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DISSERTATION

Dianthus versicolor FISCH. – Phytochemical and biological investigations of a traditional Mongolian medicinal plant

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This thesis is based on the following publications:

1. **Obmann, A.**, Tsendayush, D., Thalhammer, T., Zehl, M., Vo, T.P.N., Purevsuren, S., Narantuya, S., Kletter, C., Glasl, S.
   Extracts from the Mongolian traditional medicinal plants *Dianthus versicolor* FISCH. and *Lilium pumilum* DELILE stimulate bile flow in an isolated perfused rat liver model
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2. **Obmann, A.**, Werner, I., Presser, A., Zehl, M., Purevsuren, S., Narantuya, S., Kletter, C., Glasl, S.
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3. **Obmann, A.**, Zehl, M., Purevsuren, S., Narantuya, S., Reznicek, G., Kletter, C., Glasl, S.
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4. **Astrid Obmann**, Sodnomtseren Purevsuren, Martin Zehl, Christa Kletter, Gottfried Reznicek, Samdan Narantuya, Sabine Glasl
   HPLC Determination of flavonoid glycosides in Mongolian *Dianthus versicolor* FISCH. (Caryophyllaceae) compared to quantification by UV-spectrophotometry
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1 Introduction

Traditional medicinal systems, especially of Asian origin, have become more interesting in Western countries during the last decades. Traditional Chinese Medicine, Ayurveda and Traditional Tibetan Medicine are popular ones, which are often applied to patients in many European countries as alternative (complementary) medical treatment. Rather unknown is the Traditional Mongolian Medicine (TMM). Considering the Mongolian history there has been an increasing interest in traditional Mongolian medicine since the beginning of the 1990s. This led to scientific investigations in this ancient medical system not only within Mongolia, but also together with foreign academic institutions in Asian and Western countries. Among the new academic partners were also various universities in Austria, and one of them is the Department of Pharmacognosy in Vienna [1].

1.1 Traditional Mongolian Medicine

Literature about TMM is scarce and mostly written in Mongolian or Russian. However, some books are also edited in English and some interesting information about the Mongolian health system and the use of traditional medicine during the last decade is compiled in reports available in online-databases [2, 3, 4, 5]. The next chapters provide a comprehensive overview about the available literature and refer to the following topics: Historical background of TMM, principles of TMM, traditional medicinal preparations, the Mongolian health care system, and the health situation in Mongolia.

1.1.1 Historical background of TMM

TMM developed from Mongolian folk medicine and was highly influenced by the Traditional Tibetan Medicine and Ayurveda. During the 13th century, Mongolia came under the missionary influence of Lamaism. Its sciences of healing, which initially were just healing practises, were established and developed gradually. Medical practitioners were called from Tibet to Mongolia as court physicians

and independent medical schools developed in Mongolia, even though under Tibetan names [6]. Traditional Mongolian medical knowledge was transmitted over generations in various ways: through private practitioners who got their medical knowledge from the elders and through educational and faculty systems which were 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 scientific studies for scholars of various subjects [7]. In 1921 Mongolia declared independence from China and, in 1924, the Mongolian People's Revolutionary Party established the People's Republic of Mongolia. The Communists' system of the Soviet Union gained more and more influence on Mongolian politics, and in 1936 the Mongolian republic entered into an alliance with the Soviets. This communist influence caused dramatic changes in political and social structures. Lamaism, the religion which had dominated until then was banned officially. In 1937 the destruction of monasteries and persecution of monks started and led to a collapse of religious institutions and, subsequently, also to a come down of traditional medicine [1]. Finally, the last Tibetan pharmacy in Mongolia was closed officially, and Western medicine became the only permitted possibility of medical treatment [2]. For a long time it was forbidden to practise the ancient Mongolian medicine. However, traditional medicine was still popular and used secretly, especially in the treatment of chronic diseases. After the political changes in the Soviet system at the beginning of the 1990s the restrictions were lifted and Mongolian medicine became officially recognized as its own Mongolian heritage [1]. During the 1990s the state policy of the new Mongolian government focused on the revival of Buddhism and TMM. A new Manba Datsan including a hospital and training centre with the aim to restore TMM in the country was founded [7]. In 1991 the Otoch Manramba Medical College at this respective Manba Datsan was established in order to provide training courses in traditional Mongolian medicine. This institution aims at combining traditional methods of treatment with the modern achievement of biomedicine. Besides training activities, the college is pursuing research and maintains regular contact with other medical research institutions, e.g. in India, Japan, USA, Switzerland, UK, Germany, and Austria [7]. Another institution, the School of Traditional Medicine of the Health Sciences University of Mongolia, offers all

degrees of education in traditional medicine and promotes research in this field. As a consequence of those efforts, today, various hospitals and clinics apply western as well as traditional methods. Furthermore, many small clinics, which offer special traditional treatments, have been established throughout the country during the last decades. By 2007, the number of people who received traditional medicinal health care reached 23.6% of the total population [2].

1.1.2 Principles of Traditional Mongolian Medicine

The basics of TMM follow mainly the guidelines of Traditional Tibetan Medicine and Tibetan Buddhism. Although there exists a variety of ancient medical treatises written by Mongolians, traditional doctors accept the main Tibetan medical treatise, the *rgyud bzhi* (Dürben Ündüsün in Mongolian), literally "The Four Roots", as the basis of their medical knowledge [1, 6]. According to traditional medicine, the human body is seen as an entity and health relies on the equilibrium of the three life sustaining principles, named in Mongolian *hii* (Tibetan *rlung*), *šar* (Tibetan *mkhris pa*) and *badgan* (Tibetan *bad kan*). Any disturbance in the equilibrium of the three principles results in a disease. Therefore, traditional medical treatment focuses on the re-establishment of this equilibrium. Pulse reading, tongue examination, urine check (smell, taste, and colour) and interrogation of the patient serve as diagnosis parameters. It has to be kept in mind that traditional methods of diagnosis do not necessarily correlate with diseases as classified in Western medicine. In general, TMM differentiates 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. E.g. fever, localisation of pain, and yellow skin are mentioned as symptoms which indicate a liver involvement. The therapy of diseases, according to traditional medicine, focuses first on changes in the patient's diet and behaviour in daily life. Other therapeutic measures are medication, massages, blood-letting, steam baths, acupuncture, and moxibustion. The medical preparations are usually complex mixtures of a varying number of ingredients, mainly plants but also minerals and animal drugs. In general, one ingredient is specified as the leading one, while the remaining ones support the main ingredient in its efficacy. A specific nature and particular

qualities as well as secondary qualities, which influence the three life sustaining principles in the human body, are assigned to each ingredient. In general, hot diseases are treated with medicines having a cooling nature, whereas cold diseases require a treatment with medicines of warming qualities. Furthermore, it is very important, that the qualities and second qualities of the ingredients are well balanced because otherwise the medicine may have an adverse effect on the patient instead of a favourable one [1].

1.1.3 Traditional Mongolian medicinal preparations

The three main types of traditional medicines in Mongolia are available in the form of *talkh*, *tan*, and *pills*. The *tan* preparation is a traditional medicinal drug preparation at a pulverization level of 0.5-2 mm. It consists of either a single component or a combination of several ingredients. The ingredients are selected separately and cleaned, then blended and pulverized. This kind of preparation is boiled or macerated with water, milk or soup. In 2006 about 32% of the total amount of traditional medicines was *tan* preparations [2]. The *talkh* preparation, which is the most common form of traditional preparations (50% of total preparations in 2006), consists of a single component or a mixture of ingredients but, compared to *tan* preparations, the pulverization level is 0.125-0.315 mm. *Talkh* preparations are applied topically or internally, with water, milk or soup [2]. For the preparation of *pills* the finely powdered medicine is mixed with a suitable excipient such as water, honey, molasses or other liquids. *Pills* can be coated or uncoated and are usually taken with water, milk or soup. *Pill* preparations play a minor role and amounted only to 6% of the total number of manufactured preparations in 2006 [2]. 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. Today, the mode of preparing medicines has mostly changed. A large amount of traditional remedies is produced in the big centres of traditional medicine and Mongolian companies using modern production methods. Ancient sutra books as well as more recent formula handbooks are used by traditional medicinal drug manufactures for the production of the preparations. In 2006 a total of 36

traditional medicinal drug manufactures were registered officially in Mongolia. They represent 16% of the total number of drug manufactures and can be found in public and private sectors [2]. Among the private traditional medicinal drug manufactures, for example, is the Training Centre of Mongolian Traditional Medicine Manba Datsan in Ulaanbaatar. Modern equipment allows the production of traditional *tan* and *talkh* preparations, *pills*, mixtures, extracts and ointments. 120 types of *tan*, *talkh*, and *pills* are produced using traditional and alternative formulas and technologies [2]. The needed plants, animal drugs or minerals are collected by the locals in the Mongolian countryside or are purchased at various markets in and outside Mongolia. Such traditional medicines are sold within the Republic of Mongolia by traditional medicinal drug wholesalers. Some of those products are even exported [1]. In 2001 public and private drug manufactures produced 4.8 tons of traditional medicinal drugs. In comparison to that, the total amount of traditional medicinal drugs produced in 2006 increased by 60% up to 7.8 tons. This reflects the increasing importance of traditional medical treatment in Mongolia. In 1965 Mongolia started the implementation of a quality control system founding the Mongolian State Central Inspection Laboratory. Today, this institution and the State Inspection Agency are responsible for monitoring and standardizing traditional preparations with many ingredients to ensure their quality. Mechanisms of regulation include the Mongolian drug law (1998), an approved list of standardized medicinal raw materials as well as inspection guidelines for traditional medicinal materials and formulas (2003). Furthermore, validated procedures for analyses of active ingredients by TLC or HPLC are very important. According to the policy on improving the Mongolian Traditional Medicine new model guidelines of inspection are going to be developed similarly to the international guidelines. E.g., the microbiological quality is evaluated by the enumeration of total bacteria and fungi contamination of traditional drugs. All traditional medicinal drug manufactures are expected to provide medicinal raw materials for inspection by the State Inspection Agency Central Laboratory. Nevertheless, further improvement of standardization and quality inspection of traditional medicines is required to ensure a high quality [2].

1.1.4 The Mongolian health care system

Information about the Mongolian health care system can be found mainly in reports prepared for the WHO, such as the “Health indicators Mongolia” [3] or the review “Health Systems in Transition” [4]. The following passages give an insight into the development of the Mongolian health care system since the beginning of the 1990s. However, some details remain unclear due to a lack of information.

With the economic transition after the political changes in the former Soviet Union the state expenditure for the social sector in Mongolia fell and the quality of health care decreased dramatically as the health care system could not maintain itself. During this transition period international aid and donor's assistance helped to compensate the withdrawal of Soviet financial and social support. The resource gap in the social system in the early 1990s was filled by introduction of user fees and in 1994 by the introduction of a compulsory health insurance. The system is now financed primarily through the state budget for fixed costs, while variable costs are covered by the Health Insurance Fund (HIF). Since 1993, there has been a series of attempts to develop a package of "essential" and "complementary" health care services. Now the "essential" package of services, which includes medical emergency and ambulance services, and treatment for certain diseases requiring long-term care, is provided free of charge, whether or not a patient is covered by health insurance. All other services, generally curative and diagnostic, are included in the "complementary" package, which is financed by the HIF [4]. According to the report “Health indicators Mongolia” [3] the Mongolian health care system is characterized by three levels of care: primary health care is provided mainly by family group practises in Ulaanbaatar, in *aimag* centres, and in *soum* and *inter-soum* hospitals. Secondary care takes place in district general hospitals in Ulaanbaatar and in *aimag* general hospitals. Tertiary care is provided in major hospitals and specialized professional centres in Ulaanbaatar [3]. Unfortunately, it is not specified which services are exactly included in the three levels of care. Traditional medical clinics and supervision rooms were established in the capital and *aimag's* central hospitals according to the implementation of the Ministry of

Health (MoH) policy – Development of Mongolian Traditional Medical Service from 1991-1995. The aim was to increase the number of traditional specialists in the capital and in *aimags'* hospital services and to facilitate the organization of basic traditional medical training in the capital, *aimags* and districts. The first clinical traditional wards with 50 beds and 8 supervision rooms were opened in the State Clinical Central Hospital in 1989. The hospital division was later expanded to become the Traditional Medicinal Scientific Technology Corporation with 120 beds in 1998. The Hepatological Clinical Centre of Traditional Medicine, the Traditional Medical Training Centre Manba Datsan, and 110 private sector hospitals were established in 1991 [2]. 51.1% of the traditional medical hospitals budgets are sourced from a mixture of funders. Since 2002 the number of traditional hospitals in Ulaanbaatar has increased up to 79 in 2006. An important topic is the education of traditional medical doctors and nurses. Since the late 1990s traditional medical doctors have been educated in public and private universities in Mongolia. The Department of Traditional Medicine was founded in 1989 by the decision of the Health Sciences University of Mongolia (HSUM) with 3 lectures and 24 students. In 1993 the first traditional medical doctors graduated. The School of Traditional Medicine was established as one of the 7 independent institutes of HSUM in 2000. In two other public institutions, Darkhan city's Medical college and Govi-altai's Medical college, traditional medical doctor assistants and nurses are educated. Otoch Manramba Medical University was established in 1991 as the first private traditional medical university. In total, for the years 1991-2008, 2102 traditional medical doctors have been graduated, and all over Mongolia 331 traditional medical doctors are working in the public health sector [2].

1.1.5 Health situation in Mongolia

Since the beginning of the 1990s an increasing prevalence of lifestyle-related chronic diseases has been observed and has become an important public health issue. The number of people suffering from cardiovascular diseases, cancer, injuries, and poisonings has increased, while deaths from communicable and respiratory diseases have declined. However, respiratory and gastrointestinal diseases still dominate the morbidity pattern. Infectious

diseases, like HIV, sexually transmitted infections (STI), tuberculosis (TB), viral hepatits, and zoonotic diseases, which are related primarily to risk factors, such as behaviour, lifestyle choices, and living conditions, are showing a tendency to increase. The Mongolian Steps Survey on the Prevalence of Non-Communicable Disease Risk Factors 2006, conducted by the Ministry of Health, revealed that 90.6% of the surveyed population had at least one risk factor for developing a non-communicable disease (NCD), 20.7% had three or more risk factors or were at high risk. The government of Mongolia has been active in population health education campaigning, but health behaviours remained largely unchanged. Although the majority of respondents knew about the negative impact of alcohol on health, drinking and smoking rates are high. It could be argued that the high levels of alcohol consumption and smoking are closely related to the socioeconomic problems of the transitional period, including poverty and unemployment [4]. However, a survey in 2005 showed the prevalence of smokers to be 28%, among them 24.2% daily smokers. In addition, 23% of the surveyed population reported low levels of physical activity. The National Programme on NCD Control and Prevention for 2006-2015 aims to reduce risk factors, thus contributing to a reduction in NCD morbidity and mortality [5]. In 2008 the leading causes for outpatient morbidity were diseases of the respiratory system, the digestive system, the genitourinary system, and the circulatory system. The incidence of those diseases were in general higher in rural than in urban areas. The predominant causes of hospitalization in *soum* and *aimag* hospitals were diseases of the genitourinary and respiratory system, whereas the residents of Ulaanbaatar were mainly admitted because of diseases of the digestive and circulatory systems. Moreover, 25.6% of the patients with diseases in the digestive system had liver problems. 13.7% suffered from cholecystis. Compared to the figures of 2000, the number of patients with liver problems increased from 18.9% to 25.6%, whereas the number of patients with cholecystis decreased from 14.6% to 13.7% in 2008 [3]. As medicinal plants are often employed for the treatment of diseases related to the digestive system, scientific research on the used remedies and traditional preparations is required. This was one of the reasons for the starting of the Austrian-Mongolian research-cooperation on traditional Mongolian medicinal plants.

1.2 Liver and bile flow

The liver plays an important role in the metabolism and has a number of functions in the body, among them detoxification, protein-biosynthesis, and glycogen storage. It is the largest glandular organ in the human body and produces the bile which aids in the digestion via emulsification of lipids. The organ is located on the right side of the abdominal cavity beneath the diaphragm, and it is divided into two lobes of unequal size. On the lower, concave, side of the liver (*porta hepatis*) two important blood vessels are entering: the hepatic artery (*arteria hepatica*) and the portal vein (*vena porta*). The venous blood, brought via the portal vein from the stomach, small intestine, and related organs, contains digested nutrients and covers about 75% of the liver's blood supply whereas the hepatic artery blood, enriched in oxygen, contributes to the blood supply only to 25%. Furthermore the bile ducts (*ductus hepatici*) are leaving the liver via the *porta hepatis*. The liver tissue is composed of lobules of 1-2 mm size, which are separated by weak connective tissue. Each lobule consists of hepatocytes, which possess a wide spectrum of different enzymes, and carry out most of the liver functions. The hepatocytes, forming characteristic tissue structures, are nerved by capillary vessels (sinusoids). Their wall is formed by two cell types, namely endothelial cells and kupffer cells. Kupffer cells are specialized macrophages and part of the reticuloendothelial system [8].

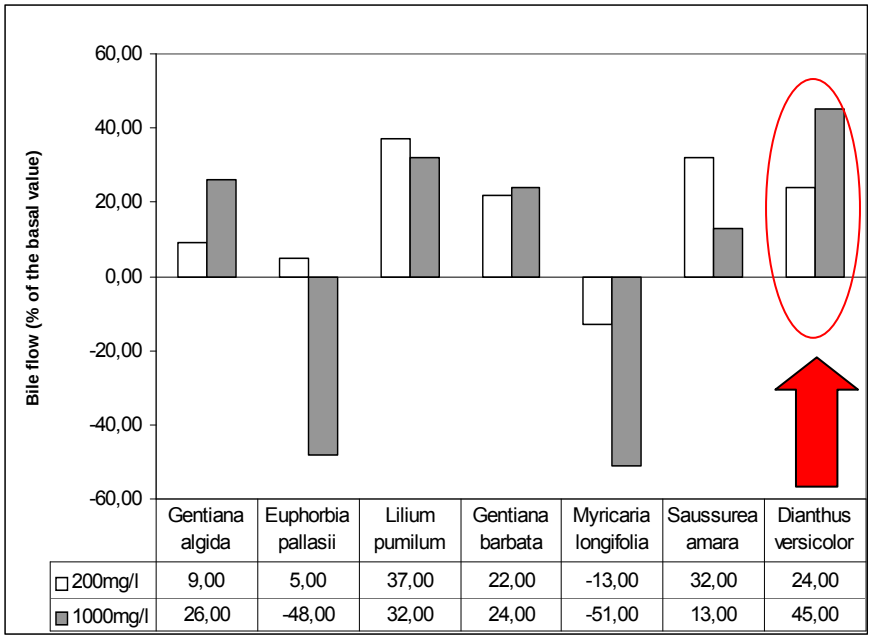
The bile is produced by hepatocytes and secreted into bile canalicula which start in the centre of the liver lobules and merge in the periphery of the lobules, forming the left and right hepatic ducts. The hepatic ducts merge near the *porta hepatis* and form the so called common hepatic duct (*ductus hepaticus communis*). The cystic duct (*ductus cysticus*) joins with the common hepatic duct and forms the common bile duct (*ductus choledochus*). Bile can either drain directly via the common bile duct into the duodenum or be temporarily stored in the gallbladder via the cystic duct [8].

Biliary secretion (choleresis) relies on two mechanisms. On the one hand, excretion of conjugated bile acids, bilirubin and organic ions leads to an osmotic gradient which is responsible for the passive water movement into the canalicula (bile salt dependent bile flow). On the other hand, an osmotic

gradient is developed by an active Na^+-transport from hepatocytes into the canalicula or the excretion of other osmotically active compounds which is also followed by an increased water flow into the hepatic duct (bile salt independent bile flow). On its way through the hepatic duct the bile is modified by reabsorption or secretion of electrolytes, which has an impact on the additional flow or the reabsorption of water. The bile consists of conjugated bile acids, cholesterol, phospholipids (lecithin), enzymes, and anorganic ions (Na^+, Cl^-, HCO_3^-). The production of bile is stimulated by secretin, a peptide hormone, and bile acids itself [8]. Bile acids are amphipathic steroidal compounds derived from the enzymatic catabolism of cholesterol, which is the most important route to eliminate cholesterol from the body. Bile acids are conjugated at their terminal carboxyl group with either glycin (mainly in humans) or taurin (mainly in rodents). This amidation increases their amphipathic character making them more hydrophilic and better excretable into the bile. In the intestinal lumen bacterial enzymes dehydroxylate bile acids, and a part of these secondary bile acids is absorbed from the intestine and recirculates entero-hepatically. The detergent properties of bile acids aid in the solubilisation of cholesterol in bile and of dietary fats and cholesterol in intestinal fluid, a prerequisite for their intestinal absorption [9]. The complex anatomical structure of the liver and the biliary tree makes clear that liver and biliary tract diseases and their clinical manifestations can not be classified easily. Furthermore, it has to be considered that its unique dual blood supply makes the liver an intermediate filter of most of the venous drainage of the abdominal viscera. This often leads to secondary hepatic involvement in a number of extrahepatic diseases and makes the liver a relatively common site of solid tumor metastases [10].

A reduced function of the liver and the gallbladder is generally associated with an impairment of the bile flow. Plant extracts, exhibiting bile-flow-stimulating effects, may resolve disturbances in the hepato-biliary system caused by a reduced bile secretion (cholestasis). Phytotherapy, as applied in Western countries, suggests a variety of plants to cure disorders of stomach, liver and the biliary system. Discomfort in the stomach, bloating, lack of appetite, nausea and mild diarrhoea or constipation are improved by the intake of choleretic compounds such as artichoke leaf extract which stimulates bile secretion and shows hepatoprotective effects in animal models [11].

In cooperation between the Department of Pharmacognosy, University of Vienna, the Health Sciences University of Mongolia, and the Institute of Pathophysiology, Medical University of Vienna about 20 plants were selected which are most frequently used in the therapy of liver disorders according to TMM. Methanolic and aqueous extracts of these plants were screened for their effect on the bile flow in the model of the isolated perfused rat liver (Fig. 1) [1]. Such experiments are not only suitable to detect choleretic effects but provide also a possibility for investigations of metabolites secreted into the bile. Thus, extracts from the Mongolian medicinal plant *Saussurea amara* were identified as potent choleretic agents [12]. Furthermore, an aqueous extract of *D. versicolor* showed a dose dependent effect on the bile flow in the tested concentrations (Fig.1).



	Gentiana algida	Euphorbia pallasii	Lilium pumilum	Gentiana barbata	Myricaria longifolia	Saussurea amara	Dianthus versicolor
□200mg/l	9,00	5,00	37,00	22,00	-13,00	32,00	24,00
■1000mg/l	26,00	-48,00	32,00	24,00	-51,00	13,00	45,00

Fig. 1 Influence of aqueous extracts from selected Mongolian medicinal plants on bile flow tested in two different concentrations in the model of the isolated perfused rat liver. (chapter 2.4.1 p. 27) *D. versicolor* is indicated by the red arrow.

Due to these results *D. versicolor* was chosen for intensive phytochemical investigation and further biological testing presented within this PhD thesis.

1.3 *Dianthus versicolor* FISCH.

Dianthus versicolor FISCH. belongs to the plant family of the Caryophyllaceae. It is distributed mainly in Asia, e.g. Siberia, Kazakhstan, Mongolia (except the Southern provinces), and China, but also in Eastern Europe, e.g. Ukraine [13, 14].

The plant is a perennial herb with a height of 20-50 cm and branched stems. The sessile lineal-lanceolate leaves in opposite position are 13-18 mm long and 3-7 mm wide. Flowers can stand solitary or in groups of two or three. The calyx is a tube of 13-18 mm length and 3-5 mm width. The petals are 20-25 mm long and of purple colour [15]. On the inner side of each petal a wing-like appendage is visible, and five brownish nectaries are located at the base of the stamens. The plant grows on rocky ground, pebbles or in the steppe.

Fig. 2 *Dianthus versicolor*; photo: Christa Kletter, Dept. of Pharmacognosy, Vienna

Until now literature on anatomical investigations of *D. versicolor* is not available. First microscopic analyses show anatomical structures which are similar to *Dianthus superbus*. On the leaf numerous short unicellular trichomes with

thickened cell wall and warty cuticle are visible. The stomata appear diacytic, and epidermis cells show bead-like thickenings. Oxalate druses appear in leaf, stem and sepals. On the sepals and petals uni- to multicellular hairs are found. The epidermis of the ovary is partly turned into a sclerenchyma with papillose style [16]. To confirm these first results detailed analyses concerning the distribution of microscopic characters in different tissues of the plant are required. Therefore further investigations on a greater number of herbarium specimens are in progress [17].

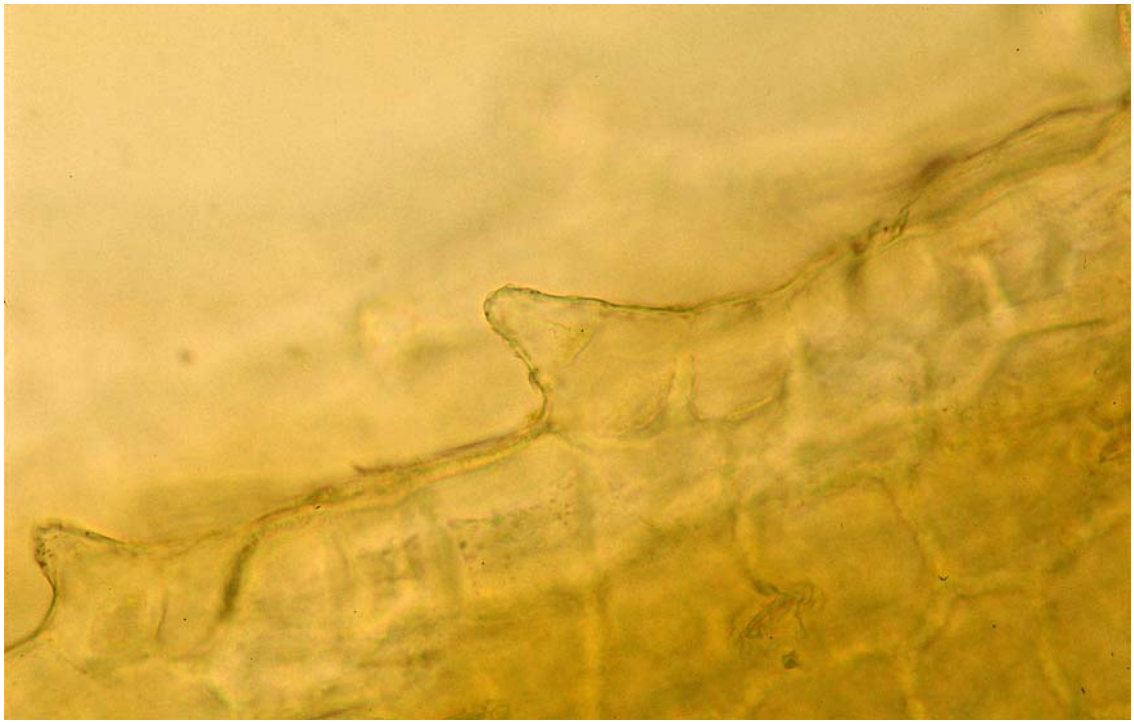


Fig. 3 Microscopic investigation of a *D. versicolor* leaf; the surface view (x 400) shows short unicellular hairs; photo: Christa Kletter, Dept. of Pharmacognosy, Vienna

In traditional medicine the aerial parts (flowers, stems, and leaves) are used. Data about the chemical composition of this plant are scarce. Boguslavskaya et al. described two flavonoid-C-glycosides, namely chrysoeriol-6-C-syn-α-D-glucopyranoside and chrysoeriol-6-C-anti-α-D-glucopyranoside [18]. Ma et al. identified seven new and nine known triterpenoid saponins in *D. versicolor* [19]. Some of those compounds showed inhibitory effects on various cell-lines. Furthermore, alkaloids, cumarines, and ascorbic acid are said to be contained in the aerial parts of this plant [15]. *D. versicolor* (in Mongolian *alag bashir*) is described as beneficial in chronic pain. It influences the blood pressure and is used to treat cardiovascular diseases by traditional physicians. Due to its

property to increase the contractility of the uterus, *D. versicolor* is used against bleeding after birth and against strong bleeding during menstruation in Mongolian, Tibetan, and Russian medicine. It is furthermore known to be beneficial in pneumonia, typhoid fever, and poisoning. Its actions include diuretic and anti-inflammatory effects, and, despite its staunching properties, it may cause bleeding when overdosed. Beside these indications, *D. versicolor* is also used against liver diseases in various prescriptions by traditional health institutions [20]. For example, prescription number 10 according to the traditional Mongolian physician Dr. Natsagdorj is used for the treatment of liver ailments. It contains 5 ingredients, among them 25% *D. versicolor*. Another prescription, number 5, consists of 25 ingredients, but contains only 3% *D. versicolor* [20]. As described in chapter 1.1.2, p. 5-6, the choice of the proper prescription for the treatment of a certain disease depends on the exact diagnosis. According to the traditional Mongolian physician Dr. Natsagdorj *D. versicolor* is also used as a substitute of *D. superbus* L. (in Mongolian *Javhaalag bashir*). This fact is in accordance with literature [15], which attributes similar qualities to both plants, *D. versicolor* and *D. superbus*. According to the "Report of market research on Mongolian traditional medicinal drugs" prepared in September 2007 for the WHO, *D. versicolor* ranks among the 45 most common domestic herbal drugs traded in Mongolia. With an average price of about € 3,- per kilogram on the local markets, it belongs to one of the most expensive domestic drugs in Mongolia [2].

1.4 Aim of the thesis and overview of publications

As traditional Mongolian medicine has become more important since the last two decades, scientific research is necessary to prove the efficacy of the therapeutic methods and the medicinal plants applied to patients. Phytochemical data about *D. versicolor* are scarce, as mentioned in chapter 1.3 p. 14-16. Therefore, the aim of this thesis was first the bioassay-guided fractionation of a *D. versicolor* aqueous extract, applying chromatographic techniques. In the next step the fractions, which showed a positive impact on the bile-flow in the model of the isolated perfused rat liver, should be characterized phytochemically employing TLC, HPLC-DAD and HPLC-MS^n. This approach should lead to the isolation, identification, and structure elucidation of active compounds by multidimensional matching employing LC-MS^n, GC-MS, UV-, and NMR-spectroscopy. Publication 1 [21], pp. 127-133, comprises the investigations of *D. versicolor* extracts and fractions in the model of the isolated perfused rat liver. In publication 2 [22], pp. 30-85, the isolation and structure elucidation of flavonoid-glycosides, which are supposed to contribute to the choleretic effect, is described in detail. Furthermore, as a basis for quality control, appropriate analytical methods including applications for the quantification of the flavonoid-glycosides should be established. This was realized by the development of an analytical HPLC-DAD method using isovitexin-7-O-glucoside as external standard and is described in publication 3 [23], pp. 87-112. Additionally, in publication 4 [24], pp. 113-125, the quantification of flavonoids applying a validated HPLC-DAD method especially developed for the separation of very polar compounds is compared to quantification by UV-spectrophotometry.

2 Material and methods

This chapter provides a short overview of the methods applied in this thesis. Further information is given in the experimental sections of the four publications included in section 3, pp. 29-133.

2.1 Plant material

The aerial parts of *D. versicolor* were collected in the years 2003-2005 in different regions in Mongolia (Fig. 4). The plant material was identified by E. Ganbold, State University of Mongolia, Ulaanbaatar. Voucher specimens are deposited at the Institute of Botany, State University of Mongolia, Ulaanbaatar, Mongolia and the Department of Pharmacognosy, University of Vienna, Vienna, Austria (Table 1, p. 19).

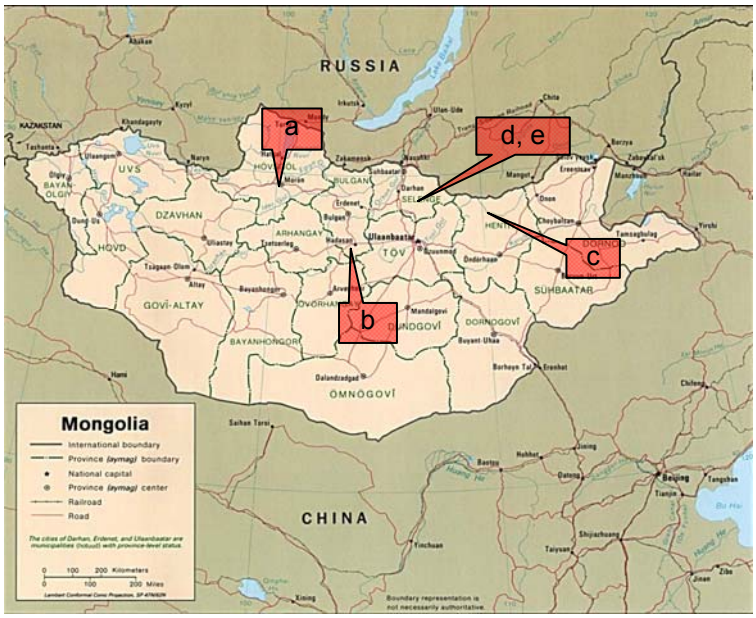


Fig. 4 Map of Mongolia indicating the collections sites of different *D.versicolor* samples; assignments correspond to the samples listed in Table 1, p. 19

Due to the fact, that each experiment required the sacrifice of an animal, biological investigations were carried out exclusively on sample **a**. Based on these results a bioassay-guided fractionation was conducted with the aim of the

isolation of new compounds. All five samples were compared phytochemically by TLC and HPLC-analysis showing qualitative similarity but quantitative differences regarding the flavonoid pattern.

Sample	Collection number	Collection site and year	Herbarium number (Dept. of Pharmacognosy)	Herbarium number (Inst. of Botany)
a	53/04/mon	Khubsgul August 2004		424
b	52/04/mon	Bulgan August 2004	03/04/mon/H	
c	02/05/mon	Khentii August 2005		H 20050805
d	05/03/mon	Selenge July 2003	14/03/mon/H	
e	06/03/mon	Selenge July 2003	14/03/mon/H	

Table 1 Samples **a-e** of *D. versicolor* collected in the years 2003-2005 in Mongolia (see Fig. 4, p. 18)

2.2 Extraction, fractionation and isolation

2.2.1 Preparation of the aqueous extract (OWE)

According to the traditional way of intake (chapter 1.1.3, p 6) a special aqueous extract, named "Original Water Extract" (OWE) was prepared. 100 g of the dried aerial parts of *D. versicolor* (sample **a**) were extracted with 2.5 L of water, adjusted to pH 2 with trifluoroacetic acid (TFA), for 1 h at 40°C by shaking gently, in order to simulate the acid pH of the stomach. After the extraction process, the highly volatile TFA was removed under reduced pressure at a temperature of 45°C and the resulting solution was freeze dried yielding 21.9 g of OWE (DER 1:0.213). This OWE served for the biological investigations in the isolated perfused rat liver (chapter 2.4.1, p. 27; publication 1 [21], chapter 3.3, pp. 127-133) as well as for the fractionation and isolation of compounds (Fig. 7, p 23, publication 2, chapter 3.1. pp. 30-85). A scheme of the extraction and fractionation by SPE is given in Fig. 5.

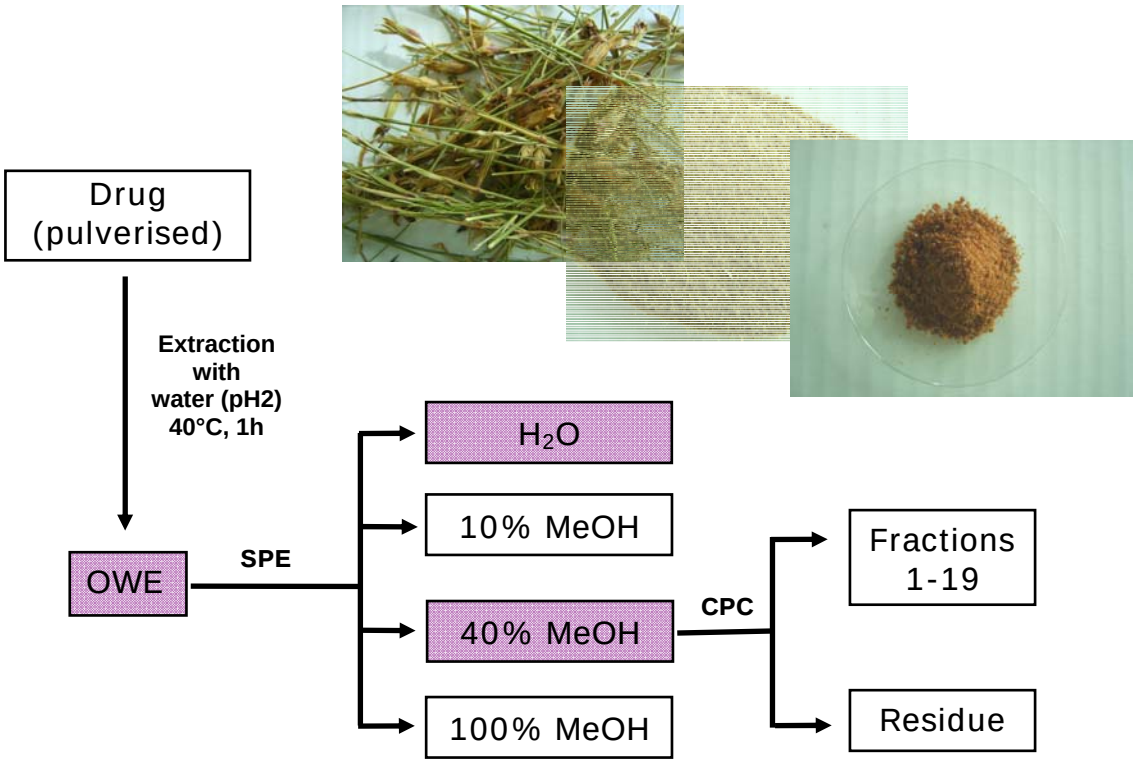


Fig. 5 Extraction-scheme of the aerial parts from *D. versiolor* and fractionation of the OWE by SPE and CPC; the purple colour indicates testing in the isolated perfused rat liver (chapter 2.4.1, p. 27; publication 1, chapter 3.3, pp. 127-133)

2.2.2 Solid phase extraction (SPE)

Solid phase extraction was used for the purification and fractionation of the OWE. A total amount of 2.4 g of OWE was applied on C 18 cartridges and eluted with different ratios of MeOH/H_2O mixtures. The four resulting fractions were either lyophilized or dried under reduced pressure at a maximum temperature of 45°C. The method is described in detail in the experimental sections of publications 1 [21] and 2 [22] (chapter 3.3, pp. 127-133, chapter 3.1. pp. 30-85) and the fractionation scheme is given in Fig. 5, chapter 2.2.1, p 20.

2.2.3 Centrifugal partition chromatography (CPC)

Centrifugal partition chromatography is a chromatographic technique based on liquid-liquid partition between stationary and mobile phase. In contrast to common column chromatography this technique allows the fast separation of compounds without any loss of substance due to adsorptive effects of

compounds to the stationary phase. In this study CPC was used for further fractionation of 80 mg of the SPE derived 40% MeOH subfraction. The applied method is described in detail in the experimental section of publication 2 [22] (chapter 3.1. pp. 30-85) the fractionation scheme is given in Fig. 5 (chapter 2.2.1 p. 20).

2.2.4 Column chromatography (CC)

Column chromatography was employed for fractionation of the OWE using Polyamide [25]. For further fractionation of the resulting subfractions Sephadex LH-20 was used as stationary phase and different ratios of MeOH/H_2O mixtures served as mobile phase. Similar fractions were unified after TLC analysis (chapter 2.3.1, p. 24 and chapter 3.4.1 pp. 134-137). The method is described in detail in the experimental section of publication 2 [22] (chapter 3.1. pp. 30-85). An overview of the different CC-systems and the fractionation is given in Fig. 6.

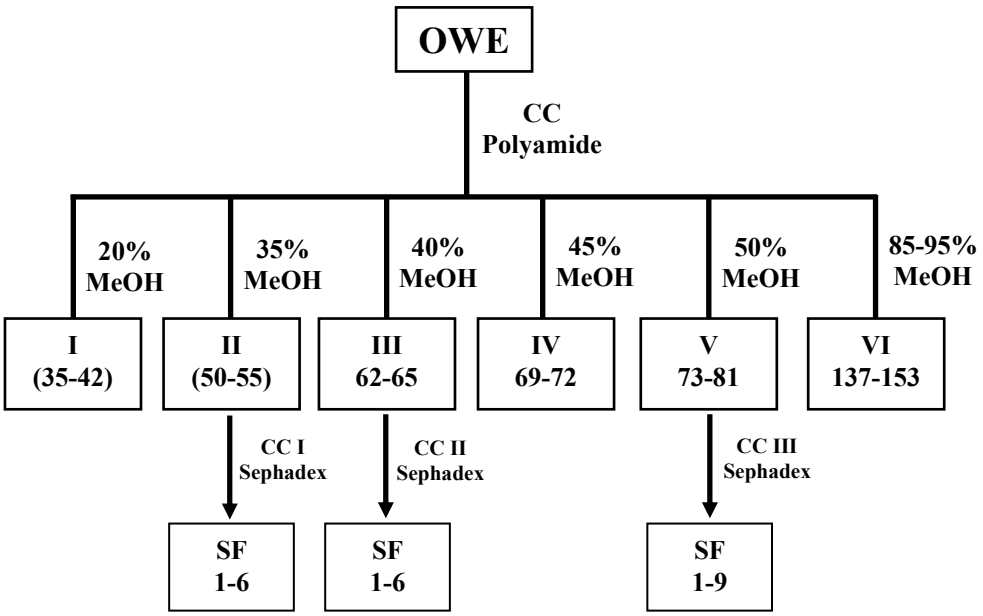


Fig. 6 Scheme of fractionation of the OWE (2.2.1, p 19) by CC on Polyamide and Sephadex LH-20.

2.2.5 Semipreparative high performance liquid chromatography (HPLC)

Semipreparative HPLC was employed for the isolation of the compounds **1**-**7** (Fig. 31, p. 152) from various fractions derived either by CPC or CC (chapter 2.2.3 p. 20 and 2.2.4, p. 21). The method is described in detail in the experimental section of publication 2 [22] (chapter 3.1. pp. 30-85). In brief, separations were carried out applying a low pressure gradient using doubly distilled water (A) adjusted to pH 2.8 with TFA and MeCN (B) at a flow rate of 1.0 mL/min. A 5µm Thermo Aquasil® C_{18} column (250 x 4.6 mm) served as stationary phase. Aqueous and aqueous/methanolic solutions (up to 40% MeOH) at a concentration of 2-5% served as sample solutions. As a consequence of the high structural similarity of the compounds in some cases baseline separation could not be achieved but with an injection volume of 10 µl the resolution was satisfying. The following scheme (Fig. 7) comprises the various procedures resulting in the isolation of nine flavonoid glycosides.

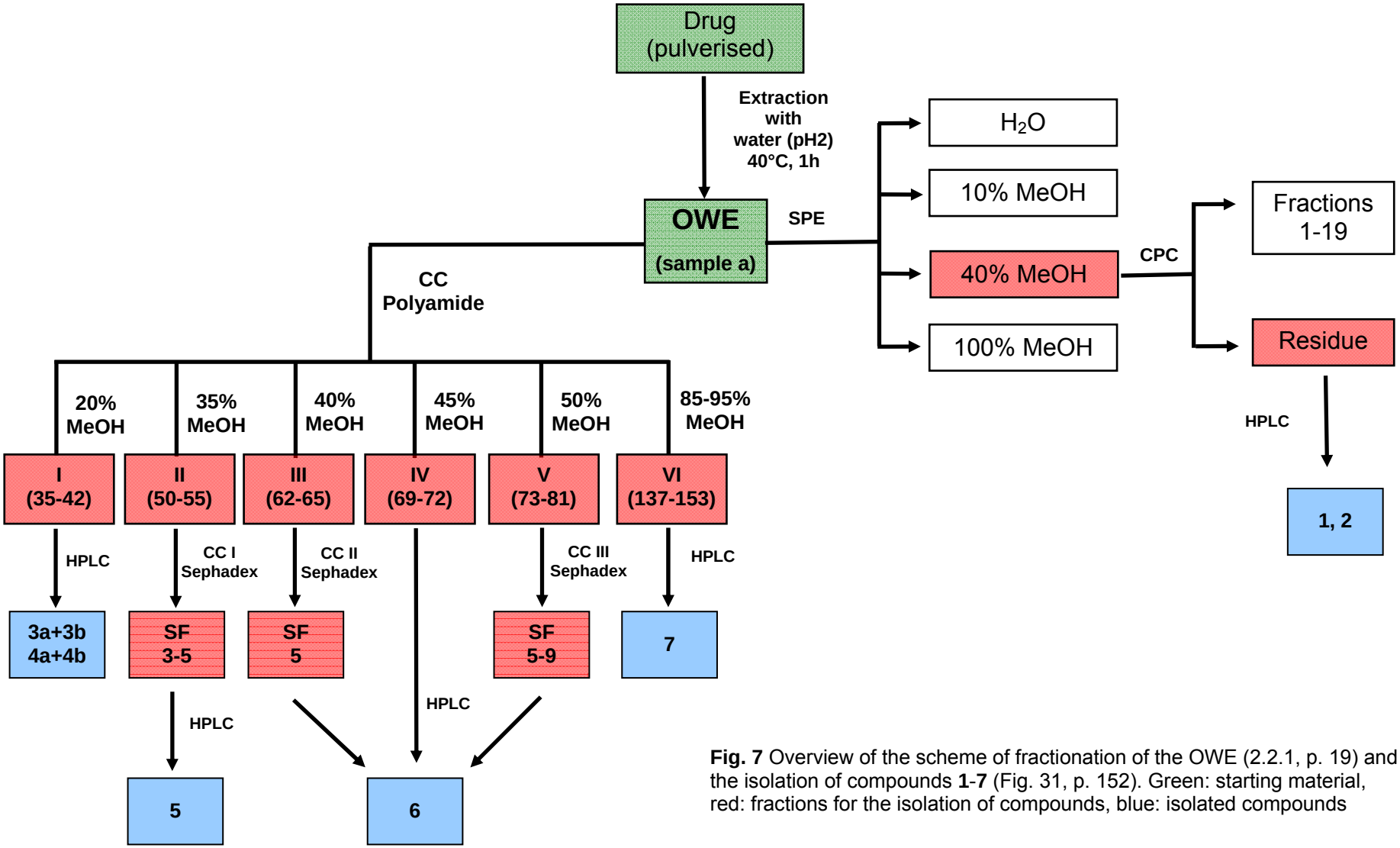


Fig. 7 Overview of the scheme of fractionation of the OWE (2.2.1, p. 19) and the isolation of compounds **1**-**7** (Fig. 31, p. 152). Green: starting material, red: fractions for the isolation of compounds, blue: isolated compounds

2.3 Analytical methods

2.3.1 Thin layer chromatography (TLC)

TLC was used for analytical purposes to get a quick overview about the flavonoids in the OWEs of different *D. versicolor* samples. It served also for a rapid comparison of CPC or CC derived fractions, and based on these analyses similar fractions were unified. Furthermore, this technique was applied for the identification of free sugars contained in the samples and for the identification of monosaccharides after acid hydrolysis of the isolated flavonoid-glycosides in comparison with reference substances. Silica gel coated aluminium sheets served as stationary phase for all analyses whereas different solvent mixtures were used as mobile phase. System 1 was suitable for the separation of flavonoid glycosides whereas system 2 was used for flavonoid aglycones. System 3 served for the separation of sugars. Flavonoids became visible after spraying with a solution of natural product reagent followed by PEG 400 under UV 366nm. For detection of sugars diphenylamine-aniline reagent was used. Zones became visible under daylight after heating the plate at 105-110°C for 10 min.

The different TLC-systems are summarized in table 3, the method is described in detail in publications 1 [21] and 2 [22] (chapter 3.3, pp. 127-133 and chapter 3.1. p. 30-85).

System	**1**	**2**	**3**
Stationary phase	Silica gel		
Mobile phase	EtOAc/HCOOH/ CH_3COOH/H_2O (100/11/11/26)	CH_2Cl_2/cyclohexane/ HCOOH/ethylformiate (35/30/5/30)	CH_2Cl_2/CH_3COOH_{conc}/ MeOH/H_2O (60/32/12/8)
Sample volume	5-10µl	5-10µl	5-10µl
Detection	Natural product reagent/PEG 400	Natural product reagent/PEG 400	Diphenylamine-aniline reagent

Table 2 TLC systems 1-3 used for the separation of flavonoids and sugars

2.3.2 High performance liquid chromatography (HPLC)

HPLC was used for analytical as well as for semipreparative purposes (chapter 2.2.5 p. 22). Various stationary and mobile phases were tested in order to find the best system for the separation of the flavonoids contained in *D. versicolor* samples. Slightly different methods were developed for the quantification of the flavonoids by HPLC-DAD and HPLC-MS^n-analysis. The methods used for analytical purpose required changes in the dimensions of the column, the flow rate, the linear gradient, and the column oven temperature. The method development is described in detail in chapter 3.4.2 pp. 138-147. Further details about the analytical HPLC methods can be found in the experimental sections of publications 1, 2, 3, and 4 [21, 22, 23, 24] (section 3, pp. 29-133).

2.3.3 Gas chromatography-mass spectrometry (GC-MS)

GC-MS was used for the identification of the sugar part of the isolated flavonoid-glycosides including the determination of the absolute configuration of the monosaccharides. These analyses demanded a special sample preparation, which is described in detail in the experimental section of publication 2 [22] (chapter 3.1. pp. 30-85) together with the exact instrument parameters of the GC-MS analyses. In case of the disaccharides the linkage between the two sugar moieties was of interest. In addition to NMR experiments, a method described by de Bettignies-Dutz et al. [26] which was previously applied successfully on saponins, was used for this purpose. In brief, the isolated flavonoid glycoside was permethylated, followed by acid hydrolysis with Kiliani reagent (1 mL HCl_{conc}/3.5 mL CH_3COOH/5.5mL H_2O). After liquid-liquid partition (EtOAc/H_2O), the aqueous layer was evaporated to dryness and derivatized with N-methyl-N-trimethylsilyl-trifluoroacetamide prior to GC-MS analysis. According to de Bettignies-Dutz [27] the sugars show different fragment ions depending on the substitution of OH-groups either with CH_3-residues or trimethylsilyl-residues (TMS). Therefore, different linkages can be distinguished in comparison to reference substances. Quercetin-3-O-rutinoside (rutoside) served as reference for rhamnosyl-1→6-glucoside. The results of these analyses are compiled in chapter 3.4.3, pp. 147-150.

2.3.4 UV-spectrophotometry (UV)

UV-spectrophotometry was applied successfully for the determination of the total flavonoid content in the crude drug as well as in the OWE of *Dianthus versicolor*. Based on the monograph Passiflorae herba from the European Pharmacopoeia, the method was slightly modified and is described in detail in publications 1 [21] and 4 [24] (chapter 3.3, pp. 127-133 and chapter 3.2. pp. 113-125). Furthermore, UV-spectroscopy was used for the identification of compounds **4a/4b** and **5** (Fig. 31, p. 152) via the application of shift reagents ($AlCl_3$/HCl and NaOMe) [28] in addition to NMR experiments as described in publication 2 [22] (chapter 3.1. p. 30-85).

