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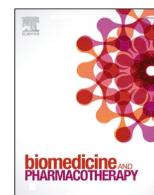

Aryltetralin lignans from *Hyptis brachiata* inhibiting T lymphocyte proliferation

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A B S T R A C T

Increased activation and proliferation of T lymphocytes plays an essential role in the development of chronic inflammation and autoimmune diseases. Currently used immunosuppressive drugs often do not provide long-lasting relief of symptoms and show a gradual loss of efficacy over time, and are accompanied by various side effects. Therefore, novel immunosuppressive lead substances are needed. For this purpose, an in-house library consisting of 600 extracts of plants from Panama was screened for inhibition of human T lymphocyte proliferation. As one of the hits, an ethyl acetate extract from the aerial parts of *Hyptis brachiata* (Lamiaceae) exhibited strong inhibitory effects. Subsequent investigation resulted in the isolation of seven aryltetralin lignans, five arylnaphthalene lignans, two flavonoids, three triterpenes, and cinnamyl cinnamate. Aryltetralin lignans inhibited T lymphocyte proliferation in a concentration-dependent manner without induction of apoptosis. No relevant inhibition was observed for the arylnaphthalene lignans, flavonoids, and triterpenes. Additional cell cycle arrest investigations revealed that isolated aryltetralin lignans potently inhibited cell division in G2/M phase similarly to podophyllotoxin. Multifluorescence panel analyses of the extract also showed weak suppressive effects on the production of IL-2 and TNF-α. Therefore, preparations made out of *H. brachiata* could be further explored as an interesting herbal alternative in the treatment of autoimmune diseases.

1. Introduction

Autoimmune dysfunction affects 5% of the population and refers to a disorder in which the body's immune tolerance is impaired, resulting in a reaction to the body's own cells and tissues [1]. Autoimmune diseases are treated symptomatically, as no causal therapy is currently available [2]. Moreover, therapy with current drugs suffers from several limitations, such as various side effects, nonresponding, increased susceptibility to infections, and high treatment costs [3–6]. Therefore, continued research into new therapies is mandatory.

Enhanced activation and proliferation of T lymphocytes is known to play a crucial role in the pathogenesis of autoimmune diseases and chronic inflammation, such as type I diabetes, rheumatoid arthritis, psoriasis, or multiple sclerosis [7]. Therefore, targeted therapies that specifically enhance inhibitory pathways in lymphocytes are an attractive approach to augment disease resolution in an attempt to effectively treat human autoimmune diseases [8]. In this context, we started a project aimed at the discovery of new plant-derived immune-modulating substances which specifically inhibit the activation and proliferation of human T lymphocytes. For this purpose, an in-house library consisting of 600 extracts of plants from Panama was screened for potential T lymphocyte inhibition. As one of the hits, an ethyl acetate extract from the aerial parts of *Hyptis brachiata* Briq. (Lamiaceae) exhibited strong inhibitory effects.

H. brachiata is native to Central America. In Venezuela the infusion of the whole plant is traditionally used as an antiseptic, a wound-healing

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agent, and as a treatment of ulcers and cancers [9]. Pharmacological data on *H. brachiata* are scarce. Recently, an ethyl acetate extract of the aerial parts showed moderate inhibition of aberrant AKT signaling in MM121224 human melanoma cells, and the methoxylated flavonoid sideritoflavone was identified as active compound [10]. Apart from that very little is known about chemical constituents from *H. brachiata*. In a study on the essential oil obtained from the leaves of *H. brachiata,* various compounds such as α-humulene, germacrene A, germacrene D, *E*-caryophyllene, and γ-cadinene were identified [11].

We report here on a comprehensive phytochemical profiling of *H. brachiata*, and on the identification of the constituents inhibiting T lymphocyte proliferation. The active aryltetralin lignans were further investigated in a cell cycle arrest assay. In addition, the impact of the extract and isolated substances on functional T lymphocyte suppression was explored.

