

**Tímea Bencsik Pharm D**

**Comparative Histological, Phytochemical, Microbiological, and  
Pharmacological Characterization of Some *Lythrum salicaria* L. Populations**

Ph.D. thesis

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## I. Introduction

*Lythrum salicaria* L. (purple loosestrife) is native to Europe and Asia, but nowadays it is also widely distributed in North America, Northwest Africa, and Northeastern Australia. It was a well-known and frequently used medicinal plant already in the ancient times. Extracts of the dried aerial parts of this plant were traditionally used in the treatment of diarrhoea, chronic intestinal catarrh, stomach aches, haemorrhoids, eczema, varicose veins, and bleeding gums. Nowadays, *Lythri herba*, the flowering aerial part of this plant, is an official drug in the Hungarian Pharmacopoeia (Ph. Hg. VIII.). Today, its application is less important but even more and more *in vitro* and *in vivo* pharmacological investigations are carried out to prove the ethnopharmacological observations (e.g., antidiarrhoeal or haemostyptic effects) and discover new therapeutic purposes (e.g., against hypercholesterolemia or atherosclerosis). Recently, *L. salicaria* has been reported to have astringent, antidiarrhoeal, hypoglycemic, antioxidant, anti-inflammatory, antinociceptive, antifungal, antibacterial, and calcium antagonistic properties, which are attributed to the major groups of its compounds, i.e. tannins and flavonoids. *L. salicaria* extracts also showed inhibitory effect on human acyl-CoA-cholesterol acyltransferase *in vitro* (due to their triterpene components) suggesting that they might be effective in the prevention and treatment of hypercholesterolemia or atherosclerosis. Additionally, its polysaccharide-polyphenolic conjugates showed antitussive and bronchodilatory effects on guinea-pig.

*L. salicaria* is a traditionally used medicinal plant, and many of its effects have been confirmed by recent investigations. To find and investigate new medicinal plants and to prove the efficacy of ancient medicinal plant taxa are important tasks recently. Most of the active substances of drugs are derived from natural sources. The former and new challenges, e.g. the antibiotic resistance, have necessitated a continued search for new active compounds.

## II. Aims

According to the previously mentioned directions of recent researches on medicinal plants, the specific aims of this study were:

- to collect and investigate some Hungarian populations of *L. salicaria* living at different areas (drainage ditches, mesotrophic wet meadows, and lake edges).
- to measure some histological parameters of leaves, flowers and stems of *L. salicaria* and to investigate the histological variations among populations with various ecological conditions.
- to investigate the phytochemical variations of the above mentioned *L. salicaria* populations.
- to compare the polyphenol contents of different plant parts (leaf, flower, stem) of *L. salicaria* populations.

- to identify and quantify some polyphenolic compounds of *L. salicaria* extracts by UHPLC-MS.
- to prove antimicrobial effects of *L. salicaria* with different microbiological tests and microbes.
- to evaluate the pharmacological effects of *L. salicaria* extracts on guinea pig ileum and to try to find whether the identified phenolic compounds could be responsible for these effects or not.

### **III. Investigation of the selected habitats of *L. salicaria* populations**

This chapter summarizes the weather and soil conditions of the selected habitats.

#### **III. 1. Materials and methods**

##### ***III. 1. 1. Selection and description of habitats and ecological and phytosociological character of *L. salicaria****

Whole herbs of 12 *L. salicaria* populations were collected from different ecological habitats in south-west Hungary (Baranya and Somogy counties) in summer of 2010 and 2011: drainage ditches (Szentlőrinc, Szigetvár, Kaposvár, Cserénfa), mesotrophic wet meadows (Csebény, Kacsóta, Almamellék, Szentlászló), and lake edges (Lake of Almamellék, Deseda, Malomvölgy, and Sikonda). Herbs were dried at room temperature for 3 weeks, ground, and stored in dark. The geographical coordinates and altitudes were measured by a GPS Navigation System (VayteQ N720BT).

Phytosociological relevés were conducted on the 12 selected areas in September, 2011, and the species co-occurring with *L. salicaria* were determined by the coenologist Dr. Róbert Pál. The size of individual plots was 1 m<sup>2</sup>, in a square shape.

##### ***III. 1. 2. Weather conditions of the habitats***

The monthly precipitation was measured in Szentlászló, as well as data of monthly precipitation, average temperature, and sunshine could be asked from the Hungarian Meteorological Service (Országos Meteorológiai Szolgálat - OMSZ) in the case of the habitats of Szigetvár, Kaposvár, and Pécs.

##### ***III. 1. 3. Soil investigation***

The soil samples (0.5 kg/habitat) were collected in the selected areas near the plant samples from the above 10 cm layer of the ground soil in July 2010, and July and August 2011. The foreign elements (coming either of vegetable, animal or mineral origin) were removed from the samples, then they were dried and ground. The pH values were measured with DZS-708 Multi-parameter Analyzer and WTW SenTix 60 combination electrode. The content of nitrate, nitrite, sulphate, phosphate, potassium, and ammonium ions were detected by Quantofix test strips

(Macherey Nagel, Germany). The "free" water content was determined by gravimetric method and expressed in m/m%. The samples were measured, dried for 3 weeks at room temperature and then measured again. Physisorbed water content was not investigated.

### **III. 2. Results and discussion**

#### ***III. 2. 1. Selection and description of habitats and ecological and phytosociological character of *L. salicaria****

The altitude of the habitats varied from 120 m (Szigetvár) to 185 m (Lake Sikonda, in Mecsek Hills). The number of the species varied between 9 (Szigetvár) and 28 (Cserénfa) within the plots. The most frequently occurring species were *Calystegia sepium* (L.) R. BR. (in 8 habitats), *Ranunculus repens* L. (in 6 habitats), *Carex acutiformis* EHRH., *Elymus repens* (L.) GOULD, *Solidago gigantea* AITON, *Symphytum officinale* L. (in 5 habitats), *Carex riparia* CURTIS, *Epilobium hirsutum* L., *Equisetum arvense* L., *Erigeron annuus* (L.) PERS., *Galium mollugo* L., *Glechoma hederacea* L., *Lycopus europeus* L., *Potentilla reptans* L., *Taraxacum officinale* WEBER ex WIGGERS, and *Urtica dioica* L. (in 4 habitats).