2.3.5 Mass-spectrometry (MS)

Mass-spectrometry is one of the most important analytical methods for structure elucidation. Depending on the mode of ionisation and on the detection unit of a mass spectrometer comprehensive information about a chemical structure can be obtained. High resolution mass-spectrometry (e.g. ESI-TOF) allows the determination of the exact molecular mass of a compound whereas the fragmentation pattern obtained by MS^n experiments (e.g. ESI-IT, ESI-QQQ) provides detailed information about certain structural elements of the molecule. In this thesis MS was used in combination with HPLC and GC. LC-MS^n analyses were performed on an HPLC-ESI-IT system in the negative mode. To obtain high resolution masses an HPLC-coupled MicrOTOF-Q mass spectrometer with ESI ion source was used in the negative mode. Detailed information about the applied methods can be found in publication 2 [22] (chapter 3.1. pp. 30-85). GC-MS analyses were employed for the identification of sugar moieties in the flavonoid glycosides as described in chapter 2.3.3, p. 25 and in the experimental section of publication 2 [22] (chapter 3.1. p. 30-85).

2.3.6 Nuclear magnetic resonance spectroscopy (NMR)

NMR is certainly the most important spectroscopic technique applied in structure elucidation. It facilitates the investigation of the chemical environment

of certain nuclei – in organic chemistry usually ^{1}H and ^{13}C nuclei– and their interactions with vicinal atoms. Besides the 1D techniques ^{1}H and ^{13}C spectroscopy a number of 2D experiments provides the possibility of establishing the chemical structure of an investigated compound. In this study ^{1}H, dqfCOSY (double quantum filtered correlation spectroscopy, H, H correlation), HSQC (heteronuclear single quantum coherence, vicinal H, C correlations), HSQC-TOCSY (initial HSQC pulse followed by a TOCSY – total correlation spectroscopy sequence) and HMBC (heteronuclear multiple bond cohrerence, H, C long range coupling via 2-4 bonds) experiments were conducted. NMR-experiments were carried out in cooperation with Prof. Armin Presser, Institute of Pharmaceutical Sciences, University of Graz. The method is described in detail in the experimental section of publication 2 [22] (chapter 3.1., p. 30-85).

2.4 Biological methods

2.4.1 Isolated perfused rat liver

The isolated perfused rat liver was chosen as an ex-vivo model for the detection of choleretic effects in the aqueous extract and fractions prepared from the aerial parts of *D. versicolor*. These investigations were carried out in cooperation with Prof. Theresia Thalhammer, Institute for Pathophysiology, Medical University of Vienna. The method is described in detail in publication 1 [21] (chapter 3.3, p. 127-133)

2.4.2 Isolated organ preparations

As mentioned in the introduction (chapter 1.3. p. 14) according to Ligaa [15] *D. versicolor* is used as a uterus constringing agent against strong bleeding after birth and during menstruation. Furthermore, *D. versicolor* is said to have an influence on the blood pressure and to be used for the treatment of cardiovascular diseases. In order to investigate the effects of the OWE on the uterus, the heart, arteria pulmonalis, terminal ileum, and the aorta, tests on guinea-pig derived isolated organs were conducted. Those were performed by

Birgit Weisz-Pecher [29] at the Department of Pharmacology and Toxicology, University of Vienna in cooperation with Prof. Lemmens-Gruber. The OWE was tested in three different concentrations (1, 3, and 9 mg/mL) and evaluated regarding the force of contraction. For further investigation of the mechanism behind, smooth muscle cells were isolated from the arteria pulmonalis and the uterus. In order to investigate a possible influence on the intracellular Ca^{2+} concentration, the cells were treated with 0.3, 3, and 9 mg/mL of the aqueous extract solution. Details regarding these investigations can be found in the diploma thesis of Birgit Weisz-Pecher [29].

3 Results

This chapter is divided into four sections describing the results of the thesis. At the beginning of each section a brief overview of the publications, either submitted or published, and a report on what has been achieved by Astrid Obmann is given.

3.1 Preparative section

The isolation and structure elucidation of nine flavonoid-glycosides from the OWE of *Dianthus versicolor* is described in the following manuscript **"Flavonoid *C*- and *O*-glycosides from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH."**, which is in preparation to be submitted to the journal *Chemical & Pharmaceutical Bulletin* [22]. The isolation and structure elucidation of compounds **1**-**6** (Fig. 31, p. 152) was carried out by Astrid Obmann at the Department of Pharmacognosy. Compound **7** (Fig. 31, p. 152) was isolated by Tina Radovic [25] under the supervision of Astrid Obmann who identified the compound. Compound **8** was isolated by Zita Swoboda [30] under the supervision of Astrid Obmann who identified the compound. HR-ESI-MS was measured at the Department of Nutritional Sciences, University of Vienna. NMR spectra were recorded at the Institute of Pharmaceutical Sciences, University of Graz.

Flavonoid *C*- and *O*-glycosides from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH.

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Abstract

Eighteen flavonoids were identified in an aqueous extract of the aerial parts of *Dianthus versicolor*, a plant used in traditional Mongolian medicine against liver diseases. The flavonoid *C*- and *O*-glycosides isoorientin-7-*O*-rutinoside, isoorientin-7-*O*-rhamnosyl-galactoside, isovitexin-7-*O*-rutinoside, isovitexin-7-*O*-rhamnosyl-galactoside, isoscoparin-7-*O*-rutinoside, isoscoparin-7-*O*-rhamnosyl-galactoside, isoscoparin-7-*O*-galactoside, and isoorientin-7-*O*-galactoside were isolated and structurally elucidated. Their structures were established on the basis of extensive spectroscopic techniques including LC-UV-DAD, LC-MS^n, LC-HRMS, 1D and 2D NMR, and by GC-MS analysis after hydrolysis. Furthermore, isovitexin-7-*O*-glucoside (saponarin), isovitexin-2"-*O*-rhamnoside, apigenin-6-glucoside (isovitexin), luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, as well as the aglycones luteolin, apigenin, chrysoeriol, diosmetin, and acacetin were identified by TLC and LC-DAD-MS^n comparison to reference substances or literature data. All compounds are reported for *D. versicolor* for the first time. Most of them have not been described for the genus *Dianthus* until now.

Keywords: *Dianthus versicolor*, Caryophyllaceae, ethnopharmacognosy, traditional Mongolian medicine, isolation, structure elucidation, flavonoids

1. Introduction

The genus *Dianthus* L. (Caryophyllaceae) includes more than 300 species distributed mainly in Eurasia (Angiosperm Phylogeny Website, 2010). It is a summer flowering shrub and contains many narrow endemics, especially in Europe (Valente et al., 2010). Flavonoids seem to be a relevant class of compounds for this genus, as shown by a number of publications from the late seventies up to now. However, among the 300 species only 14 are referred to in literature. 34 flavonoids have been described so far - a summary of those compounds, the corresponding species and references are given in Table 1.

In our investigations we focus on *Dianthus versicolor* FISCH.[1], one of the five *Dianthus* species growing in Mongolia according to Grubov (2001). *D. versicolor* is a purple to pink flowering herb distributed all over the country except the Southern provinces close to the Chinese border (Grubov, 2001; Boldsaikhan, 2004). The aerial parts are used in traditional Mongolian medicine for various purposes. *D. versicolor* is described as beneficial in chronic pain (Boldsaikhan, 2004), it influences uterus motility (Boldsaikhan, 2004), and is applied to treat liver diseases (Kletter et al., 2008). The chemical composition of *D. versicolor* is not well studied; publications on phytochemistry and biological activity are scarce. Two Russian publications from the early eighties report on flavonoids but lack any NMR data (Boguslavskaya et al., 1983a; Boguslavskaya et al., 1983b). A paper published just recently by Ma et al. (2009) presents seven new and nine known triterpenoid saponins isolated from an ethanolic-aqueous (80%) extract of the aerial parts. For nine of these compounds cytotoxic activities against various cell lines were shown (Ma et al., 2009).

With regard to the use of *D. versicolor* in traditional Mongolian medicine as a cure against liver impairment, we performed experiments in the isolated perfused rat liver in

[1] The denomination of the species bases on Grubov (2001)

a recently published study (Obmann et al., 2010). For the aqueous extract and an enriched fraction, bile flow enhancing properties were recorded without any signs of acute hepatotoxicity. The aqueous extract as well as the enriched fraction contained mainly flavonoids, which seem to contribute to the favourable effect of *D. versicolor* on the gastrointestinal tract (Obmann et al., 2010). In the present paper we report on the isolation and structural characterisation of 18 flavonoids from the aerial parts of *D. versicolor*, seven of them are new to the best of our knowledge.

2. Results and Discussion

The separation of compounds **1-16** was achieved by HPLC. The differences in the polarity of the constituents required the development of three different gradient systems which provided the separation within acceptable retention times (see Fig. 1A-C). Compounds **1, 2, 3a, 3b, 4a, 4b, 5,** and **6** were isolated and structurally elucidated applying HR-MS, UV, and NMR. Their UV-spectra, which were recorded online during analytical HPLC (Fig. 1A), are typical for flavonoids (Markham, 1982). Structural differences are apparent at position C-3' (Ring B) of the aglycones: apigenin (3'-H) is the basic structure for **3a** and **3b**, whereas for **1**, **2**, and **6** the aglycone is luteolin (3'-OH), and for **4a**, **4b**, and **5** it is chrysoeriol (3'-OCH_3) (see Fig. 2). All compounds are *C*-glycosides linked in position C-6 of the aglycone. This was confirmed by NMR analysis: the recorded NMR data are typical for a *C*-glucoside (Leitão and Delle Monache, 1998). In addition, the HMBC spectra showed a clear correlation between H-1'' and C-6. The combined informations indicated that the basic *C*-monoglycosides are isovitexin, isoorientin, and isoscoparin. This was confirmed by HPLC-UV analysis of the EtOAc fraction after acid hydrolysis in comparison with reference substances. Moreover, all compounds showed characteristic fragmentation patterns in the LC-MS^n analysis: MS^2 experiments of compounds **5** and **6** revealed a neutral loss of 162 Da

suggesting the occurrence of an *O*-linked hexose (Qimin et al., 1991). For compounds **1**, **2**, **3a**, **3b**, **4a**, and **4b** a neutral loss of 308 Da was found, suggesting an *O*-linked disaccharide moiety consisting of hexose (neutral loss: 162 Da) and deoxyhexose (neutral loss: 146 Da) (Qimin et al., 1991). In MS^3 experiments on the de-*O*-glycosylated fragment ions of each compound, typical losses of 120 and 90 Da, which is due to the cross-ring cleavage of the *C*-glycosylic moiety (Qimin et al., 1991), were observed. 2D NMR experiments indicated an *O*-linkage of the hydrolysable moiety – either a monosaccharide or a disaccharide – in position 7 of the aglycone. Acid hydrolysis and subsequent GC-MS analysis revealed the occurrence of the hexoses glucose or galactose. The deoxyhexose was identified as rhamnose. Correlations of H-1''' to C-7 and H-1'''' to C-6''' in the HMBC spectra suggested a rhamnosyl-(1→6)glucose (compounds **1**, **3a**, **4a)** and a rhamnosyl-(1→6)galactose unit (**2**, **3b**, **4b**) in position 7 of the aglycone (Fig. 3). Compounds **5** and **6** were determined as 7-*O*-galactosides. NMR data indicated *β*-orientation for C-1 of all glucoses and galactoses, and *α*-orientation for C-1 of the rhamnose-moieties. The absolute configuration of the sugars was determined by the preparation of chiral derivatives as described by Reznicek et al. (1993). After acid hydrolysis of the glycosides, the monosaccharides were subjected to reaction with (R)-(-)-2-BuOH and subsequent derivatization with *N*-methyl-*N*-trimethylsilyl-trifluoracetamide (MSTFA). The diastereomeric butylglycosides were analyzed by GC-MS revealing D-configuration for glucose and galactose, and L-configuration for rhamnose.

Compound **1** was obtained as a yellow amorphous powder, $[\alpha]^{20}_{D}$ -48. HR-ESIMS experiments showed an $[M-H]^-$ ion at m/z 755.1993 (calculated for $C_{33}H_{39}O_{20}$ 755.2040). HSQC experiments suggested the presence of two anomeric protons (δ 5.43, d, H-1''', and δ 5.58, s br, H-1'''') with corresponding ^{13}C-resonances at δ 103.5 (C 1''') and δ 102.4 (C 1''''). Another signal at δ 5.78, d, with the chemical shift value of

C 1'' at δ 74.8 indicated the presence of an additional *C*-glycosidic unit. Furthermore, 12 CH-signals, 2 CH_2-signals and 1 CH_3-signal were identified (Table 2), demonstrating the existance of two hexoses and one deoxyhexose. The chemical shifts of the carbohydrate units were in perfect accordance with the data reported for glycosides with similar glycosylation pattern (Li et al., 2005; Rayyan et al., 2005) and suggested glucose for the two hexoses and rhamnose for the deoxyhexose. The combined information from acid hydrolysis, 1D and 2D NMR experiments, and mass spectrometry of **1** revealed the flavonoid-*C*-monoglycoside isoorientin (luteolin-6-glucoside) as basic skeleton. This was confirmed by HPLC retention time and UV spectra in comparison to the authentic reference. Characterization of the *O*-linked disaccharide unit was performed by derivatization and subsequent GC-MS analysis, proving glucose and rhamnose as constituents. Therefore the structure of **1** is confirmed as a luteolin-6-*C*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (isoorientin-7-*O*-rutinoside). This compound has been described before from *Triticum aestivum* (Julian et al., 1971), however, the respective paper lacks any MS and NMR data. For *D. versicolor* this compound is described for the first time.

Compound **2** yielded a slightly yellow amorphous powder, $[\alpha]^{20}_{D}$ -33. HR-ESIMS experiments showed an $[M-H]^-$ ion at m/z 755.1991 (calculated for $C_{33}H_{39}O_{20}$ 755.2040). NMR resonances were similar to compound **1** except for the chemical shifts of spin system H-1''' (Table 2). GC-MS analysis suggested the presence of a galactose, which was supported by NMR analysis. Furthermore, 2D NMR experiments proved the linkage of the side chain in position 7 of the aglycone. These findings confirm the structure of **2** as a luteolin-6-*C*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside (isoorientin-7-*O*-rhamnosyl-galactoside), which is a new compound.

Compounds **3a** and **3b** were obtained as a mixture (yellowish amorphous powder) in a ratio of 2:1. HR-ESIMS experiments yielded isobaric $[M-H]^-$ ions at m/z 739.1993 (calculated for $C_{33}H_{39}O_{19}$ 739.2091). NMR structure analyses suggested a *C*-6-glucosylated apigenin as basic structure (Ohkawa et al, 1998) (Table 2). In addition, the carbohydrate chains showed a high analogy to those found for **1** and **2.** This led us to the hypothesis, that compound **3a** represents a 7-*O* attached rhamnosyl-glucoside, whereas **3b** is a rhamnosyl-galactoside. This assumption was confirmed by GC-MS sugar analyses, which revealed the existence of the monosaccharides glucose, galactose, and rhamnose. In conclusion, the structure of **3a** was established as apigenin-6-*C*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (isovitexin-7-*O*-rutinoside) and the structure of **3b** as apigenin-6-*C*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside (isovitexin-7-*O*-rhamnosyl-galactoside). Compound **3a** has been isolated before from *Hordeum vulgare* var. *nudum* leaves (Ohkawa et al., 1998), but is new for *D. versicolor*. Compound **3b** is a new compound.

Compounds **4a** and **4b** were obtained as a mixture (yellow amorphous powder) in a ratio of 1.5:1. HR-ESIMS showed for both an $[M-H]^-$ ion at m/z 769.2171 (calculated for $C_{34}H_{41}O_{20}$ 769.2197). NMR data suggested a chrysoeriol-6-*C*-glucoside as basic structure due to the following findings: In comparison to the previously described NMR experiments, the data of **4a** and **4b** showed a remarkable signal at δ_H 3.88 and δ_H 3.86, respectively, and at δ_C 56.3 (Table 2), which was identified as OCH_3 group in position 3'. The NMR resonances of the sugar chains in **4a** and **4b** were almost identical to that obtained from **3a** and **3b** (Table 2). Acid hydrolysis of the mixture led to the chrysoeriol-6-*C*-glucoside skeleton (isoscoparin), which was published by Boguslavskaya et al. (1983b) for *D. versicolor*. The authors identified isoscoparin based on IR, UV, and chemical transformation studies, but did not give any NMR or MS data. GC-MS analysis of the carbohydrate unit confirmed glucose, galactose, and rhamnose

to be attached either to **4a** or **4b**. For this reason the structures were established as chrysoeriol-6-*C*-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-glucopyranoside (isoscoparin-7-*O*-rutinoside, **4a**) and chrysoeriol-6-C-*β*-D-glucopyranosyl-7-*O*-*α*-L-rhamnopyranosyl-(1→6)-*β*-D-galactopyranoside (isoscoparin-7-*O*-rhamnosyl-galactoside, **4b**). Both compounds are not published so far.

Compound **5** was obtained as a yellow amorphous powder, $[\alpha]^{20}_{D}$ -52. HR-ESIMS showed an $[M-H]^-$ ion at m/z 623.1533 (calculated for $C_{28}H_{31}O_{16}$ 623.1638). As for compounds **4a** and **4b**, ^{1}H NMR experiments displayed a characteristic ^{1}H signal at δ 3.81 with an intensity of 3H, suggesting the presence of an OCH_3-group. In the anomeric region, two different signals were observed: one anomeric proton at δ_H 5.50 with the corresponding ^{13}C signal at 104.4 and another resonance at δ_H 5.83 (δ_C 75.1). The latter resonances were in perfect accordance with the H-1 and C-1 shift values of the *C*-glucosyl moiety in all compounds discussed before. Acid hydrolysis and GC-MS analysis as described above, and 2D NMR analysis revealed the structure of compound **5** as chrysoeriol-6-*C*-*β*-D-glucopyranosyl-7-*O*-*β*-D-galactopyranoside (isoscoparin-7-*O*-galactoside), which is a new compound.

Compound **6** was isolated as a yellowish amorphous powder, $[\alpha]^{20}_{D}$ -21. HR-ESIMS showed an $[M-H]^-$ ion at m/z 609.1392 (calculated for $C_{27}H_{29}O_{16}$ 609.1461). NMR data of the aromatic part of the molecule were similar to compounds **1** and **2** (isoorientin derivatives). In contrast, only one anomeric proton at δ 5.52 with the corresponding carbon at δ 104.0 was detected. NMR resonances of the carbohydrate unit were almost identical with compound **5**. Subsequent acid hydrolysis and GC-MS analysis revealed the flavonoid-*C*-glycoside isoorientin as basic skeleton with a 7-*O*-linked galactose moiety. In conclusion, the structure of **6** was identified as luteolin-6-*C*-*β*-D-glucopyranosyl-7-*O*-*β*-D-galactopyranoside (isoorientin-7-*O*-galactoside), which is a new compound.

Compound **7** was identified as isovitexin-7-*O*-glucoside by co-chromatography with an authentic sample employing HPLC. Comparison of mass spectra and UV spectra obtained with shift reagents (NaOMe, $AlCl_3$, HCl) supported this result. **7** is known as saponarin and has been isolated from various species, among them *Saponaria officinalis* (Caryophyllaceae) and *Passiflora incarnata* (Passifloraceae) (Patel et al., 2009). For *D. versicolor* this compound has not been described so far.

Compound **8** showed an $[M-H]^-$ ion at m/z 577.2 which gave prominent fragment ions at *m/z* 457.1, 413.0, and 293.0 in MS^2 experiments. This fragmentation can be explained by a cross-ring cleavage of the C-glycosylic moiety, the Z-type cleavage of the terminal deoxyhexose unit (164 Da), and the combination of both, suggesting a 1→2 linked deoxyhexose (Qimin et al., 1991). HPLC and GC-MS analysis after acid hydrolysis revealed isovitexin as basic monoglycoside and rhamnose as attached sugar moiety. Comparison of ^1H and ^{13}C signals of the isolated compound with literature data proved **8** to be isovitexin-2"-*O*-rhamnoside, which has been previously found in *Crataegus* species (Rosaceae) (Li et al., 2005), but is new for *D. versicolor*.

Compounds **9-16** were compared to reference substances by TLC and HPLC. We identified them as apigenin-6-glucoside (isovitexin, **9**), luteolin-7-*O*-glucoside (**10**), apigenin-7-*O*-glucoside (**11**), luteolin (**12**), apigenin (**13**), chrysoeriol (**14**), diosmetin (**15**), and acacetin (**16**).

3. Concluding remarks

In summary, our phytochemical investigations on the traditional Mongolian medicinal plant *D. versicolor* led to the identification of eighteen flavonoids. The currently available phytochemical literature about flavonoids and their structures within the genus *Dianthus* are summarised in Table 1. The comparison showed that the flavonoids found in the cultivated species *D. caryophyllus* are mostly kaempferol-, kaempferide- and

quercetin-*O*-glycosides. In addition, chalcononaringenin-2'-*O*-glucoside, apigenin-6,8-diglucoside (vicenin-2), and apigenin-6-*C*-glucosyl-7-*O*-(6-malyl-glucoside) were reported. All other previously investigated species contain mainly *C*- or *O*-*mono*glycosides of apigenin, luteolin, and chrysoeriol. So far, isovitexin-4'-*O*-glucoside (isosaponarin) from *D. squarrosus* as well as from *D. pseudosquarrosus* and apigenin-6-*C*-glucosyl-7-*O*-(6-malyl-glucoside) from *D. caryophyllus* are the only *C*- and *O*-*di*glycosides.identified within the genus *Dianthus*. Remarkably, half of the *D. versicolor* flavonoids presented in this study are flavonoid-*C*- and *O*-glycosides of apigenin, luteolin, and chrysoeriol with either two or three sugar moieties. They were isolated from a flavonoid enriched fraction of an aqueous extract. This fraction had shown a favorable effect by increasing the bile flow in the model of the isolated perfused rat liver (Obmann et al., 2010). In the present paper, we characterize the main constituents in the respective biologically active fraction of *D. versicolor* as mainly new flavonoids. Nine further flavonoids were identified from the crude aqueous extract. They are known compounds but new for *Dianthus versicolor*. Isovitexin and luteolin-7-*O*-glucoside have been isolated from other *Dianthus* species before, but all other compounds presented in this paper are described for the genus *Dianthus* for the first time.

4. Experimental

4.1. Chemicals and reagents

Reagents for TLC (natural product reagent A, PEG 400) were of analytical grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA). MSTFA for the derivatization of sugars was purchased from Machery-Nagel GmbH&Co.KG (Dueren, Germany). Reference substances for GC, TLC, and HPLC were purchased from Roth (Karlsruhe, Germany) and of HPLC quality. Solvents used for extraction were of

analytical grade; those used for HPLC were of gradient grade and obtained from VWR (West Chester, Pennsylvania, USA).

4.2. General experimental procedures

Optical rotations were determined by a Polarimeter 341 (PerkinElmer Inc., Waltham, Massachusetts, USA) using MeOH as solvent in the following concentrations: Compound **1** 0.062 g/100 ml, **2** 0.015 g/100 ml, **6** 0.021 g/100 ml, **7** 0.024 g/100 ml.

UV spectra were recorded online during HPLC analyses using a Prominence SPD-M20 Diode Array Detector coupled to a Prominence LC-20AD (Shimadzu Corporation, Kyoto, Japan). For UV shift-experiments with NaOMe and $AlCl_3$/HCl, spectra were recorded in MeOH using a Beckman DU 640 spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA).

NMR spectra were recorded on a Varian Unity Inova 400, ^{1}H at 400 MHz, ^{13}C at 100 MHz, and a Varian Unity Inova 600, ^{1}H at 600 MHz, ^{13}C at 150 MHz (Varian Inc., Palo Alto, California, USA), at 24°C using solutions in pyridine-d_5. The TMS resonance was used as internal standard. ^{1}H- and ^{13}C-resonances were assigned using 1D proton and carbon experiments as well as 2D COSY, HSQC, HSQC-TOCSY, and HMBC techniques. The latter were optimized for 8 Hz heteronuclear coupling constant. Spin systems were identified in COSY, HSQC, and HSQC-TOCSY spectra. Subsequently, these spin systems and the quaternary carbons were connected by correlations found in the HMBC experiment. The relative stereochemistry was assigned by selective NOE experiments. ^{1}H- and ^{13}C-resonances are numbered as shown in Fig. 3.

LC-MS^n analyses were performed on an UltiMate 3000 RSLC series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap instrument equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany) in the negative mode. To obtain high resolution masses a MicrOTOF-Q mass spectrometer with ESI

ion source (Bruker Daltonics) connected to an UltiMate 3000 series system (Dionex) was used in the negative mode. Parameters: nebulizer 30 psi; dry temperature 350°C; dry gas 7 l/min; capillary voltage 4000 V; target mass 600 m/z; scan range 150-1000 m/z.

For HPLC a 3µm Thermo Aquasil C_{18} column (150 x 2.1 mm) and a 5µm Thermo Aquasil C_{18} column (250 x 4.6 mm) were used, the latter also for semipreparative purposes. Elutions were conducted with different low pressure gradients using doubly distilled water (A) adjusted to pH 2.8 with either TFA or HCOOH and MeCN (B). For detailed information about the different gradients see section *4.4 HPLC*.

Centrifugal partition chromatography was performed on a CCC-1000 High Speed Counter Current Chromatograph (Pharma-Tech Research Corp., Baltimore, Maryland, USA). Separation was conducted in tail to head mode using water–n-BuOH–EtOAc (2:1:2) as solvent system. TLC analyses were performed at room temperature (22°C) on silica gel coated aluminium sheets (TLC Silica gel 60 F_{254}, Merck, Darmstadt, Germany) using EtOAc–CH_3COOH_{conc}–$HCOOH_{conc}$–H_2O (100:11:11:26) as mobile phase. Flavonoid glycosides became visible under UV 366 nm after spraying with natural product reagent and PEG 400.

4.2. Plant material

Samples of *Dianthus versicolor* FISCH. (Caryophyllaceae) were collected in the Mongolian provinces Khubsgul and Khentii in summer 2004 and 2005, and identified by E. Ganbold, State University of Mongolia, Ulaanbataar. Herbarium specimens are deposited at the Institute of Botany, State University of Mongolia, Ulaanbaatar, Mongolia (herbarium numbers 424 and H 20050805).

4.3. Extraction and Isolation

The preparation of an aqueous extract and its subsequent fractionation by solid phase extraction (SPE) has been described by Obmann et al. before (Obmann et al., 2010). In brief, the dried and powdered aerial parts (100 g) were extracted with 2.5 l doubly distilled water adjusted to pH 2 with trifluoroacetic acid (TFA) at 40°C by shaking gently. This procedure simulated the traditional way of intake, where the crude pulverized drug is taken with plenty of water. After removing the highly volatile TFA under reduced pressure at a temperature of 45°C, the remaining solution was freeze dried, yielding 21.9 g of aqueous extract. A total amount of 2.4 g aqueous extract was further fractionated by SPE: per cartridge 400 mg of aqueous extract were redissolved in 1 ml water and applied on C_{18} material (cartridge: Mega Bond Elut© 60cm^3, Varian, Zug, CH) previously washed with 1 reservoir volume (RV) MeOH and conditioned with 1 RV water. The elution was started 10 min after extract application at a drop rate of 1 ml/min with 3 RV each of water, 10% MeOH (v/v), 40% MeOH (v/v) and 100% MeOH. The aqueous fraction was lyophilized, the others were dried under reduced pressure at a maximum temperature of 45°C. This procedure gave four fractions yielding 987 mg aqueous fraction, 28 mg 10% MeOH fraction, 159 mg 40% MeOH fraction and 32 mg 100% MeOH fraction. The 40% MeOH fraction (enriched in flavonoid glycosides) was further fractionated by centrifugal partition chromatography (CPC). For preparation of solvents water (800 ml), n-BuOH (400 ml) and EtOAc (800 ml) were mixed in a separation funnel. After separation of the layers, the lower phase was taken as stationary phase, and the upper phase was taken as mobile phase. 80 mg of 40% MeOH fraction were redissolved in 2 ml of a 1:2 mixture of stationary and mobile phase and injected. Separation was performed during 8 h at a flow rate of 1.5 ml/min in tail to head mode. Fractions of 1.5 ml were collected automatically. After TLC analysis, similar fractions were combined to yield 19 subfractions. After the experiment, the

stationary phase was evaporated to dryness under reduced pressure at 45°C giving 50 mg dried residue. From this residue compounds **1** (1.16 mg), **2** (1.38 mg), **3** (1.55 mg), and **4** (4.16 mg) were isolated by repeated semipreparative HPLC (gradient 4). NMR analysis revealed **3** to be a mixture of **3a** and **3b**. HPLC-MS analyses showed **4** to be a mixture of three compounds which co-eluted in the established semipreparative HPLC system (gradient 4). Therefore a different separation technique was tried, subjecting 12.3 g of the aqueous extract to column chromatography using Polyamide (Roth, particle size 0.05-0.016 mm) as stationary phase and MeOH-water in different mixing ratios as mobile phase. The resulting 177 fractions were combined to 32 subfractions according to TLC analysis, and six of them (subfractions I-VI) were used for further isolation. Subfraction I (fractions 35-42, 88 mg) eluting with 20% MeOH was further purified by semipreparative HPLC (gradient 4) and yielded again the mixtures **3a** and **3b** (3.05 mg) as well as **4a** and **4b** (4.56 mg). Subfraction VI (fractions 137-153, 43 mg) eluting with 85% MeOH was purified on Sephadex LH-20 followed by semipreparative HPLC (gradient 4) to give 1 mg of compound **7**. Subfraction II (fractions 50-55, 31 mg) was subjected to column chromatography on Sephadex LH-20. Elution with water yielded 40 fractions which were combined to 6 subfractions. Four of these subfractions were purified by semipreparative HPLC (gradient 4) and yielded compound **5** (0.78 mg). Subfraction III (fractions 62-65, 26 mg), subfraction IV (fractions 69-72, 47 mg) and subfraction V (fractions 73-81, 149 mg) underwent the same procedure as subfraction II and yielded altogether compound **6** (2 mg). Compound **8** (1.62 mg) was isolated by semipreparative HPLC (gradient 5) from a fraction obtained by column chromatography using silica gel (Merck, Darmstadt, Germany) as stationary phase and a mixture of EtOAc–MeOH (9:1) as mobile phase.

4.4. HPLC

For analytical and semipreparative purposes, different low pressure gradients were developed. All of them were carried out using doubly distilled water (A) adjusted to pH 2.8 (TFA) and MeCN (B). For LC-MS analyses, TFA was replaced by HCOOH. Each gradient included a final purging step at 95% B for 10 min. Gradient 1 (analytical) was conducted at a flow rate of 0.35 ml/min and a column oven temperature of 15°C. It started at a concentration of 2% B rising to 14% B within 23 min (rate: 0.52%/min), which was followed by an isocratic period for 27 min. Gradient 2 (analytical), at a flow rate of 1 ml/min and a column oven temperature of 25°C, started at a concentration of 15% B which was increased to 45% B within 60 min (rate: 0.5%/min). Gradient 3 (analytical) started at a concentration of 15% B rising to 20.25% B within 35 min (rate: 0.15%/min) followed by a slow increase up to 29% in 25 min (rate: 0.35%/min). The flow rate was set to 1 ml/min and a column oven temperature of 25°C was held during the analysis. Gradient 4 (semipreparative) was employed using a flow rate of 1 ml/min and a column oven temperature of 20°C. The starting concentration of 0% B was raised to 10% B within 20 min (rate: 0.5%/min), followed by a very slow increase from 10% B to 20% B within 60 min (rate: 0.17%/min), and a more rapid one from 20% B to 40% B within 20 min (rate: 1%/min). Gradient 5 (semipreparative) started at a concentration of 17% B rising up to 19.65% B within 18 min (rate: 0.15%/min), followed by an isocratic period for 7 min.

4.5. Determination of sugar moieties

To identify the sugar units and to determine their absolute configuration, the isolated flavonoid glycosides were hydrolyzed for 2 h at 100°C using Kiliani reagent (mixture of 3.5 ml CH_3COOH_{conc}, 1 ml HCl_{conc}, and 5.5 ml H_2O). The solution was extracted three times with EtOAc, and the aqueous layer containing monosaccharides was evaporated

to dryness. (R)-(-)-2-Butanol and HCl_{conc} were added to the residue in order to get the corresponding diastereomeric butylglycosides. After 15h at 100°C the solution was evaporated to dryness and the residue was prepared for GC-EI-MS analysis by derivatization with N-methyl-N-trimethylsilyl-trifluoracetamide. GC-MS analyses were performed on a GCMS-QP 2010 (Shimadzu Corporation, Kyoto, Japan) using the following parameters:

GC: Phenomenex ZB-5 capillary column (60 m x 0.25 mm, film thickness 0.25 µm), carrier gas: He 5.0, flow rate: 2.0 ml min^{-1}, split ratio 1:10; temperature gradient: 100°C to 270°C at a rate of 3°C min^{-1}; 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 monosaccharides were identified by comparison of retention times and mass spectra to authentic substances.

4.6. Compound characterization

*4.6.1. Luteolin-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**1**)*

Yellow amorphous powder; [α] -48° (*c* 0.062, $MeOH)_{20.D}$; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 755.1993 (calcd for $C_{33}H_{39}O_{20}$ 755.2040) $[M-H]^-$; R_f=0.12, colour: orange (UV 366 nm, natural product reagent); t_R=32.49 min (gradient 1)

*4.6.2. Luteolin-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**2**)*

Slightly yellow amorphous powder; [α] -33° (*c* 0.015, $MeOH)_{20.D}$; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 755.1991 (calcd for $C_{33}H_{39}O_{20}$ 755.2040) $[M-H]^-$; R_f=0.12, colour: orange (UV 366 nm, natural product reagent); t_R=33.31 min (gradient 1)

*4.6.3. Apigenin-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**3a**)*

Yellowish amorphous powder; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 739.1993 (calcd for $C_{33}H_{39}O_{19}$ 739.2091) $[M-H]^-$; R_f=0.17, colour: greenish (UV 366 nm, natural product reagent); t_R=39.58 min (gradient 1)

*4.6.4. Apigenin-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**3b**)*

Yellowish amorphous powder; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 739.1993 (calcd for $C_{33}H_{39}O_{19}$ 739.2091) $[M-H]^-$; R_f=0.17, colour: greenish (UV 366 nm, natural product reagent); t_R=39.58 min (gradient 1)

*4.6.5. Chrysoeriol-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (**4a**)*

Yellow amorphous powder; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 769.2171 (calcd for $C_{34}H_{41}O_{20}$ 769.2197) $[M-H]^-$; R_f=0.17, colour: greenish (UV 366 nm, natural product reagent); t_R=43.42 min (gradient 1)

*4.6.6. Chrysoeriol-6-C-β-D-glucopyranosyl-7-O-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside (**4b**)*

Yellow amorphous powder; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 769.2171 (calcd for $C_{34}H_{41}O_{20}$ 769.2197) $[M-H]^-$; R_f=0.17, colour: greenish (UV 366 nm, natural product reagent); t_R=43.42 min (gradient 1)

*4.6.7. Chrysoeriol-6-C-β-D-glucopyranosyl-7-O-β-D-galactopyranoside (**5**)*

Yellow amorphous powder; [α] -52° (*c* 0.021, $MeOH)_{20.D}$; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 623.1533 (calcd for $C_{28}H_{31}O_{16}$ 623.1638) $[M-H]^-$; R_f=0.21, colour: greenish (UV 366 nm, natural product reagent); t_R=41.02 min (gradient 1)

*4.6.8. Luteolin-6-C-β-D-glucopyranosyl-7-O-β-D-galactopyranoside (**6**)*

Yellowish amorphous powder, [α] -21° (*c* 0.024, $MeOH)_{20.D}$; ^{1}H and ^{13}C NMR (Table 2); –HR-ESIMS *m/z* 609.1392 (calcd for $C_{27}H_{29}O_{16}$ 609.1461) $[M-H]^-$; R_f=0.19, colour: orange (UV 366 nm, natural product reagent); t_R=31.08 min (gradient 1)

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Appendix A. Supplemenary data

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

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Table 1 Flavonoids within the genus *Dianthus* L.

Species	Compounds	Literature
Dianthus caryophyllus (various cultivars)	Kaempferide-3-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	Curir et al., 2001
	Kaempferide-3-O-[2^G-β-D-glucopyranosyl]-β-rutinoside	Curir et al., 2005
	3-[6-O-(α-L-arabinopyranosyl)-β-D-glucopyranosyl]-quercetin (Peltatoside) 3,5,7,2'-Tetrahydroxyflavon (Datiscetin)	Curir et al., 2003
	Kaempferol-3-(6'''-rhamnosyl-2'''-glucosyl-glucoside) Kaempferol-3-(6'''-rhamnosyl-2'''-(6-malyl-glucosyl)-glucoside) Apigenin-6-C-glucosyl-7-O-(6-malyl-glucoside)	Fukui et al., 2003
	Chalcononaringenin 2'-O-glucoside	Yoshida et al., 2004 Ogata et al., 2004
	Kaempferol-3-O-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranoside Kaempferol-3-O-rutinoside	Galeotti et al., 2008a
	Apigenin-6,8-di-C-β-D-glucopyranoside	Galeotti et al., 2008b
	Kaempferol-3-O-β-D-glucopyranosyl-(1→2)-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	Galeotti et al., 2008a
	Kaempferol-3-O-[α-L-rhamnopyranosyl-(1→6)]-β-D-glucopyranoside	Galeotti et al., 2008b
	Kaempferol-3-O-β-D-glucopyranosyl-(1→2)-O-[α-L-rhamnopyranosyl- (1→6)]-β-D-glucopyranoside	Galeotti et al., 2008b Galeotti et al., 2008a
Dianthus arenarius *Dianthus crinitus* *Dianthus tetralepsis* [2]	Luteolin-6-glucoside (isoorientin) Luteolin-8-glucoside (orientin) Apigenin-6-glucoside (isovitexin) Apigenin-8-glucoside (vitexin) Luteolin-4'-glucopyranoside Apigenin-4'-glucopyranoside	Boguslavskaya et al., 1983a
Dianthus hoeltzeri *Dianthus acicularis*	Apigenin-6-C-syn-α-D-glucopyranoside (neoavroside) Apigenin-6-C-anti-α-D-glucopyranoside (isoneoavroside)	Boguslavskaya et al., 1983a
Dianthus squarrosus	Apigenin-6-C-glucosyl-4'-O-glucoside (isosaponarin)	Boguslavskaya et al., 1983a

[2] *D. tetralepsis* is a synonym of *D. crinitus* (Germplasm Resources Information Network, 2010)

Dianthus dicolor [3] *Dianthus ramosissimus* [4] *Dianthus versicolor*	Chrysoeriol-6-C-syn-α-D-glucopyranoside Chrysoeriol-6-C-anti-α-D-glucopyranoside	Boguslavskaya et al., 1983a Boguslavskaya et al., 1983b
Dianthus superbus	Luteolin-6-glucoside (isoorientin) Luteolin-8-glucoside (orientin)	Seraya et al., 1978
Dianthus pseudosquarrosus [5]	Apigenin-6-glucoside (isovitexin) Apigenin-8-glucoside (vitexin) Apigenin-6-glucosyl-4'-O-glucoside (isosaponarin) Luteolin-7-O-glucoside Luteolin-7-O-diglucoside Luteolin-5-O-glucoside	Darmograi and Khimenko, 1978
Dianthus platyodon	Quercetin-3-O-rutinoside (rutin) Kaempferol-3-O-(β-D-glucopyranosyl-β-D-glucopyranoside)	Boguslavskaya, 1976a
Dianthus deltoides	Apigenin-6-C-syn-α-D-glucopyranoside (neoavroside) Apigenin-6-C-anti-α-D-glucopyranoside (isoneoavroside) Chrysoeriol Luteolin Luteolin-4'-O-β-D-glucopyranoside Chrysoeriol-4'-O-β-D-glucopyranoside	Boguslavskaya and Beletskii, 1978 Boguslavskaya et al., 1976b

[3] *D. dicolor* is probably a spelling mistake, instead, *D discolor* exists according to IPNI, which is also stated as *D. chinensis* (The International Plant Name Index, 2010a)

[4] *D. ramosissimus* is referred to as *D. ramosissimum* according to Grubov (2001)

[5] *D. pseudosquarrosus* is also stated as *D. arenarius* f. *pseudosquarrosus* (The International Plant Name Index, 2010b)

Table 2. ^{1}H and ^{13}C NMR data for **1**, **2**, **3a**, **3b**, **4a**, **4b**, **5**, **6** in pyridine-d_5 (*J* in Hz)

	1		**2**		**5**		**6**	
position	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
2	165.7, qC		165.8, qC		164.9, qC		165.3, qC	
3	104.3, CH	6.86, br s	104.5, CH	6.87, br s	104.5, CH	6.95, s	104.0, CH	6.94, s
4	183.0, qC		183.1, qC		183.3, qC		183.1, qC	
5	161.4, qC		161.5, qC		161.2, qC		161.5, qC	
6	112.0, qC		112.0, qC		112.1, qC		111.9, qC	
7	164.2, qC		164.5, qC		164.3, qC		164.1, qC	
8	94.5, CH	7.02, br s	94.8, CH	7.04, br s	95.3, CH	7.27, s	94.7, CH	7.09, s
9	157.6, qC		157.7, qC		157.5, qC		157.4, qC	
10	106.6, qC		106.7, qC		106.4, qC		106.4, qC	
1’	122.6, qC		122.8, qC		122.4, qC		122.8, qC	
2’	114.5, CH	8.00, br s	114.6, CH	7.99, d (2.1)	110.5, CH	7.57, br s	114.6, CH	7.88, d (2.0)
3’	147.8, qC		147.7, qC		149.2, qC		148.0, qC	
4’	152.1, qC		152.1, qC		153.0, qC		152.2, qC	
5’	117.3, CH	7.43, d (8.4)	117.4, CH	7.44, d (8.4)	117.1, CH	7.23, d (8.6)	116.7, CH	7.25, d (8.3)
6’	120.1, CH	7.56, d (8.4)	120.4, CH	7.56, dd (8.4, 2.1)	121.5, CH	7.55, d (8.6)	119.5, CH	7.46, d (8.3)
OCH_3					56.2, CH_3	3.81, s		
6C-Glc-1’’	74.8, CH	5.78, d (9.6)	75.0, CH	5.77, d (9.7)	75.1, CH	5.83, d (10.0)	74.7, CH	5.81, d (9.7)
2	72.9, CH	5.16, m	73.0, CH	5.12, t (9.1)	73.2, CH	5.20, t (9.3)	72.8, CH	5.22, m
3	80.9, CH	4.40, t (9.1)	81.1, CH	4.37, t (9.0)	81.2, CH	4.40, t (9.4)	80.8, CH	4.42, m
4	71.5, CH	4.57, t (9.2)	71.7, CH	4.44, m	71.5, CH	4.56, m	71.1, CH	4.56, m
5	83.0, CH	4.11, m	83.3, CH	4.12, m	83.0, CH	4.10, m	82.6, CH	4.09, m
6	62.0, CH_2	4.48, m	62.3, CH_2	4.51, m	62.7, CH_2	4.47, m	62.1, CH_2	4.50, m
		4.61, dd (11.7, 4.1)		4.57, m		4.61, m		4.62, m
7O-Glc-1’’’	103.5, CH	5.43, d (7.5)						
2	75.1, CH	4.19, t (8.5)						
3	77.7, CH	4.26, m						
4	71.3, CH	3.92, t (9.3)						
5	77.3, CH	4.28, m						
6	67.9, CH_2	4.10, m						
		4.83, m						
7O-Gal-1’’’			104.8, CH	5.36, d (7.7)	104.4, CH	5.50, d (7.6)	104.0, CH	5.52, d (7.6)
2			72.7, CH	4.79, m	72.9, CH	4.83, t (8.7)	72.5, CH	4.86, m
3			74.5, CH	4.26, m	75.0, CH	4.25, dd (9.7, 3.6)	74.5, CH	4.31, m
4			70.4, CH	4.42, m	70.3, CH	4.56, m	69.9, CH	4.62, m
5			76.4, CH	4.34, m	78.3, CH	4.32, m	77.9, CH	4.36, m
6			68.2, CH_2	4.46, m	62.3, CH_2	4.47, m	61.7, CH	4.46, m
				4.49, m		4.61, m		4.62, m
Rha-1’’’’	102.4, CH	5.58, br s	102.7, CH	5.52, br s				
2	72.0, CH	4.80, m	72.3, CH	4.80, m				
3	73.0, CH	4.73, dd (8.9, 3.4)	73.2, CH	4.71, m				
4	73.8, CH	4.31, t (9.2)	74.0, CH	4.32, t (9.1)				
5	70.0, CH	4.35, m	70.1, CH	4.35, m				
6	18.5, CH_3	1.63, d (6.0)	18.7, CH_3	1.67, d (5.8)				

	3a		**3b**		**4a**		**4b**	
position	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)	δ_C, mult.	δ_H (*J* in Hz)
2	165.3, qC		165.3, qC		165.2, qC		165.2, qC	
3	104.2, CH	6.87, s	104.2, CH	6.87, s	104.4, CH	6.92, s	104.4, CH	6.92, s
4	183.2, qC		183.2, qC		183.2, qC		183.2, qC	
5	161.5, qC		161.5, qC		161.6, qC		161.6, qC	
6	112.1, qC		112.1, qC		112.1, qC		112.1, qC	
7	164.3, qC		164.5, qC		164.3, qC		164.4, qC	
8	94.9, CH	7.12, s	94.9, CH	7.12, s	94.8, CH	7.16, s	94.8, CH	7.16, s
9	157.6, qC		157.6, qC		157.7, qC		157.7, qC	
10	106.7, qC		106.7, qC		106.6, qC		106.6, qC	
1’	122.2, qC		122.2, qC		122.4, qC		122.4, qC	
2’	129.4, CH	8.03, m	129.4, CH	8.03, m	110.4, CH	7.57, m	110.4, CH	7.57, m
3’	117.5, CH	7.47, m	117.5, CH	7.47, m	149.2, qC		149.2, qC	
4’	163.2, qC		163.2, qC		152.9, qC		152.9, qC	
5’	117.5, CH	7.47, m	117.5, CH	7.47, m	117.6, CH	7.58, m	117.6, CH	7.58, m
6’	129.4, CH	8.03, m	129.4, CH	8.03, m	121.9, CH	7.81, m	121.9, CH	7.81, m
OCH_3					56.3, CH_3	3.88, s	56.3, CH_3	3.86, s
6C-Glc-1’’	75.1, CH	5.79, d (9.7)	75.1, CH	5.78, d (9.7)	74.9, CH	5.80, d (9.7)	75.0, CH	5.79, d (9.7)
2	73.1, CH	5.18, t (9.1)	73.0, CH	5.13, t (9.1)	73.0, CH	5.19, t (9.2)	72.9, CH	5.15, t (9.2)
3	81.1, CH	4.40, m	81.1, CH	4.37, m	81.1, CH	4.39, m	81.1, CH	4.37, m
4	71.6, CH	4.58, t (9.1)	71.7, CH	4.46, m	71.6, CH	4.57, t (9.4)	71.6, CH	4.47, t (9.4)
5	83.2, CH	4.11, m	83.2, CH	4.11, m	83.1, CH	4.10, m	83.1, CH	4.10, m
6	62.3, CH_2	4.47, m	62.3, CH_2	4.49, m	62.2, CH_2	4.47, m	62.2, CH_2	4.47, m
		4.61, m		4.58, m		4.60, m		4.60, m
7O-Glc-1’’’	103.8, CH	5.50, d (7.5)			103.6, CH	5.51, d (7.4)		
2	75.3, CH	4.20, t (8.5)			75.3, CH	4.20, t (8.4)		
3	77.9, CH	4.27, m			77.8, CH	4.22, m		
4	71.3, CH	3.98, t (9.3)			71.2, CH	3.98, t (9.2)		
5	77.8, CH	4.31, m			77.7, CH	4.24, m		
6	68.0, CH_2	4.17, m			67.8, CH_2	4.18, m		
		4.78, m				4.74, m		
7O-Gal-1’’’			104.7, CH	5.42, d (7.6)			104.5, CH	5.43, d (7.6)
2			72.7, CH	4.77, m			72.6, CH	4.78, t (8.6)
3			74.3, CH	4.26, m			74.4, CH	4.28, m
4			70.2, CH	4.46, m			70.0, CH	4.45, m
5			76.2, CH	4.35, m			76.0, CH	4.31, m
6			67.8, CH_2	4.41, m			67.4, CH_2	4.35, m
				4.51, m				4.53, m
Rha-1’’’’	102.8, CH	5.59, br s	102.6, CH	5.48, br s	102.6, CH	5.59, br s	102.4, CH	5.49, br s
2	72.4, CH	4.74, m	72.4, CH	4.74, m	72.3, CH	4.70, m	72.3, CH	4.70, m
3	73.0, CH	4.60, m	73.0, CH	4.60, m	72.9, CH	4.57, m	72.9, CH	4.57, m
4	74.3, CH	4.26, m	74.3, CH	4.26, m	74.2, CH	4.24, m	74.2, CH	4.24, m
5	70.1, CH	4.35, m	70.1, CH	4.35, m	69.9, CH	4.36, m	69.9, CH	4.36, m
6	18.7, CH_3	1.63, d (6.0)	18.8, CH_3	1.67, d (6.0)	18.7, CH_3	1.63, d (6.1)	18.7, CH_3	1.67, d (6.1)

Figure 1. HPLC-analysis of the aqueous extract of *Dianthus versicolor* employing gradient 1 (1A), gradient 2 (1B), and gradient 3 (1C). Peak numbers correspond to the compounds listed in Figure 2.