2. Material and methods

2.1. General chromatographic procedures

Flash chromatography was carried out on a PuriFlash® 4100 system equipped with an UV detector and fraction collector (Interchim, Montluçon, France).

Preparative HPLC was done on a Preparative LC/MSD System (Agilent Technologies, Santa Clara, CA, USA) consisting of a binary pump (1260 Prep Bin Pump), a quaternary pump (Infinity II 1290), a PDA detector (1100 Series), and a 6120 single quadrupole MS detector. A SunFire Prep C18 OBD column (5 μm, 150 × 30 mm i.d., Waters, Milford, MA, USA), equipped with a C18 Prep Guard Cartridge (10 × 30 mm i.d.) was used. MeCN and water, both containing 0.1% formic acid (FA), was used as mobile phase for all preparative separations. A flow rate of 20 mL/min was applied. A 1290 Infinity II Valve Drive manual injection system (Agilent Technologies) was used for injection. Data acquisition and processing was done by ChemStation software (Agilent Technologies).

For semi-preparative separations an Agilent HP1100 Series HPLC instrument (Agilent Technologies, Santa Clara, CA, USA) equipped with a binary pump (G1312A bin pump), auto sampler (G1357A WPALS), column oven (G1316A COLCOM), and a diode array detector (G1315A DAD), or an Alliance 2690 HPLC system equipped with a DAD 996 detector (Waters, Milford, MA, USA) were used. Chromatography was performed either on a SunFire C18 column (5 μm, 150 × 10 mm i.d., Waters) or on a ReproSIL-Pur 120 C18-AQ column (3 *μ*m, 150 ×10 mm i.d., Dr. Maisch GmbH, Ammerbuch-Entringen, Germany), both equipped with a guard column (10 × 10 mm i.d.). The mobile phases used consisted of MeCN or MeOH and water, all containing 0.1% FA. The flow rate was 4 mL/min. Data acquisition and processing was performed using ChemStation (Agilent Technologies, Santa Clara, CA, USA) or Empower software (Waters, Milford, MA, USA).

Microfractionation and HPLC-PDA-ELSD-ESIMS analyses were performed on a LC-MS 8030 system (Shimadzu, Kyoto, Japan) consisting of a degasser, auto-sampler, quaternary pump (LC-20 CE), a column oven (CTO-20AC), a PDA detector (SPD-M20A) connected via a T-split to a triple quadrupole MS and an ELSD 3300 detector (Alltech, Deerfield, IL, USA). Separations were either carried out on a SunFire C18 column (3.5 μm, 150 × 3 mm i.d., Waters, Milford, MA, USA), or on a ReproSIL-Pur 120 C18-AQ column (3 *μ*m, 150 × 3 mm i.d., Dr. Maisch GmbH, Ammerbuch-Entringen, Germany), both equipped with a guard column (10 × 3 mm i.d.). The mobile phases consisted of MeCN or MeOH and water, all containing 0.1% FA. The flow rate was 0.4 mL/min. The LabSolutions software (Shimadzu) was used for data acquisition and processing.

TLC was performed on silica gel 60 F254-coated aluminum plates (ALUGRAM Xtra Nano-SIL G, Macherey-Nagel, Düren, Germany). Detection was with UV 254 nm, UV 366 nm, and after spraying with 1% ethanolic vanillin and 10% sulfuric acid in EtOH followed by heating at 110 °C.

Silica gel 60 (0.040–0.063 mm) used for flash chromatography was obtained from Merck (Darmstadt, Germany). HPLC-grade solvents MeCN, MeOH (Avantor, Radnor, PA, USA), and water from a Milli-Q water purification system (Merck Millipore, Billerica, USA) were used for HPLC separations. HPLC-grade formic acid (FA) and DMSO were obtained from Scharlau (Scharlab S.L., Spain). Technical grade EtOAc, MeOH, and *n*-hexane (Rheuss Chemie, Tägerig, Switzerland) were redistilled before use for extraction and MPLC. Rosmarinic acid was purchased from Sigma (Darmstadt, Germany).

