90
CS: thickness	75	120	90
CS: palisade parenchyma thickness	75	125	90
CS: spongy parenchyma thickness	75	125	90
CS: upper cuticula thickness	75	125	80
CS: lower cuticula thickness	75	125	80
CS: upper epidermis thickness	75	125	80
CS: lower epidermis thickness	75	125	80
Corolla			
IS: trichome type 3 length	25	95	70
IS: trichome type 3 width	25	95	70
IS: trichome type 4 length	25	95	70
IS: trichome type 4 width	25	95	70
IS: glandular trichome 4 diameter	25	105	69

OS: trichome type 5 length	24	n.a.	70
OS: trichome type 5 width	25	n.a.	70
OS: trichome type 6 length	25	110	70
OS: trichome type 6 width	25	110	70
OS: trichome type 7 length	25	110	70
OS: trichome type 7 width	25	110	70
OS: glandular trichome 5 diameter	18	105	70
OS: glandular trichome 6 diameter	18	105	70
Gynoecium			
Ovary: trichome type 8 length	14	n.a.	45
Ovary: trichome type 8 width	15	n.a.	45
Ovary: glandular trichome 7 diameter	10	82	14
Ovary: glandular trichome 8 diameter	n.a.	89	52
Style: width	20	98	55
Stigma: length	10	47	15
Stigma: width	10	51	15
Androeceium			
Filament: trichome type 9 length	20	105	70
Filament: trichome type 9 width	20	105	70
Filament: trichome type 10 length	n.a.	93	51
Filament: trichome type 10 width	25	94	70
Filament: glandular trichome 9 diameter	9	103	56
Pollen diameter	21	100	62

Table 3 Established microscopic characters of *L. cardiaca*, *L. japonicus*, *L. sibiricus*. US=upper side, LS=lower side, CS=cross section, IS=inside, OS=outside; n.a.=not applicable

Statistical analyses were performed with GraphPad Prism software version 4.03. One-way analysis of variance (ANOVA) with Bonferroni's post hoc test was used to determine statistical significance in Figure 4. Unpaired t-test was used to determine statistical significance in Figure 3 and Figure 5. Two-way analysis of variance with Bonferroni's post hoc test was used to determine statistical significance in figure 1. Data groups with $p < 0.05$ were considered statistically significant. The given values were calculated as mean \pm SD of all measurements.

2.6 Extraction

Prior to extraction, all plant material samples were pulverized with a basic electric grinder. 2 g pulverized material of each sample were extracted with methanol by accelerated solvent extraction, using an ASE 200 system from Dionex (Sunnyvale, CA, USA). The material was filled in stainless steel extraction cells. Each cell was extracted three times each with 20 mL methanol, intermitted by a tube-cleaning step with acetone between every new cycle.

Extractions were carried out at 40°C and under a pressure of 10 MPa. Resulting liquid extracts were evaporated to dryness under reduced pressure at 40°C. The drug to extract ratios (DER) of the dry methanol extracts are given in Table 2.

2.7 HPTLC analyses

High performance thin layer chromatography (HPTLC) was used for comparative fingerprint analyses and for detection of leonurine in the samples. HPTLC silica gel 60 F₂₅₄ glass plates (Merck, Darmstadt, Germany) served as stationary phase. As mobile phase served a mixture of ethylacetat, acetic acid, formic acid and water (100:11:11:26). Sample solutions were prepared by re-dissolving the respective extract in methanol at a concentration of 30 mg/mL. Pure reference compounds were used at a concentration of 10 mg/mL. All extracts and the reference compound leonurine were acidified with 2 µL concentrated formic acid to improve solubility of leonurine. Using an automated TLC sampler (ATS4, CAMAG, Berlin, Germany), samples were applied in bands, 130 mm above the lower edge of the HPTLC plate. For detection of polyphenols and iridoids, 5 µL sample were applied, for detection of stachydrine and leonurine, 15 µL. HPTLC development was performed by CAMAG development device ADC2, which was set to the following parameters: 48% humidity (K₂CO₃), temperature 24°C, 20 minutes saturation with saturation pad, total step time 59 minutes. Developed plates were visualized using a CAMAG visualizer at daylight, 245 nm and 366 nm before derivatization. Derivatization methods varied, depending on the to-be-detected compounds. For detection of iridoids, HPTLC plates were derivatized with anisaldehyde-sulfuric acid reagent (anisaldehyde, glacial acetic acid, methanol, sulfuric acid 0.5:10:85:5 v/v/v/v) and subsequently put 7 min on a heating plate adjusted to 100°C. Phenylpropanoids were detected with natural product reagent A (1% methanolic solution of diphenylboric acid-β-aminoethylester complex) and alkaloids with modified Dragendorff reagent (bismuth oxynitrate 0.085 g, sulphuric acid 1.75 mL, glacial acetic acid 1 mL, 40% potassium iodide solution 5 mL, water ad 50.0 mL). Derivatization was performed by dipping the plate in the respective reagent, using CAMAG chromatogram immersion device III. After derivatization, plates with Dragendorff reagent were visualized at daylight, plates with anisaldehyde reagent or natural product reagent A at 366 nm.

3. Results

This section holds only this information, which is necessary for the distinction of the three *Leonurus* species. A summary of all distinction characters is given in Table 4 including their mean values \pm SD. Additionally, since LJ's and LS's leaves and flowers have not been anatomically described before, a detailed anatomical characterization of these two *Leonurus* species is outlined in the supplementary information.

Characteristic	<i>L. cardiaca</i> [mean \pm SD]	<i>L. japonicus</i> [mean \pm SD]	<i>L. sibiricus</i> [mean \pm SD]
Trichome type 1 (upper leaf side) length [μ m]	418 \pm 139	103 \pm 36	112 \pm 55
Trichome type 2 (lower leaf side) length [μ m]	425 \pm 172	168 \pm 43	70 \pm 26
Trichome type 1 (upper leaf side) density [Nr./10 cm ²]	9 \pm 5	32 \pm 19	21 \pm 10
Trichome type 2 (lower leaf side) density [Nr./10 cm ²]	32 \pm 13	44 \pm 33	16 \pm 9
Trichome type 5 (corolla outside) length [μ m]	1560 \pm 296	n.a.	933 \pm 192
Trichome type 3 (corolla inside) length [μ m]	482 \pm 62	277 \pm 55	660 \pm 158
Trichome type 8 (ovary) length [μ m]	571 \pm 250	n.a.	207 \pm 68

Table 4 Summary of relevant differentiation characters of *L. cardiaca*, *L. japonicus* and *L. sibiricus*, expressed as means \pm SD. For determination of characters see section 2.4 and 2.5. n.a.=not applicable.

3.1 Differences in the anatomy of leaves

The leaves of the three *Leonurus* species are very similar. The lower side is always brighter in color than the upper side of the leaf. Both leaf sides are pillose. On the lower side, trichomes are mostly located on the veins, on the upper leaf side strictly in between the veins. Variations between the three species become visible when leaf surfaces are examined under the light microscope. LC, LJ and LS have similar trichome types, but they differ in length and density. These non-glandular trichomes are tapered and thick walled with a warty cuticula. In LS and LJ they mostly consist of one to two uniseriate cells, whereas trichomes of LC consist of up to four uniseriate cells. The densest pilosity on both leaf sides is found in LJ. The ratio of trichome density between upper and lower side of the leaf is different in all three species. In LC and LJ, the leaf upper side is less pillose than the lower side, a difference which is more pronounced in LC. On the other hand, this ratio is reversed in LS: the upper leaf side is more pubescent than the lower side. Detailed numbers are given in Table 4.

As far as length is concerned, LC possesses the longest non-glandular trichomes on both leaf sides, measuring approximately $420 \pm 139 \mu\text{m}$. In comparison, LS's trichomes are $70 \pm 25 \mu\text{m}$ and $112 \pm 55 \mu\text{m}$ and LJ's trichomes $168 \pm 43 \mu\text{m}$ and $103 \pm 36 \mu\text{m}$ long on the lower and upper leaf side, respectively. Figure 1 shows a comparison of trichome length (panel A) and density (panel B) of the leaves' lower and upper side of LC, LJ and LS (for determination of trichome density and length see section 2.4 and 2.5).

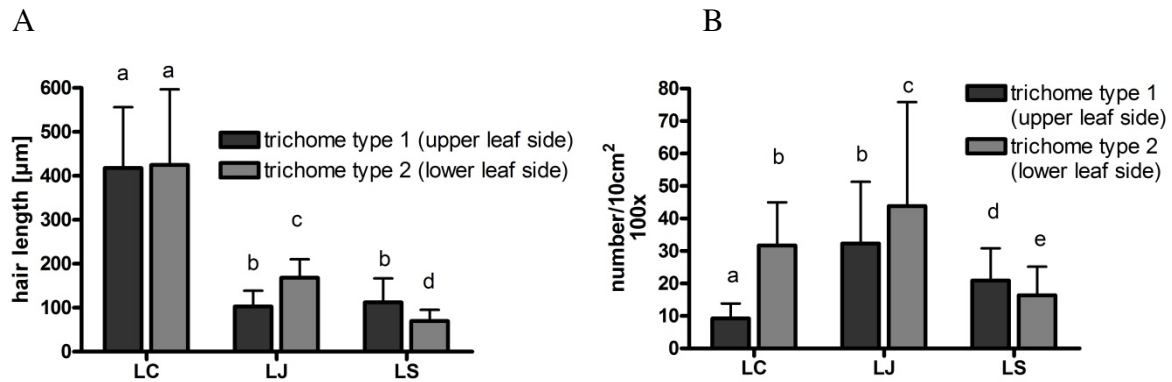


Figure 1

3.2 Differences of the flowers

3.2.1 Corolla

The corolla of Lamiaceae is generally composed of two lips – an upper lip and a lower lip. The lower lip of *Leonurus* consists of three lobes, from which the middle lobe's shape is important for the distinction between LS and LJ. In LS the form of the middle lobe is simply oblong. LJ reveals a middle lobe which is subdivided into two big separate lobes. The latter form an obtuse angle and surmount the two lateral lobes of the lower lip (see Figure 2).



Figure 2

When examining the petals under the light microscope, several types of trichomes and numerous crystals in the epidermis cells become apparent. In all three species, the petals' outside is pillose with non-glandular and glandular trichomes as well as glandular scales, whereas the inside is less pubescent. LC and LS possess long, thin walled, multicellular, uniseriate trichomes with rounded ends on the outside of the upper lip. These trichomes do not exist on the lower lip. In case of LS, these trichomes are $933 \pm 192 \mu\text{m}$ long and composed of up to seven uniseriate cells. LC's multicellular trichomes comprise up to 15 individual cells and reach a length of $1560 \pm 296 \mu\text{m}$. LJ lacks this characteristic completely (see Figure 3). Its outer surface's trichomes are shorter, thinner, composed of only two cells and are therefore not comparable with the multicellular trichomes of LC and LS.

The tube of all three species shows a similar property: on the flowers' inside, approximately 5 mm above the lower edge of the tube, a hairy annulus of densely arranged, unicellular trichomes is situated, which resembles a brush border. These trichomes are thin walled and, in some cases, contain solitary crystals or crystal needles, accumulated to a cluster. The length of the brush border trichome is different in all three species (see Figure 4): $660 \pm 158 \mu\text{m}$ on LS, $482 \pm 68 \mu\text{m}$ on LC and $277 \pm 55 \mu\text{m}$ on LJ.

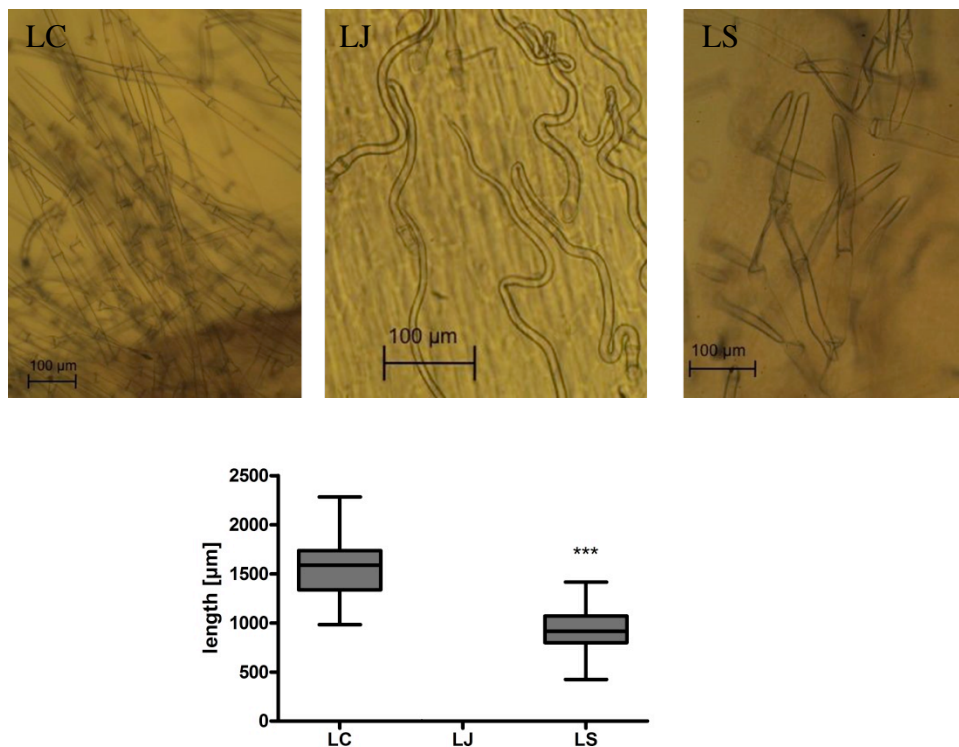


Figure 3

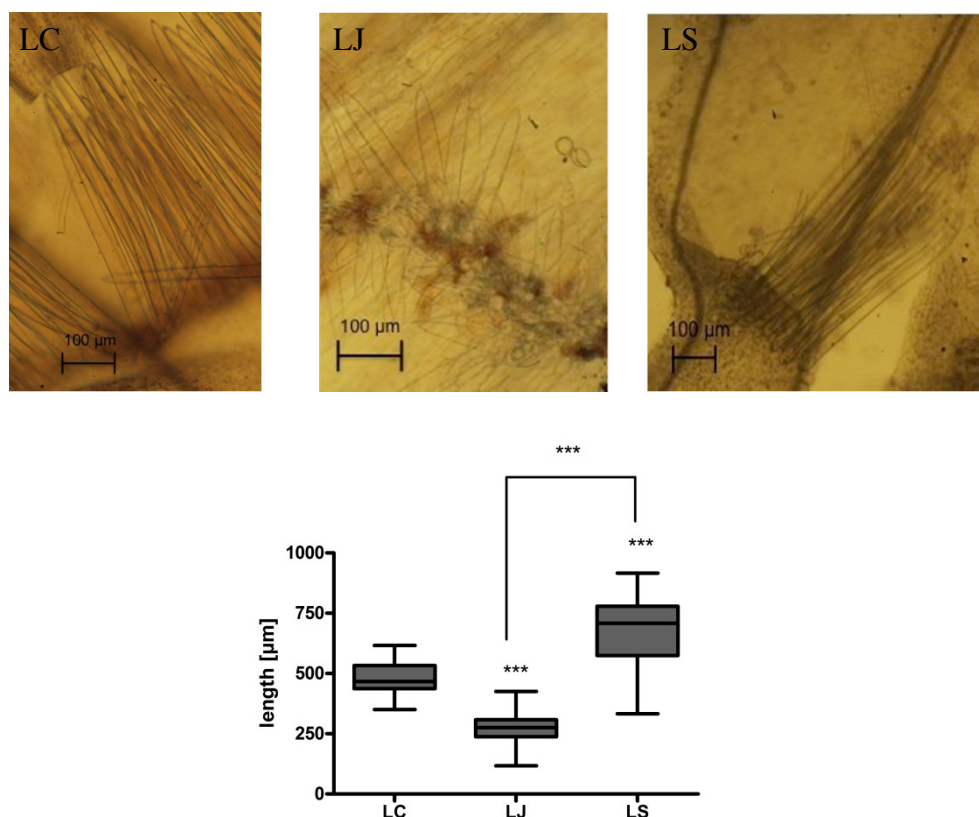


Figure 4

3.2.2 Ovary

The outer surface of the ovaries in all three *Leonurus* species show glandular trichomes and glandular scales. LC's and LS's ovaries also display non-glandular trichomes with different lengths, whereas LJ does not possess such trichomes on its ovary. The ovaries of LC and LS are separable by the number of cells the trichomes are composed of and by the trichomes' length. LC's trichomes are composed of two to four cells, reaching lengths of $571 \pm 250 \mu\text{m}$. In comparison, LS's trichomes are shorter, measuring $207 \pm 68 \mu\text{m}$ and comprising only one to two cells (see Figure 5).

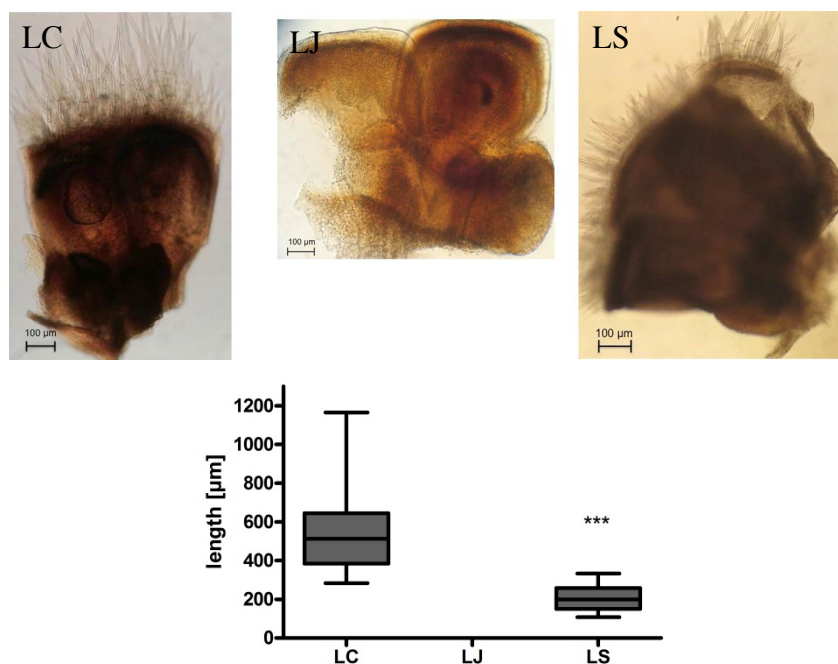


Figure 5

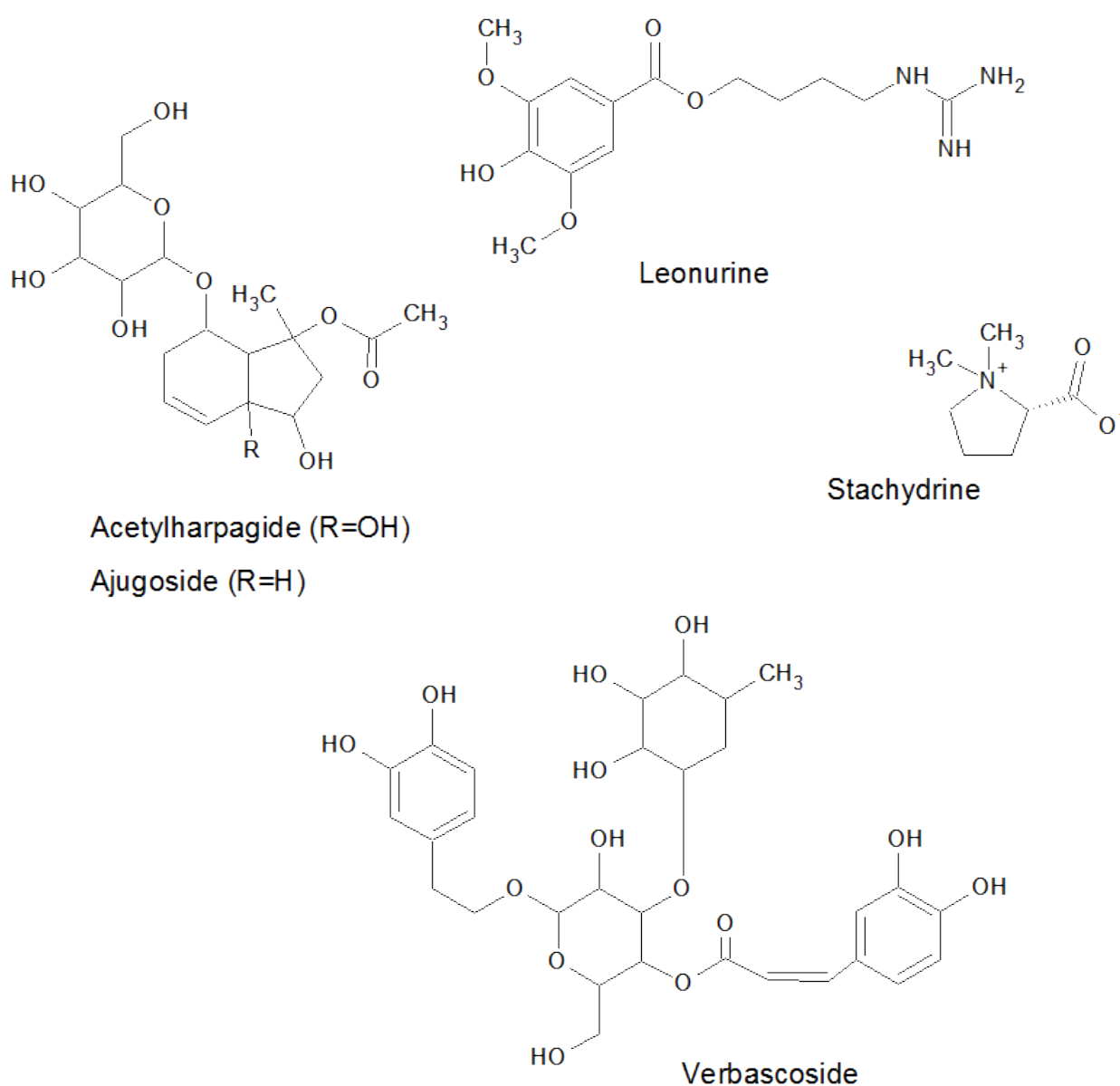
3.3 Differences in the HPTLC fingerprint

HPTLC served for comparison of the phytochemical fingerprint and for monitoring the presence of certain reference compounds. Three chromatograms were produced, each with the same mobile and stationary phase, but with varying detection reagents (see section 2.6). The chromatogram of Figure 6A was derivatized with natural product reagent A, thereby visualizing polyphenols like flavonoids or caffeoylquinic acid derivatives. As reference substance served verbascoside, which shows a bright blue fluorescing band at $R_f=0.53$. Earlier studies of ours show that this phenylpropanoid is one of the major constituents in LS (Pitschmann et al., 2015). This secondary metabolite is also found in all LC samples, but in none of the LJ samples, which makes the presence or absence of verbascoside a valid differentiation criterion between LJ and LS or LC. The polypyhenolic patterns of LS and LC are generally very similar and differ from each other only in a few selected bands.

