APPLICATION OF SUPERCRITICAL FLUIDS IN THE STUDY OF *TANACETUM PARTHENIUM* L.: FROM EXTRACTION TO ANALYSIS

PhD thesis

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Introduction

Medicinal plants have been used to treat human diseases since ancient times. Nowadays an herbal renaissance is happening all over the world. In the patients' point of view, the herbal products symbolize safety compared to synthetics. People are returning to natural products with the hope of safety, security and most importantly, less side-effects. The herbal medicine expertise was grounded in empirical knowledge that has been transferred from generation to generation. This centuries phytochemical and pharmacological research can give a validated, evidence based background for the application. Plant extracts are complex matrices with numerous different compounds, the isolation of the putative biologically active components in most cases is not a viable option, because there is a synergistic effect between them or at least a sort of constituents can contribute to the biological response. This makes the development of reproducible extraction and sophisticated and validated analytical methods necessary.

The application of novel solvents like supercritical fluids are highly desirable. In the last 17 years (2000-2017) more than 350 studies have been published about supercritical fluid extraction (SFE), concerning the extracts of plant species. The extraction with supercritical carbon dioxide was the first alternative for the widely used Soxhlet extraction, applying organochlorine solvents for sample preparation. SFE has many advantages compared to the traditional and popular extraction techniques, such as liquid-liquid extraction, solid phase extraction and purge/trap method. In the food, pharmaceutical, and fine chemical industries, supercritical fluid extraction is an often applied method. The other utilization of supercritical fluids is the area of chromatography. Although supercritical fluid chromatography (SFC) is an old chromatographic technique, the most commonly used chromatographic tools are still gas chromatography (GC) and liquid chromatography (LC). The first SFC systems which were specifically designed to manage supercritical fluids appeared in the recent years. This technological development has caused a dramatic uplift in the number of researches, publications and probable application of the method. The concurrent presence of semipolar and apolar components in a mixture, can be a good model system both for SFE and SFC to prove its effectiveness and selectivity.

Tanacetum parthenium L. has been used as medicinal plant for over 2000 years due to its pharmacological activities, particularly the migraine prophylactic effect. Feverfew contains a diverse range of compounds, including sesquiterpenoids, flavonoids and essential oil. The sesquiterpene lactone, parthenolide is considered to be the major active constituent of the

plant, however the pharmacological benefits of the lipophilic flavonoids and some of the volatile oil components cannot be neglected. Feverfew has COX-2, 5-HT releasing and NF-κB inhibitory effects besides other pharmacological activities. The biologically active constituents of feverfew have lipophilic, semipolar qualities. For a migraine therapeutic effect, the molecules have to cross the blood brain barrier, which can happen through passive or active transport. The preselection of the compounds can be done by using special extraction methods, like supercritical fluid extraction. Through the modification of the fluids polarity, the degree of the lipophilic character can be regulated. The extracted lipophilic/hydrophilic parthenolide, its derivates and the lipophilic flavonoids have a higher plausibility to be bioavailable at the site of the effect, in the central nervous system. Their concurrent present in feverfew makes the plant an exceptional model for methods using supercritical fluids, like supercritical fluid extraction and supercritical fluid chromatography and for the blood-brain barrier specific artificial membrane permeability assay.

Objectives

Feverfew is a well-known medicinal plant with large literature and research background. The primary aim of our work was to reevaluate and to complement the phytochemical and pharmaceutical information about the herb. Considering the fact, that nowadays the rapid and environmental friendly methods are desirable, and the semipolar nature of the simultaneously present molecules in the plant, we aimed to use techniques applying supercritical fluids.

1. The primary aim was to optimize a supercritical fluid extraction method of feverfew in order to maximize the extraction yield and parthenolide content of the extracts. Our objective was to plan a detailed experimental program of the supercritical fluid extraction conditions (temperature, pressure, cosolvent content) in order to evaluate their individual and combined effects. We intended to compare the optimalised SFE method to other conventional extraction methods like Soxhlet and ultrasound assisted extraction.
2. We aimed to develop a validated qualitative and quantitative supercritical fluid chromatographic method for the determination of the parthenolide content of the feverfew extracts prepared by supercritical fluid and conventional extraction methods.