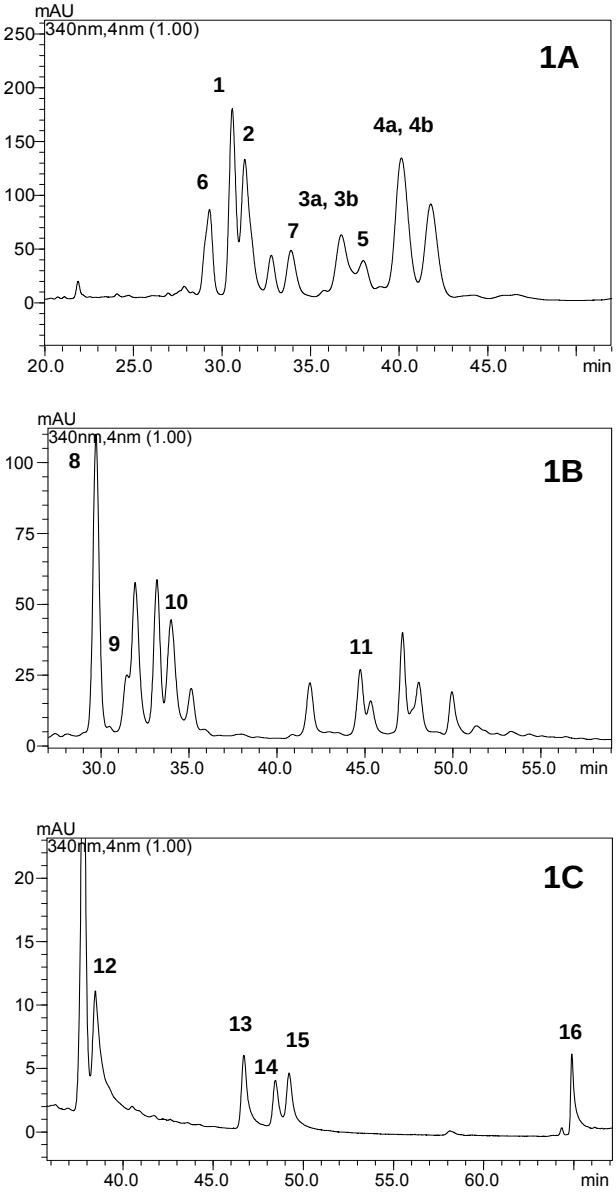
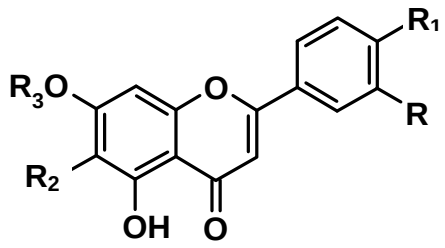
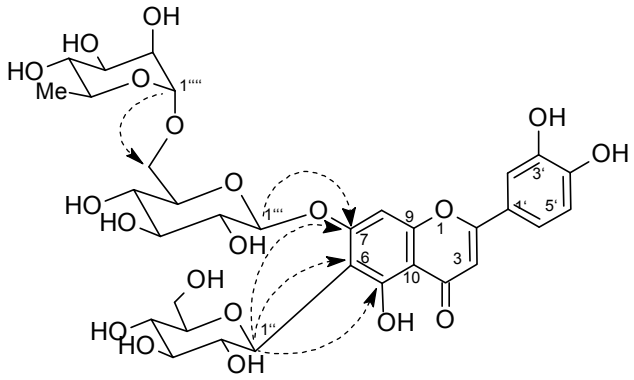


Figure 2. Chemical structures of compounds **1-16** (Gal: galactose, Glc: glucose, Rha: rhamnose)



	R	R_1	R_2	R_3	Common name
1	OH	OH	Glc	Rha(1→6)Glc	Isoorientin-7-O-rutinoside
2	OH	OH	Glc	Rha(1→6)Gal	Isoorientin-7-O-rhamnosyl-glactoside
3a	H	OH	Glc	Rha(1→6)Glc	Isovitexin-7-O-rutinoside
3b	H	OH	Glc	Rha(1→6)Gal	Isovitexin-7-O-rhamnosyl-galactoside
4a	OCH_3	OH	Glc	Rha(1→6)Glc	Isoscoparin-7-O-rutinoside
4b	OCH_3	OH	Glc	Rha(1→6)Gal	Isoscoparin-7-O-rhamnosyl-galactoside
5	OCH_3	OH	Glc	Gal	Isoscoparin-7-O-galactoside
6	OH	OH	Glc	Gal	Isoorientin-7-O-galactoside
7	H	OH	Glc	Glc	Isovitexin-7-O-glucoside (saponarin)
8	H	OH	Rha(1→2)Glc	H	Isovitexin-2"-O-rhamnoside
9	H	OH	Glc	H	Apigenin-6-glucoside (isovitexin)
10	OH	OH	H	Glc	Luteolin-7-O-glucoside
11	H	OH	H	Glc	Apigenin-7-O-glucoside
12	OH	OH	H	H	Luteolin
13	H	OH	H	H	Apigenin
14	OCH_3	OH	H	H	Chrysoeriol
15	OH	OCH_3	H	H	Diosmetin
16	H	OCH_3	H	H	Acacetin

Figure 3. Characteristic HMBC correlations of **1**



Flavonoid C- and O-glycosides from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH.

Astrid Obmann,[1] Ingrid Werner, [1] Armin Presser, [2] Martin Zehl, [1] Zita Swoboda, [1] Sodnomtseren Purevsuren,[3] Christa Kletter, [1] and Sabine Glasl [1]

[1]Department of Pharmacognosy, University of Vienna, Althanstrasse 14, 1090 Vienna, Austria

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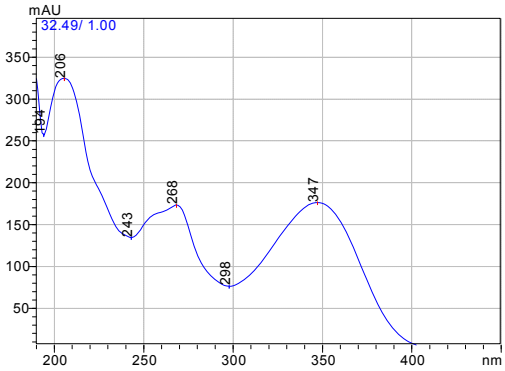
Supplementary data

Table of contents:

Fig S1. UV spectra of compounds **1-6** and **8** recorded online in 14% MeCN (**1-6**) or 31% MeCN (**8**)

1:



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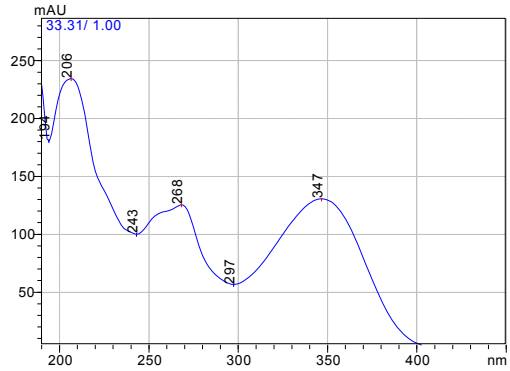

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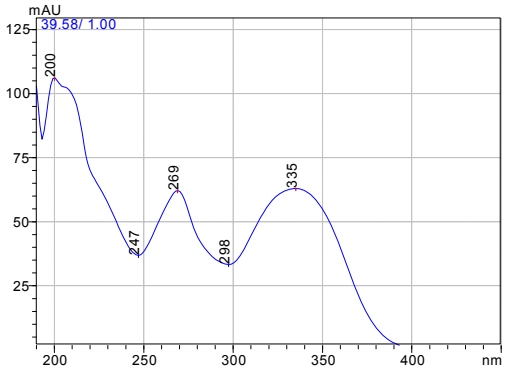

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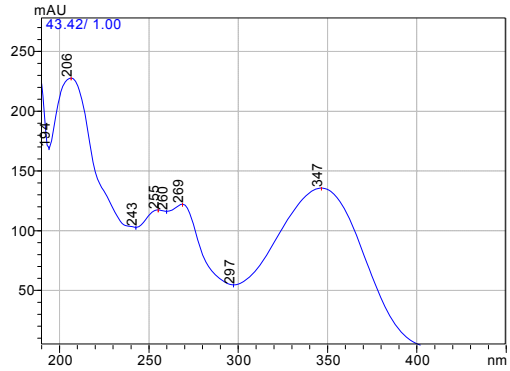

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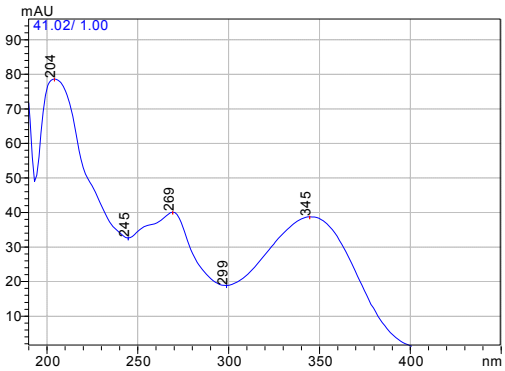

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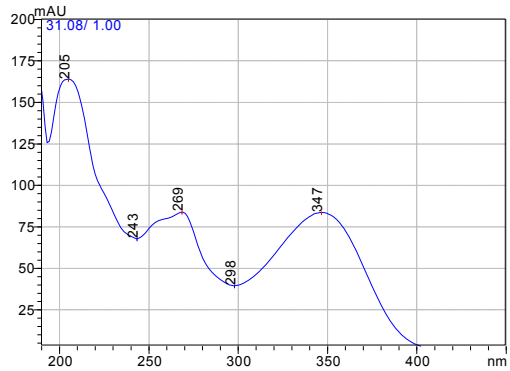

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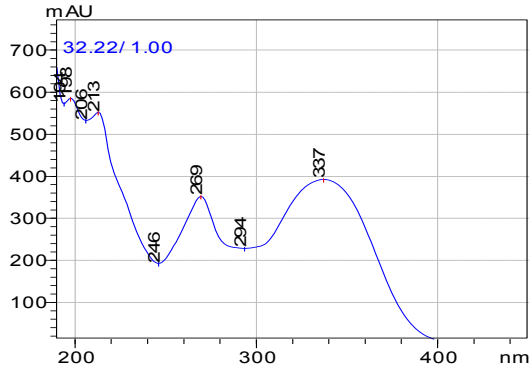


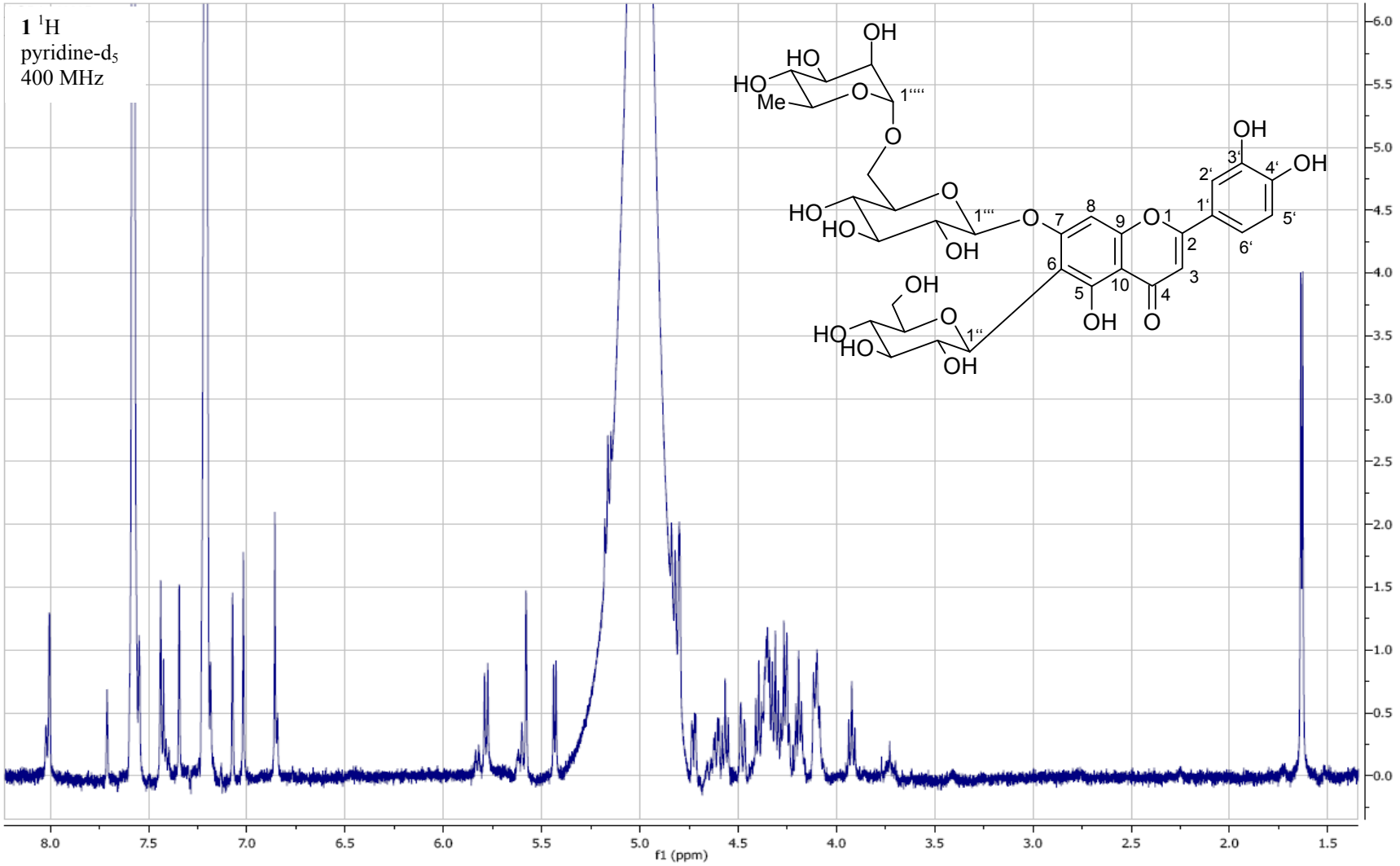
Table S1. UV data of **1-6** and **8**

Cpd	λ_{max} (nm)	λ_{min} (nm)
1	256 (sh), 268, 347	243, 298
2	256 (sh), 268, 347	243, 297
3a+3b	269, 335	247, 298
4a+4b	255 (sh), 269, 347	243, 260, 297
5	256 (sh), 269, 345	245, 299
6	256 (sh), 269, 347	243, 298
8	269, 337	245, 294

Table S2. MS^n data of compounds **1-8**:

Cpd	[M+H]+	[M-H]-	Main Fragment Ions (>10%) in -DDA
1	757.2	755.3	MS2 [755.3]: 447.1, 356.9, 326.9, 298.9 MS3 [447.1]: 356.9, 326.9, 298.9 MS3 [326.9]: 298.9
2	757.2	755.3	MS2 [755.3]: 755.3, 635.2, 489.0, 447.1, 357.0, 326.9, 298.9 MS3 [635.2]: 399.0, 326.9, 298.9, 298.0 MS3 [447.1]: 356.9, 326.9, 299.0 MS3 [326.9]: 298.9
3a +**3b**	741.2	739.3	MS2 [739.3]: 431.1, 310.9 MS3 [431.1]: 340.9, 310.9, 282.9 MS3 [310.9]: 282.9
4a+**4b**	771.2	769.3	MS2 [769.3]: 461.1, 340.9, 297.9 MS3 [461.1]: 371.0, 341.0, 297.9 MS3 [340.9]: 297.9
5	625.2	623.3	MS2 [623.3]: 503.2, 461.1, 371.0, 341.0, 297.9 MS3 [461.1]: 371.0, 340.9, 297.9 MS3 [341.0]: 325.9, 312.9, 297.9
6	611.2	609.3	MS2 [609.3]: 447.1, 357.0, 326.9, 298.9 MS3 [447.1]: 356.9, 326.9, 299.0 MS3 [326.9]: 298.9
7	595.2	593.3	MS2 [593.3]: 473.1, 431.1, 341.0, 310.9, 282.9 MS3 [431.1]: 340.9, 310.9, 282.9 MS3 [310.9]: 282.9
8	579.1	577.2	MS2 [577.2]: 457.1, 413.0, 293.0 MS3 [457.1]: 311.0, 282.0 MS3 [413.1]: 293.0 MS3 [293.0]: 174.9

Figure S2. ^{1}H NMR spectrum of **1**



S5

Figure S3. DQFCOSY of **1**

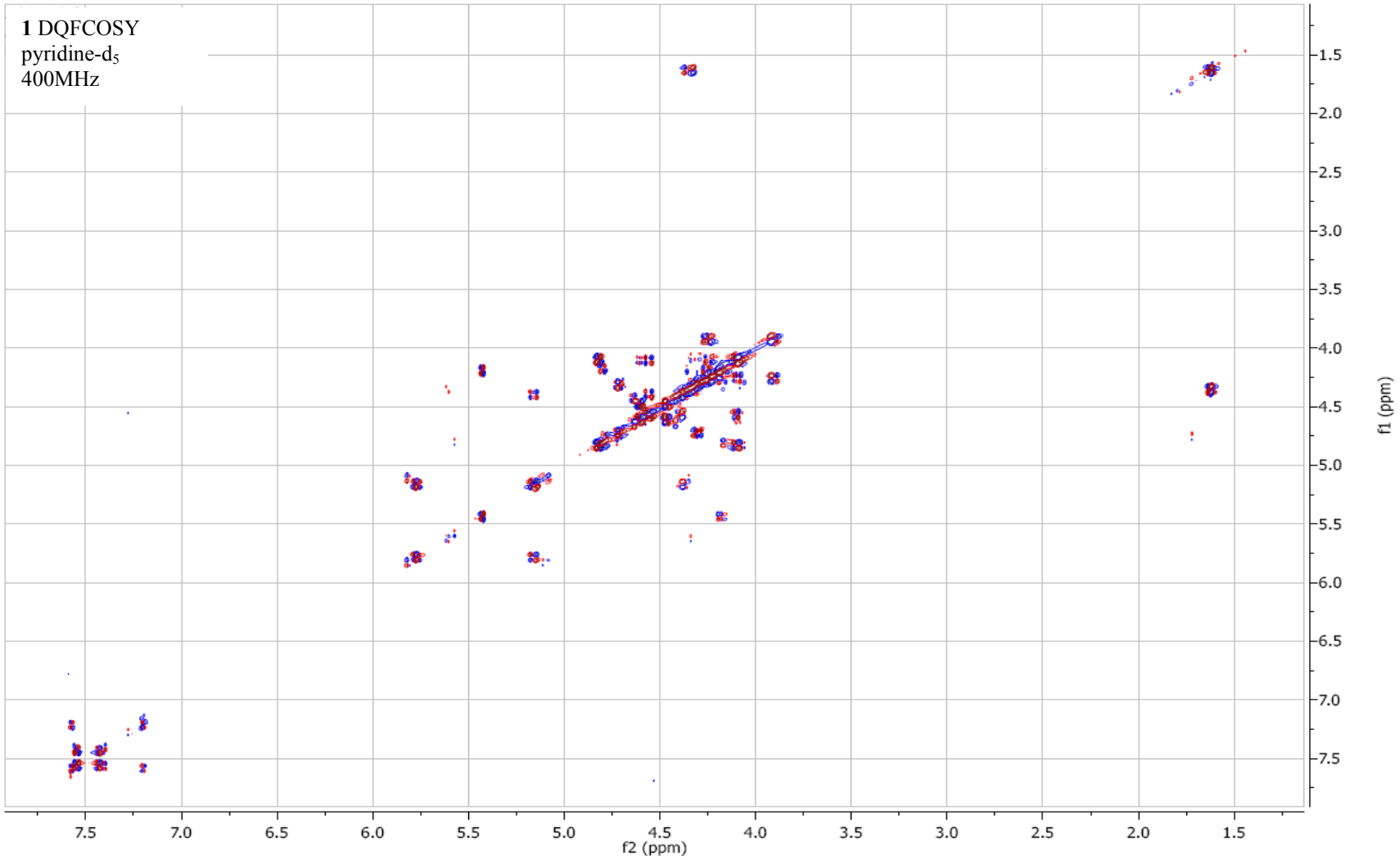


Figure S4. HSQC of **1**

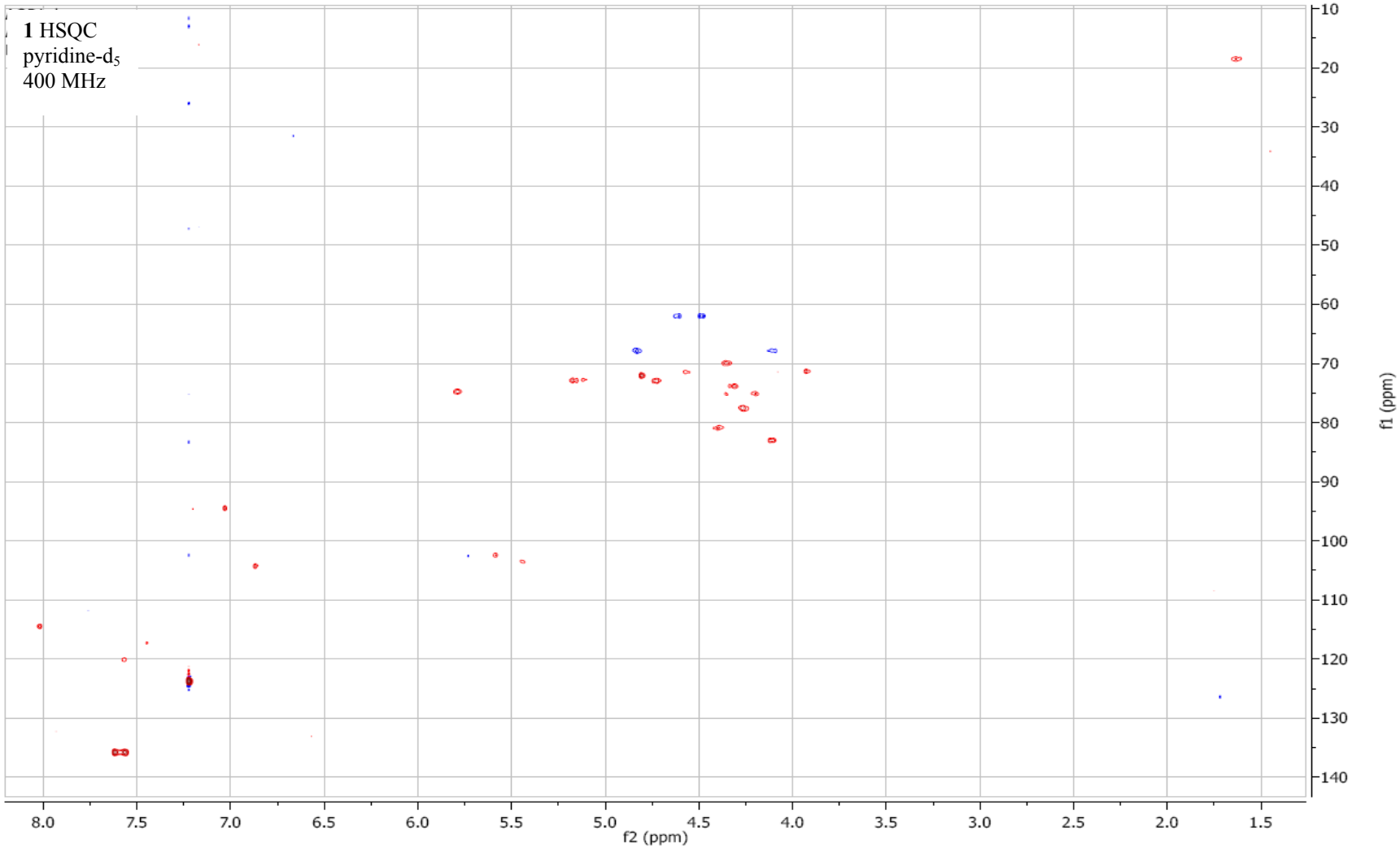


Figure S5. HMBC of **1**

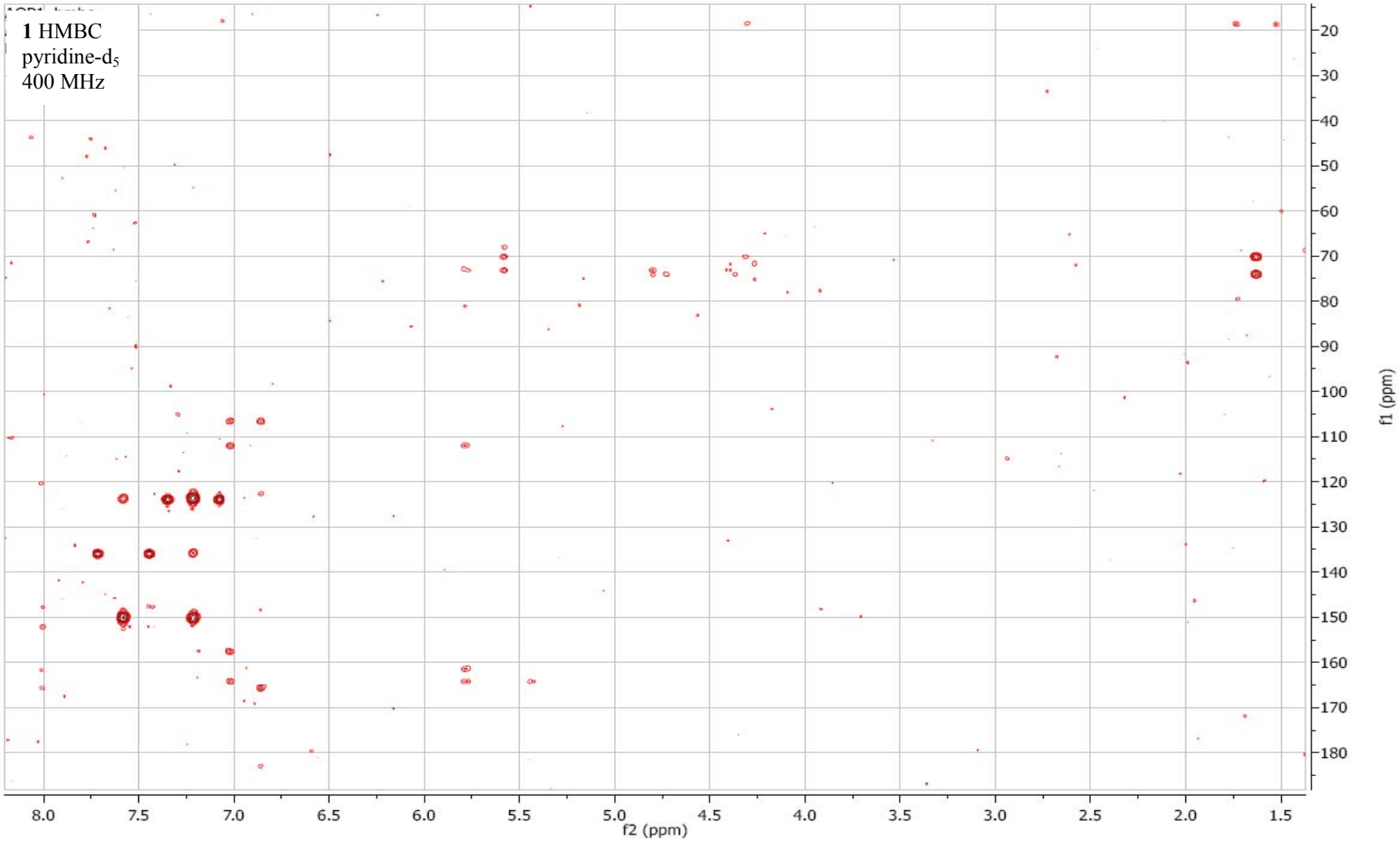
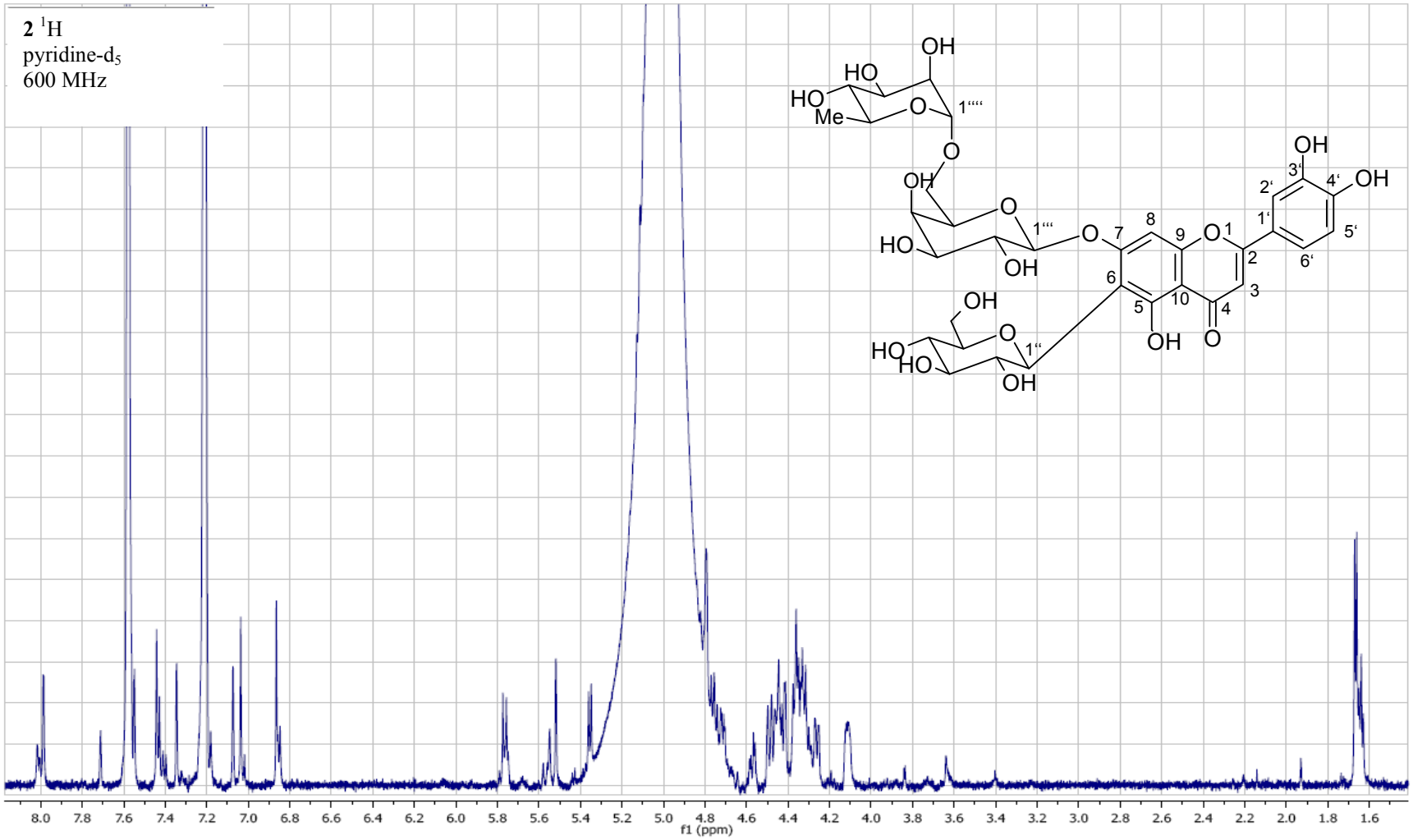


Figure S6. ^{1}H NMR spectrum of **2**



S9

Figure S7. HSQC of **2**

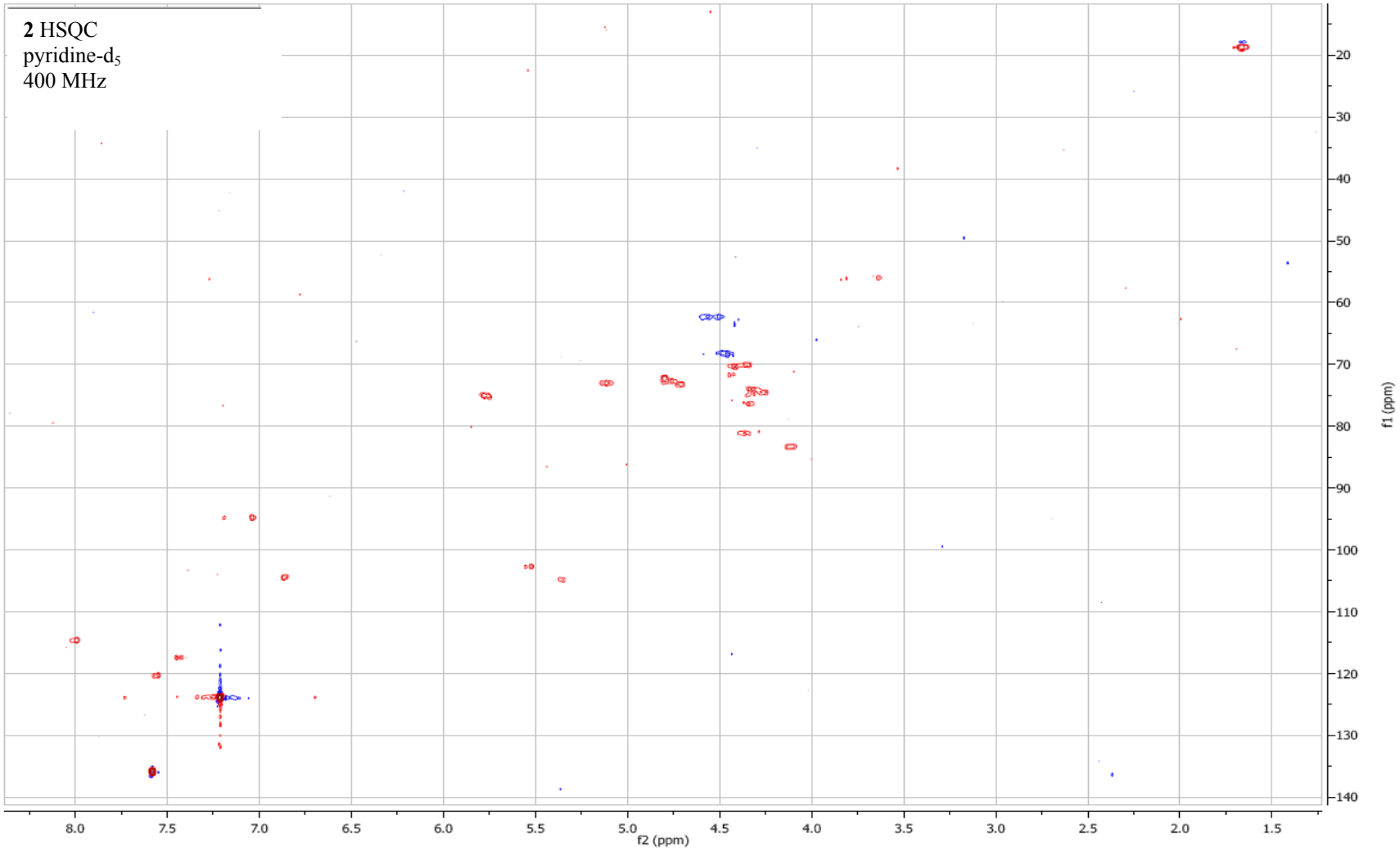


Figure S8. HMBC of **2**

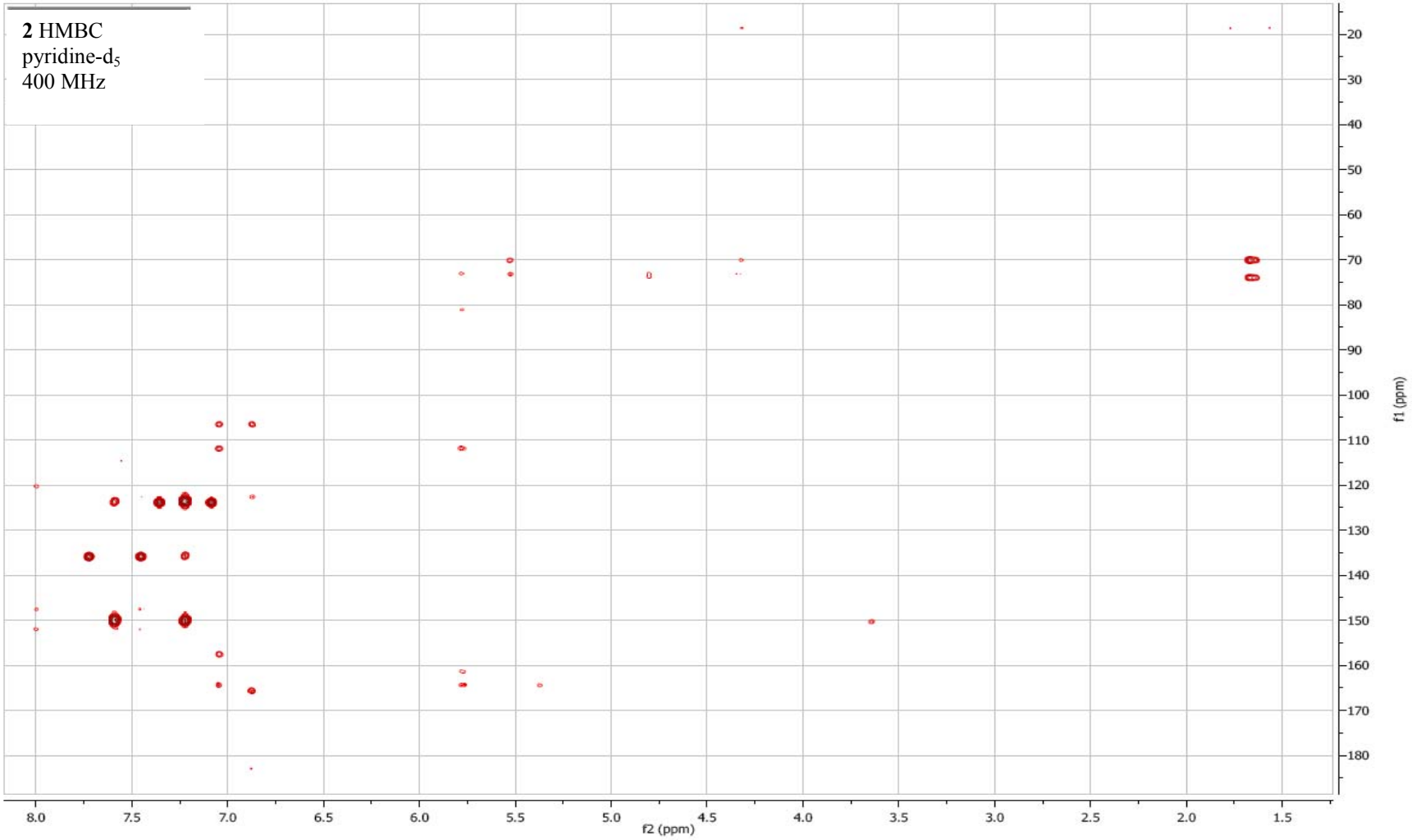
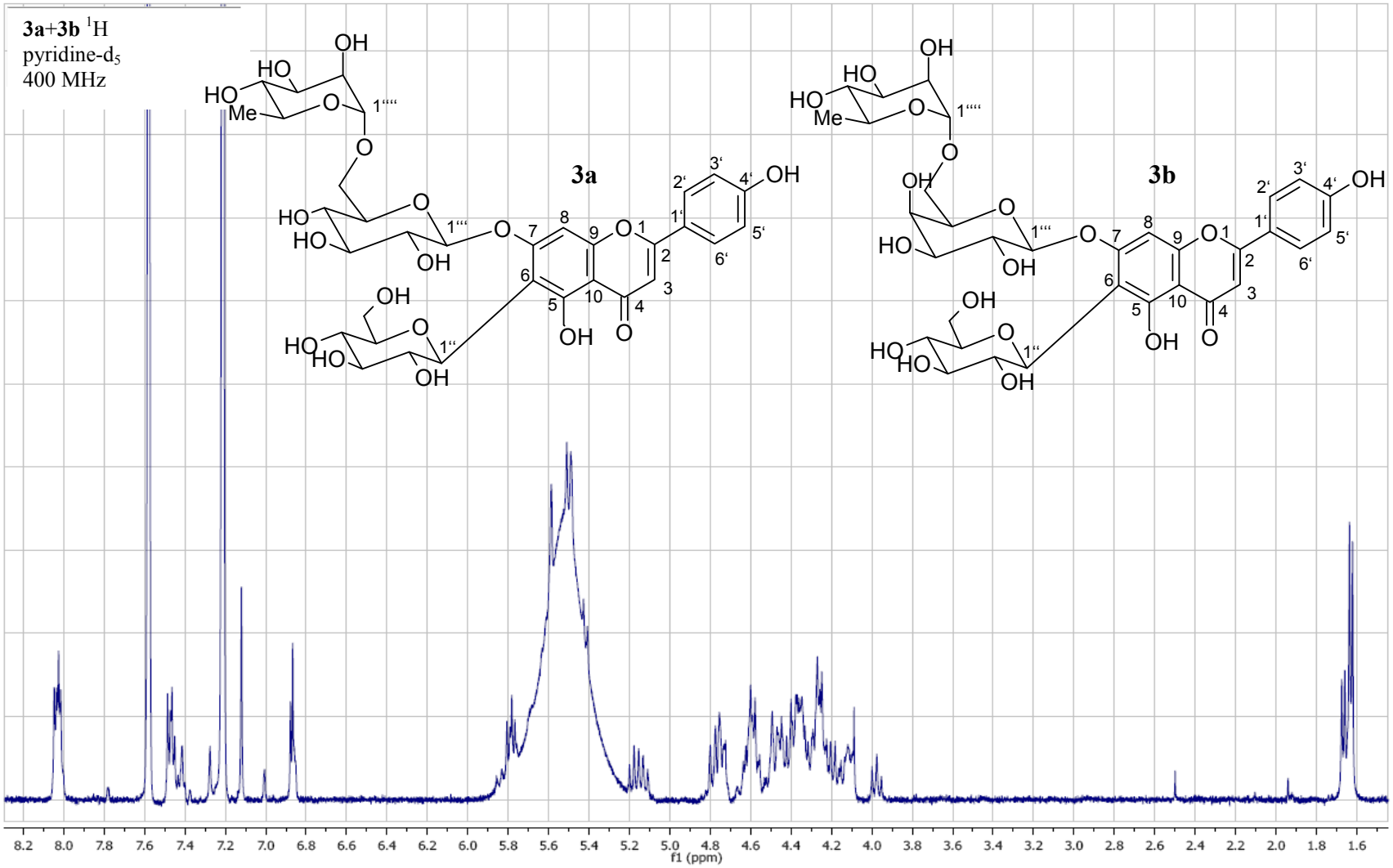