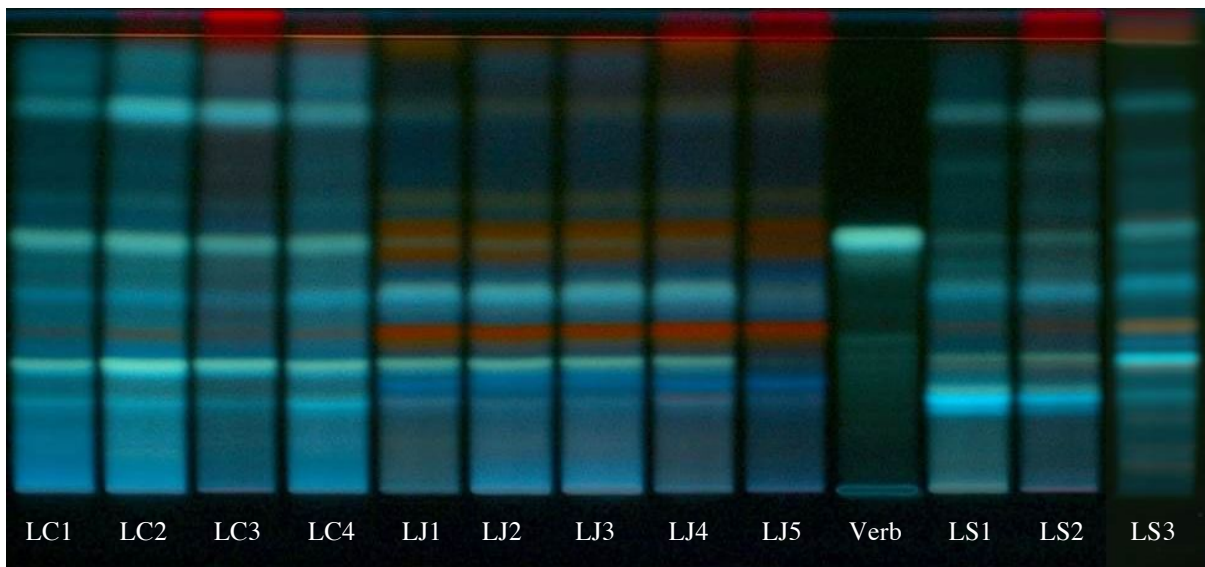
All samples derivatized with modified Dragendorff reagent (Fig. 6B) showed an intense brown band at $R_f=0.14$. This compound is stachydrine. Among all secondary metabolites of LJ, stachydrine has been most extensively studied (Shang et al., 2014). On the same chromatogram, all LJ samples show another, in comparison to stachydrine brighter colored band at $R_f=0.52$, which LC and LS do not display. This is the alkaloid leonurine. It's presence or absence in LC has been subject to discussion in the past. In this study we provide a further report for the absence of leonurine in LC. Leonurine has also been described to be part of

LS's secondary metabolite's profile (Reuter and Diehl, 1971). However, it could not be detected in any of the three LS samples used in our study. Additionally, the absence of leonurine in the three LS extracts was checked by comparing retention times and UV spectra obtained by HPLC-DAD (see chromatograms in Figure S5).

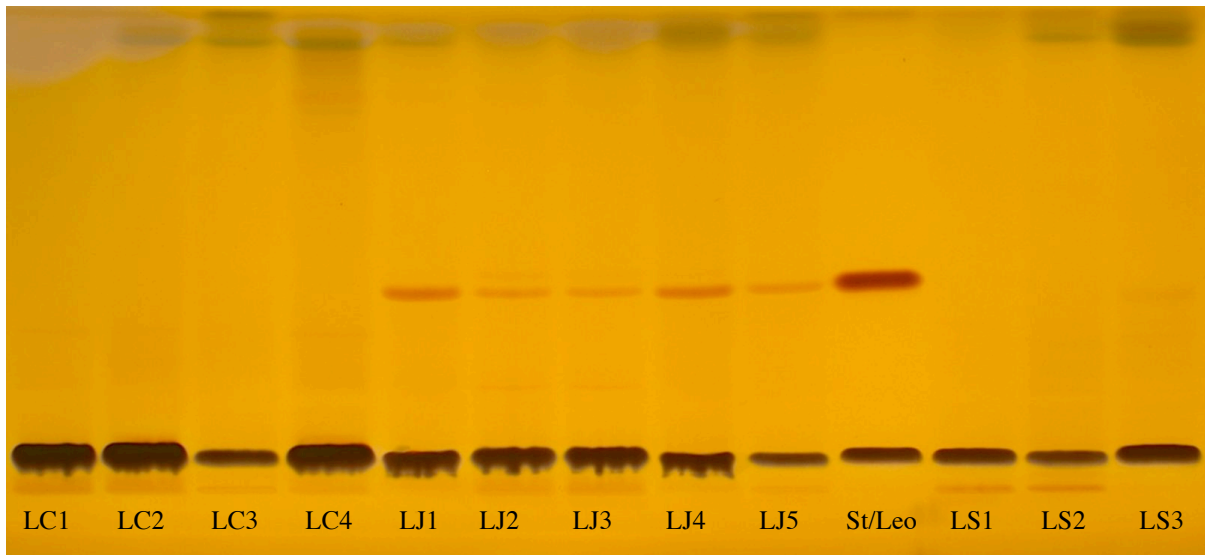
The third HPTLC detection method, anisaldehyde-sulfuric acid reagent, was used to visualize iridoids (Fig. 6C). As reference compounds served acetylharpagide, appearing as yellow-green band at $R_f=0.18$ and ajugoside, appearing as dark red fluorescing band at $R_f=0.29$. These two iridoids are marker substances of LS (Pitschmann et al., 2015). They are also present in a lesser degree in LC, but not to be detected in LJ.



A



B



C

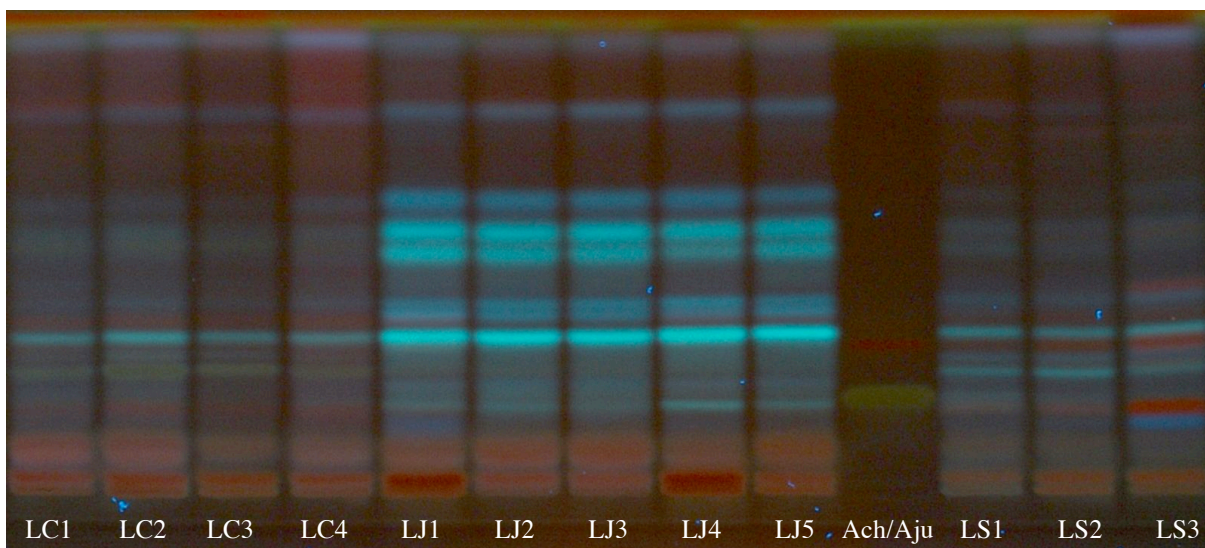


Figure 6

4. Discussion

Leonurus sibiricus and *Leonurus japonicus* resemble each other strongly in their habitus. Being first described independently they are often confused or mistakenly used as synonyms in phytochemical and biological studies and subsequently in many books referring to the recent literature. Additionally, pharmacopoeias holding a monograph of LJ are not specific enough in their plant identification requirements. Characteristics of LJ described in the Chinese pharmacopoeia, for instance, are also applicable to LS. The Japanese Pharmacopoeia even lists both species in the same monograph, Leonuri Herba, probably being not aware of the substantial chemical differences. In the Japanese monograph, a TLC identification method is proposed, which is based on a non-defined compound, which is assumed to be stachydrine. Indeed, this betaine is part of the phytochemical fingerprint of LS as well as LJ and is therefore not suited for identity tests. Companies offering LJ material rely on specifications provided by the respective pharmacopoeia. If the requirements are too vague, potentially adulterated drug material is supplied to the end-consumer, which would explain deviating results in scientific studies.

Another alkaloid has been reported for *Leonurus* – leonurine. Hayashi (1962), and Reuter and Diehl (1971) independently isolated and described leonurine in LS. In their work, Reuter and Diehl mention that the used plant material was obtained from Vietnam, where it is named Ich-mao-thao or yakumoso in Japanese. However, when translated correctly, this name means *Leonurus japonicus* (Bensky et al., 2004). Thus, the true identity of the plant investigated by Reuter and Diehl remains doubtful and may be in fact LJ. Since then, many studies and books (Rätsch, 1998; WHO, 2013) cited Hayashi's and Reuter's discovery of leonurine in LS. We describe for the first time the absence of leonurine in LS, based on analyses of three independent samples, undermining the hypothesis that Reuter and Diehl may have worked with *Leonurus japonicus*, which contains leonurine. If our results can be replicated with a larger set of LS samples, preferably originating from different geographical regions, the absence of leonurine in LS constitutes a distinctive parameter for the discrimination between LS and LJ. Leonurine has also been described as secondary metabolite of LC. Recently it has been suggested that these reports are untrue (Monschein et al., 2014; Narukawa et al., 2014), which is further strengthened by concurring results in our study. In none of the four investigated LC samples was leonurine to be detected. Our outlined leonurine and stachydrine detection method using modified Dragendorff reagent on HPTLC plates is described for the first time, thereby providing a quick, fairly sensitive and cost

effective differentiation procedure between LJ and LS or LC, which is well suited to be adapted in any pharmacopoeia.

Further chemical differences of LJ, LS and LC lie in their polyphenolic fingerprint. As has been previously suggested by Monschein et al. (2014), the pattern of flavonoids, phenylpropanoids and chlorogenic acid derivatives in LJ differs greatly from that of LC, making a confusion very improbable. LS and LC, however resemble each other closely in their chemistry. For the differentiation between those two *Leonurus* species, anatomical and morphological properties need to be considered. The most striking difference lies in the form and length of the flower's lower lip. This is not only true for the distinction between LS and LC, but also for that of LJ. The shape of all three species' lower lip is sufficiently specific to serve as discriminative character when the wild plant is collected in the field. If, however, the plants are obtained from drug companies, the material is usually cut and morphological details may not be recognizable. In this case, anatomic characteristics need to be examined. For that scenario, non-glandular multicellular uniseriate trichomes situated outside on the upper corolla lips' of LC and LS represent a suitable feature (see Figure 3), since LJ does not possess them at all. Between LS and LC they vary in length. The same is true for the pilosity of the ovaries: LC's trichomes are longer than LS's but missing in LJ (see Figure 5). Trichomes of different kinds are also to be found on both leaf sides. The length and density ratio in between the species, but also between upper and lower side of the leaf are further characteristics on which identification can be based on.

To conclude, we show that the anatomy and the chemical composition of LS deviate from that of LJ and are characteristic for the respective species, supporting recognition as separate species, as previously proposed (Harley and Paton, 2001; Krestovskaja, 1992). The described morphological features of the flowers allow for quick and easy macromicroscopic identification, whereas anatomical and chemical characteristics are applicable in laboratory quality control. Availability of these characters are a requirement for proper usage of either species in TCM and TMM. Additionally, the presented anatomical and chemical differences between LJ and LC may serve for development of a monograph of LJ in the European Pharmacopoeia.

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Fig. 1. Length (A) and density (B) of trichome type 1 (upper leaf side) and trichome type 2 (lower leaf side) of *Leonurus cardiaca* (LC), *Leonurus japonicus* (LJ) and *Leonurus sibiricus* (LS). For sample sizes see Table 3. Density was assessed by counting the trichomes on a 100 cm² area using a drawing tubus. Bars represent mean \pm SD, two-way ANOVA, Bonferroni's post-test. Different superscript letters represent statistically significant ($p < 0.05$) differences between the respective data.

Fig. 2. Macroscopic view of corollae of *Leonurus cardiaca* (LC), *Leonurus sibiricus* (LS) and *Leonurus japonicus* (LJ).

Fig. 3. Length of trichome type 5 on the outside of the corolla's upper lip of *Leonurus cardiaca* (LC), *Leonurus japonicus* (LJ) and *Leonurus sibiricus* (LS). For sample sizes see Table 3. Unpaired t-test, *** $p < 0.001$.

Fig. 4. Length of trichome type 3 on the corolla's inside of *Leonurus cardiaca* (LC), *Leonurus japonicus* (LJ) and *Leonurus sibiricus* (LS). For sample sizes see Table 3. One-way ANOVA, Bonferroni's post-test, *** $p < 0.001$

Fig. 5. Length of trichome type 8 on the ovaries of *Leonurus cardiaca* (LC), *Leonurus japonicus* (LJ) and *Leonurus sibiricus* (LS). For sample sizes see Table 3. Unpaired t-test, *** $p < 0.001$

Fig. 6. HPTLC analyses of four *Leonurus cardiaca* samples (LC1-LC4), five *Leonurus japonicus* samples (LC1-LC5) and three *Leonurus sibiricus* samples (LS1-LS3, see Table 2). Plates derivatized with natural product reagent are shown under UV=366 nm (A), modified with Dragendorff reagent at daylight (B) and derivatized with anisaldehyde-sulfuric acid reagent under UV=366 nm (C). Verb=verbascoside, St/Leo=stachydrine and leonurine, ach/aju=acetylharpagide and ajugoside.

Untangling the *Leonurus* confusion – microscopic and phytochemical differentiation of *L. sibiricus* L., *L. japonicus* HOUTT. and *L. cardiaca* L.

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Supplementary information

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1. Anatomical description of LS and LJ based on analyses performed within this study

The following anatomic description applies to both LS and LJ. Species-specific characters are treated in the main article and only referenced here. Length and width are given as mean \pm SD.

1.1 *Leaves*

bifacial; epidermis and cuticula of the lower surface thicker than on the upper one (Fig. S1a), epidermis cells generally anticlinally wavy; hypostomatic, stomata anomocytic (Fig. S1b); pubescent at both surfaces: mainly on the veins at the lower surface, but intercostally at the upper surface, non-glandular trichomes one or two celled, tapered and thick walled, with warty cuticula (Fig. S1a), bent on veins (for species-specific quantitative differences see main article); glandular trichomes with unicellular stalk and four-celled head on both leaf surfaces, $22 \pm 2 \mu\text{m}$ in diameter (Fig. S1a and Fig. S1b), glandular scales $40 \pm 5 \mu\text{m}$ in diameter, composed of 8-12 secreting cells, found on the lower surface only; with solitary crystals or crystal needles dispersed in the mesophyll (Fig. S1a).

1.2 *Corolla*

upper and lower lip are the same length as the tube; upper lip entire and helmet-shaped; lower lip with three lobes (see main article for species-specific shapes); four and three kinds of trichomes on the outside of the upper lip in LS and LJ, respectively. Type 1: one- to two celled, wound, non-glandular trichome, $500 \pm 170 \mu\text{m}$ in length (Fig. S2a). Type 2 (only on LS): multicellular, uniseriate non-glandular trichome, $933 \pm 192 \mu\text{m}$ in length (Fig. S2b), about twice as wide as type 1. Type 3: glandular trichome with unicellular stalk and four-celled head. Type 4: glandular scale with 8-12 secreting cells; inside of the tube a hairy annulus of densely arranged, unicellular, straight trichomes situated ca. 5 mm above its basis (Fig. S2c) (see main text for species-specific differences in trichome length); lower lip numerous glandular trichomes and short papillae, papillae become elongated towards the throat where they form ca. $80 \pm 30 \mu\text{m}$ long trichomes (Fig. S2d); solitary crystals, druses or crystal needles in every epidermis cell and sometimes within trichomes (Fig. S2e); epidermis cells elongated.

1.3 *Androecium*

stamen with three types of trichomes, lower third of the filament with unicellular trichomes rounded top, upwards followed by very thin-walled trichomes, collapsing, twisting and tearing easily (Fig. S3); occasionally, glandular trichome type of corolla and leaves are found

on the filament and the anthers, anthers' surface well-appointed with glandular scales of corolla type.

1.4 Gynoecium

Ovary of LS pubescent with non-glandular trichomes, measuring $207 \pm 68 \mu\text{m}$ in length, glabrous in LJ (see also main article), with glandular trichomes and glandular scales of the type described for leaves and corolla; style $206 \pm 30 \mu\text{m}$ in width; stigma with two flanks, $580 \pm 150 \mu\text{m}$ long and $170 \pm 41 \mu\text{m}$ wide (measured on the basis (Fig. S4)).

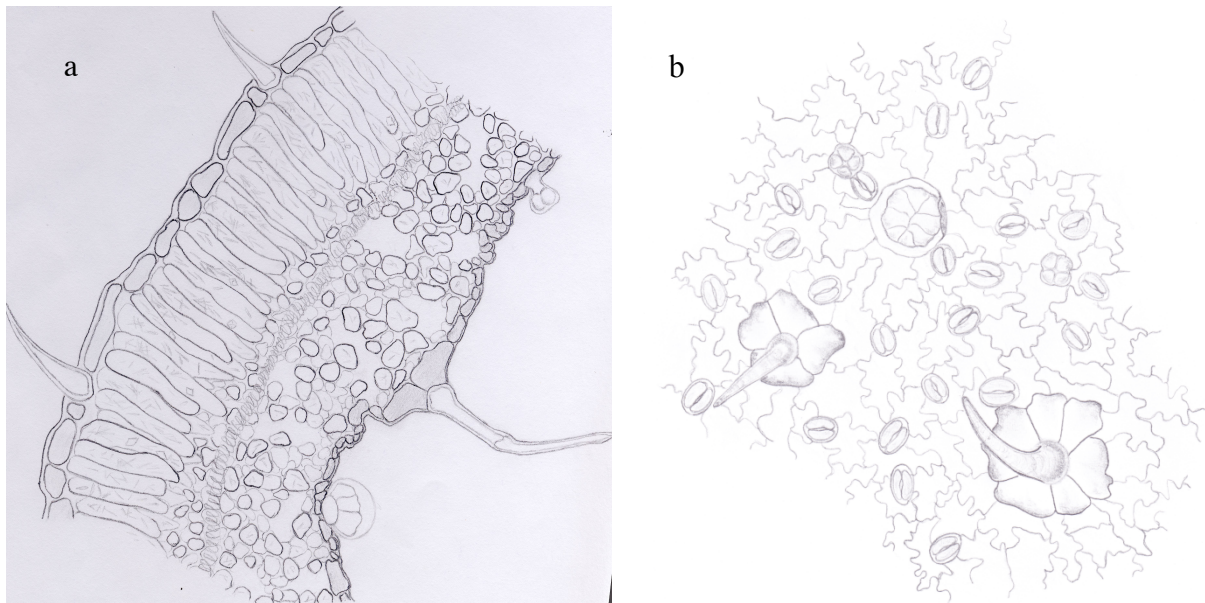


Figure S1. Cross section (a) and surface view (b) of LJ leaf.

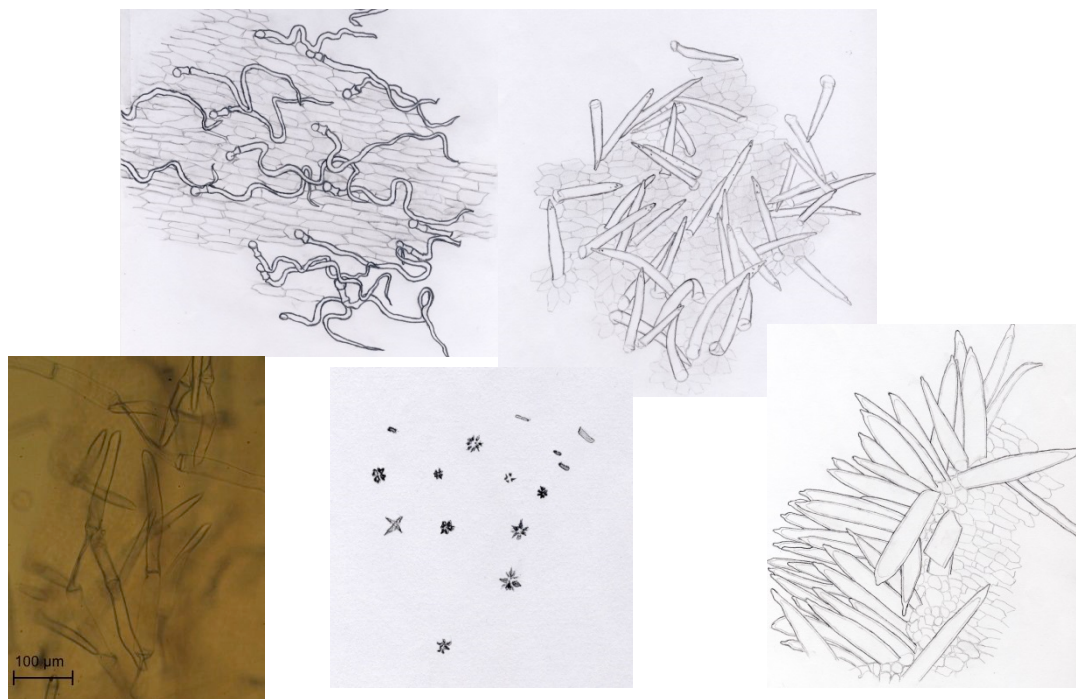


Figure S2. Different non-glandular trichomes (a-d) and crystals (e) of LS and LJ. Trichomes of panel (b) are only found in LS.

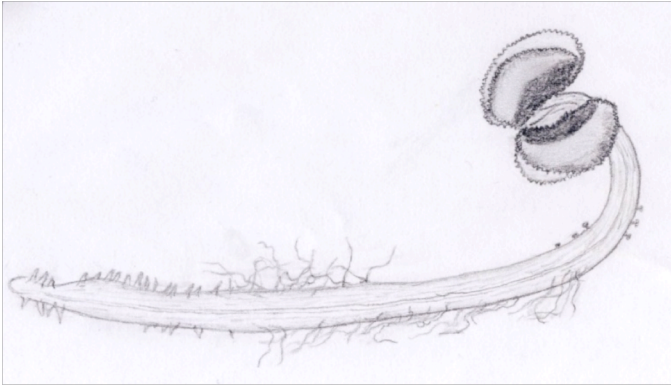


Figure S3. Stamen of LJ.