3. For the identification of simultaneously present co-extracts, mass spectrometry is acquired to gain structural information about the compounds, with particular attention on the lipophilic flavonoids.

4. Postulating that other components beside parthenolide take place in the activity, our objective was to screen compounds possessing positive permeability rates through the blood-brain barrier (BBB+) in the supercritical fluid extracts of *T. parthenium*. We aimed to isolate these BBB+ components and identify them.

5. In order to gain information about the volatile oil of feverfew, steam distillation was chosen to prepare samples for the further analysis. An HS-SPME-GC/MS method was applied for the qualitative and quantitative analysis of the essential oil samples and supercritical fluid extracts. The main, potentially biologically active component of the essential oil is camphor beside camphene and *trans*-chrysanthenyl acetate based on literature data. Our objective was to develop and validate a supercritical fluid chromatographic method for the quantitative study of the camphor content of the volatile oil. As comparison, the camphor content of the supercritical fluid extracts was also aimed.

Materials and methods

Plant material

Tanacetum parthenium L. seeds were collected from the medicinal plant collection of the National Botanical Garden in Vácrátót (Hungary, 2011), Botanical Garden of the University of Debrecen (Hungary, 2011), Botanical Garden of Bonn (Germany, 2011).

The seedlings were raised in Érd and collected before and during flowering. The blooming samples were manually separated to flower heads and leaves. The samples originated from the Vácrátót seedlings were investigated in experiments applying supercritical fluids. Only the essential oil and camphor content was determined from all three originations. After grinding, the size of the particles was between 40 and 50 Mesh. Voucher specimens are deposited in the Department of Pharmacognosy, Semmelweis University, Budapest.

Extraction and sample preparation

Analytical scale supercritical fluid extraction

Supercritical extractions were carried out using a system consisting of a Jasco PU-2080 CO2 pump with a Peltier cooling system for the delivery of carbon dioxide and a Jasco PU-2080 pump, linked to a Jasco MX-2080-32 Dynamic mixer for the addition of modifier. A 1 ml Jasco extraction vessel was mounted in the column position in a CO-2060 plus intelligent column thermostat. A Jasco BD-2080 back-pressure regulator was applied to adjust the pressure in the system. The 1 ml extraction vessel was packed with (ca. 0.5 g) plant material and the optimal parameters were investigated in regard to the amount and composition (parthenolide content) of the extract. The solute leaving the extractor was introduced through a pressure-reducing valve, where the product was collected. The extractions were carried out for over 1hour, the solvent flow rate was 0.4 ml/min. The extracts were filtered through Phenex-RC 15 mm, 0.2 μm syringe filters (Gen-Lab Ltd, Budapest, Hungary).

Experimental design on the SFE

The relationship between the measured responses and the individual and combined effects was analyzed with response surface methodology. In the extraction process a factorial experiment using a full 3^3 design was followed. The influence of temperature (40, 60 and 80°C), pressure (at three levels between 10 and 30MPa in 10MPa increments) and modifier addition (0%, 5% and 10% ethanol) on the extraction yield and parthenolide content in the extracts of the different blooming stages and parts was studied. The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction.

Two other independent variables, solvent flow rate (0.4 ml/min) and extraction time (1h) were kept constant.

27 experiments were performed for the Design of Experiment (DOE) and additional runs were performed to check the trueness and the reproducibility of the experiments and the model.

Soxhlet extraction

Soxhlet extraction was performed using laboratory-scale apparatus. The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction.

