Reprinted from:
Journal of Ethnopharmacology 136 (2011) 444-451

ISSN 0378-8741

ELSEVIER

Journal of ETHNO-PHARMACOLOGY

Ein wässriger Extrakt von Betula pendula-Blättern hemmt das Wachstum und die Teilung von aktivierten Lymphozyten

Journal of Ethnopharmacology 136 (2011) 444–451

Inhaltsverzeichnisse über ScienceDirect verfügbar

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Ein wässriger Extrakt von Betula pendula-Blättern hemmt das Wachstum und die Teilung von aktivierten Lymphozyten

Carsten Gründemann[a,*], Christian W. Gruber[b,1], Anke Hertrampf[a], Martin Zehl[b], Brigitte Kopp[b], Roman Huber[a]

[a] *Center for Complementary Medicine, Department of Environmental Health Sciences, University Medical centre Freiburg, Breisacher Str. 115B, 79106 Freiburg, Germany*
[b] *Department of Pharmacognosy, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria*

ARTIKELINFORMATION

Schlüsselwörter:
Betula pendula
Flavonoide
Arthritis
Lymphozyten
Apoptose
Anthroposophische Medizin

ZUSAMMENFASSUNG

Ethnopharmakologische Relevanz: Extrakte aus den Blättern von *Betula pendula* werden traditionell zur Behandlung von Patienten mit rheumatoider Arthritis (RA) oder Osteoarthritis eingesetzt.
Zielsetzung der Studie: Wir untersuchten *in vitro* die antiproliferativen Effekte eines wässrigen Extrakts von *Betula pendula* (BPE)-Blättern auf primäre humane Lymphozyten, da aktivierte Lymphozyten bei der Entstehung und Manifestierung der RA eine bedeutende Rolle spielen.
Material und Methoden: Lymphozytenproliferation und -teilung wurden über die mitochondriale Dehydrogenase-Aktivität sowie per Markierung mit dem membrangängigen Fluoreszenz-Farbstoff Carboxyfluoresceindiacetatsuccinimidylester (CSFE) gemessen. Die Analyse der Apoptose erfolgte anhand durchflusszytometrischer und photometrischer Verfahren durch Oberflächenfärbung von Phosphatidylserin und die intrazelluläre Aktivierung der Effektor-Caspasen 3 und 7. Dabei wurde gegen Methotrexat verglichen. Zusätzlich wurde der Effekt des Extrakts auf den Zellzyklus mittels DNA-Färbung mit Propidiumjodid geprüft. Im Rahmen der Bioassays wurden BPE-Konzentrationen von 10–160 µg/ml untersucht. Um die polyphenolischen Inhaltsstoffe des Birkenblattextrakts zu identifizieren, wurde eine phytochemische Analyse mittels LC-MS und HPLC vorgenommen.
Ergebnisse: Die Extrakte von *Betula pendula*-Blättern hemmten dosisabhängig und signifikant das Wachstum und die Teilung von aktivierten T-Lymphozyten, nicht jedoch die der ruhenden T-Lymphozyten ($CD8^+$: 40 µg/ml: 45 %; 80 µg/ml: 60 %; 160 µg/ml: 87 %) ($CD4^+$: 40 µg/ml: 33 %; 80 µg/ml: 54 %; 160 µg/ml: 79 %). Die Hemmung der Lymphozytenproliferation durch Apoptoseinduktion (verglichen mit der unbehandelten Kontrolle: 40 µg/ml: 163 %; 80 µg/ml: 240 %; 160 µg/ml: 384 %) und das Ausmaß der Zellzyklus-Arretierung waren mit Methotrexat vergleichbar. LC-MS-Analysen zeigten, dass der Extrakt verschiedene Quercetin-Glycoside enthält.
Schlussfolgerung: Die Ergebnisse unserer Untersuchung bieten eine rationale Grundlage für die Anwendung des *Betula pendula*-Extrakts zur Therapie von Erkrankungen des Immunsystems wie der rheumatoiden Arthritis durch Reduktion der Anzahl von proliferierenden inflammatorischen Lymphozyten.

Journal of Ethnopharmacology 136 (2011) 444–451

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

An aqueous birch leaf extract of *Betula pendula* inhibits the growth and cell division of inflammatory lymphocytes

Carsten Gründemann[a,*], Christian W. Gruber[b,1], Anke Hertrampf[a], Martin Zehl[b], Brigitte Kopp[b], Roman Huber[a]

[a] *Center for Complementary Medicine, Department of Environmental Health Sciences, University Medical centre Freiburg, Breisacher Str. 115B, 79106 Freiburg, Germany*
[b] *Department of Pharmacognosy, University of Vienna, Althanstr. 14, A-1090 Vienna, Austria*

ARTICLE INFO

Article history:
Received 20 December 2010
Received in revised form 18 April 2011
Accepted 11 May 2011
Available online 17 May 2011

Keywords:
Betula pendula
Flavonoids
Arthritis
Lymphocytes
Apoptosis
Anthroposophic medicine

ABSTRACT

Ethnopharmacological relevance: Leaf extracts of *Betula pendula* have been traditionally used for the treatment of patients with rheumatoid arthritis (RA) or osteoarthritis.
Aim of the study: We investigated the anti-proliferative capacity of an aqueous leaf extract of *Betula pendula* (BPE) on human primary lymphocytes *in vitro*, because activated lymphocytes play a major role in the initiation and maintenance of RA.
Materials and methods: Lymphocyte proliferation and cell division was measured by the activity of mitochondrial dehydrogenases and by using the membrane-permeable dye carboxyfluorescein diacetate succinimidyl ester (CFSE), respectively. Apoptosis was analyzed by surface staining of phosphatidylserine and intracellular activation of effector caspases 3 and 7 in comparison to the drug methotrexate using flow cytometric and photometrical analysis. In addition, the impact of the extract on cell cycle distribution was investigated by propidium iodide staining of DNA. For the bioassays BPE concentrations of 10–160 μg/mL were investigated. A phytochemical analysis, using LC–MS and HPLC, was conducted to identify the polyphenolic constituents of the birch leaf extract.
Results: Leaf extracts of *Betula pendula* inhibited the growth and cell division ($CD8^+$: 40 μg/mL: 45%; 80 μg/mL: 60%; 160 μg/mL: 87%) ($CD4^+$: 40 μg/mL: 33%; 80 μg/mL: 54%; 160 μg/mL: 79%) of activated, but not of resting T lymphocytes in a significant dose-dependent manner. The inhibition of lymphocyte proliferation due to apoptosis induction (compared to untreated control: 40 μg/mL: 163%; 80 μg/mL: 240%; 160 μg/mL: 348%) and cell cycle arrest was comparable to that of methotrexate. LC–MS analyses showed that the extract contains different quercetin-glycosides.
Conclusion: Our results give a rational basis for the use of *Betula pendula* leaf extract for the treatment of immune disorders, like rheumatoid arthritis, by diminishing proliferating inflammatory lymphocytes.