Compared to literature data, the following co-occurring species are common in Hungary and North America: *Carex* spp., *Solidago* spp., *Urtica dioica*, *Typha latifolia*, *Phalaris arundinacea*, *Salix* spp., *Alisma plantago-aquatica*, *Rubus* spp., *Convolvulus arvensis*, *Solanum dulcamara*, and *Cirsium arvense*.

#### ***III. 2. 2. Weather conditions of the habitats***

In both 2010 and 2011, the least precipitation was measured in Pécs. The highest precipitation could be observed July 2011; the lowest ones were measured in July 2010 and August 2011. The highest average temperature could be measured in Pécs in all examined months. Values of montly sunshine duration were available only from Pécs; the sum of them was higher in 2011 than in 2010.

#### ***III. 2. 3. Soil investigation***

In agree with the literature data, our *L. salicaria* samples were found mostly in basic soils (except for Cserénfa having neutral soil) and wet habitats, it seems there was no nutrient deficiency in either habitat, and most of the plants lived in full light. The highest soil pH value was measured in Szigetvár (8.03), the lowest one in Cserénfa (6.95). The average "free" water content was high in all of the soil samples. In Lake Sikonda and Lake Malomvölgy, soil samples could not be collected directly around the plant, because it was too deep to reach the bottom of the lake.

### **IV. Morphology and histology**

*L. salicaria* can vary in some morphological features according to its habitat. This chapter summarizes the histological results obtained from different plant parts of this plant collected at the selected twelve populations.

#### **IV. 1. Materials and methods**

**Histological preparation.** Studied plant materials (stem, leaf, bract, and flower) in each population were fixed in the mixture of 96% ethanol : glycerine : distilled water (1:1:1). It was followed by dehydration in ethanol series; then samples were embedded in synthetic resin (Technovit 7100). Longitudinal and transverse sections (10-15  $\mu\text{m}$  thick) were prepared by a rotation microtome (Anglia Scientific 0325), and stained with toluidine blue (0,02%).

**Cleared preparations.** The structure of the trichomes (shape, cell number) was investigated in cleared preparations. The fixed samples were stored in 5% potassium hydroxide for 72 hours, then in 5% sodium hypochlorite, until each sample lost its colour. Then they were rinsed with distilled water, and dehydrated in ethanol series. The cleared leaf pieces were fixed in Canada balsam.

**Programs used for evaluation and Statistical analysis.** Slides were studied by Nikon Eclipse 80i and Opton Anxiovert 35 microscopes, Olympus 12 MP digital camera, SPOT BASIC 4.0, and Olympus Cell D programs. Histological data were measured by Image Tool 3.00 program. Data were presented as mean values. For the analysis of the primary data Microsoft Excel 2010 Software was used in all experiments. Statistical evaluation of the data was performed with the SPSS 13.0 for Windows (SPSS Inc., Chicago, Illinois, USA).

#### **IV. 2. Results and discussion**

The average height of the plants was between 80 and 185 cm in 2010 (average of all populations: 135 cm), and between 60 and 155 cm in 2011 (average of all populations: 100 cm). The plants grew higher in 2010 than in 2011 in all habitats probably due to the different weather conditions ( $p < 0.005$ ), resulting in smaller biomass production.

##### *Stem*

The ratio of the xylem and phloem was similar in all samples collected from various habitats (xylem: 91.0-95.0% in thick stems and 88.0-93.0% in thin stems), except for the sample collected in Kacsóta, where the ratio of the xylem was only 74.0% maybe due to the lack of optimal conditions. This theory may be supported by the fact, that *L. salicaria* extincted from this habitat in 2011. The epidermal cells of the thick stem were elongated and flat, while those of the thin stem were nearly isodiametric. There was no difference in the size of  $\text{Ca}(\text{COO})_2$  crystal druses between the thicker and thinner stems.

##### *Leaf and bract*

Thickness of the leaves measured in the top, middle, and base of the leaf blade varies between 112  $\mu\text{m}$  (Lake Sikonda) and 195  $\mu\text{m}$  (Kacsóta), and that of the bracts varies from 102  $\mu\text{m}$  (Kaposvár) to 159  $\mu\text{m}$  (Kacsóta) in all plant samples. The length of trichomes changes from 157  $\mu\text{m}$  (Cserénfa) to 384  $\mu\text{m}$  (Szentlőrinc) in the bracts, and from 67  $\mu\text{m}$  (Cserénfa) to 224  $\mu\text{m}$  (Lake