Dried and milled plant samples (10.0 g each) were extracted with chloroform at 60ºC and methanol at 90ºC, each for 4 hours. The extracts were evaporated to dryness under reduced pressure at 50ºC, then dissolved in supergradient grade acetonitrile (3.0 ml) and filtered through a Phenex RC 15 mm, 0.2 μm syringe filter (Gen-Lab Ltd., Budapest, Hungary).

Ultrasonic extraction

Ultrasound assisted extraction was carried out on the fresh and dried leaves collected before flowering, leaves collected during flowering and flower heads (10.0 g each). The plants raised from the seeds of the National Botanical Garden in Vácrátót were used for the extraction.

The plant samples were extracted with chloroform at room temperature for 30 minutes. The extracts were evaporated to dryness under reduced pressure at 50ºC, then dissolved in supergradient grade acetonitrile (3.0 ml) and filtered through a Phenex RC 15 mm, 0.2 μm syringe filter (Gen-Lab Ltd., Budapest, Hungary).

Steam distillation of *Tanacetum parthenium* L.

The determination of essential oil in *Tanacetum parthenium* L. was carried out by steam distillation in a special apparatus described in the Pharmacopoea Hungarica VII. (Ph. Hg. VII.) [1987]. The quantity of the essential oil was determined after 3 hours of distillation.

Chromatographic methods

Quantitative analyses by Ultra Performance Convergence Chromatography

Quantification of parthenolide and camphor in the feverfew supercritical fluid extracts was performed by the external standard method. The chromatographic analysis was performed using a Waters ACQUITY UltraPerformance Convergence Chromatography (UPC²) system, equipped with a binary solvent delivery pump, an auto-sampler, a column oven, a diode array detector and a back-pressure regulator.

Determination of parthenolide

The quantitative analysis of parthenolide was performed using an Acquity UPC² BEH C18 column (100mmx3mm, 1.7 μm, Waters, MA, USA). The eluents were supercritical carbon dioxide (A) and acetonitrile (B). An isocratic program was applied: 0-5 min. 18% B with a flow rate of 2 ml/min. The column temperature was 45°C and the detection wavelength was 210 nm during the analysis, UV spectra were recorded from 200 nm to 400 nm. The back pressure was set to 2000 psi. Empower 3 software was used for the data processing.

The UPC^2 method was validated for accuracy, linearity, limit of detection, limit of quantification, repeatability and intermediate precision in accordance with ICH guidelines.

Determination of camphor content

The quantitative determination of camphor in the extracts was performed using an Acquity UPC² BEH-2EP column (100mmx3mm, 1.7 µm, Waters, MA, USA). The eluents were supercritical carbon dioxide (A) and isopropanol (B). A gradient program was applied 0-10 min 0% →10% B with a flow rate of 2 ml/min. The column temperature was 50°C and the detection wavelength was 290 nm during the analysis, UV spectra were recorded from 200 nm to 400 nm with a resolution of 2.4 nm, the sampling rate was 20 points/min The back pressure was set to 2000 psi. Empower 3 software was used for the data processing.

The UPC^2 method was validated for accuracy, linearity, limit of detection, limit of quantification, repeatability and intermediate precision in accordance with ICH guidelines.

SemiPrep-HPLC

SemiPrep-HPLC was carried out on a Hanbon AS20005 semi-preparative HPLC system consisting of a NP7005C preparative pump, a NU3000C UV detector, a preparative injector and a high pressure gradient mixer. The samples were separated on a Phenomenex Luna C18 column (150 x 10.0 mm, 5µm), the UV detection occurred at 254 nm. The following gradient elution program was applied, where eluent A was 0.1 % (v/v) trifluoroacetic acid in water, eluent B was 0.1 % (v/v) trifluoroacetic acid in (acetonitrile: water = 95: 5): 0 min: 30 % (v/v) B; 30 min: 100 % (v/v) B; 5 min: 100 % (v/v) B.