2.2. General procedures for physicochemical characterization

NMR spectra were recorded on a Bruker Avance III NMR spectrometer operating at 500.13 MHz for ^{1}H and 125.77 MHz for ^{13}C at 23 °C, or on a Bruker Avance NEO NMR spectrometer operating at 600.15 MHz for ^{1}H at 25 °C (both Bruker BioSpin, Rheinstetten, Germany). ^{1}H NMR, COSY, HSQC, HMBC, NOESY, and ROESY spectra were measured in a 1 mm TXI, a 5 mm BBO, or a 3 mm TCI cryoprobe. Spectra were recorded in $CDCl_3$ (Sigma-Aldrich, St. Louis, MO, USA) or DMSO-d_6 (ARMAR Chemicals, Döttingen, Switzerland). Data were analyzed using Bruker Topspin 3.5 and ACD/Labs NMR Workbook suites software. Chemical shifts are reported as δ values (ppm) using the solvent signal (δ_H 2.50; δ_C 39.51 for DMSO-d_6 or δ_H 7.27; δ_C 77.00 for $CDCl_3$) as internal reference; coupling constants (*J*) are given in Hz.

Optical rotations were measured in $CHCl_3$ or MeOH (1 mg/mL) on a JASCO P-2000 polarimeter (Brechbühler AG, Schlieren, Switzerland) equipped with a 10 cm temperature-controlled microcell and a sodium light source (589 nm).

2.3. Plant material

Aerial parts of *Hyptis brachiata* Briq. were collected in August 1990 in El Valle de Antón, El Pinar, Coclé Province (Panama) by CIFLORPAN. The taxonomic identity was confirmed by Carmen Galdames, botanist at CIFLORPAN, and a voucher specimen (no. 89710) deposited at the herbarium of the University of Panama. A voucher specimen is also available at the Division of Pharmaceutical Biology, Department of Pharmaceutical Sciences, University of Basel (no. 1181).

2.4. HPLC-based activity profiling

The EtOAc extract of *H. brachiata* aerial parts stored in our in-house library was used for HPLC-based activity profiling. Microfractionation was carried out by analytical RP-HPLC on an LC-MS 8030 system (Shimadzu, Kyoto, Japan) connected to an FC 204 fraction collector (Gilson, Mettmenstetten, Switzerland) adapted for 96-deep-well plates. Three injections of the extract (10 mg/mL in DMSO) were performed: 2 × 30 μL (corresponding to 600 μg of extract) using only a PDA detector for collection of microfractions, and 1 × 10 μL with PDA-ELSD-ESIMS detection without collection. As mobile phase MeCN and water was used, both containing 0.1% FA. The gradient was 5–100% MeCN in 30 min, followed by a final hold for 5 min at 100% MeCN. Fractions of 1 min each were collected from 0 to 35 min. Microfractions of two successive injections were collected into the same wells of a 96-deep-well plate. The plate was dried in a Genevac EZ-2 evaporator (Genevac, Ipswich, England).

The dried microfractions were re-suspended in 25 μL of DMSO for use in the proliferation assays. 1 μL of this dilution was used in 100 μL of cell suspension (2 * 10^{6} cells/mL) as maximal concentration, and 1:3 titrations were performed for a total of six concentrations. With this approach the relative activity of the fractions was assessed in a concentration-dependent manner. The HPLC-PDA-ELSD-ESIMS chromatograms were correlated with the activity of the fractions to generate the activity profile.

2.5. Large scale extraction and isolation

The ground plant material (712 g) was percolated with EtOAc (9 L), followed by MeOH (17 L) as previously reported [10]. Evaporation under reduced pressure yielded 9.4 g of EtOAc (1.3%) and 27.4 g of MeOH extract (3.8%).