1. Introduction

Rheumatoid arthritis (RA) is a common chronic systemic inflammatory disorder that affects approximately 1% of the population worldwide (Harris, 1990). Its predominant symptoms include pain, stiffness, and swelling of peripheral joints. The synovial membrane of patients with arthritis is characterized by hyperplasia, increased vascularity, and a strong infiltrate of peripheral inflammatory cells from the blood, primarily lymphocytes. They regulate cell-mediated immune-inflammation and maintain activation of macrophages and synovial fibroblasts, transforming them into tissue-destructive effector cells (Choy and Panayi, 2001). Peripheral T lymphocytes are the dominant cell types in the synovial filtrate and there is at least a partial therapeutic effect of T-cell depletion for patients suffering from RA (Berner et al., 2000).

Several effective therapies, including methotrexate, which act through the induction of apoptosis of proliferating cells (e.g. T-lymphocytes), are in use to treat patients with RA (Genestier et al., 1998). Despite the existence of effective conventional medications, approximately 60–90% of patients with arthritis use alternative therapies to avoid side-effects (Rao et al., 1999).

One of these traditionally used therapies includes herbal remedies of members of the Betulaceae family, which grow mainly in northern Europe (Demirci et al., 2004). Leaf extracts of *Betula pendula* Roth, also well known as the European white birch or silver birch, are traditionally used all over Europe to treat rheumatism and arthritic diseases (Havlik et al., 2010; Saric-Kundalic et al., 2010). Studies suggest that extracts from *Betula pendula* have mild

* Corresponding author. Tel.: +49 761 270 83220; fax: +49 761 270 83230.
E-mail address: carsten.gruendemann@uniklinik-freiburg.de (C. Gründemann).
[1] Current address: Medical University of Vienna, Centre for Physiology and Pharmacology, Schwarzspanierstr. 17, A-1090 Vienna, Austria.

0378-8741/$ – see front matter © 2011 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2011.05.018

diuretic effects by inhibiting endopeptidases (Schilcher and Rau, 1988; Major, 2002), and anti-inflammatory capacity (Tunon et al., 1995; Trouillas et al., 2003). They were also used for the supportive treatment of rheumatic diseases in anthroposophic medicine (Pieroni and Gray, 2008; Girke, 2010).

Because peripheral blood lymphocytes play an important role in the perpetuation of the autoimmune processes in RA and the maintenance of these cells might be caused by the dysregulation of proliferation and apoptosis, we investigated the influence of an extract of *Betula pendula* on primary human lymphocytes in comparison to the synthetic anti-arthritis drug methotrexate *in vitro*.

2. Materials and methods

2.1. Aqueous Betula pendula extract preparation

The investigated aqueous *Betula pendula* extract (BPE) is an injectable plant extract of birch leaves of *Betula pendula* Roth which is marketed by Abnoba GmbH (Pforzheim, Germany) as an officially registered preparation (*Betula folium* D3 Abnoba, batch-no. 706 A41 was used for the investigations) according to Section 38/39 of the German Drug Law. The extract is for legal reasons manufactured according to method no. 32 of the German Homeopathic Pharmacopoeia (GHP). The harvested birch leaves are extracted by maceration in a patented pressing machine under protective atmosphere to avoid any oxidation. The finished BPE contains 1.88 mg of fresh plant material, corresponding to an anhydrous mass of 0.63 mg, per mL. The finished product is sterile filtered, filled in glass ampoules and released for sale if all compulsory controls meet the current specifications for solutions for injection of the European Pharmacopoeia (EP). All specifications for parenteral medications were fulfilled according to the EP. Good Manufacturing Practise (GMP) and quality control according to the EP is monitored from the German authorities (Federal Institute for Drugs and Medical Devices; BfArM). This includes proof of plant source identity and absence of contamination with heavy metals, pesticides, aflatoxins and microorganisms. Ampoules from the sales stock were sent to our laboratory in Freiburg, Germany, where the cell biological experiments were performed. For each experiment, a fresh ampoule of the *Betula pendula* extract was used and concentrations of 10, 20, 40, 80 and 160 μg/mL were tested.

2.2. Preparation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of adult donors obtained from the Blood Transfusion Centre (University Medical Center Freiburg, Freiburg, Germany). All experiments conducted on human material were approved by the Ethics committee of the University Freiburg. Venous blood was centrifuged on a LymphoPrep™ gradient (density: 1.077 g/cm^3, 20 min, 500 × *g*, 20 °C; Progen, Heidelberg, Germany). Afterwards cells were washed twice with medium and cell viability and concentration was determined using the trypan blue exclusion test.

2.3. Cell culture and primary human material

PBMC were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS (PAA, Coelbe, Germany), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen, Karlsruhe, Germany). The cells were cultured at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere.

2.4. Activation of peripheral blood mononuclear cells

PBMC (10^5) were stimulated with anti-human CD3 (clone OKT3) and anti-human CD28 (clone 28.6) mAbs (both from ebioscience, Frankfurt, Germany) for 96 h in the presence of medium, methotrexate (1 mg/mL; Sigma–Aldrich, Taufkirchen, Germany) and Triton-X 100 (0.5%; Carl Roth, Karlsruhe, Germany) as controls or different concentrations of BPE, respectively. After cultivation the cells were assessed as described below.

2.5. Determination of cell proliferation

Cell proliferation rate was determined in three independent experiments by the WST-1 assay, commercially available from Roche (Mannheim, Germany) and was accomplished according to the manufacturer's instructions. The assay is based on the cleavage of the tetrazolium salt WST-1 by cellular enzymes to a formazan dye. The absorbance was measured with an ELISA reader (Tecan, Crailsheim, Germany) at a wavelength of 450 nm.

2.6. Cell division tracking using CFSE

PBMC were harvested and washed twice in cold PBS and resuspended in PBS at a concentration of 5×10^6 cells/mL. CFSE (carboxyfluorescein diacetate succinimidyl ester, 5 mM; Sigma, Taufkirchen, Germany) was diluted 1/1000 and incubated for 10 min at 37 °C. The staining reaction was stopped by washing twice with complete medium. Afterwards, the PBMC were additionally stained with anti-human CD4 and anti-human CD8 mAbs (both from ebioscience, Frankfurt, Germany) and cell division progress was analyzed from three independent experiments using flow cytometry.

2.7. Flow cytometric estimation of cell cycle distribution

Cellular DNA content of three independent experiments was measured after permeabilization of PBMC by fixation with 70% ethanol and staining of the DNA with PI master mix (40 μg/mL propidium iodide, 100 μg/mL RNase; both from Sigma, Taufkirchen, Germany and PBS), allowing the identification of the generally called "G_0/G_1"-, "S"-, "G_2"-phase peaks of cells on a DNA content frequency histogram. Analysis was performed by flow cytometry. The software Modifit© (Verity Software House, Topsham, USA) was used to analyze the DNA frequency histograms and to estimate the proportions of cells in particular phases of the cell cycle.

2.8. Determination of PBMC apoptosis and necrosis using annexin V and propidium iodide staining

The levels of apoptosis were determined in three independent experiments using the annexin V-FITC Apoptosis Detection kit (ebioscience, Frankfurt, Germany) according to the manufacturer's instructions. After annexin V staining, propidium iodide (PI; ebioscience, Frankfurt, Germany) was added and the cells were incubated for additional 10 min in the dark, followed by a flow cytometric analysis to determine the amount of apoptosis and necrosis.