Characterization of phenolics in the *Tanacetum parthenium L.* supercritical fluid extracts by HPLC-DAD-MS/MS

The HPLC-DAD-MS/MS experiments were performed on an Agilent 1200 HPLC system (G1379B degasser, G1312B binary gradient pump, G1367C autosampler, G1316B column thermostat and G1315C diode array detector) coupled with an Agilent 6410 triple quadrupole mass spectrometer equipped with an ESI ion source (Agilent Technologies, Waldbronn, Germany). The Masshunter B.04.01 software was used for qualitative analyses.

The samples were separated on a Kinetex-XB C18 column (150×4.6 mm, 2.6 µm; Phenomenex, Torrance, CA, USA) maintained at 45°C. The injection volume was 30 µL. The following gradient elution program was applied at a flow rate of 1.0 mL/min; where eluent A was 0.1 % (v/v) trifluoroacetic acid in water, eluent B was 0.1 % (v/v) trifluoroacetic acid in (acetonitrile: water = 95: 5): 0 min: 5 % (v/v) B; 0 min: 28 % (v/v) B; 40 min: 80 % (v/v) B; 55 min: 100 % (v/v) B; 65 min: 100 % (v/v) B. Chromatograms were acquired at 210±5 nm and 254±5 nm. Triple quadrupole mass spectrometric parameters were as follows: Ion source: ESI, positive and negative ion mode, Drying gas (N_2) temperature: 350 °C; Drying gas (N_2)

flow rate: 13 l/min; Nebulizer gas (N_2) pressure: 40 psi; Fragmentor voltage: 135 V; Capillary voltage: 4000 V.

Full-scan mass spectra were acquired over the *m/z* range of 50-1000 (1 scan/sec). Collision energy was changed between 10–50 eV, according to differences in molecule structures (high purity N_2 was used as collision gas).

For structural characterization of the compounds retention times, molecular masses and calculated molecular formulas, UV and mass spectral data were compared to literature data and to those of authentic standards, where available.

Solid Phase MicroExtraction (SPME)

Air-dried plant material (0.5 g) or 50 µl essential oil was put into vials (20 ml headspace) sealed with a silicon/PTFE septum prior to SPME-GC/MS analysis. Sample preparation using the static headspace solid phase microextraction (sHS-SPME) technique was carried out with a CTC Combi PAL (CTC Analytics AG, Zwingen, Switzerland) automatic multipurpose sampler using a 65 µM StableFlex polydimethyl siloxane/divinyl benzene (PDMS/DVB) SPME fibre (Supelco, Bellefonte, PA, USA). After an incubation period of 5 min at 100ºC, extraction was performed by exposing the fiber to the headspace of the 20 mL vial containing the sample for 10 min at 100ºC. The fiber was then immediately transferred to the injector part of the GC/MS, and desorbed for 1 min at 250ºC. The SPME fiber was cleaned and conditioned in a Fiber Bakeout Station in pure nitrogen atmosphere at 250ºC for 15 min after desorption.

Gas Chromatography coupled with Mass Spectrometry (GC-MS)

The analyses were carried out with an Agilent 6890N/5973N GC-MSD (Santa Clara, CA, USA) system equipped with an Agilent HP-5MS capillary column (30m × 250µm× 0.25µm). The GC oven temperature was programmed to increase from 60ºC (3 min isothermal) to 200ºC at 8ºC/min (2 min isothermal), from 200–230ºC at 10ºC/min (5 min isothermal) and finally from 230–250ºC at 10 ºC/min (1 min isothermal). High purity helium was used as carrier gas at 1.0 mL/min (37 cm/s) in constant flow mode. The injector temperature was 250°C and the split ratio was 1:50. The analyses were conducted using a mass selective detector operated in electron ionization mode at 70 eV, full scan mode coupled with a quadrupole mass analyzer (41–500 amu at 3.2 scan/s). The data were evaluated using MSD ChemStation D.02.00.275 software (Agilent). The identification of the compounds was carried out by comparing retention times and recorded spectra with the NIST 05 library.