The EtOAc extract (9.3 g) was separated by flash chromatography. The sample was dissolved, mixed with 35 g of silica gel 60 (0.040–0.063 mm) and dried, prior to loading onto a self-packed silica 60 glass column (0.040–0.063 mm, column size: 46 × 7 cm, i.d.). A gradient of EtOAc in *n*-hexane [2% (0–10 min), 2–70% (10–450 min), 70–100% (450–570 min), 100% (570–615 min)], followed by MeOH in EtOAc [0–50% (615–735 min), 50–100% (735–750 min), 100% (750–810 min)] was applied at a flow rate of 20 mL/min. A total of 808 fractions were collected and combined into 28 fractions (F1–F28) based on TLC analysis.

Further separation of F19 (206 mg) by preparative HPLC with a gradient of 30–100% MeCN in water in 30 min afforded 8 fractions (F19.1–F19.8). Follow-up purification of F19.1 (4.2 mg) by semi-preparative HPLC on a ReproSil-Pur 120 C18-AQ column eluted with a gradient of 30–60% MeCN in water in 30 min yielded compound **2** (1.9 mg, t_R 14.7 min). Compound **1** (25.2 mg, t_R 13.3 min) was isolated by semi-preparative HPLC from F19.2 (37.2 mg) on a ReproSil-Pur 120 C18-AQ column eluted with 38% MeCN in water. Further separation of F19.4 (12 mg) using a combination of semi-preparative HPLC with a SunFire C18 column with 37% MeCN in water, followed by semi-preparative HPLC using a ReproSil-Pur 120 C18-AQ column with 48% MeCN in water afforded a crude lignan mixture F19.4.3.1 (10.6 mg) and **17** (1.3 mg, t_R 10.2 min). F19.4.3.1 was further purified using semi-preparative HPLC on a SunFire C18 column with a gradient of 45–70% MeOH in water in 30 min, yielding **3** (8.7 mg, t_R 17.4 min) and **4** (2.4 mg, t_R 18.6 min). Separation of F19.7 (11.8 mg) by semi-preparative HPLC on a ReproSil-Pur 120 C18-AQ column with a gradient of 47–62% MeCN in water in 30 min gave **15** (5.5 mg, t_R 14.9 min) and **10** (6.3 mg, t_R 15.8 min). F19.8 (10.3 mg) was further purified over a semi-preparative SunFire C18 HPLC column with 45% MeCN in water resulting in **9** (5.7 mg, t_R 19.1 min). F20 (707 mg) was further fractionated by preparative HPLC applying a gradient of 30–100 MeCN in water in 30 min to yield fractions F20.1–F20.4. Fraction F20.3 (35.0 mg) was purified by semi-preparative HPLC on a ReproSil-Pur 120 C18-AQ column using a gradient of 35–75% MeCN in water in 30 min yielding fraction F20.3.4 (22.4 mg). Further purification of F20.3.4 by semi-preparative HPLC on a ReproSil-Pur 120 C18-AQ column using a gradient of 55–75% MeOH in water in 30 min gave **7** (5.2 mg, t_R 11.8 min) and **8** (16.3 mg, t_R 18.6 min). Fractionation of F21 (249 mg) by preparative HPLC with a gradient of 5–100% MeCN in water in 30 min gave 21 fractions F21.1–F21.21. F21.21 was identified as **13** (18.6 mg, t_R 29.6 min). Further separation of F21.11 (6.4 mg) by semi-preparative HPLC with a SunFire C18 column and a MeCN gradient in water of 30–85% in 30 min afforded **12** (1.7 mg, t_R 9.7 min), **11** (0.5 mg, t_R 16.8 min), and **18** (0.6 mg, t_R 30.0 min). Further separation of F21.12 (7.1 mg) using semi-preparative HPLC with a SunFire C18 column with 36% MeCN in water afforded **5** (3.3 mg, t_R 11.1 min). Fraction F21.13 (17.9 mg) was further purified using semi-preparative HPLC with a gradient of 30–60% MeCN in water in 30 min on a ReproSil-Pur 120 C18-AQ column to yield **6** (2.5 mg, t_R 15.2 min) and **16** (8.9 mg, t_R 15.9 min). Purification of F21.16 (21.5 mg) by semi-preparative HPLC on a SunFire C18 column with a gradient of 42–62% MeCN in water in 30 min yielded **14** (4.7 mg, t_R 15.5 min). NMR and HPLC purity of the compounds was $> 90\%$ except for **11** and **14** (both approximately 85%), for **13** (approximately 80%), and for **15** (approximately 75%). The impurity in compounds **11**, **13**, and **15** was an unidentified triterpene.