Figure S4. Part of style and stigma of LJ.

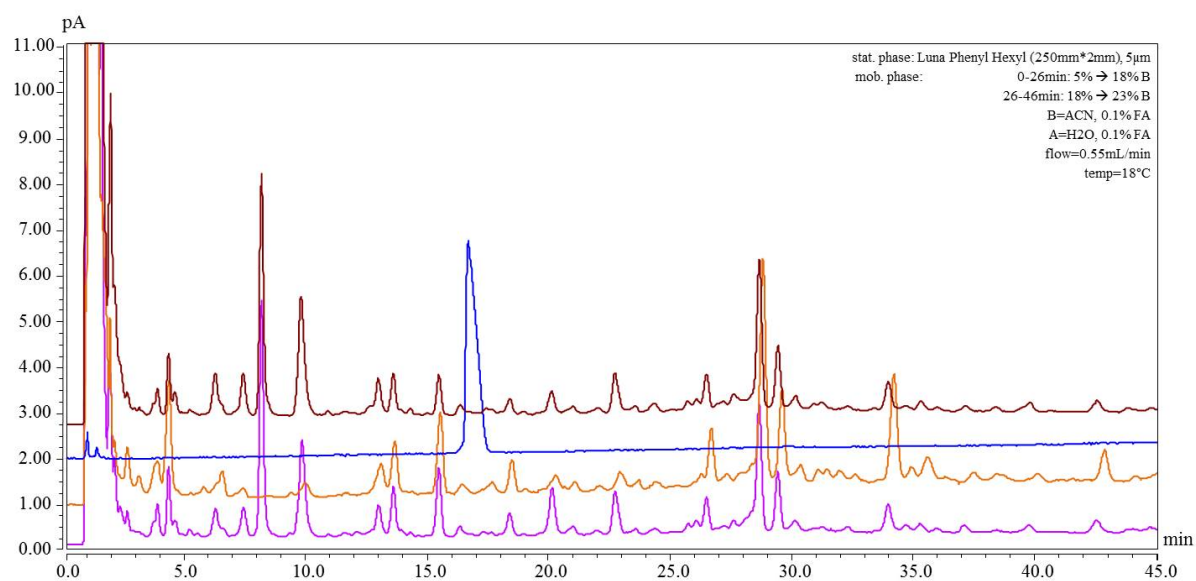


Figure S5. Comparison of HPLC chromatograms: LS2 (pink), LS3 (orange), leonurine (blue), LS1 (brown).

4.4 *J. regia*: *in vitro* PTP1B inhibition and increase of glucose-uptake

4.4.1 Preparatory work

As introduced in chapter 3.1.2 (page 34), at the beginning of this investigation stood the screening for the potential glucose-uptake enhancing activity of six plants, which are used against diabetes mellitus in Austrian folk medicine. These plants were *Arctium lappa* L. (AL), *Equisetum arvense* L. (EA), *Juglans regia* L. (JR), *Plantago lanceolata* L. (PL), *Ribes nigrum* L. (RN) and *Sambucus nigra* L. (SN).

For this purpose, the plants were extracted (see chapter 3.2.2, page 37) and chlorophyll-containing extracts were purified from the same (see Figure 3.3, page 37) to avoid false positive results in the bioassays (Picker et al., 2014). The effectiveness of chlorophyll depletion was verified by HPTLC using derivatization reagent 3 (see Table 3.2, page 39). Chlorophyll shows an intense red fluorescing band at $R_f=1$ under UV=366 nm. Absence of this band was considered evidence of successful chlorophyll separation. Chlorophyll-depleted extracts are denominated with the additive "wC" (without chlorophyll), e.g. JRwC. Figure 4.6 (page 120) shows chromatograms by HPTLC of extracts of EA (4.6a), JR (4.6b) and PL (4.6c). The first lane of each chromatogram shows the crude methanol extract, the second lane the extract depleted from chlorophyll and the third lane the separated chlorophyll. As evident of the missing red zone at $R_f=1$ under UV=366 nm in the respective second lane, the chlorophyll depletion process was effective in all three cases.

Obtained chlorophyll-depleted extracts (EAwC, JRwC, PLwC) and methanol extracts of AL, RN, SN were tested on the glucose-uptake influencing effect in C2C12 myocytes (see page 78 f) and PTP1B inhibition (see page 79). Panel (a) in Figure 4.7 (page 121), shows the basal (white bars) and insulin-stimulated (black bars) glucose-uptake of the mentioned extracts at concentrations of 25 $\mu\text{g}/\text{mL}$ compared to negative control (=solvent DMSO). Out of the six tested extracts, only *J. regia* significantly increased the basal as well as the insulin-stimulated glucose-uptake.

For the PTP1B inhibition assay (panel (b) in Figure 4.7, page 121), extracts were tested

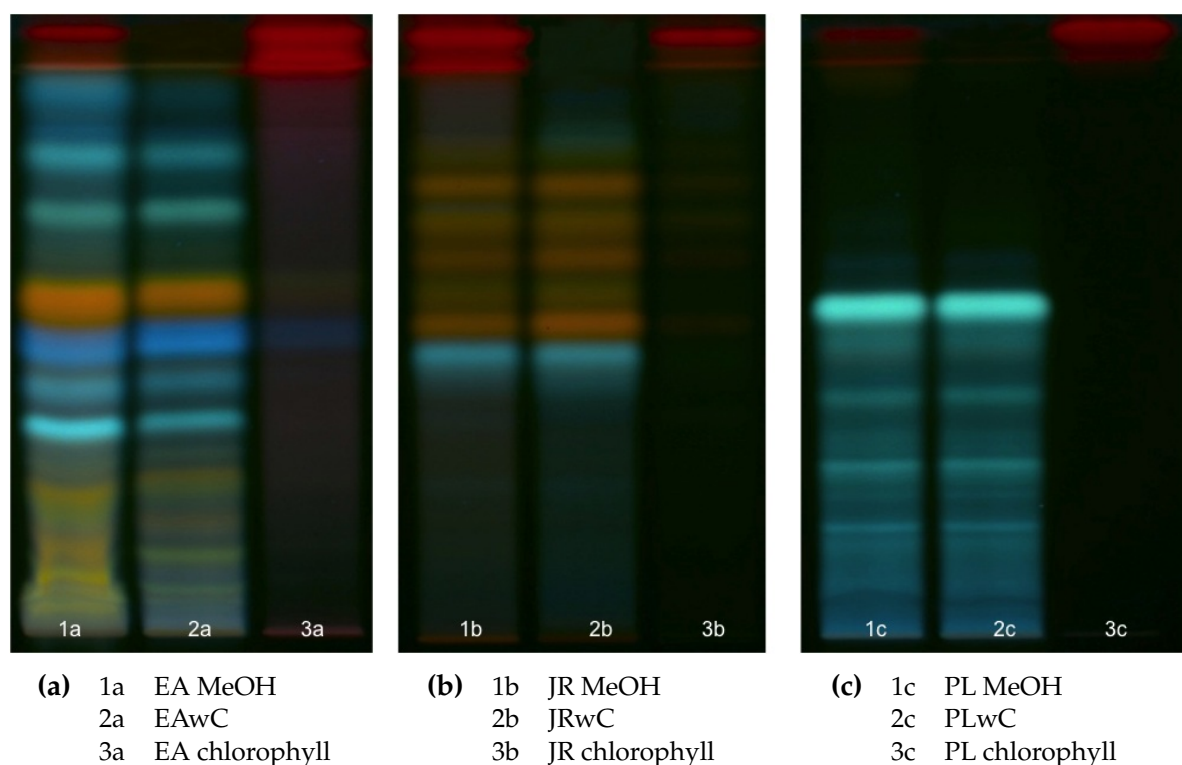
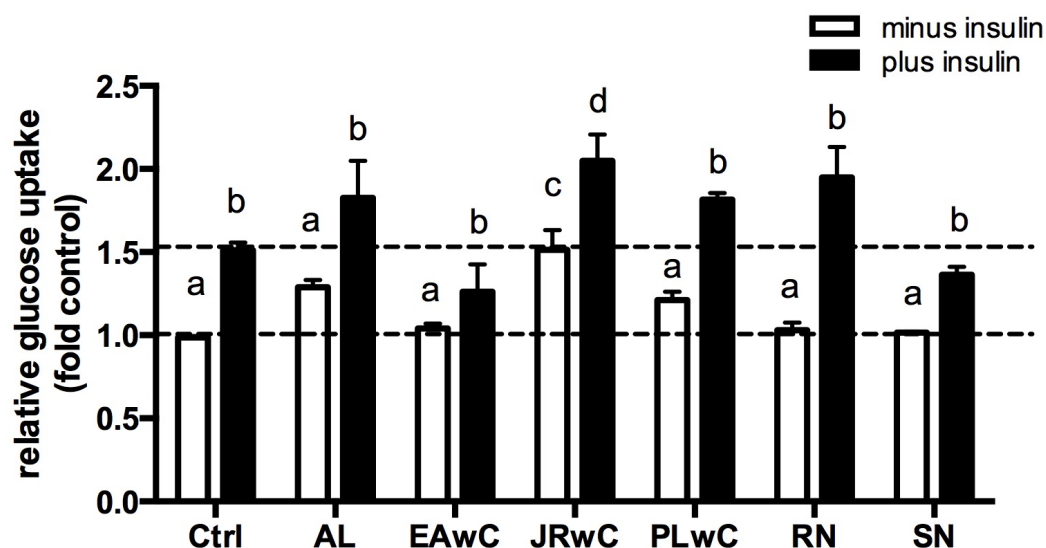
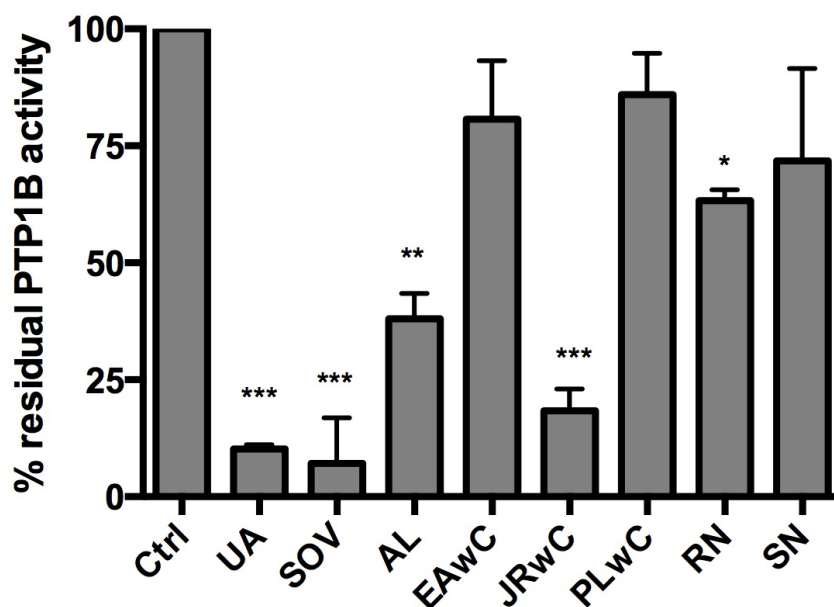


Figure 4.6.: Analyses of chlorophyll depletion of *Equisetum arvense* (EA) (a), *Juglans regia* (JR) (b) and *Plantago lanceolata* (PL) (c) extracts by HPTLC and derivatization reagent 3 (see Table 3.2, page 39). wC=without chlorophyll

at concentrations of 25 $\mu\text{g/mL}$. Ursolic acid 30 μM (UA) and sodium orthovanadate 10 μM (SOV) served as positive control. Compared to the negative control DMSO, JR inhibited PTP1B most potently, followed by AL and RN. The other three plants did not show PTP1B inhibitory activity. In these first two screenings of the six Austrian plants, JR achieved best activity, which prompted its further investigation.



(a) Glucose-uptake assay (see page 78 f) of methanol extracts at a concentration of 25 $\mu\text{g}/\text{mL}$. Negative control=DMSO (Ctrl). wC=without chlorophyll. Different superscript letters represent statistically significant ($p < 0.05$) differences, two way ANOVA/Bonferroni



(b) PTP1B inhibition (see page 79) of extracts at concentrations of 25 $\mu\text{g}/\text{mL}$. Negative control=DMSO (Ctrl), positive control=ursolic acid 30 μM (UA) and sodium orthovanadate 10 μM (SOV). wC=without chlorophyll. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, one way ANOVA/Bonferroni

Figure 4.7.: *In vitro* activity of AL (=Arctium lappa), EA (=Equisetum arvense), JR (=Juglans regia), PL (=Plantago lanceolata), RN (=Ribes nigrum) and SN (=Sambucus nigra) extracts (see section 3.2.2, page 37). (a) Glucose-uptake assay in C2C12 myocytes, (b) Colorimetric PTP1B inhibition enzyme assay. Bars represent mean \pm SD. Experiments were performed by Elke Heiss, Department of Pharmacognosy, University of Vienna

EFFECT OF TANNIN SEPARATION

It is known from literature, that tannins may increase the glucose-uptake rate (Muthusamy et al., 2008). To ensure that tannins present in JR do not mask the activity of other constituents, they were removed from JRwC by the tannin separation method introduced in Figure 3.4 (page 38). Since tannins may be visualized on TLC using anisaldehyde as derivatization reagent (Wagner et al., 1983), the success of the tannin separation procedure was verified by TLC using derivatization reagent 2 (see table 3.2, page 39). Figure 4.8 (page 122) shows the detannified JR extract (JRwCT) on TLC in lane 2 in comparison to the not-detannified extract (JRwC) in lane 1. It is apparent that polar substances ($R_f < 0.3$), which form green or brown bands with anisaldehyde reagent, got depleted in course of the tannin separation procedure.



Figure 4.8.: Analysis of the effectiveness of detannification procedure of JRwC by TLC and derivatization reagent 2 (see Table 3.2, page 39). Lane 1=JRwC, lane 2=JRwCT

HPLC analyses on RP18 corroborated this finding: chromatograms of JRwC and JRwCT, detected at UV=300nm, a wavelength where polyphenols are typically detected, differed from each other by the intensity of peaks eluting at high amounts of water in the mobile phase. Peaks before minute 23 (see Figure 4.9, page 123) got significantly depleted by the tannin separation procedure. The rest of the chromatogram (after minute 23) remained mainly unchanged.

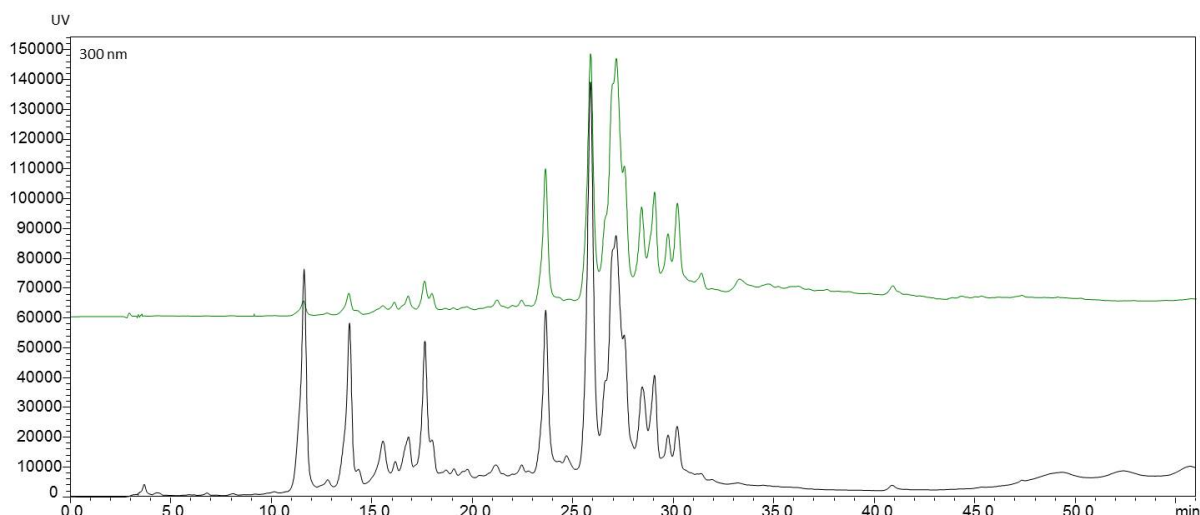


Figure 4.9.: Analyses of JRwC (black) and JRwCT (green) by HPLC method 3 (Table 3.4, page 41) at 300 nm

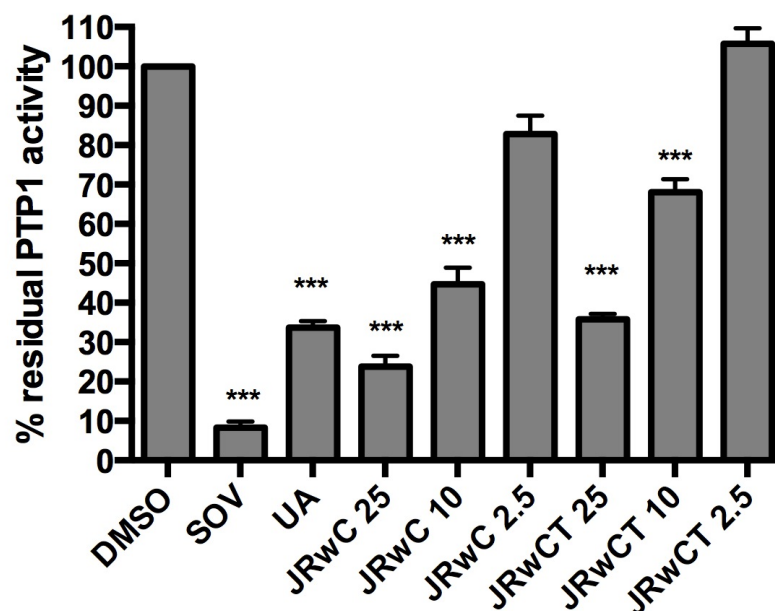


Figure 4.10.: PTP1B inhibition (see page 79) of JRwC and JRwCT at concentrations of 2.5, 10 and 25 $\mu\text{g/mL}$. Negative control=DMSO, positive control=ursolic acid 30 μM (UA) and sodium orthovanadate 10 μM (SOV). wC=without chlorophyll, wCT=without chlorophyll and tannins. *** $p < 0.001$, one way ANOVA/Bonferroni. Experiment was performed by Dr. Elke Heiss, Department of Pharmacognosy, University of Vienna

The detannified extract was, together with its mother fraction JRwC, tested in a concentration range of 2.5 to 25 $\mu\text{g/mL}$ on its PTP1B inhibitory potential. PTP1B was inhibited by JRwCT dose dependently (see Figure 4.10, page 123). In comparison to the not detannified extract, JRwC, however, JRwCT was less active in all three concen-

trations. Compounds depleted by the detannification process therefore contribute to the PTP1B inhibition effect observed in JRwC, but are not the sole active principle.

4.4.2 Published paper

Dereplication of JRwC and JRwCT resulted in the identification of several flavonoids, quinic acid derivatives and a trihydroxynaphthalene-hexoside. The influence of these compounds on PTP1B has been described in earlier studies (Zhang et al., 2010a; An et al., 2003). Bioactivity-guided fractionation was therefore not performed. The glucose-uptake enhancing and PTP1B inhibiting potential of the whole JR extract, alongside its chemical characterization is published in the "Journal of Ethnopharmacology". The paper and supplementary information are provided in the following pages.

The wording of methods described in the following paper may resemble publications of co-authors, where similar methods were applied.



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Ethnopharmacological communication

Walnut leaf extract inhibits PTP1B and enhances glucose-uptake *in vitro*

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ABSTRACT

Ethnopharmacological relevance: Walnut, *Juglans regia* L. (Juglandaceae), is one of the medicinal plants used to treat diabetic symptoms in Austrian folk medicine. The air-dried green leaves are either used as aqueous decoctions or liquor preparations and are consumed on a daily basis. We investigated the hypoglycemic effect of a methanolic *Juglans regia* leaf extract on glucose uptake, protein tyrosine phosphatase 1B (PTP1B) inhibition and peroxisome proliferator-activated receptor gamma (PPAR γ) activation.

Material and methods: Hypoglycemic activity was assessed by glucose-uptake in C2C12 myocytes, inhibition of PTP1B and activation of PPAR γ . Phytochemical characterization of the extract was carried out by LC–MS and GC–MS.

Results: Methanolic *Juglans regia* leaf extract enhanced the glucose uptake rate in C2C12 myocytes at concentrations of 25 μ g/mL compared to untreated cells. This activity may partly be explained by the inhibition of PTP1B but not PPAR γ agonism. LC–MS analyses revealed chlorogenic acid (**1**), 3-p-coumaroylquinic acid (**2**), a trihydroxynaphthalene-hexoside (**3**), as well as eight flavonoids (**4–11**) as main phenolic constituents in the active extract.

Conclusions: The finding that *Juglans regia* leaf extract enhances glucose uptake and inhibits PTP1B provides an *in vitro*-based rationale for the traditional use of walnut leaf preparations against elevated blood-glucose levels.

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1. Introduction

Diabetes mellitus is a metabolic disorder characterized by high blood glucose levels (hyperglycemia) resulting from defects in the body's ability to produce or respond to insulin. Long-term hyperglycemia is associated with severe micro- and macrovascular damage, whose prevention is the main aim of hypoglycemic pharmacotherapy (Fowler, 2007). Due to – partly counterproductive – side effects of current clinical treatment and the continuously rising number of patients (Wild et al., 2004), new, more efficient therapeutic principles are needed. Inhibitors of protein-tyrosine phosphatase 1B (PTP1B), a cytosolic enzyme, not only increase cellular response to insulin, but also elevate leptin signaling and are therefore a promising strategy for the treatment of diabetes mellitus and obesity (Thareja et al., 2012).

In search of new lead compounds, traditional medicines proved to constitute a valuable source (Patwardhan and Mashelkar, 2009). In this regard, *Juglans regia* L. (Juglandaceae), the common walnut, represents an interesting candidate for scientific investigations, since it is used in different traditional medicines for a variety of indications (Taha and Al-wadaan, 2011). In Austrian folk medicine, water decoctions and liquor preparations of the foliage are known as traditional remedy against diabetic symptoms (Gerlach, 2007). The positive effect of *Juglandis folium* (walnut leaf) extracts on blood glucose levels and lipid profiles has been scientifically proven by numerous animal studies (Asgary et al., 2008; Mohammadi et al., 2011) as well as by a recent human clinical trial (Hosseini et al., 2014). The activity was attributed to the antioxidant capacity of the polyphenols present in walnut leaves (Amaral et al., 2004; Pereira et al., 2007). The mechanism of action, however, remained speculative. The present study aims to fill this gap by evaluating the influence of *Juglans regia* leaf extract in *in vitro* functional (glucose uptake) and target-oriented (PTP1B and PPAR γ) models related to insulin sensitivity. Phytochemical characterization ensures similar qualitative extract composition as in previous studies.