Figure S9. ^{1}H NMR spectrum of **3a**+**3b**



S12

Figure S10. DQFCOSY of **3a**+**3b**

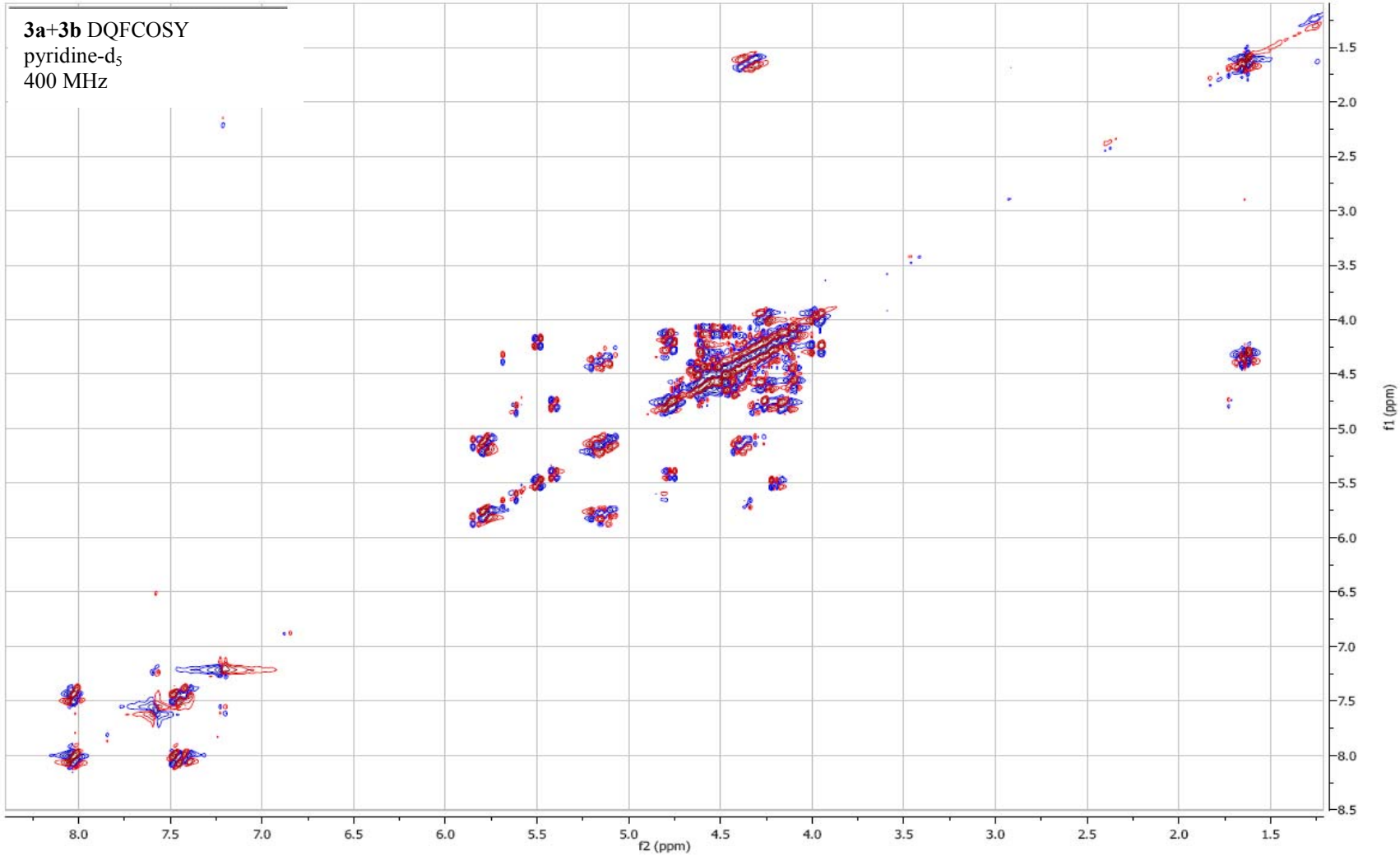


Figure S11. HSQC of **3a**+**3b**

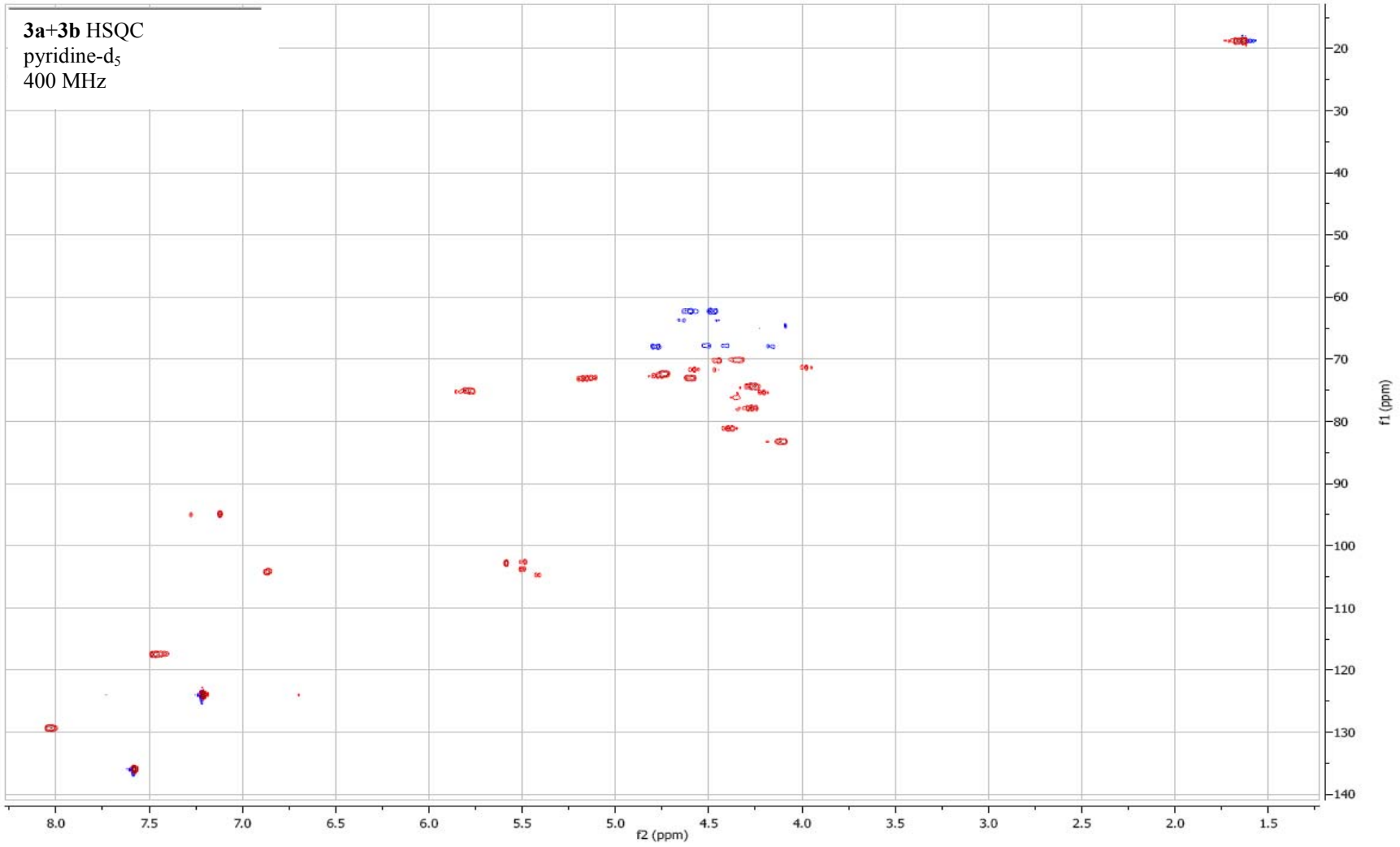


Figure S12. HMBC of **3a**+**3b**

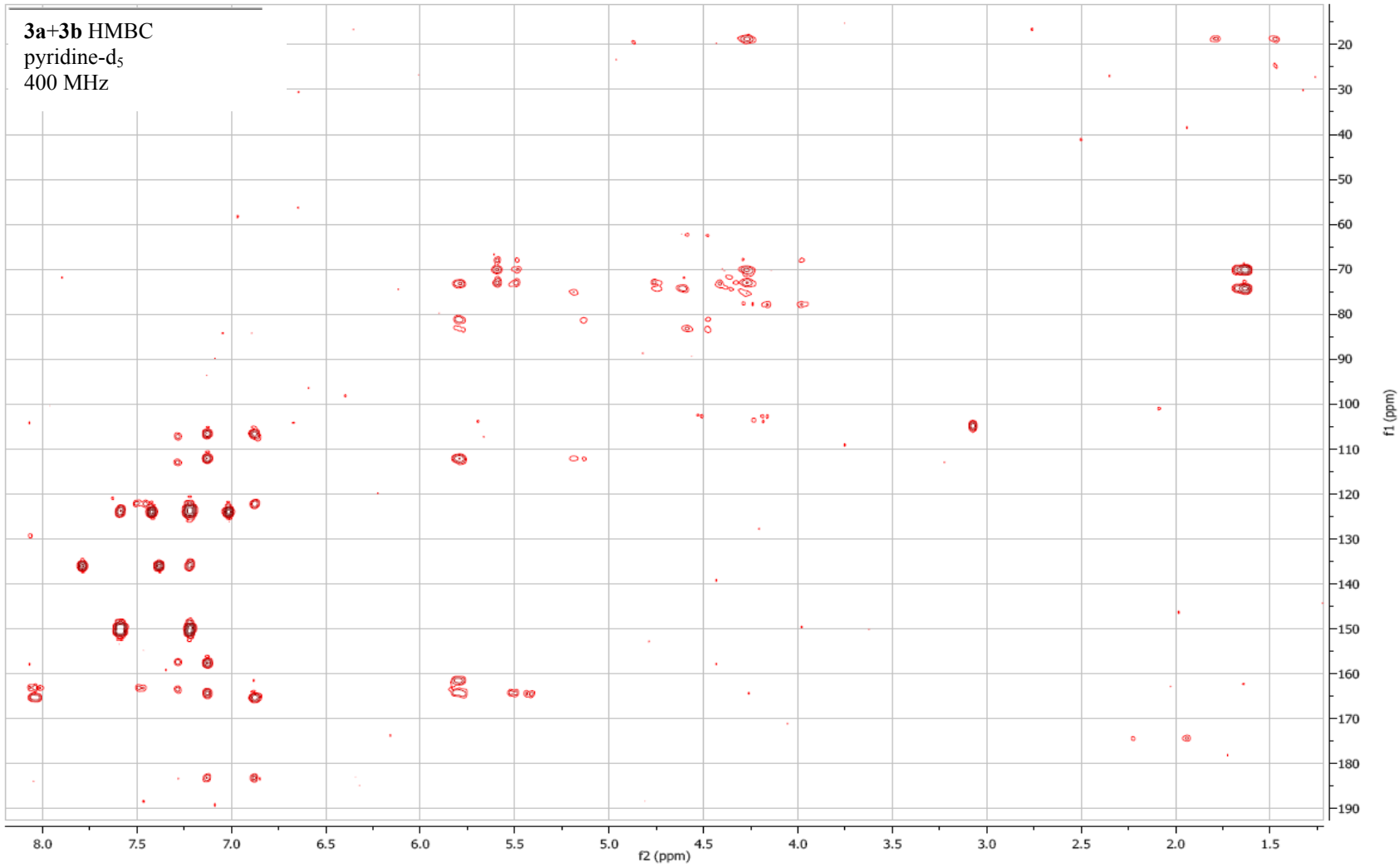


Figure S13. ^{1}H NMR spectrum of **4a**+**4b**

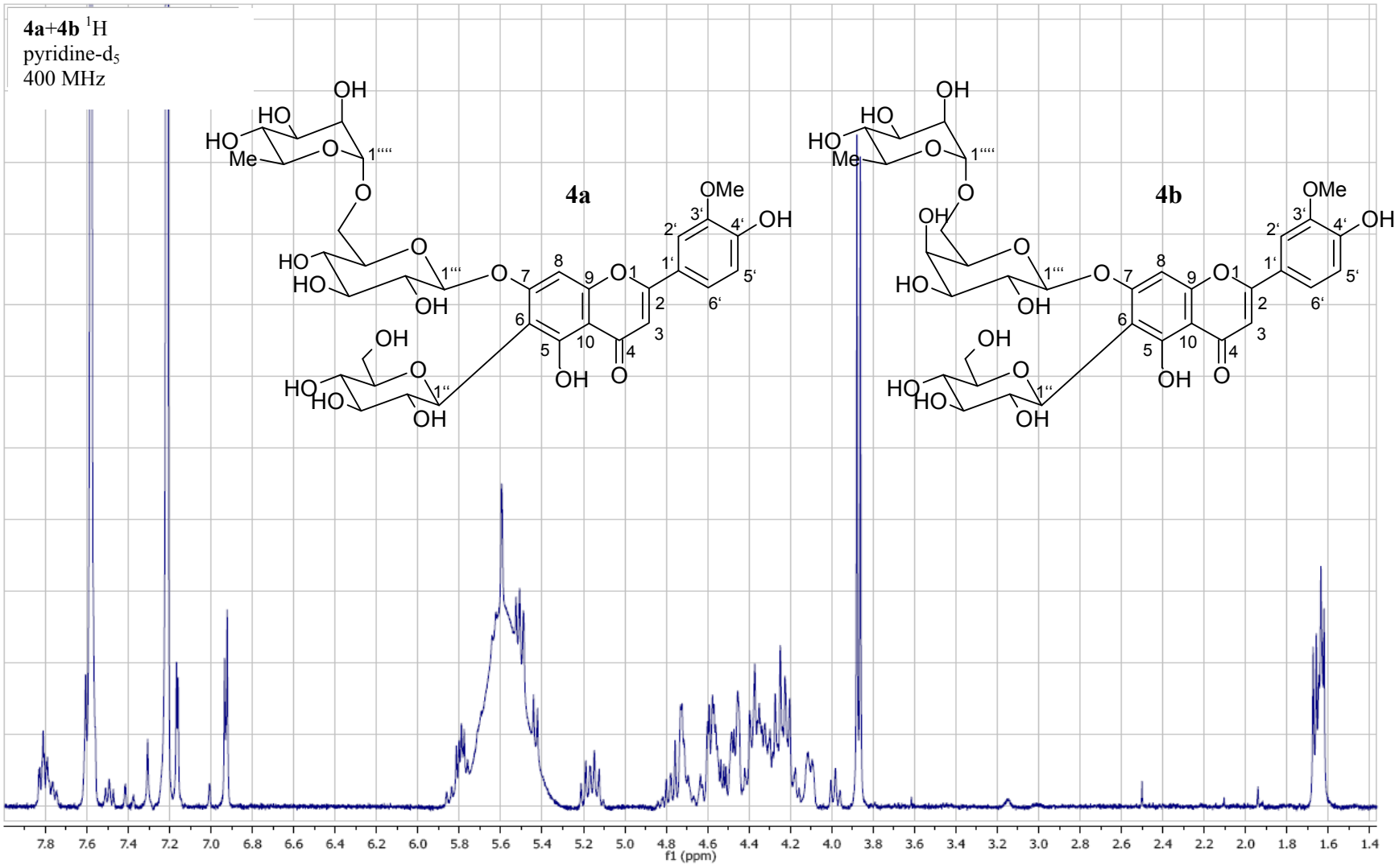
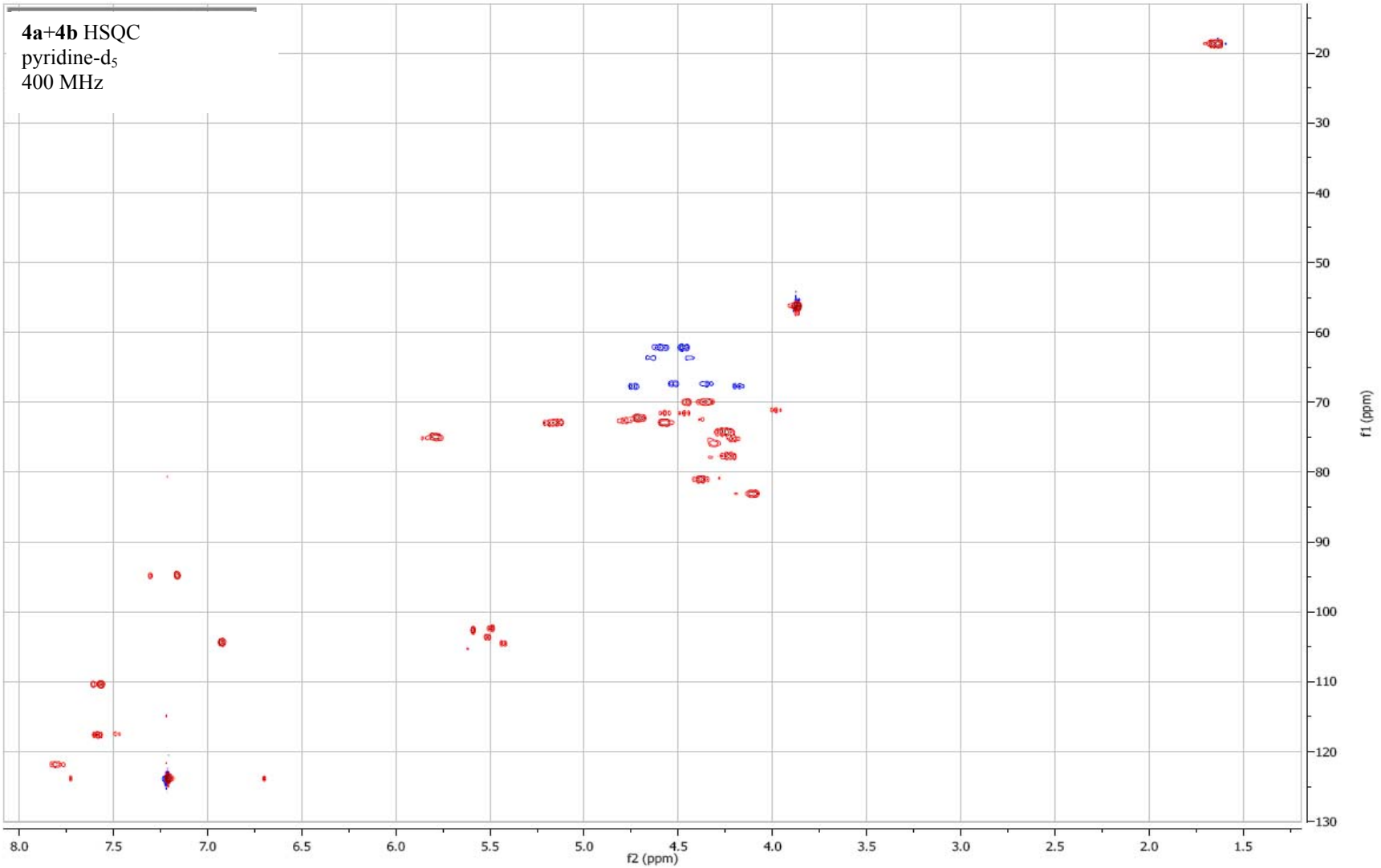


Figure S14. DQFCOSY of **4a**+**4b**

Figure S15. HSQC of **4a**+**4b**



S18

Figure S16. HSQC-TOCSY of **4a**+**4b**

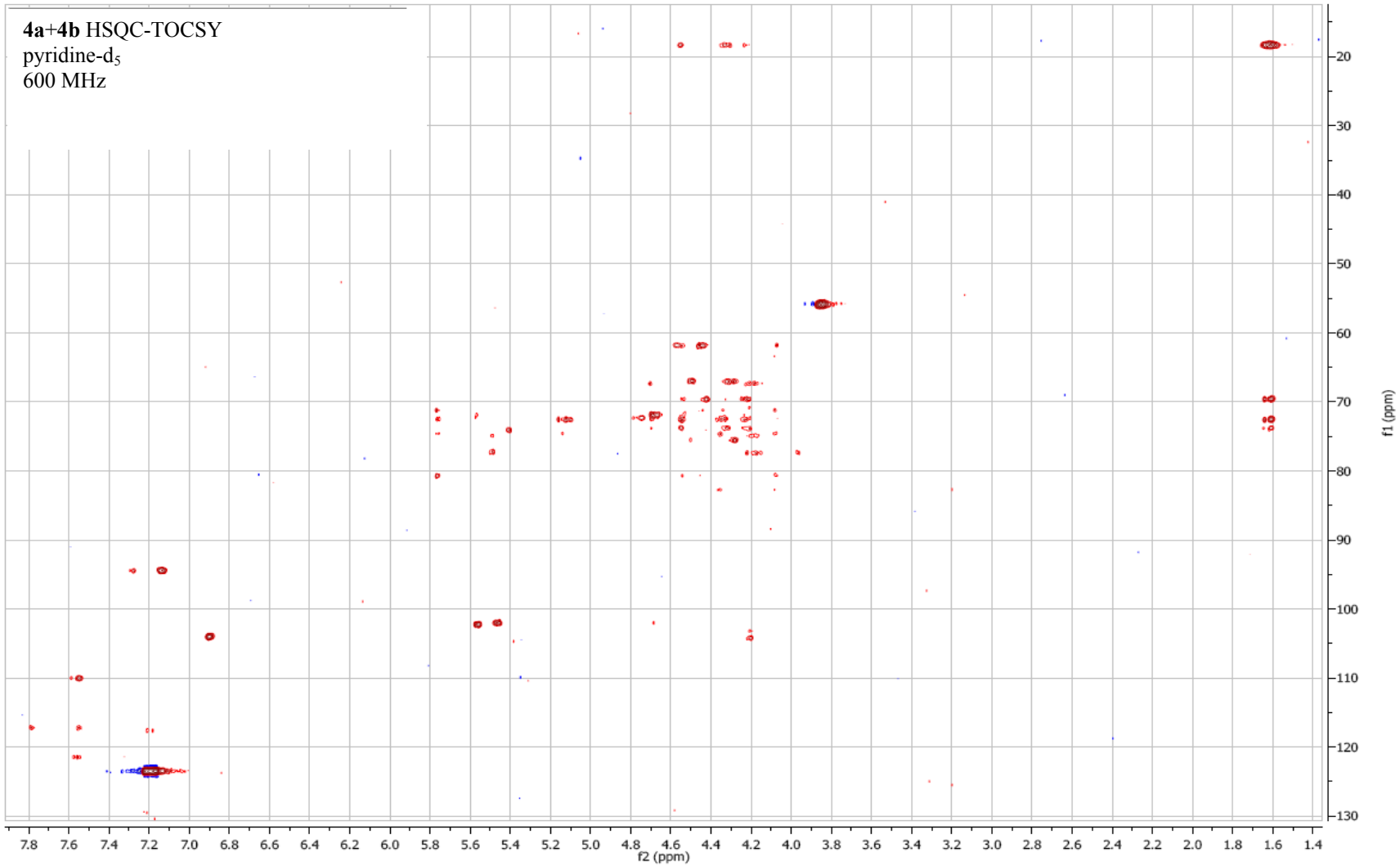


Figure S17. HMBC of **4a**+**4b**

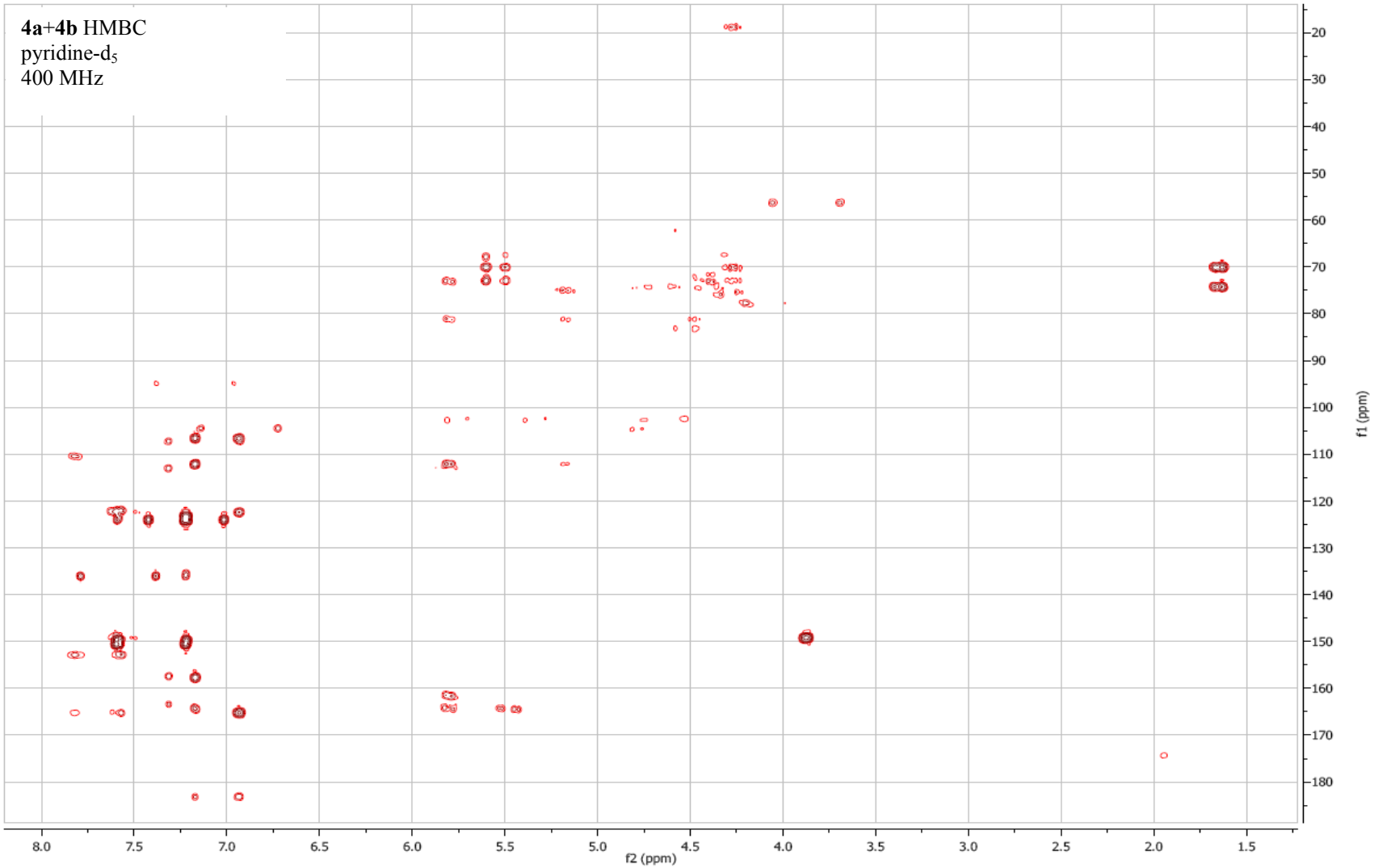


Figure S18. ^{1}H NMR spectrum of **5**

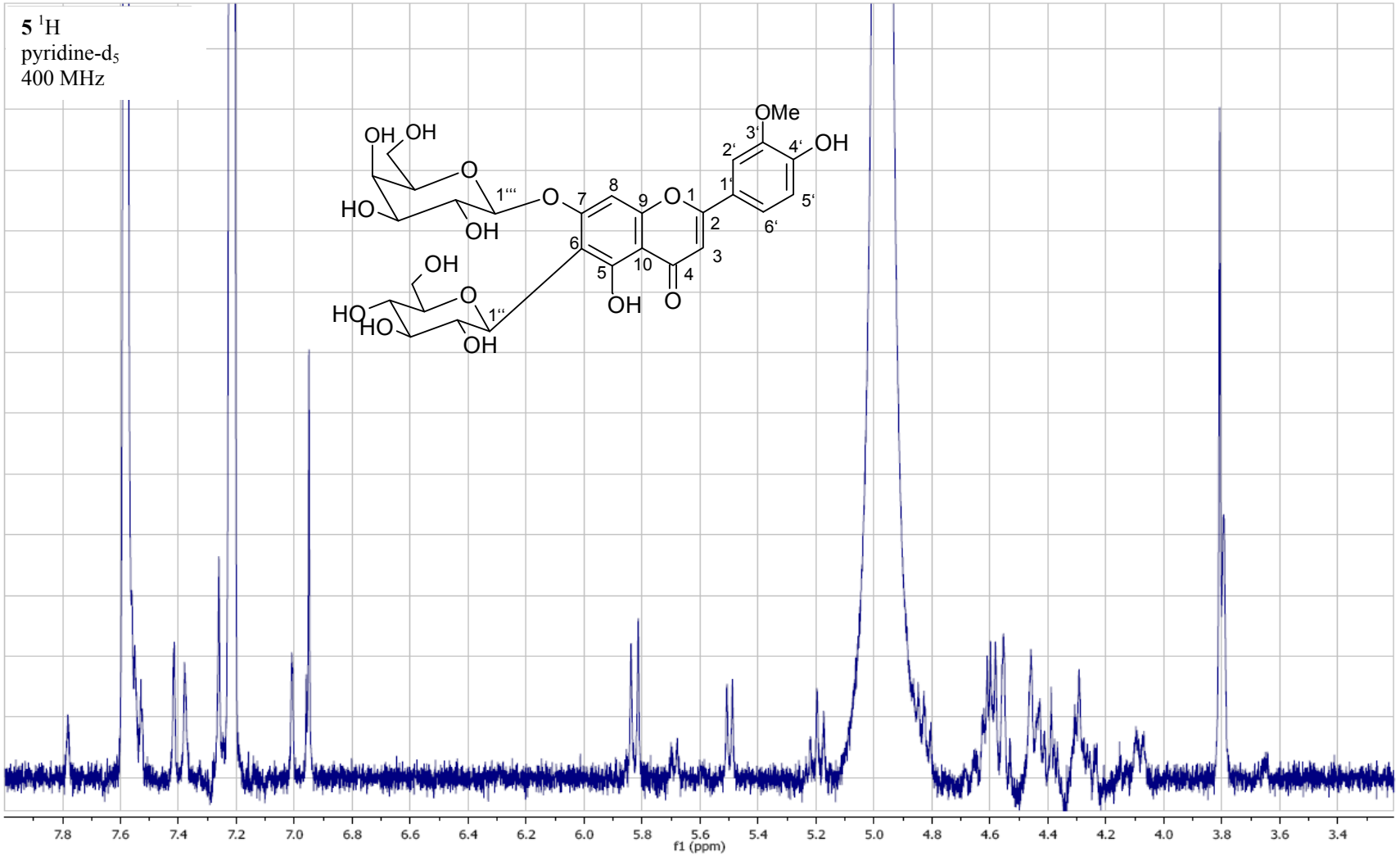


Figure S19. GCOSY of **5**

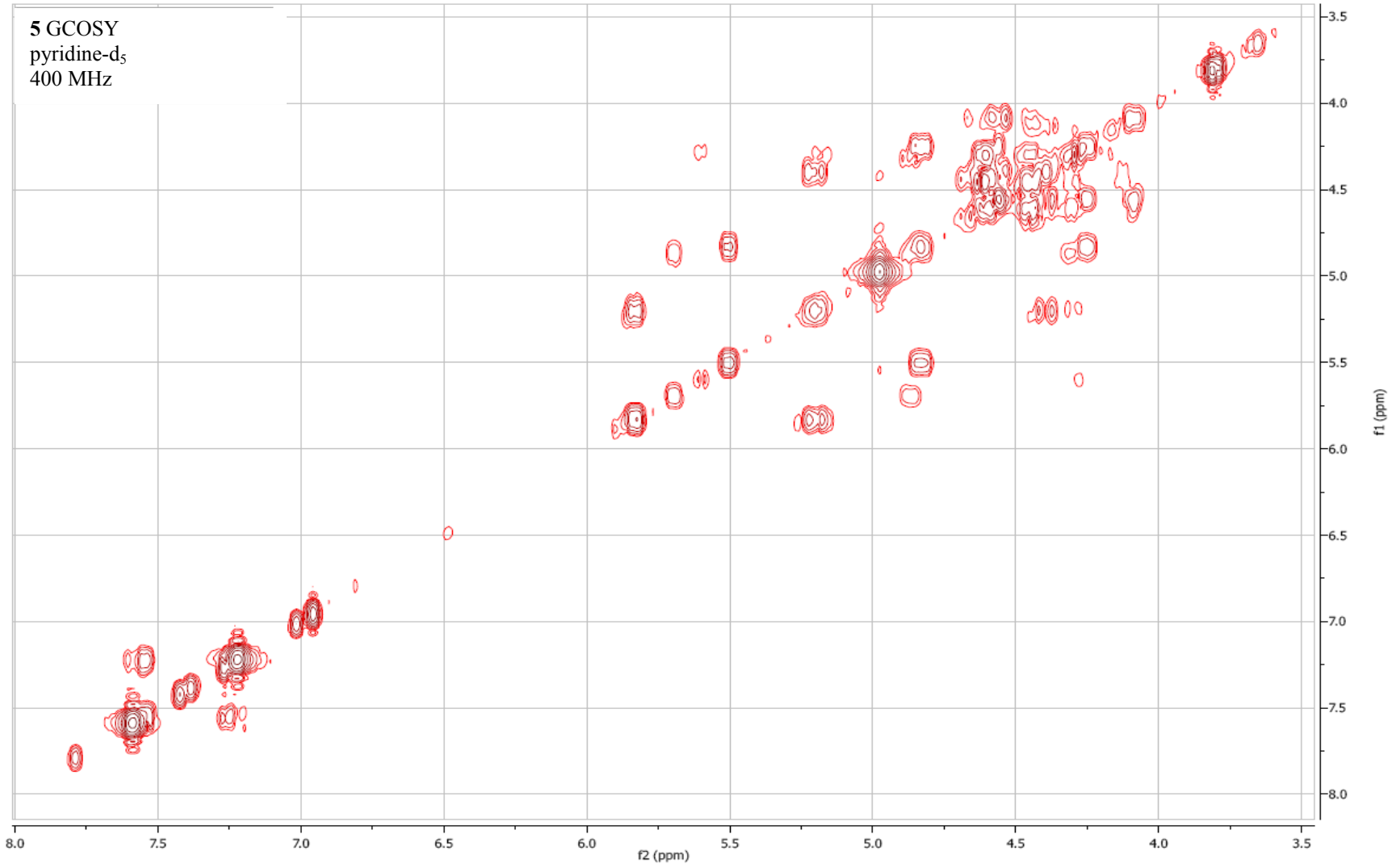


Figure S20. HSQC of **5**

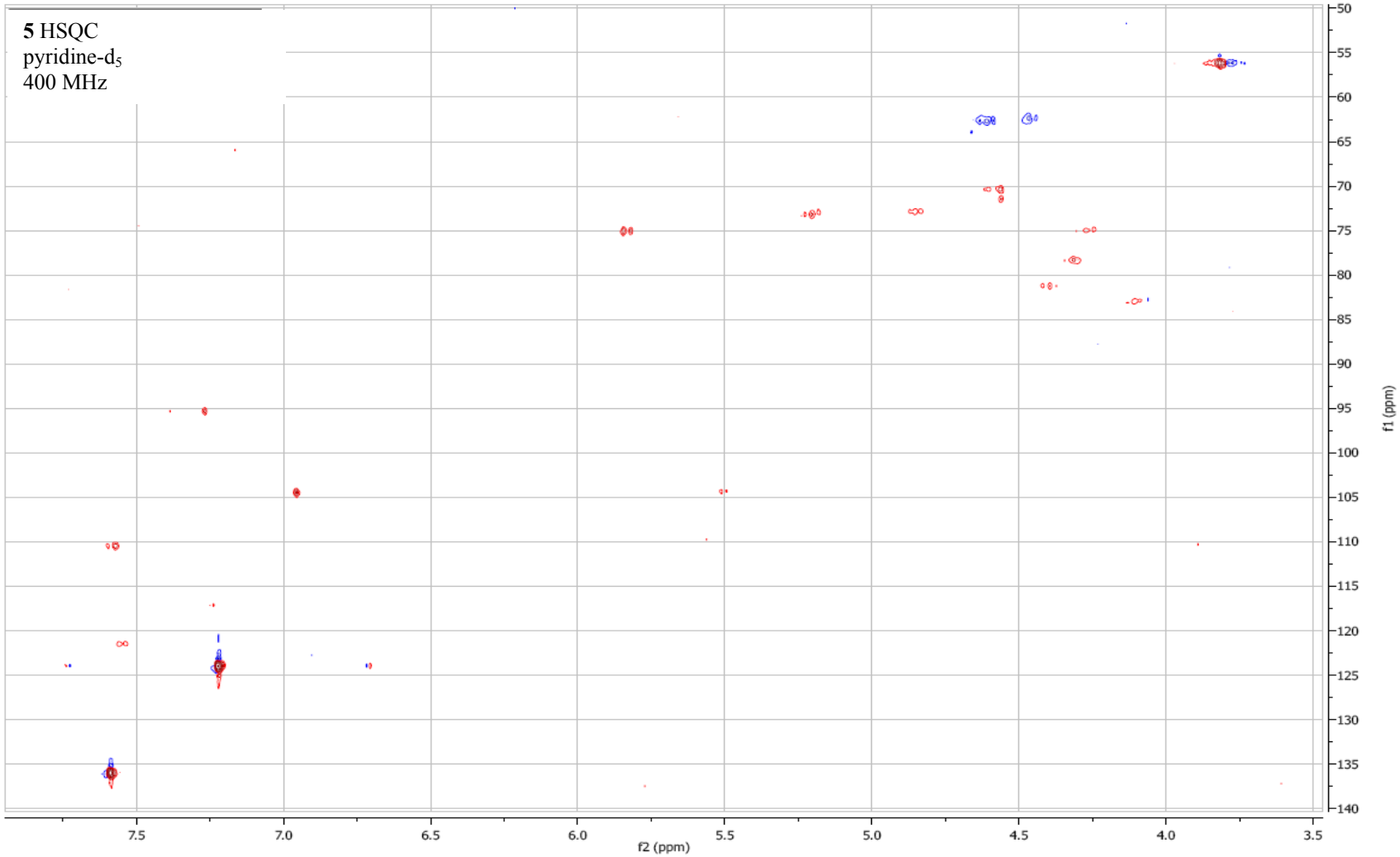


Figure S21. HMBC of **5**

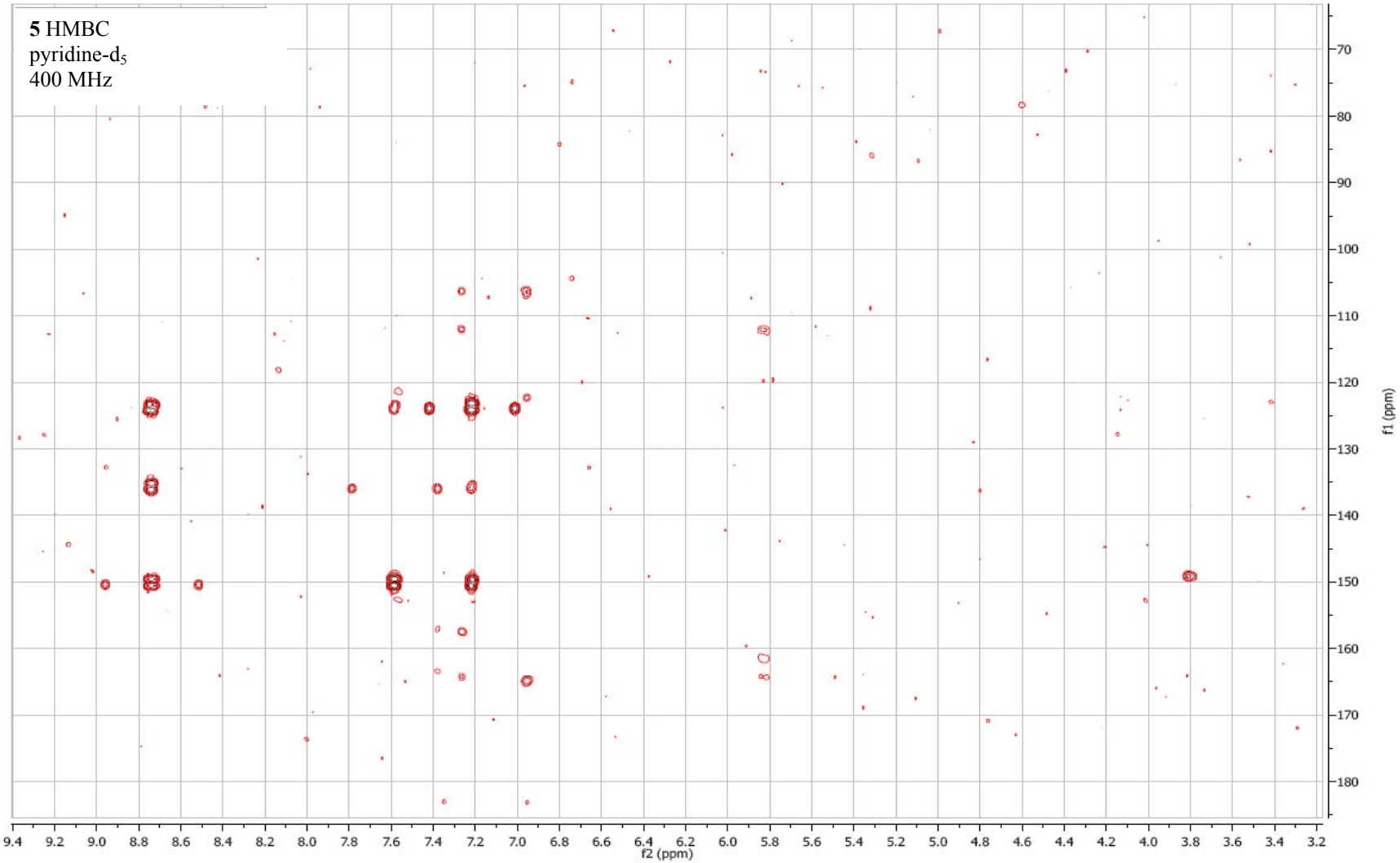


Figure S22. ^{1}H NMR spectrum of **6**

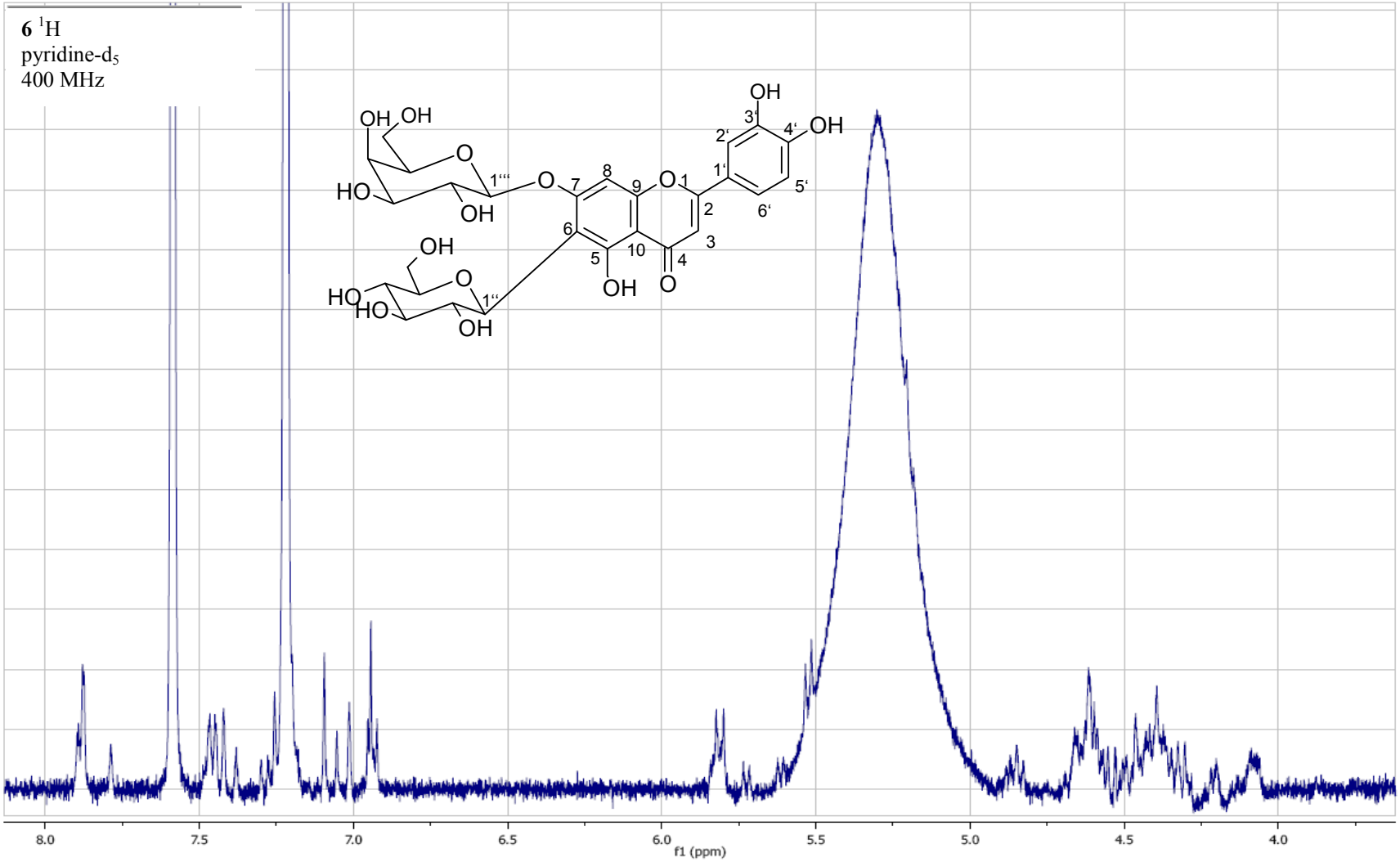


Figure S23. DQFCOSY of **6**

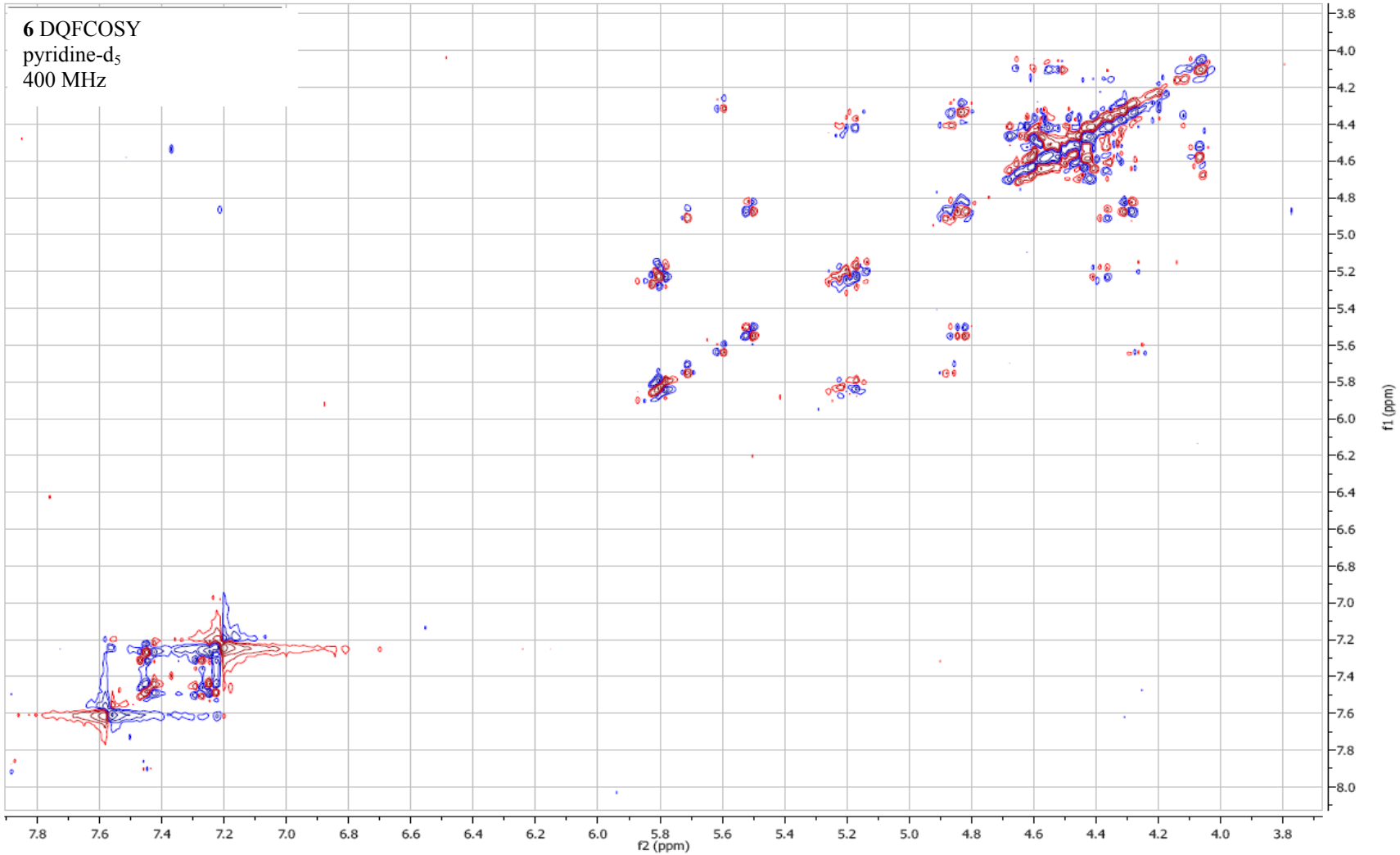


Figure S24. HSQC of **6**

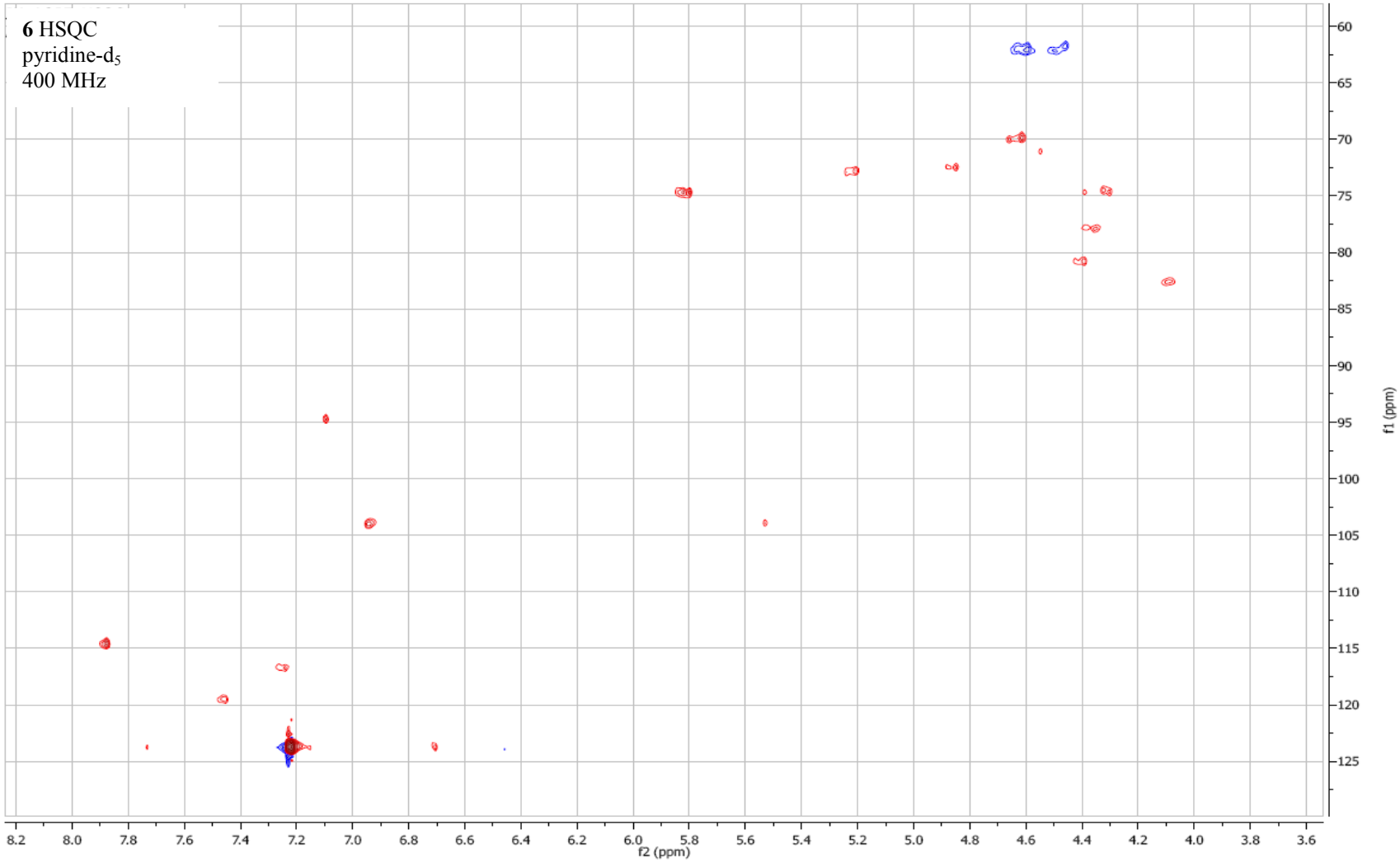
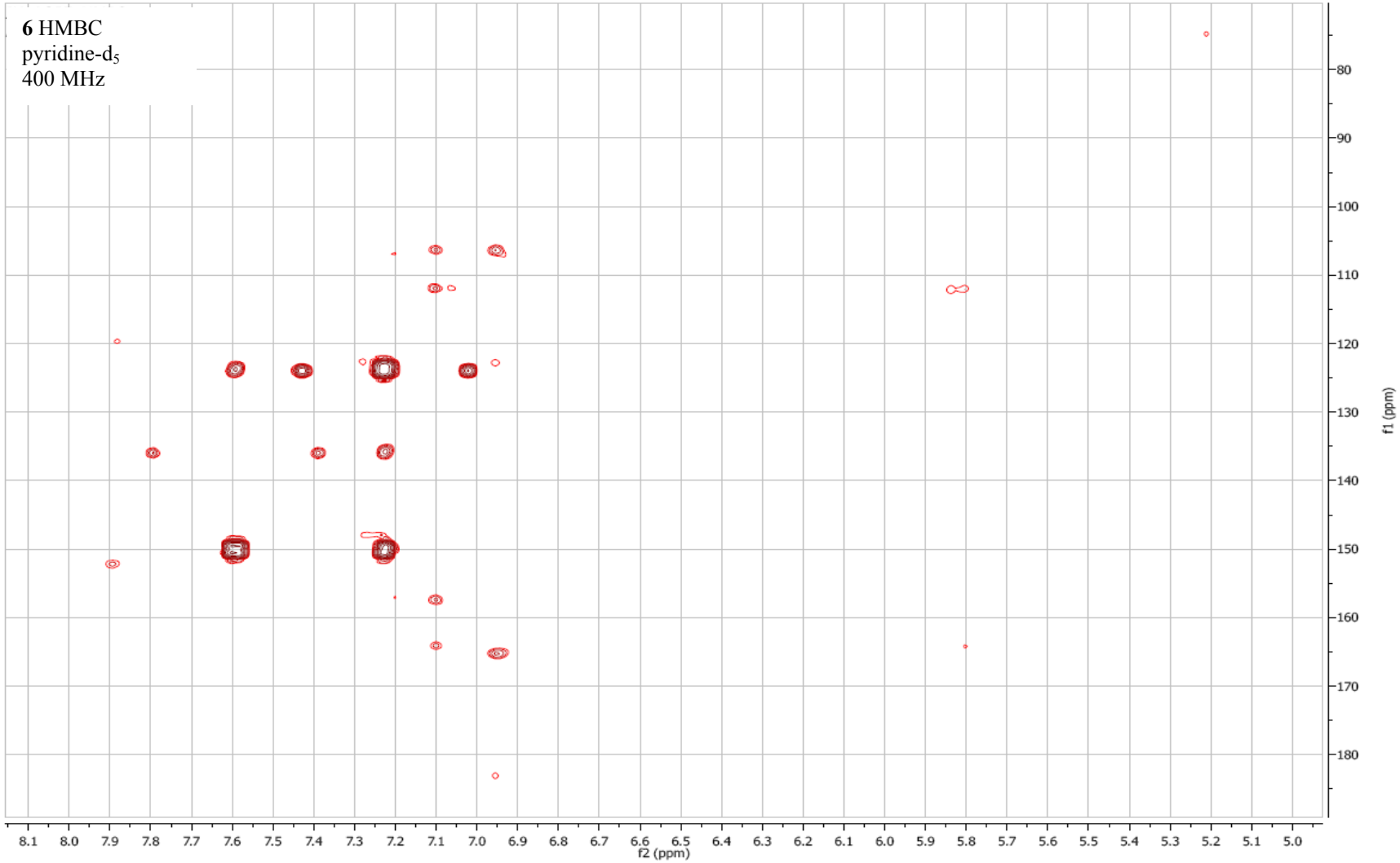


Figure S25. HMBC of **6**



3.2 Analytical section

The quantification of flavonoid-glycosides by HPLC-DAD and UV-spectrophotometry is described in the following manuscripts **"Quantification of flavonoid glycosides from the traditional Mongolian medicinal plant *Dianthus versicolor* FISCH."**, accepted for publication in *Journal of Separation Science* [23] on 17th November 2010 and **"HPLC-Determination of flavonoid glycosides in Mongolian *Dianthus versicolor* Fisch. (Caryophyllaceae) compared to quantification by UV-spectrophotometry"**, in preparation to be submitted for publication to *Phytochemical analysis* [24]. The development and validation of the analytical HPLC-DAD methods as well as the sample analyses and calculations were carried out by Astrid Obmann at the Department of Pharmacognosy, University of Vienna. HPLC-MS^n-analyses and interpretation of the MS-spectra were performed at the Department of Pharmacognosy by Martin Zehl together with Astrid Obmann. The quantification of flavonoids by UV-spectrophotometry was done by Astrid Obmann.

Quantification of flavonoid glycosides in an aqueous extract from the traditional Mongolian medicinal plant *Dianthus versicolor* FISCH.

Astrid Obmann[1], Martin Zehl[1], Sodnomtseren Purevsuren[2], Samdan Narantuya[2], Gottfried Reznicek[1], Christa Kletter[1], Sabine Glasl[1]

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Running title: Quantification of flavonoids in *Dianthus versicolor*

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Keywords: *Dianthus versicolor* / Flavonoids / Traditional Mongolian Medicine / Identification (LC/MS) / Quantification (LC/DAD)

Abstract

An HPLC-DAD method was established in order to investigate dried aerial parts of *Dianthus versicolor* FISCH. (Caryophyllaceae), a plant used in traditional Mongolian medicine against liver impairment. Aqueous extracts were separated on an Aquasil® C_{18} column with a linear gradient of acetonitrile (ACN) and water (adjusted to pH 2.8 with formic acid) as mobile phase. LC-IT-MS facilitated the assignment of 26 flavonoids, among them a series of rare C-glycosylated as well as O-glycosylated derivatives, which are assumed to be the active principles. Quantification was performed and validated using isovitexin-7-O-glucoside (saponarin) as external standard. The method showed good linear behaviour ($r^2 \geq 0.9999$) over the investigated concentration range (0.007-3.5 mg/mL). The good precision of the method allowed the successful qualitative and quantitative analysis of flavonoid-glycosides in the aqueous extracts prepared from five different *D. versicolor* samples. Depending on the origin of the samples, the total flavonoid content was found to vary considerably from 0.41% to 3.30% in the aqueous extracts and from 0.07 to 0.57% in the crude drug. In addition, the relative composition of the various flavonoids was found to differ strongly. These results highlight the need for proper quality control for this herbal drug.

1 Introduction

Dianthus versicolor FISCH. (Caryophyllaceae) is a medicinal plant well known in traditional Mongolian medicine. In Mongolian literature it is reported that it influences the blood pressure and is used to treat cardiovascular diseases by traditional physicians [15]. Due to its property to increase the contractility of the uterus, *D. versicolor* is used against bleeding after birth and against strong bleeding during menstruation [15]. It is furthermore known to be beneficial in pneumonia, typhoid fever, and poisoning [2]. Its actions include diuretic and antiinflammatory effects, and, despite its staunching properties, it may cause bleeding when overdosed [3]. Another indication, although scientifically less studied, comprises impairments of the liver as reported by a very reputed traditional health institution [4]. Support for this use was obtained by recent investigations in the model of the isolated perfused rat liver [6]. According to the "Report of market research on Mongolian traditional medicinal drugs" prepared in September 2007 for the WHO [3], *D. versicolor* ranks among the 45 most common domestic herbal drugs traded in Mongolia. With an average price of about € 3,- per kilogram on the local markets [3], it belongs to one of the most expensive domestic drugs in Mongolia. Scientific data about its pharmacology and phytochemistry are scarce. The plant has been shown to contain a series of pentacyclic triterpenoid saponins [5]. Our investigations have shown that an aqueous extract and a flavonoid-enriched fraction of *D. versicolor* cause an increase of bile flow in the isolated perfused rat liver [6]. Bioactivity-guided fractionation led to the isolation and structure elucidation a number of flavonoids [7]. However, to date neither methods for qualitative analysis of these active constituents nor methods to quantify the flavonoids in *D. versicolor* have been reported.

In this paper, five plant samples of *D. versicolor*, collected in different years and different Mongolian regions, are compared by TLC and HPLC. TLC analysis provides a

quick overview and showed strong variations in the relative concentration of the main flavonoids in the five aqueous extracts. Metabolite profiling was performed by LC-MS^n which enabled the identification of flavonoid-glycosides with up to three sugar moieties. In order to quantify these substances, a new HPLC-DAD method was developed using saponarin as external standard.

The validated method allows the simultaneous quantitative determination of 26 flavonoids in *D. versicolor* and was successfully applied to compare the flavonoid pattern of five different samples.

2 Materials and methods

2.1 Chemicals and reagents

The reagents for TLC were natural product reagent A (diphenylboric acid-2-amino-ethylester) and PEG 400. They were of analytical grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA). Saponarin was purchased from Roth (Karlsruhe, Germany; batch 21896936, HPLC purity 100% according to enclosed certificate). Solvents used for extraction were of analytical grade, those used for HPLC were of gradient grade and obtained from VWR (West Chester, Pennsylvania, USA).