In addition, a minor peak at t_R 12.2 min in the HPLC chromatographic trace of the EtOAc extract was identified as rosmarinic acid (**19**) based on UV and MS data and chromatographic comparison with a commercial reference. Rosmarinic acid was a major peak in the UV 254 nm trace of the MeOH extract (Data not shown).

2.6. Ethics approval statement

All adult, healthy blood donors gave written informed consent for blood collection. The blood samples were obtained in an anonymized and coded form, without any visible ID number, from the central blood donation of the University Hospital in Basel. The work therefore does not fall within the scope of the Swiss Human Research Act. Thus, no ethics vote by the Ethics Committee Central and Northwestern Switzerland is required for the methods used to work with the blood samples.

2.7. Preparation and cultivation of human peripheral lymphocytes

Isolation of PBMCs was performed by centrifugation over a LymphoPrep™ gradient (1.077 g/cm^3, 20 min, 500 g, 20 °C; Progen). White blood cells were isolated and washed twice with phosphate-buffered saline (PBS, GE Healthcare). Cultivation was performed in an incubator at 37 °C, 5% CO_2, and 95% air atmosphere using RPMI 1640 medium supplemented with 10% fetal calf serum (FCS, GE Healthcare Life Sciences), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Sigma-Aldrich).

2.8. Treatment with extracts and compounds

Isolated cells were treated with extracts (0.3–100 µg/mL) and compounds (0.01–100 µM). Cyclosporine (5 µg/mL; Sandimmun®), staurosporine (0.3 µM; Tocris), and vinblastine (0.03 µM; Tocris) were used as controls. Commercial podophyllotoxin (Sigma-Aldrich) was also included as a reference substance and was named **5** in the manuscript. All cells were stimulated with soluble CD3 and CD28 monoclonal antibodies (mAbs) (eBioscience) at 100 ng/mL, except the unstimulated controls. Experiments were repeated at least three times.

2.9. T lymphocyte proliferation assay

Isolated PBMCs (5 *10^6 cells/mL) were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 µM: Sigma-Aldrich) for 10 min at 37 °C with carboxyfluorescein diacetate succinimidyl ester (CFSE; 5 µM: Sigma-Aldrich). The staining was stopped by addition of RPMI complete medium. Excess of CFSE was removed by washing. Cells (2 * 10^6 cells/mL) were treated for 72 h and cell proliferation was analyzed by CytoflexS (Beckman Coulter) and FlowJo software (BD Biosciences).

2.10. Cell death assay

Isolated PBMCs (2 * 10^6 cells/mL) were treated for 72 h and then stained with Annexin V-FITC (eBioscience) as recommended by the manufacturer's instructions. The percentage of apoptotic cells was determined using CytoflexS (Beckman Coulter) and FlowJo software (BD Biosciences).

2.11. Cell cycle assay

Isolated PBMCs (2 * 106 cells/mL) were treated for 72 h and subsequently washed with 4 °C cold PBS. Cells were re-suspended in 3 mL ice cold 70% ethanol and fixed overnight at −20 °C. After fixation, cells were washed and stained in 200 µL staining solution (1 µL propidium iodide (1 mg/mL; Sigma-Aldrich) in 199 µL PBS) for 30 min at room temperature in the dark. The measurement was performed with a CytoflexS (Beckman Coulter).