Nuclear Magnetic Resonance (NMR)

All NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 3.2 C/Chempack 5.1 were used for structure identifications. The complete resonance assignments were established from direct $^{1}H–^{13}C$, long-range $^{1}H–^{13}C$, and scalar spin–spin connectivities derived from 1D ^{1}H, ^{13}C, 1D TOCSY, $^{1}H–^{1}H$ gCOSY, zTOCSY experiments, respectively. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The ^{1}H chemical shifts were referenced to the applied NMR solvents CD_3OD (δ (CD_2HOD) = 3.310 ppm) and ^{13}C chemical shifts were referenced to 49.00 ppm while in $CDCl_3$ the ^{1}H chemical shifts were referenced to (δ H = 7.260 ppm) and ^{13}C chemical shifts to 77.160 ppm, respectively.

Blood-Brain Barrier Specific Artificial Membrane Permeability Assay (PAMPA-BBB)

The solutions of the *Tanacetum* extract and the individual compounds were prepared with dimethyl sulfoxide (DMSO) at concentrations of 50.0 mg/mL and 10.0 mM, respectively. These were diluted with PBS (Phosphate Buffered Saline; pH = 7.4) to obtain the donor solutions (295.0 µL buffer+5.0 µL DMSO solution), then shaken for an hour at room temperature in a 96-well polypropylene plate (Agilent, Waldbronn, Germany), and filtrated after (Vacuum Manifold, Millipore). A parallel artificial membrane permeability assay (PAMPA) system was used to determine the effective permeability (P_e) for the compounds of interest.

Each well of the top plate (96-well polycarbonate based filter donor plates (Multiscreen™-IP, MAIPN4510, pore size 0.45 µm; Millipore)) was coated with 5 µL of porcine polar brain lipid extract (PBLE) solution (16.0 mg PBLE + 8.0 mg cholesterol dissolved in 600.0 µL *n*-dodecane), then 150.0 µL of the filtrate was placed on the membrane. The bottom plate (96-well polytetrafluoroethylene (PTFE) acceptor plates (Multiscreen Acceptor Plate, MSSACCEPTOR; Millipore)), was filled with 300.0 µL buffer solution (0.01 M PBS buffer, pH= 7.4). The donor and acceptor plates were fit, and then the sandwich system was incubated at 37 °C for 4 hours in a Heidolph Titramax 1000. After the incubation the PAMPA plates were separated and the compound concentrations in the donor ($C_{D(t)}$) and acceptor ($C_{A(t)}$) solutions, as well as in the donor solution at zero time point ($C_{D(0)}$) were determined by HPLC-DAD.

Results

Comparison of supercritical fluid and conventional extraction methods of *Tanacetum parthenium* L.

Supercritical fluid extraction is a highly desirable method, because it is fast, environmental friendly and can be customized with the variation of the extraction parameters for the dissolution of specific extractable compounds. We developed a supercritical fluid extraction method, where *Tanacetum parthenium* L. leaves collected before flowering, leaves collected during flowering and flower heads were investigated. A full factorial 3^3 experimental design was followed to maximize the total extraction yield and the parthenolide content of the supercritical fluid extracts. Based on the statistical results, the models represented the experimental data sufficiently for all three samples. It was confirmed that all the individual factors had significant positive or negative effect, influencing the extraction. One of the main effecting component was the ethanol content of the fluid. The modification in the fluids polarity caused an increase in the extraction yield, enhancing the extraction of more polar components, however the larger solvent power also meant an increase for the parthenolide content of the extracts, worsening the selectivity and the efficiency.

Total extraction yield of the supercritical fluid extracts

The Pareto chart of the effects showed, that all the individual factors have a significant effect on the extraction yield. The temperature had a higher influence on the extraction, than the pressure. Positive effect of pressure on the extraction is consistent with the increasing solubility of compounds by the increasing extraction pressure at constant temperature. At high pressures, the solubility of compounds increases by increasing temperature. This crossover effect, usually occurring from 20 to 35 MPa, is caused by the competing effects of the reduction in solvent density and the increase of the vapor pressure. The ethanol content of the supercritical fluid is also among the three most significant factors. The interaction between pressure and temperature and the quadratic effect of these two variables are also notable.