2.2 Plant material

Aerial parts of five *D. versicolor* FISCH. batches **(a-e)** were collected in different Mongolian provinces. The plant material was identified by E. Ganbold, Health Sciences University, Mongolia. The denomination of the species bases on Grubov [8]. Voucher specimens are kept in the Department of Pharmacognosy, University of Vienna, Austria, herbarium numbers 03/04/mon/H (**b**), 14/03/mon/H (**d**, **e**) and in the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia, herbarium numbers 424 (**a**)

and H 20050805 (**c**). For details concerning region and year of collection see supporting information (see Table S1).

2.3 Preparation of extracts

For phytochemical investigations aqueous extracts of all five *D. versicolor* samples were prepared according to a method we had described earlier [6]. Briefly, 10 g of the dried aerial parts were powdered and extracted with 250 mL water (pH 2, adjusted with trifluoroacetic acid) for 1h at 40°C by shaking gently. This procedure is intended to resemble the physiologic processes taking place after the consumption of the crude herbal drug which is taken together with a certain amount of water. After extraction the highly volatile trifluoroacetic acid was removed under reduced pressure at a maximum temperature of 45°C and the remaining solution was lyophylised. The yields are given as drug to extract ratio (DER).

2.4 TLC analysis

TLC served as a quick method to compare the flavonoid fingerprints of the *D. versicolor* samples. Silica gel 60 F_{254} coated aluminium sheets (Merck, Darmstadt, Germany) were used as stationary phase. The mobile phase was a mixture of ethylacetate, formic $acid_{conc}$, acetic $acid_{conc}$ and water (100:11:11:26 v/v/v/v). Visualisation was performed by spraying with a 1% methanolic solution of natural product reagent A followed by a 5% ethanolic solution of PEG 400 under UV 366nm.

2.5 HPLC-DAD and HPLC-DAD-MS^n conditions

Qualitative and quantitative HPLC-DAD analyses of the aqueous extracts were conducted on a Prominence LC-20AD coupled to a Prominence SPD-M20 Diode Array Detector (Shimadzu Corporation, Kyoto, Japan). For data analysis the software

LabSolutions version 1.25, LC-Postrun (Shimadzu, Kyoto, Japan) was used. Peaks were integrated automatically by the method of vertical separation applying the following parameters: the minimum peak width was set at 5 sec, at a slope of 1000 µV/min, and a minimum peak area of 14450 units according to the LOQ for saponarin. The wavelength was set at 340 nm for detection in accordance with the maximum absorption of the flavonoids measured by DAD (see Fig. S1). An Aquasil® C_{18} column (4.6 mm x 250.0 mm, 5µm, Thermo Fisher Scientific, Waltham, Massachusetts) served as stationary phase. Doubly distilled water adjusted to pH 2.8 with formic acid (A) and ACN (B) were used as mobile phase at a flow rate of 1.0 mL/min. Separations were carried out at a column oven temperature of 15°C. The developed gradient could not provide baseline separation of the polar compounds but allowed an adequate separation of all constituents in a reasonable time: 15% B to 23.75% B within 58 min (rate: 0.15%/min) followed by an increase up to 29% B within 15 min (rate: 0.35%/min) (see Fig. 1).

The LC-MS 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 as described above. The eluent flow was split roughly 1:8 before the ESI ion source, which was tuned as follows: capillary voltage: +3.7 kV (i.e. negative ion mode), nebulizer: 30 psi (N_2), dry gas flow: 8 L/min (N_2), and dry temperature: 340 °C. The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode where each MS scan (m/z 120-1000, average of 5 spectra) was followed by MS^2, MS^3 and MS^4 scans (m/z 40-1000, average of 3 spectra, isolation window of 4 Th, fragmentation amplitude of 1.0 V) of the two most intense precursor ions in the previous scan (provided that they were detected with sufficient intensity).

Sample preparation: 0.1 g of the aqueous extracts was redissolved in 5.0 mL of methanol-water (40:60, v/v). The solution was treated in the ultrasonic bath for 10 min at room temperature. After centrifugation at 13500 rpm for 10 min, 40 µL of the sample solution was subjected to HPLC analysis.

3 Results and discussion

3.1 General

The aim of our research was to provide appropriate methods for the qualitative and quantitative analysis of the flavonoids in the Mongolian medicinal plant *D. versicolor*. Such tools represent the basis for the quality control of this traditionally used medicinal herb and its remedies.

The TLC-comparison (see Fig. S2) of the five samples showed two types of fingerprints differing in the number and ratio of detected flavonoids. Samples **a** and **b** were characterised by spots up to R_f values of 0.28, whereas in samples **c**, **d**, and **e** additional fluorescent bands were located in a much less polar range from R_f 0.32 to 1.0. The HPLC analyses confirmed this result (see Fig. 1). The respective peaks were identified or characterised by LC-MS (see Table 1 and section 3.2). The quantification method was developed using saponarin as external standard (see section 3.3).

3.2 Characterisation of compounds

Based on the HPLC fingerprint, 26 major flavonoid constituents were selected for the quantitative analysis. The structures of 13 compounds have been elucidated following bioactivity-guided fractionation in a previous study [7], and are shown in Fig. 2. Identification of the remaining components was based on their characteristic UV and MS^n spectra [9].

According to TLC and HPLC analyses, the samples **a** and **b** are mainly characterised by highly polar flavonoids (Fig. 1 and Table 1, compounds **1-11**). Nine of them have earlier been isolated by our group and found to be *C*- and *O*-glycosides of apigenin, luteolin, and chrysoeriol. They all possess a *C*-glycopyranosyl group at position 6 and an additional mono- or disaccharide bound to the hydroxyl-group at position 7 (Fig. 2). Compound **4**, which was detected as $[M-H]^-$ ion at *m/z* 725.2, showed a predominant neutral loss of 116 Da in MS^2 that is attributed to the loss of a malyl-group. Further CID experiments on the resulting fragment ion at *m/z* 609.2 yielded spectra that are identical to those of luteolin-6-glucopyranosyl-7-O-galactopyranoside (**1**), allowing the identification of compound **4** as a luteolin-*C*-hexosyl-*O*-hexoside malyl-ester (Table 1 and Fig. S3). Analogously, compound **11** was identified as a malyl-ester of apigenin-*C*-hexosyl-*O*-hexoside. Apigenin-6-*C*-glucosyl-7-O-(6-malyl-glucoside), together with several malyl-esters of glycosylated anthocyanins, have previously been isolated from *D. caryophyllus* [10].

In sample **c**, those compounds could also be detected, but were of much lower abundance. Instead, a series of less polar flavonoids predominated, which were identified as either *C*- or *O*-glycosides of apigenin, luteolin, and 3'- or 4'-methylluteolin (Fig. 1 and Table 1, compounds **12-26**). *C*-hexosides showed typical neutral losses of 90 and 120 Da due to cross-ring cleavage of the carbohydrate moiety ($^{0,3}X_0^-$ and $^{0,2}X_0^-$ ions, respectively), whereas *O*-linked deoxyhexosyl-hexosides, hexosides, and hexuronides are characterized by the neutral loss of 308, 162, and 176 Da (Y_0^- ions), respectively [11]. The aglyca were identified by comparison of the respective mass spectra with an in-house library containing mass data of 57 flavonoids (Fig. S4).

The remaining two samples, **d** and **e**, contained all the above mentioned compounds at comparable levels.

3.3 Quantification of flavonoids

3.3.1 Optimisation of chromatographic conditions

As is evident from Table 1 and Figure 2, the main flavonoid components in the aqueous extracts of *D. versicolor* are structurally highly similar, with some of them differing only in the isomerism of a sugar unit. Consequently, the chromatographic separation is highly challenging and a practical method must be a compromise between the achieved degree of separation and a reasonable analysis time. For the same reason existing methods for the quantification of flavonoids in other *Dianthus* species [12] could not be applied. Various stationary phases were tested such as Phenomenex® Luna Phenyl-Hexyl, LiChrospher® 100 RP-18e, and Aquasil® C18, with the latter leading to most satisfactory results. Trials at various column temperatures, above and below room temperature, showed 15°C to be optimal. This gradient is a compromise providing separation of most of the 26 compounds, although with rather low resolution for some peak pairs, at acceptable retention times.

3.3.2 Method validation

The method was validated with respect to linearity, accuracy and precision following the ICH guidelines on validation of analytical methods [13].

3.3.2.1 Calibration curve, LOD and LOQ

Isovitexin-7-O-glucoside (saponarin) was chosen as external standard. It is present in all investigated samples of *D. versicolor* and commercially available. As a flavonoid diglycoside with a molecular weight of 594 Da it matches very well with the other flavonoids found in *D. versicolor*, whose molecular weights ranged between 432 Da and 770 Da (see Table 1). For assessing the linearity, six different concentrations of

saponarin ranging from 0.007-3.5 mg/mL were examined in triplicates. The least square line and the correlation coefficient were calculated and showed the method to be linear (r^2>0.9999; linear regression equation y = 22279996x - 42847). The standard deviation of the y-intercept (s_a) was calculated to be 72352.

The LOD and LOQ for saponarin under the chromatographic conditions were determined at the *S/N* of 3 and 10, showing values of 0.05 µg/mL and 0.17 µg/mL, respectively.

3.3.2.2 Response factors

The content of each compound to be quantified was calculated considering the peak areas of the analytes and those of the external standard as well as the concentration of the sample and the standard solution. Since the majority of the quantified compounds was not commercially available, the response factors were calculated based on the ratios of the molecular weights. It has been shown that the response factors of compounds with the same chromophor (see Fig. S1) are directly proportional to their molecular weights [14]. Consequently, the molecular weight of each analyzed compound was compared to the molecular weight of the standard saponarin (594 Da). This procedure follows the European Pharmacopoeia which provides mathematical corrections as described above, e. g. for the quantification of Ginkgo folium by HPLC [15]. The obtained response factors ranged between 0.73 and 1.30 (see Table S2) and were included in the above mentioned calculation. Therefore, this method allows not only the determination of the total flavonoid content but also the estimation of single compounds.

3.3.2.3 Accuracy

The accuracy of this newly developed method was investigated by comparison to an established UV spectrophotometric method from the European Pharmacopoeia that was

slightly modified as published earlier [6]. For this, samples **a** and **c** have been analysed with both methods in triplicates. The total flavonoid content as determined by the HPLC method was 107.3% for sample **a** and 91.9% for sample **c** compared to the UV method.

3.3.2.4 Precision

For determination of the inter- and intra-day variability, nine solutions of the aqueous extract of sample **a** were individually prepared. Three samples were analyzed per day in three consecutive days. Variations were expressed by the relative standard deviations (RSD) which were less than 2.0% and demonstrated a good precision and repeatability of the proposed method. The results are summarised in Table 3.

3.4 Sample analysis

The established HPLC method was applied to determine the flavonoid content in the aqueous extracts of five different *D. versicolor* samples. Those aqueous extracts were prepared by mimicking gastric conditions (see section 2.3). The flavonoid contents in the corresponding crude drugs were recalculated on basis of the yields given as DER and represent minimum values since the extraction efficiency was not evaluated (see Table 2). The HPLC fingerprints (Fig. 1) showed a comparable qualitative composition of the five samples but revealed substantial quantitative differences. For example, the content of luteolin-6-glucopyranosyl-7-O-galactopyranoside (**1**) ranges from 0.02% in samples **b** and **d** to 0.26% in sample **c**. Apigenin-6-glucopyranosyl-2"-O-rhamnoside (**12**) could only be quantified in the samples **c** (0.70%), **d** (0.05%), and **e** (0.02%), but was below the LOD in the samples **a** and **b**. In general, the extracts of samples **a** and **b** contained predominantly flavonoid glycosides of higher polarity with two or three sugar moieties attached, whereas in samples **c**, **d**, and **e** mainly monoglycosides were detected. The aqueous extracts of samples **a** and **c** were characterised by high total flavonoid

contents (1.91% and 3.30 %, respectively), whereas the total contents of the others were remarkably low, ranging from 0.41% to 0.52%. This might be due to differences in habitat and climate between the five collection sites. These results highlight the need for proper quality control for this herbal drug.

4 Concluding remarks

In the present study, a new HPLC-UV method was developed for the quantification of 26 flavonoids in the aqueous extract of *D. versicolor* using saponarin as external standard. The examined aqueous extracts had been shown to give a positive impact on bile secretion [6]. Therefore, the quantified flavonoids can be regarded as markers for the quality assessment of *D. versicolor*. The method was validated and showed good linearity, accuracy, and precision. Thus, it is highly suitable for the quality control of this herbal drug.

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The authors have declared no conflict of interest.

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Figure 1. HPLC comparison of five different *D. versicolor* aqueous extracts. The indicated numbers correspond to the substances listed in Table 1.

Figure 2. Chemical structures of the known flavonoid glycosides in *D. versicolor*

Legend: Gal: β-D-galactose, Glc: β-D-glucose, Rha: α-D-rhamnose

Table 1. Compounds **1-26** listed according to the HPLC fingerprints (Fig. 1) including molecular mass and MS^n fragmentation patterns. Compounds denoted in italics are tentatively identified based exclusively on UV- and MS-data.

	Compound Name or Proposed Structure	**M_r**	**$[M-H]^-$**	**Main Fragment Ions (>10% rel. Int.)**
1	Luteolin-6-glucopyranosyl-7-*O*-galactopyranoside	610	609.2	MS2 [609.2]: 489.1, 447.1, 357.0, 327.0, 299.0 MS3 [447.1]: 357.0, 327.0, 299.0 MS3 [327.0]: 298.9
2	Luteolin-6-glucopyranosyl-7-*O*-rutinoside	756	755.3	MS2 [755.3]: 635.1, 447.1, 357.0, 327.0, 298.9 MS3 [447.1]: 357.0, 327.0, 299.0 MS3 [327.0]: 298.9
3	Luteolin-6-glucopyranosyl-7-*O*-rhamnopyranosyl-galactopyranoside	756	755.3	MS2 [755.3]: 635.1, 447.1, 357.0, 327.0, 298.9 MS3 [447.1]: 357.0, 327.0, 299.0 MS3 [327.0]: 298.9
4	*Luteolin-C-hexosyl-O-hexoside malyl-ester*	726	725.2	MS2 [725.2]: 609.2 MS3 [609.2]: 489.1, 447.1, 357.1, 327.0, 299.0 MS4 [447.1]: 357.0, 327.0, 299.0 MS4 [327.0]: 298.9
5	Apigenin-6-glucopyranosyl-7-*O*-glucopyranoside	594	593.2	MS2 [593.2]: 473.1, 431.1, 341.1, 311.0, 296.9, 283.0 MS3 [431.1]: 341.0, 311.0, 282.9 MS3 [311.0]: 282.9
6	Apigenin-6-glucopyranosyl-7-*O*-rutinoside	740	739.3	MS2 [739.3]: 431.1, 310.9 MS3 [431.1]: 341.0, 310.9 MS3 [310.9]: 282.9
7	Apigenin-6-glucopyranosyl-7-*O*-rhamnopyranosyl-galactopyranoside	740	739.3	MS2 [739.3]: 431.1, 310.9 MS3 [431.1]: 341.0, 310.9 MS3 [310.9]: 282.9
8	Chrysoeriol-6-glucopyranosyl-7-*O*-galactopyranoside	624	623.2	MS2 [623.2]: 503.1, 461.1, 371.1, 341.1, 297.9 MS3 [461.1]: 370.9, 341.0, 297.9 MS3 [341.1]: 326.0, 297.9
9	Chrysoeriol-6-glucopyranosyl-7-*O*-rutinoside	770	769.3	MS2 [769.3]: 461.1, 371.1, 341.0, 298.0 MS3 [461.1]: 371.0, 341.0, 297.9 MS3 [340.9]: 312.9, 297.9
10	Chrysoeriol-6-glucopyranosyl-7-*O*-rhamnopyranosyl-galactopyranoside	770	769.3	MS2 [769.3]: 461.1, 371.1, 341.0, 298.0 MS3 [461.1]: 371.0, 341.0, 297.9 MS3 [340.9]: 312.9, 297.9

11	*Apigenin-C-hexosyl-O-hexoside malyl-ester*	710	709.2	MS2 [709.2]: 593.2, 431.1, 311.0 MS3 [593.2]: 473.1, 431.1, 341.0, 311.0, 282.9 MS3 [431.1]: 341.0, 310.9, 282.9 MS4 [310.9]: 282.9
12	Apigenin-6-glucopyranosyl-2"-*O*-rhamnoside	578	577.2	MS2 [577.2]: 457.1, 413.1, 292.9 MS3 [413.1]: 292.9 MS3 [292.9]: 248.8, 238.7, 236.8, 220.9, 219.8, 174.7, 172.7, 116.9
13	Apigenin-6-glucoside	432	431.1	MS2 [431.1]: 341.0, 311.0, 283.1 MS3 [341.0]: 323.0, 310.9, 294.9, 282.9, 281.9, 280.9, 268.9 MS3 [311.0]: 282.9
14	*Luteolin-O-(deoxyhexosyl-hexoside)*	594	593.2	MS2 [593.2]: 284.9 MS3 [284.9]: 266.8, 242.8, 241.8, 240.8, 222.8, 216.8, 212.8, 200.7, 198.8, 196.7, 174.8, 170.7, 168.8, 150.7, 132.8
15	*Chrysoeriol-C-hexosyl-2''-O-deoxyhexoside*	608	607.2	MS2 [607.2]: 443.1, 323.0, 308.0 MS3 [443.1]: 323.0, 308.0 MS3 [323.0]: 307.9 MS4 [307.9]: 279.9
16	Luteolin-7-*O*-glucoside	448	447.1	MS2 [447.1]: 284.9 MS3 [284.9]: 266.9, 256.9, 256.0, 242.7, 240.8, 212.8, 200.7, 198.7, 197.8, 196.6, 174.7, 168.9, 150.7
17	*Luteolin-O-hexuronide*	462	461.1	MS2 [461.1]: 284.9 MS3 [284.9]: 243.0, 240.9, 216.9, 201.9, 200.7, 198.9, 197.9, 197.0, 174.9, 154.7, 132.9
18	*Chrysoeriol-C-hexoside*	462	461.1	MS2 [461.1]: 371.1, 341.0, 298.0 MS3 [341.0]: 312.9, 297.9
19	*Apigenin-O-(deoxyhexosyl-hexoside)*	578	577.2	MS2 [577.2]: 268.9 MS3 [268.9]: 224.7, 223.8, 196.8, 182.7, 180.7, 150.7, 116.9
20	*3'- or 4'-Methylluteolin-O-(deoxyhexosyl-hexoside)*	608	607.2	MS2 [607.2]: 298.9, 283.9 MS3 [298.9]: 283.9 MS3 [283.9]: 255.7
21	Apigenin-7-*O*-glucoside	432	431.1	MS2 [431.1]: 268.8
22	*Apigenin-O-hexuronide*	446	445.1	MS2 [445.1]: 268.9, 174.7 MS3 [268.9]: 224.8, 200.8, 180.8, 158.7, 148.8, 116.8
23	*3'- or 4'-Methylluteolin-O-(deoxyhexosyl-hexoside)*	608	607.2	MS2 [607.2]: 298.9, 283.9 MS3 [298.9]: 283.9 MS3 [283.9]: 255.9

24	*3'- or 4'-Methylluteolin-O-hexoside*	462	461.1	MS2 [461.1]: 446.0, 313.0, 298.9, 298.0, 284.9, 283.9, 282.9 MS3 [446.0]: 312.9, 296.9, 284.9, 283.9, 282.9, 254.8, 243.7 MS3 [298.9]: 283.9
25	*3'- or 4'-Methylluteolin-O-glucuronide*	476	475.1	MS2 [475.1]: 298.9, 283.9 MS3 [298.9]: 283.9 MS4 [283.9]: 255.8
26	*3'- or 4'-Methylluteolin-O-hexoside*	462	461.1	MS2 [507.1] ($[M+HCOO]^-$ ion): 461.0, 298.9 MS3 [461.0]: 298.9 MS3 [298.9]: 283.9

Table 2. Total flavonoid contents of five *D. versicolor* samples

Sample	Aqueous extract	Crude drug	DER
a	1.91 (± 0.03)	≥ 0.40 (± 0.01)	1:0.213
b	0.41 (± 0.03)	≥ 0.07 (± 0.00)	1:0.163
c	3.30 (± 0.05)	≥ 0.57 (± 0.02)	1:0.168
d	0.50 (± 0.03)	≥ 0.08 (± 0.00)	1:0.158
e	0.52 (± 0.03)	≥ 0.08 (± 0.00)	1:0.162

Legend:
The total flavonoid contents are given in % ± SD of the mean. The flavonoid contents are calculated as the sum of the 26 individually quantified flavonoids. The total flavonoid contents of the crude drug are calculated considering the respective DER and represent minimum values since the extraction efficiency was not evaluated.

Table 3. Precision of the quantification method

Sample	Flavonoid content (%)	Intraday RSD (%)	Interday RSD (%)
a (n=9)	1.85 1.90 1.92	1.91	
	1.90 1.89 1.90	0.30	
	1.92 1.96 1.92	1.19	1.22

Figure 1. HPLC comparison of five different *D. versicolor* aqueous extracts. The indicated numbers correspond to the substances listed in Table 1

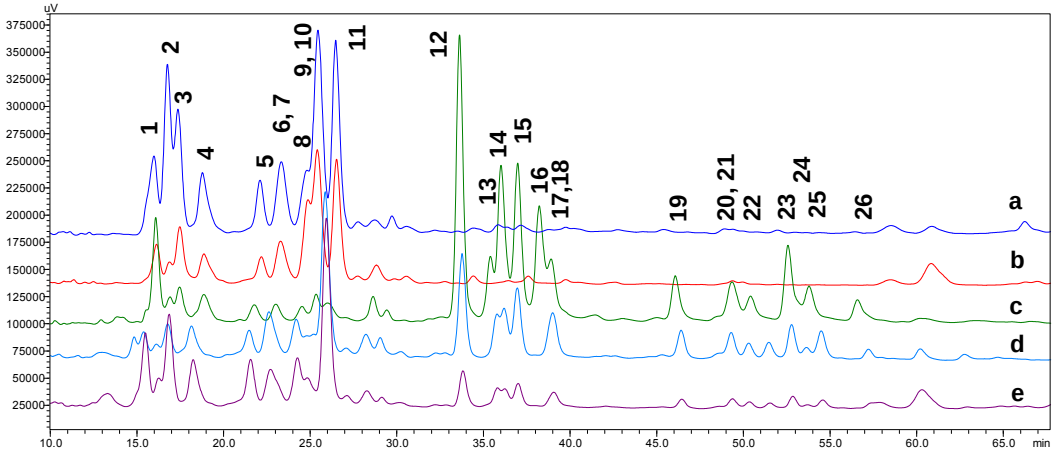
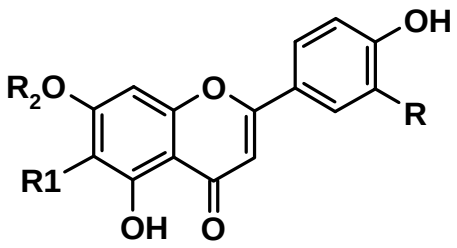


Figure 2. Chemical structures of the known flavonoid glycosides in *D. versicolor* (Gal: β-D-galactose, Glc: β-D-glucose, Rha: α-D-rhamnose)



	R	R_1	R_2	Common name
1	OH	Glc	Gal	Isoorientin-7-O-galactoside
2	OH	Glc	Rha(1→6)Glc	Isoorientin-7-O-rutinoside
3	OH	Glc	Rha(1→6)Gal	Isoorientin-7-O-rhamnosyl-galactoside
5	H	Glc	Glc	Isovitexin-7-O-glucoside (saponarin)
6	H	Glc	Rha(1→6)Glc	Isovitexin-7-O-rutinoside
7	H	Glc	Rha(1→6)Gal	Isovitexin-7-O-rhamnosyl-galactoside
8	OCH_3	Glc	Gal	Isoscoparin-7-O-galactoside
9	OCH_3	Glc	Rha(1→6)Glc	Isoscoparin-7-O-rutinoside
10	OCH_3	Glc	Rha(1→6)Gal	Isoscoparin-7-O-rhamnosyl-galactoside
12	H	Rha(1→2)Glc	H	Isovitexin-2"-O-rhamnoside
13	H	Glc	H	Isovitexin
16	OH	H	Glc	Luteolin-7-O-glucoside
21	H	H	Glc	Apigenin-7-O-glucoside

Quantification of flavonoid glycosides from the traditional Mongolian medicinal plant *Dianthus versicolor* FISCH.

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Supporting information

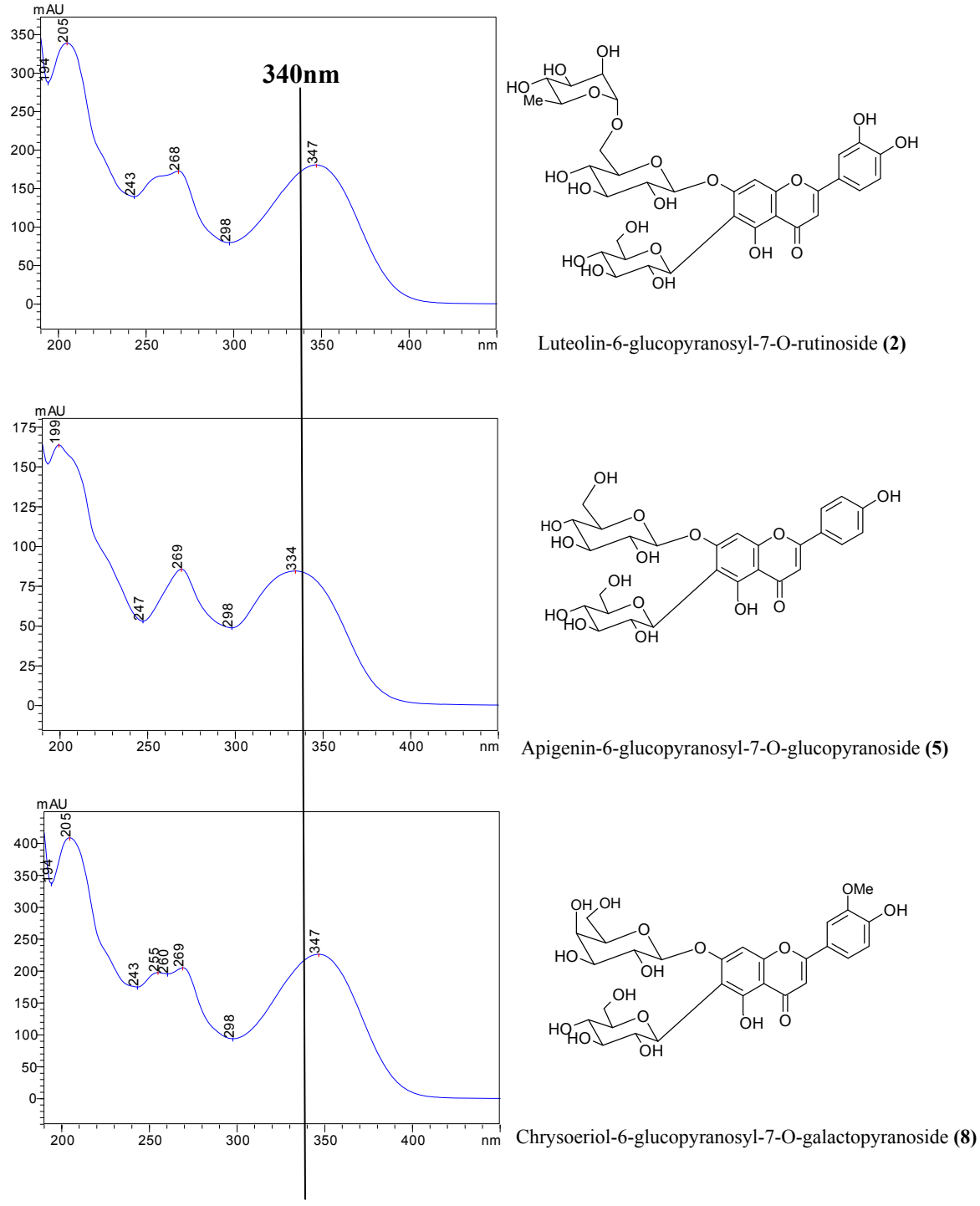

Figure S1. UV-spectra and structures of the compounds **2**, **5**, and **8**. The three chromophores differ only by the presence of an additional OH- or OCH_3-group in position 3'. 340 nm represents a mean value of the maxima (334 nm and 347 nm) and was therefore chosen as detection wavelength.

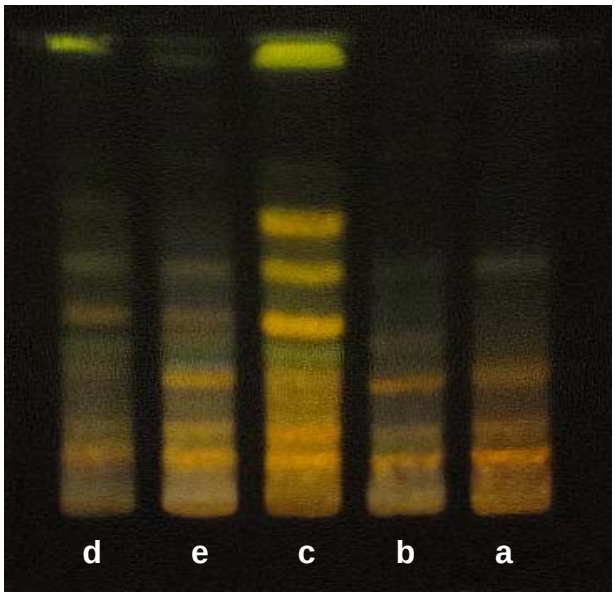

Figure S2. TLC fingerprints of the five *D. versicolor* aqueous extracts.

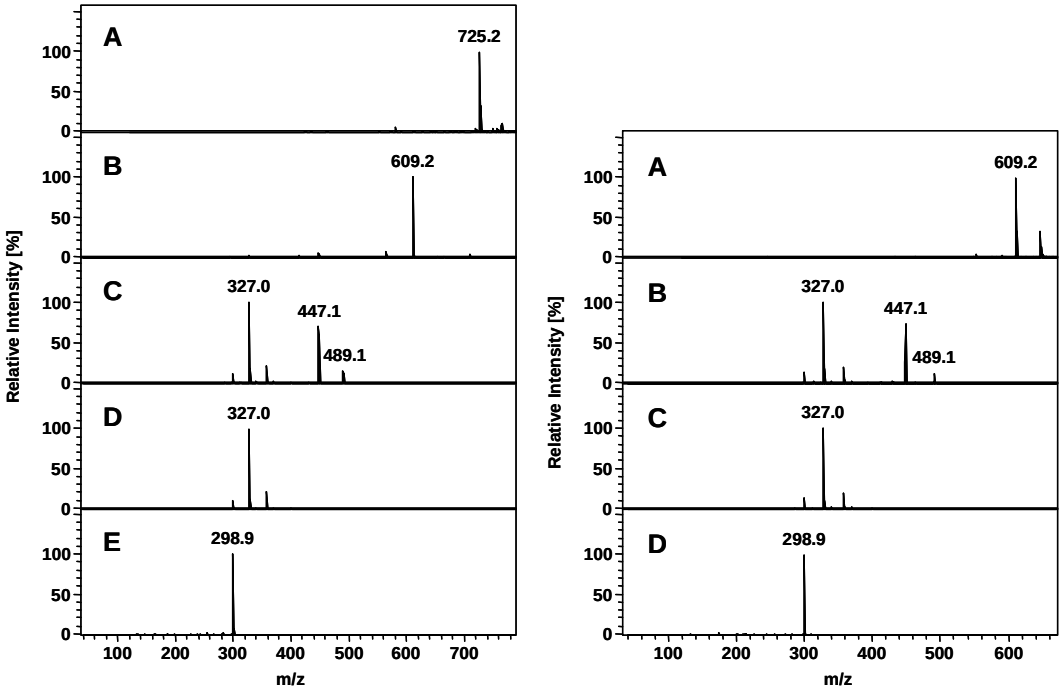

Figure S3. Multistage mass spectra (LC-ESI-IT-MS^n) of compound 4 (luteolin-*C*-hexosyl-*O*-hexoside malyl-ester) and luteolin-6-glucopyranosyl-7-*O*-galactopyranoside (1). Left panel: (A) MS^1, (B) MS^2 (725.2 →), (C) MS^3 (725.2 → 609.2 →), (D) MS^4 (725.2 → 609.2 → 447.1 →), and (E) MS^4 (725.2 → 609.2 → 327.0 →) mass spectra of compound **4**. Right panel: (A) MS^1, (B) MS^2 (609.2 →), (C) MS^3 (609.2 → 447.1 →), and (D) MS^3 (609.2 → 327.0 →) mass spectra of luteolin-6-glucopyranosyl-7-*O*-galactopyranoside (**1**). Note that, following cleavage of the malyl-group from **4**, the two compounds show identical fragmentation patterns.

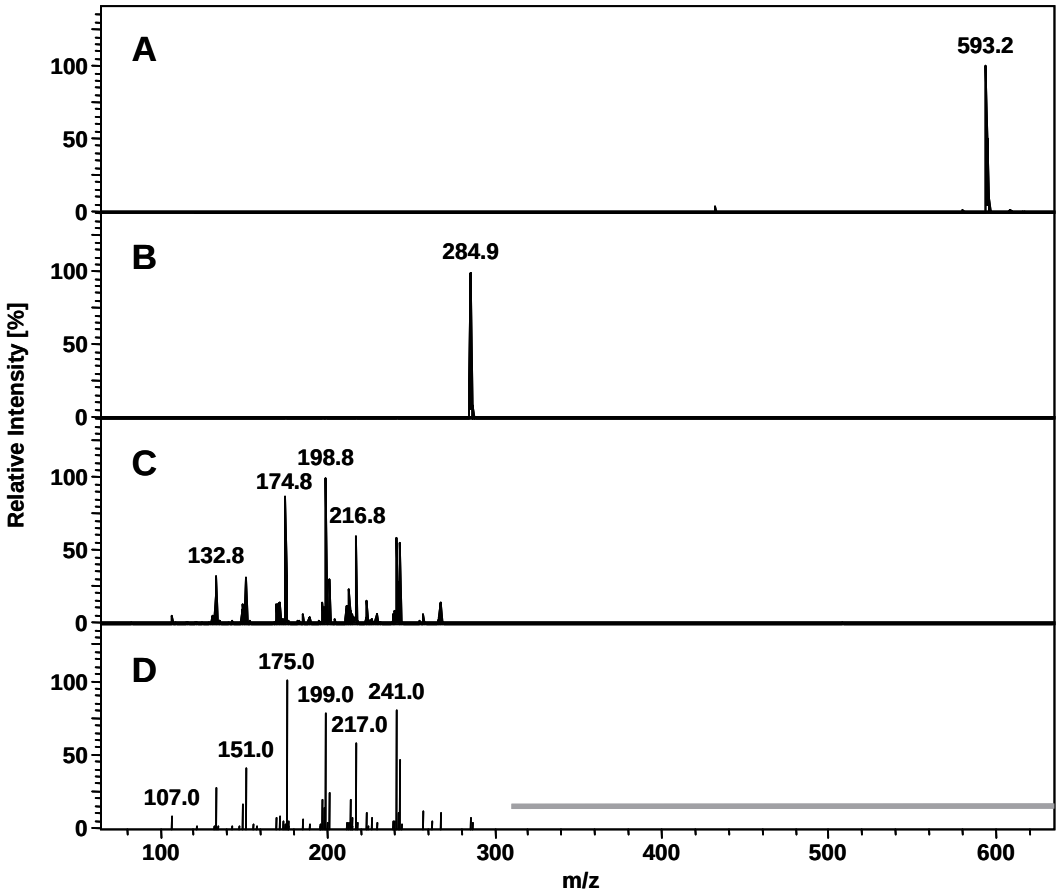

Figure S4. Multistage mass spectra (LC-ESI-IT-MS^n) of compound 14 (luteolin-*O*-(deoxyhexosyl-hexoside)). (A) MS^1, (B) MS^2 (593.2 →), and (C) MS^3 (593.2 → 284.9 →) mass spectra of compound **14**. The aglycon was identified by matching the MS^3 spectrum of **14** (C) to the MS^3 (447.0 → 284.9 →) mass spectrum of luteolin-4'-*O*-glucoside (D) from an in-house library containing MS data of 57 flavonoids.

Sample	Collection number	Collection site and year	Herbarium number (Dept. of Pharmacognosy, University of Vienna)	Herbarium number (Inst. of Botany, Mongolian Academy of Sciences, Ulaanbaatar)
a	53/04/mon	Khubsgul, August 2004		424
b	52/04/mon	Bulgan, August 2004	03/04/mon/H	
c	02/05/mon	Khentii, August 2005		H 20050805
d	05/03/mon	Selenge, July 2003	14/03/mon/H	
e	06/03/mon	Selenge, July 2003	14/03/mon/H	

Table S1. Plant material

Sample a (aqueous extract)			
Compound	**Content (%)**	**Response factor**	**Corrected content (%)**
1	0.10	1.03	0.10
2	0.22	1.27	0.28
3	0.21	1.27	0.27
4	0.05	1.22	0.07
5	0.05	1	0.05
6+7	0.13	1.25	0.17
8	0.11	1.05	0.12
9+10	0.46	1.3	0.59
11	0.22	1.2	0.27
Total content (%)			**1.91**

Sample c (aqueous extract)			
Compound	**Content (%)**	**Response factor**	**Corrected content (%)**
1	0.25	1.03	0.26
3	0.08	1.27	0.10
9+10	0.07	1.3	0.09
12	0.72	0.97	0.70
13	0.14	0.73	0.10
14	0.42	1	0.42
15	0.43	1.02	0.44
16	0.34	0.75	0.26
17+18	0.17	0.78	0.13
19	0.14	0.97	0.14
20+21	0.17	0.88	0.15
22	0.12	0.75	0.09
23+24	0.28	0.9	0.25
25	0.13	0.80	0.10
26	0.09	0.78	0.07
Total content (%)			**3.30**

Table S2. Contents of single compounds and total flavonoid content in the aqueous extracts of samples **a** and **c**.

Content (%) = $Mass_{Standard}$ * $Peakarea_{Analyte}$ * 100 / $Mass_{Extract}$ * $Peakarea_{Standard}$

Corrected content (%) = Content * Response Factor

HPLC Determination of flavonoid glycosides in Mongolian *Dianthus versicolor* FISCH. (Caryophyllaceae) compared to quantification by UV-spectrophotometry

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Abstract:

Introduction – *Dianthus versicolor*, a plant used in traditional Mongolian medicine, is known to contain flavone-6-C-7-O-di- and triglycosides. Reliable and accurate analytical methods are needed for their determination in the crude drug and extracts thereof.

Objective – To provide a validated HPLC-DAD method especially developed for the separation of polar flavonoids and to compare the obtained data with those evaluated by UV-spectrophotometry.

Results – Separations were carried out on an Aquasil® C_{18} column (4.6 mm x 250.0 mm) with acetonitrile and water (adjusted to pH 2.8 with formic acid) as mobile phase. Linear gradient elution was employed using rutoside as internal standard with linear behaviour in a concentration range of 0.007-3.5 mg/mL. Accuracy was determined by spiking the crude drug with saponarin resulting in recoveries between 92% and 102%. The method allows the quantification of highly polar flavonoid glycosides and the determination of their total content. The latter was evaluated by 0.45% for the crude drug, by 1.73% for the aqueous extract and by 16.29% for the enriched fraction. In case of an identical flavonoid glycoside spectrum in the extracts UV spectrophotometry was shown to match the contents determined by HPLC. It was proven that threefold sonication represents a time-saving, effective and cheap method for the extraction of the polar flavonoid glycosides.

Conclusion – The HPLC method represents a powerful technique for the quality control of *D. versicolor*. Extraction experiments showed sonication to be superior to refluxing. UV-spectrophotometry may be regarded as acceptable alternative to HPLC.

Keywords: *Dianthus versicolor* FISCH.; Caryophyllaceae; Traditional Mongolian Medicine; flavonoids; HPLC-UV-DAD; spectrophotometric quantification

Introduction

Dianthus versicolor FISCH. (Caryophyllaceae) is one of the five *Dianthus* species growing in Mongolia (Grubov, 2001) and is used in traditional Mongolian medicine (Kletter, 2008). Its indications are manifold and include e. g. inflammation, pneumonia, typhoid fever, poisoning, impairments of the liver, and womb´s bleeding (Boldsaikhan 2004; Ligaa 2006; Report of Market Research on Mongolian Traditional Medicinal Drugs 2007; Dr. Damdinsuren Natsagdorj, Otoch Manramba, Institute of Traditional Mongolian Medicine, Ulaanbaatar, personal communication). Recent investigations have shown an aqueous extract of *D. versicolor* as well as a flavonoid enriched fraction thereof to enhance bile secretion in the isolated perfused rat liver (Obmann et al. 2010a). The constituents were identified as C- and O-glycosylated apigenin-, luteolin-, and chrysoeriol-derivatives (Obmann et al. 2010b). The quantification of the flavone-6-C-7-O-glycosides in this aqueous extract was performed by HPLC and external standardisation (Obmann et al. 2010b). The occurrence of drug samples containing a big amount of highly polar flavonoids required the development of an alternative system. For this purpose the extraction procedure was optimized. A new HPLC-UV-DAD method was developed and validated using rutoside as internal standard. Furthermore we determined the content of the highly polar flavonoid glycosides in the crude drug, in the aqueous extract and in the enriched fraction (see Fig. 1). In order to provide an additional simple and low-cost procedure for quantification in laboratories which are possibly not equipped with an HPLC device, we employed UV-spectrophotometry. The results obtained by HPLC were compared to the UV method in order to give evidence about the comparability of the two methods.

Experimental

Material, chemicals and reagents

Reagents for UV-spectrophotometric quantification (boric acid, oxalic acid) were of analytical grade and purchased from Sigma Aldrich (St. Louis, Missouri, USA). Reference substances purchased from Roth (Karlsruhe, Germany) were of HPLC

quality. Solvents used for extraction were of analytical grade, those used for UV-spectrophotometry and HPLC were of gradient grade and obtained from VWR (West Chester, Pennsylvania, USA).

Plant material

Aerial parts of *Dianthus versicolor* were collected in the Mongolian province of Hövsgöl, August 2004. The plant material was identified by E. Ganbold, Health Sciences University, Mongolia. A voucher specimen (herbarium specimen number 424) is deposited at the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia.

Spectrophotometric procedure

Instrumentation. Spectrophotometric measurements were performed on a Beckman DU 640 spectrophotometer (Beckman Instruments Inc., Fullerton, California, USA) equipped with 1 cm quartz cells.

Stock solutions. For the preparation of stock solutions the crude drug, the dry aqueous extract, and the enriched fraction were treated as follows:

Crude drug: 0.5 g of the dry pulverized plant material was extracted with 40 mL methanol-water (40:60, v/v) for 30 min under reflux. The extract solution was filtered and the residue was re-extracted repeating the extraction procedure two times for 10 min. After filtration the solutions were unified and diluted to 100.0 mL with methanol-water (40:60, v/v).

Dry aqueous extract: 0.2 g of the lyophilized aqueous extract was re-extracted with 40 mL methanol-water (40:60, v/v) for 10 min by sonication. After filtration, 40 mL of fresh solvent were added to the pellet, which was treated a second time by sonication for 10 min. The resulting solutions were combined and diluted to 100.0 mL.

Enriched fraction: 0.02 g of the dry material was dissolved in 100.0 mL methanol-water (40:60, v/v).

Compensation liquid. 5.0 mL of the stock solution were evaporated to dryness, and the residue was redissolved in 10 mL methanol-acetic $acid_{conc}$ (1+10, v/v). Instead of the reagent (see sample preparation) 10 mL of formic $acid_{conc}$ were added, and the solution was further diluted to 25.0 mL with acetic $acid_{conc}$.

Sample preparation. 5.0 mL of each stock solution were evaporated to dryness, and the residue was redissolved in 10 mL methanol-acetic $acid_{conc}$ (1+10, v/v). 10 mL of reagent (25 g/L boric acid and 20 g/L oxalic acid in formic $acid_{conc}$) were added, and the solution was further diluted to 25.0 mL with acetic $acid_{conc}$. After 30 min, the absorption of the sample at 401 nm was measured against the compensation liquid. The percentage content of flavonoids was calculated and expressed as 8-*C*-glucosyl-apigenin (vitexin) based on the specific absorbance of vitexin, i. e. 628 (European Pharmacopoeia, 2008). Each assay was carried out with at least three different sample weights.

HPLC analysis

Sample preparation of the crude drug. 0.2 g of the crude drug was extracted three times with 20 mL of methanol-water (40:60, v/v) by sonication at room temperature for 10 min. The solutions were combined and after addition of 50 µL of the standard solution (20.0 mg/ 5 mL rutin in MeOH) evaporated to dryness under reduced pressure at a maximum temperature of 45°C. The residue was redissolved in 5.0 mL of methanol-water (40:60, v/v). From this solution 40 µl were subjected to HPLC-analysis after centrifugation.

Sample preparation of the aqueous extract. The aqueous extract was obtained as previously described (Obmann et al. 2010a). In brief, 10 g of the powdered aerial parts were extracted with 250 mL water (pH 2, adjusted with trifluoroacetic acid) for 1h at 40°C by shaking gently. This procedure is intended to resemble the physiologic processes taking place after the consumption of the crude herbal drug which is taken together with a certain amount of water. After extraction the highly volatile trifluoroacetic acid was removed under reduced pressure at a maximum temperature of 45°C. The remaining solution was lyophylized and yielded 2.16 g of the aqueous extract. For HPLC analysis 0.1 g of the aqueous extract was redissolved in 5.0 mL of methanol-water (40:60, v/v) followed by an addition of 50 µL of the standard solution (17.83 mg/ 5 mL rutin in MeOH). After centrifugation at 13500 rpm for 10 min 40µl of the sample solution were subjected to HPLC analysis.

Sample preparation of the enriched fraction. The enriched fraction was obtained by solid phase extraction of the aqueous extract on RP18 cartridges as described earlier (Obmann et al. 2010a). For HPLC analysis to 0.01 g of the dried fraction 50µl of standard solution (17.5 mg/ 5 mL rutin in MeOH) were added and diluted to 0.5 mL

with methanol-water (40:60, v/v). After centrifugation at 13500 rpm for 10 min 20µl of the sample solution were subjected to HPLC analysis.

Analytical conditions. Quantification of flavonoids was conducted on a Shimadzu Prominence LC-20AD coupled to a Shimadzu Prominence SPD-M20 Diode Array Detector (Shimadzu Corporation, Kyoto, Japan). For data analysis the software LabSolutions version 1.25, LC-Postrun (Shimadzu, Kyoto, Japan) was used. Peaks were integrated automatically by the method of vertical separation applying the following parameters: the minimum peak width was set at 5 sec, at a slope of 1000 µV/min, and a minimum peak area of 9900 units according to the LOQ for rutoside. The wavelength was set at 340 nm for detection in accordance with the maximum absorption of the flavonoids measured by DAD. An Aquasil® C_{18} column (4.6 mm x 250.0 mm, 5µm, Thermo Fisher Scientific, Waltham, Massachusetts) served as stationary phase. Doubly distilled water adjusted to pH 2.8 with formic acid (A) and ACN (B) were used as mobile phase at a flow rate of 1.0 mL/min. Separations were carried out at a column oven temperature of 15°C. A low pressure gradient was used starting at 0% B and raising up to 10% B within 20 min (0.5%/min) followed by three further gradient steps: First a very slow increase from 10% B to 20% B within 60 min (0.17%/min), then from 20% B to 40% B within 20 min (1%/min) and finally from 40% B up to 95% B within 10 min (5.5%/min).

Results and Discussion

Chromatographic separation

Among the various C_{18} materials that were tested for the separation of the 11 flavone-6-C-7-O-glycosides Aquasil® with a particle size of 5 µm and a column dimension of 4.6 mm x 250 mm turned out to be most appropriate. On the one hand this stationary phase achieved the best separation and on the other hand columns of this dimension are still most common in laboratories. In order to shorten the retention time analyses were carried out on Aquasil® C_{18} material with a particle diameter of 3 µm at column dimensions of 2.1 mm x 150 mm. However, as this resulted in a remarkably lower resolution of the compounds **2**, **3**, **6**+**7** and **8** we decided to choose the long column with a higher separation efficiency but long time of analysis. Compounds **6** / **7** and

compounds **9** / **10** represent pairs of substances which differ only by a glucose and galactose unit, respectively (see Fig. 1 and Fig. 2). They co-elute and are determined together.

In order to be independent from the injection by an autosampler or from concentration effects occurring during sample preparation we focussed on the development of a method with an internal standard. Rutoside (quercetin-3-*O*-rutinoside, see Fig. 1) was chosen as internal standard because it is cheap and similar to the compounds investigated but not contained in *Dianthus versicolor*. As a flavonoid diglycoside with a molecular weight of 610 it matches very well with the molecular weights we found for the flavonoids in *Dianthus versicolor*.

Effects of extraction assay and optimisation of extracting conditions

The extraction procedure and selection of the proper solvent are crucial factors in quantitative phytochemical analysis. Two different extraction assays, refluxing and ultrasonication, were applied to investigate the effectiveness of the extraction procedure. Sonication turned out as the more powerful extraction technique (see Fig. 3). Singular ultrasonication for 10 min at room temperature achieved the same content as three fold heating under reflux which took in total 50 min. All together we obtained a maximum total content after treating the plant material for three times in the ultrasonic bath. The first extraction step provides a yield of 89%, the second step yields another 9% and the third time 2% of the total content (see Fig. 3). The results show that threefold ultrasonication represents a time-saving, effective and cheap method for the extraction of the flavonoid-glycosides from *Dianthus versicolor*.

Method validation, calibration curves, accuracy and precision

Out of the flavonoid-glycosides to be analysed only compound **5** (saponarin) was commercially available. Therefore, we determined the response factor for saponarin in comparison to the internal standard rutoside and calculated the response factors of the other compounds by taking their molecular weight into account. It has been shown that the response factors of compounds with the same chromophor are directly proportional to their molecular weights (Reznicek et al. 1996). The correctness of this approach implies the selection of an appropriate detection wavelength at which all constituents

are registered equally. The analytes in *Dianthus versicolor* represent C- and O-glycosides of apigenin, luteolin (3'-OH-apigenin) and chrysoeriol (3'-OCH_3-apigenin) whose absorption maxima range between 334 nm and 347 nm (Obmann et al. 2010b). Therefore, the detection wavelength for quantification was set at 340 nm. We experimentally evaluated the response of the three aglycones at 340 nm by injecting equal amounts and found high similarity of the molar responses with a maximum deviation of 5.9%.

For assessing the linearity six different concentrations of rutoside and saponarin ranging from 0.007-3.5 mg/mL were examined in triplicates. The least square lines and the correlation coefficients were calculated and proved the method to be linear (rutoside: r^2>0.9998; linear regression equation: y=14858531x – 223566; saponarin: r^2>0.9999; linear regression equation y = 22279996x - 42847).

The response factor of saponarin was determined by mixing varying amounts (0.007-3.5 mg/mL) with 0.07 mg/mL rutoside. The average response factor of saponarin was evaluated by 0.72 (af_r). On basis of this response factor the response factors of all other identified compounds were evaluated arithmetically as described above. Accordingly, the response factors of compounds **1-11** are given in Table 1. Amounts were calculated by the following equation: $af_r/MW_{(saponarin)}*MW_{(compound)}$.