2.11.1. Analysis of the activation status and cytokine production of human T lymphocytes

Isolated PBMCs were treated with anti-CD3 and anti-CD28 mAbs for 40 h before restimulation and afterwards re-stimulated with phorbol-12-myristate-13-acetate (PMA, 50 ng/mL; Sigma-Aldrich) and ionomycin (1 µg/mL; Sigma-Aldrich) (except the unstimulated control) for further 4 h. To block the Golgi apparatus, cells were treated with GolgiPlugTM (1 µL/mL; BD Biosciences) and GolgiStopTM (0.65 µL/mL; BD Biosciences) and incubated simultaneously due to the restimulation process. The cell surface was stained with two separate panels (panel 1: CD3-APC AlexaFluor750, CD4-AlexaFluor700, CD69-PC7; panel 2: CD3-APC Alexa Fluor750, CD8-AlexaFluor700, CD69-PC7 (all Abs from Beckman-Coulter)) for 30 min at room temperature in the dark. Cells were re-suspended in 100 µL Cytofix/Cytoperm solution for 15 min at 4 °C. Cells were intracellularly stained with two different panels (panel 1: IFN-γ FITC (Beckman-Coulter), IL-2 APC (BD), TNF-α PE (Beckman-Coulter), IL-21 BV421 (BD); panel 2: IFN-γ FITC, TNF-α PE, MIP1-β BV421 (all Abs from Beckman-Coulter)) for 30 min at 4 °C. Afterwards, the cells were fixed with 2% paraformaldehyde (PFA; Electron Microscopy Sciences) for 10 min at 4 °C. Analysis was performed using a CytoflexS flow cytometer (Beckman Coulter) and FlowJo software (BD Biosciences).

2.12. Data analysis

For statistical analysis, data were processed with PRISM software (version 9.3.1 for PC, GraphPad Software). The Shapiro-Wilk test was used to test for a normal distribution. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA, followed by Dunnet́s T3 post-hoc test. Values are presented as mean ± standard deviation differences from controls (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.).

3. Results

3.1. Effect of H. brachiata EtOAc extract on proliferation and apoptosis of human T lymphocytes

An in-house library consisting of 600 extracts of plants collected in Panama was screened at a single concentration of 20 µg/mL for inhibition of human T lymphocyte proliferation using carboxyfluorescein succinimidyl ester (CFSE) staining and flow cytometric analysis. The active extracts were subsequently tested for suppression of T lymphocyte division at six concentrations ranging from 0.3 to 100 µg/mL. Among the hits, an ethyl acetate extract obtained from the aerial parts of *H. brachiata* significantly inhibited the proliferation of human T lymphocytes in a concentration range from 1 to 100 µg/mL (Fig. 1). Apoptosis induction in the concentration range was excluded by annexin V-FITC staining (Fig. 1). Cytotoxicity of the vehicle (DMSO) was also tested. The data showed that the overall inhibitory effects of only the highest tested concentrations (100 µg/mL for extract and 30 µM for single substances) were mediated by the cytotoxicity of the DMSO solvent. The data are provided as supplementary information (Fig. S1). To identify the active constituents, the EtOAc extract was submitted to HPLC-based activity profiling, a procedure which combines analytical HPLC separation with on-line recorded spectroscopic data and with biological information obtained in parallel from microfractions collected from the column effluent. Separation of the extract into 35 one-minute microfractions and subsequent testing of the fractions on T lymphocytes proliferation revealed a main active time window between 16 and 22 min (Fig. 2). Several peaks were detected which could be tentatively assigned to lignans, flavonoids, and triterpenes based on their UV and MS spectra, and with the aid of chemotaxonomic data.