Malomvölgy) in the leaves. The longer trichomes of the bracts play important role in the protection of the flowers. The cell number of the trichomes is regularly one to two in the leaves and one to three in the bracts except for the samples collected from Lake Malomvölgy and Szigetvár having also three to four cells in the leaves, and samples collected from Szentlőrinc having also four to five cells in the bracts. The size of the epidermal cells was highly variable in all plant parts and in all populations. Leaf epidermal cells were higher on the adaxial than the abaxial surface in all samples. Palisade cells of the leaves were variable in their height (37-71  $\mu\text{m}$ , Lake Sikonda – Kaposvár) and width (13-17  $\mu\text{m}$ , Kacsóta – Szentlászló), similarly to the measured extreme data of spongy cells in the cell height (16-23  $\mu\text{m}$ , Szigetvár – Szentlászló) and width (16-25  $\mu\text{m}$ , Szigetvár – Lake Malomvölgy). Size of palisade cells in bracts were similar in all populations (average: 36  $\times$  14  $\mu\text{m}$ ) except for Lake Sikonda (53  $\mu\text{m}$   $\times$  16  $\mu\text{m}$ ), where they were extremely high. Spongy cells were the highest at Cserénfa (19  $\mu\text{m}$ ) and the lowest at Szigetvár (14  $\mu\text{m}$ ) in the bracts. The measured parameters of  $\text{Ca}(\text{COO})_2$  crystals were similar to each other, except for the fact that these crystals were wider than high in the stems and the leaves, while they were rather isodiametric in the bracts. In the stem, the average height and width of crystals for all populations was 23  $\times$  31  $\mu\text{m}$ . The size of crystals in the leaves was 28  $\times$  30  $\mu\text{m}$ , and that of the bracts was 23  $\times$  25  $\mu\text{m}$ .

### *Flowers*

The flowering period lasts from June to September. During sample collection, it was found that flowering started earlier in 2010 than in 2011. We were able to collect whole flowering herbs in all habitats in July 2010, but there were some habitats, where the plants have not started to flower in July 2011 yet, in spite of the fact that we collected the samples in the same calendar week (27<sup>th</sup> and 34<sup>th</sup> weeks) in both years. This phenomenon may be due to different weather conditions.

A central vascular bundle was observed in the inflorescence axis branching at the base of each flower and running along the whole length of the flowers. Sepals are composed of 4 or 6 cell layers with flattened epidermal cells and unicellular trichomes on the abaxial surface. The base and the top of the sepals consist of spongy cells exclusively, whereas the middle part can be characterised by both spongy and palisade cells together with numerous intercellular spaces. In our samples, the petals were composed of only 1-2 cell layers. The filament of the stamen has a collateral closed bundle in central position. The ovary is divided by the septum bearing ovules on both sides (axile placentation). The vascular bundle of the septum is surrounded exclusively by spongy cells at the top and the base. The annular nectary is located in the valley between the receptacle and the ovary. Trichomes and intercellular cavities could not be observed in the structure of the ovary. The average thickness of the wall of the ovary is composed of 4-5 cell layers.

## **V. Phytochemical evaluation of *L. salicaria***

In this chapter, the polyphenol composition of the twelve populations of *L. salicaria* was investigated with official methods of the Ph. Hg. VIII. (measurements of the total polyphenol, tannin, and flavonoid content, as well as TLC analysis) and UHPLC-MS.

### **V. 1. Materials and methods**

#### ***V. 1. 1. Total polyphenol and tannin content***

Ph. Hg. VIII. prescribes the determination of total tannin content of *Lythri herba* which could not be less than 5.0% of tannins expressed as pyrogallol and calculated with reference to the dried drug. The correlation between the polyphenol content of the populations in 2010 and 2011 was evaluated by Past 2.17 Program with One-way-ANOVA method.

#### ***V. 1. 2. Total flavonoid content***

Ph. Hg. VIII. prescribes different spectrophotometric methods for identification of total flavonoid content of drugs containing *O*-glycosides (Method 1) and *C*-glycosides (Method 2). Although *L. salicaria* contains mainly *C*-glycosides, at first, we carried out some preliminary measurements, because we were intended to compare which method gives better results, and then we used this method for measurement of the samples.

#### ***V. 1. 3. TLC***

Ph. Hg. VIII. prescribes TLC for identification and quality assurance of most of the herbal drugs, as also in the case of *Lythri herba*. Ph. Hg. VIII. recommends the application of only chlorogenic acid, hyperoside, rutin, and vitexin as reference compounds, but we also used orientin. Our aim was to check whether there are any qualitative differences among the flavonoid pattern of the drug parts and the habitats.

#### ***V. 1. 4. Qualitative-quantitative LC-MS analysis of *L. salicaria* extracts***

We were interested in the evaluation of some polyphenolic compounds, whether they could be responsible for pharmacological (spasmogenic) effects of *L. salicaria* extracts, so different solvents (hexane, chloroform, ethyl acetate, 50% ethanol in water) were used and extracts were analysed by UHPLC-MS. In the case of microbiological studies, the most potent extract was the 50% ethanol in water, and therefore only this extract was analysed by UHPLC-MS.

The flowering branches used in the microbiological experiments were collected from Kaposvár in August 2010, and those of used in the pharmacological experiments were collected from Szigetvár in August 2011.

The extracts used in the microbiological experiments were prepared as follows: 3.00 g drug was extracted three times with 30 mL 50% ethanol in water

with ultrasonic water bath at 40°C, the solution was filtered, evaporated by vacuum-distillation, and the concentration was set to be 10 mg dried extract/1.0 mL 50% ethanol in water (50% ethanol Microb.).

The extracts used in the pharmacological experiments were prepared as follows: 0.50 g drug was extracted with 10.0 mL solvent (hexane, chloroform, ethyl acetate, or 50% ethanol in water) using ultrasonic water bath at 40°C for 3 min. The solution was filtered, evaporated by vacuum-distillation, and diluted to 10.0 mL with 50% ethanol in the case of the ethanol extract and with DMSO in the case of the hexane, chloroform, and ethyl acetate extracts. DMSO was used to ensure the blending of the latter extracts with the organ bath. The qualitative-quantitative analyses were carried out on these extracts by UHPLC-MS. Components were identified by comparison of their retention times, UV spectra and mainly by the mass of their deprotonated molecules ( $[M-H]^-$ ) with those of the standards and the published literature data.