2.9. Caspase-3/7 assay

The caspase-3 and -7 activities were measured from three independent experiments using the protocol supplied by Promega (Mannheim, Germany). The assay is based on a luminogenic caspase-3/7 substrate, which contains the tetrapeptide sequence DEVD for caspases. Following caspase cleavage, the substrate for luciferase (aminoluceferin) is released, resulting in the luciferase reaction and the production of light. Hereby, the luminescence is proportional to the amount of caspase activity and was measured using a microplate reader (Tecan, Crailsheim, Germany).

2.10. Data analysis

For statistical analysis, data were exported to SigmaPlot® (Systat Software Inc., Erkrath, Germany). Data are presented as mean + standard deviation and statistical significance was determined by Student's paired *t*-test. *P* values <0.05 (*) were considered statistically significant and <0.01 (**) were considered highly statistically significant, compared to the respective control.

2.11. Preparation of Betula pendula extract for phytochemical analysis

The BPE content of 24 phials was combined (24 mL). The combined aqueous solution was fractionated and concentrated with solid phase extraction (SPE) using Strata C18-E (55 μm, 70 Å), 2 g/12 mL Giga Tubes (Phenomenex, Germany). The SPE cartridges were washed twice with one cartridge volume of acetonitrile and equilibrated three times with 1 vol. of aqueous 0.1% formic acid (FA). The aqueous *Betula pendula* solution containing 0.1% FA was applied to the cartridge, washed three times with 10% acetonitrile/90% H_2O containing 0.1% FA and eluted twice with 60% acetonitrile/40% H_2O containing 0.1% FA. Excess solvent was reduced by roto-evaporation and the *Betula pendula* fraction was dried to powder by lyophilisation. The dry *Betula pendula* powder was dissolved in 0.5 mL H_2O (pH 2.8 with FA) and analyzed by LC–MS.

2.12. Liquid chromatography–mass spectrometry (LC–MS)

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 on a Hypersil BDS-C18 column (4.0 mm × 250 mm, 5 μm, Agilent, Santa Clara, CA, USA) at 20 °C and a flow rate of 1.0 mL/min. Water (pH 3.1 with FA) and acetonitrile were used as mobile phase A and B, respectively. The following gradient program was used: 14% B (0 min), 19% B (20 min), 95% B (22 min) and 95% B (29 min). The eluent flow was split roughly 1:8 before the ESI ion source, which was operated as follows: capillary voltage: 3.7 kV, 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* 80–750, average of 5 spectra) was followed by MS^2 scans (*m*/*z* 40–750, average of 3 spectra, isolation window of 4 Th, fragmentation amplitude of 1.0 V) of the two most intense precursor ions, and MS^3 scans (*m*/*z* 40–750, average of 3 spectra, isolation window of 4 Th, fragmentation amplitude of 1.0 V) of the two most intense fragment ions in each MS^2 scan. The flavonoid aglyca were identified by spectrum matching to an in-house library containing the MS^n spectra of 57 reference flavonoids using ESI compass 1.3 for HCT (Bruker Daltonics). Finally, four of the identified components were confirmed by comparison of the retention times, UV- and MS-spectra with the reference compounds quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-rhamnoside (quercitrin), and quercetin-3-*O*-arabinoside (avicularin) (all from Carl Roth, Karlsruhe, Germany).

2.13. Semi-quantitative analysis of the flavonoid glycosides

The content of the major flavonoid glycosides in the SPE fraction was determined by HPLC-DAD using a simple two-point calibration with hyperoside as an external standard. The analyses were conducted on a Shimadzu prominence system equipped with a SIL-20AC HT auto sampler, an LC-20AD pump, a CTO-20 AC column oven, and an SPD-M20A diode array detector (Shimadzu Austria GmbH, Vienna, Austria). The column and the elution conditions were identical to LC–MS. A stock solution of hyperoside (purity >99% by HPLC, Carl Roth) was prepared by dissolving 0.96 mg in 1 mL DMSO and completion to 5.0 mL with methanol. The two calibration solutions with 38.4 and 7.68 μg/mL were obtained by stepwise 5-fold dilution with methanol. Ten microlitres of the SPE fraction and the two calibration solutions were injected in triplicate. The chromatograms at 354 ± 2 nm were used to determine the peak areas.

3. Results

3.1. Birch leaf extract reduced the growth of activated human PBMC

For a first characterization, we evaluated the effect of BPE on cell growth and performed proliferation assays with increasing concentrations of BPE (10–160 μg/mL) on stimulated and resting human PBMC in comparison to methotrexate (Fig. 1). As shown in Fig. 1A, BPE significantly reduced the proliferation of

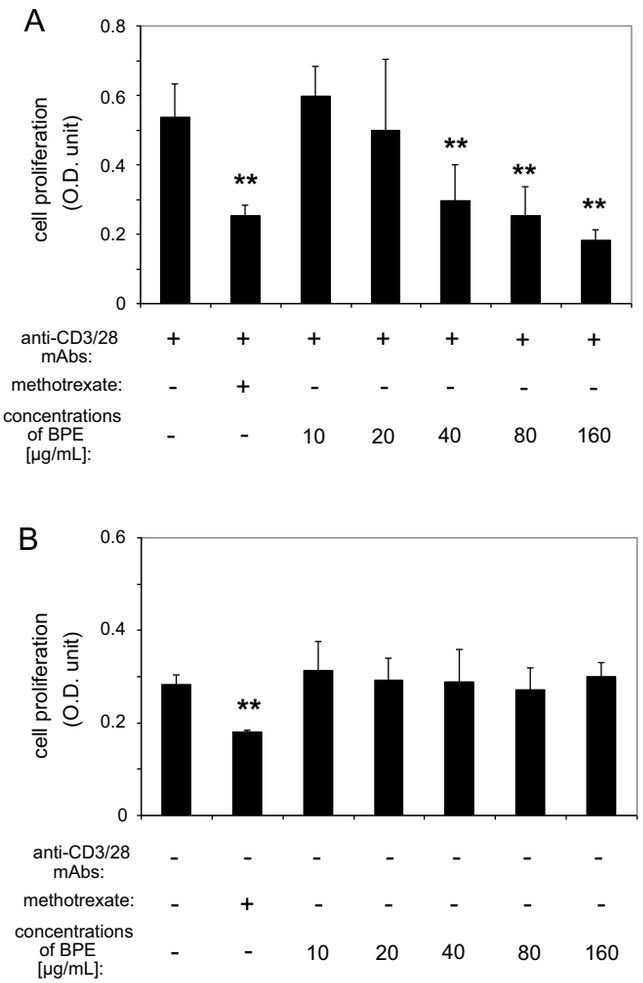

Fig. 1. Effects of the *Betula pendula* extract on cell proliferation of human peripheral blood mononuclear cells. Isolated human PBMC either activated with anti-CD3/CD28 mAbs (A) or unstimulated (B), were cultured in the presence of medium, methotrexate (1 mg/mL) or *Betula folium* extract (10–160 μg/mL). The cells were further analyzed for cell viability using the WST-1 assay and quantified by spectrophotometric analysis. Data are presented as mean and standard deviation (SD) of three independent experiments. The asterisks represent significant differences from untreated stimulated cells (*$P < 0.05$, **$P < 0.01$).