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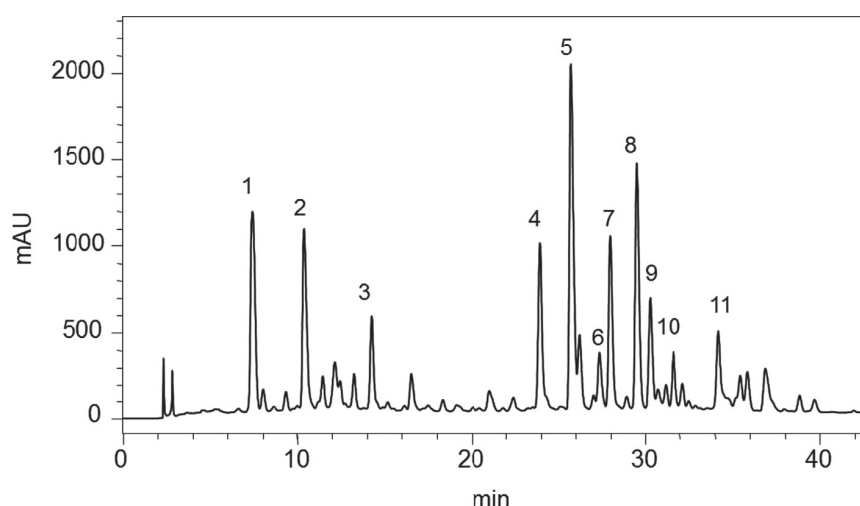
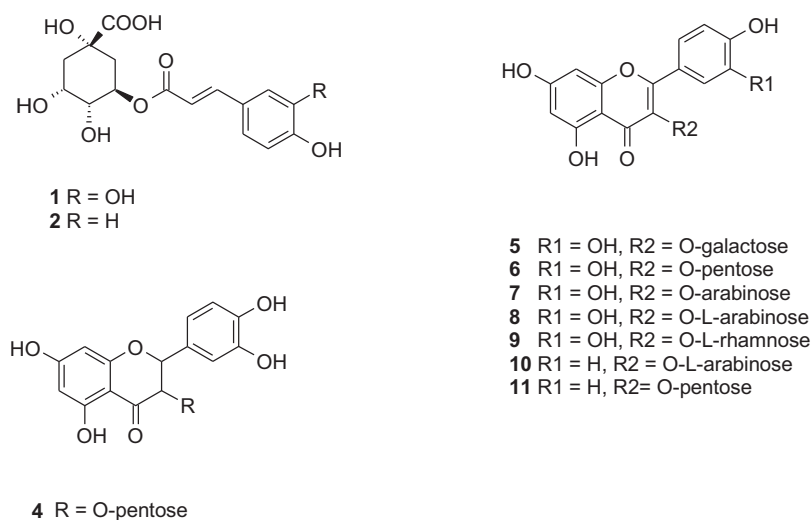


Fig. 1. HPLC chromatogram (300 nm) of JR: (1) chlorogenic acid, (2) 3-p-coumaroylquinic acid, (3) trihydroxynaphthalene-hexoside, (4) dihydroquercetin-pentoside, (5) hyperoside, (6) quercetin-pentoside, (7) avicularin, (8) quercetin-L-arabinoside, (9) quercetin-L-rhamnoside, (10) kaempferol-L-arabinoside, and (11) kaempferol-pentoside.

2. Material and methods

2.1. Chemicals

(S)-(+)-2-butanol with a purity of 99% was purchased from Sigma Aldrich (St. Louis, MO, USA). Reference substances for GC and HPLC were of HPLC quality and purchased from Carl Roth (Karlsruhe, Germany). Solvents used for extraction were of analytical grade; those used for HPLC were of gradient grade and obtained from VWR (West Chester, PA, USA).

2.2. Plant material

Dried, cut *Juglans regia* leaf material was obtained from Kottas Pharma GmbH (Vienna, Austria). The lot number of the material was 10240212. Prior to extraction, the leaves were pulverized with a basic electric grinder and stored at room temperature, protected from light.

2.3. Extraction and extract-purification

Pulverized *Juglans regia* leaves were extracted three times with methanol, using an ASE 200 system from Dionex (Sunnyvale, CA, USA). Extractions were carried out at 40 °C under a pressure of 10 MPa with an acetone tube cleaning step between every cycle. Resulting liquid extracts were combined and evaporated to dryness under reduced pressure at 40 °C. The drug to extract ratio was 4–6:1.

For chlorophyll depletion, the crude extract was re-dissolved in CH₂Cl₂ and partitioned with equal volumes of H₂O/MeOH (1:1). The upper layer was separated and evaporated to dryness to yield a chlorophyll free extract (JR).

2.4. Cell culture, assessment of glucose uptake rate and PTP1B inhibition assay

Settings were used as previously in (Heiss et al., 2012) and can also be found in the supplementary information.

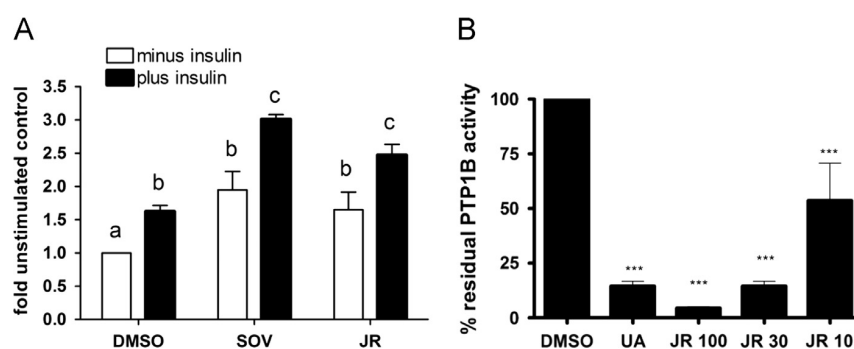


Fig. 2. *In vitro* activity of JR on glucose uptake (A) and PTP1B (B). A Basal and insulin (100 nM)-stimulated ^3H -deoxyglucose uptake by C2C12 myocytes was assessed after incubation of cells with solvent or 25 $\mu\text{g}/\text{mL}$ JR for 48 h; SOV=sodium orthovanadate (10 μM), positive control; DMSO=negative control; B JR was tested at 100 $\mu\text{g}/\text{mL}$, 30 $\mu\text{g}/\text{mL}$ and 10 $\mu\text{g}/\text{mL}$ for its PTP1B inhibitory potential in a colorimetric *in vitro* enzyme assay; UA=ursolic acid (10 μM), positive control; DMSO=negative control; statistics: bars represent mean \pm SD, $n=3$, one-way ANOVA, Bonferroni's post-test; A: different superscript letters represent statistically significant ($p < 0.05$) differences between the respective data; B: *** $p < 0.001$.

2.5. HPLC–DAD and HPLC–DAD–MSⁿ analyses

Qualitative HPLC–DAD analyses of the extract were conducted on a Prominence LC-20AD instrument coupled to a Prominence SPD-M20 Diode Array Detector (Shimadzu, Kyoto, Japan). The wavelength was set to 300 nm for detection. The data were analyzed by the software LabSolutions version 1.25 (Shimadzu). As stationary phase served a Luna C18 column (4.0 \times 250 mm², 5 μm ; Phenomenex, Torrance, CA). Doubly distilled water adjusted to pH 2.8 with formic acid (A) and methanol (B) were used as mobile phase. Gradient elution started at 25% B and increased to 60% B in 40 min. 10 μL of the extract were injected, at a concentration of 30 mg/mL. The flow rate was 1 mL/min and the temperature was set to 25 $^{\circ}\text{C}$. Three flavonoids (see Fig. 1, compounds 8, 9 and 10) were isolated by the same HPLC method, only the concentration of the injected sample was raised to double the amount.

Tentative identification of the main constituents was facilitated by HPLC–DAD–MS. Settings were similar as in (Zehl et al., 2011) and are given in detail in the supplementary information.

2.6. Statistical methods and data analysis

All values are expressed as the means \pm SD. Statistical analyses were performed with GraphPad Prism software version 4.03. One-way and two-way ANOVA with Bonferroni's post hoc test were used to determine statistical significance for PTP1B, PPAR γ and glucose uptake assay, respectively. Data groups with $p < 0.05$ were considered as statistically significant.

3. Results

3.1. HPLC-fingerprint of walnut leaf extract comprises several phenolic compounds

Qualitative analysis of the JR methanol extract by HPLC shows the presence of numerous phenolic compounds (see Fig. 1). The identification of the eleven main constituents in JR was based on their LC–MSⁿ spectra. That way, five compounds were tentatively assigned to chlorogenic acid (1), trihydroxynaphthalene-*O*-hexoside (3), dihydroquercetin-*O*-pentoside (4), quercetin-*O*-pentoside (6), and kaempferol-*O*-pentoside (11). The presence of hyperoside (quercetin 3- β -galactoside) (5) and avicularin (quercetin 3- α -*L*-arabinofuranoside) (7) was further verified by comparison with reference compounds, and compound 2 was assigned as 3-*p*-coumaroylquinic acid by comparison with literature data (Plazonic et al., 2009). Finally, the

flavonoids 8–10 were isolated and their sugar moiety, after hydrolysis and derivatization, identified by GC–MS. Thereby, these compounds were identified as quercetin-*O*-*L*-arabinoside (8), quercetin-*O*-*L*-rhamnoside (9), and kaempferol-*O*-*L*-arabinoside (10). This qualitative composition is in line with literature data (Amaral et al., 2004), except that compounds 4 and 10 are described for the first time in *Juglans regia* leaves. Specific data about compound identification can be found in the supplementary information.

3.2. Walnut leaf extract shows hypoglycemic and insulin-sensitizing activity *in vitro*

To investigate a potential anti-hyperglycemic effect of JR *in vitro*, we performed a glucose uptake assay in C2C12 myotubes. At a concentration of 25 $\mu\text{g}/\text{mL}$, JR increased both the basal and the insulin-stimulated glucose uptake rate up to 1.5 fold compared to control cells (see Fig. 2A).

Next, we determined the effect of JR on PTP1B and PPAR γ , two proteins known to boost glucose uptake and insulin signaling. JR appeared to be a potent PTP1B inhibitor. In a concentration range between 10 and 100 $\mu\text{g}/\text{mL}$, JR inhibited PTP1B in a concentration-dependent manner. DMSO served as negative, ursolic acid (10 μM , UA) as positive control (see Fig. 2B). Activation of PPAR γ could not be achieved at 10, 30 and 100 $\mu\text{g}/\text{mL}$ of JR (see Fig. S1). This suggests that the observed enhanced glucose uptake in our cell-based assays and effects in *in vivo* models performed by others upon exposure to JR may be – at least partly – caused by inhibition of PTP1B, but most likely not by PPAR γ agonism.

4. Discussion

Based on ethnopharmacologic reports and previous *in vivo* data, we attempted to achieve first mechanistic hints of the hypoglycemic activity of *Juglans regia* leaves by using *in vitro* assays and to chemically characterize the used extract. We showed that a methanolic *Juglans regia* leaf extract increases the glucose uptake of myocytes *in vitro*. One possible target, PTP1B, was inhibited, whereas another potential target, PPAR γ , was not affected by the plant extract. HPLC analyses of this extract confirm the presence of several flavonoids, chlorogenic acid derivatives and a trihydroxynaphthalene-*O*-hexoside, as stated before by others. To the best of our knowledge, dihydroquercetin-pentoside and kaempferol-pentoside are being described for the first time in the flavonoid-pattern of this species. Of note and in line with our findings, similar flavonoids and naphthoquinones originating from different plants also elicited inhibition of PTP1B in previous reports (Ahn et al., 2002; Islam et al., 2013).

In conclusion, our results provide an *in vitro*-based rationale for the traditional use of walnut leaf preparations against elevated blood-glucose levels. Application of accordant extracts to patients might be useful in the management of diabetes mellitus due to inhibition of PTP1B.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.jep.2014.02.017>.

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Walnut leaf extract inhibits PTP1B and enhances glucose uptake *in vitro*

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Supplementary Information

Files in this data supplement

Material and Methods: Cell culture, assessment of glucose uptake rate, *in vitro* enzyme assay, PPAR reporter gene assay, HPLC-MSⁿ analyses, Determination of sugar moieties,

Table S1

Figure S1

Figure S2

Material and Methods

Cell culture

C2C12 myoblasts (ATCC) were maintained under sub-confluent conditions in DMEM medium supplemented with 10% FBS, glutamine, and penicillin/streptomycin under 5% CO₂ environment at 37 °C. For differentiation into myotubes, cells (passage number <15) were seeded in 24-well plates (assessment of glucose uptake) and grown until confluency. Differentiation was then initiated by changing to DMEM supplemented with 2% horse serum. After 96 hours, cells were considered fully differentiated and used for experiments within 2 days.

Assessment of the Glucose Uptake Rate

C2C12 myotubes were treated with extracts for 48 hours before they were serum starved for 4 hours and stimulated with 100 nM insulin as indicated for 30 minutes in standard Krebs-Ringer HEPES buffer, pH 7.4. Then glucose uptake was assessed in the presence of 0.1 mM 2-deoxyglucose (DOG) spiked with 1 μ Ci/mL tritium-labelled 3 H-DOG (NEN, Austria) for 10 minutes at room temperature. Then, cells were rinsed with ice cold PBS and lysed with 0.05 N NaOH. Aliquots of the lysates were analysed in a Perkin Elmer TriCarb 2100 liquid scintillation counter and used for determination of the protein content (Bradford assay). The glucose uptake rate for each sample was determined as the amount of incorporated radiolabelled glucose per minute uptake time, normalized to the cellular protein content.

In vitro Enzyme Assay

PTP1B activity (human recombinant enzyme from R&D) was determined using 2 mM para-nitrophenylphosphate (pNPP) in 50 mM MOPS, pH 6.5, as a substrate. The known PTP inhibitors sodium orthovanadate (SOV, 10 μ M) and/or ursolic acid (UA, 10-30 μ M) were used as positive controls. Fractions were first dissolved in DMSO and diluted to a final concentration of 1% DMSO in 100 μ L overall assay volume. The inhibitory action towards PTP1B was measured in 96-well format in the presence of 1 mM dithiothreitol. The reactions were subjected to kinetic absorbance readings at 405 nm for 30 min in a Tecan/SUNRISETM photometer. Subsequently, the reaction was stopped with 10 M NaOH and the absorbance again measured at 405 nm.

PPAR γ luciferase reporter gene transactivation

HEK-293 cells (ATCC, USA) were cultured in DMEM with phenol red, supplemented with 100 U/mL benzylpenicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine, and 10% FBS. Cells were

grown in 75 cm² flasks containing 10 mL medium in cell culture incubators maintained at 37 °C and 5% CO₂. Before experiments, cells were seeded in 10 cm dishes at a density of 6×10^6 cells/dish, incubated for 18 h, and transfected by the calcium phosphate precipitation method (Graham and Van der Eb, 1973) with 4 µg of the luciferase reporter plasmid (tk-PPREx3-luc), 4 µg of PPAR γ expression plasmid, and 2 µg green fluorescent protein plasmid (pEGFP-N1, Clontech, Mountain View, CA) as internal control. After 6 h, the transfected cells were re-seeded in 96-well plates (5×10^4 cells/well) containing DMEM without phenol red, with 100 U/mL benzylpenicillin, 100 µg/mL streptomycin, 2 mM L-glutamine, and 5% charcoal-stripped FBS. Cells were treated as indicated (figure legends) for 18 h. At the end of the incubation period, cells were lysed with a reporter lysis buffer (E3971, Promega, Madison, USA). Luciferase activity was evaluated using a GeniosPro plate reader (Tecan, Grödig, Austria). The luminescence signals were normalized to the EGFP-derived fluorescence, to account for differences in the transfection efficiency or cell number.

LC-MS

Analyses were performed on an UltiMate 3000 RSLC-series system (Dionex) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). HPLC separation was carried out as described in the main text (2.8 HPLC-DAD and HPLC-DAD-MSⁿ analyses). The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: ± 3.7 kV, nebulizer: 26 psi (N₂), dry gas flow: 9 L/min (N₂), and dry temperature: 340 °C. Positive and negative ion mode multistage mass spectra up to MS⁴ were obtained in automated data-dependent acquisition (DDA) mode using helium as collision gas, an isolation window of 4 Th, and a fragmentation amplitude of 1.0 V. The aglycone part of all mentioned flavonoids was identified by spectrum matching to

an in-house library containing the MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics).

Determination of sugar moieties

To identify the glycosylation units and to determine their configuration, the isolated flavonoid glycosides **8-10** were hydrolysed for two hours with Kiliani reagent (a mixture of 5.5 mL H₂O, 3.5 mL of CH₃COOH_{conc.}, and 1 mL HCl_{conc.}) at 100 °C. The solution was partitioned with EtOAc three times. The aqueous phase, containing the monosaccharides, was evaporated to dryness. In order to obtain the corresponding diastereomeric butyl glycosides as acetals, (S)-(+)-2-butanol and HCl_{conc.} were added and the reaction mixture was kept at 100 °C for 15 hours. The solution was evaporated to dryness, the reaction product re-dissolved in pyridin and derivatized with hexamethyldisilacane and trimethylsilyl chloride. Before injection into the GC-MS, the solution was kept at 80 °C for 10 minutes. GC-MS analyses were performed on a QP2010 GC-MS (Shimadzu, Kyoto, Japan) using the following parameters:

GC: Phenomenex ZB-5 capillary column (65 m × 0.25 mm, film thickness 0.25 μm); carrier gas: He 5.0; flow rate: 2.17 mL/min; split ratio 1:10; temperature gradient: 100-270 °C at a rate of 3 °C/min; injector and interface temperature: 270 °C;

MS: ion source temperature: 250 °C; electron-impact ionization at 70eV, scan range: m/z 40 - 500. The monosaccharides were identified by comparison of retention times and mass spectra to authentic substances.

Table S1. Proposed structure, MS, and UV data of the secondary plant metabolites identified in the *Juglans regia* extract.

#	Proposed Structure	[M-H] ⁻	Main Fragment Ions (>10% Rel. Int.)	UV (λ _{max} in nm)	References
1	Chlorogenic acid	353.0	MS ² [352.7]: 190.7 (100), 178.7 (48), 134.8 (11) MS ³ [178.7]: 134.8 (100)	204, 325	(Amaral et al., 2004)
2	3- <i>p</i> -Coumaroylquinic acid	337.0	MS ² [336.7]: 162.7 (100) MS ³ [162.7]: 118.9 (100)	196, 312	(Amaral et al., 2004) (Plazonic et al., 2009)
3	Trihydroxynaphthalene- <i>O</i> -hexoside	337.0	MS ² [336.8]: 174.7 (100) MS ³ [174.7]: 93.3 (100)	196, 215, 306, 325, 340	-
4	Dihydroquercetin- <i>O</i> -pentoside ^a	435.0	MS ² [434.9]: 302.7 (85), 284.7 (100) MS ³ [302.7]: 284.7 (100), 124.9 (12) MS ³ [284.7]: 256.6 (13), 242.7 (13), 240.7 (100), 216.7 (27), 198.7 (37), 174.7 (89)	216, 291	PMC-link_out
5	Hyperoside ^{a,b} (quercetin 3-β-galactoside)	463.0	MS ² [462.9]: 300.6 (100), 299.7 (28) MS ³ [300.6]: 270.6 (100), 254.6 (79), 178.6 (59), 150.7 (56)	271, 365	(Amaral et al., 2004)
6	Quercetin- <i>O</i> -pentoside ^a	433.0	MS ² [432.8]: 300.6 (100), 299.7 (25) MS ³ [300.6]: 272.7 (12), 270.6 (80), 254.6 (60), 178.6 (100), 150.7 (83), 107.0 (20)	256, 355	(Amaral et al., 2004)
7	Avicularin ^{a,b} (quercetin 3-α-L-arabinofuranoside)	433.0	MS ² [432.8]: 300.6 (99), 299.7 (100) MS ³ [299.7]: 270.6 (100), 254.6 (56), 178.7 (16), 150.7 (18)	223, 254, 352	(Amaral et al., 2004)
8	Quercetin- <i>O</i> -L-arabinoside ^a	433.0	MS ² [432.8]: 300.7 (100), 299.7 (18) MS ³ [300.7]: 272.7 (15), 270.6 (45), 254.6 (41), 228.7 (12), 178.6 (100), 150.7 (93), 107.0 (14)	230, 260, 347	(Amaral et al., 2004)
9	Quercetin- <i>O</i> -L-rhamnoside ^a	447.0	MS ² [446.9]: 300.6 (100), 299.7 (36) MS ³ [300.6]: 272.7 (12), 270.6 (100), 254.7 (60), 178.6 (64), 150.7 (67)	255, 348	(Amaral et al., 2004)
10	Kaempferol- <i>O</i> -L-arabinoside ^a	417.0	MS ² [416.8]: 284.6 (28), 283.7 (100), 254.7 (45) MS ³ [283.7]: 255.6 (15), 254.6 (100), 226.7 (12)	266, 347	(Amaral et al., 2004)
11	Kaempferol- <i>O</i> -pentoside ^a	417.0	MS ² [416.8]: 284.6 (100), 283.7 (40), 254.7 (20) MS ³ [284.6]: 255.6 (16), 254.7 (100), 226.6 (19)	265, 346	(Amaral et al., 2004)

^a The aglycone part of the flavonoids was identified by spectrum matching to an in-house library containing the MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics, Bremen, Germany).

^b These constituents were confirmed by comparison of the retention times, UV- and MSⁿ-spectra with reference compounds.

Fig. S1. PPAR γ luciferase reporter gene transactivation assay with JR 100 $\mu\text{g/mL}$, 30 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$. Pio= Pioglitazone (5 μM), positive control; DMSO= negative control; (bars represent mean+ SD, n= 3, one-way ANOVA, Bonferroni's post-test, *** p < 0.001).