## **V. 2. Results and discussion**

### ***V. 2. 1. Total polyphenol and tannin content***

Each sample met the requirements of the Ph. Hg. VIII. The statistical analysis showed that the total polyphenol content of the populations ( $p = 0.012$ ) was significantly higher in 2011 than in 2010, which might be caused by the weather conditions. There was no significant difference between July and August 2010 ( $p = 0.822$ ), July and August 2011 ( $p = 0.971$ ), July 2010 and 2011 ( $p = 0.063$ ), as well as August 2010 and 2011 ( $p = 0.091$ ), respectively. Polyphenol and tannin contents were higher in the flowering top than in the other organs. In 2010, the measured total polyphenol values ranged from 1.2 to 27.3% (8.3-27.3% in the flowering top, 5.3-23.3% in the leaves, and 1.2-9.9% in the stems). Total tannin values varied between 1.0 and 21.9% (6.6-21.9% in the flowering top, 3.9-20.9% in the leaves, and 1.0-8.4% in the stems). In 2011, the total polyphenol values ranged from 4.7 to 36.8% (13.5-36.8% in the flowering top, 8.4-28.7% in the leaves, and 4.7-9.6% in the stems). Total tannin values varied between 3.2 and 25.7% (9.0-25.7% in the flowering top, 6.1-22.0% in the leaves, and 3.2-7.2% in the stems).

### ***V. 2. 2. Total flavonoid content***

Despite the *C*-glycoside content of *Lythri herba*, greater values of total flavonoids were measured with Method 1 except for one occasion; therefore this method was used in our study. The statistical analysis showed that the total flavonoid content of the populations was significantly higher ( $p = 0.007$ ) in July 2011 than in July 2010 in all plant parts ( $p = 0.027$ ,  $0.028$ , and  $0.006$  in the stem, leaves, and flowers, respectively), which might be caused by different weather conditions. The monthly average precipitation was about three times higher, but the monthly average temperature was a little lower in July 2011 compared to July 2010. The flavonoid content of the populations was higher in August 2010 than in

July 2010, but there was no significant difference between July and August 2011. The highest flavonoid content was measured in the leaves, followed by the flowering branches and stems. In 2010, the detected values ranged from 0.0 to 2.7% (0.3-2.7% in the leaves, 0.1-0.7% in the flowering tops, and 0.0-0.2% in the stems). In 2011, the detected values ranged from 0.1 to 2.9% (0.2-2.9% in the leaves, 0.2-0.9% in the flowering tops, and 0.1-0.3% in the stems).

### V. 2. 3. TLC

The following flavonoids could be identified: orientin (yellow,  $R_f \sim 0.62$ ), isoorientin (yellow,  $R_f \sim 0.5$ ), vitexin (light green,  $R_f \sim 0.72$ ) and isovitexin (light green,  $R_f \sim 0.44$ ). All parts of the plant contained all of these flavonoid components but in different amounts. In agreement with the results of the total flavonoid measurements, our observation was that the greatest amount of flavonoids could be found in the leaves followed by flowering branches, and the smallest amounts of them are in the stems. There was no qualitative difference between the habitats. Rutin, hyperoside, chlorogenic acid, and caffeic acid could not be detected in the samples by TLC.

### V. 2. 4. Qualitative-quantitative LC-MS analysis of *L. salicaria* extracts

The yield of dried extracts of hexane, chloroform, ethyl acetate, and 50% ethanol in water were 11.1 mg/mL, 13.5 mg/mL, 28.2 mg/mL, and 90.5 mg/mL, respectively. Only the 50% ethanol extract contained all of the selected standards. Ellagic acid could be detected in the largest amount followed by isoorientin and orientin. Catechin, caffeic acid, hyperoside, and rutin could not be detected in the hexane, chloroform, and ethyl acetate extracts.

## VI. Antimicrobial activities of *L. salicaria* extracts

We selected disk and agar diffusion methods as well as tube dilution method to examine the antibacterial activity of *L. salicaria* extracts. The activity of the sample solution can be estimated from the diameter of the inhibition zones in the disk and agar diffusion methods. The aim of our microbiological experiments was to confirm the antimicrobial effects of *L. salicaria* extracts published previously in the literature, and to compare the results of the most frequently used microbiological techniques.

### VI. 1. Materials and methods

**Preparation of extracts.** See Chapter V. 1. 4.

**Disk (DDM) and agar diffusion method (ADM).** Mueller-Hinton agar (Oxoid, UK) was used; the tested microorganisms were *Staphylococcus aureus* ATCC 29213, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 90028, MRSA 4262, *Staphylococcus epidermidis* 122228, *Bacillus subtilis*, *Micrococcus luteus*, and a multi-drug resistant *Pseudomonas aeruginosa*. These test microorganisms were maintained on Mueller-

Hinton agar in the Institute of Medical Microbiology and Immunology at University of Pécs. From each microorganism a solution was prepared, which corresponds to  $>10^5$  colony-forming units (cfu)/mL. In DDM, 20  $\mu$ L of leaf, flower, and stem extracts were applied to the sterile paper disks (diameter: 5 mm), dried and arranged equidistant on the agar surface in sterile Petri dishes (diameter: 8 cm); in ADM, 50 and 100  $\mu$ L of leaf and flower extracts prepared with 50% ethanol in water were measured into the holes made in the agar using sterile metal borers. The inhibition zones were measured after 48 hours incubation at 37°C (except for *C. albicans*, incubated at 30°C), and the effect was calculated as a mean of triplicate tests.