The LOD and LOQ for rutoside and saponarin under the chromatographic conditions were determined at the *S/N* of 3 and 10, respectively. The LOD for rutoside and saponarin were 0.2µg/mL and 0.05 µg/mL, and the LOQ were 0.7 µg/mL and 0.17 µg/mL, respectively.

The accuracy of the method was investigated by means of recovery experiments spiking the crude drug with different concentrations of saponarin dissolved in methanol. Known saponarin amounts were added at low (50% of the known amount in the sample), medium (same amount as in the sample) and high (150% of the known amount in the sample) levels. Analyses were performed in triplicates. The recoveries were evaluated by 91.8% (± 7.2% RSD), 92.1% (± 7.4 RSD) and 101.9% (± 4.1 RSD).

The intermediate precision of the developed method was determined by preparing independently nine methanolic-aqueous extracts of the crude drug. The analyses were performed on different days with a relative standard deviation (RSD) of 4.8% (see Table 1) which demonstrated a good precision of the method.

Sample analyses

The above described HPLC method allows the determination of the single polar flavonoid glycosides on the one hand and the quantification of the total content on the other hand (see Table 1). The total content of the flavonoid glycosides in the crude drug amounted to 0.45%. The aqueous extract represents the way of intake by mimicking gastric conditions during the extraction process. This extraction procedure resulted in a concentration of polar flavonoids up to an amount of 1.73%. Further purification of this aqueous extract by SPE yielded an enriched fraction which has been shown to increase the bile secretion in the isolated perfused rat liver (Obmann et al. 2010a). The total content of flavonoid glycosides in this fraction was determined by 16.29% with the compounds **2**, **3**, **9** and **10** at the highest concentrations. We compared these results to the values obtained by UV-spectrophotometry which respresents a quick and cheap method to determine the content of all flavonoids, either glycosides or aglycones. The respective method has been published earlier for *D. versicolor* (Obmann 2010a). It was developed following the monograph "Passiflorae herba" of the European Pharmacopoeia (European Pharmacopoeia, 2008), because the basic structures of the main flavonoids in *Passiflora* (Patel, 2009, Quimin, 1991) resemble to those isolated from *Dianthus versicolor*. For the crude drug a content of 0.75% was determined. This corresponds to more than the 1.5fold of the value obtained by HPLC and may be explained by the fact that aglycones which are co-extracted by methanol-water were not compassed by the HPLC method. In comparison, the contents determined by both methods in the aqueous extract and in the enriched fraction were in good agreement. For the aqueous extract a value of 1.73% analysed by HPLC corresponded very well with 1.78% determined by UV-spectrophotometry. Also the contents of the enriched fraction fit together with 16.59% determined by UV-spectrophotometry and 16.29% evaluated by HPLC.

Conclusion

The presented data contribute to the scientific investigation of herbal drugs applied in traditional Mongolian medicine. The techniques described in this paper may be regarded as basic methods for quality control of *D. versicolor*. The proposed HPLC method shows good linearity, accuracy and precision and achieves a better separation of the polar flavonoid glycosides than an earlier published method. Alternatively, a simple

UV spectrophotometric method was employed. It was slightly modified according to a monograph of the European Pharmacopoeia and showed contents which matched very well the values obtained from HPLC-DAD analysis. UV-spectrophotometry may be regarded as acceptable alternative in case that no HPLC device is available. In terms of extraction efficiency sonication was superior to refluxing. Threefold sonication with methanol 40% represents a time-saving, effective and cheap method for the extraction of the polar flavonoid glycosides from *Dianthus versicolor*.

Acknowledgements

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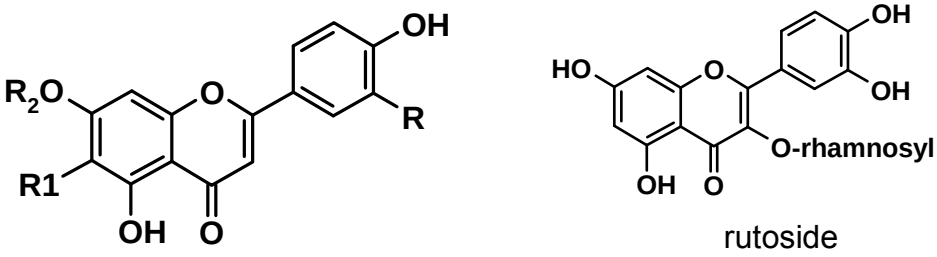
Table 1. Content of flavonoids in the crude drug, an aqueous extract and a flavonoid enriched fraction of *Dianthus versicolor* determined by HPLC-DAD and UV-spectrophotometry

Compound number		**Crude drug (n=9)**	**Aqueous extract (n=9)**	**Enriched fraction (n=3)**
	Response factor	**Single compounds (mean %, ± SD)**		
1	0.74	0.03 (± 0.003)	0.09 (± 0.002)	0.97 (± 0.065)
2	0.91	0.05 (± 0.005)	0.25 (± 0.007)	2.30 (± 0.028)
3	0.91	0.04 (± 0.004)	0.24 (± 0.007)	2.20 (± 0.029)
4	0.88	0.03 (± 0.003)	0.06 (± 0.002)	0.78 (± 0.072)
5	0.72	0.02 (± 0.001)	0.04 (± 0.002)	0.58 (± 0.062)
6+7	0.90	0.05 (± 0.003)	0.15 (± 0.013)	1.53 (± 0.016)
8	0.88	0.03 (± 0.005)	0.11 (± 0.009)	1.13 (± 0.067)
9+10	0.93	0.08 (± 0.004)	0.54 (± 0.015)	4.45 (± 0.541)
11	0.86	0.12 (± 0.005)	0.25 (± 0.007)	2.36 (± 0.089)
Total content HPLC (mean %, ± RSD)		0.45 (± 4.79)	1.73 (± 2.45)	16.29 (± 4.11)
Total content UV (mean %, ± RSD)		0.75 (± 2.68)	1.78 (± 2.90)	16.59 (± 1.43)

Figure 1. Chemical structures of flavonoid glycosides in *D. versicolor* (Gal: β-D-galactose, Glc: β-D-glucose, Rha: α-D-rhamnose, Hex: hexose) and of the internal standard rutoside



	R	R_1	R_2	Common name
1	OH	Glc	Gal	Isoorientin-7-O-galactoside
2	OH	Glc	Rha(1→6)Glc	Isoorientin-7-O-rutinoside
3	OH	Glc	Rha(1→6)Gal	Isoorientin-7-O-rhamnosyl-galactoside
4	*OH*	*Hex*	*Hex-malyl*	*Luteolin-C-hexosyl-O-hexoside malylester*
5	H	Glc	Glc	Isovitexin-7-O-glucoside (saponarin)
6	H	Glc	Rha(1→6)Glc	Isovitexin-7-O-rutinoside
7	H	Glc	Rha(1→6)Gal	Isovitexin-7-O-rhamnosyl-galactoside
8	OCH_3	Glc	Gal	Isoscoparin-7-O-galactoside
9	OCH_3	Glc	Rha(1→6)Glc	Isoscoparin-7-O-rutinoside
10	OCH_3	Glc	Rha(1→6)Gal	Isoscoparin-7-O-rhamnosyl-galactoside
11	*H*	*Hex*	*Hex-malyl*	*Apigenin-C-hexosyl-O-hexoside malylester*

Figure 2. HPLC-fingerprints of the crude drug extracted with MeOH/water (40/60, v/v) (1), the aqueous extract (2), and the flavonoid enriched fraction (3). The peak numbers correspond to the compounds listed in Fig xx. Peak 12 represents the internal standard rutin.

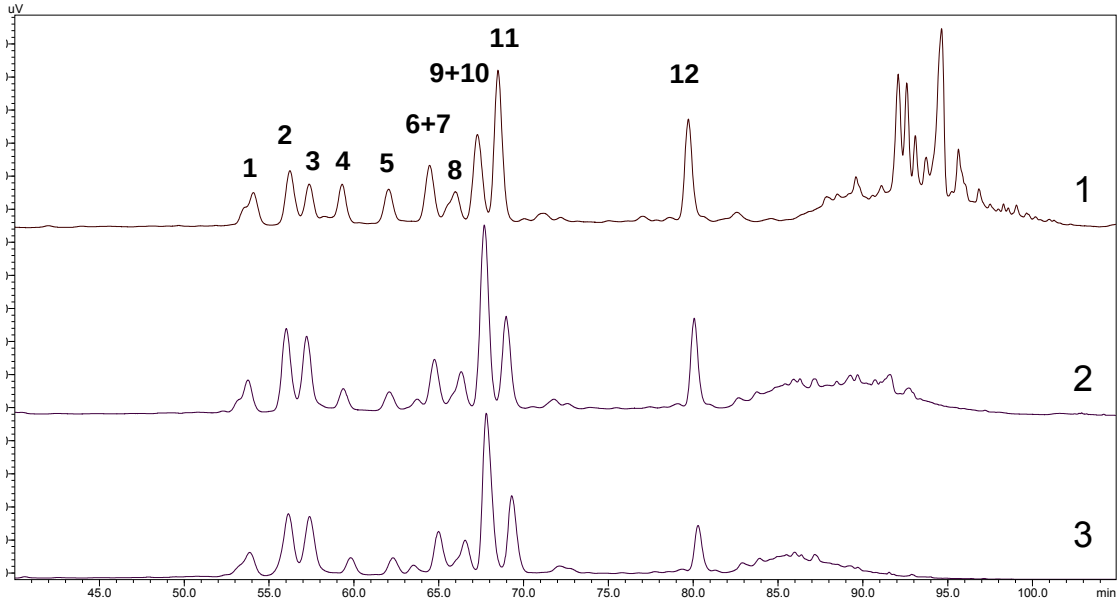
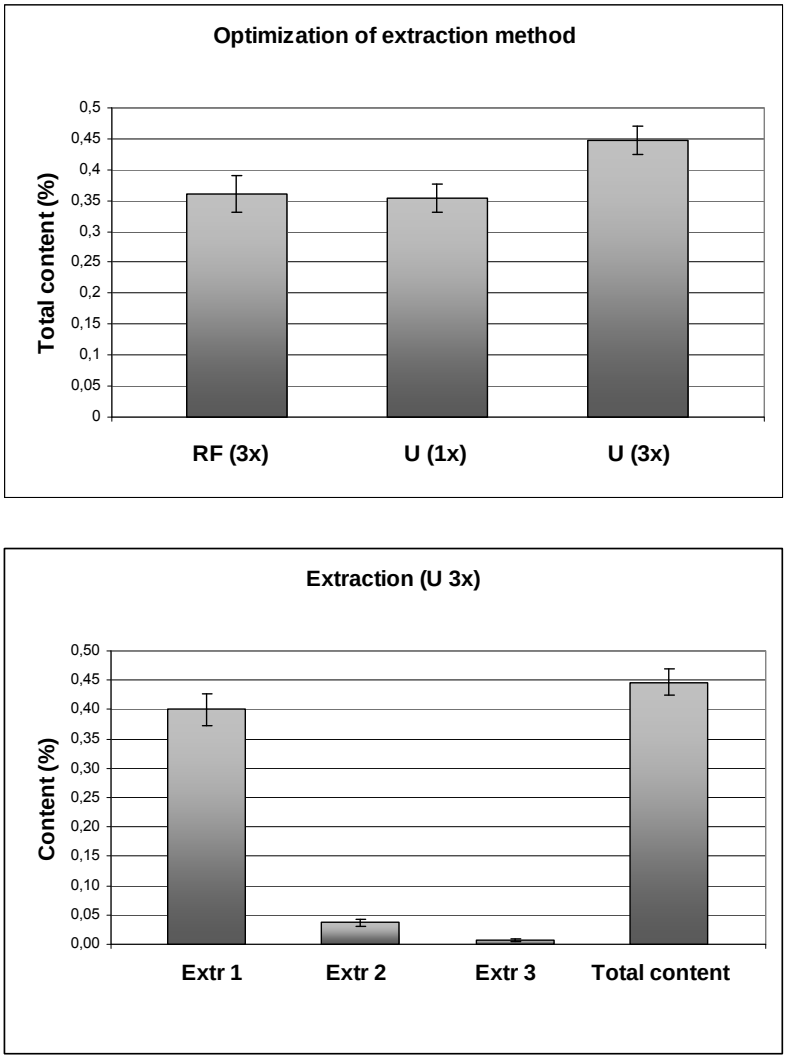


Figure 3. Optimization of the extraction method



Legend: The upper panel compares the total content of flavonoids after extraction with a mixture of MeOH/water (40/60, v/v) under reflux (RF 3x, 30 min each), extraction for one time by ultrasound (U 1x, 10 min), and extraction for three times by ultrasound (U 3x). For each of the extraction modes three individual samples were tested. The lower panel compares the three extraction steps (Extr 1, Extr 2, Extr 3) by ultrasound (U 3x).

3.3 Biological section

In this section the biological investigation of aqueous extracts and fractions of *D. versicolor* are described. The influence on the bile flow was examined in the model of the isolated perfused rat liver. Those results are summarized in the publication **"Extracts from the Mongolian traditional medicinal plants *Dianthus versicolor* FISCH. and *Lilium pumilum* DELILE stimulate bile flow in an isolated perfused rat liver model"** published in *Journal of Ethnopharmacology* [21].

The aqueous extract of *D. versicolor* and fractions thereof were prepared and phytochemically analysed by Astrid Obmann. The quantification of flavonoids was done by Astrid Obmann. Phytochemical analyses of *Lilium pumilum* were performed by Ines Offenmüller under supervision of Astrid Obmann and are part of her Diploma thesis. The liver perfusions were carried out at the Institute of Pathophysiology, Medical University of Vienna by the group of Theresia Thalhammer. Test solutions were prepared by Astrid Obmann. The evaluation of the results in the isolated perfused rat liver was done by Astrid Obmann together with Theresia Thalhammer and Sabine Glasl.

The results of the investigations on isolated organ preparations are contained in the diploma thesis of Birgit Weisz-Pecher [29], the effects on the uterus are shown on the poster **"Uterus activity of the Mongolian medicinal plant *Dianthus versicolor* FISCH."** [31] (Appendix, p.167). Extracts and fractions for those analyses were prepared by Astrid Obmann, the investigations on the isolated organ preparations were carried out by Birgit Weisz-Pecher and Pakiza Rawnduzi at the Department of Pharmacology and Toxicology, University of Vienna.

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Extracts from the Mongolian traditional medicinal plants *Dianthus versicolor* FISCH. and *Lilium pumilum* DELILE stimulate bile flow in an isolated perfused rat liver model☆

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ABSTRACT

Aim of the study: *Dianthus versicolor* (Caryophyllaceae) and *Lilium pumilum* (Liliaceae) are two medicinal plants used in traditional Mongolian medicine to treat hepatic and gastrointestinal disorders. In this study aqueous (AE) and methanolic (ME) extracts of *Dianthus versicolor* and *Lilium pumilum* were investigated for their influence on the bile flow. The aqueous extracts of both plants were tested in absence and presence of 10 μM taurocholic acid at three different concentrations (100, 250, and 500 mg/L). The aqueous extract of *Dianthus versicolor* was further purified in order to locate the active principles. Two resulting fractions, one enriched in flavonoids and the other in sugars, were investigated for their influence on the bile flow in absence of taurocholic acid at 10, 20, and 40 mg/L. The aqueous extracts of both plants were analysed qualitatively by LC-MS^n and quantitatively by UV-spectrophotometry.
Materials and methods: The bile flow experiments were performed in the isolated perfused rat liver. The compounds were identified by LC-DAD-MS^n and TLC using references. The UV-spectrophotometric analysis was based on the monograph "Passiflorae herba" of the European Pharmacopoeia, and the total flavonoid contents were calculated and expressed as vitexin.
Results: AE and ME of both plants increased the bile flow dose-dependently (between 9% and 30%), and no hepatotoxic effect was seen even during longer perfusions. Stimulation of bile secretion was comparable in the presence and in the absence of taurocholic acid. The flavonoid fraction of *Dianthus versicolor* increased the bile flow by 18% ($p < 0.05$) at 40 mg/L, which was comparable to the positive control cynarin. The phytochemical investigations of the *Dianthus versicolor* AE (total flavonoid content 1.78%) revealed the presence of the isovitexin derivative saponarin. In the AE of *Lilium pumilum* (total flavonoid content 1.04%) the flavonoids rutoside, kaempferol-3-*O*-rutinoside, and isorhamnetin-3-*O*-rutinoside were detected.
Conclusions: The results show that choleresis under extract application is due to a stimulation of the bile-salt-independent bile flow which might be caused by the osmotic power of the extracts (hydrocholeresis). The flavonoids seem to contribute to the bile-flow-stimulating effect of *Dianthus versicolor*. Both plants exhibit a considerable choleretic effect that contributes to their use in traditional Mongolian medicine against gastrointestinal disorders.

1. Introduction

Liver diseases and gastrointestinal disorders of different etiology, e.g. viral infection, dietary factors, alcohol abuse, are frequently observed in the Mongolian population (Ebright et al., 2003; Bolormaa et al., 2008). In many cases, treatment with traditional Mongolian medicine, in which extracts from indigenous plants are used, is highly effective in improving the patients' condition.

☆ The results in this paper concerning *Dianthus versicolor* are part of the PhD thesis of Astrid Obmann.

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In order to get a better understanding of the active principles in Mongolian medicinal plant extracts, we focussed on two species, namely *Dianthus versicolor* Fisch. (Caryophyllaceae) and *Lilium pumilum* Delile (Liliaceae). Both species are used in traditional Mongolian medicine to treat liver diseases and gastrointestinal disorders (Kletter et al., 2008). A reduced function of the liver and the gallbladder is generally associated with an impairment of the bile flow. Some plant extracts have shown to restore bile secretion (Holtmann et al., 2003) and improve discomfort in the stomach, bloating, lack of appetite, nausea, and mild diarrhoea or constipation. The modes of action behind these activities are not fully understood, but two possible mechanisms may contribute: on the one hand, the extracts could trigger an increased transport of bile acids, which results in a better digestion of fats accompanied by an improving general condition. On the other hand, osmotically active compounds excreted into the bile are the driving force to stimulate bile secretion by causing an increased water flow (hydrocholeresis, Graf, 1983). To detect such choleretic effects, we examined the influence of *Dianthus versicolor* and *Lilium pumilum* extracts on the bile flow in the rat liver perfusion model (Glasl et al., 2007). The isolated perfused rat liver is a suitable system to measure the basal hepatic bile flow, which is independent from bile salts and represents a measure of liver basal exocrine functions. Furthermore, by employing radioactive labelled taurocholic acid, this system offers the opportunity to quantify the bile-salt-dependent flow and bile salt excretion (Kroker et al., 1978). Taurocholic acid was used due to its strong choleretic properties as a physiological constituent of bile. It has been applied as a model substance in a number of bile secretion studies (Suchy et al., 1997; St-Pierre et al., 2001). Induction of the transport of bile acids into the bile entails stimulation of the bile-salt-dependent bile flow. Stimulation of either component, the bile-salt-independent or the bile-salt-dependent bile flow, results in an increase of the total bile secretion.

Different extracts of the two species were prepared and monitored in the isolated perfused rat liver. Qualitative and quantitative phytochemical investigations comprised HPLC coupled to MS and UV-DAD as well as spectrophotometry.

2. Materials and methods

2.1. Chemicals

^{3}H-Taurocholic acid (185 GBq/mmol) and scintillation fluid (Emulsifier Safe) were purchased from Perkin-Elmer (Boston, MA). Taurocholic acid (sodium salt hydrate, purity >95%) was obtained from Sigma–Aldrich (Vienna, Austria). All other chemicals and solvents purchased from Merck (Darmstadt, Germany) were of analytical or gradient grade and used without further purification.

2.2. Plant material

The aerial parts of *Dianthus versicolor* were harvested in the Mongolian province Khubsgul in summer 2004 (collection number 53/04/mon), those of *Lilium pumilum* were collected in the province Bulgan in summer 2003 (collection number 19/03/mon). The species were identified by E. Ganbold, Mongolia. Herbarium specimens of *Lilium pumilum* (herbarium number 32/03/mon/H) are deposited at the Department of Pharmacognosy, University of Vienna, Austria. A reference specimen for *Dianthus versicolor* from the province Khubsgul is kept in the Institute of Botany, Mongolian Academy of Sciences, Ulaanbaatar, Mongolia (herbarium number 424).

2.3. Preparation of extracts

Aqueous extract (AE): 10 g powdered dried plant material (drug) was suspended in 250 mL water, and, after adjustment to pH 2 with trifluoroacetic acid, the suspension was shaken gently at 40 °C to simulate gastric conditions. Trifluoroacetic acid was used instead of HCl because it could be removed by vacuum evaporation after extraction. The extracts were obtained by subsequent freeze-drying. The yields are given as drug to extract ratio (DER) and amounted to 1:0.213 for *Dianthus versicolor* and 1:0.232 for *Lilium pumilum*.

Methanolic extract (ME): Initially, 40 g of powdered dried plant material were extracted with light petroleum to deplete chlorophyll. Subsequently, the drug powder was extracted with ethyl acetate to separate the apolar compounds. Finally, this pre-extracted plant material was treated with methanol (*Dianthus*: DER 1:0.127; *Lilium*: DER 1:0.096). The methanolic solution was evaporated to dryness under reduced pressure. The drug to solvent ratio was 1:10 for each extraction step, and the extractions were performed for 1 h by ultrasound at room temperature. The drug material was completely dried up between the respective extraction steps by spreading it under a flue at room temperature.

2.4. Fractionation of Dianthus versicolor AE

The aqueous extract of *Dianthus versicolor* was purified by solid phase extraction yielding two main fractions: a flavonoid-enriched fraction (FF) and a sugar-enriched fraction (SF). 400 mg aqueous extract (AE) of *Dianthus versicolor* were redissolved in 1 mL water and applied to a C_{18} cartridge (Mega Bond Elut® 60 cm^3, Varian, Zug, CH), previously conditioned with 1 reservoir volumen (RV) methanol and 1 RV water. The elution was started 10 min after application of the extracts at a flow rate of 1 mL/min with 3 RV water, followed by 3 RV 10% (v/v) methanol, 3 RV 40% (v/v) methanol, and 3 RV 100% methanol. The aqueous fraction was lyophilized, and the other fractions were dried under reduced pressure at 45 °C. A total amount of 2.4 g AE was subjected to this procedure and yielded 987 mg sugar-enriched aqueous fraction (SF) and 159 mg flavonoid-enriched 40% methanolic fraction (FF). Both fractions were further studied, whereas the 10% methanolic fraction (28 mg) and the 100% methanolic fraction (32 mg) were precluded from further testing due to the low yields.

2.5. Thin layer chromatography (TLC)

For all analyses silica gel 60 F_{254} coated aluminium sheets (Merck, Darmstadt) were used as stationary phase. The monosaccharides were separated with the mobile phase dichloromethane–acetic acid$_{conc}$–methanol–water (60:32:12:8). The flavonoids were analysed by a mixture of ethyl acetate–acetic acid$_{conc}$–formic acid$_{conc}$–water (100:11:11:26). To detect reducing sugars, aniline–diphenylamine–phosphoric acid$_{conc}$ (0.5 g diphenylamine dissolved in 0.5 mL aniline, diluted with 20 mL acetone and 4 mL phosphoric acid$_{conc}$) was used as spraying reagent. Differently coloured sugar stains became visible after heating at 100–105 °C (Merck, 1970). The flavonoids were detected under UV 366 nm after spraying with natural product reagent (1% in methanol) and polyethylene glycol 400 (5% in methanol).

2.6. High pressure liquid chromatography (HPLC)

HPLC-UV-DAD analyses were performed on a Prominence LC-20AD coupled to a Prominence SPD-M20 Diode Array Detector (Shimadzu Corporation, Kyoto, Japan). Water used for HPLC was adjusted to pH 2.8 with HCOOH$_{conc}$. MeOH or MeCN were used as organic solvents.

Lilium pumilum: For the sample solution 0.2 g of the AE were dissolved in 10 mL water, 10 μL thereof was injected. As stationary phase served a 3.5 μm Zorbax SB C_8 column (4.6 mm × 75 mm) held at 25 °C by a column oven. The flow rate of the mobile phase was set to 0.7 mL/min. The gradient started at 20% MeOH raising up to 40% within 50 min (rate: 0.4%/min).

Dianthus versicolor: For the sample solution 0.2 g of the AE were dissolved in 10 mL water, 5 μL thereof was injected. As stationary phase served a 3 μm Thermo Aquasil C_{18} column (2.1 mm × 150 mm) held at 15 °C by a column oven. The flow rate of the mobile phase was set to 0.35 mL/min. The gradient started at 2% MeCN raising up to 14% within 23 min (rate: 0.52%/min) followed by 27 min of isocratic elution at 14% and subsequent raise to 95% within 1 min.

HPLC-MS^n analyses were performed on an UltiMate 3000 RSLC-series system (Dionex, Germering, Germany) coupled to a 3D quadrupole ion trap instrument equipped with an orthogonal ESI source (HCT, Bruker Daltonics, Bremen, Germany). HPLC separation was carried out as described above. In the case of *Lilium pumilum*, the eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: negative ion mode; capillary voltage: 4.0 kV; nebulizer (N_2): 30 psi (*Lilium*) or 50 psi (*Dianthus*); dry gas flow (N_2): 7 L/min (*Lilium*) or 10 L/min (*Dianthus*); and dry temperature: 350 °C (*Lilium*) or 365 °C (*Dianthus*). The mass spectrometer was operated in an automated data-dependent acquisition (DDA) mode where each MS scan (*m/z* 150–1000) was followed by MS^2 scans (*m/z* 40–1000, isolation window of 4 Th, fragmentation amplitude of 0.7 V) of the two most intense precursor ions and MS^3 scans (*m/z* 40–1000, isolation window of 4 Th, fragmentation amplitude of 1.0 V) of the one or two most intense fragment ions in each MS^2 scan.

2.7. Spectrophotometric quantification of the flavonoids

Preliminary results led to the conclusion that the main flavonoids in *Dianthus versicolor* were apigenin derivatives of the isovitexin type. The total flavonoid content in the aqueous extract was determined according to the monograph "Passiflorae herba" of the European Pharmacopoeia (European Pharmacopoeia, 2008), because the main flavonoids in *Passiflora* are vitexin derivatives. Briefly, 0.2 g of the dry AE (either *Dianthus* or *Lilium*) were re-extracted with 40 mL methanol–water (40:60, v/v) for 10 min by sonification. After filtration, 40 mL of fresh solvent were added to the pellet, which was treated a second time by sonification for 10 min. The resulting solutions were combined and diluted to 100.0 mL. From this stock solution, two solutions were prepared in parallel to obtain the sample solution and the compensation liquid. For each solution, 5.0 mL of the stock solution were evaporated to dryness, and the residue was redissolved in 10 mL methanol–acetic $acid_{conc}$ (1 + 10, v/v). Sample solution: 10 mL of reagent (25 g/L boric acid and 20 g/L oxalic acid in formic $acid_{conc}$) were added, and the solution was further diluted to 25.0 mL with acetic $acid_{conc}$. Compensation liquid: 10 mL of formic $acid_{conc}$ were added, and the solution was further diluted to 25.0 mL with acetic $acid_{conc}$. After 30 min, the absorption of the sample at 401 nm was measured against the compensation liquid. The percentage content of flavonoids was calculated and expressed as 8-C-glucosyl-apigenin (vitexin) based on the specific absorbance of vitexin, i.e. 628 (European Pharmacopoeia, 2008).

2.8. Liver perfusion

Male Sprague–Dawley rats (body weight: 180–270 g; liver weight: 10–15 g) were purchased from the Department of Animal Research and Genetics of the Medical University of Vienna. Animals were housed in a room with constant temperature and humidity under a 12 h light-dark cycle with free access to water and food. The study was approved by the committee for animal welfare at the institution (number of approval 66.009/64-C/GT/2007).

Single pass liver perfusion experiments were carried out using the techniques previously described (Jäger et al., 2003) on anesthetised animals according to the protocol approved by the committee of the institution. Before the excision of the liver, glass canulas for the influent and effluent perfusate were applied to the portal vein and the vena cava inferior, respectively. For the collection of bile, a canula was inserted into the bile duct. Perfusions were conducted using Krebs–Henseleit bicarbonate buffer (KHB) pH 7.4, equilibrated with 95% O_2/5% CO_2 at a constant flow rate of 35 mL/min. The temperature of the perfusion cabinet and perfusion medium was kept at 37 °C. Single bile drops falling from the bile duct (approx. 8 μL per drop) were collected.

Perfusion in the absence of taurocholic acid: After 30 min of pre-perfusion at the beginning of each experiment, perfusions were performed with increasing extract concentrations for a period of 10 min each, intermitted by intervals of 10 min purging with pure KHB. The bile flow was determined by drop frequency and liver weight given in μL per g liver per min. AE and ME were first dissolved in DMSO (10 μL), followed by addition of water (800 μL). This stock solution (530 mg/mL) was added to the KHB giving final concentrations of 100, 250, and 500 mg/L. 35 mg each of the flavonoid and the sugar fraction were redissolved in 20 μL DMSO and 180 μL water. This stock solution (175 mg/mL) was added to the KHB giving final concentrations of 10, 20, and 40 mg/L.

Perfusion in the presence of ^{3}H-taurocholic acid: After a pre-perfusion period of 30 min with KHB, 10 μM ^{3}H-taurocholic acid was added. After another 15 min, extracts were added and perfused for 10 min with each concentration. Between applications of the different extract concentrations, rinsing with KHB containing ^{3}H-taurocholic acid was performed for 10 min. Single bile drops were collected continuously to measure the excretion of ^{3}H-taurocholic acid in the bile, and each single drop was mixed with 5 mL Emulsifier Safe scintillation fluid. Liquid scintillation counting was performed on a Beckman Coulter LS 6500 multi-purpose scintillation counter (Beckman, Fullerton, CA). After perfusion, histological examinations of liver sections were done on a Zeiss microscope (Zeiss, Darmstadt, Germany) at a magnification of 100× and 400×.

2.9. Statistical analysis

All experiments were performed at least in triplicate. Data are expressed as mean ± S.D. Significance ($p < 0.05$) was calculated by applying one-way analysis of variance with the Bonferroni multiple comparison test (GraphPad Prism 5 software, La Jolla, CA).

3. Results

To study the influence of *Dianthus versicolor* and *Lilium pumilum* extracts on the bile flow in the isolated perfused rat liver, we prepared two different extracts: an aqueous extract according to the common intake of crude drugs as prescribed in traditional Mongolian medicine, and a methanolic extract, which covers a different polarity spectrum of the constituents. This was intended to direct us towards the group of compounds which contributes to the favourable activity of these plants in patients with digestive problems. The perfusion experiments summarised in Table 1 comprised trials without bile acids added to the perfusion medium in order to scrutinize the effects on the bile-salt-independent bile flow (IBF). Aqueous extracts (AE) and methanolic extracts (ME) of both plants, *Dianthus versicolor* and *Lilium pumilum*, were screened for their effect on the IBF. In the other set of experiments, studies on

Table 1
Increase of the bile flow in % of the basal flow after addition of extracts or pure substances to the perfusion buffer in absence of taurocholic acid (independent bile flow).

	Concentration		Bile-salt-independent	
	mg/L	μM	Increase (%)	S.D.
Dianthus				
Aqueous crude extract	100		11	6
	250		22*	7
	500		30*	6
Methanolic extract	100		18*	3
	250		19*	4
	500		24*	9
Flavonoid fraction	10		2	1
	20		9	3
	40		18*	5
Lilium				
Aqueous crude extract	100		13	5
	250		20*	6
	500		28*	7
Methanolic extract	100		23*	7
	250		24*	8
	500		26*	9
Reference				
Cynarin	10	19	6	3
	20	39	14	5
	40	78	23*	10
Reference				
Apigenin-7-O-glucoside	5	12	22	12
	10	23	30	1
	20	46	39*	9
Bile acid				
Taurocholic acid	2.5	5	48*	9
	5.0	10	75*	14
	10.0	20	116*	23

Control values are calculated as the mean of the bile flow during the last 3 min in the periods before a certain concentration of the extract was added (see black bars in Fig. 1).
* Significant vs. respective controls ($p < 0.05$).

the bile-salt-dependent bile flow (DBF) were performed in presence of ^{3}H-taurocholic acid in the perfusion medium. For these experiments only the aqueous extracts of *Dianthus versicolor* and *Lilium pumilum* were investigated (see Table 2). In all experiments, a pre-perfusion period of 30 min with KHB was applied before the extract (in IBF experiments) or ^{3}H-taurocholic acid (in DBF experiments) was added to the perfusion medium. The pre-perfusion was done

Table 2
Effects of different doses of *Dianthus versicolor* and *Lilium pumilum* aqueous extracts on bile flow and ^{3}H-taurocholic acid excretion in the isolated perfused rat liver.

Extract mg/L	^{3}H-Taurocholic acid excretion		Bile flow
	nmol/(g_{liver} min)	% of control	% increase
Dianthus			
100	22.5 (±0.9)	101 (±4)	8 (±6)
250	21.9 (±0.7)	99 (±3)	16 (±7)
500	19.7 (±0.9)	89 (±4)*	17 (±3)*
Lilium			
100	23.9 (±0.9)	102 (±4)	14 (±8)
250	24.1 (±1.1)	103 (±5)	18 (±6)
500	23.1 (±1.2)	98 (±5)	23 (±7)*

After 30 min perfusion with KHB, 10 μM ^{3}H-taurocholic acid was added to the perfusion medium. The ^{3}H-taurocholic acid excretion in absence of the extracts amounted to 22.3 nmol/(g_{liver} min) for *Dianthus versicolor* and 23.4 nmol/(g_{liver} min) for *Lilium pumilum*. After 15 min of perfusion in presence of 10 μM ^{3}H-taurocholic acid, 100 mg/L of the aqueous extract was applied for 10 min, followed by 10 min perfusion with 10 μM ^{3}H-taurocholic acid alone. Thereafter, 250 and 500 mg/L of the aqueous extract were applied for 10 min, with a 10 min rinsing period in between.
* Significant vs. respective controls ($p < 0.05$), see legend of Fig. 2.

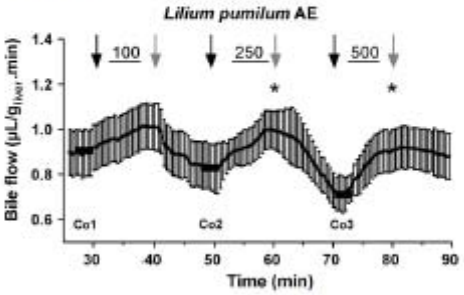

Fig. 1. Time course of bile flow changes during application of *Lilium pumilum* aqueous extract (100, 250, and 500 mg/L). Application of extracts and washing periods are indicated by black and grey arrows, respectively. Control values are calculated as the mean of the bile flow during the last 3 min in the periods before a certain concentration of the extract was added (see black bars: Co1, Co2, Co3). Bile flow changes were calculated from the maxima of the graph during the last 3 min under extract perfusion (grey arrows). All perfusions were done at least in triplicates. * Significant vs. respective controls ($p < 0.05$).

to deplete the bile from endogeneous compounds secreted by the liver, e.g. bile acids. Indeed, previous studies showed that in the isolated perfused rat liver 5 min after onset of the perfusion with KHB the release of liver-derived bile salts approaches zero (Boyer and Klatskin, 1970; Graf et al., 1972).

3.1. Perfusions in the absence of bile acids

As shown in Fig. 1, application of 100, 250, and 500 mg/L AE from *Lilium pumilum* resulted in a dose-dependent increase of bile flow over the perfusion period. To calculate the percentage of maximal stimulation, the mean of the bile flow values measured during the last three 3 min before extract application (see Fig. 1, black arrows) was set to 100% (control). Maximal stimulation of bile secretion was 13%, 20%, and 28%, respectively. Stimulation of the bile secretion was reversible: the secretion rate dropped back or even below the baseline during the washing periods (see Fig. 1). This small but continuous decline in bile secretion rates during the perfusion is also observed in control experiments in which only KHB is applied. This "normal" decline averages to 5–8% after 60 min of perfusion and is likely to be caused by a slight but continuous depletion of osmotically active compounds, e.g. glutathione or glucuronic acid, from the liver (Graf, 1983). Therefore, the pronounced decrease in bile secretion after application of 250 mg/L *Lilium pumilum* AE could be caused by the depletion of glucuronic acid. Glucuronidation is needed to produce more hydrophilic, better excretable metabolites (e.g. of flavonoids). This was previously shown for the hepatobiliary excretion of the isoflavone genistein (Jäger et al., 1997).

Similar to the choleretic effect of *Lilium pumilum* AE, a dose-dependent choleretic effect was observed after application of 100, 250, and 500 mg/L *Dianthus versicolor* AE (maximal stimulation of 11%, 22%, and 30%, respectively, see Table 1). Comparative studies using the methanolic extracts of both plants showed that their stimulatory effects on the bile flow were more or less similar to that observed with the aqueous extracts. As shown in Table 1, *Dianthus versicolor* ME stimulated the bile flow dose-dependently by 18%, 19%, and 24%. *Lilium pumilum* ME showed similar effects of the three doses. Our further studies focussed on the aqueous extracts in order to elucidate their effects on bile-salt-dependent bile secretion.

3.2. Perfusion in the presence of taurocholic acid

To determine the effects of the plant extracts on the bile-salt-dependent bile flow (DBF) and biliary bile salt excretion, perfusions

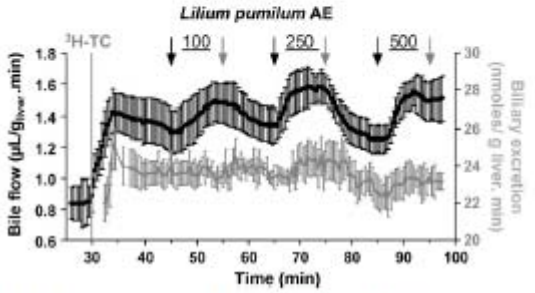

Fig. 2. Time course of bile flow changes during application of *Lilium pumilum* aqueous extract in the presence of 10 µM ^{3}H-taurocholic acid (black graph). Control values are calculated as the mean of the bile flow during the last 3 min in the periods before a certain concentration of the extract was added (see black bars). Bile flow changes were calculated from the maxima of the graph during the last 3 min under extract perfusion. All perfusions were done at least in triplicates. ^{3}H-taurocholic acid was added after 30 min of pre-perfusion and was present throughout the whole experiment. Grey graph: excretion of ^{3}H-taurocholic acid into bile. *Significant vs. respective controls ($p < 0.05$).

were done in presence of 10 µM ^{3}H-taurocholic acid. Calculations were performed as described in Section 3.1. As expected taurocholic acid stimulated bile secretion dose-dependently by 49%, 75%, and 116% (see Table 1). The black curve in Fig. 2 shows the time-dependent bile flow changes after application of 100, 250, and 500 mg/L *Lilium pumilum* AE. As already observed for IBF, *Lilium pumilum* AE led to an increase in the bile secretion by 14%, 18%, and 23%. Similarly, *Dianthus versicolor* AE stimulated the bile flow by 8%, 16%, and 17% (Table 2). The excretion of taurocholic acid remained more or less unchanged during the application of *Lilium pumilum* AE (see Fig. 2, grey curve). The slight fluctuations that are seen in these experiments suggest that changes are caused rather by solvent drag than by a significant effect on the bile acid transporters. This pattern is also observed after application of *Dianthus versicolor* AE (see Table 2). Therefore, we assume that the dose-dependent increase in bile flow under extract application is mainly due to a stimulation of the basal hepatic bile flow, which is independent from bile salts. Consequently, other experiments with AE-derived fractions (see Section 3.4) were not continued in the presence of taurocholic acid.

3.3. Phytochemical investigations

The phytochemical investigations focussed on the AE of *Dianthus versicolor* and *Lilium pumilum*. For the qualitative screening HPLC was coupled with UV-DAD and ion trap MS revealing apigenin-, kaempferol-, isorhamnetin-, and quercetin-diglycosides. The quantitative determinations were conducted by UV-spectrophotometry following the monograph "Passiflorae herba" of the European Pharmacopoeia.

3.3.1. Identification of flavonoids

The LC-UV-DAD analyses of the two aqueous extracts are shown in Fig. 3. In parallel, multistage MS-data were recorded in the negative mode. The combined information revealed the diglycosides quercetin-3-*O*-rutinoside (rutoside, **1**), kaempferol-3-*O*-rutinoside (**2**), and isorhamnetin-3-*O*-rutinoside (**3**) for *Lilium pumilum*. In *Dianthus versicolor* the apigenin-*C*-glycosyl isovitexin-7-*O*-glucoside (saponarin, **4**) was identified. The main flavonoids in *Dianthus versicolor* are apigenin- and luteolin-derivatives – most of them are new and part of a presently submitted publication (Obmann et al., submitted for publication). The structures of compounds **1**–**4** are given in Fig. 4. In Fig. 3 the corresponding peaks are

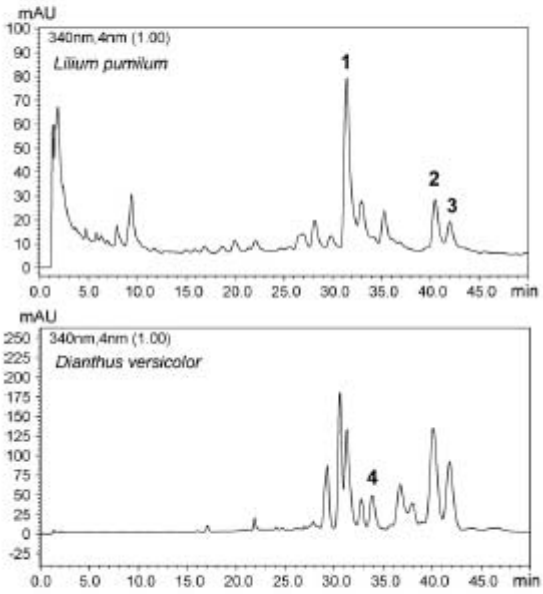

Fig. 3. Aqueous extracts of *Lilium pumilum* and *Dianthus versicolor* after LC-DAD-MS analysis detected at 340 nm (peak numbers correspond to structures given in Fig. 4). Deprotonated molecular ions $[M-H]^-$: *m/z* 609 (**1**), *m/z* 593 (**2**), *m/z* 623 (**3**), *m/z* 593 (**4**).

assigned, and the legend comprises the respective deprotonated molecular ions. The identity of the flavonoids **1**–**4** was confirmed by co-chromatography with authentic substances on silica gel (TLC) and RP (HPLC).

3.3.2. Determination of the flavonoid content

According to MS-data, the AE of *Dianthus versicolor* was expected to contain mainly flavonoid-*C*-glycosides. As passionflower also mainly consists of this type of flavonoids (Patel et al., 2009) the UV-spectrophotometric determination followed the monograph "Passiflorae herba" of the European Pharmacopoeia. The amount

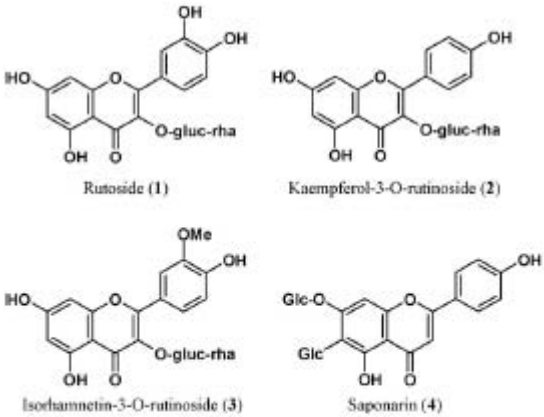

Fig. 4. Structural formulae of flavonoids **1**–**3** (*Lilium pumilum*) and **4** (*Dianthus versicolor*).

of total flavonoids in the AE of *Dianthus versicolor* was 1.78 ± 2.9 (RSD)%, and the content in the AE of *Lilium pumilum* amounted to 1.04 ± 10.0 (RSD)%. The total flavonoid values were calculated and expressed as vitexin.

3.4. Effect of Dianthus versicolor fractions on the bile secretion

Additional experiments focussed on *Dianthus versicolor* because the plant is among the 45 most common medicinal plants from domestic Mongolian markets (Report of Market Research on Mongolian Traditional Medicinal Drugs, 2007). The aqueous extract from *Dianthus versicolor* was further fractionated by solid phase extraction, and yielded two fractions enriched in either flavonoids (FF) or sugars (SF) see Section 2.4. In our study we found a significant choleretic effect of the *Dianthus versicolor* flavonoid fraction, which was rich in apigenin- and luteolin-derivatives. As shown in Table 1, 40 mg/L of this flavonoid fraction increased the bile flow by 18 ± 5% ($p < 0.05$). A comparable value was achieved by the AE although at a six-fold higher dose (250 mg/L). Previously identified choleretic compounds in plant extracts (Glasl et al., 2007), such as the flavonoid apigenin-7-*O*-glucoside and the dicaffeoylquinic acid cynarin, were used in control experiments. A stimulation of 39 ± 9% at 20 mg/L was recorded for apigenin-7-*O*-glucoside, and 23 ± 10% for cynarin at 40 mg/L (see Table 1). This suggests that flavonoids indeed contribute to the choleretic effect of *Dianthus versicolor*.

The sugar fraction obtained after solid phase extraction of *Dianthus versicolor* AE was enriched mainly in glucose and fructose according to TLC analysis. As expected, up to 40 mg/L of the sugar fraction did not influence the bile flow.

3.5. Liver toxicity

No apparent acute toxic effects of the extracts were seen after microscopic inspection of the liver. Neither *Dianthus versicolor* nor *Lilium pumilum* extracts caused a change in the normal liver architecture and in the hepatocyte structure.

4. Discussion and conclusion

We show that in the isolated perfused rat liver, aqueous and methanolic extracts of *Dianthus versicolor* and *Lilium pumilum* cause a dose-dependent increase in bile flow. The effective stimulation of the bile secretion by a flavonoid-enriched fraction further suggests that these constituents contribute substantially to the bile-flow-stimulating effect of the plants.

In traditional Mongolian medicine people take the crude drug (pulverised dried plant material) together with several glasses of water, and therefore, we focussed on the aqueous extracts. To simulate the effect of the acidic gastric juice the aqueous extraction of the dried plant material was performed at acidic pH. In order to cover a more lipophilic spectrum of compounds, methanolic extracts of both plants were prepared. The aqueous as well as the methanolic preparations of both plants caused a pronounced stimulation of the bile secretion. This indicates that choleretically active constituents with a wide spectrum of polarity are present. The main flavonoids found in *Dianthus versicolor* aqueous extract were C- and *O*-glycosylated apigenin-derivatives. Indeed, we show that a fraction enriched in these flavonoids is largely responsible for the stimulation of bile secretion. Apigenin-glycosides were also isolated previously from *Saussurea amara* (L.) DC. – another plant used in traditional Mongolian medicine – and was found to cause choleresis (Glasl et al., 2007).

In order to elucidate the mode of action underlying the choleretic effect of *Dianthus versicolor* and *Lilium pumilum*, experiments in absence and presence of taurocholic acid were performed. As no significant stimulatory effect on taurocholic acid excretion was observed, an influence of these plant extracts on hepatic bile acid transporters and consequently on DBF seems unlikely. However, it cannot be excluded that unknown compounds interact with the active transport of taurocholic acid at higher extracts' concentrations. The mechanism behind the stimulation in bile secretion remains unclear. Most likely the increased bile flow is due to an increased osmotic pressure and additional water flow into the bile (hydrocholeresis) caused either by active constituents or active metabolites. This hypothesis has to be confirmed by metabolization studies.

With respect to unfavourable effects, we have observed that these extracts do not inhibit taurocholic acid secretion, at least for concentrations up to 500 mg/L. This is in accordance with the histological findings that these plant extracts lack acute toxicity on the liver in the given doses.

The fact that *Dianthus versicolor* and *Lilium pumilum* extracts show an impact on the isolated liver explains, at least in part, their beneficial effect in patients treated with traditional Mongolian preparations. The results from this study warrant further in vivo tests.

Acknowledgements

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3.4 Additional analyses and optimization of methods

This chapter mentions additional TLC and GC-MS analyses as well as the optimization of HPLC-methods which is not described in detail in publications 1-4 [21, 22, 23,24].

3.4.1 TLC

TLC was used to get a quick overview of compound classes in the OWE of *D. versicolor*. In first trials the aqueous extracts of five different samples (Table 1, p. 19) were compared in a system suitable for flavonoid-glycosides (system 1, chapter 2.3.1 p. 24, table 3). These investigations showed that the aqueous extracts of samples **a** and **b** were rich in highly polar compounds characterized by Rf-values up to 0.28 (Fig. 8). In the samples **c**, **d**, and **e** less polar flavonoids (Rf > 0.4) were detected in addition to the polar ones known from samples **a** and **b**. Differences in the types of aglycones were indicated by appearance of orange (luteolin-type) or greenish (apigenin-type, chrysoeriol-type) spots after spraying with natural product reagent/ PEG 400, which is highly suitable for the detection of phenolic compounds, and detection under UV 366 nm.

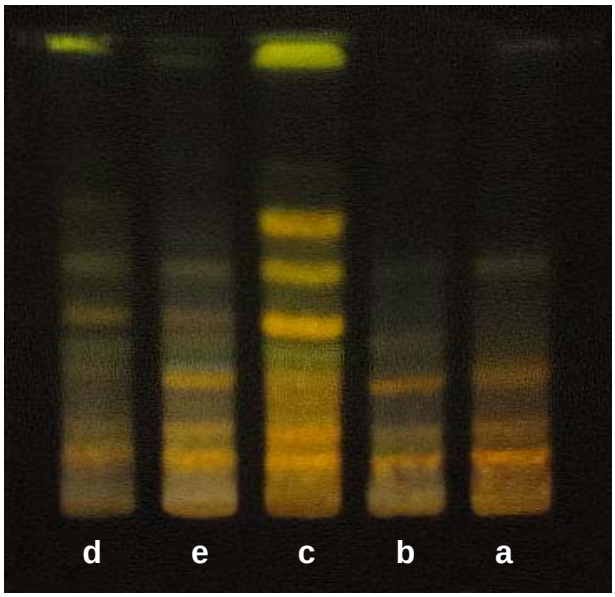


Fig. 8 TLC analysis of the OWE (chapter 2.2.1, p. 19) of samples **a**-**e** applying system 1 (chapter 2.3.1 p. 24, table 3) detected with natural product reagent/PEG 400, UV 366 nm

Based on these results, in addition to the HPLC method for the quantification of flavonoids in all five samples described in publication 3 [23] an alternative method was developed for the analysis of the OWEs of samples **a** and **b** [24].

System 1 was furthermore used for the analysis of fractions obtained by SPE (Fig. 9). The analysis clearly shows, that flavonoids were enriched in the fraction eluting with 40% MeOH which we needed for the isolation of compounds **1**-**7** (Fig. 5, 7, and 31).

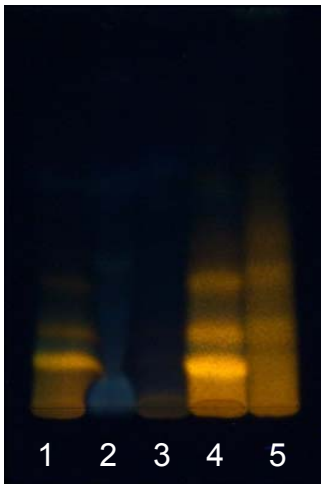


Fig. 9 TLC analysis of OWE (1) (chapter 2.2.1, p. 19) and fractions obtained by SPE eluting with H_2O (2), 10% MeOH (3), 40% MeOH (4), and 100% MeOH (5) applying system 1 (chapter 2.3.1 p.24, table 3) detected with natural product reagent/PEG 400, UV 366 nm

Moreover, CPC or CC derived fractions (Fig. 7, p. 23) were unified according to their similarity in the TLC-fingerprint obtained after analysis in system 1 (Fig. 10 and 11).