The flower heads differed from the other two samples, because the most significant factor was the ethanol content of the supercritical fluid. It is well known that the modifier changes the polarity of the supercritical solvent, thus solubility of both valuable and non-valuable co-extracts may increase. Pressure and temperature also have a significant positive effect. The extraction yield did not stabilize within the studied ranges, but reached a maximum value at 30 MPa, 80°C and 10% ethanol content.

Parthenolide content of the supercritical fluid extracts

The Pareto charts have shown that the ethanol content of the fluid individually and also in quadratic form had the most significant, however negative effect on the extraction of parthenolide. All the other linear and quadratic impacts had a statistically significant positive effect. In all three cases the temperature had more significant influence on the extraction, than the pressure, also in quadratic form. The flower heads contained the highest amount of parthenolide (0.604%). Higher parthenolide content was observed in the leaves collected before flowering than in the leaves collected during flowering.

The critical values of the extraction of the leaves collected before and during flowering and the flower heads are the same (7% EtOH, 22MPa, 64°C).

Conventional extraction methods

Soxhlet extraction

Conventional methods such as Soxhlet extraction are the most commonly applied technics for the recovery of parthenolide and other constituents from *Tanacetum parthenium* L. The highest total extraction yield was reached the leaves before flowering, the maximum parthenolide content was observed of the flower heads. When methanol was used as organic phase, the results were only approximately 60-70% as of the results using chloroform in both of the observed cases in all three samples

Ultrasound assisted extraction

A bottle stirring method in ultrasonic bath was carried out for the determination of the total extraction yield and parthenolide content in fresh and dried samples of feverfew. Chloroform was used, as organic phase, because of the semipolar quality of parthenolide. Literature data and the results of the performed Soxhlet extraction were also confirming the application of chloroform in the extraction. The extracts made from fresh plant material, are modeling the parthenolide content of the glandular thrichomes, since no grinding was applied, so the deeper cell structure of the plant was not erupted.

Comparison of supercritical fluid extraction and conventional methods

Conventional methods, like Soxhlet and ultrasound assisted extraction are still the first choice for the separation of compounds from plant matrices. Supercritical fluid extraction is a fast, environment friendly selective and effective alternative for the widely applied sample preparation processes using organochlorine solvents. The results show, that the supercritical fluid extraction method produced the highest amount of parthenolide, in the highest

concentration, which confirms its effectiveness and selectivity of the method. Only the ultrasound assisted bottle stirring method of the fresh leaves produced similar outcome, which is persistent with the fact that parthenolid is placed in the glandular thrichomes.

Characterization of sesquiterpene lactones and phenolics in the supercritical fluid extracts of *Tanacetum parthenium* L.

HPLC-DAD-MS/MS

The results from the characteristic UV and MS spectra designated seven terpenoid type structure beside parthenolide and thirteen flavonoid type structure in the supercritical fluid extract of feverfew. Luteolin, apigenin, axillarin, parthenolide and casticin were identified by comparison of their chromatographic and spectrometric data to authentic standards, while the structural characterization of the other eight flavonoid derivatives was based merely on the evaluation of their (-) ESI-MS/MS fragmentation data.

The flavonoid derivates were tentatively identified as two isomers of methylquercetin, a methoxyflavone, a dihydroxyquercetin, a dimethylquercetin, a trihydroxy-trimethylflavone and a dihydroxy-trimethoxyflavone. Additionally, costunolide (compound 7), dihydro-β-cyclopyrethrosin (compound 8), a tanacetol A isomer (compound 13), tanaphillin (compound 18), 3-β-Hydroxyanhydroverlotorin (compound 19), seco-tanapartholide A (compound 20), seco-tanapartholide B (compound 21), hispidulin (compound 22) has been tentatively identified.