**Evaluation of minimum inhibitory (MIC) and bactericidal concentrations (MBC) by tube dilution method.** 1.0 mL broth (in the case of *C. albicans*, the broth contained 3% glucose) was measured into 10 tubes, then 1.0 mL plant extract (10 mg/mL) or antibiotic solution (200  $\mu$ g/mL) was added to the first tube and a twofold dilution was performed through the series of tubes. 10  $\mu$ L of bacterial culture ( $A_{600\text{ nm}} \approx 0.1$ , which corresponds to  $>10^5$  cfu/mL) were added to each tube. After a 24 hours incubation at 37°C (except for *C. albicans*, incubated at 30°C), the bacterial cultures were spread on the surface of Mueller-Hinton agar. The lowest inhibitory and bactericidal concentrations were evaluated after a 48 hours incubation period at 37°C (except for *C. albicans*, incubated at 30°C). The number of bacterial colonies was compared to the untreated control. MIC is the lowest concentration of an antimicrobial substance that could inhibit the visible growth of bacteria. MBC is the lowest concentration of an antimicrobial substance required to kill all germs in a tube.

## VI. 2. Results and discussion

**DDM.** *S. aureus*, MRSA, *S. epidermidis*, and *M. luteus* were sensitive to the 50% ethanol in water and distilled water extracts. The greatest inhibition zones were produced by the flowering branches then the leaves and finally the stems. *S. epidermidis* was the most sensitive bacterium. The solvents caused no inhibition by themselves. The hexane and chloroform extracts of *L. salicaria* failed to show any activity against the investigated microbial strains. Reason of this phenomenon is probably, that polyphenols, which are thought to be responsible for the antimicrobial effect, are less soluble in nonpolar solvents compared to the polar ones.

**ADM.** All of the selected strains except for *B. subtilis* were sensitive to 50% ethanol in water extract. The Gram positive *Staphylococcus* strains were the most sensitive. The inhibition zones could be evaluated even 2 weeks after the application.

**MIC and MBC.** All of the selected strains were sensitive to 50% ethanol in water extract of *L. salicaria* in appropriate concentrations. The antibiotic resistant *S. aureus* and *P. aeruginosa* were similarly sensitive than the *S. aureus* and *P. aeruginosa* having no antibiotic resistance. Compared to the antibiotics, *L.*

*salicaria* approximates mostly the effectiveness of vancomycin against MRSA. The other antibiotics can be more than 1000 times potent than our extracts.

We can conclude that the antimicrobial effect of *L. salicaria* is dose-dependent. The higher doses were given (in the different methods) the more microbes were sensitive to the extract. Our experiments confirmed that *L. salicaria* extracts are effective against *S. aureus*; moreover, methicillin-resistant *S. aureus* also showed sensitivity to these plant extracts. We also confirmed that *M. luteus* and *B. subtilis* are sensitive to *Lythrum* extracts. To the best of our knowledge, this is the first time, when antimicrobial effects of *Lythrum* extracts have been proven on MRSA, *S. epidermidis*, and a multidrug-resistant *P. aeruginosa*. In the literature, we found only one article stating that *L. salicaria* is ineffective against *C. albicans*, our result confirms the opinion of most of the authors, i.e. it is effective.

## **VII. Pharmacological study of *L. salicaria* extracts on guinea pig ileum preparation**

The purpose of our work was to investigate the effect and the mechanism of action of different extracts of *L. salicaria* on isolated guinea pig ileum. The extracts of flowering tops were used in the experiments, because they were more active in the preliminary tests than the leaf. The 50% ethanol in water extract was tested both in the absence and presence of various drugs having specific mechanisms of action. The guinea pig small intestine was chosen as an isolated organ capable of detecting a whole range of neuronal and smooth muscle inhibitory and excitatory effects. In order to attempt to explain the background of the obtained results, phytochemical analyses of the extracts were also carried out (See Chapter V. 2. 4.).

### **VII. 1. Materials and methods**

The studies were approved by the Ethics Committee on Animal Research of University of Pécs according to the Ethical Codex of Animal Experiments, and the licence was given (licence no.: BA 02/2000-7/2006).

**Preparation of extracts.** See Chapter V. 1. 4.

**Animals and preparation.** Short-haired, coloured guinea pigs of both sexes, weighing 400-600 g were killed by stunning and bled out. The pre-terminal ileum was excised and put in Krebs' solution. Whole segments of the ileum (approx. 3 cm in length) were made up as longitudinally-oriented preparations that were put into organ baths containing 5 mL of Krebs' solution and kept at 37°C with the help of a circulating thermostat (Experimetria, Budapest, Hungary). The bathing fluid was bubbled with a mixture of 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The preparations were connected to isotonic lever transducers (Hugo Sachs Elektronik/Harvard Instruments, March-Hugstetten, FRG). Movements of the tissues were recorded on compensographic ink writers. The load on the tissues was 7 mN. Experiments were commenced after 40 min equilibration of the preparations.

Contractions were compared to and expressed as percentage of the maximal longitudinal spasm evoked by histamine (0.5  $\mu\text{M}$ ) administered at the beginning of the experiment and washed out carefully. *L. salicaria* extracts were administered twice, each for 15 min, with a washout period of 30 min.

**Drugs and contact times.** The following concentrations and contact times were used for pre-treatments:  $\alpha,\beta$ -meATP, 15 + 15  $\mu\text{M}$ , 10 min each; PPADS (50  $\mu\text{M}$ ) plus suramin (100  $\mu\text{M}$ ), 20 min; chloropyramine (0.2  $\mu\text{M}$ ), 15 min; indomethacin (2  $\mu\text{M}$ ), 15 min; atropine (0.5  $\mu\text{M}$ ), 15 min; tetrodotoxin (0.5  $\mu\text{M}$ ), 15 min; atropine plus tetrodotoxin, 15 min. capsaicin (10  $\mu\text{M}$ ) was administered for 10 min for producing tachyphylaxis (desensitization), but it was removed from the organ bath and a 40-min washout period was allowed to the preparations to recover from its smooth muscle-depressant effect.

**Electrical field stimulation.** Electrical field stimulation was delivered by a high-performance stimulator (Experimetria, Budapest, Hungary) through platinum wire electrodes placed in the bathing solution above and below the preparation, at a distance of 4 cm from each other. Parameters of electrical field stimulation were as follows: 60 V amplitude, 0.1 ms pulse width, single shocks at a frequency of 0.05 Hz.