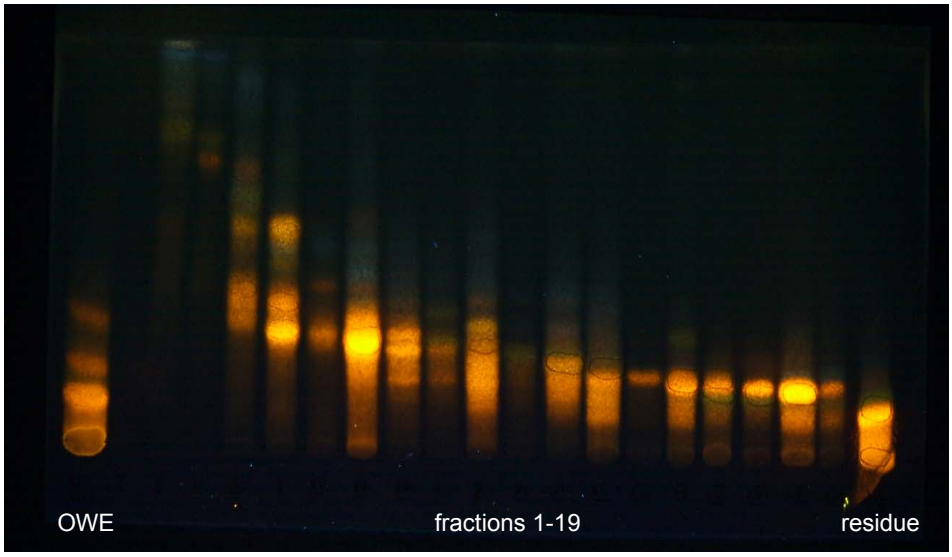


Fig. 10 TLC analysis of the OWE (chapter 2.2.1, p. 19), the unified fractions 1-19 and the residue obtained by CPC (chapter 2.2.3, p. 20 and publication 2 [22] chapter 3.1. pp. 30-85) applying system 1 (chapter 2.3.1, p. 24 table 3) detected with natural product reagent/PEG 400, UV 366 nm

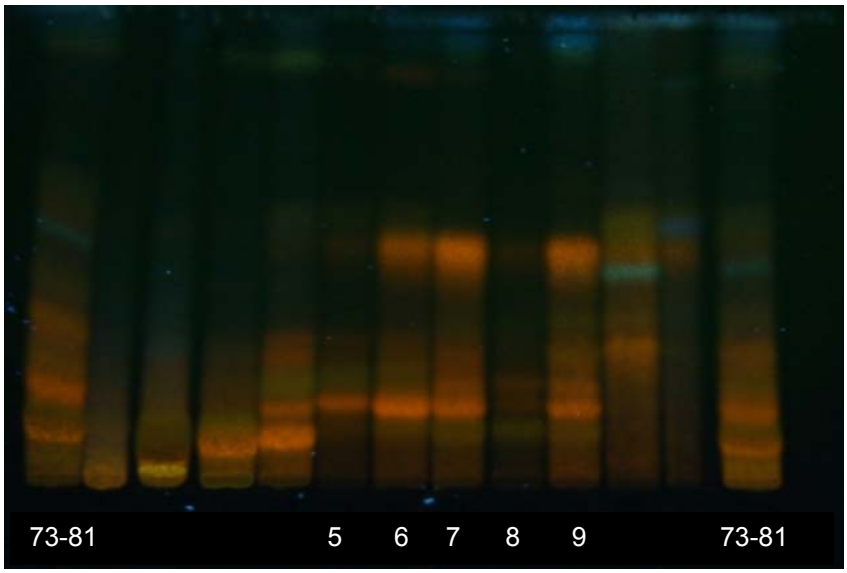
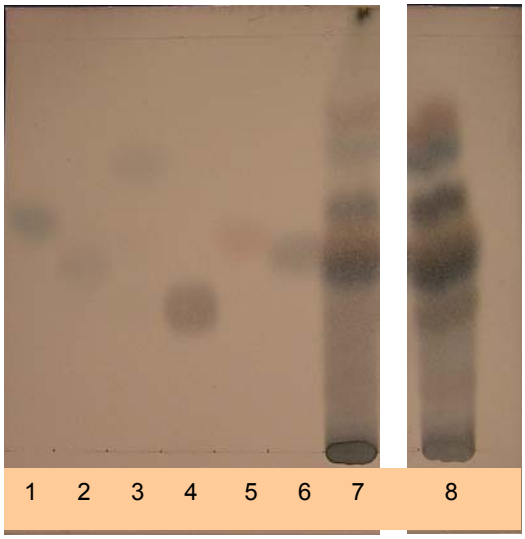


Fig. 11 TLC analysis of the unified fraction 73-81 obtained by CC of the OWE (chapter 2.2.1, p. 19) on polyamide (chapter 2.2.4, p. 21 and publication 2 [22] chapter 3.1. p. 30-85) and further fractions thereof obtained by CC on Sephadex LH 20 (chapter 2.2.4, p. 21 and publication 2 [22] chapter 3.1. p. 30-85, Fig. 6, p. 21 and Fig. 7, p. 23) applying system 1 (chapter 2.3.1 p. 24, table 3) detected with natural product reagent/PEG 400, UV 366 nm

For the analysis of the flavonoid aglycones in samples **a**-**e** (Table 1, p. 19) a more apolar system (system 2, chapter 2.3.1 p. 24, table 3) was used (Fig. 12). According to TLC-analysis samples **c**, **d**, and **e** contained the aglycones apigenin, luteolin, and chrysoeriol. This was confirmed by HPLC-analysis (publication 2 [22], chapter 3.1. p. 29-85).

Fig. 12 TLC analysis of the OWE (chapter 2.2.1, p. 19) of samples **a**, **c**, **d**, and **e** in comparison to the reference substances luteolin (1), apigenin (2), kaempferol (3), and chrysoeriol (4) applying system 2 (chapter 2.3.1 p. 24, table 3) detected with natural product reagent/PEG 400, UV 366 nm

For the identification of monosaccharides in the OWE of sample **a** and for the identification of the glycosidic part of isolated compounds after acid hydrolysis a third TLC-system was applied (system 3, chapter 2.3.1 p. 24, table 3). Comparison with reference substances led to the identification of glucose, fructose and saccharose in the OWE of sample **a** (Fig. 13a), which was also confirmed by GC-MS analyses. The sugar moieties of three isolated flavonoid glycosides were identified as glucose, galactose, and rhamnose (Fig. 13b). The results obtained by TLC were confirmed by GC-MS (chapter 3.4.3, pp. 147-150).



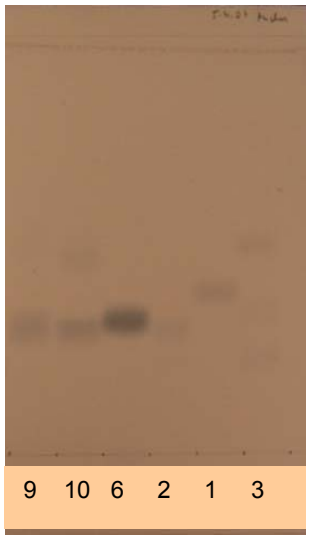


Fig. 13a (left) TLC analysis of the OWE (chapter 2.2.1, p. 19) of sample **a** (7) and the aqueous fraction obtained by SPE (8) in comparison to the reference substances arabinose (1), galactose (2), rhamnose (3), saccharose (4), fructose (5), and glucose (6) applying system 3 (chapter 2.3.1 p. 24, table 3) detected with diphenylamine-aniline-reagent after heating to 105-110°C

Fig. 13b (right) TLC analysis of the aqueous fraction of compound **6** (9) and the mixture of compounds **1**+**2** (10) after acid hydrolysis (chapter 2.3.3. p. 25) in comparison to the reference sugars glucose (6), galactose (2), arabinose (1), and rhamnose (3) applying system 3 (chapter 2.3.1 p. 24, table 3) detected with diphenylamine-aniline-reagent after heating to 105-110°C

3.4.2 Optimization of HPLC-methods

HPLC was employed for the qualitative and quantitative analysis of OWEs and fractions of *D. versicolor*. We hyphenated this technique with DAD or MS and developed suitable methods according to the respective analytical question.

3.4.2.1 Establishment of an analytical HPLC-method

For the method development the OWE of sample **a** (Table 1, p. 19) which served for the isolation of compounds **1-7** (Fig. 31, p. 152) was chosen. Throughout the method development various parameters such as stationary phase, linear gradient and temperature were varied. Based on the results of TLC analysis, which revealed the presence of flavonoid glycosides, for first experiments an RP-18e column (LiChrospher® 100 RP-18e, 250 x 4.6 mm, 5 µm particles) was chosen. But even very flat gradients with a starting concentration of 0% organic solvent (B) did not result in a satisfactory resolution (Fig. 14).

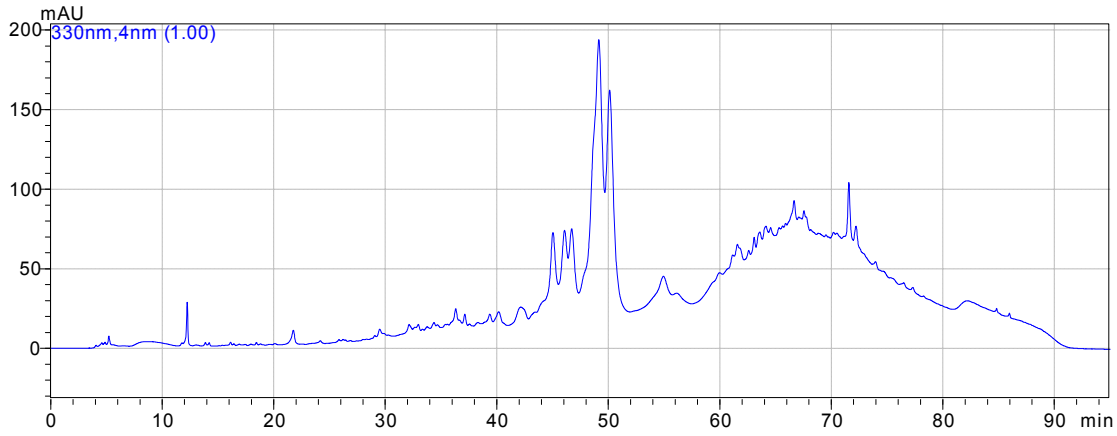


Fig. 14 HPLC analysis of the OWE (sample **a**, chapter 2.2.1, p. 19) on LiChrospher ® 100 RP-18e applying the following linear gradient: 0→15 % B within 30 min, 15% isocratic for 20 min, 15→40% B within 25 min, 40→100% B within 10 min at a flow rate of 1mL/min and a column oven temperature of 25°C; detected at 330nm

The second tested stationary phase was Phenomenex® Luna-Phenyl-hexyl (250 x 2.1 mm, 5µm particles), which should facilitate special interactions between the aromatic part of the analytes and the phenyl-rests of the stationary phase

and, therefore, provide a better separation. This column showed better results than the RP-18e column but there was still need for improvement (Fig. 15).

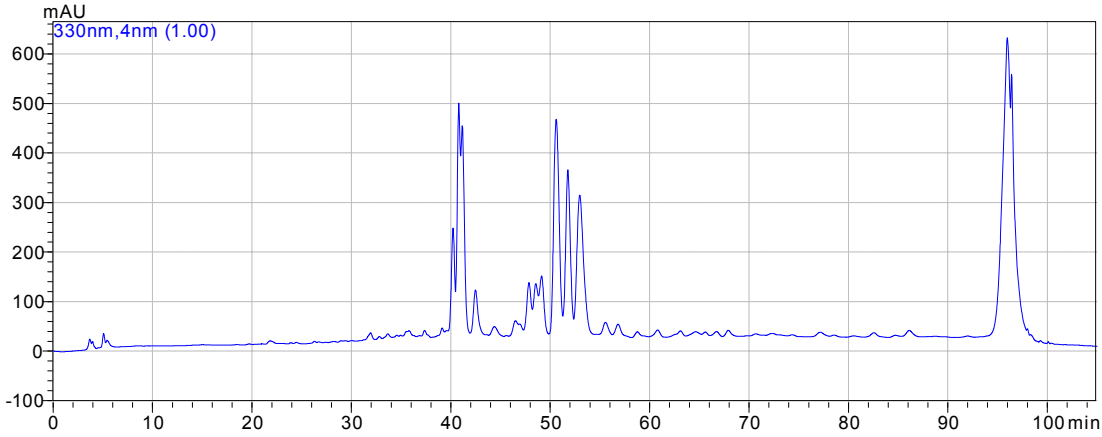


Fig. 15 HPLC analysis of the OWE (sample **a**, chapter 2.2.1, p. 19) on Phenomenex® Luna Phenyl-hexyl applying the following linear gradient: 0→10% B within 20 min, 10→12% within 5 min, 12→20% B within 60 min, 20→100% B within 10 min at a flow rate of 0.2 mL/min and a column oven temperature of 25°C; detected at 330nm

In order to overcome this problem the column oven temperature was changed to values above and below room temperature (Fig. 16).

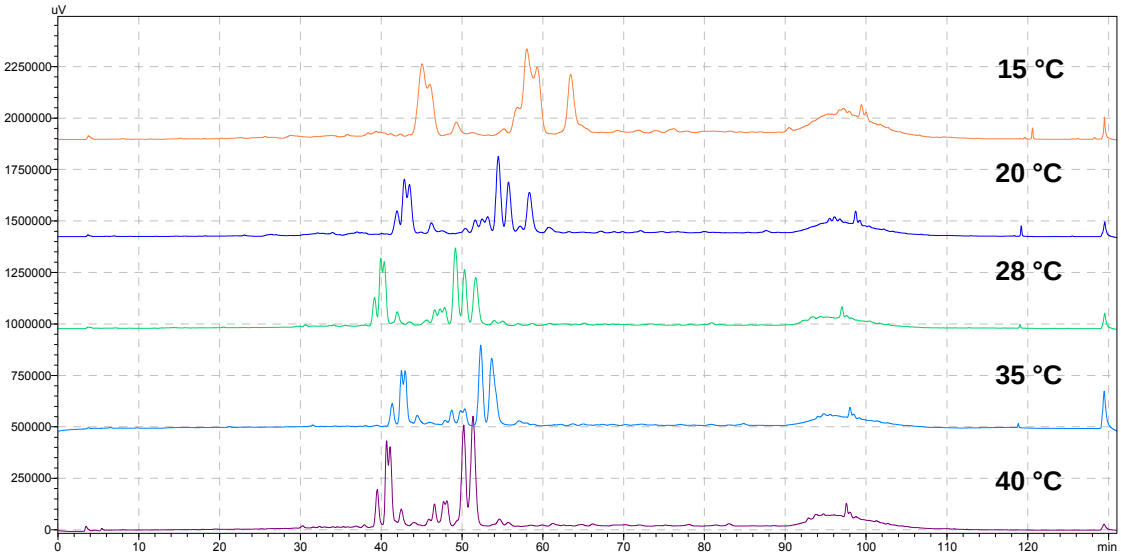


Fig. 16 HPLC analysis of the OWE (sample **a**, chapter 2.2.1, p. 19) on Phenomenex® Luna Phenyl-hexyl applying the following linear gradient: 0→10% B within 20 min, 10→12% within 5 min, 12→20% B within 60 min, 20→100% B within 10 min at a flow rate of 0.2 mL/min and various column oven temperatures; detected at 340nm

In general, higher temperatures shortened the retention times, whereas lower temperatures delayed the elution of the compounds. The experiments showed that a temperature of 15°C led to a good separation of the compound eluting

after 62-63 min from those eluting earlier, but the resolution of all other compounds was bad. On the other hand, higher temperatures (up to 40°C) resulted in a good separation of the compound eluting at 39 min. The optimal oven temperature for using this column seemed to be 28 °C.

Nevertheless, the separation on Luna-Phenyl-hexyl was not satisfying, and therefore, a third column, Aquasil® C18 (250 x 4.6 mm, 5 µm particles), was tested. This stationary phase is characterized by C_{18} chains with polar embedded groups and should therefore be suitable for the separation of polar compounds. Indeed, this column showed the best results (Fig.17).

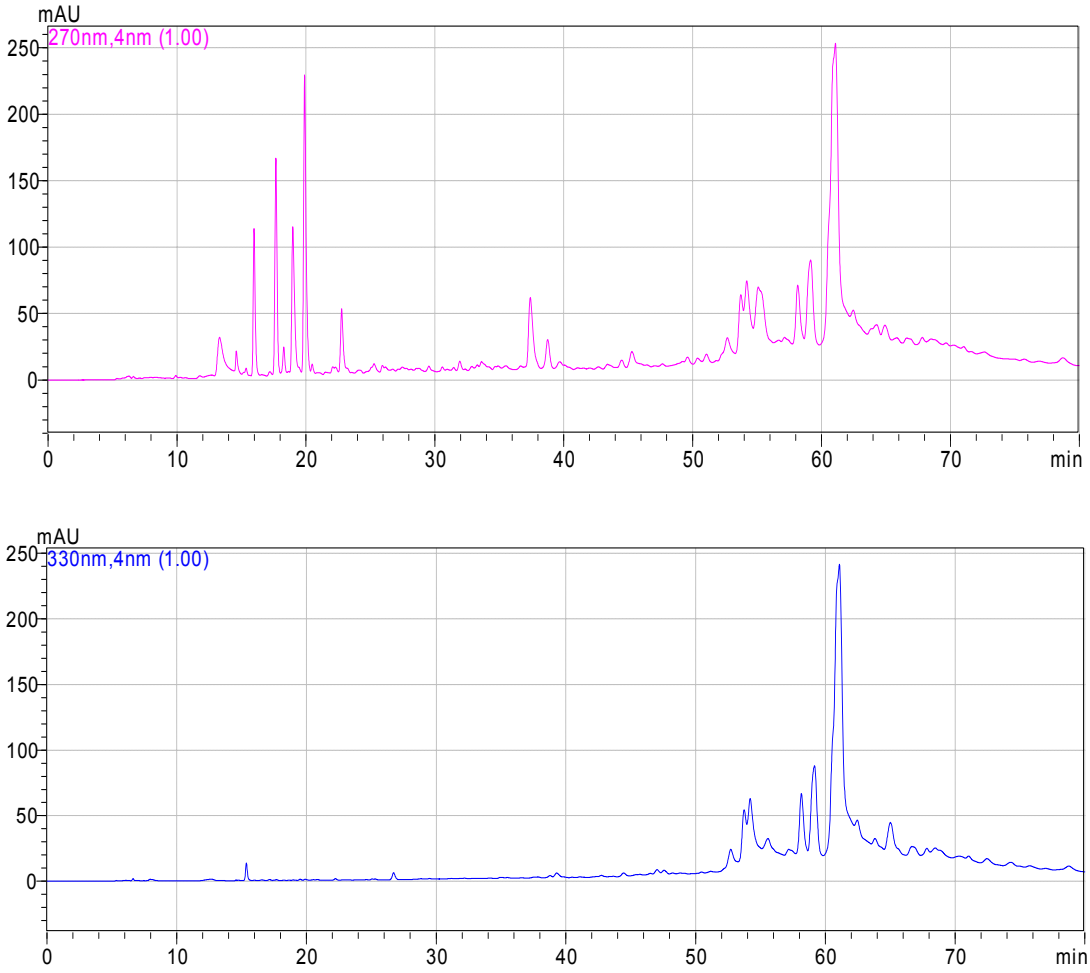


Fig. 17 HPLC analysis of the OWE (sample **a**, chapter 2.2.1, p. 19) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→23% within 40 min, 23 isocratic for 15 min, 23→40% B within 10 min, 40→100% B within 5 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 270 nm (upper panel) and 330 nm (lower panel)

The next step was the optimization of the linear gradient. Based on the trials on RP-18e and phenyl-hexyl material a starting concentration of 0% of solvent B was chosen and the steepness of the gradient was gradually decreased

resulting in the following optimal composition: 0% B to 10% B within 20 min (rate: 0.5%/min), 10% B to 20% B within 60 min (rate: 0.17%/min), 20% B to 40% B within 20 min (rate: 1%/min) and finally from 40% B up to 95% B within 10 min (rate: 5.5%/min) (Fig. 18).

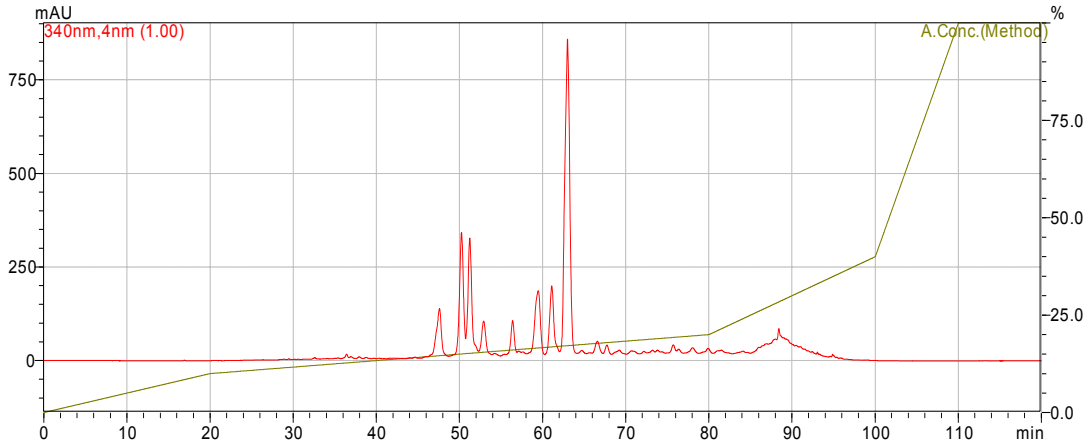


Fig. 18 HPLC analysis of the OWE (chapter 2.2.1, p. 19) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 340 nm

This method was used for the qualitative analysis of fractions obtained by SPE (Fig. 19), CPC, and CC and for the semipreparative isolation of compunds **1**-**7** (Fig. 20-23).

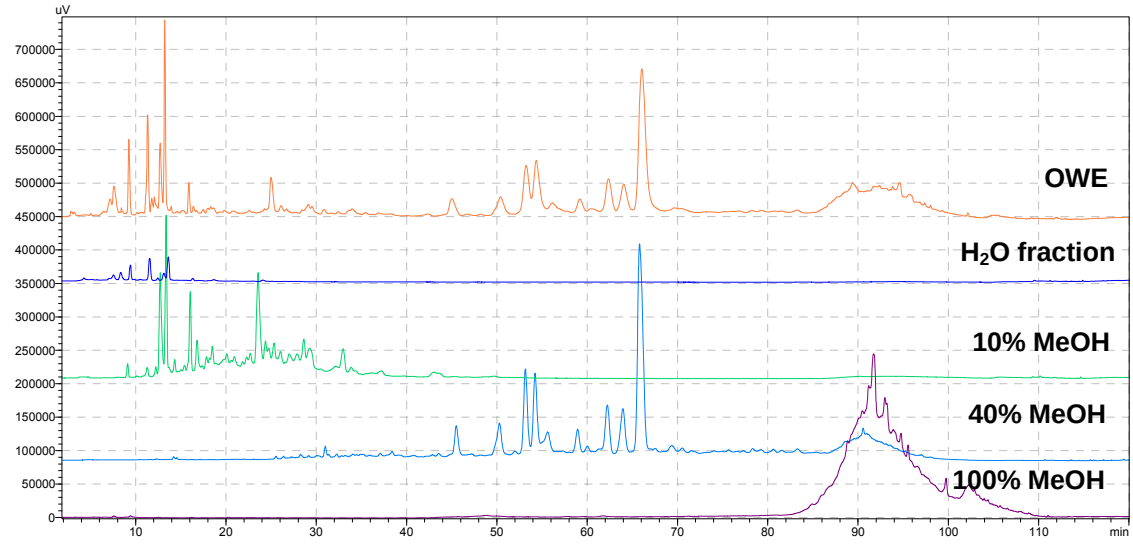


Fig. 19 HPLC analysis of the OWE (chapter 2.2.1, p. 19) and fractions thereof obtained by SPE (chapter 2.2.2, p. 20) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 340 nm

Fig. 20 shows the HPLC analysis of the fraction “residue” (Fig. 7, chapter 2.2.3, p. 23 and publication 2 [22], chapter 3.1. p. 30-85). It served for the isolation of compounds **1** and **2** by semipreparative HPLC (publication 2 [22], chapter 3.1. p. 30-85)

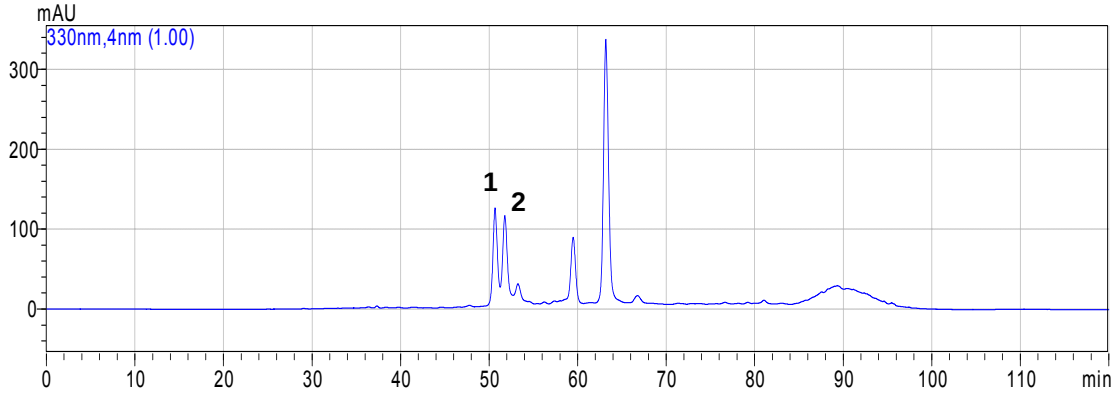


Fig. 20 HPLC analysis of the fraction “residue” (Fig. 7, chapter 2.2.3, p. 23 and publication 2 [22] chapter 3.1. p. 30-85) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 330 nm;

Fig. 21 shows the HPLC analysis of fraction I (35-42) (Fig. 7, chapter 2.2.4, p. 23 and publication 2 [22] chapter 3.1. p. 30-85), which served for the isolation of the isobaric compounds **3a**, **3b** and **4a**, **4b** (publication 2 [22], chapter 3.1. p. 30-85).

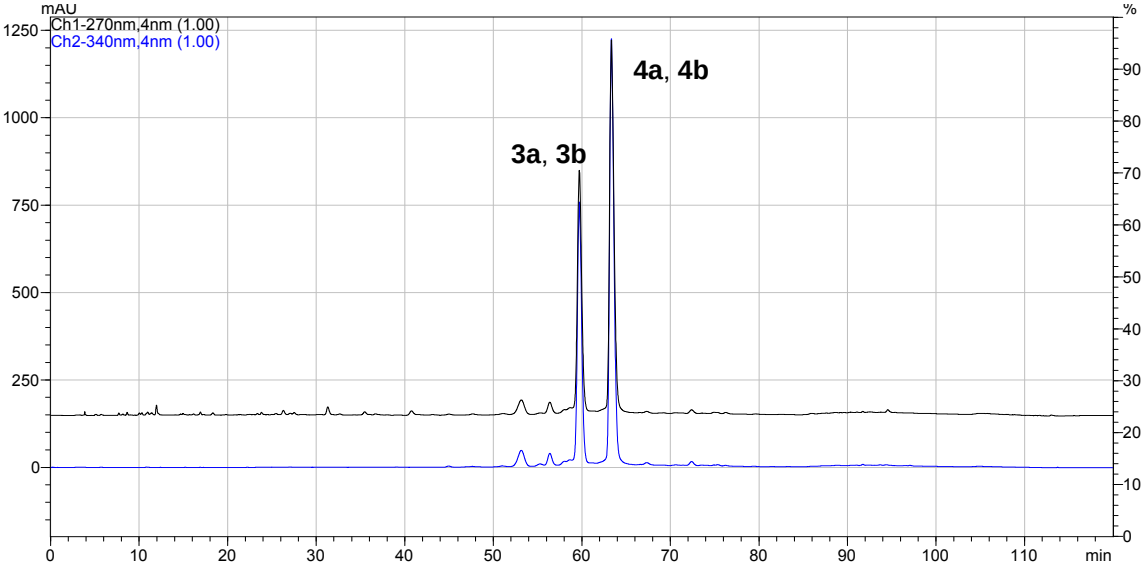


Fig. 21 HPLC analysis of fraction I (35-42) (Fig. 7, chapter 2.2.4, p. 23) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 270 and 340 nm;

Fig. 22 shows the HPLC analysis of fractions 2-6 obtained by CC of fraction II (50-55) (Fig. 7, p. 23) on Sephadex LH-20. Fractions 3, 4, and 5 served for the isolation of compound **5** (red ring) by semipreparative HPLC (publication 2 [22], chapter 3.1. p. 30-85).

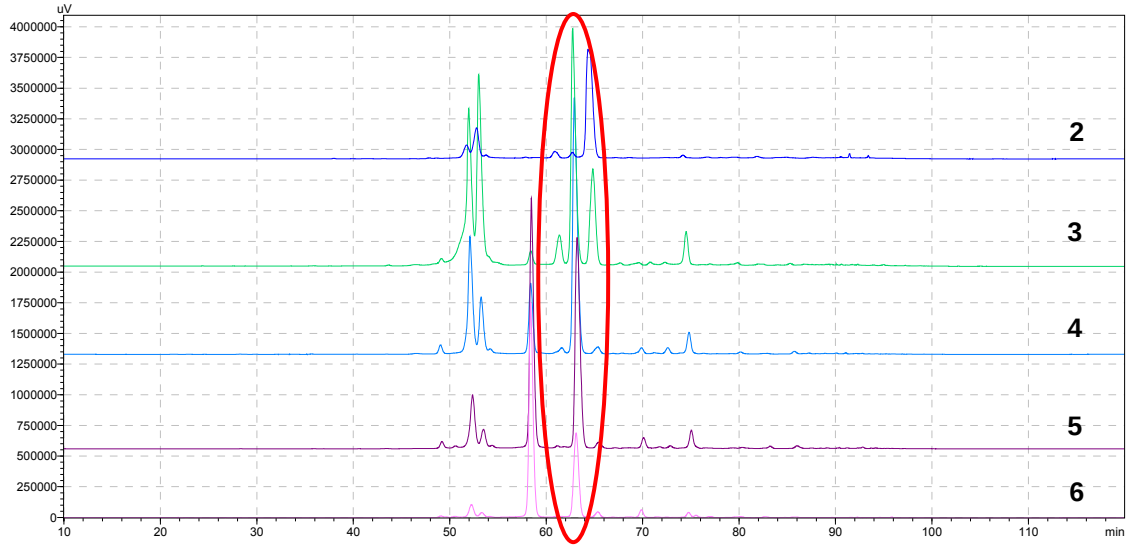


Fig. 22 HPLC analysis of fractions 2-6 (Fig. 7, chapter 2.2.4, p. 23) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 340 nm

Fig. 23 shows the HPLC analysis of fractions 5-9 obtained by CC of fraction V (73-81) (Fig. 7, chapter 2.2.4, p. 23) on Sephadex LH-20. These fractions served for the isolation of compound **6** (black ring) by semipreparative HPLC (publication 2 [22], chapter 3.1. p. 30-85).

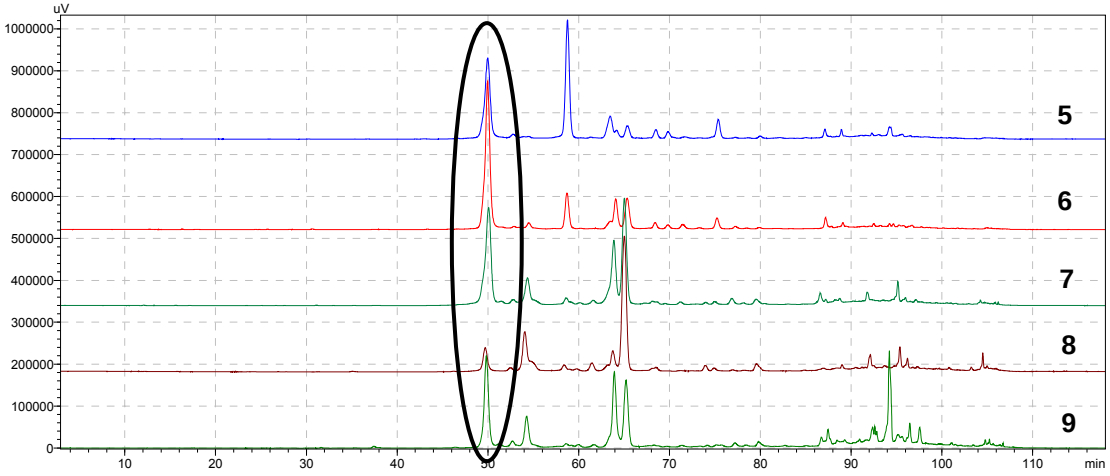


Fig. 23 HPLC analysis of fractions 5-9 (Fig. 7, p. 23) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and a column oven temperature of 25°C; detected at 340 nm

Even though the separation of the flavonoid glycosides contained in the OWE of sample **a** seemed to be satisfying LC-MS analyses carried out later showed that the peak eluting at 66 min was a mixture of at least two compounds.
As described before variations on column oven temperature were performed at 15°C and 25°C (Fig. 24).

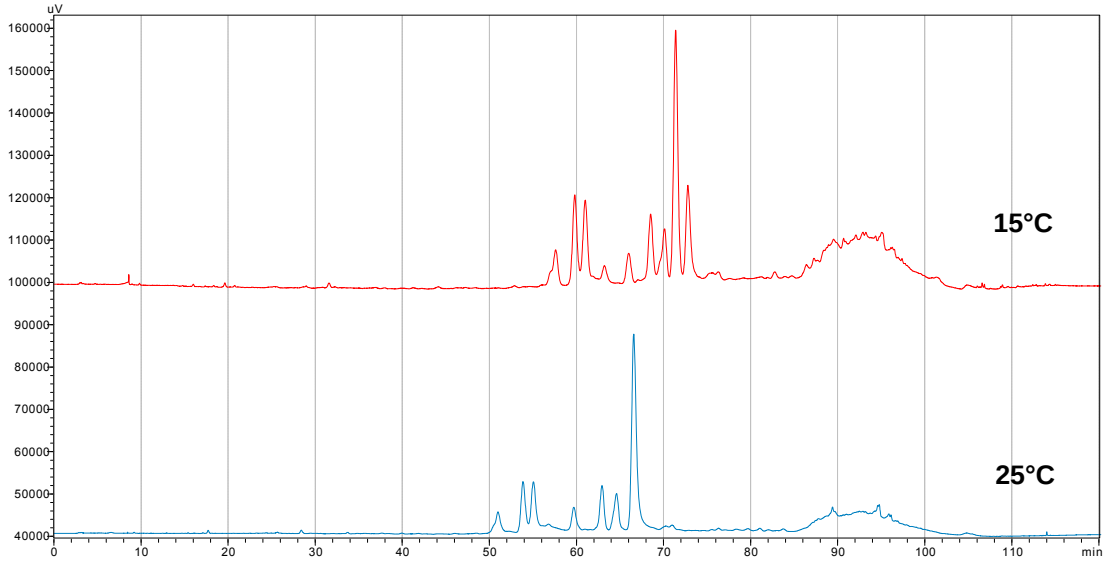


Fig. 24 HPLC analysis of the OWE (chapter 2.2.1, p. 19) on Aquasil® C 18 applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and column oven temperature of 15 and 25°C; detected at 340 nm

According to those analyses a column oven temperature of 15°C showed an acceptable separation, which provides the possibility to quantify single compounds. A comparison of the OWEs of samples **a**-**e** led to the conclusion that the application of this method was useful only for the quantification of flavonoids in the OWE and the crude drug of sample **a**. This application using rutoside as internal standard is described in publication 4 [24], pp. 113-125. Furthermore, a disadvantage was the quite long time of analysis (121 min). Therefore, the dimensions of the Aquasil® C 18 column were changed from 250 x 4.6 mm, 5µm particles to 150 x 2.1 mm, 3µm particles in order to shorten the retention times. As a consequence of the change in column parameters the linear gradient and the flow rate had to be optimized. After a couple of trials the following parameters provided an acceptable separation at a reasonable duration for qualitative analyses within 51 min: 2% B to 14% B within 23 min

(rate: 0.52%/min), followed by an isocratic period of 27 min (Fig. 25). The flow rate of 0.35 mL/min allowed direct coupling to MS and reduced the consumption of solvents by 86%.

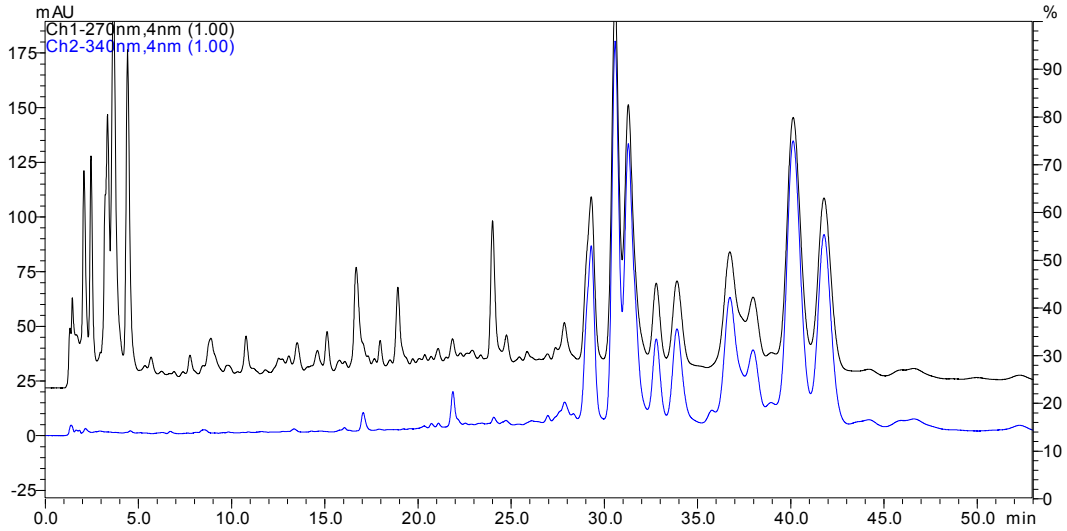


Fig. 25 HPLC analysis of the OWE (chapter 2.2.1, p. 19) on Aquasil® C 18 (3µm particles) applying the following linear gradient: 2→14% B within 23 min followed by an isocratic period of 23 min at a flow rate of 0.35 mL/min and column oven temperature of 15°C; detected at 270 and 340 nm

Unfortunately, for the quantification of single compounds the resolution was not sufficient for all compounds.

3.4.2.2 Quantification of flavonoids by HPLC-UV

A comparison of the OWEs of samples **a**, **c**, **d**, and **e** showed that the method described in publication 4 [24] was not suitable for all samples (Fig. 26).

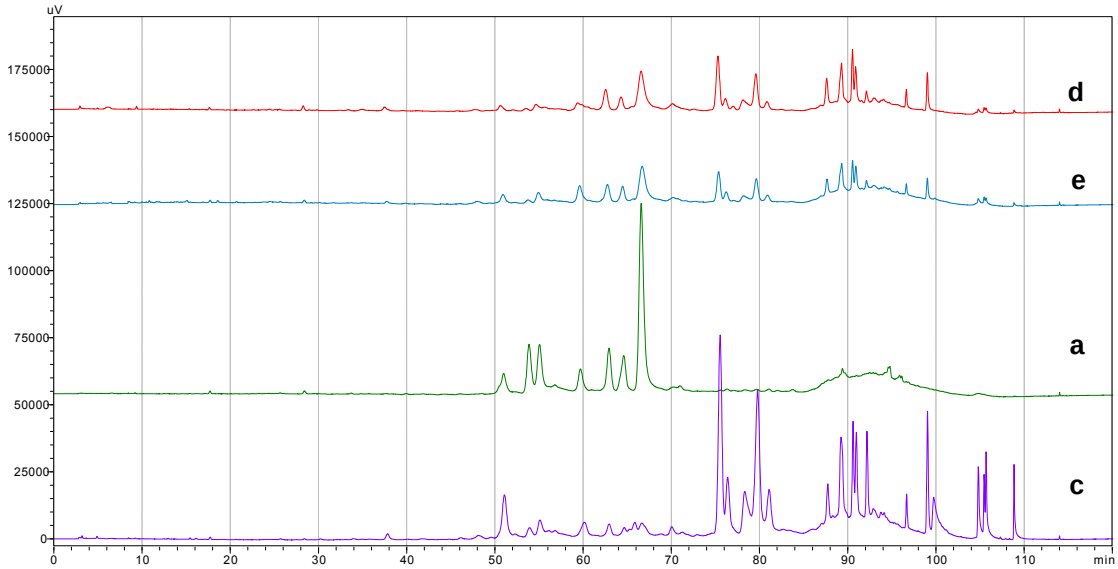


Fig. 26 HPLC analysis of the OWEs (chapter 2.2.1, p. 19) of samples **a**, **c**, **d**, and **e** on Aquasil® C 18 (5µm particles) applying the following linear gradient: 0→10% B within 20 min, 10→20% within 60 min, 20→40% B within 20 min, 40→95% B within 10 min at a flow rate of 1 mL/min and column oven temperature of 15°C; detected at 340 nm

For this, a method which has been developed for analysis of sample **c** [30] was adopted by varying the gradient and the column oven temperature (Fig. 27).

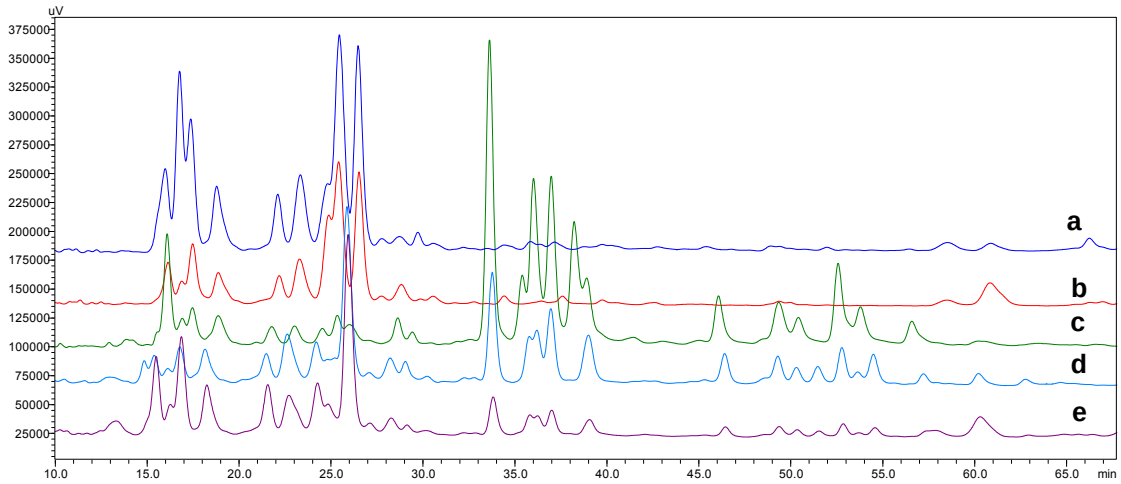


Fig. 27 HPLC analysis of the OWEs (chapter 2.2.1, p. 19) of samples **a**-**e** on Aquasil® C 18 (5µm particles) applying the following linear gradient: 15→23.75% B within 58 min, 23.75→29% within 15 min at a flow rate of 1 mL/min and a column oven temperature of 15°C; detected at 340 nm

This newly developed gradient is a compromise providing separation of most of the compounds, although with rather low resolution for some peak pairs, at acceptable retention times and is described in publication 3 [23] (chapter 3.2, pp. 87-112).

3.4.3 Determination of sugar moieties

GC-MS analyses after acid hydrolysis with Kiliani reagent (chapter 2.3.3. p. 25) led to the assessment of the absolute configuration of the monosaccharides which is described in detail in publication 2 (chapter 3.1. pp. 30-85). This technique had been applied successfully before for the determination of sugar moieties in saponins [32] and phenolic derivatives [33]. Fig. 28 shows the GC-MS analysis of the diastereomeric butylglycosidic residues of compound **4a**+**4b** in comparison to the identically treated reference substances rutoside and hyperoside (quercetin-3-O-galactoside) containing D-glucose, D-galactose, and L-rhamnose.

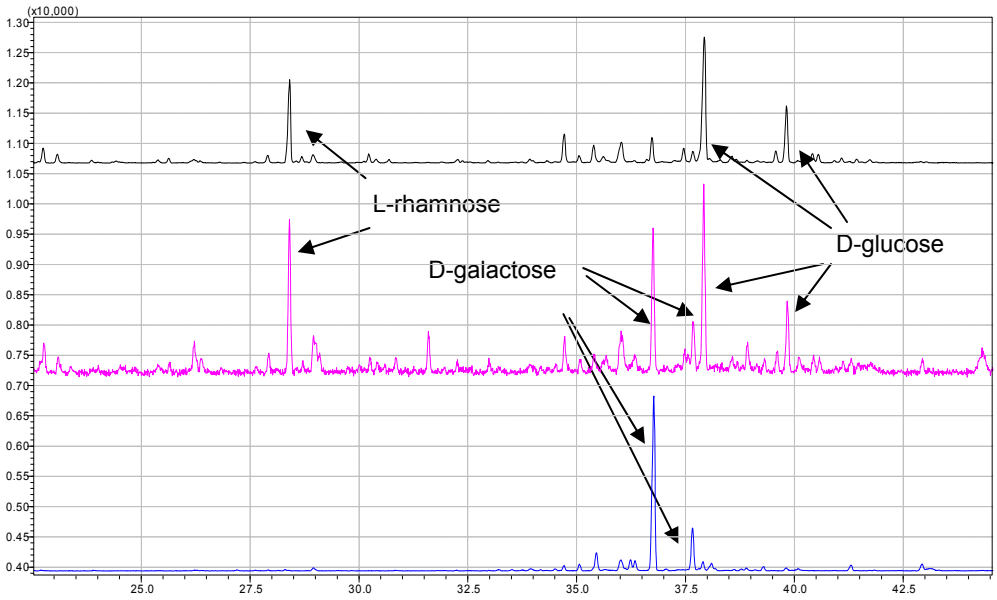


Fig. 28 GC-MS analysis of the diastereomeric butylglycosidic residues of compound **4a**+**4b** (pink curve) in comparison to the identically treated references rutoside (black curve) and hyperoside (blue curve) according to the method described in publication 2 [22] (chapter 3.1. p. 30-85)

Furthermore, the exact linkage of the sugar units within the disaccharide moiety of the isolated compounds was determined following the method described in chapter 2.3.3, p 25. According to the nomenclature of specific fragment series developed by Kochetkov and Chizov [34, 35] the intensities of the J, F/G, H, and K series were compared. The fragments show different molecular masses depending on their substituent which can either be an $–OCH_3$ or –OTMS residue, and the most intensive fragment in each of these series represents the substituent of a particular carbon atom [26]. Furthermore, after hydrolysis the equilibrium of the two anomeric forms of a monosaccharide is reached resulting in the appearance of peak pairs in the chromatogram. The ratio of the peak areas of the two corresponding peaks is characteristic e.g. the ratios for 2,3,4-tetramethyl-1,6-TMS-glucose and 2,3,4-tetramethyl-1,6-TMS-galactose are almost 1:1, whereas 2,3,4-Tetramethyl-1-TMS-rhamnose shows a main peak eluting prior to a very small second peak (ratio almost 90:10). Mixtures of the compounds **1**+**2**, **3a**+**3b** and **4a**+**4b** were analyzed using rutoside as a reference substance. Fig. 29, p. 149 (upper panel) shows the total ion current (TIC, black) and the extracted ion currents (EIC) m/z 88 (pink, $-OCH_3$ at C_2 and C_3), m/z 101 (blue, $-OCH_3$ at C_2 and C_4), and m/z 133 (brown, -OTMS at C_1 and $–OCH_3$ at C_3). The mass spectra of the derivatized monosaccharides appearing as peak pairs at 17.7 /17.9 min and 26.1/26.6 min are shown in Fig. 29 (middle and lower panel) and confirm the above mentioned fragments as the most abundant of the respective series of rhamnosyl-1→6-glucoside representing the glycosidic part of rutoside.

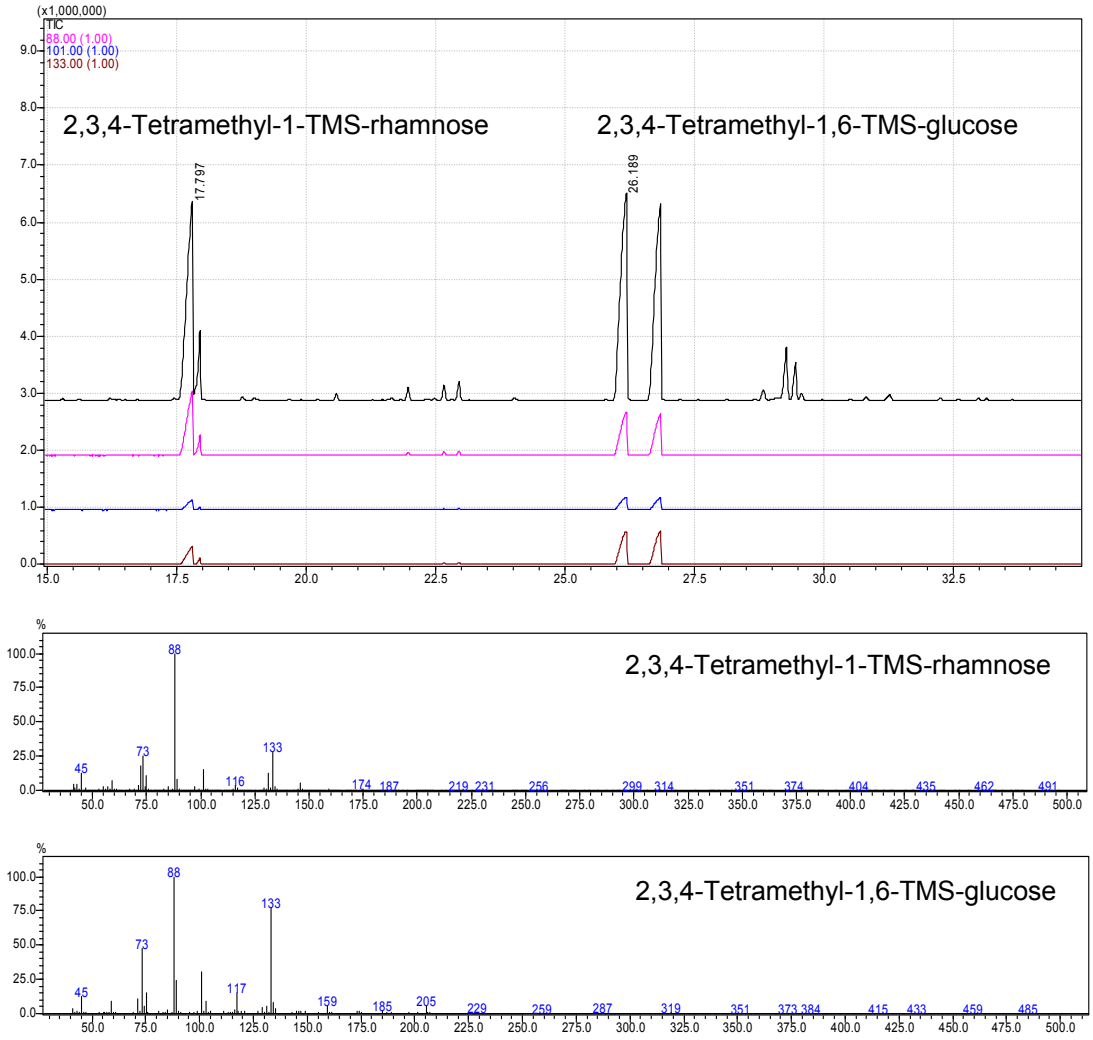


Fig. 29 GC-MS analysis of rutoside, treated according to the method described in chapter 2.3.3, p. 25 showing the TIC and EIC (upper panel) and the EI-MS spectra of the compounds eluting at 17.7 min (middle panel) and 26.1 min (lower panel)

The GC-MS analysis of the derivatized sugar units of compounds **1** and **2** is shown in Fig. 30. The chromatogram shows peak pairs at retention times identical to those of 2,3,4-tetramethyl-1-TMS-rhamnose (t_R=17.8/18.1 min) and 2,3,4-tetramethyl-1,6-TMS-glucose (t_R=26.1/26.6), obtained from the reference rutoside. The ratios of the peak areas of the two anomers are in accordance with literature [27] and suggest a 1→6 linkage for the rhamnosyl-glucoside of compound **1**. The peaks appearing at (t_R=22.7/23.1) are supposed to be 2,3,4-tetramethyl-1,6-TMS-galactose and suggest a 1→6 linkage for the rhamnosyl-galatoside in compound **2**.