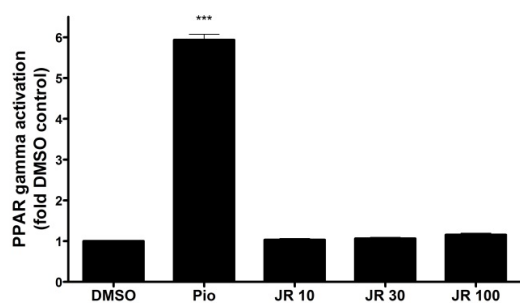
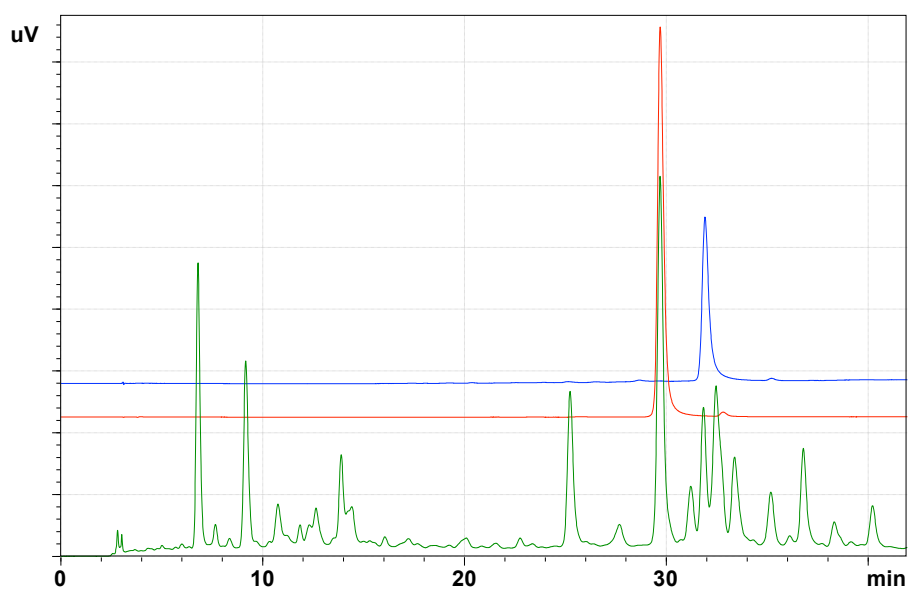
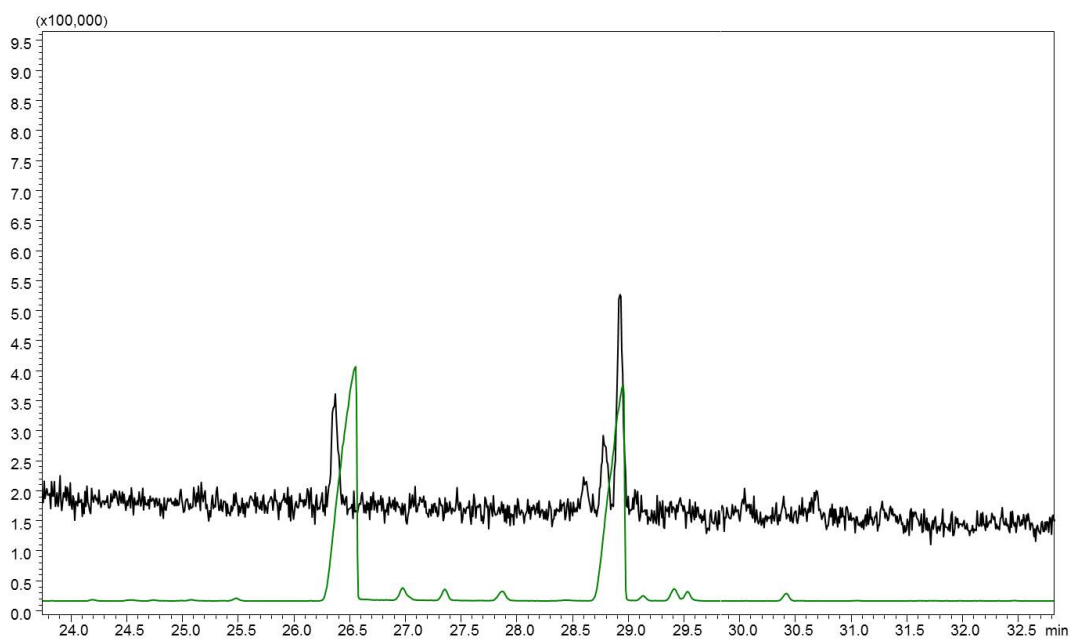


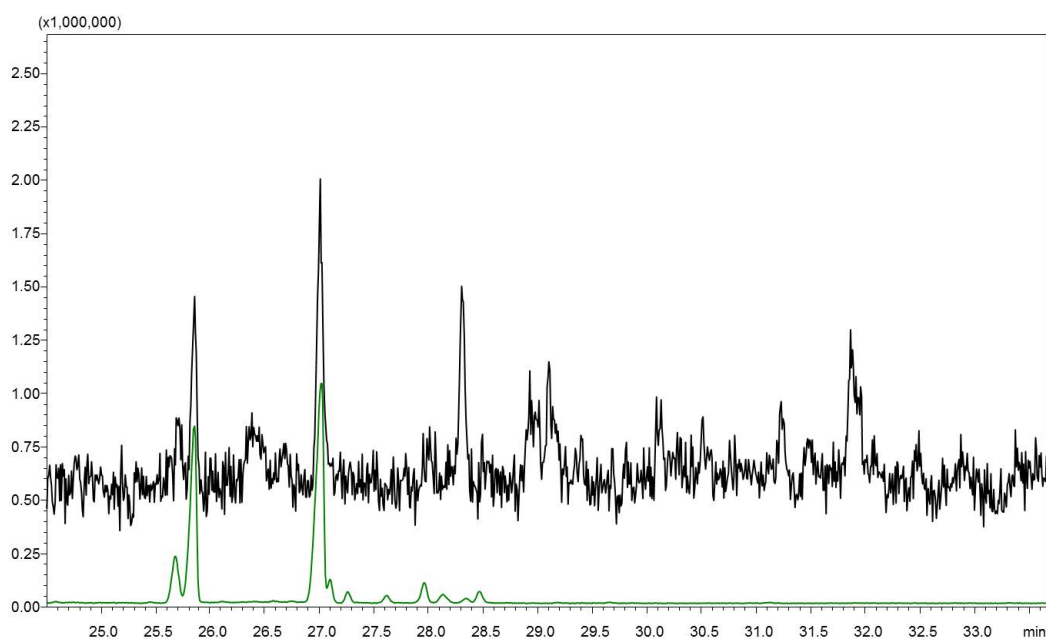
Fig. S2. HPLC chromatogram (300 nm) of JR (green), hyperoside (red) and avicularin (blue)



4.4.3 Additional data



(a) Analysis of L-rhamnose (green) and compound "peak 9" by GC.



(b) Analysis of L-arabinose (green) and compound "peak 10" by GC.

Figure 4.11.: GC chromatograms of isolated flavonoid 9 (a) and flavonoid 10 (b) of JR (see Figure 1, page 126 and Table S1, page 133) and authentic reference substances L-rhamnose and L-arabinose, respectively. For derivatization and GC parameters see section 3.5.2 (page 44)

The identification of the sugar moieties of the isolated flavonoids **9-10** (see page 56)

was not discussed in detail in the publication. For the sake of completeness, GC-chromatograms of the glycosidic residues are added in Figure 4.11 (page 135). The isolated compounds were derivatized as described in chapter 3.5.1 (page 43) and analyzed by GC-MS (chapter 3.5.2, page 44). Authentic reference substances L-arabinose and L-rhamnose were processed the same way. The assignment of the flavonoid glycosides was based on comparison with chromatograms and MS data of the authentic reference substances.

5 Discussion and conclusion

Long before insulins have been synthesized or the breakthrough of oral anti-diabetics occurred, hyperglycemia was treated with plants or plant-derived formulations. Information on the choice of plants and their preparations can be found in testimonies of different traditional medical systems, but many ascribed effects lack modern scientific evidence. In this thesis, the traditional application of two plants, which are used in different ethnomedicines against diabetes mellitus, is probed. The plants of choice are *Juglans regia* L. as representative of the Austrian folk medicine and *Leonurus sibiricus* L., representing the Traditional Mongolian Medicine (TMM). Since TMM is largely unknown to the Western world, but is still the major medical care provider in Mongolia, the development and main characteristics of TMM are discussed in a review, published in "Phytochemical Reviews" (see section 1.2, page 8 ff.).

The bigger part of the work explores different aspects of *L. sibiricus*. This Lamiaceae species ranks among the ten most often applied plants in Mongolia and finds itself in numerous traditional recipes. Its aerial parts are traditionally used against high fever, diarrhea and symptoms of diabetes mellitus. Since usage of TMM in Mongolia has increased in the last decades due to political changes, officials in Mongolia are striving to scientifically prove the medical efficacy of the used plants. In cooperation with the Mongolian National University of Medical Sciences, Ulaanbaatar, the aerial parts of *L. sibiricus* were collected in the summers of 2008, 2009 and 2014 for microscopic, phytochemical and molecular-biological investigations at the Department of Pharmacognosy, University of Vienna, Austria.

The influence of *L. sibiricus* on the glucose metabolism is tested in an *in vitro* system using C2C12 myocytes. At a concentration of 10 $\mu\text{g/mL}$, a *L. sibiricus* methanol extract (LS MeOH) enhances the insulin-stimulated glucose-uptake in C2C12 myocytes. A negative regulator of insulin signaling, protein tyrosine phosphatase 1B (PTP1B), is inhibited by the same extract, suggesting this to be one of the targets involved in the increased glucose-uptake process. Bioassay-guided fractionation of the active extract located the primal activity to an iridoid enriched fraction, mainly composed of acetyl-

harpagide and ajugoside. A synergistic effect of the active fraction's compounds is assumed, since the solitary metabolites do not show activity.

L. sibiricus' main secondary metabolites are phenylpropanoids and iridoid derivatives, namely verbascoside, lavandulifolioside, acetylharpagide and ajugoside. These marker compounds are quantified by HPLC-CAD in LS MeOH, LS BuOH (an active sub-fraction of LS MeOH) and in the crude *L. sibiricus* drug, using theophylline as internal standard. The combined iridoid and phenylpropanoid content is assessed to be 0.4% and 1.6%, respectively in the active LS MeOH extract. The used quantitation method is validated according to ICH (International Committee on Harmonization) guidelines and shows good accuracy and precision, thereby providing a detailed method for quality control. The manuscript summarizing the effects on the *in vitro* glucose metabolism and the quantitation of *L. sibiricus*' major compounds is under revision in "Phytochemical analysis" and can be found in section 4.1 (page 47 ff).

To show a broader perspective of *L. sibiricus*' influence on diabetes, its effects on the insulin release of INS-1E insulinoma cells are tested in cooperation with the Paracelsus Medizinische Privatuniversität in Salzburg. An aqueous *L. sibiricus* extract (LS OWE) fosters cell proliferation and increases insulin secretion of INS-1E insulinoma cells by closure of K_{ATP} channels, depolarization of the membrane and increase of Ca_i . The corresponding paper is published in the "Journal of Ethnopharmacology". Since the major part of this work was done by Sabine Schmidt, Paracelsus Medizinische Privatuniversität, the published work is added in appendix D (page 161 ff). Phytochemical analyses of the insulin release increasing LS OWE extract (performed in the course of two diploma theses (see section 4.2, page 89)) identify stachydrine as major constituent. Even though stachydrine is contained in considerable amounts in LS OWE, it does not contribute to the activity on insulinoma cells, as it is not active when tested separately. The phytochemical fingerprint of LS OWE using reversed phase chromatography shows caffeoylquinic acid derivatives, several glycosylated flavonoids and phenylpropanoids in minor concentration. Generally, the qualitative composition of LS MeOH and LS OWE is similar, with differences in quantities. The small contents of lavandulifolioside and verbascoside in *L. sibiricus* aqueous extracts, which, in comparison, represent main metabolites in the methanol extract, is surprising, given their highly glycosylated state. This circumstance, however, is monitored in three indepen-

dent samples (see section 4.1, page 47 ff).

Together, the reported effects of *L. sibiricus* on insulin release in insulinoma cells and on glucose-uptake in myocytes provide a good example for the multiple facets of phytotherapy. Due to the great number of compounds, plant extracts act on various targets simultaneously, thereby providing a wholistic therapy option. In case of *L. sibiricus*, the findings provide explanations for the traditional use of *L. sibiricus* formulations in the treatment of diabetes mellitus in TMM.

The identity of *L. sibiricus* is subject to controversial discussion in current scientific literature. Some studies declare it to be a distinct species, while others propose that *L. sibiricus* is a synonym for a different *Leonurus* species, *Leonurus japonicus* HOUTT., a plant regularly used in TCM. Both species grow in the same region, but are traditionally used for different indications. Therefore, the question of correct identification needs to be addressed, especially since *L. japonicus* is about to become adopted in the European Pharmacopeia, next to *Leonurus cardiaca* L., as second *Leonurus* species. To clarify the situation between *L. sibiricus* and *L. japonicus* and to discriminate them from *L. cardiaca*, means for identification using microscopy and HPTLC fingerprints are provided for all three species.

Microscopic analyses of several reference specimens of *L. sibiricus* and *L. japonicus* reveal clear differences in their anatomy and micro-morphology, thereby refuting the previously proposed hypothesis that these two species are the same. The most striking macro-microscopic difference of the herba drugs lies in the shape of the lower corolla lip. The middle lobe of *L. japonicus*' lower lip is divided into two additional lobules. *L. sibiricus* and *L. cardiaca* lack this feature. Their middle lobe is entire and not further divided. Further anatomical differences lie in the presence, dimensions and density of trichomes on the leaves, corollae and ovaries. A comparison of the phytochemical fingerprints using HPTLC undermines the results seen in the microscopic analyses. The polyphenolic pattern differs greatly between *L. sibiricus* and *L. japonicus*. Verbascoside, a phenylpropanoid, may be used as marker, being present in *L. cardiaca* and *L. sibiricus*, but not in *L. japonicus*. The alkaloid leonurine, although reported in literature for all three species, is only contained in *L. japonicus*. Its absence in *L. sibiricus* is described for the first time, based on results from three independent biological samples. A ratifica-

tion of this result using a larger set of samples is desirable. Stachydrine on the other hand, which is often used as marker for identity tests of *L. japonicus* is contained in all three species and therefore not suited to discriminate them. All microscopic data and evaluations by HPTLC, with which a clear distinction between *L. sibiricus*, *L. japonicus* and *L. cardiaca* becomes possible, are summarized in section 4.3 (page 91 ff) and prepared for submission to the "Journal of Ethnopharmacology".

In addition to the investigations of the TMM plant *L. sibiricus*, a representative of the Austrian folk medicine, *Juglans regia* L., is studied with respect to its *in vitro* insulin-sensitizing properties. The hypoglycemic effect of its leaves has been shown previously in literature by *in vivo* studies on rats and by a small human clinical trial. The mechanism of action, however, remained speculative. In the manuscript provided in section 4.4.2 (page 124) and published in the "Journal of Ethnopharmacology", first mechanistic explanations of the hypoglycemic action of walnut leaves are offered by the inhibition of PTP1B. Another important target, peroxisome proliferator receptor gamma (PPAR γ) is not activated by walnut leaves and therefore not involved in the blood glucose lowering effect. Dereplication of the walnut leaf methanol extract adds two flavonoids to the chemical fingerprint.

To conclude, this work provides a number of methods for the quality control of two plants, *L. sibiricus* and *J. regia*, which are traditionally applied in the Traditional Mongolian Medicine and Austrian folk medicine, respectively, against diabetic conditions. In three publications, *L. sibiricus*' microscopic and phytochemical characteristics as well as its influence on glucose-homeostasis are discussed. By proofing *L. sibiricus*' insulin-sensitizing and insulin-releasing properties in *in vitro* models, the traditional use is ratified, thereby warranting *in vivo* studies in the future. The described anatomical features of *L. sibiricus* allow a clear distinction to other *Leonurus* species and represent the first microscopic report of *L. sibiricus*. Analyses by HPTLC display a quick method for identity tests, while a validated quantitation method provides detailed quantitative ratios of main secondary metabolites in *L. sibiricus*. Together, these techniques serve as state-of-the art methods for the quality control of this extensively used herbal drug. Furthermore, the previously reported blood glucose-lowering activity of *J. regia*

leaves is corroborated with the models used in this thesis and explains the effect, at least partly, by PTP1B inhibition.

6 Summary

This thesis deals with the investigation of two plants, *Leonurus sibiricus* L. and *Juglans regia* L.. These plants are used against diabetic disorders in the Traditional Mongolian Medicine (TMM) and Austrian folk medicine, respectively. A review on the development and main characteristics of TMM introduces this heretofore largely unknown medicinal system in chapter 1.2.2 (page 9 ff). In three further publications, phytochemical and partly anatomical features of the plants are investigated as well as their effects on selected targets of the glucose metabolism.

The focus lies on *L. sibiricus*. Its methanol extract enhances the *in vitro* glucose-uptake in myocytes and inhibits PTP1B, whereby the traditional use is corroborated. The main secondary metabolites, acetylharpagide, ajugoside, lavandulifolioside and verbascoside, detected in three independent samples, are designated as chemical markers for *L. sibiricus*' quality control. A validated HPLC-CAD method is provided, with which a precise quantitation of these marker compounds is possible (section 4.1, page 47 ff).

In accordance with monographs in modern pharmacopoeias, the methods for *L. sibiricus*' quality control are complemented by a representative HPTLC fingerprint for quick chemical identity tests and by anatomical characteristics. Additionally, to avoid misidentifications and confusions with *Leonurus japonicus* HOUTT. and *Leonurus cardiaca* L., the two most prevalent *Leonurus* species, specific distinction criteria are given. These lie in the shape of the corolla, the existence of trichomes on different organs and the presence of the chemical compounds leonurine and ajugoside (chapter 4.3, page 91 ff).

The goal of investigations on *J. regia* is to provide further understanding of its blood glucose lowering activity, which has been shown in literature by previous *in vivo* studies. Inhibition of PTP1B, a negative regulator of insulin signaling, is firstly revealed as possible mechanism for the hypoglycemic effect of walnut leaf extracts. The involvement of another important target, PPAR γ is excluded. Phytochemical investigations sustain findings of previous studies and widen the chemical fingerprint by two flavonoid derivatives (section 4.4.2, page 124 ff).

7 Deutsche Zusammenfassung

Die vorliegende Arbeit untersucht zwei Arzneipflanzen, *Leonurus sibiricus* L. und *Juglans regia* L.. Aus der Traditionellen Mongolische Medizin (TMM) und der Österreichischen Volksmedizin sind Anwendungen der jeweiligen Pflanze bei Diabetes mellitus überliefert. Ein Review über die Charakteristika von TMM stellt dieses bisher wenig bekannte Medizinalsystem in Kapitel 1.2.2 (Seite 9 ff) vor. In drei weiteren Publikationen werden Effekte auf den Glukosestoffwechsel, phytochemische und teilweise mikroskopische Merkmale besagter Pflanzen erörtert.

Der Fokus liegt bei *L. sibiricus*. Ein Methanolextrakt erhöht *in vitro* die Glukoseaufnahme in Muskelzellen und hemmt PTP1B, wodurch die traditionell-überlieferte Wirkung bekräftigt wird. Die Hauptmetaboliten Acetylharpagid, Ajugosid, Lavandulifoliosid und Verbascosid, detektiert in drei unabhängigen Proben, werden als Markersubstanzen zur Qualitätskontrolle herangezogen. Eine validierte HPLC-CAD Methode ermöglicht die präzise Quantifizierung dieser Markersubstanzen (Kapitel 4.1, Seite 47 ff).

In Anlehnung an Arzneibuchmonographien werden die Methoden zur Qualitätskontrolle von *L. sibiricus* durch einen repräsentativen HPTLC-fingerprint und anatomische Merkmale erweitert. Um Verwechslungen mit *Leonurus japonicus* HOUTT. und *Leonurus cardiaca* L., den meist verwendeten *Leonurus*-Arten, vorzubeugen, werden zusätzlich Unterscheidungscharakteristika beschrieben. Diese Charakteristika bestehen in der Form der Corolla, der Anwesenheit von Trichomen auf verschiedenen Organen und der Detektion von Leonurin und Ajugosid (Kapitel 4.3, Seite 91 ff).

Die Untersuchungen an *J. regia* sollen die in der Literatur beschriebene hypoglykämische Wirkung genauer analysieren. Die gezeigte Hemmung von PTP1B durch einen Walnussblattextrakt liefert erstmals eine mögliche Erklärung für den blutzuckersenkenden Effekt. Die Beteiligung eines anderen wichtigen Targets, PPAR γ , kann ausgeschlossen werden. Phytochemische Analysen der Walnussblätter bestätigen frühere Resultate und erweiterten den chemischen Fingerprint mit der Zuordnung zwei weiterer Flavonoide (Kapitel 4.4.2, Seite 124 ff).

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A Curriculum vitae

Personal Data

Anna Pitschmann

Dates of birth: 27.04.1986 in Vienna

Citizenship: Austrian

Marital status: married



School and Education

1992-1996 Elementary school in Laxenburg, Austria

1996-2004 Grammar school in Mödling, Austria
school certificate examination - Matura, passed with distinction

University studies & career

2004-2009 Master studies in Pharmacy at the University of Vienna, Austria. Passed with distinction. Title of master thesis: "Phytochemische Untersuchungen an einem Dichlormethanextrakt der mongolischen Arzneipflanze *Euphorbia pallasii* TURCZ."

since 2009 PhD studies in Pharmacy at the University of Vienna, Austria

2009-2011 "Praedoc" assistant at the University of Vienna, Austria

2011-2013 Research associate specialist at the University of Wisconsin - Madison, USA

2013-2015 External lecturer in courses of microbiology and plant microscopy at the University of Vienna, Austria

Activity

2005-2011 Pharmaceutical co-worker at "Marien-Apotheke Breitenfurt", Austria

2007-2011 Several tutor-jobs at the University of Vienna, Austria

08/2007 Summerschool in Beijing, China: "Chinese herbal drugs"

09/2010 Summerschool in Seggau, Austria: "Chromatographie und Kopplung mit Massenspektrometrie"

since 2015 Secretary general of "Österreichische Gesellschaft für Phytotherapie"

Awards

2014 PhD completion grant, Faculty of Life Sciences, University of Vienna

B List of publications

1. Pitschmann, A., Zehl, M., Atanasov, A., Dirsch, V.M., Heiss, E., and Glasl, S. (2014). Walnut leaf extract inhibits PTP1B and enhances glucose-uptake in vitro. *Journal of Ethnopharmacology*, 152:599-602. IF=2.939, Q1 (Plant sciences, Integrative & complementary medicine)
2. Singh, C.K., Pitschmann, A. and Ahmad, N. (2014), Resveratrol-zinc combination for prostate cancer management. *Cell Cycle*, 13:1867-1874. IF=5.006, Q2 (Cell biology)
3. Pitschmann, A., Purevsuren, S., Obmann, A., Natsagdorj, D., Gunbilig, D., Naran-tuya, S., Kletter, C., and Glasl, S. (2013). Traditional Mongolian Medicine: history and status quo. *Phytochemistry Reviews*, 12:943-959. IF=2.894, Q1 (Plant sciences)
4. Schmidt, S., Jakab, M., Jav, S., Streif, D. Pitschmann, A., Zehl, M., Purevsuren, S., Glasl, S., and Ritter, M. (2013). Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells. *Journal of Ethnopharmacology*, 150:85-94. IF=2.939, Q1 (Plant sciences, Integrative & complementary medicine)

Publications in preparation:

5. Pitschmann, A., Zehl, M., Heiss, E., Purevsuren, S., Urban, E., Dirsch, V.M., and Glasl, S. Quantification of phenylpropanoids and iridoids in insulin-sensitizing extracts of *Leonurus sibiricus* L. (Lamiaceae). *Phytochemical Analysis*, under revision. IF=2.450, Q2 (Analytical chemistry)
6. Pitschmann, A., Waschulin, C., Sykora, C., Purevsuren, S., and Glasl, S. Untangling the *Leonurus* confusion - microscopic and phytochemical differences of *L. sibiricus* L., *L. japonicus* HOUTT. and *L. cardiaca* L.. *Journal of Ethnopharmacology*, in preparation. IF=2.939, Q1 (Plant sciences, Integrative & complementary medicine)

C Presented posters

WALNUT LEAVES AGAINST DIABETES MELLITUS?