**Statistical analysis.** For the analysis of the primary data Microsoft Excel 2010 Software was used. Statistical evaluation of the data was performed with Microsoft Excel WinSTAT. Ileum contractions were expressed in mean  $\pm$  SEM as percentage of the maximal longitudinal spasm triggered by histamine (0.5  $\mu\text{M}$ ). Mann-Whitney test was used for two independent samples, Kruskal-Wallis test was used for more than two independent samples and Wilcoxon's signed rank test was used for two related samples.

## VII. 2. Results and discussion

The hexane, chloroform, ethyl acetate and 50% ethanol in water extracts (10  $\mu\text{L}$ /5 mL organ bath) produced contractile responses ( $n = 5-7$ ) on guinea-pig ileum preparations. The largest contractions were elicited by the 50% ethanol in water extract. The effect was concentration-dependent. The spasmogenic effect partially faded away without washing the tissue with fresh bathing fluid. The magnitude of contractions induced by electrical stimulation was not significantly influenced by the presence of extracts. The solvents by themselves caused no response. None of the pre-treatments applied could completely abolish the contractile responses due to the plant extract showing that more mechanisms are involved in its spasmogenic effect. The contractile response of the ethanol extract was reduced strongly, but not completely, in the presence of the nonselective muscarinic antagonist atropine, indicating that muscarinic receptor activation could partially explain the contractile response. Since the response was not significantly inhibited by tetrodotoxin (a  $\text{Na}^+$  channel blocker that fully suppresses the cholinergic 'twitch' response), it seems probable that cholinergic stimulation of the *L. salicaria* extract is a post-junctional phenomenon, i.e. takes place at the level of

the smooth muscle. Responses due to the 50% ethanol in water extract were also significantly reduced by the cyclooxygenase inhibitor indomethacin and the purinergic P<sub>2</sub> receptor inhibitor PPADS *plus* suramin, the former suggesting that the contractile responses are also mediated through prostanoids. A weak inhibitory effect of the P<sub>2</sub> purinoceptor antagonists might indicate some involvement of a purinergic mechanism, although endogenous purinoceptor agonists may positively modulate cholinergic excitation. Overall, the inhibitory effects of fairly highly-dosed indomethacin or the purinoceptor antagonists were only moderate. This could indicate some release of prostanoids and an ATP-like substance by the extract (in addition to a contractile effect of unknown origin) and/or, alternatively, a positive modulation of the contractile effect of the extract by prostanoids or ATP-like purinergic agonists. Pre-treatment of the tissues with chloropyramine, a histamine (H<sub>1</sub>) receptor blocker, did not alter the response to the plant extract, demonstrating that no histamine receptor activation has a role in the contractile responses. Pre-treatments with  $\alpha,\beta$ -meATP, a purinergic P<sub>2X</sub> receptor agonist and desensitizer, and capsaicin, that damages the afferent nerve endings, elicit no changes in the responses demonstrating that these pathways are also not involved in the contractile mechanism.

The 50% ethanol in water extract was found to be rich in polyphenols. Ellagic acid could be detected in the highest amount, followed by isoorientin and orientin. Hexane, chloroform and ethyl acetate extracts also showed spasmogenic effects while catechin, caffeic acid, hyperoside and rutin could not be detected in these extracts. For this reason we do not think that these compounds play a part in the mechanism of the spasmogenic effect. Further isolation, purification and pharmacological analysis of the different components are necessary to identify the components which can be responsible for the antispasmodic and spasmogenic effects. Cholinergic receptor stimulants are used against gastrointestinal atony. The present results may suggest that a diluted extract of *L. salicaria p.o.* could be used as a mild stimulant of gastrointestinal motility.

## VIII. Novel findings

- This study was the first which investigated populations of *Lythrum salicaria* in south-west Hungary.
- Our histological examinations showed that the leaf epidermal cells were higher on the adaxial than the abaxial surface in all samples, and the trichomes of the bracts were longer than the trichomes of the leaves.
- Based on the results of the phytochemical investigations, we can state that all selected specimens contained high amounts of tannins, and therefore the collection of all of them can be suggested for therapeutic purposes from this point of view.
- The flowering tops contained higher amounts of tannins than the leaves, while the leaves contained higher amounts of flavonoids than the flowers.
- TLC examination showed that all parts of the plant (flowers, leaves, stems) contain the same flavonoid compounds, but in different concentration.
- To the best of our knowledge, this is the first time, that catechin, hyperoside, rutin, luteolin, and apigenin has been detected in samples of *L. salicaria*, however in low amount.
- We applied first the tube dilution method for the investigation of the antimicrobial effects of *L. salicaria*.
- We confirmed that *L. salicaria* extracts are effective against *S. aureus*, moreover, methicillin-resistant *S. aureus* also showed sensitivity to these plant extracts. Results of other authors are ambiguous in the case of *Escherichia coli*, *Pseudomonas aeruginosa*, and *Candida albicans*, but in our experiments they were also sensitive to *Lythrum* extracts. To the best of our knowledge, this is the first time, when antimicrobial effects of *Lythrum* extracts have been proven on MRSA, *S. epidermidis*, and a multidrug-resistant *P. aeruginosa*.
- In the pharmacological experiments, we found that the contractile response of the *Lythrum* extract was reduced strongly, but not completely, in the presence of the nonselective muscarinic antagonist atropine, indicating that muscarinic receptor activation could partially explain the contractile response. We suppose that cholinergic stimulation of the *L. salicaria* extract is a post-junctional phenomenon, i.e. takes place at the level of the smooth muscle.