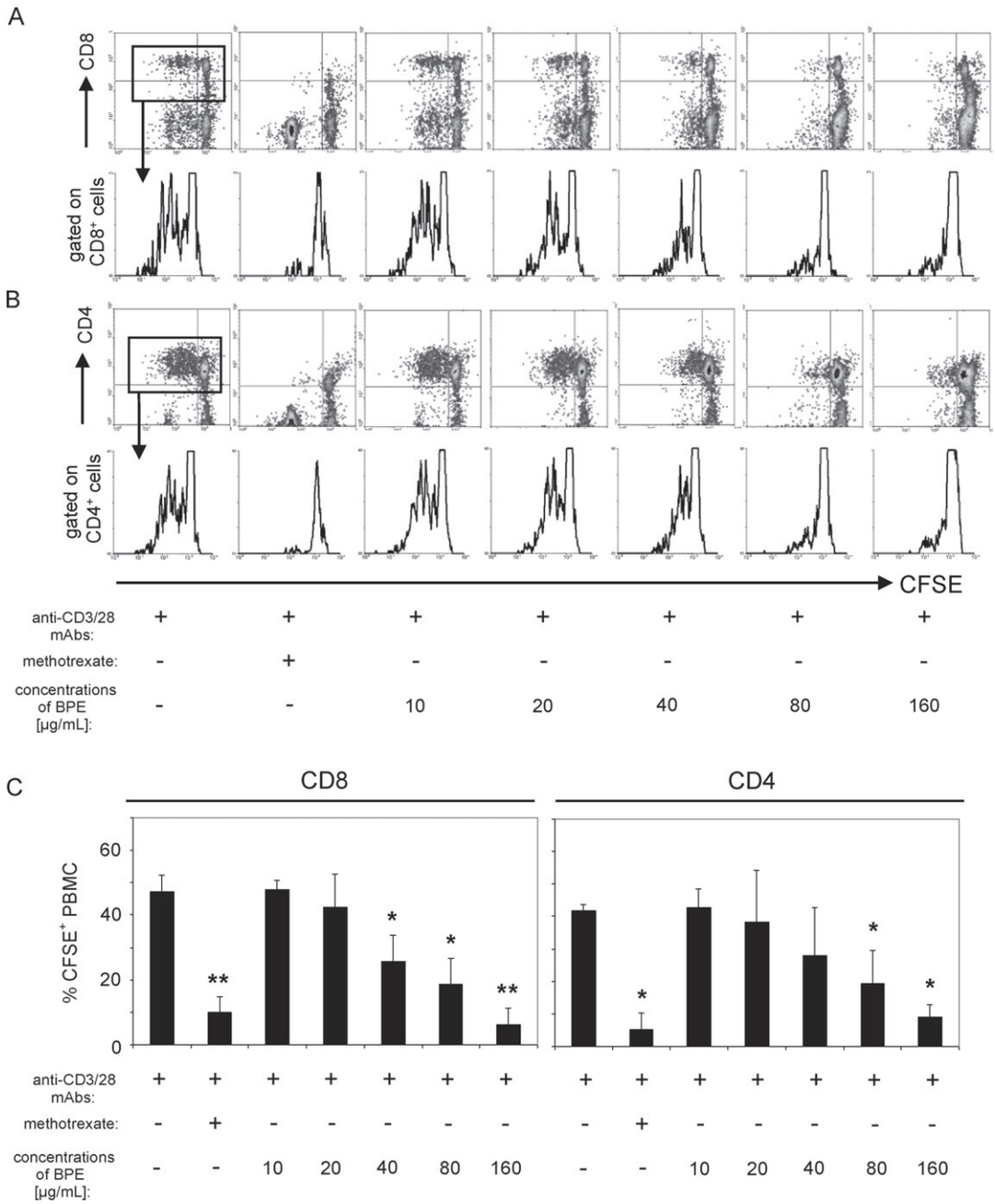

Fig. 2. Effects of the *Betula pendula* extract on cell division of peripheral human blood mononuclear cells. CFSE-labeled primary human PBMC were antibody-activated (anti-CD3/CD28 mAbs) and cultured in the presence of medium, methotrexate (1 mg/mL) or different concentrations of *Betula pendula* extract (10–160 µg/mL). Cell division analyses were assessed by flow cytometry and pictured as representative dot plots (upper panel) and histograms (lower panel) in (A) for $CD8^+$ PBMC and (B) for $CD4^+$ PBMC. Results are summarized for CD8 (left graph) and CD4 (right graph) in (C) and data are presented as mean and standard deviation (SD) of three independent experiments. The asterisks represent significant differences from untreated stimulated cells ($^*P < 0.05$, $^{**}P < 0.01$).

antibody-activated PBMC in a dose-dependent manner (40 µg/mL: 0.29 ± 0.10; 80 µg/mL: 0.25 ± 0.08 and 160 µg/mL: 0.18 ± 0.03) compared to untreated control (0.54 ± 0.1). The effects were comparable to that of methotrexate (1 mg/mL: 0.25 ± 0.09). There was no effect of BPE on proliferation of resting PBMC (Fig. 1B).

3.2. Birch leaf extract inhibited cell division of activated human PBMC

We further evaluated the impact of BPE on cell division of activated PBMC using flow cytometric-based analysis of CFSE-labeled cells (Fig. 2). The CFSE label is inherited by daughter cells after cell division and each dividing cell loses fluorescent intensity. The data shown in Fig. 2C indicate that the BPE treatment at concentrations of 40, 80 and 160 µg/mL caused a dose-dependent cell division inhibition of PBMC, especially of $CD8^+$ (40 µg/mL: 25.7%; 80 µg/mL: 18.7%; 160 µg/mL: 6.2%), as well as of $CD4^+$ (40 µg/mL: 27.9%; 80 µg/mL: 19.3%; 160 µg/mL: 8.9%) PBMC subpopulation in comparison to untreated cells ($CD8^+$: 47.1%; $CD4^+$: 41.7%). In the presence of methotrexate, which was used as a positive control, the cells showed a comparably strong inhibition of cell division ($CD8^+$: 10.1%; $CD4^+$: 5.2%).