PAMPA-BBB, Semi-Prep-HPLC, NMR

The whole *T. parthenium* extract was subjected to the PAMPA-BBB analysis without purification or prefractionation. Among the flavonoid constituents, a trihydroxy-trimethylflavone and a dihydroxy-trimethoxyflavone were shown to have BBB permeability potential comparable to that of parthenolide. The optimal application conditions of the supercritical fluid extraction enhanced the coextraction of compounds with similar polarity. This explains the presence of BBB positive lipophilic flavonoids in the extract. These flavonoids may contribute to the overall CNS activity of the plant, so the further identification of these were necessary.

For the isolation of the compounds capable of crossing the blood brain barrier, SemiPreparative HPLC method was applied. The structural identification of these compounds was performed with NMR spectroscopy. Consequently, the trihydroxy-trimethylflavone compound was identified as aceronin and sudachitin, which only differ from each other in the

position of a methoxy/hydroxyl group and the dihydroxy-trimethoxyflavone was identified as nevadensin.

PAMPA-BBB of the newly identified compounds

The purified compounds were subjected individually to PAMPA-BBB measurements again together with two flavonoid aglycones, luteolin and apigenin, two methylated flavonoids axillarin and casticin and the sesquiterpene lactone, parthenolide. The $logP_e$ values of sudachitin/aceronin and parthenolide measured in the extract and as individual compounds were not significantly different. Conversely, nevadensin showed higher permeability potential when measured in the extract than as purified compound. This might be attributed to pharmacokinetic interactions occurring among the constituents in the extract. According to the results luteolin, apigenin and casticin also fell in the BBB+ region, although these constituents were not detected on the acceptor side of the PAMPA when the whole extract was investigated. Although axillarin was present in the extract in an amount comparable to nevadensin, it was not detected on the acceptor side of the PAMPA neither in the whole extract nor when measured as individual compound, which confirms axillarin being BBB-.

Qualitative analysis of the essential oil and supercritical fluid extract of feverfew

The essential oil and supercritical fluid extracts were analyzed with HS-SPME-GC/MS method. 11 different compounds were identified according to their retention times and mass spectrums. The volatile constituents in the essential oil were α-pinene, camphene, p-cymene, limonene, γ-terpinene, camphor, chrysanthenyl acetate, bornyl acetate, β-caryophyllene, β-farnesene and germacrene D. The compounds were present in all six essential oil samples in different distribution. The supercritical fluid extract of feverfew contained only 7 compounds, these were camphene, p-cymene, limonene, γ-terpinene, camphor, chrysanthenyl acetate and germacrene D. The main compounds in all samples were camphor and chrysanthenyl acetate.

Camphor content of the essential oil and supercritical fluid extract from *Tanacetum parthenium* L.

The camphor content of the essential oils from the aerial parts of the three feverfew samples from different origin was analyzed by the previously validated convergence chromatographic method. The essential oil content of the leaves (from Debrecen, Budaörs and Vácrátot) amounted from 0.59 to 0.69g/100g drug. The flower heads yielded from 0.32 to 0.39g/100g drug. The highest amount of camphor was found in the leaves (178.2, 146.08, 224.11 mg/100g drug).