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**X. This doctoral thesis is based on the following publications and presentations:**

Original articles

- I. **T. Bencsik**, L. Barthó, V. Sándor, N. Papp, R. Benkó, A. Felinger, F. Kilár, Gy. Horváth. (2013): Phytochemical evaluation of *Lythrum salicaria* L. extracts and their effects on guinea pig ileum. *Natural Product Communications* 8: 1247-1250. (IF: 0.956)
- II. **T. Bencsik**, Gy. Horváth, N. Papp. (2012): A réti füzény (*Lythrum salicaria* L.) biológiai jellemzői és gyógyászati értéke. *Botanikai Közlemények* 99: 195–210.
- III. **T. Bencsik**, Gy. Horváth, N. Papp. (2011): Variability of total flavonoid, polyphenol and tannin contents in some *Lythrum salicaria* L. populations. *Natural Product Communications* 6: 1417-1420. (IF: 1,242)

Posters

- I. **T. Bencsik**, V. Sándor, L. Barthó, P. Molnár, F. Kilár, A. Felinger, Gy. Horváth. *Lythrum salicaria* L. kivonatok *in vitro* farmakológiai és fitoanalitikai vizsgálata. MET Elválasztástudományi Vándorgyűlés, Hungary, Hajdúszoboszló, 2012. November 7-9., Abstract: P-3, p. 72.
- II. **T. Bencsik**, N. Papp. *Lythrum salicaria* L. populációk polifenol- és cserzőanyag-tartalmának értékelése. XII. Magyar Gyógynövény Konferencia. Hungary, Szeged, 2011. május 5-7. Abstract: Gyógyszerészet Supplementum LV. S24.
- III. **T. Bencsik**, N. Papp. Variability of flavonoid content in some *Lythrum salicaria* L. populations. CIPAM 2011. Italy, Cagliari, 2011. April 13-15. Abstract: T1P8, p. 142.
- IV. **T. Bencsik**, Á. Farkas, N. Papp. A *Lythrum salicaria* L. hisztológiai értékelése. Greguss Pál Növényanatómiai Szimpózium. Hungary, Szeged, 2010. október 21.

Oral presentations

- I. **T. Bencsik**, L. Barthó, B. Kocsis, Gy. Horváth. *Lythrum salicaria* kivonatok farmakológiai és mikrobiológiai jellemzése. Magyar Gyógyszerésztudományi Társaság Gyógynövény Szakosztályának Előadói ülése. Hungary, Lajosmizse, 2012. június 8.
- II. **T. Bencsik**. *Lythrum salicaria* L. populációk fitokémiai és mikrobiológiai vizsgálata. X. Clauder Ottó Emlékverseny. Hungary, Budapest, 2011. október 13-14. Abstract: p. 19.
- III. **T. Bencsik**, Á. Farkas, N. Papp. Adatok a réti füzény (*Lythrum salicaria* L.) szövettanához. Magyar Biológiai Társaság Pécsi Csoportjának 236. szakülése. Hungary, Pécs, 2011. március 10.

## Participation in the development of a herbal product

AcneSolve<sup>®</sup> – containing extract of *Lythrum salicaria* (2012)

## List of publications not related to the topic of this thesis

### Original articles

- I. N. Papp, K. Birkás-Frendl, **T. Bencsik**, Sz. Stranczinger, D. Czégényi (2014): Survey of traditional beliefs in the Hungarian Csángó and Székely ethnomedicine in Transylvania, Romania. *Revista Brasileira de Farmácia. In press* (IF: 0.676)
- II. P. Felső, Gy. Horváth, **T. Bencsik**, R. Godányi, É. Lemberkovics, A. Böszörményi, K. Böddi, A. Takátsy, P. Molnár, B. Kocsis (2013): Detection of antibacterial effect of essential oils on outer membrane proteins of *Pseudomonas aeruginosa* by Lab-on-a-chip and MALDI-TOF/MS. *Flavour and Fragrance Journal* 28: 367-372. (IF: 1.824)
- III. A. Végh, **T. Bencsik**, P. Molnár, A. Böszörményi, É. Lemberkovics, K. Kovács, B. Kocsis, Gy. Horváth (2012): Composition and antipseudomonal effect of essential oils isolated from different Lavender species. *Natural Product Communication*. 7(10): 1393-1396. (IF: 0.956)
- IV. M. Poór, S. Kunsági-Máté, **T. Bencsik**, J. Petrik, S. Vladimir-Knežević, T. Kőszegi (2012): Flavonoid aglycones can compete with ochratoxin A for human serum albumin: a new possible mode of action. *International Journal of Biological Macromolecules*, 51(3): 279-83. (IF: 2.502)
- V. N. Papp, **T. Bencsik**, K. Németh, K. Gyergyák, A. Sulc, Á. Farkas (2011): Histological study of some *Echium vulgare* L., *Pulmonaria officinalis* L. and *Symphytum officinale* L. populations. *Natural Product Communications*, 6 (10) 1475-1478. (IF: 1,242)

### Posters

- I. P. Felső, K. Böddi, Gy. Horváth, **T. Bencsik**, R. Godányi, É. Lemberkovics, A. Böszörményi, A. Takátsy, F. Kilár, P. Molnár, B. Kocsis. Néhány illóolaj bakteriális külső membránfehérjékre gyakorolt hatásának vizsgálata. MET Elválasztástudományi Vándorgyűlés, Hungary, Hajdúszoboszló, 2012. November 7-9., Abstract: P-11, p. 80.
- II. H. Békésiné Kallenberger, **T. Bencsik**, Á. Farkas, L. Balogh, N. Papp. A *Fallopia sachalinensis* és *F. × bohemica* fajok összehasonlító szövettani vizsgálata (Comparative histological study of *Fallopia sachalinensis* és *F. × bohemica*). XIV<sup>th</sup> Hungarian Plant Anatomy Symposium. Hungary, Pécs, 2012. szeptember 28. Abstract: P3. pp. 45-46.
- III. Á. Farkas, R. Filep, **T. Bencsik**, E. Scheidné Nagy Tóth. Összehasonlító szövettani vizsgálatok *Cotoneaster* taxonok nektáriumstruktúrájára vonatkozóan (Comparative histological studies on the nectary structure of *Cotoneaster* taxa). XIV<sup>th</sup> Hungarian Plant Anatomy Symposium. Hungary, Pécs, 2012. September 28. Abstract: P12. pp. 63-64.