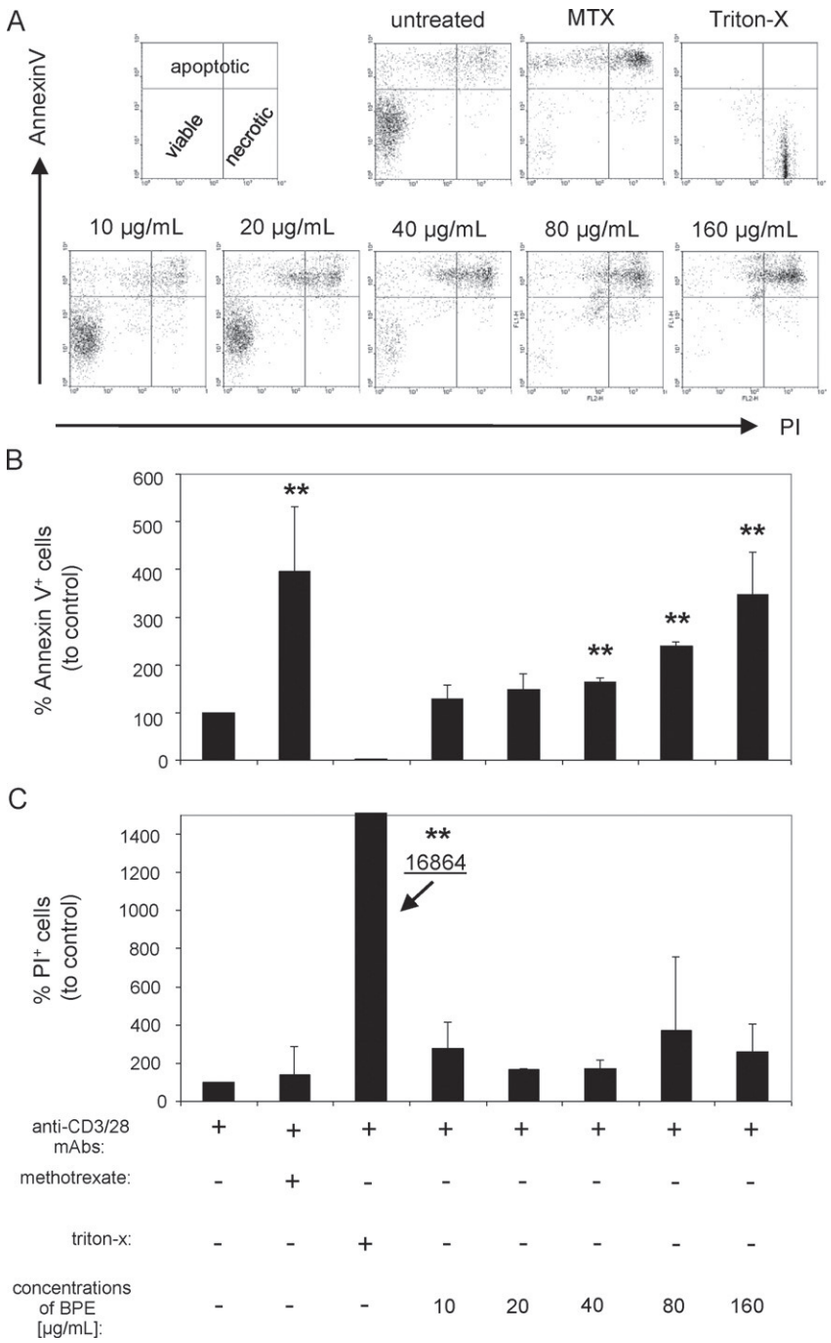

Fig. 3. Effects of the *Betula pendula* extract on apoptosis and necrosis of peripheral human blood mononuclear cells. After activation of human primary PBMC with anti-CD3/CD28 mAbs in the presence of medium, methotrexate (1 mg/mL), Triton-X 100 (0.5%) or different concentrations of *Betula pendula* extract (10–160 μg/mL), the cells were stained with annexin V and propidium iodide (PI) to assess the percentages of viable (annexin V^-/PI^-), apoptotic (annexin V^+/PI^-) and necrotic (annexin V^-/PI^+) cells. The cells were analyzed by flow cytometry and representative results a depicted in (A). Results from analysis of apoptotic (B) and necrotic (C) cells are summarized and data are presented as mean and standard deviation (SD) of three independent experiments. The asterisks represent significant differences from untreated stimulated cells alone ($^*P < 0.05$, $^{**}P < 0.01$).

3.3. Birch leaf extract induced apoptosis in human PBMC

To determine whether the BPE-mediated reduction of cell growth and inhibition of cell division of activated PBMC was of apoptotic or necrotic origin, we first combined the annexin V and propidium iodide staining (Fig. 3). This allowed the discrimination between viable (annexinV^-/PI^-), apoptotic (annexinV^+/PI^+) or necrotic (annexinV^-/PI^+) cells. After exposure of PBMC to BPE, the data revealed a concentration-dependent increase of apoptotic$^+$ cells (Fig. 3B) (40 μg/mL: 163.3%; 80 μg/mL: 239.9%; 160 μg/mL 348.1%, compared to untreated cells), whereas the percentages of necrotic cells was still low (Fig. 3C).Activation of caspases, a family of cysteine proteases, is an essential step in various forms of apoptosis. The activation of effector caspases 3 and 7 was confirmed by photometrical analysis, which showed strong pro-apoptotic activity of methotrexate-treated activated PBMC (compared to untreated control:605.8%, Fig. 4). The data further showed a dose-dependent pro-apoptotic effect of BPE in comparison to untreated controls (see Fig. 4) (40 μg/mL: 322.1%; 80 μg/mL: 480 μg/mL and 160 μg/mL: 531.9%).

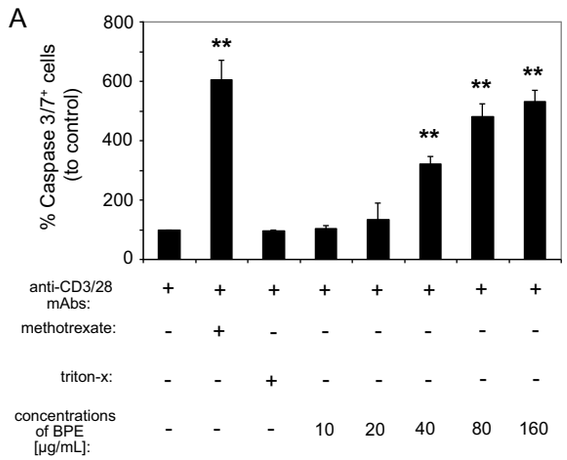

Fig. 4. Effects of the *Betula pendula* extract on caspase 3/7 activity of peripheral human blood mononuclear cells. The influence of medium, methotrexate (1 mg/mL), Triton-X 100 (0.5%) or different concentrations of *Betula pendula* extract (10–160 μg/mL) on caspase 3/7 activity of anti-CD3/CD28 mAbs activated human primary PBMC was measured by luciferase reaction assay using a microtiter plate reader. Data are presented as mean and standard deviation (SD) of three independent experiments. The asterisks represent significant differences from stimulated cells alone (*$P < 0.05$, **$P < 0.01$).