Possible mechanism and active principle

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ABSTRACT

Walnut (*Juglans regia* - JR) is one of the medicinal plants used against diabetes mellitus in Austrian folk medicine. The anti-diabetic effect of *Folium Juglandis* has also been scientifically proven by several *in vivo* studies in mouse models. The present study is designed to scrutinize the active anti-diabetic principle of JR leaves and to achieve first hints of its physiological mechanism of action. The chlorophyll-free methanol extract of JR leaves (JRwC) enhanced the basal glucose uptake in C2C12 myocytes by 1.5 times at concentrations of 25 µg/mL compared to untreated cells. Compared with the solvent DMSO, this extract elicited more than doubled insulin-stimulated cellular glucose uptake rate. These effects could partly be explained due to the inhibition of protein tyrosine phosphatase 1B (PTP 1B). JRwC inhibited PTP 1B by about 80% at a concentration of 25 µg/mL. Further separation of this fraction resulted in decrease and dispartment of activity, suggesting synergy between the individual components of JRwC. LC-MS analyses of the active JRwC extract led to the identification of chlorogenic acid, cumaroylquinic acid and naphthoquinone-hexoside, as well as eight flavonoids. Two of the flavonoids were identified as hyperoside and avicularin by comparison with reference compounds. The sugar moiety of three further flavonoids was determined by trimethylsilyl-derivatisation and GC-MS analyses leading to quercetin-L-arabinoside, quercetin-L-rhamnoside and kaempferol-L-arabinoside.

INTRODUCTION

- Diabetes mellitus is a metabolic disorder characterized by hyperglycemia
- Prevalence is increasing at alarming rate all over the world
- Current clinical treatment cannot cure or prevent secondary complications
- Effective new therapy needed
- *Juglans regia* L. (JR) has been traditionally used in Austrian folk medicine against elevated blood-glucose levels as tea infusions and liquors
- Dried leaves extract showed anti-diabetic *in vivo* activity

AIM

- Characterize and find active anti-diabetic principle of *Juglans regia* leaves
- MeOH-extract by bioassay-guided fractionation
- Elucidate physiological mechanism of action

METHODS

Extraction of JR leaves with methanol at 40°C, ASE®200 (Dionex)
HPLC. *Stat. phase*=Luna C18 (Phenomenex); *mob. phase*= ddH₂O pH2.8; w/ formic acid (A), MeOH (B); gradient elution 25% B to 60% B in 40 min.

Determination of sugar moieties. 2h hydrolysis with Kiliani-reagent. Partition with EtOAc. Add S-(+)-Butanol and HCl₂ to evaporated polar phase for 15h. Dry, solve in Pyridin and add HMDS and TMCS. Inject into GC-MS.

Assessment of Glucose uptake rate. 48h - treatment of C2C12 myotubes with JRwC. 4h serum starvation, 30min insulin stimulation. Glucose-uptake assessment in the presence of 3H-DOG. Lysis of cells, counted in scintillation counter.

In vitro enzyme assay. PTP1B activity was determined using 2mM pNPP in 50mM MOPS, pH 6.5 as a substrate. JRwC was diluted to 1% DMSO in 100 µL overall assay volume. The inhibitory action towards the phosphatases was measured in 96-well format in the presence of 1mM PTP1B. The reactions were subjected to kinetic absorbance readings at 405nm for 30min. Subsequently, the reaction was stopped with 10M NaOH, and the absorbance was again measured at 405 nm.

RESULTS

➤ HPLC-fingerprint of active fraction

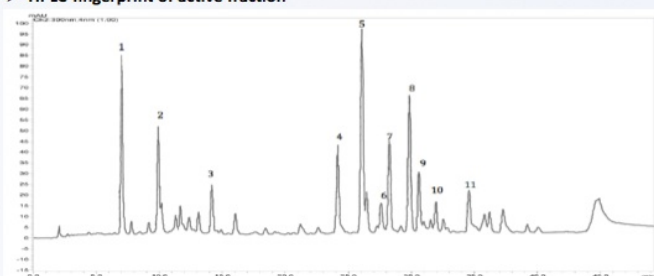
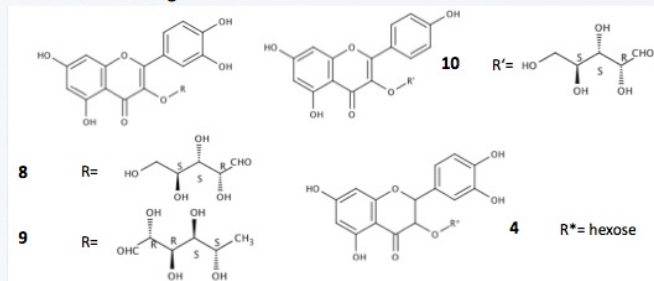


Figure 1: HPLC-analysis of JRwC: (1) chlorogenic acid, (2) cumaroylquinic acid, (3) naphthoquinone-hexoside, (4) dihydroquercetin-hexoside, (5) hyperoside, (6) quercetin-pentoside, (7) avicularin, (8) quercetin-L-arabinoside, (9) quercetin-L-rhamnoside, (10) kaempferol-L-arabinoside, (11) kaempferol-pentoside

➤ Identification of sugar moieties



➤ Enhanced glucose uptake

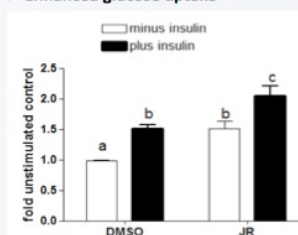


Figure 2: Glucose uptake of JRwC, neg. control= DMSO. n=3, ANOVA

➤ PTP1B inhibition – dose response

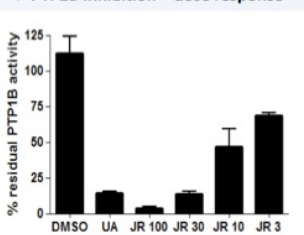


Figure 3: Colorimetric *in vitro* PTP 1B inhibition assay of JRwC. UA= pos. control, DMSO= neg. control

ACKNOWLEDGEMENTS

We thank Raul Jimenez Heredia for excellent support regarding the *in vitro* PTP 1B enzyme assay. Part of this work was financed by the Hochschuljubiläumsstiftung der Stadt Wien (HJS 1755/2010 to EHH).

TRADITIONAL MONGOLIAN MEDICINE & Antidiabetic activity of *Leonurus sibiricus* L.

Anna Pitschmann, Martin Zehl, Elke Heiss, Sabine Glasl

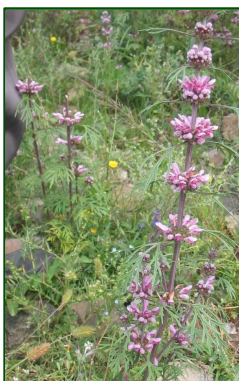
Department of Pharmacognosy - University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

Traditional Mongolian Medicine – TMM

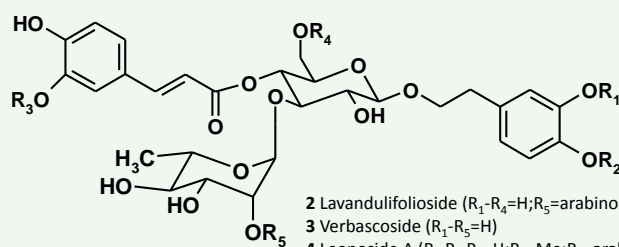
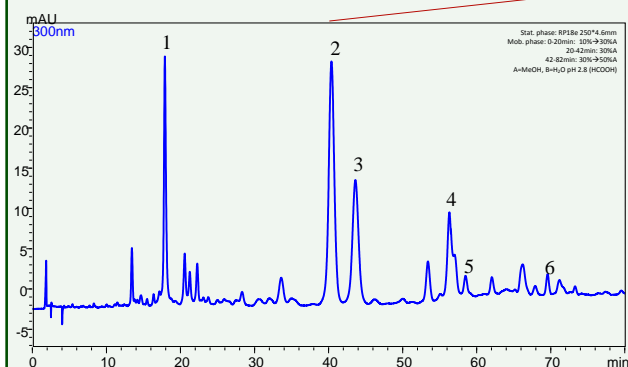
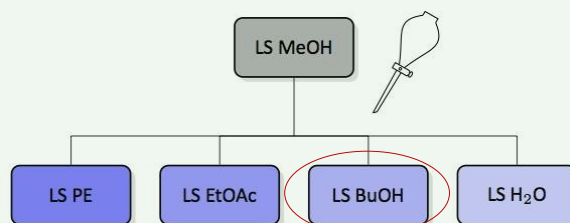
History Medical methods have been practiced since ancient times in Mongolia. In the 16th century, Lamaism became the leading religion in Mongolia and Tibetan Buddhism largely influenced the daily life and TMM. Education of traditional practitioners took place in monasteries. When Lamaism was banned during the Soviet supremacy in the early 20th century, the medical system collapsed and was forbidden. After recent political changes in the 1990ies, TMM finds new updraft. In course of this new development, scientific evidence for the traditionally used plants is sought.

- Knowledge passed on over generations
- Education in „Manba Datsans“ and universities
- Diagnosis: reading pulse, tongue and urine examination
- Treatment with traditional preparations, moxibustion and massage
- Medical preparations are complex mixtures of plants, minerals and animal drugs
- Medicinal plants were collected by the practitioner in the past. Today, traditional medicine pharmacies supply herbal medicine

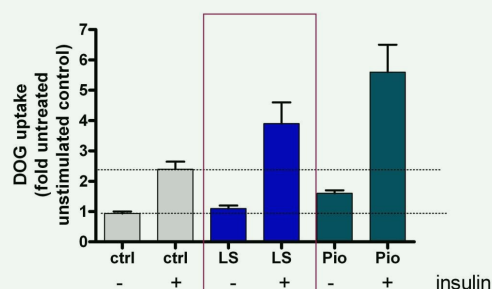
Leonurus sibiricus L. against diabetes mellitus



Leonurus sibiricus L. (LS) belongs to the Lamiaceae family and is distributed in Southeast Asia and Mongolia. In Mongolian literature, LS is mentioned as a treatment for typical symptoms of diabetes mellitus. This study investigates the effect of LS on an *in vitro* model related to insulin sensitivity.



- 2 Lavandulifolioside ($R_1-R_4=H; R_5=arabinose$)
- 3 Verbascoside ($R_1-R_5=H$)
- 4 Leonoside A ($R_1, R_2, R_4=H; R_3=Me; R_5=arabinose$)
- 5 Leucosceptoside A ($R_1, R_2, R_4, R_5=H; R_3=Me$)
- 6 Leonoside B ($R_1, R_3=Me; R_2, R_4=H; R_5=arabinose$)



The dried herbal material was pulverized, extracted with methanol and fractionated by lq/lq partition with four different solvents. The HPLC fingerprint of the butanolic extract states the presence of several phenylpropanoids (**2-6**) and a caffeoylquinic acid derivative (**1**). Preliminary chemical characterization was assessed by LC-MSⁿ. The butanolic extract of LS, rich in polyphenols, increases the insulin-stimulated glucose uptake of C2C12 myocytes *in vitro* at concentrations of 10 µg/mL by 2 fold compared to control. This suggests that the LS extract acts as insulin sensitizer.

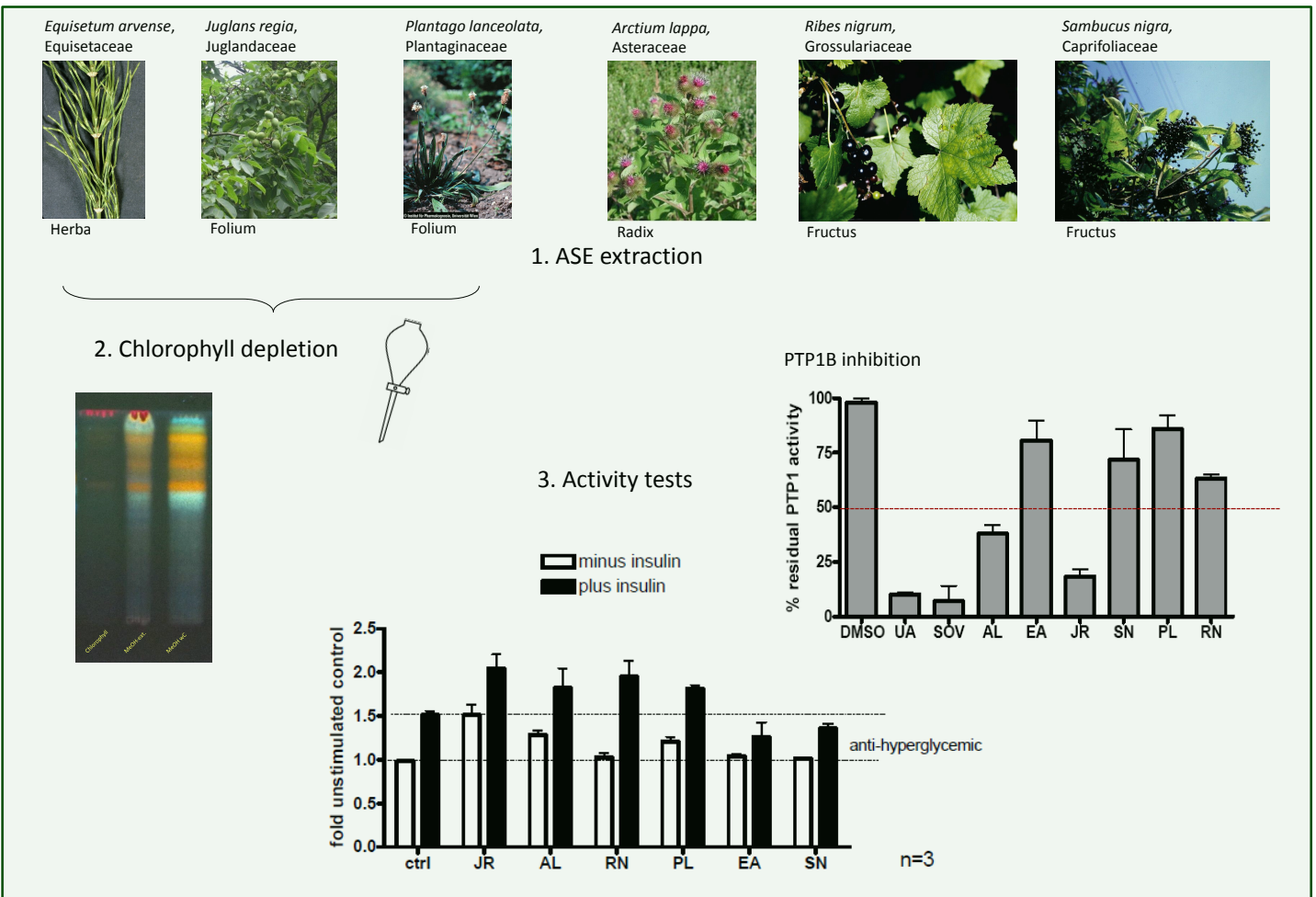
Conclusions Our results indicate that LS preparations might be useful in the management of diabetes mellitus by increasing insulin sensitivity, which warrants *in vivo* studies in the future.

Searching for anti-diabetic activity in plants from Austrian folk medicine

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Abstract Diabetes mellitus is a metabolic disorder characterized by hyperglycemia and is accompanied by several secondary complications. The Volksmed databank¹ provides a list of 58 plants that are traditionally used against typical diabetic symptoms in Austrian folk medicine. The aim of this study is to test a selection of six plants for anti-hyperglycemic activity and inhibition of PTP1B, a phosphatase and major negative regulator of insulin signaling. These plants were *Arctium lappa* (AL), *Equisetum arvense* (EA), *Juglans regia* (JR), *Plantago lanceolata* (PL), *Ribes nigrum* (RN) and *Sambucus nigra* (SN).



Methods In the first step, methanol extracts of AL, EA, JR, PL, RN and SN were produced by accelerated solvent extraction. If present, chlorophyll was separated by liquid/liquid partition with CH₂Cl₂. Chlorophyll depletion was confirmed by TLC and the absence of a characteristic red fluorescent band under UV 366 nm. Extracts were then subjected to *in vitro* functional (glucose uptake in C2C12 myocytes) and target-oriented (PTP1B inhibition) test systems for potential anti diabetic activity.

Results Four of the examined plant extracts, JR, AL, PL and RN, elicited increased glucose uptake in C2C12 myocytes at concentrations of 25 µg/mL compared to control DMSO. EA and SN showed no reproducible positive effect. Out of these six plant extracts, PTP1B was most potently inhibited by JR, followed by AL at concentrations of 10 µg/mL. Ursolic acid (UA) and sodium orthovanadate (SOV) served as positive controls. The other four plant extracts showed no PTP1B inhibition.

Conclusion Our results provide a first step towards scientific evidence for the use of JR, AL, PL and RN preparations against typical diabetic symptoms. In case of AL and JR this activity could, at least partly, be attributed to the inhibition of PTP1B².

[1] Saukel J, Kubelka W (1993) *Sci Pharm* 61:14.
[2] Pitschmann A et al (2014) *J Ethnopharmacol* 152:599-602.

D Insulin secretory activity of *L. sibiricus*

The publication contained in this section was largely accomplished by cooperation partners of the Paracelsus Medizinische Privatuniversität in Salzburg. It is therefore not added in the main text. It is, nevertheless, an important and interesting contribution to *L. sibiricus*' activity on glucose-homeostasis.

The wording of methods described in the following paper may resemble publications of co-authors, where similar methods were applied.



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Extracts from *Leonurus sibiricus* L. increase insulin secretion and proliferation of rat INS-1E insulinoma cells



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ABSTRACT

Ethnopharmacological relevance: Traditional Mongolian medicine (TMM) uses preparations from herbs as one form of medication for the treatment of a diversity of diseases including diabetes mellitus (DM). We evaluated the effect of extracts from the plant *Leonurus sibiricus* L. (LS), used in TMM to treat typical symptoms of type 2 DM, on insulin secretion, electrophysiological properties, intracellular calcium concentration and cell proliferation of INS-1E insulinoma cells under standard cell culture conditions (SCC; 11.1 mM glucose).

Materials and methods: Insulin secretion was measured by ELISA, electrical properties were assessed by whole cell patch clamping, intracellular calcium concentration (Ca_i) by Fluo-4 time lapse imaging, insulin receptor expression was verified by RT-PCR and cell proliferation assessed by CellTiter-Glo® cell viability assay.

Results: Insulin released from INS-1E cells into the culture medium over 24 h was significantly increased in presence of 500 mg/L aqueous LS extract (LS OWE) as well as methanolic LS extract (LS MeOH/H₂O) but not in the presence of the butanol-soluble extract (LS MeOH/BuOH). Acute application of LS OWE resulted in a depolarization of the cell membrane potential paralleled by an initial increase and subsequent decline and silencing of action potential frequency, by KATP channel inhibition, persisting depolarization and an increase in Ca_i . The electrophysiological effects were comparable to those of 100 μ M tolbutamide, which, however failed to elevate insulin secretion under SCC. Furthermore all LS extracts stimulated INS-1E cell proliferation.

Conclusions: The finding that extracts from *Leonurus sibiricus* L. enhance insulin secretion and/or foster cell proliferation may provide possible explanations for the underlying therapeutic principles in the empirical use of LS-containing formulations in DM and DM-related disorders as applied in TMM.

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Abbreviations: AP, action potential; Ca_i , intracellular calcium concentration; DM, Diabetes mellitus; T2DM, type 2 diabetes mellitus; DMrd, DM-related disorders; GLUT-4, glucose transporter isoform 4; IK_{ATP} , ATP-sensitive K^+ conductance/ currents; IR, insulin receptor; LS, *Leonurus sibiricus* L.; LS MeOH/BuOH, *Leonurus sibiricus* purified methanolic extract; LS MeOH/H₂O, *Leonurus sibiricus* water-soluble part of the methanolic extract; LS OWE, *Leonurus sibiricus* original water extract; SCC, standard cell culture condition; T2DM, Type 2 Diabetes mellitus; TMM, Traditional Mongolian Medicine; TOL, tolbutamide; V_{mem} , cell membrane potential; WHO, World Health Organization.

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1. Introduction

Driven by socio-economic needs empiricism-based traditional healing methods have gained worldwide attention with respect to evidence based scientific evaluation of their medical efficiency, unraveling the mode(s) of action(s) as well as identification of active constituents. However, national pharmacopoeias or other regulations regarding formulae, analytical composition, physical constants, main chemical properties and mode(s) of preparation of traditional herbal medicines do not exist in the majority of countries practicing traditional medicines (WHO, 2005). Traditional Mongolian medicine (TMM), using herbs as one form of medication, exists for more than 2500 years. 200–250 medicinal plants are commonly used and more than 800 plants have been registered in Mongolia as medicinal plants. TMM has developed

from traditional Tibetan medicine by replacing shamanistic practices with medical theories, techniques and medications (Ligaa, 1996).

In the year 2010 worldwide approximately 285 million people were affected by Diabetes mellitus (DM) with type 2 (T2DM) making up about 90% of the cases (Melmed et al., 2011). The incidence of T2DM is increasing rapidly (Wild et al., 2004), and the disease is a major socio-economic cost factor for all health care systems (WHO, 2013). Since antiquity, DM or DM-related disorders (DMrD) have been treated with herbal medicines, such as plants and their extracts. In Mongolia, different medicinal plants are used in the treatment of DM or DMrD, such as the prescription BAIMAI-SAN, which consists of 19 different ingredients (Liu et al., 2011). However, to date, only a small number of scientific studies have addressed the application of medicinal plants, traditionally used in TMM, to treat DM and the underlying mechanisms of action still have to be identified.

T2DM is characterized by insulin resistance of peripheral tissues and defective insulin secretion which results from intrinsic β -cell dysfunction and reduced β -cell mass (Bonner-Weir and O'Brien, 2008; de Koning et al., 2008). Studies demonstrated a 63% reduction in islet mass of T2DM patients as compared to normo-glycemic control subjects and a loss of β -cell mass even at early stages of the disease due to increased rates of apoptosis (Butler et al., 2003). Both, the peripheral tissues as sites of insulin action and the site of hormone production itself, the β -cell, are possible targets for medicinal plant constituents used in the treatment of T2DM.