- IV. Gy. Horváth, **T. Bencsik**, R. Godányi, P. Felső, É. Lemberkovich, A. Böszörményi, A. Takátsy, P. Molnár, B. Kocsis. Lab-on-a-chip: a technique for detection of antibacterial effect of essential oils on outer membrane proteins. 43<sup>rd</sup> International Symposium on Essential Oils (ISEO2012). Lisbon, Portugal, 2012. September 5-8. Abstract: P.207. p. 251.
- V. A. Sulc, **T. Bencsik**, N. Papp. *Symphytum officinale* L. populációk összehasonlító szövettani jellemzése. XII. Magyar Gyógynövény Konferencia. Hungary, Szeged, 2011. május 5-7. Abstract: Gyógyszerészet Supplementum LV. S32.
- VI. N. Papp, Gy. Horváth, **T. Bencsik**, E. Turcsi, J. Deli, P. Molnár. *Euphorbia* taxonok karotinoid analízise. XII. Magyar Gyógynövény Konferencia. Hungary, Szeged, 2011. május 5-7. Abstract: Gyógyszerészet Supplementum LV. S31.
- VII. N. Papp, A. Sulc, **T. Bencsik**. Histological variability of *Symphytum officinale* L. populations. CIPAM 2011. Italy, Cagliari, 2011. April 13-15. Abstract: T1P34, p. 168.
- VIII. F. Budán, I. Szabó, K. Birkás-Frendl, Gy. Boris, **T. Bencsik**, N. Papp. A népgyógyászatban tumor kezelésére alkalmazott élelmiszer- és fűszernövények kemopreventív hatásainak irodalmi háttere. Fiatal Higénikusok Fóruma. Hungary, Debrecen, 2010. május 27-29. Abstract: Egészségtudomány LIV., p. 99.
- IX. N. Papp, K. Birkás-Frendl, Gy. Boris, V. Vántus, K. Csepregi, **T. Bencsik**, É. Vojkovich, D. Vincz, V. Bóna, I. Farkas, S. Bartha, L. Gajdos, I. Fancsali, T. Grynaeus, K. Csedő. Etnobotanikai kutatások a Pécsi Tudományegyetemen. XX. Tudományos Ülésszak Romaniaia, Kézdivásárhely, 2010. április 22-24. Abstract: Orvostudományi Értesítő 83: 41.
- X. N. Papp, K. Birkás-Frendl, Gy. Boris, É. Vojkovich, **T. Bencsik**, T. Grynaeus. Gyógynövények a népi bőrgyógyászatban. Congressus Pharmaceuticus Hungaricus XIV., Hungary, Budapest, 2009. November 13-15. Abstract: Gyógyszerészet Supplementum I. 2009/11. p. 110.

Oral presentations (the presenting author is underlined)

- I. Gy. Horváth, Á.M. Móricz, O. Péter, E. Kira, **T. Bencsik**, A. Böszörményi, É. Lemberkovics, B. Kocsis. Application of TLC-bioautography for detecting the antibacterial activity of essential oils. 44th ISEO, Budapest, Hungary. 2013. September 8-12. Abstract: O-11.
- II. F. Budán, **T. Bencsik**, Gy. Boris, K. Birkás-Frendl, I. Fancsali, Á. Farkas, Z. Gyöngyi, N. Papp. Synergies of anticarcinogenic and chemopreventive effects of edible herbs from populer medicine survey to secondary transmitter molecules (Kemopreventív élelmiszernövények hatásainak szinergiái - népgyógyászati felméréstől a másodlagos jelátvivő molekuláig). International Conference of Preventive Medicine and Public Health. Hungary, Pécs, 19-20 November 2010. Abstract: Egészségtudomány, LIV.(3):106. (2010)
- III. N. Papp, **T. Bencsik**, R. Molnár, R. Filep, Gy. Horváth, Á. Farkas. Gyógynövények hisztológiai jellemzői – Kutatásaink a pécsi Farmakognóziái Tanszéken. Greguss Pál Növényanatómiai Szimpózium. Hungary, Szeged, 2010. október 21.
- IV. Gy. Horváth, Á. Farkas, N. Papp, **T. Bencsik**, R. Molnár, P. Molnár, L. Gy. Szabó. Gyógynövénykutatás a Mecseken és környékén: múlt, jelen, jövő. MGYT Gyógynövény Szakosztályának előadóülése. Hungary, Lajosmizse, 2010. szeptember 17.
- V. N. Papp, Á. Farkas, Gy. Horváth, **T. Bencsik**, R. Molnár, L. Gy. Szabó, P. Molnár. Bemutatkozik a Melius Gyógynövénykert. Magyar Biológiai Társaság Pécsi Csoportjának 231. szakülése. Hungary, Pécs, 2010. szeptember 18.
- VI. **T. Bencsik**, N. Papp. A VIII. Magyar Gyógyszerkönyv néhány új gyógynövénye. Magyar Biológiai Társaság Pécsi Csoportjának 220. szakülése. Hungary, Pécs, 2009. március 18.
- VII. **T. Bencsik**. A VIII. Magyar Gyógyszerkönyv új gyógynövényei és gyógyászati felhasználásuk. PTE ÁOK Házi Tudományos Diákköri Konferencia. Hungary, Pécs, 2009. február 19-21. Abstract: p. 60.