3.4. Birch leaf extract halted the cell cycle of activated human PBMC

Besides the induction of apoptosis, the observed decrease in cell proliferation of the activated PBMC after BPE treatment could also be a consequence of cell cycle arrest. Therefore, we analyzed the effect of BPE on cell cycle progression using flow cytometry. Fig. 5 shows that at 40, 80 and 160 μg/mL, BPE caused a dose-dependent arrest in the G_0/G_1 phase (Fig. 5A) (untreated: 85.5%; 40 μg/mL: 93.6%; 80 μg/mL: 96.4%; 160 μg/mL: 97.6%) and a concomitant reduction in the number of cells in the S phase (Fig. 5B) (untreated: 11.8%; 40 μg/mL: 3.9%; 80 μg/mL: 2.6%; 160 μg/mL: 1.9%) and G_2/M phase (Fig. 5C) (untreated: 2.7%; 40 μg/mL: 1.03%; 80 μg/mL: 0.85%; 160 μg/mL: 0.45%) of the cell cycle compared to untreated cells. Methotrexate showed similar effects (G_0/G_1: 96.4%; S: 2.7%; G_2/M: 0.9%).

3.5. Phytochemical analysis of the birch leaf extract

For further interpretation of the observed effects of the BPE, it is important to know it's molecular composition. For this purpose, we carried out sample cleanup by SPE followed by LC–MS analysis, and identified several flavonoid-glycosides. The major phenolic components in the SPE extract were found to be quercetin-monoglycosides (see Fig. 6 and Table 1). Four

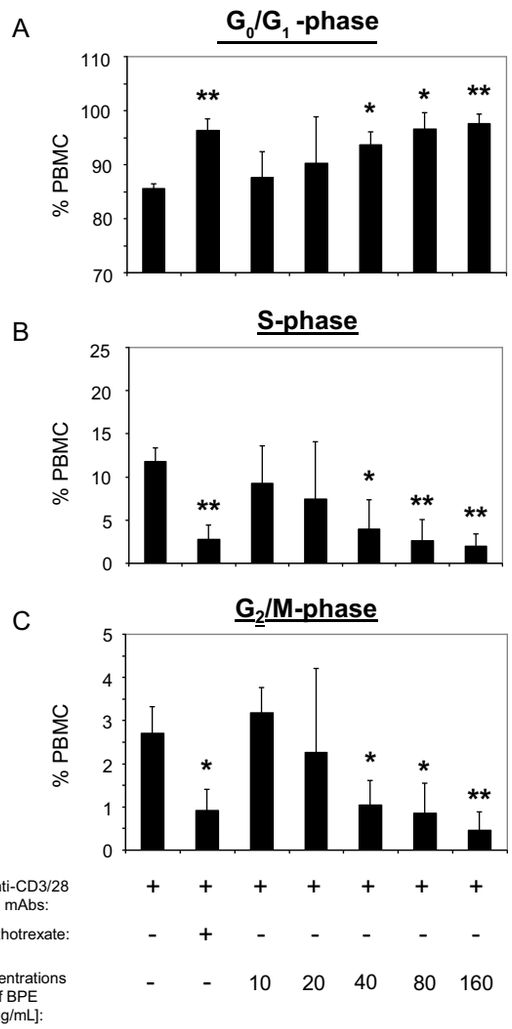

Fig. 5. Effects of the *Betula pendula* extract on cell cycle distribution of peripheral human blood mononuclear cells. Flow cytometric analysis of DNA histogram of human primary PBMC treated with anti-CD3/CD28 mAbs in the presence of medium, methotrexate (1 mg/mL), Triton-X 100 (0.5%) or different concentrations of *Betula pendula* extract (10–160 μg/mL). (A) G_0/G_1 sub-phase, (B) S sub-phase and (C) G_2/M sub-phase distribution of cell cycle (%) from three independent experiments. Data presented are mean ± SD and the asterisks represent significant differences from untreated stimulated cells (*$P < 0.05$, **$P < 0.01$).

of these constituents were confirmed by comparison of the retention times, UV- and MS^n-spectra with the reference compounds quercetin-3-*O*-galactoside (hyperoside), quercetin-3-*O*-glucoside (isoquercitrin), quercetin-3-*O*-arabinoside (avicularin), and quercetin-3-*O*-rhamnoside (quercitrin), which is in agreement

Table 1
Flavonoid glycosides identified by LC–MS and semi-quantified by HPLC-DAD.

#	t_R (min)	$[M-H]^-$ (*m/z*)	Proposed structure	Main fragment ions (>10% Rel. Int.)	SPE fraction conc. (μg/mL)
1	12.9	477.1	Quercetin-*O*-hexuronide	MS^2 [477.1]: 301.0 MS^3 [301.0]: 178.8, 150.9, 107.1	17.2 ± 0.7
2	13.7	477.1	Quercetin-*O*-hexuronide	MS^2 [477.1]: 301.0 MS^3 [301.0]: 178.8, 150.9, 107.0	n.d.
3	14.1	463.1	Quercetin-3-*O*-galactoside (hyperoside)	MS^2 [463.1]: 300.9 MS^3 [300.9]: 270.9, 254.9, 178.9, 150.9	25.7 ± 1.1
4	14.9	463.1	Quercetin-3-*O*-glucoside (isoquercitrin)	MS^2 [463.1]: 300.9 MS^3 [300.9]: 270.9, 254.9, 178.9, 150.9, 107.1	2.7 ± 0.1
5	18.0	433.1	Quercetin-3-*O*-arabinoside (avicularin)	MS^2 [433.1]: 300.1 MS^3 [300.1]: 270.9, 254.9, 178.8, 150.9	7.8 ± 0.3
6	20.6	447.1	Quercetin-3-*O*-rhamnoside (quercitrin)	MS^2 [447.1]: 300.9 MS^3 [300.9]: 270.9, 254.9, 178.8, 150.9, 107.0	3.4 ± 0.1

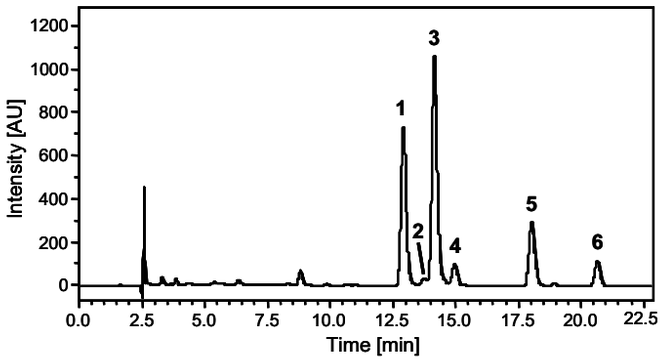

Fig. 6. The flavonoid glycosides in *Betula pendula* extract. HPLC chromatogram showing the DAD response at 354 ± 2 nm. Proposed structures of corresponding compounds 1–6 can be found in Table 1.

to previous results (Pietta et al., 1989). In addition, a high and a low abundant quercetin-*O*-hexuronide were identified. Based on the literature data on *Betuala pendula*, the more abundant of these two is most likely quercetin-3-*O*-glucuronide (Pietta et al., 1989).