Conclusions

1. For the preselection and enrichment of the plausibly bioavailable compounds of *Tanacetum parthenium* L. supercritical fluid extraction was applied. The optimum conditions of the SFE of feverfew were determined, in order to maximize the parthenolide recovery of the extracts. The experiments were carried out at different temperature (40°C, 60°C and 80°C), pressure (10MPa, 20MPa and 30MPa) and modifier content (0%, 5% and 10% ethanol). The design of experiment (DOE) was based on a full factorial 3^3 design. Three feverfew samples were investigated (leaves collected before flowering, leaves collected during flowering and flower heads). For the extraction yield no optimum was found. The critical values of parthenolide content were 7 % EtOH, 22 MPa and 64 °C. Our results indicated, that the flower heads contained the highest amount of parthenolide (0.604% wt.). It was confirmed, that the optimum conditions of the extraction, where the maximum parthenolide content and extract yield can be reached, do not coincide.
2. A simple and rapid isocratic Ultra Performance Convergence Chromatographic (UPC^2) method was developed for the qualitative and quantitative analysis of *Tanacetum parthenium* L. supercritical fluid extracts and parthenolide which was proved to be reliable and accurate. The method was successfully validated with advantages of high resolution, sensitivity, very good reproducibility and short analysis time. Therefore, it could be concluded that the developed UPC^2 methods can be successfully utilized for the analysis of the feverfew extracts.
3. Complexity of the extracts was proved by tentative identification of the compounds using HPLC-DAD-MS/MS methods. 7 terpenoids beside parthenolide were identified, namely costunolide, dihydro-β-cyclopyrethrosin, a tanacetol A isomer, tanaphillin, 3-β-hydroxyanhydroverlotorin, seco-tanapartholide A, seco-tanapartholide B Twelve flavonoid components were detected in the extract. Besides apigenin, luteolin, casticin and axillarin, eight further methylated flavonoids were identified.
4. An *in vitro* parallel artificial membrane permeability assay for the blood-brain barrier (PAMPA-BBB) was successfully utilized for the selection of brain-penetrable compounds of the extracts.

- Beside the main BBB positive component parthenolide, minor sesquiterpene lactones and some methylated flavonoids were enriched in the acceptor side.
- Based on the NMR analysis of the isolated methylated flavonoids sudachitin/aceronin isomers and nevadensin were identified as BBB positive flavonoids.
- The presence of sudachitin and nevadensin were previously proven in the Asteraceae family, but neither of the three flavonoids were found in *Tanacetum parthenium* L. before. The components were also tested individually in the PAMPA-BBB model to investigate the occurring pharmacokinetic interactions. The $logP_e$ values of sudachitin/aceronin and parthenolide measured in the extract and as individual compounds were not significantly different, but nevadensin showed higher permeability potential when measured in the extract than as purified compound. Luteolin, apigenin, axillarin and casticin were found to be BBB negative.

5. The essential oil yield of three *Tanacetum parthenium* L. samples was studied.
    - Leaves collected during flowering and flower heads were investigated. Our studies supported the presumption that the leaves collected during flowering contained the highest amount of volatile oil.
    - The essential oil and supercritical fluid extracts were analyzed with HS-SPME-GC/MS method. 11 volatile compounds were identified in the essential oil and 7 in the supercritical fluid extract. The main compounds in all samples were camphor and chrysanthenyl acetate.
    - A simple and rapid method was developed and validated for the qualitative and quantitative analysis of the camphor content in steam distilled samples and supercritical fluid extracts of *Tanacetum parthenium* L. The UPC^2 method was validated for selectivity, accuracy, repeatability and intermediate precision, linearity, limit of detection, limit of quantification and robustness. The validation was successful with advantages of high resolution, sensitivity and very good reproducibility and the method was proven to be reliable and accurate. Very short sample preparation and analysis time have been achieved, the latter with a remarkable 0.785 min retention of camphor. The highest camphor content was measured in the essential oil of the leaves during

flowering and the lowest in the supercritical fluid extracts. The presence of nonvolatile apolaric and semipolaric compounds in the extracts beside the volatile terpenoids gives preference to the chromatographic method using supercritical fluid.

Publications

Publications related to the thesis

Végh K, Riethmüller E, Hosszú L, Darcsi A, Müller J, Alberti Á, Tóth A, Béni Sz, Könczöl Á, Balogh Gy T, Kéry Á. (2018). Three newly identified lipophilic flavonoids in *Tanacetum parthenium* supercritical fluid extract penetrating the Blood-Brain Barrier. J Pharm Biomed Anal, 149:488-493.

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