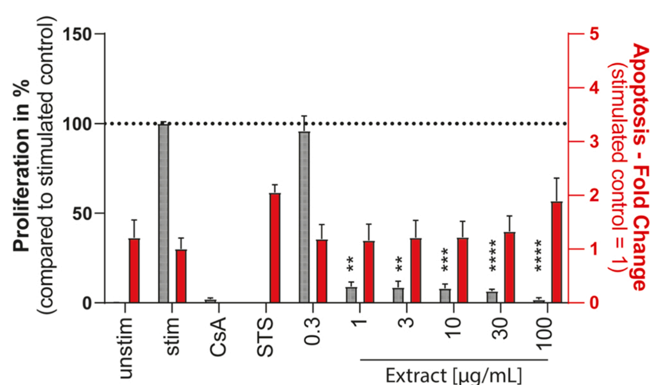

Fig. 1. Effect of the *H. brachiata* EtOAc extract on T lymphocyte proliferation and apoptosis. T lymphocyte proliferation is shown in gray (left y-axis) and apoptosis induction in red (right y-axis). Primary human lymphocytes were stained either with CFSE or Annexin V-FITC stainings in proliferation or apoptosis experiments, respectively. Lymphocytes were stimulated with anti-CD3 and anti-CD28 mAbs (except the unstimulated control) and incubated for 72 h in the presence of medium (unstimulated (unstim), stimulated (stim)), cyclosporine A (CsA; 4.16 µM), staurosporine (STS, 0.3 µM), or *H. brachiata* EtOAc extract (µg/mL). Both, proliferation and apoptosis induction were analyzed by flow cytometry. Only the proliferation results were normalized to stimulated control. Results are depicted as mean ± standard deviation. n = 3; *p < 0.05; **p < 0.01; ***p < 0.005; ****p < 0.001. The Shapiro-Wilk test was used to test for a normal distribution. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA, followed by Dunnet́s T3 post-hoc test.

3.2. Isolation of compounds, and effect on proliferation and apoptosis induction of human T lymphocytes

For preparative isolation, a larger amount of aerial parts was extracted with EtOAc. Targeted fractionation of the extract by a combination of chromatographic methods resulted in the isolation of 18 compounds, including seven aryltetralin lignans (**1**–**7**), five arylnaphthalene lignans (**8**–**12**), three triterpenes (**13**–**15**), two flavonoids (**16**–**17**), and cinnamyl cinnamate (**18**). Compounds were identified with the aid of ESI-MS and NMR analysis. The absolute configuration was established by comparison of optical rotation data with literature values. In addition, rosmarinic acid (**19**) was identified in the extract from its UV and MS data, and by chromatographic comparison with a commercial reference sample (Figs. 2 and 3). Physicochemical and spectroscopic data of compounds **1**-**18** are provided as Supplementary Information (Tables S1-S18).

The aryltetralin lignans **1**–**4** and **7**, arylnaphthalene lignans **8**–**10**, triterpenes **14** and **15**, and flavonoids **16** and **17** were obtained in sufficient amounts and purity to be tested for T lymphocyte proliferation inhibition and apoptosis induction. The lignan **5** identified as podophyllotoxin was isolated as a minor substance of insufficient purity. Therefore, a commercially available reference of **5** was used for all bioassays. Except for **4**, all aryltetralin lignans significantly inhibited proliferation in a concentration-dependent manner at concentrations of 0.1 µM for **1** and **2**, 0.3 µM for podophyllotoxin (**5**) and **7**, and 3 µM for **3** (Fig. 4A). Apoptosis correlated with proliferation inhibition, but these effects were weak and not significant. (Fig. 4 A). No effects on T lymphocyte proliferation were observed for arylnaphthalene lignans, triterpenes, and flavonoids (Fig. 4B).

3.3. Effect of H. brachiata EtOAc extract and aryltetralin lignans on the cell cycle of human T lymphocytes

To characterize the proliferation inhibition by the extract and the aryltetralin lignans in more detail, cell cycle arrest analyses were performed. The distribution of stimulated T lymphocytes in different cell