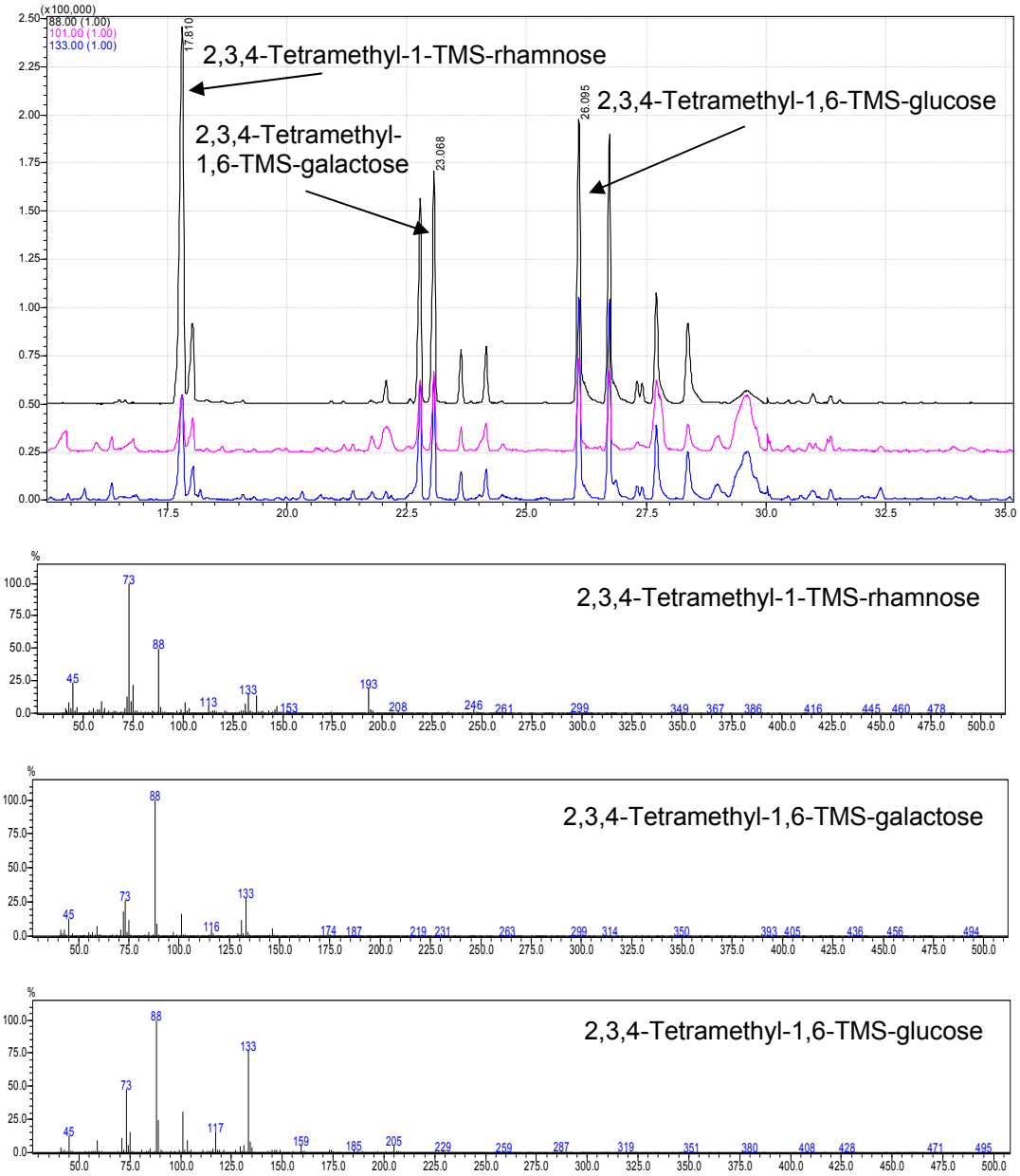


Fig. 30 GC-MS analysis of compounds **1**+**2**, treated according to the method described in chapter 2.3.3, p. 25 showing EIC (upper panel) and the EI-MS spectra of the compounds eluting at 17.8 min, 22.7 min, and 26.1 min (lower panels)

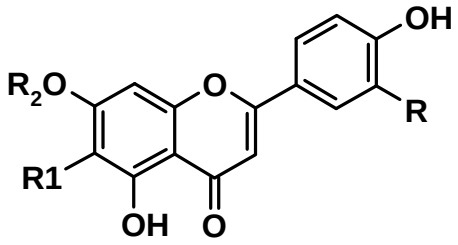
The mixtures of **3a**+**3b** and **4a**+**4b** showed identical patterns to those obtained for compounds **1**+**2**. The above described results were confirmed by HMBC analyses (publication 2 [22], chapter 3.1. pp. 30-85).

4 Discussion and conclusion

Dianthus versicolor is a plant used traditionally in Mongolian medicine against liver impairments. As traditional medicine has become more important since the last two decades in Mongolia, increasing scientific research is required to prove the efficacy of the therapeutic methods and the medicinal plants applied to patients. In cooperation between the HSUM, the Department of Pharmacognosy of the University of Vienna and the Medical University of Vienna the plant was investigated for its bile flow stimulating activities. An aqueous extract, specially prepared according to the traditional way of intake, was examined in the model of the isolated perfused rat liver in concentrations of 100, 250, and 500 mg/L. It caused an increase of the bile salt independent bile flow (hydrocholeresis) showing a slight dose dependency. The bile salt dependent bile flow remained unaffected. In order to locate the active principles the extract was subsequently fractionated by SPE and two of the resulting fractions, one enriched in flavonoids and one enriched in sugars were tested in the model. The flavonoid enriched fraction, tested in concentrations of 10, 20, and 40 mg/mL, increased the bile flow, which was comparable to the positive control cynarin, a dicaffeoyl-quinic-acid well known for choleretic activity [12, 36]. On the other hand, the sugar fraction did not show any effect. It is known that choleretic plant extracts, e.g. from yarrow and artichoke, are beneficial for the treatment of digestive problems related to a reduced function of liver and bile flow [37, 38, 39]. Therefore, our findings could explain, at least in part, the beneficial effects of this plant on patients treated with traditional preparations. Furthermore the flavonoids are suggested to contribute substantially to the effect on the bile flow. Indeed, flavonoids e.g. apigenin-7-O-glucoside, which is contained in choleretic plants e.g. *Achillea millefolium* s.l., *Cynara scolymus*, *Saussurea amara* have been shown to increase the bile flow in this model before [12, 39, 40, 41]. However, it has to be kept in mind that the applied test system is an ex vivo model and further in vivo studies are required to confirm these results.

Further phytochemical investigations were focussed on the flavonoids. The SPE derived 40% MeOH fraction was fractionated by CPC, and one of those fractions, purified by semipreparative HPLC led to the isolation of isoorientin-7-

O-rutinoside **(1)** and isoorientin-7-O-rhamnosyl-glactoside **(2)**. Furthermore, the OWE was fractionated via CC on Polyamide. Fractions thereof were subsequently fractionated on Sephadex LH-20 and finally purified by semipreparative HPLC. This resulted in the isolation of isovitexin-7-O-rutinoside **(3a)**, isovitexin-7-O-rhamnosyl-galactoside **(3b)**, isoscoparin-7-O-rutinoside **(4a)**, isoscoparin-7-O-rhamnosyl-galactoside **(4b)**, isoscoparin-7-O-galactoside **(5)**, isoorientin-7-O-galactoside **(6)**, and isovitexin-7-O-glucoside **(7)** (Fig. 31).



	R	R_1	R_2	Common name
1	OH	Glc	Rha(1→6)Glc	Isoorientin-7-O-rutinoside
2	OH	Glc	Rha(1→6)Gal	Isoorientin-7-O-rhamnosyl-galactoside
3a	H	Glc	Rha(1→6)Glc	Isovitexin-7-O-rutinoside
3b	H	Glc	Rha(1→6)Gal	Isovitexin-7-O-rhamnosyl-galactoside
4a	OCH_3	Glc	Rha(1→6)Glc	Isoscoparin-7-O-rutinoside
4b	OCH_3	Glc	Rha(1→6)Gal	Isoscoparin-7-O-rhamnosyl-galactoside
5	OCH_3	Glc	Gal	Isoscoparin-7-O-galactoside
6	OH	Glc	Gal	Isoorientin-7-O-galactoside
7	H	Glc	Glc	Isovitexin-7-O-glucoside (saponarin)

Fig. 31 Structures of the isolated compounds **1**-**7**; Glc: glucose, Gal: galactose, Rha: rhamnose

Their structures were matched multidimensionally on the basis of extensive spectroscopic and spectrometric investigations including LC-DAD, LC-MS^n, LC-HR-ESI-MS, 1D and 2D NMR, and by GC-MS analysis after hydrolysis. Especially the structure elucidation of **3a** and **3b**, as well as **4a** and **4b** was challenging, because the compounds were obtained as mixtures of the isobaric forms, differing only in the nature of the 7-O-attached hexose. The occurrence of two or three sugar moieties within one molecule and the relatively small sample amounts (1.5 - 4.5 mg) made the interpretation of the NMR spectra rather difficult. In order to support the identification of sugar moieties and the linkage of monosaccharides within the molecules GC-MS after hydrolysis of the permethylated flavonoid glycosides was employed. The method was applicable also to even less than 1 mg of compound. The isolated compounds are all C- and O-glycosides, which is rather rare within the genus *Dianthus*, according to

the available phytochemical literature [22]. Among the isolated compounds isovitexin-7-O-glucoside **(7)** and isovitexin-7-O-rutinoside **(3a)** are well known compounds and have been isolated before [42, 43]. Isoorientin-7-O-rutinoside **(1)** has also been mentioned in literature [44], but it is the first time, that full NMR data and the absolute configuration of the sugar moieties is presented. The other compounds are isolated and structurally elucidated for the first time. Furthermore the known flavonoid-glycosides isovitexin-2''-O-rhamnoside, isovitexin, apigenin-7-O-glucoside, luteolin-7-O-glucoside, apigenin, luteolin, chrysoeriol, diosmetin, and acacetin were identified [30]. None of those compounds has been described for *D. versicolor* so far.

In order to establish analytical methods, which could serve as tool for quality control of this traditionally used Mongolian drug, different samples of *D. versicolor* were compared. The samples were collected in different years and different locations in Mongolia. Aqueous extracts were prepared and analyzed by TLC and HPLC. All of them showed a qualitatively similar flavonoid fingerprint but revealed differences in the quantity of flavonoids. For the quantification of the flavonoid glycosides an HPLC-DAD method was developed and validated concerning linearity, accuracy, and precision using isovitexin-7-O-glucoside as external standard. This method allows the determination of the content of single compounds as well as the total flavonoid content and showed total flavonoid contents ranging from 0.41 % to 3.30 % for the analyzed samples. Samples **a** and **b** contained an exclusively high content of polar flavonoid-di- and -triglycosides. The HPLC method described above can be seen as compromise providing an acceptable separation within a reasonable time of analysis. In order to reach a better separation of the polar compounds, an alternative method was developed and validated using rutoside as internal standard. Showing good linearity, accuracy, and precision this method is suitable for the analysis of samples containing very polar compounds. Finally, a quite simple UV-spectrophotometric method, based on the European pharmacopoeia monograph of “Passiflorae herba”, was modified for the determination of the total flavonoid content of *D. versicolor*. The crude drug of sample **a**, the respective aqueous extract, and the flavonoid enriched fraction were compared, and correlation between the total flavonoid contents assessed

by HPLC and UV-spectrophotometry was found. Therefore, the methods described in this thesis can be regarded as basic methods for quality control of this traditional Mongolian drug.

The results obtained from the tests on isolated organ preparations (uterus, papillar muscle, arteria pulmonalis, aorta, and terminal ileum) must be seen as preliminary, although the observed uterus constringing potential seems to relate to the indications described by Ligaa [15]. However, flavonoids are not responsible for these findings, because they are known for their spasmolytic activity, and a lot of medicinal plants, e.g., yarrow, chamomile, are used for this beneficial effect [37]. Therefore, other polar ingredients must be the active principle and should be further investigated.

To conclude, this work provides first phytochemical investigations on the traditional Mongolian medicinal plant *D. versicolor*. With regard to the traditional use for the treatment of liver disorders the focus was put on the analysis of flavonoids, which contribute to the beneficial effect. The qualitative and quantitative analyses applying various chromatographic, spectrometric, and spectroscopic techniques allowed a comprehensive phytochemical characterization of this plant and led to the isolation and structural elucidation of new compounds. However, the flavonoids represent only one class of compounds contained in the complex mixture of this plant's extract. Therefore, further investigations are encouraged.

5 Summary

The aim of this thesis was the phytochemical investigation of the Mongolian medicinal plant *Dianthus versicolor* accompanied by the testing for its activity on the bile flow. It is used in traditional medicine for various indications, among them for the treatment of liver and gastrointestinal disorders.

An aqueous extract, prepared according to the traditional way of intake, was tested in the model of the isolated perfused rat liver in order to examine its influence on the bile flow. Different concentrations led to an increase of the bile flow showing a slight dose dependency. The extract was subsequently fractionated by solid phase extraction and one of the fractions, enriched in flavonoids showed an influence on the bile flow. This effect was comparable to the positive control cynarin, which is known for its cholerectic activity. Furthermore, the extract was examined on isolated organ preparations from the uterus, aorta, heart, arteria pulmonalis and terminal ileum and showed a uterus-constringing activity.

Further fractionation of the enriched flavonoid fraction by centrifugal partition chromatography or column chromatography and purification by semipreparative HPLC led to the isolation of nine flavonoid-C- and O-glycosides. Their structures were established on the basis of extensive spectroscopic and spectrometric investigations including LC-DAD, LC-MS^n, LC-HR-ESI-MS, 1D and 2D NMR, and by GC-MS analysis after hydrolysis. Seven of the isolated structures are new, and have not been described for *D. versicolor* so far.

For the quantification of the flavonoids different HPLC-DAD methods were established and validated using external or internal standards. As alternative a quite simple UV-spectrophotometric method was developed. It based on a monograph from the European Pharmacopoeia and was slightly modified, showing comparable data to those obtained from HPLC-DAD analysis.

The qualitative and quantitative analyses allowed a detailed phytochemical characterization of the flavonoids contained in the aqueous extract of this plant and led to the isolation and structural elucidation of new compounds.

6 Zusammenfassung

Das Ziel dieser Arbeit war die phytochemische Untersuchung der mongolischen Arzneipflanze *Dianthus versicolor*, die in der tradtionellen Medizin gegen Lebererkrankungen und gastrointestinale Beschwerden verwendet wird.
Unterschiedliche Konzentrationen eines wässrigen Extraktes führten im Modell der isolierten perfundierten Rattenleber zu einer Steigerung des Gallenflusses, was als Hinweis auf eine Stimulierung der Leber gesehen werden kann. In weiterer Folge wurde das Extrakt aktivitätsgeleitet fraktioniert, wobei eine flavonoidreiche Fraktion eine dem Cynarin vergleichbare choleretische Wirkung zeigte. Darüber hinaus wurde das wässrige Extrakt an verschiedenen glattmuskulären Organpräparaten getestet und wies in höherer Dosierung eine Uterus-kontrahierende Wirkung auf.

Eine weiterführende Fraktionierung der Flavonoid-Fraktion mittels CPC, CC und semipräparativer HPLC resultierte in der Isolierung von neun Flavon-C- und O-glykosiden. Die Strukturaufklärung dieser Verbindungen gelang unter Anwendung diverser spektroskopischer und spektrometrischer Verfahren, wie LC-DAD, LC-ESI-MS^n, LC-HR-ESI-MS, 1D und 2D-NMR sowie GC-MS nach Hydrolyse. Sieben der isolierten Verbindungen sind neue Strukturen und für *D. versicolor* noch nicht beschrieben. Für die Analytik der Flavonoide wurden geeignete HPLC-Systeme ausgearbeitet, die die Quantifizierung mit internem oder externem Standard erlauben. Als Alternative zur Quantifizierung mittels HPLC wurde eine UV-spektrophotometrische Methode, basierend auf der Monographie „Passionsblumenkraut“ der Pharmacopoeia Europea, erstellt, welche vergleichbare Ergebnisse liefert.

Die im Rahmen dieser Arbeit durchgeführten qualitativen und quantitativen Analysen tragen wesentlich zur Chrarakterisierung der in *D. versicolor* enthaltenen Flavonoid-Glykoside bei. Darüber hinaus gelang die Isolierung und Strukturaufklärung neuer Substanzen.

7 References

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Additional references cited in the publications in section 3 can be found within the respective manuscripts.

8 Curriculum vitae & List of publications

Name: Astrid Erika Obmann
Date of birth: 14th April 1981
City of birth: Klagenfurt, Austria
Nationality: Austria

Education:

Since 10/2005	Doctoral study of Natural Sciences at the Department of Pharmacognosy, University of Vienna
25.5.2005	Graduation: „Fachprüfung für den Apothekerberuf“
7/2004	Graduation: “Magistra der Pharmazie (Mag. pharm.)”
10/1999-04/2004	Diploma Study of Pharmacy at the University of Vienna
17.6.1999	Graduation:„Matura“
1991-1999	Secondary school: „BG und BRG Völkermarkterring“, Klagenfurt
1987-1991	Elementary school: “Volksschule der Ursulinen”, Klagenfurt

Work experience:

Since 9/2010	AGES PharmMed (Institute Marketing Authorisation & Lifecyle Management)
Since 10/2008	“Lehrbeauftragte” at the Department of Pharmacognosy, Vienna: - Gewinnung und instrumentelle Analytik (PR) - Pharmazeutische Qualität biogener Arzneimittel (PR) - Allgemeine Mikrobiologie und Hygiene (PR) - Botanische Exkursionen für Pharmazeuten (EX)
19.7.2006-1.10.2008	Scientific assistant („Assistentin in Ausbildung“) at the Department of Pharmacognosy, Vienna
6.6.2005-31.5.2006	Employed pharmacist (16-24h/week), Apotheke „Zum Hl. Josef“, 1020 Vienna
1.6.2004-31.5.2005	“Aspirantenjahr”, St. Georg Apotheke, 1220 Vienna

Further teaching at the University of Vienna:

WS 2001/02, WS 2002/03	„Tutorin“: Übungen aus Hygiene u. Mikrobiologie
WS 2005/06, SS 2006	„Tutorin“: Übungen „Pharmazeutische Qualität biogener Arzneimittel“ and „Gewinnung und instrumentelle Analytik“
WS 2008/09-WS 2010/11	„Lehrbeauftragte“: Universitätslehrgang „Pharmazeutisches Qualitätsmanagement“ (Mikrobiologie)

Publications in peer reviewed journals:

Astrid Obmann, Damba Tsendayush, Theresia Thalhammer, Martin Zehl, Thanh Phuong Nha Vo, Sodnomtseren Purevsuren, Samdan Narantuya, Christa Kletter, Sabine Glasl
Extracts from the Mongolian Traditional Medicinal Plants *Dianthus versicolor* FISCH. and *Lilium pumilum* DELILE Stimulate Bile Flow in an Isolated Perfused Rat Liver Model
Journal of Ethnopharmacology 2010, 131 (5), 555-561

Astrid Obmann, Ingrid Werner, Armin Presser, Martin Zehl, Sodnomtseren Purevsuren, Christa Kletter, Sabine Glasl
Flavonoid C- and O-glycosides from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH.
Chemical & Pharmaceutical Bulletin, 2010, in preparation

Astrid Obmann, Zita Swoboda, Martin Zehl, Sodnomtseren Purevsuren, Christa Kletter, Sabine Glasl
Quantification of flavonoid glycosides from the traditional Mongolian medicinal plant *Dianthus versicolor* FISCH.
Journal of separation science, accepted for publication on 17th November 2010

Astrid Obmann, Sodnomtseren Purevsuren, Martin Zehl, Christa Kletter, Sabine Glasl
HPLC Determination of flavonoid glycosides in Mongolian *Dianthus versicolor* FISCH. (Caryophyllaceae) compared to quantification by UV-spectrophotometry
Phytochemical Analysis, 2010, in preparation

Short lectures:

Obmann A.
***Dianthus versicolor* - Phytochemical Investigations**
Mongolia 800th Anniversary Symposium, 4th April 2006, Vienna (Austria)

Obmann A., Kletter C., Thalhammer T., Glasl S.
***Dianthus versicolor* – Phytochemische Untersuchungen einer mongolischen Arzneipflanze**
Young Researcher Meeting, 2007, March 30-31, Münster (Germany)

Obmann A., Thalhammer T., Kletter C., Glasl S.
Flavonoid glycosides in the Mongolian Medicinal plant *Dianthus versicolor* FISCH. and their effect on bile flow
PSE Young Scientists Meeting, 2008, March 26-29, Bad Herrenalb (Germany)

Astrid Obmann, Amarsanaa Badgaa, Christa Kletter, Theresia Thalhammer, Peter Eckl, Sabine Glasl
Activity of plants used in Traditional Mongolian Medicine
Plants from High Altitude – Phytochemistry and Bioactivity, 2009 April 26-29, Obergurgl (Austria)

Posterpresentations:

Obmann A., Reither Ch., Reznicek G.
Analytik der Flavonoide und Phenolcarbonsäuren aus *Thymus vulgaris*
19. Wissenschaftliche Tagung der ÖPhG, 2006, April 20-22, Innsbruck (Austria)

Obmann A., Radovic T., Kletter C., Glasl S.
HPLC-MS-Analysis of Flavonoid-C-Glycosides in the Mongolian Medicinal Plant *Dianthus versicolor*
55th Annual Meeting and International Congress of the Society for Medicinal Plant Research, 2007, Sept. 2-6, Graz (Austria)

A. Obmann, B. Weisz-Pecher, P. Rawnduzi, Ch. Kletter, R. Lemmens-Gruber, S. Glasl
Uterus activity of the Mongolian medicinal plant *Dianthus versicolor* FISCH.
21. Wissenschaftliche Tagung der ÖPHG, 2009, April 16-18, Vienna (Austria)

Obmann A, Presser A, Kletter Ch, Thalhammer T, Glasl S
Phytochemical Analysis and Biological Activity of the Flavonoids from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH.
57th International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 2009, August 16-20, Geneva (Switzerland)

Obmann A, Mraz B, Kubasa B, Zehl M, Kletter Ch, Glasl S
Phytochemical profiling of the Mongolian medicinal plant *Myricaria longifolia* EHRENB.
58th International Congress & Annual Meeting of the Society for Medicinal Plant and Natural Product Research, 2010 August 29 - September 2, Berlin (Germany)

Appendix

Department of Pharmacognosy universität wien

Analytik der Flavonoide und Phenolcarbonsäuren aus *Thymus vulgaris*

Astrid Obmann, Charlotte Reither, Gottfried Reznicek

Department für Pharmakognosie der Universität Wien,
Althanstrasse 14, A-1090 Wien, Österreich

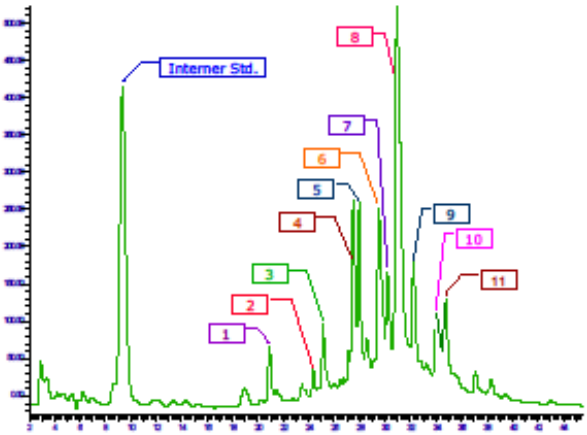
Einleitung

Thymus vulgaris und daraus hergestellte Extrakte werden vor allem zur Behandlung von Atemwegserkrankungen (Bronchitis, Keuchhusten und Katarrhe der oberen Luftwege verwendet. Zur Beurteilung der Qualität der Arzneidroge wird im Europäischen Arzneibuch nur das ätherische Öl herangezogen, das expektorierende und antimikrobielle Wirkung zeigt [1]. Die ebenfalls enthaltenen nichtflüchtigen phenolischen Inhaltsstoffe (Flavonoide, Phenolcarbonsäuren) können wesentlich für die bronchospasmolytische [2] als auch antiphlogistische Wirksamkeit [3] verantwortlich gemacht werden, weshalb eine entsprechende Analytik dieser Stoffgruppen zur umfassenden Qualitätsbeurteilung der Arzneidroge und daraus hergestellten Extrakten notwendig ist.

Ergebnisse und Diskussion

Zur Ausarbeitung einer HPLC-Methode wurde ein **Thymiantrockenextrakt**, Chargennummer 98765 (2396/98) von Bionorica AG (Neumarkt, BRD) zur Verfügung gestellt. Dieser wurde im Ultraschallbad in wässriger Essigsäure (pH 4) gelöst (10 mg/ml), zentrifugiert und der Überstand für die HPLC-Analyse verwendet. Nach Versuchen an zahlreichen stationären Phasen (Kieselgel RP-8, RP-18, Aquasil C18, Polyethylenglykol (PEG), verschiedene Polar RP-Phasen) und entsprechenden Fließmitteln konnten an **Phenomenex Luna Phenyl-hexyl** mit Acetonitril/Methanol/wässrige Essigsäure (pH 4) als mobile Phase eine signifikant bessere Trennung der **Flavonoide und Phenolcarbonsäuren** und ausgezeichnete Peaksymmetrien erreicht werden [4, 5].

Peak	R_t (min)	Bezeichnung	% im Extrakt
1	27,8	Vicenin-2	1,8
2	30,9	Eriodictyol-7-O-galaktosid	0,7
3	34,1	6-OH-Luteolin-7-O-glucuronid	2,7
4	34,7	Rosmarinsäure-3'-O-glucosid	5,4
5	35,6	Luteolin-7-O-glucosid	5,4
6	37,5	Luteolin-7-O-glucuronid	8,4
7	38,5	Eriodictyol-7-O-glucuronid	2,9
8	39,1	Rosmarinsäure	15,4
9	42,6	Apigenin-7-O-glucuronid	2,6
10	44,4	Phenolcarbonsäure	2,1
11	46,5	Phenolcarbonsäure	3,2



Die einzelnen Peaks konnten mittels UV-Absorption bei 270 nm und 330 nm, **UV-Spektren, Massenspektrometrie, Cochromatographie** mit authentischen **Vergleichssubstanzen** bzw. **Isolierung** und **NMR-Spektroskopie** eindeutig zugeordnet werden [4 - 7]. Die Quantifizierung erfolgte mittels **internem Standard Homoprotocatechusäure** (3,4-Dihydroxyphenylessigsäure), wobei alle Flavonoide als Luteolin-7-O-glucosid und alle Phenolcarbonsäuren als Rosmarinsäure berechnet wurden (Tab. 1). Die relative Standardabweichung bei der Quantifizierung der einzelnen Komponenten betrug etwa 8%.

Experimentelles

Gerät: Perkin-Elmer series 200 LC pump, PE series 200 autosampler, PE series 200 DAD, PE series 200 column oven, PE series 200 vakuum degasser
Steuer- und Auswertesoftware: Perkin-Elmer Turbochrom Navigator 6.1.2.0.1
Stationäre Phase: Phenomenex Luna Phenyl-hexyl, 5 µm, 2x250 mm **Ofentemperatur:** 25°C
Mobile Phase: Zusammengesetzt linearer Gradient
LM A: Acetonitril / Methanol (80+20) + Eisessig (1,6 ml/l)
LM B: Wässrige Essigsäure (pH 4)
Fluss: 0,18 ml/min **Detektion:** UV 270 nm **Injektionsvolumen:** 10 µl Extraktlösung

Zeit (min.)	LM A (%)	LM B (%)
0	10	90
20	33	67
15	33	67
16	80	20
1	100	0

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19. Wissenschaftliche Tagung der Österreichischen Pharmazeutischen Gesellschaft, 20. - 22. April 2006, Innsbruck, Österreich

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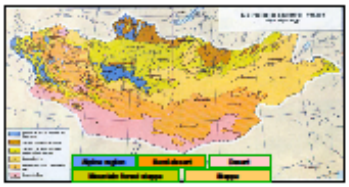
HPLC-MS-Analysis of Flavonoid-C-Glycosides in the Mongolian Medicinal Plant *Dianthus versicolor*

Obmann A., Radovic T., Kletter C., Glasl S.

Department of Pharmacognosy University of Vienna, Althanstraße 14, 1090 Vienna, Austria



Introduction:

Dianthus versicolor FISCH. is used in Traditional Mongolian Medicine against liver diseases together with other Mongolian plants. Until now chrysoeriol-C-glycosides were found in the plant [1]. Our first investigations of an aqueous extract (OWE) by TLC indicated the occurrence of further flavonoids. The aim of our work is to identify known flavonoid-C-glycosides and to elucidate new structures using HPLC-MS among other analytical techniques.

Experimental:

The plant material was collected in Hubsgul aimag (Mongolia) and dried, for phytochemical investigations only aerial parts of the plant were used. For preparation of the OWE the plant material was powdered and extracted with water (pH 2, trifluoroacetic acid) at 40°C for 1 h by shaking gently. To purify the extract and concentrate the flavonoids Solid Phase Extraction (SPE) was used as appropriate method using RP18 cartridges and methanol-water mixtures of different polarity. The 40% MeOH-fraction increased the bile flow in the perfused isolated rat liver [2]. HPLC-analyses were performed on a Shimadzu liquid chromatograph (LC-20 AD). HPLC-ESI-MS experiments were carried out with a Perkin Elmer liquid chromatograph (Series 200) coupled to a PE Sciex API 150 EX single quadrupole instrument.

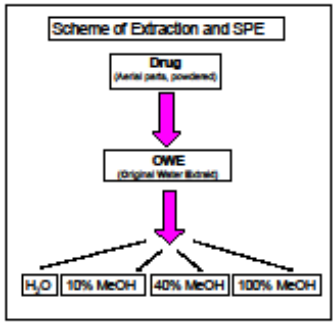
Parameters:

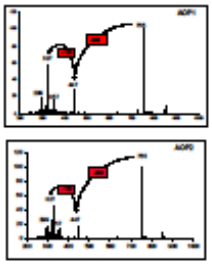
HPLC:

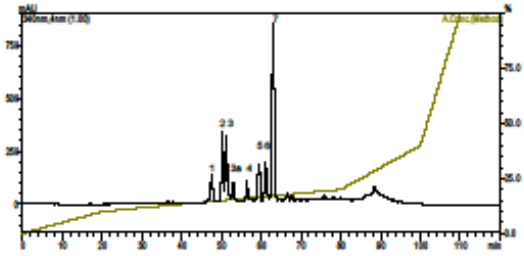
Stationary phase: Aquasil C_{18}
Mobile phase: Solvent A ACN
Solvent B H2O (pH 2.8 TFA)
Gradient: 0-20 min 0% A → 10% A
20-60 min 10% A → 20% A
60-100 min 20% A → 40% A
100-110 min 40% A → 100% A
110-120 min 100% A
Flow: 1 ml/min
Detection: UV-DAD

ESI-MS:
PE Sciex API 150 EX
Scan mode: negative, 150-1000 amu / 2 sec

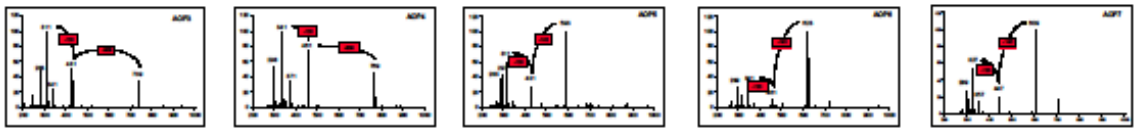
Nebulizer gas (NEB)	12.0
Curtain gas (CUR)	8.0
Ion spray voltage (IS)	-4500
Temperature (TEM)	400
Declustering potential (DP)	-150
Focusing potential (FP)	-300
Entrance potential (EP)	-10





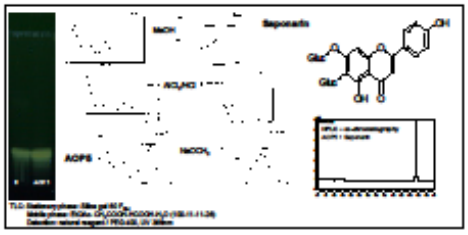


Peak	Quasi molecular ion [M-H]⁻	Fragments (m/z)
1 (AOP7)	609	447; 357; 327; 313; 299; 285
2 (AOP1)	755	447; 357; 327; 313; 299; 285
3 (AOP2)	755	447; 357; 327; 313; 299; 285
4 (AOP5)	593	431; 311; 297; 283
5 (AOP3)	739	431; 341; 311; 283
6 (AOP6)	623	461; 341; 312; 298; 269
7 (AOP4)	769	461; 371; 341; 298



Identification of AOP5:

According to retention time, mass- and UV-spectra AOP5 was supposed to be apigenin-6,7-*O*-diglucoside (saponarin). To confirm this hypothesis a small amount of this compound was isolated by semipreparative HPLC and compared to pure saponarin by TLC and UV-experiments using shift reagents [3]. The isolated substance showed the same R_f-value and fluorescence colour after detection with natural reagent A at UV 366nm. The reaction to UV-shift reagents was identical with the pure substance, therefore the compound was identified as apigenin-6,7-*O*-diglucoside.



Conclusion:

Aquasil C_{18} provides a good separation of the main flavonoids occurring in *Dianthus versicolor*. ESI is a soft ionisation method and allows the determination of the molecular masses. Our experiments indicate that the flavonoids of *Dianthus versicolor* are triglycosides (AOP1, AOP2, AOP3 and AOP4) and diglycosides (AOP5, AOP6 and AOP7).This hypothesis is confirmed by the fragmentation patterns showing the fragments [M-H-308]⁻ or [M-H-162]⁻ which indicate the separation of either a disaccharid (hexose+desoxyhexose) or a hexose. The fragments m/z 447 (isoorientin, orientin), m/z 431(isovitexin, vitexin) and m/z 461(chrysoeriol, diosmetin) represent the corresponding C-monoglycosides. The fragment [M-H-120]⁻ is characteristic for flavonoid-C-glycosides [4]. AOP5 was identified as apigenin-6,7-*O*-diglucoside.

References: [1] Bogustavskaya, L.I. et al. (1983) Khim. Prir. Soedin., 6: 783-4;
[2] Glasl S., Tsendayush D. et al. (2007) Planta Med. 73: S9-86
[3] Radovic T. , 2007, diploma thesis, University of Vienna, in preparation
[4] Pemp, E., 2005, PhD thesis, University of Vienna

55th Annual Meeting and International Congress of the Society for Medicinal Plant Research
Sept 2 - 6, 2007 Graz, Austria

universität wien

Department of Pharmacognosy

Uterus activity of the Mongolian medicinal plant *Dianthus versicolor* FISCH.

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Introduction:

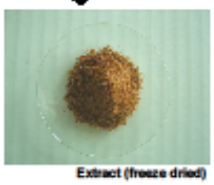
Dianthus versicolor FISCH. is used in Mongolia as uterus constricting agent. In folk medicine it is recommended after birth in order to remove the rest of the placenta and during lochia [1]. Aqueous preparations of the aerial parts are applied successfully in humans and animals. According to traditional prescriptions an aqueous extract was prepared and tested on isolated preparations of the guinea pig.

Dianthus versicolor

Drug (packed)

Drug (aerial parts)

Drug (pulverized)



Extract (freeze dried)

Experimental:

For the preparation of the extract 100 g of the dried aerial parts of *Dianthus versicolor* were pulverized and extracted with 2500 mL of water (pH2, trifluoroacetic acid) for 1h at 40°C by shaking gently to simulate gastric conditions. After removing the volatile acid under vaccum the solution was freeze dried and yielded 21,3 g aqueous extract (DER: ~ 5:1).

Force of contraction (f_c) and spontaneous frequency of contraction (f) were recorded in different isolated preparations (uterus, arteria pulmonalis, aorta, papillary muscle, right atrium and terminal ileum). The measurements were performed isometrically in Krebs-Henseleit Solution (37°C) at three different concentrations (1 mg/mL, 3 mg/mL, 9 mg/mL). An appropriate resting tension was applied to allow maximal contractility of the preparations. After a control period the extract was added cumulatively every 45 min after steady-state had been reached. [2]

Results:

Fig. 1: *Original recording, isolated myometrial strip*

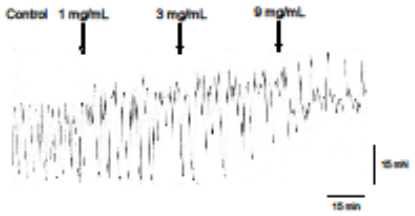


Fig. 2: *Effect of the extract on: f_c (green bars), resting tension (green striped bars) and f (red bars); n=4*

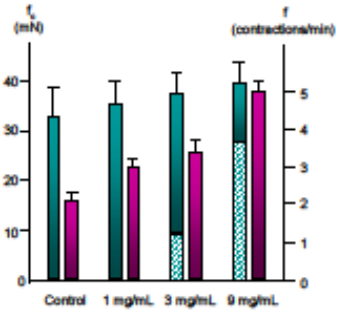
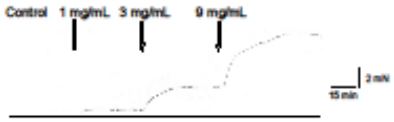


Fig. 3: *Original recording, isolated ring of the arteria pulmonalis*



Conclusion:

The aqueous extract of *Dianthus versicolor* concentration-dependently increased f_c and f of the uterus (Fig.1 and Fig.2). At 9 mg/mL even the resting tension was enhanced markedly (Fig. 1 and Fig. 2). In contrast, effects on other smooth muscle preparations were negligible except the arteria pulmonalis, which showed a remarkable increase in f_c at the highest concentration tested (Fig.3). Summing up, the contractile effect of *D. versicolor* on the uterus was confirmed. Higher doses of the aqueous extract might additionally increase the contractility of the arteria pulmonalis. Therefore, the proper dosage plays an important role.

References: [1] Boldsaikhan B. Encyclopedia of Mongolian medicinal plants. Vol. 1, 2nd ed., Mongolian University of Science & Technology, System Science Research Institute, Ulaanbaatar, 2004.
[2] Weisz-Pecher B. (2009) Untersuchung der Wirkung eines Extraktes aus *Dianthus versicolor* auf die Kontraktilität des Uterus sowie Herz und glattmuskulären Organen, Diplomarbeit, Universität Wien.

Presented at the 21st Scientific Congress of the Austrian Pharmaceutical Society
April 16th to 18th 2009, Vienna, Austria

Phytochemical Analysis and Biological Activity of the Flavonoids from the Mongolian Medicinal Plant *Dianthus versicolor* FISCH.

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Map of Mongolia indicating the origin of *Dianthus versicolor* 53/04 and 02/05 samples

Dianthus versicolor

Introduction:

Dianthus versicolor is used in Traditional Mongolian Medicine against various liver diseases and gastrointestinal disorders together with other plants. Until now triterpenoid saponins and chrysoeriol-C-glycosides are described in the plant [1,2]. Aqueous (OWE), methanolic extracts and fractions obtained by solid phase extraction (SPE) were tested in the isolated perfused rat liver in order to check the influence of this plant on bile flow and to verify its beneficial effect in traditional use.

To find active principles the plant was investigated phytochemically and six flavonoids were isolated and structurally elucidated, three more were characterized by HPLC-MS/MS. Additionally, the total flavonoid content was determined by a spectrophotometric method comparing samples of two different origins.

Experimental:

Preparation of extracts and fractions:

Aerial parts were powdered and extracted with water (pH 2, TFA) to simulate the traditional way of intake where the crude drug is taken together with a certain amount of water. The resulting extract was fractionated by SPE (C_{18} cartridges) yielding H_2O, 10% MeOH, 40% MeOH, 100% MeOH fractions (Fig. 1).

Isolated perfused rat liver:

Liver perfusion experiments were carried out using techniques described before [3] according to the protocol approved by the committee of the institution. Extracts and fractions (H_2O, 40% MeOH) were tested in three different concentrations (Fig. 2, n=3).

Isolation and structure elucidation:

Flavonoids were isolated from the 40% MeOH fraction using CPC, CC (Polyamide, Sephadex LH-20) and semipreparative HPLC. Structures were elucidated using different spectroscopic (UV, 1D and 2D NMR) and spectrometric (LC-MS/MS) techniques. By GC-MS the monosaccharides glucose, rhamnose and galactose were found to be attached to the C-monoglycosides isoscoparin, isoorientin and isovitexin (Fig. 3).

Quantification of flavonoids:

Known compounds were quantified by HPLC on Aquasil C_{18} using a MeCN/H_2O (pH 2.8, TFA) gradient and quercetin-3-O-rutinoside as internal standard (Fig. 3). For determination of the total flavonoid content a spectrophotometric method was established following the European Pharmacopoeia [4]

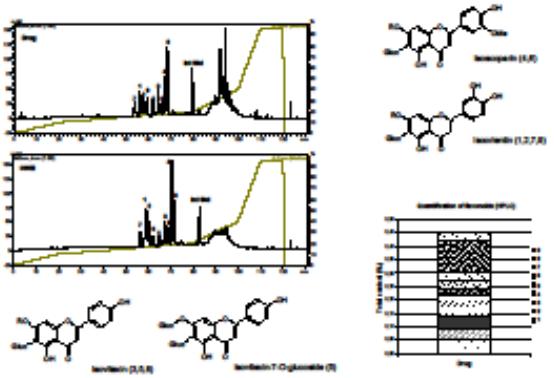

Fig. 3 Quantification of flavonoids by HPLC and structures (R = sugars)

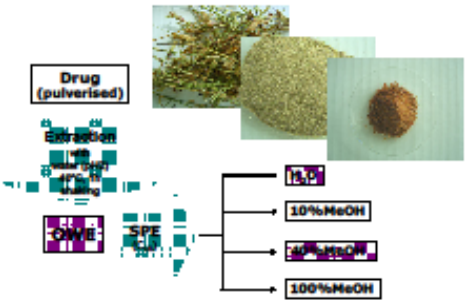

Fig. 1 Preparation of OWE and fractions

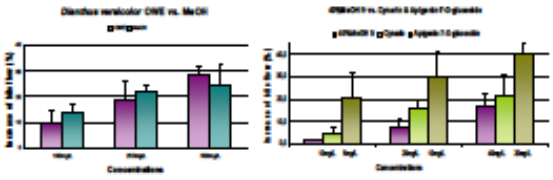

Fig. 2 Isolated perfused rat liver

Results:

Aqueous and methanolic extracts from *Dianthus versicolor* show a dose dependent influence on bile flow. The choleretic effect of a flavonoid enriched fraction applied in higher concentrations is comparable to cynarin. From this fraction six flavonoids were isolated and structurally elucidated, three more were characterised by HPLC-MS/MS and UV. The total flavonoid content in samples of two different origins was determined by UV-spectrophotometry. The crude drugs contained 0.75% and 1.19% flavonoids, whereas the OWEs showed amounts of 1.78% and 3.59%, respectively. In summary flavonoids seem to contribute to the choleretic effect of *Dianthus versicolor* and confirm the traditional use in Mongolia against hepatobiliary diseases.

References: [1] Bogusławskaya, L.I. et al. (1983) Khim. Prir. Soedin., 6: 703-4. [2] Ma, L. et al. (2009) J. Nat. Prod., doi:10.1021/np800598e. [3] Glasl S., Thendayath D. et al. (2007) Planta Med. 73: S9-66. [4] European Pharmacopoeia, 6th edition, Strasbourg: EDQM; 2008:CD-ROM

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August 16th to 20th 2009, Geneva, Switzerland

universität wien

Department of Pharmacognosy

Phytochemical profiling of the Mongolian medicinal plant *Myricaria longifolia* Ehrenb.

Myricaria longifolia

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Introduction

Myricaria longifolia Ehrenb. (Tamaricaceae) is used in traditional Mongolian medicine to heal fever, poisoning [1, 2], and liver diseases. It is an ingredient of various prescriptions consisting of several herbal components. Aqueous extracts have been shown to inhibit the growth of liver carcinoma cells (HepG2), breast cancer cells (MCF-7) [3], and primary rat hepatocytes [4]. The same extracts caused damage of the isolated liver during perfusion experiments [5]. In order to find out more about the active ingredients the plant extracts were investigated phytochemically employing TLC, LC-DAD-MS^n and NMR-analysis.

Experimental

Two extracts were prepared using either selectively the dried leaves (aqueous leaf extract, ALE) or all aerial parts (aqueous extract, AE) of *M. longifolia*. The powdered plant material was extracted with H_2O (pH 2, TFA) in order to simulate the traditional way of intake. Furthermore, the ALE was fractionated by CC using Sephadex LH-20 as stationary phase and a MeOH/H_2O gradient as mobile phase. TLC was carried out on silica gel as stationary phase using a mobile phase optimized for the separation of flavonoid-glycosides. LC-DAD-MS analyses of extracts and fractions were performed on a Dionex UltiMate 3000 RSLC-series system coupled to a Bruker Daltonics HCT ESI-IT. NMR experiments were conducted on a Varian Unity Inova 400 in DMSO-d_6.

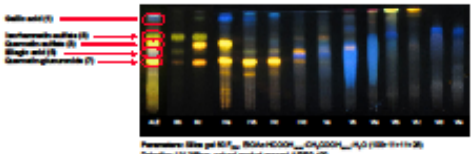

Fig. 1 TLC analysis of the aqueous leaf extract (ALE) and selected fractions obtained by CC

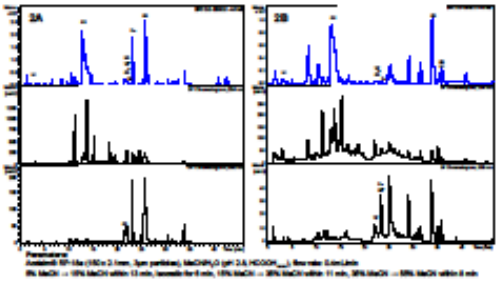

Fig. 2 LC-DAD-MS analysis of the ALE (3A) and the AE (2B), peak assignment according to Table 1

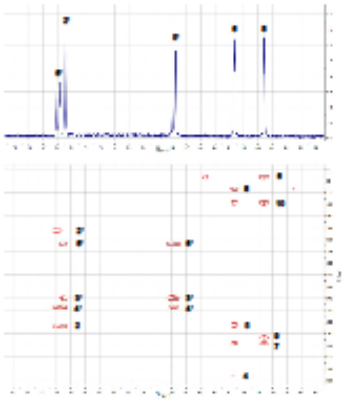

Fig. 4 ^{1}H and HMBC spectra of quercetin-3-O-sulfate recorded in DMSO-d_6, resonances are numbered as given in the formula (Fig 5., 3)

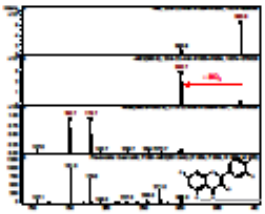

Fig. 3 MS^{1+} spectra of quercetin-3-O-sulfate

Compound	Rt [min]	[M-H]	Proposed structure
1	3.0	169	Gallic acid
2	12.5–14.0	377	Dimethylgallic acid-sulfate
3	16.8–20.0	381	Quercetin-3-O-sulfate
4	18.0–22.0	381	Ellagic acid-sulfate
5	22.1	301	Ellagic acid
6	23.0	609	Rutin
7	23.1	477	Quercetin-glucuronide
8	23.0–28.0	395	Isorhamnetin-sulfate
9	30.7	397	Rhamnetin-sulfate
10	36.0	315	Rhamnetin

Table 1 Phenolic compounds of *M. longifolia*

Results and Conclusion

The phytochemical screening of aqueous *M. longifolia* extracts allowed the identification of gallic acid, ellagic acid, rhamnetin, and rutin by comparison with reference substances. Additionally, LC-MS^n-analyses suggested the presence of rhamnetin-, isorhamnetin-, quercetin-, ellagic acid-, and dimethylgallic acid-sulfates and quercetin-glucuronide. Quercetin-3-O-sulfate was isolated by semipreparative HPLC. Its structure was confirmed by 1D and 2D NMR experiments, the results were in accordance with literature data [6, 7]. However, the active principles of *M. longifolia* responsible for the cytotoxic activity on various cell lines have not been discovered so far. Therefore, further investigations are required.

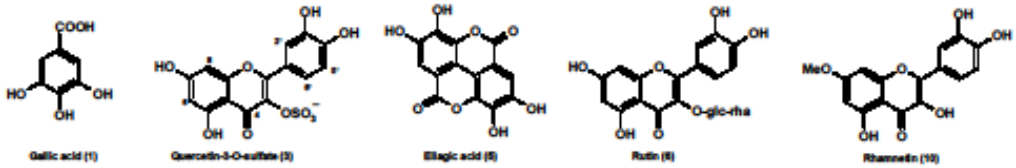

Fig. 5 Structures of identified compounds

References: [1] Baldakhan B. (2004) Encyclopedia of Mongolian medicinal plants. Ulaanbaatar. [2] Ligaa, U., Davaasuren, B., Ninjil, N. (2005) Medicinal plants of Mongolia used in western and eastern medicine. Ulaanbaatar. [3] Holec, N. (2005) Diploma thesis. University of Vienna. [4] Vogl, C. et al. (2009) Sci. Pharm. 77: 265. [5] Kletter, Ch. et al. (2008) Sci. Pharm. 76: 49-63. [6] Barron, D. et al. (1988) Phytochemistry 25, 7, 1719-1721 [7] Jones, D.J.L. et al. (2005) Bioorganic and Medicinal Chemistry 13, 6727-6731

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August 29th to September 2nd 2010, Berlin, Germany