Two genera, *Dryopteris* and *Filix*, used in Mongolia as medicinal plants, have been reported to ameliorate diabetic symptoms. Diabetic rats treated with extracts of these plants showed improved plasma glucose responses as assessed by oral glucose tolerance test and decreased glucose clearance rates during low-dose euglycemic clamp studies were markedly improved by a single treatment with these extracts. However, insulin levels were not changed in animals treated with the plant extracts. The authors concluded that the effect on plasma glucose levels was due to an improvement in insulin resistance triggered by an activation of the insulin-signaling pathway in skeletal muscle and therefore an increased amount of the glucose transporter GLUT-4 present in the plasma membrane (Khoorkhor et al., 2007). Effects on GLUT-4 translocation to the plasma membrane have also been described for other herbal constituents, such as the stilbenoid resveratrol (Penumathsa et al., 2008). On the β -cell level, marrubiin, an organic extract from *Leontis leonurus* R.Br. has been shown to increase insulin secretion in INS-1 cells both at 11.1 mM and 33.3 mM glucose, and to alleviate diabetic symptoms in a rat obesity animal model (Mnonopi et al., 2012). Furthermore, nuciferine, the main alkaloid from leaves of *Nelumbo nucifera*, a plant used in traditional Vietnamese medicine that exerts anti-diabetic effects, stimulates insulin secretion in isolated islets from CD1 mouse pancreas and INS-1E cells (Nguyen et al., 2012).

According to the Report of Market Research on Mongolian Traditional Medicinal Drugs, 2007, *Leonurus sibiricus* L. (LS) is one of the most common traditional medicinal raw materials originating from domestic markets (HSUM and MoH, 2007). It is used to treat menstrual irregularities, amenorrhea, malaria and hypertension (Taehakkyo and Yon'guso, 1998), and is known for several beneficial properties, which have been investigated in a number of scientific studies. LS was shown to exert anti-inflammatory activity by decreasing levels of inflammatory cytokines secreted from human mast cells (Shin et al., 2009). Moreover, LS can induce expression of nitric oxide and tumor necrosis factor- α in mouse peritoneal macrophages (An et al., 2008) and stimulate the murine uterus in vitro (Shi et al., 1995). Furthermore, it was shown to exert antibacterial activity (Ahmed et al., 2006), to have analgesic as

well as anti-inflammatory activity in rats (Islam et al., 2005), and to possess potent antioxidant capacity (Lee et al., 2010). In Mongolian literature, LS is mentioned as a treatment for typical symptoms of T2DM such as thirst ((Khaidav and Chojamts, 1965) and personal communication with Prof. Narantuya Samdan, WHO). It is also used to treat DM in the traditional medicine of Bangladesh (Mohammed Rahmatullah et al., 2010). As atherosclerosis is a well-known diabetic complication, it is of interest that treatment with a LS herb extract has been shown to affect the atherogenic process. Thus, C57BL/6 mice on an atherogenic diet supplemented with a LS herb extract displayed decreased plasma cholesterol levels, reduced plasma triglyceride levels, and an increased HDL-cholesterol concentration compared to animals receiving the atherogenic diet alone (Lee et al., 2010). LS also leads to a reduction of intracellular reactive oxygen species, which play an important role in the etiology of atherosclerosis (Singh and Jialal, 2006). Furthermore, it decreases the expression of adhesion molecules such as the lectin-like ox-LDL receptor (LOX-1) (Lee et al., 2010). LOX-1 may mediate the incorporation of ox-LDL into endothelial cells, a process supposed to promote endothelial dysfunction, the first stage of atherosclerosis (Vita et al., 1990). Additionally, LOX-1 expression was shown to be elevated in proatherogenic settings such as T2DM. Through its different effects on atherosclerosis development, LS may therefore retard the progression of T2DM and its resulting morbidities.

These observations, as well as the ancient tradition of using LS to treat the symptoms of DM and DMrD in TMM, led us to investigate a potential effect of LS on the production site of insulin, the pancreatic β -cell. Three extracts were monitored: an aqueous extract (LS OWE), corresponding to the traditional way of administration, a purified methanolic extract (LS MeOH/BuOH), and the water-soluble part of the methanolic extract (MeOH/H₂O).

2. Materials and methods

2.1. Plant material

The aerial parts of *Leonurus sibiricus* were harvested at Tsetserleg in the Mongolian province Archangai (latitude 47° 28' 8.22"; longitude 101° 26' 32.14") in summer 2009 (collection number 01/09/mon). The species was identified by E. Ganbold, Mongolia. A reference specimen is kept at the herbarium of the School of Pharmacy, Health Sciences University of Mongolia, Ulaanbaatar, Mongolia (herbarium number 10080805).

2.2. Preparation of plant extracts

For this study, three different extracts were prepared. As LS is an ingredient of several traditional prescriptions, which are usually taken as crude powder together with a glass of water, we prepared an aqueous crude extract (original water extract, LS OWE), which should reflect this common administration in traditional Mongolian medicine. In parallel, an extract was prepared using methanol as polar organic solvent. The use of methanol provides enrichment of less polar compounds and a softer handling during the purification process compared to the acidic aqueous solutions (shorter evaporation times, evaporation at lower temperatures). In order to obtain purified fractions of high polarity, the dissolved methanolic extract was partitioned between solvents of different polarity, yielding the two extracts used for our analysis, the butanol-soluble extract (LS MeOH/BuOH), and the water-soluble extract (LS MeOH/H₂O).

LS OWE: 5 g powdered dried plant material (drug) were suspended in 125 mL water, and, after adjustment to pH 2 with trifluoroacetic acid, the suspension was shaken gently at 40 °C for

2 h to simulate gastric conditions. Trifluoroacetic acid was used because it could be removed by vacuum evaporation after extraction. The extracts were obtained by subsequent freeze-drying. The yield (2.1 g) is given as drug to extract ratio (DER 2.4:1).

LS MeOH/BuOH and LS MeOH/H₂O: 80 g of the powdered plant material were extracted by accelerated solvent extraction (ASE 200 Accelerated Solvent Extractor, Dionex). Each of the eight extraction chambers were filled with 10 g powder and treated with methanol. Each chamber underwent three extraction cycles consisting of 5 min heat up time, 2 min static time, 10% flush volume, and 60 s nitrogen purge. All resulting solutions were combined and dried under reduced pressure at 40 °C. The DER of the resulting raw methanolic extract amounted to 6:1. It was re-dissolved in methanol and purified by liquid–liquid extraction using three solvents of different polarity consecutively. The extraction with light petroleum was performed to deplete chlorophyll. The remaining methanolic layer was partitioned against dichloromethane followed by water-saturated butanol. The whole procedure resulted in four extracts after evaporation of the solvents under reduced pressure: the light petroleum extract (DER 23:1), and the dichloromethane extract (DER 38:1) were not further investigated; the butanol extract (LS MeOH/BuOH; DER 48:1), and the aqueous layer which remained after the partition with water saturated butanol (LS MeOH/H₂O; 17:1) were used for the study. For experiments extracts were dissolved in double distilled water (LS OWE and LS MeOH/H₂O) or methanol (LS MeOH/BuOH) to receive stock solutions at concentrations of 50 mg/ml (LS OWE and LS MeOH/H₂O) or 500 mg/ml (LS MeOH/BuOH). Respective MeOH-solvent controls were used for all experimental approaches in this study.

2.3. Chemicals and reagents

All salts and chemicals used for the preparation of experimental solutions and cell culture media were p.a. grade.

2.4. Cell culture

INS-1E cells were grown under standard cell culture conditions (SCC) in RPMI 1640 medium containing 11.1 mM D-glucose, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 10% fetal calf serum (FCS), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 250 ng/mL amphotericin B at 37 °C, 5% CO₂ and 95% air. Subcultures were established twice a week by trypsin/EDTA treatment. Cells between passages 90 and 110 were used (Merglen et al., 2004).

2.5. Insulin ELISA

For ELISA experiments, cells were seeded in poly-D-lysine coated 96-well plates (Sarstedt) at a density of 8,000 cells/well and grown under standard culture conditions for 48 h prior to the start of the experiments. To avoid FCS-induced cross-reaction of serum insulin, experiments were performed in serum-free RPMI 1640 medium. Cells were incubated for 24 h in serum-free medium containing 500 mg/L LS OWE, LS MeOH/H₂O, or MeOH/BuOH. Untreated and solvent-treated cells served as controls. The insulin released into the medium during the 24-hour (LS) or 2-hour (tolbutamide) incubation period was measured using a rat insulin ELISA kit (Mercodia) according to the manufacturer's instructions. Subsequently, cell numbers were assessed by using CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) (see cell proliferation assays in this section). Data (ng insulin/mL medium) were converted to ng insulin/cells in the respective well and further to ng insulin/100,000 cells.

2.6. Cell membrane potential and intracellular Ca²⁺ recordings

INS-1E cells were seeded on poly-D-lysine-coated glass coverslips and used for patch clamp or Ca²⁺ imaging after 24–48 h. The coverslips were transferred to a recording chamber and mounted on a Nikon TE2000-U inverted microscope. Experiments were performed at room temperature. The extracellular solution contained (in mM): 135 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 HEPES-free acid (FA), 5 glucose, pH 7.4 (adjusted with NaOH), 290 mOsm/kg. Osmolalities were measured with a vapor pressure osmometer (Wescor). Solution exchange was performed with a gravity-flow perfusion system at a flow rate of 1–2 mL/minute. LS OWE (500 mg/L) and/or tolbutamide (50 or 100 μ M) were added to the extracellular solution as indicated.

Patch clamp recordings were performed in the conventional whole-cell patch clamp configuration. Patch electrode resistances were 3–5 M Ω . An EPC-10 amplifier and PatchMaster/FitMaster software (HEKA) were used for data acquisition and analysis. Membrane potential recordings were performed in the zero-current clamp mode. The pipette solution contained (in mM) 130 KCl, 4 MgCl₂, 2 CaCl₂, 20 HEPES-FA, 10 EGTA. The pH was titrated to 7.2 with KOH (296 mOsm/kg). For voltage clamp recordings of the K_{ATP} conductance an intracellular solution containing (in mM) 4 KCl, 2 MgCl₂, 10 HEPES-FA, 10 EGTA, 4 Mg-ATP, 43 mannitol, with a pH 7.3 (KOH) and 296 mOsm/kg was used. K_{ATP} currents were measured during 500-ms pulses to –80 and –60 mV at 10-s intervals from a holding potential of –70 mV as previously described (Hambrock et al., 2007; Jakab et al., 2008). In this range of potentials the membrane conductance is predominately determined by K_{ATP} currents (Drews et al., 1998).

For monitoring intracellular Ca²⁺, cells were loaded with Fluo-4/AM for 30 min at 37 °C followed by 30 min at room temperature. The light source equipped with a mercury short arc lamp and an integrated shutter (LEJ Leistungselektronik Jena GmbH) was coupled to the microscope (Nikon TE2000-U) via a liquid light guide. The light was passed through a filter cube comprising a 340–380 nm excitation filter, a 440 nm dichroic mirror and a 435–485 nm emission filter. 16-bit gray-scale images were captured for 200 ms at 10-second intervals with a cooled CCD camera (SensiCam, pco). Camera and shutter were controlled by TILLvisION software, which was also used for analysis. For each experiment, all cells in the field of vision were analyzed (approximately 20 cells). Data are presented as fluorescence intensities obtained from background-subtracted images.

2.7. RT-PCR of rat insulin receptor

Total RNA was extracted from INS-1E cells using RNeasy reagent (ams biotechnology) based on phenol/chloroform extraction and isopropanol precipitation according to the manufacturer's recommendations. 1 μ g of isolated RNA was used for the reverse transcriptase (RT) reaction with oligo-dT18 primers performed with the first strand cDNA synthesis kit (Fermentas). mRNA levels of rat insulin receptor were determined by PCR (35 cycles, annealing temperature 61 °C) using specific primers (GenBank accession # NM_017071.2; forward: 5'-CATGAATCCAGCAACTTGATGTGC-3', reverse: 5'-CAGGTGTGTGCCCCCTCGGATGT-3'), yielding a 172-bp fragment. PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. Cell proliferation assays

For the assessment of the effect of LS extracts on cell proliferation, INS-1E cells were seeded in poly-D-lysine-coated standard 96-well plates at a density of 8000 cells/well. Cells were grown for 48 h before incubation with LS extracts for further 24 and 48 h,

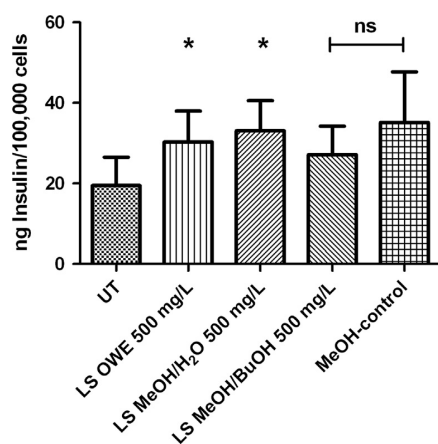


Fig. 1. Levels of insulin released from INS-1E insulinoma cells during 24 h of culture with LS extracts (500 mg/L) measured by ELISA. A statistically significant difference is evident in cells treated with 500 mg/L LS OWE as well as with LS MeOH/H₂O ($n=5$), compared to basal secretion in untreated cells, whereas the MeOH/BuOH extract is without effect, compared to its MeOH-solvent control (* $p < 0.05$; ns, not significant; unpaired t -test).

respectively. Cell numbers were assessed by using CellTiter-Glo[®] Luminescent Cell Viability Assay (Promega) following the manufacturer's instructions at three different time points, i.e. at time point zero (the time point of the addition of the LS-extracts to the cells), as well as 24 and 48 h after addition of LS-extracts. The cell numbers at the time point zero were set to 100% and the cell numbers assessed at the other two time points were normalized to these values.

2.9. LC-MS analyses

HPLC-ESI-MSⁿ analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap mass spectrometer equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). HPLC separation was carried out on a LiChrospher 100 RP18e column, 4.6 × 250 mm, 5 μm (Agilent, CA, USA) at 25 °C using water (pH 2.8 with formic acid) and methanol as mobile phase A and B, respectively. The flow rate was 1.0 mL/min and the following gradient program was used for the LS OWE sample: 10% B (0 min), 50% B (80 min), and 95% B (81 min). The gradient program for the LS MeOH/BuOH sample was as follows: 10% B (0 min), 30% B (20 min), 30% B (42 min), 50% B (82 min), and 95% B (83 min). UV data were recorded from 190 to 400 nm. The eluent flow was split roughly 1:8 before the ESI ion source, which was operated slightly different for the LS OWE sample (capillary voltage: ± 3.7 kV, nebulizer: 32 psi (N₂), dry gas flow: 9 L/min (N₂), and dry temperature: 350 °C) and for the LS MeOH/BuOH sample (capillary voltage: + 3.7/– 4.0 kV, nebulizer: 22 psi (N₂), dry gas flow: 8 L/min (N₂), and dry temperature: 320 °C). The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode to obtain MS² and MS³ spectra (collision gas: He, isolation window: 4 Th, fragmentation amplitude: 1.0 V). The aglycone part of the flavonoids was identified by spectrum matching to an in-house library containing the MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics).

2.10. HPLC-UV-DAD analyses

HPLC-UV-DAD analyses were performed on a Prominence LC-20AD coupled to a Prominence SPD-M20 Diode Array Detector (Shimadzu Corporation, Kyoto, Japan) and to a low temperature-evaporative light scattering detector (ELSD-LT, Shimadzu Corporation, Kyoto, Japan). The

chromatographic system was identical to the one employed for the MS analyses. For the sample solution of the LS OWE 25 mg were dissolved in 100 μL aqueous methanol, 10 μL thereof were injected. For the sample solution of the LS MeOH/BuOH 1.0 mg of the MeOH/BuOH extract were dissolved in 100 μL methanol, 5 μL thereof were injected. The temperature of the ELSD was set to 40 °C at an air pressure of 360 kPa.

2.11. Statistics and data presentation

Data are expressed as mean ± SEM. Statistical analysis was carried out using unpaired or paired Student's t -test or the general linear model with one fixed and one random effect, as applicable. P values < 0.05 between groups were considered as statistically significant. Data were analyzed and plotted using GraphPad Prism version 5.0d for PC (GraphPad Software).

3. Results

3.1. Effects of *Leonurus sibiricus* extracts on insulin release under standard cell culture conditions (SCC)

To determine the effect of extracts from LS on the amount of insulin released from rat INS-1E cells, insulin levels were determined by ELISA. Insulin released into the culture medium under SCC, i.e. in the presence of 11.1 mM glucose, over 24 hours was significantly increased in presence of 500 mg/L LS OWE as well as LS MeOH/H₂O from 19.5 ± 7.0 ng to 30.3 ± 7.7 ng and 33.1 ± 7.5 ng per 100,000 cells ($n=5$), respectively (Fig. 1). No effect was observed at concentrations of 100 mg/L. In contrast, in a separate series of experiments, exposure to 100 μM tolbutamide (a concentration twice as high as necessary to elicit a maximum electrophysiological response in our experiments; see Fig. 2), which is known to enhance insulin secretion in INS-1E cells kept at 2.5 or 3.3 mM glucose, respectively (Merglen et al., 2004; Taguchi et al., 2008), did not significantly enhance insulin secretion from 15.8 ± 7.0 to 19.0 ± 9.5 ng insulin/100,000 cells under SCC conditions within 2 h ($n=6$; $p=0.27$).

3.2. Effects of *Leonurus sibiricus* extracts on INS-1E single cell electrical activity

To investigate if the increased insulin secretion induced by LS was due to stimulation of electrical activity, we performed whole-cell patch clamp recordings of the cell membrane potential (V_{mem}). As shown in Fig. 2B, LS OWE (500 mg/L) acutely and significantly depolarized INS-1E cells from -61.5 ± 1.5 mV to -31.3 ± 1.4 mV within ~1.5 min ($n=4$). In one out of five experiments LS OWE was without effect. Fig. 2A shows a recording of a spontaneously active cell before the application of LS OWE; tracing 1 depicts the electrical activity recorded under control conditions. Along with depolarizing the cell, LS OWE caused an initial increase and a subsequent decline of action potential (AP) frequency with persisting depolarization, whereby the AP amplitude gradually decreased (tracings 2 and 3). The LS OWE-induced depolarization was fully reversible (Fig. 2B); V_{mem} after wash-out of LS OWE was -66.0 ± 4.3 mV ($n=4$). Spontaneous electrical activity, however, did not recur. Subsequent application of the sulfonylurea tolbutamide (100 μM) rapidly depolarized V_{mem} to -14.5 ± 3.1 mV ($n=4$) with low amplitude APs similar to those observed during LS OWE application (Fig. 2A, tracing 4). Tolbutamide was significantly more effective in depolarizing V_{mem} than LS OWE. LS OWE (500 mg/L) applied on top of tolbutamide did not add to the effect of the sulfonylurea (-17.5 ± 5.1 mV; $n=3$; Fig. 2B).

The apparent resemblance of the LS OWE effect to the effect of tolbutamide led us to test if the herb extract might likewise act on

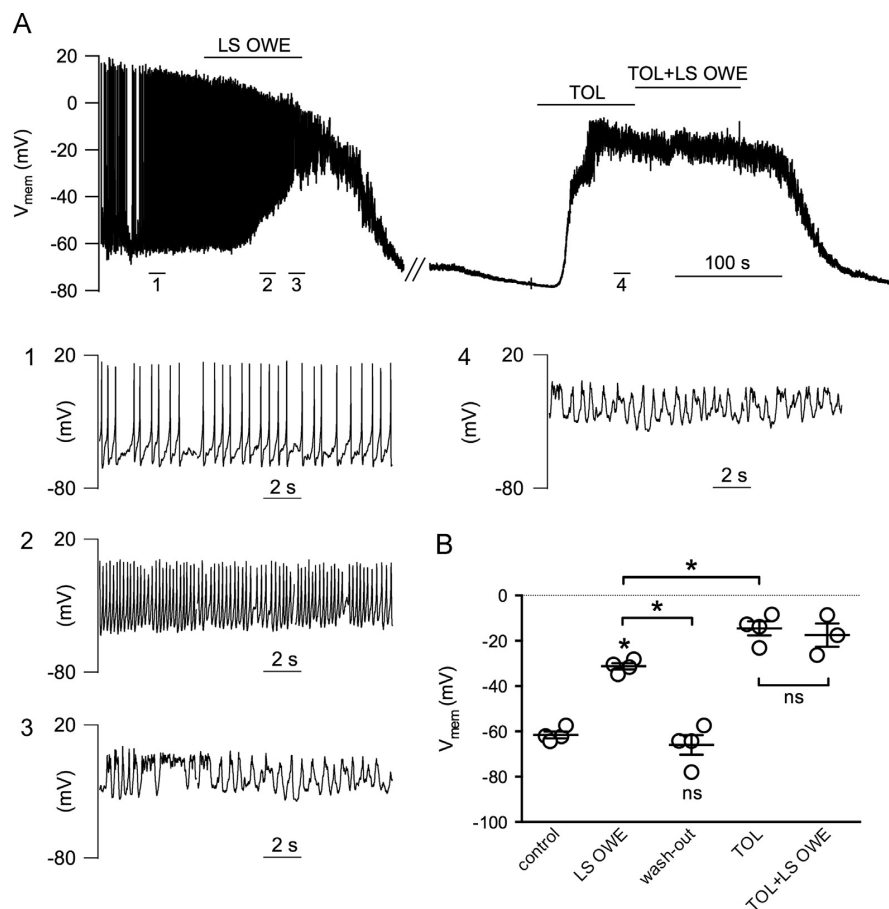


Fig. 2. LS OWE reversibly depolarizes INS-1E cells. (A) Whole-cell zero-current clamp recording in absence and presence of LS OWE (500 mg/L), during wash-out of the extract followed by the application of tolbutamide (TOL, 100 μ M), LS OWE on top of TOL and final wash-out of both substances. 1–4 show 15-second segments of the upper recording on an expanded time scale before (1), during application of LS OWE (2, 3) and tolbutamide (4), as indicated in (A). (B) Summary of patch clamp data ($n=3$, mean \pm SEM; 1way ANOVA with Bonferroni's multiple comparison post-test; * $p < 0.05$; ns, not significant).

the ATP-sensitive K^+ conductance ($I_{K_{ATP}}$). After establishing the whole-cell patch clamp configuration, V_{mem} was clamped to -70 mV and voltage pulses to -80 and -60 mV with durations of 500-ms were applied every 15 s. In this range of potentials, the whole-cell conductance of β -cells is mainly determined by $I_{K_{ATP}}$ (Drews et al., 1998). Clamping the intracellular ATP concentration by dialyzing the cytoplasm with the pipette solution yielded stable control currents within 2–5 min (Fig. 3). Similar to tolbutamide (50 μ M), LS OWE (500 mg/L) brought a significant and reversible inhibition of the K_{ATP} current, reaching a plateau after 1–2 min. An inhibitory effect of LS was observed in 3 out of 5 experiments. Exemplary time courses and current tracings are shown in Figs. 3A and B for LS and Figs. 3C and D for tolbutamide. LS OWE caused an inhibition to $62.0 \pm 8.5\%$ and $57.7 \pm 7.4\%$ of control currents at -80 and -60 mV, respectively (Fig. 3E). Tolbutamide (50 μ M) reduced $I_{K_{ATP}}$ to $34.7 \pm 7.5\%$ (at -80 mV) and $31.7 \pm 4.3\%$ (at -60 mV) of control values. The inhibition by tolbutamide measured at -60 mV was significantly stronger compared to LS OWE.