Flavonoids, in particular quercetin, have been indicated to possess immunomodulatory activity in previous studies (see Section 4), and thus may explain at least part of the activities of the BPE described above. Therefore, we conducted a semi-quantitative HPLC-DAD analysis of the major quercetin-monoglycosides identified by LC–MS. The concentrations of the constituents in the SPE fraction were determined using a simple two-point calibration with hyperoside as an external standard (Table 1). Since the UV spectra of the individual quercetin-3-*O*-glycosides are highly similar, the response factors were calculated based on the ratios of the molecular weights. The concentration in the BPE might be less up to a factor of 48 (the recovery of the SPE was not evaluated), which gives at least the following concentrations: 535 ± 22 ng/mL hyperoside, 358 ± 15 ng/mL quercetin-*O*-hexuronide, 162 ± 6 ng/mL avicularin, 70 ± 3 ng/mL quercitrin, and 56 ± 2 ng/mL isoquercitrin.

4. Discussion

Leaf extracts of *Betula pendula* are traditionally used all over Europe for the supportive treatment of patients with RA or osteoarthritis. Because peripheral lymphocytes play an important role in the perpetuation of rheumatoid arthritis, and the maintenance of these cells is caused by a dysregulation of proliferation and apoptosis, we investigated the effects of a birch leaf extract on primary human immunocompetent cells *in vitro*.

In the current study, we demonstrated that an aqueous leaf extract of *Betula pendula* inhibited the growth and cell division of activated T lymphocytes in a dose-dependent manner (40–160 μg/mL). At these concentrations the reduction on cell proliferation of peripheral blood mononuclear cells was of apoptotic origin and nearly the same in $CD4^+$ as well in $CD8^+$ lymphocyte subpopulation. Apoptosis is a form of programmed cell death that is essential for normal development, cancer prevention, resolution of inflammation (Liu and Pope, 2004) and could be detected by surface staining of phosphatidylserine and intracellular activation of effector caspases 3 and 7 using flow cytometric and photometrical analysis. The combination of annexin V and propidium iodide staining allows analyzing apoptotic and necrotic cells in parallel. Our data clearly indicate that, compared to methotrexate and detergent (Triton-X 100), which were used as positive controls for apoptosis and necrosis induction, respectively, the leaf extract of *Betula pendula* mainly induces apoptosis, whereas only a small proportion of necrotic cells were detectable. These inhibitory effects suggested that the *Betula pendula* leaf extract could be therapeutically used for the treatment of immunological disorders, like rheumatoid arthritis, by diminishing the amount of inflammatory lymphocytes. During early stages of rheumatoid arthritis, large numbers of lymphocytes are recruited from the peripheral blood in response to injury or infection. Clearance of unwanted effector cells at the end of an inflammatory response appears to be due to loss of survival signals derived from interactions with stromal cells, leading to apoptosis and subsequent phagocytosis of dead cells. In chronic inflammatory diseases, the resolution phase becomes prolonged and disordered, leading to persistence of the inflammatory lymphocyte infiltrate, tissue hyperplasia and ultimately tissue scarring. Persistence of a lymphocyte infiltrate at sites of chronic inflammation reflects a distorted homeostatic balance between factors that enhance cellularity (lymphocyte recruitment, proliferation and retention) and those that decrease cellularity (cell death and emigration) (Akbar and Salmon, 1997; Buckley, 2003). Methotrexate-induced apoptosis promotes the resolution of an inflammatory response by reducing the number of inflammatory cells (Liu and Pope, 2004).

Remarkably, non-activated T lymphocytes were resistant to apoptosis up to 160 μg/mL BPE demonstrating selectivity towards activated lymphocytes. Methotrexate-treated resting peripheral blood mononuclear cells, on the other hand, showed an inhibition on proliferation, which is in contrast to published data from Genestier et al. (1998), that showed no influence of methotrexate on resting peripheral blood mononuclear cells. This could be due to the different kind of activation stimuli or doses used in the assays.

Besides apoptosis induction, the decrease in cell proliferation was further due to an arrest in the G_0/G_1 phase and the concomitant concentration-dependent decrease (40–160 μg/mL) in the percentage of peripheral blood mononuclear cells in the S- and G_2/M phase of the cell cycle.

Members of the Betulaceae family are known to contain appreciable amounts of polyphenolic compounds (Keinänen and Julkunen-Tiitto, 1998) and several compounds of this diverse group of phytochemicals have attracted increasing interest in the past due to their physiological actions as immunomodulators (Wattel et al., 2004; Nair et al., 2006; Sternberg et al., 2008). It has been shown, that quercetin, a flavonoid aglycone, has been effective in *in vitro* models (Jackson et al., 2006) and also in animal models of rheumatoid arthritis (Guardia et al., 2001; Mamani-Matsuda et al., 2006). Furthermore, immunosuppressive effects of quercetin on dendritic cell activation and function (Huang et al., 2010) and inhibition of expression of inflammatory cytokines through attenuation of NF-κB and p38 MAPK in a human mast cell line have been shown (Min et al., 2007).

Betula pendula Roth is known to contain mainly flavonoid-glycosides, such as quercetin-3-galactoside, quercetin-3-glucoside, quercetin-3-rhamnoside and quercetin-3-glucuronide (Keinänen and Julkunen-Tiitto, 1998). In the aqueous birch leaf extract, which has been used for this study, we could confirm the presence of these typical polyphenolic compounds using LC–MS (see Table 1).

It is known from the literature, that flavonoid-glycosides given orally were absorbed and eliminated very quickly in humans (Scholz and Williamson, 2007). However, the birch leaf extract of this study is clinically used for rheumatoid arthritis as subcutaneous injection in the inflamed joint area. Therefore it may be possible, that flavonoids in relevant concentrations remain locally in the tissue for some time and can perform their immunomodulatory properties. Furthermore, our investigations show that the concentrations of this preparation can be considered as clinically relevant, because the doses that were effective *in vitro* can be obtained locally by subcutaneous injection.

The extract used in this study (*Betula folium* D3 Abnoba) is a product with marketing authorization for subcutaneous injection

in humans and the dose–response relationship in comparison to the synthetic drug methotrexate provides proof for a clinical relevance of the demonstrated effects. In single patients of our outpatient centre with RA, we have seen beneficial clinical courses after serial subcutaneous injections of the commercial birch leaf preparation (*Betula folium* D3 Abnoba) in the area of inflamed joints (unpublished observations).

5. Conclusion

In conclusion, although it's efficacy has to be confirmed in clinical placebo-controlled studies, our results provide a strong rational base for the local parental use of the leaf extract of *Betula pendula* in the treatment of immune disorders, like rheumatoid arthritis, through diminishing of proliferating inflammatory lymphocytes.

Conflict of interest

R.H. has been consultant of ABNOBA GmbH in affairs not related to this work.

Acknowledgments

C.G. and R.H. receive financial support from Software AG foundation and DAMUS e.V.

References

Akbar, A.N., Salmon, M., 1997. Cellular environments and apoptosis: tissue microenvironments control activated T-cell death. Immunology Today 18, 72–76.

Berner, B., Wolf, G., Hummel, K.M., Muller, G.A., Reuss-Borst, M.A., 2000. Increased expression of CD40 ligand (CD154) on CD4+ T cells as a marker of disease activity in rheumatoid arthritis. Annals of Rheumatic Diseases 59, 190–195.