3.3. Effect of *Leonurus sibiricus* extracts on intracellular Ca^{2+} (Ca_i)

To assess whether the altered electrical behavior of INS-1E cells under LS OWE is paralleled by a rise in Ca_i , cells were loaded with the Ca^{2+} sensitive fluorescent dye Fluo-4/AM and acutely superfused

with extracellular solution containing LS OWE (500 mg/L), tolbutamide (100 μ M) and LS OWE plus tolbutamide. On average, LS OWE and tolbutamide led to a reversible rise of Ca_i up to $30 \pm 10\%$ and $68 \pm 14\%$ ($n=3$) compared to control conditions, respectively (Fig. 4B), with a high variability of single cell responses (Fig. 4A). Peak responses were followed by a decline of the signal in the persisting presence of drugs. In two out of three experiments we applied LS OWE on top of tolbutamide, as shown in Fig. 4A, and observed that LS OWE could elicit a further Ca^{2+} peak. However, similar to the observation concerning V_{mem} recordings, there was no additive effect to the overall response of tolbutamide alone.

3.4. Insulin-receptor expression

To verify the expression of the insulin-receptor (IR) in INS-1E cells, which is the prerequisite for an auto/paracrine effect of the hormone, we performed RT-PCR. As shown in Fig. 5 the expected amplicon (172-bp fragment) can be detected using primers specific for the rat IR α -subunit.

3.5. Influence of *Leonurus sibiricus* extracts on cell proliferation

Insulin acts as a growth factor in β -cells. Given the expression of insulin-receptors in INS-1E cells, an altered proliferative behavior

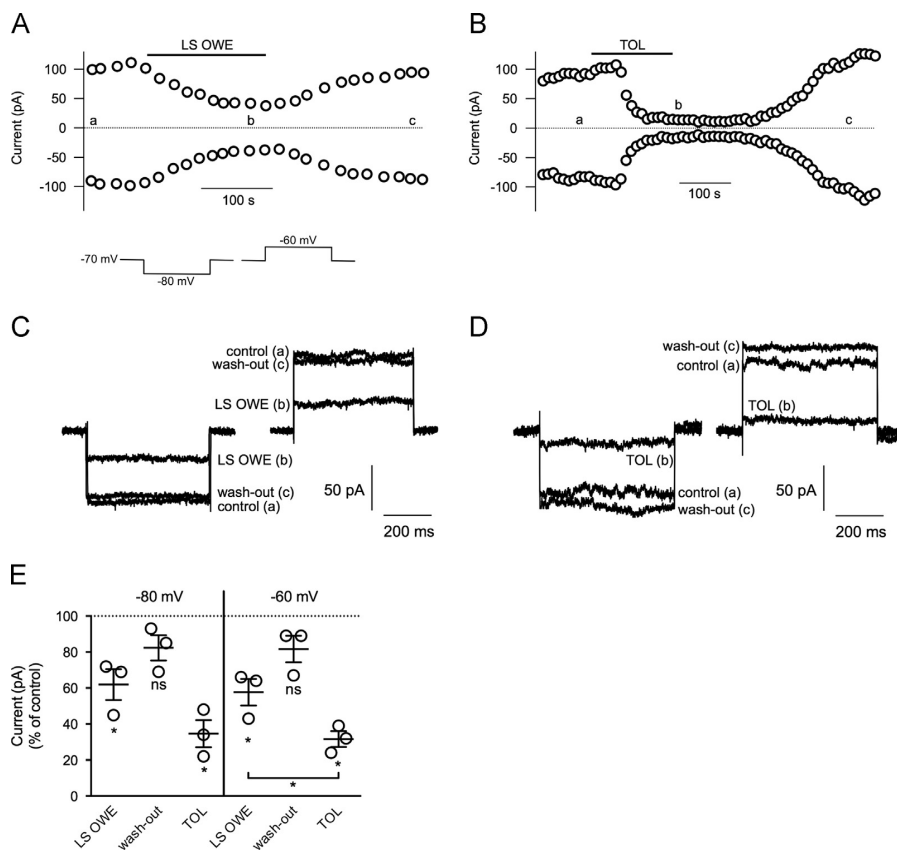


Fig. 3. Inhibition of the ATP-sensitive K^+ current (I_{KATP}) in INS-1E cells by LS OWE (whole-cell patch clamp recordings). Voltage pulses to -80 and -60 mV with durations of 500-ms were applied every 15-seconds from a holding potential of -70 mV (insert). (A+C) I_{KATP} recorded in the absence and presence of LS OWE (500 mg/L) or tolbutamide (TOL, 50 μ M). Each data point represents the average current during a 500-ms voltage pulse. Tracings were zero-offset subtracted so that currents recorded at -80 and -60 mV appear as negative and positive deflections, respectively. (B+D) Corresponding original current tracings at the time-points a (control), b (presence of LS OWE or TOL) and c (wash-out) as indicated in A+C. (E) Scatter blot summarizing the results of 3 individual experiments. Data are shown as % of control currents. * $p < 0.05$ (unpaired t -tests).

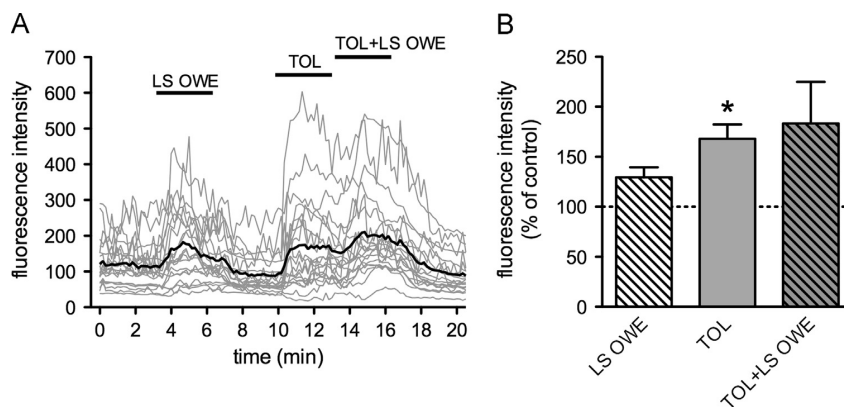


Fig. 4. LS OWE induces an acute and reversible rise in intracellular Ca^{2+} (Ca_i). Cells were loaded with Fluo-4/AM and the fluorescence intensity was measured in the absence or presence of drugs as indicated (500 mg/L LS OWE; 100 μ M tolbutamide, TOL). Images were acquired at 10-s intervals. (A) Responses of single cells (gray tracings) and mean response of all 20 cells in the field of view (black tracing) of an individual experiment. (B) Bar graph showing the average of the mean responses of 3 individual experiments (LS OWE and TOL, $n=3$; paired t -test; TOL+LS OWE, $n=2$; * $p < 0.05$). Data are given as % change in fluorescence intensity compared to control conditions.

of INS-1E cells, caused by the increased amounts of insulin secreted from the cells due to LS treatment, is plausible. We therefore assessed the effect of the three LS extracts, OWE, MeOH/ H_2O and

MeOH/BuOH (500 mg/L each) on cell proliferation 24 and 48 h after treatment. The data are shown in Fig. 6. All extracts significantly increased the number of cells compared to untreated cells or the

respective methanol control (0.1%). After 24 h, LS OWE increased the number of cells to $173 \pm 10\%$, LS MeOH/H₂O to $173 \pm 9\%$ compared to $149 \pm 4\%$ of untreated cells, while LS MeOH/BuOH had no significant effect ($176 \pm 11\%$) compared to the 0.1% methanol-control ($152.5 \pm 2.6\%$). After 48 h, LS OWE caused an increase in cell number to $215.4 \pm 5.2\%$, LS MeOH/H₂O to $218.9 \pm 7.1\%$ compared to untreated cells ($189.1 \pm 1.9\%$). LS MeOH/BuOH also enhanced cell proliferation compared to the control ($223.8 \pm 7.5\%$ vs. $194.1 \pm 2.3\%$).

3.6. Phytochemical characterization of the of *Leonurus sibiricus* plant extracts

The main phenolic constituents in the LS MeOH/BuOH extract were identified as chlorogenic acid, flavonoid glycosides and most prominently glycosylated phenyl ethanoids such as verbascoside and lavandulifolioside by LC-MS (see structural formulae, Fig. 7, and Table 1). These typical phenyl ethanoids were not detected in the LS OWE. The latter was characterized by a high content of stachydrine, which was identified by LC-MS and confirmed by TLC analysis comparing the R_f values with a reference compound after detection with Dragendorff reagent. After depletion of stachydrine, quercetin glycosides and derivatives of phenolic carbonic acids

were found as minor phenolic components of the LS OWE (data not shown).

As stachydrine was found to be present in LS OWE in high amounts, we tested whether stachydrine alone was the active compound accounting for the augmented insulin secretion from LS OWE-treated INS-1E cells. However, the sole application of stachydrine had no effect on insulin secretion in this cell line (3 individual experiments, data not shown).

4. Discussion

In the present study we determined whether extracts from *Leonurus sibiricus* L., a plant used in the treatment of DM and/or DMrd in TMM, affect pancreatic β -cell function. We have chosen rat INS-1E cells as a T2DM cell culture surrogate, as these cells are maintained at 11.1 mM glucose as standard cell culture condition (SCC), thus mimicking a hyperglycemic metabolic situation. If pre-incubated at glucose concentrations of 2.5 mM, these cells exhibit a dose-dependent glucose-induced insulin release with a plateau phase at 15–20 mM and a 50% effective concentration at 10.4 mM (Merglen et al., 2004), which is close to the SCC glucose concentration of 11.1 mM as used in this study. Therefore the experimental conditions chosen still allow for the detection of at least a shallow elevation of insulin release, while simultaneously simulating T2DM conditions. While under these conditions 100 μ M tolbutamide had no stimulatory effect on insulin release within 2 h of treatment, exposure of the cells to aqueous extracts from LS for 24 h resulted in an increased secretion. This effect was observed for OWE and MeOH/H₂O extracts, whereas the MeOH/BuOH extract showed no effect (Fig. 1). This suggests that the active compound(s) in LS responsible for the stimulation of insulin secretion is/are very polar and highly soluble in water. According to the conventional stimulus-secretion-coupling model insulin secretion is driven by elevated concentrations of plasma glucose and other physiological secretagogues like amino acids, triglycerols and free fatty acids. Glucose uptake and metabolism results in an increased ATP/ADP ratio thereby closing of ATP-sensitive K⁺ (K_{ATP} ; KIR 6.2/ SUR1) channels, depolarization of the cell membrane potential (V_{mem}) and stimulation of insulin exocytosis by Ca²⁺ influx through voltage-dependent Ca²⁺ channels (Ashcroft et al., 1989; Henquin, 2000; Satin et al., 1995). In adherence to that model, we tested the effect of LS OWE on the electrical behavior of INS-1E cells. Acute application of LS OWE resulted in closure of

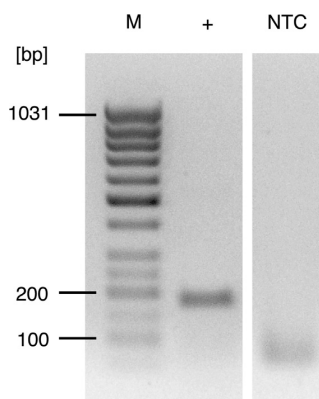


Fig. 5. Expression of the insulin-receptor (IR) in INS-1E cells. The sample containing cDNA template (+) yields a specific amplification product for the IR α -subunit (172 bp). NTC, non-template control; M, 50-bp DNA size marker. (1.5% agarose gel, ethidium bromide staining).

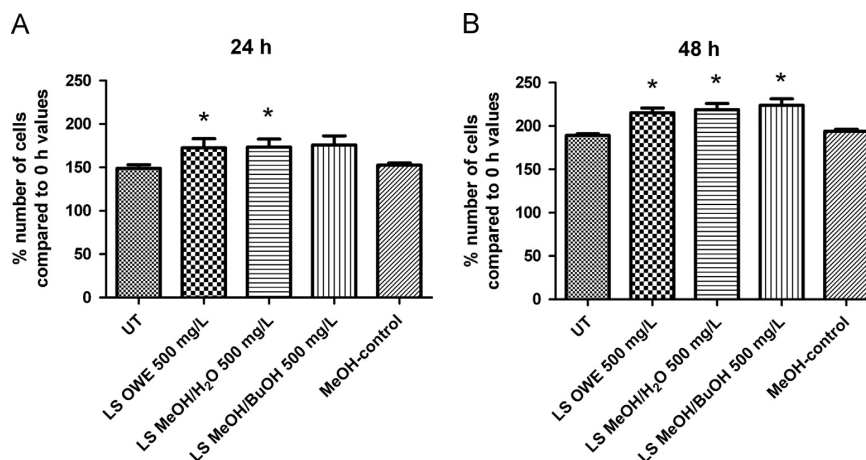


Fig. 6. Increased proliferation rates of INS-1E cells treated with LS OWE, MeOH/H₂O and MeOH/BuOH (500 mg/L) after 24 (A) and 48 (B) hours. * Statistically significant difference compared to control conditions ($p < 0.05$, Mann-Whitney test or unpaired t -test with Welch's correction).

K_{ATP} channels, and depolarization of V_{mem} , which was paralleled by an initial increase and subsequent decline of action potential (AP) frequency with persisting V_{mem} depolarization, gradually decreasing amplitudes and silencing of the APs. Washout of LS OWE caused a repolarization of V_{mem} without spontaneous electrical activity. Subsequent application of tolbutamide elicited a depolarization with low amplitude APs similar to those observed during application of LS OWE. This behavior was not further modified upon additional application of LS OWE (Fig. 2). LS OWE- and tolbutamide-induced depolarizations of V_{m} were paralleled by an increase in Ca_i (Fig. 4), most likely reflecting the opening of voltage-activated Ca^{2+} channels. In further experiments we could show that, similar to tolbutamide, LS OWE inhibits K_{ATP} channels (Fig. 3). In a previous study on the effect of the phytoestrogen resveratrol on the electrophysiological and secretory behavior of INS-1E cells, we found that the compound inhibits K_{ATP} channels (IK_{ATP}), L- and T-type voltage-dependent Ca^{2+} channels and swelling-activated Cl^- currents (IC_{islet}, IC_{glucose}, IC_{swell}) (Jakab et al., 2008). Even though both, hyperpolarizing IK_{ATP} as well as depolarizing Ca^{2+} - and Cl^- currents, were inhibited by

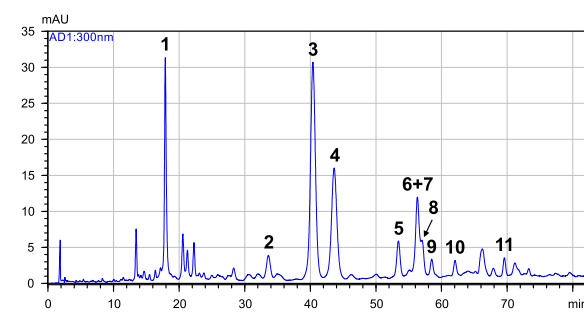


Fig. 7. HPLC chromatogram of the LS MeOH/BuOH extract showing the DAD response at 300 ± 2 nm. The extract was also analyzed by LC-MS and the proposed structures of corresponding compounds 1–11 can be found in Table 1.

resveratrol, the net effect was an inhibition of electrical activity (APs) and insulin release. Since LS OWE represents a mixture of compounds, it is likely that it exerts multiple effects on different ion conductances – probably with different temporal and dose-response profiles. The net effect of LS OWE is a depolarization and stimulated insulin release, for which the block of K_{ATP} channels provides a plausible explanation. However, we cannot exclude that the effect of LS on V_{mem} and Ca_i are due to additional effects on other ion conductances (e.g., inhibition of further K^+ currents, or activation of depolarizing ion conductance(s) like Ca^{2+} - or Cl^- channels, or a combination thereof). It has previously been shown that aqueous extracts from *Leonurus cardiaca*, another species of the genus *Leonurus* used to treat tachyarrhythmia and other cardiac disorders, act as a mixed inhibitor of ICa_L- and IK_r-channels as well as a modulator of If- channels (Ritter et al., 2010). The natural polyphenolic flavonoid quercetin has recently been found to stimulate insulin secretion in INS-1 cells and rat isolated pancreatic islets by direct activation of L-type calcium channels (Bardy et al., 2013). Since quercetin-O-hexosyl hexoside and quercetin-O-deoxyhexosyl hexoside are constituents of the LS extract (Table 1 and Fig. 7), it may be assumed that the observed effects of LS on insulin secretion/proliferation may at least in part arise from a comparable effect. In mouse pancreatic islet cells the mitochondrial uncoupling protein 2 (UCP2) could be identified as a target of genipin, a compound found in *Gardenia jasminoides* Ellis fruits, which is used in traditional Chinese medicine to treat T2DM symptoms; the substance was shown to inhibit UCP2, to increase mitochondrial membrane potential and ATP levels, to inhibit K_{ATP} channels and to stimulate insulin secretion (Zhang et al., 2006).

Besides its metabolic action on peripheral tissues, insulin also acts in an auto/paracrine manner as e.g. shown in MIN6 cells (Muller et al., 2006), which is important for cell proliferation and survival and hence the maintenance of the β -cell mass of pancreatic islets. Recent studies have further concluded that insulin is a positive regulator of its own production (Leibiger et al., 1998; Xu et al., 2000) and β -cell-specific insulin receptor (IR) knockout mice manifest reduced islet size, progressive glucose intolerance,

Table 1

Proposed structure and MS data of the secondary plant metabolites identified in the LS MeOH/BuOH extract.

#	Proposed structure ^a	[M-H] ⁻	Main fragment ions (> 10% Rel. Int.)	References
1	Chlorogenic acid	353.1	MS ² [353.1]: 190.8	Ritter et al. (2010)
2	Quercetin-O-hexosyl hexoside ^b	625.2	MS ² [625.2]: 445.1, 300.9, 299.9, 270.9, 254.9 MS ³ [299.9]: 270.9, 254.8, 178.7, 150.7	–
3	Lavandulifolioside ^c	755.3	MS ² [755.3]: 623.2, 593.3, 461.1 MS ³ [593.3]: 461.2 MS ³ [461.1]: 315.0, 314.1, 178.8, 160.8, 144.8, 135.9, 134.9, 133.9	Krasteva et al. (2011) and Ritter et al. (2010)
4	Verbascoside ^c	623.2	MS ² [623.2]: 461.2 MS ³ [461.2]: 315.0, 160.8, 134.9	Li et al. (2012) and Ritter et al. (2010)
5	Lavandulifolioside-isomer	755.3	MS ² [755.3]: 623.2, 593.3, 461.2 MS ³ [593.3]: 461.2 MS ³ [461.2]: 315.0, 297.0, 160.8, 135.9, 134.8	Cai et al. (2005)
6	Leonoside A	769.3	MS ² [769.3]: 637.3, 607.2, 593.3, 461.2 MS ³ [637.3]: 491.1, 461.2 MS ³ [593.3]: 461.2	Calis et al. (1992)
7	Quercetin-O-deoxyhexosyl hexoside ^b	609.2	MS ² [609.2]: 300.9, 300.0, 270.9 MS ³ [300.9]: 272.9, 270.9, 254.8, 178.8, 150.7	Fernando Rolim de Almeida et al. (2008) and Hayashi et al. (2001)
8	Verbascoside-isomer	623.2	MS ² [623.2]: 461.1 MS ³ [461.1]: 315.0, 160.8, 134.9	–
9	Leucoceptoside A	637.3	MS ² [637.3]: 461.1 MS ³ [461.2]: 315.0, 297.0, 160.8, 142.8, 134.9	Tasdemir et al. (1999)
10	Leonoside A-isomer	769.3	MS ² [769.3]: 637.3, 593.3, 461.1 MS ³ [637.3]: 491.1, 461.2 MS ³ [593.3]: 461.1	–
11	Leonoside B	783.3	MS ² [783.3]: 651.3, 607.3 MS ³ [607.3]: 475.2	Calis et al. (1992)

^a The identification of the compounds is based either on direct comparison of the experimental MS data with literature data or on congruence of the MS- and UV-data with the structure of known secondary metabolites from *Leonurus sibiricus* or other *Leonurus* species.

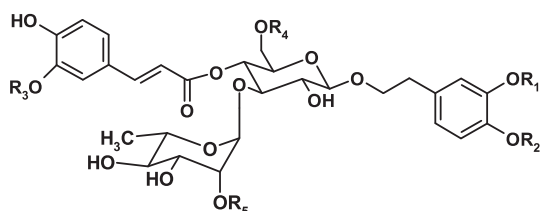
^b The aglycone part of the flavonoids as identified by spectrum matching to an in-house library containing the MSⁿ spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics, Bremen, Germany).

^c These constituents were confirmed by comparison of the retention times and UV-spectra with reference compounds.

as well as reduced insulin content (Kulkarni et al., 1999). In the current study, we show that LS extracts stimulate INS-1E cell proliferation. Since we also demonstrate the presence of the IR in INS-1E cells, the LS-boosted proliferation rates may be explained by such an auto/paracrine action of the hormone. Moreover, it cannot be excluded that LS constituents have a direct effect on cell proliferation. With respect to that it is noteworthy to mention that not only LS OWE and LS MeOH, but also LS MeBuOH was able to stimulate INS-1E cell proliferation (Fig. 6) despite its inability to increase insulin secretion (Fig. 1). Clearly the cellular signaling pathways mediating the proliferative effects of LS extracts need to be identified in further studies.

In conclusion we show that extracts from *Leonurus sibiricus* increase insulin secretion of rat INS-1E insulinoma cells that can be explained at least in part by a transiently increased electrical activity and elevation of the intracellular calcium concentration. Moreover, treatment with LS extracts stimulates INS 1-E cell proliferation. These findings may provide explanations for the empirical use of LS formulations in the treatment of diabetes mellitus and its related disorders in traditional medicine.

Structural formulae



Verbascoside=Acteoside (R_1 – R_5 =H)

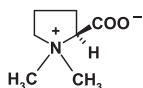
Lavandulifolioside (R_1 , R_2 , R_3 , R_4 =H; R_5 =arabinose)

Leucosceptoside A (R_1 , R_2 , R_4 , R_5 =H; R_3 =Me)

Leonoside A (R_1 , R_2 , R_4 =H; R_3 =Me; R_5 =arabinose)

Leonoside B (R_1 , R_3 =Me; R_2 , R_4 =H; R_5 =arabinose)

Stachydrine



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