Buckley, C.D., 2003. Why does chronic inflammatory joint disease persist? Clinical Medicine 3, 361–366.

Choy, E.H., Panayi, G.S., 2001. Cytokine pathways and joint inflammation in rheumatoid arthritis. New England Journal of Medicine 344, 907–916.

Demirci, B., Paper, D.H., Demirci, F., Can Baser, K.H., Franz, G., 2004. Essential oil of *Betula pendula* Roth. Buds. Evidence-based Complementary and Alternative Medicine 1, 301–303.

European Pharmacopoeia (EP), 2011. European Directorate for the Quality of Medicines & Healthcare (edqm), vols. 1 and 2, Supplements 1 and 2., 7th ed. Council of Europe, Strasbourg.

Genestier, L., Paillot, R., Fournel, S., Ferraro, C., Miossec, P., Revillard, J.P., 1998. Immunosuppressive properties of methotrexate: apoptosis and clonal deletion of activated peripheral T cells. Journal of Clinical Investigation 102, 322–328.

German Homeopathic Pharmacopoeia (GHP), 2010. Including 7th Supplement, Volume 1 and 2, Editor: Federal Institute for Drugs and Medical Devices, BfArM.

Girke, M., 2010. Innere Medizin, 1st ed. Salumed Verlag, Berlin.

Guardia, T., Rotelli, A.E., Juarez, A.O., Pelzer, L.E., 2001. Anti-inflammatory properties of plant flavonoids. Effects of rutin, quercetin and hesperidin on adjuvant arthritis in rat. Farmaco 56, 683–687.

Harris Jr., E.D., 1990. Rheumatoid arthritis. Pathophysiology and implications for therapy. New England Journal of Medicine 322, 1277–1289.

Havlik, J., de la Huebra, R.G., Hejtmankova, K., Fernandez, J., Simonova, J., Melich, M., Rada, V., 2010. Xanthine oxidase inhibitory properties of Czech medicinal plants. Journal of Ethnopharmacology 132, 461–465.

Huang, R.Y., Yu, Y.L., Cheng, W.C., OuYang, C.N., Fu, E., Chu, C.L., 2010. Immunosuppressive effect of quercetin on dendritic cell activation and function. Journal of Immunology 184, 6815–6821.

Jackson, J.K., Higo, T., Hunter, W.L., Burt, H.M., 2006. The antioxidants curcumin and quercetin inhibit inflammatory processes associated with arthritis. Inflammation Research 55, 168–175.

Keinänen, M., Julkunen-Tiitto, R., 1998. High-performance liquid chromatographic determination of flavonoids in *Betula pendula* and *Betula pubescens* leaves. Journal of Chromatography A 793, 370–377.

Liu, H., Pope, R.M., 2004. Apoptosis in rheumatoid arthritis: friend or foe. Rheumatic Disease Clinics of North America 30, 603–625.

Major, H., 2002. Untersuchungen zur Wirkungsweise von Birkenblättern (*Betulae folium*) und phenolischer Verbindungen, unter besonderer Berücksichtigung der Beeinflussung von Metallopeptidasen. Humboldt University, Berlin, Germany.

Mamani-Matsuda, M., Kauss, T., Al-Kharrat, A., Rambert, J., Fawaz, F., Thiolat, D., Moynet, D., Coves, S., Malvy, D., Mossalayi, M.D., 2006. Therapeutic and preventive properties of quercetin in experimental arthritis correlate with decreased macrophage inflammatory mediators. Biochemical Pharmacology 72, 1304–1310.

Min, Y.D., Choi, C.H., Bark, H., Son, H.Y., Park, H.H., Lee, S., Park, J.W., Park, E.K., Shin, H.I., Kim, S.H., 2007. Quercetin inhibits expression of inflammatory cytokines through attenuation of NF-kappaB and p38 MAPK in HMC-1 human mast cell line. Inflammation Research 56, 210–215.

Nair, M.P., Mahajan, S., Reynolds, J.L., Aalinkeel, R., Nair, H., Schwartz, S.A., Kandaswami, C., 2006. The flavonoid quercetin inhibits proinflammatory cytokine (tumor necrosis factor alpha) gene expression in normal peripheral blood mononuclear cells via modulation of the NF-kappa beta system. Clinical and Vaccine Immunology 13, 319–328.

Pieroni, A., Gray, C., 2008. Herbal and food folk medicines of the Russlanddeutschen living in Kunzelsau/Talacker, South-Western Germany. Phytotherapy Research 22, 889–901.

Pietta, P., Mauri, P., Manera, E., Ceva, P., 1989. HPLC determination of the flavonoid glycosides from *Betulae folium* extracts. Chromatographia 28, 311–312.

Rao, J.K., Mihaliak, K., Kroenke, K., Bradley, J., Tierney, W.M., Weinberger, M., 1999. Use of complementary therapies for arthritis among patients of rheumatologists. Annals of Internal Medicine 131, 409–416.

Saric-Kundalic, B., Dobes, C., Klatte-Asselmeyer, V., Saukel, J., 2010. Ethnobotanical study on medicinal use of wild and cultivated plants in middle, south and west Bosnia and Herzegovina. Journal of Ethnopharmacology 131, 33–55.

Schilcher, H, Rau, H., 1988. Nachweis der aquaretischen Wirkung von Birkenblättern- und Goldrutenkauauszügen im Tierversuch. Urologe B 28, 274–280.

Scholz, S., Williamson, G., 2007. Interactions affecting the bioavailability of dietary polyphenols in vivo. International Journal for Vitamin and Nutrition Research 77, 224–235.

Sternberg, Z., Chadha, K., Lieberman, A., Hojnacki, D., Drake, A., Zamboni, P., Rocco, P., Grazioli, E., Weinstock-Guttman, B., Munschauer, F., 2008. Quercetin and interferon-beta modulate immune response(s) in peripheral blood mononuclear cells isolated from multiple sclerosis patients. Journal of Neuroimmunology 205, 142–147.

Trouillas, P., Calliste, C.A., Allais, D.P., Simon, A., Marfak, A., Delage, C., Duroux, J.L., 2003. Antioxidant, anti-inflammatory and antiproliferative properties of sixteen water plant extracts used in the Limousin countryside as herbal teas. Food Chemistry 80, 399–407.

Tunon, H, Olavsdotter, C., Bohlin, L., 1995. Evaluation of anti-inflammatory activity of some Swedish medicinal plants. Inhibition of prostaglandin biosynthesis and PAF-induced exocytosis. Journal of Ethnopharmacology 48, 61–76.

Wattel, A., Kamel, S., Prouillet, C., Petit, J.P., Lorget, F., Offord, E., Brazier, M., 2004. Flavonoid quercetin decreases osteoclastic differentiation induced by RANKL via a mechanism involving NF kappa B and AP-1. Journal of Cellular Biochemistry 92, 285–295.

951000496

Elsevier GmbH
Professional Education
Hackerbrücke 6 · D-80335 München

Tel: +49-(0) 89-5383-722
Fax: +49-(0) 89-5383-725
e-mail: sonderdrucke@elsevier.de
www.elsevier.de/professional-education