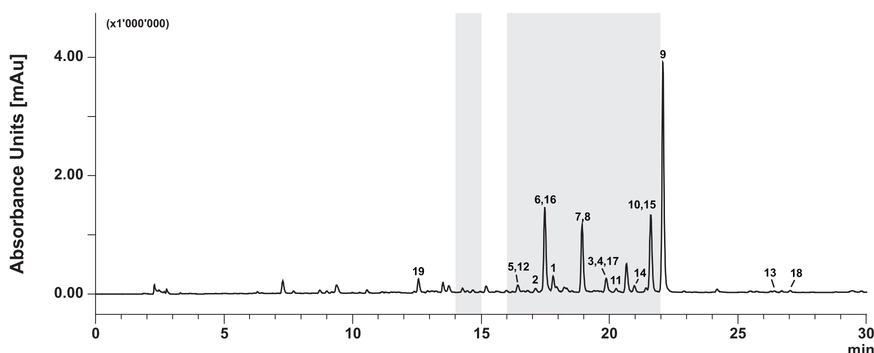

Fig. 2. HPLC-based activity profile of the *H. brachiata* EtOAc extract. HPLC-PDA chromatographic trace at 254 nm (SunFire C18, 3.5 μM. 3.0 × 150 mm i.d.). 5–100% MeCN (0.1% FA) in 30 min. The active one-minute microfractions are highlighted in grey. Numbers refer to the identified compounds.

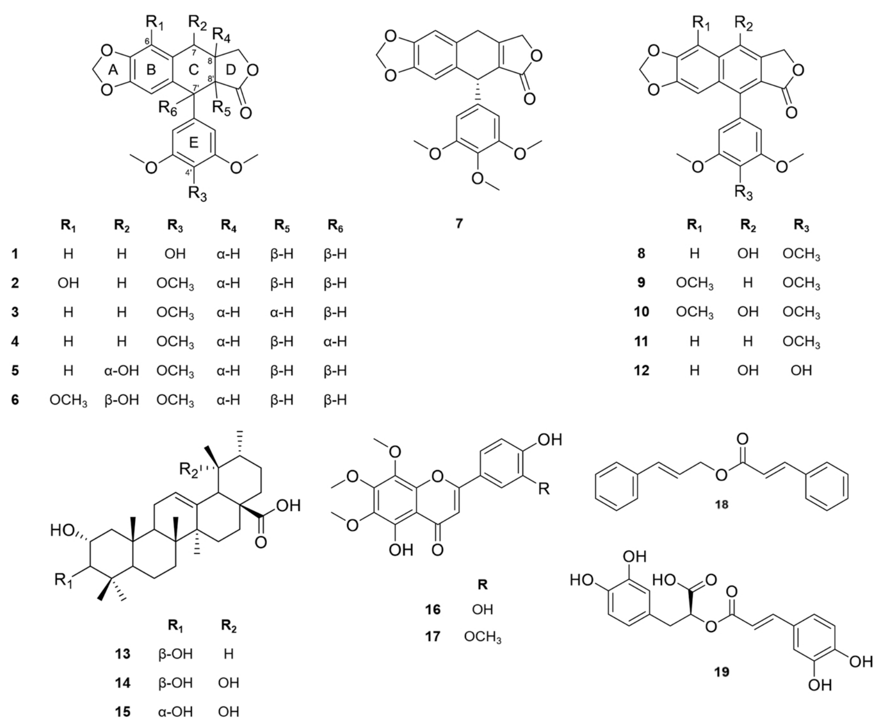

Fig. 3. Chemical structures of identified compounds.

cycle phases was observed by flow cytometric sorting analysis. Cyclosporine A and vinblastine were used as references that inhibit T lymphocyte proliferation via two different pathways to characterize the effects of our tested substances. As can be seen in Fig. 5, unstimulated cells remained mainly in G1 phase. Compared to unstimulated cells, stimulated cells showed an increased number in S and G2. The calcineurin inhibitor cyclosporine A inhibited cell division immediately after T lymphocyte activation, leading to an accumulation of cells in G1. The microtubule inhibitor vinblastine blocked cell division during mitosis and led to a higher accumulation of dividing T lymphocytes in S and G2 compared to stimulated cells (Fig. 5). At concentrations between 0.003 and 0.3 μg/mL the *Hyptis* extract showed a concentration-dependent increase of of T lymphocytes with a G2 phase arrest. However, this trend decreased again at higher concentrations (3–30 μg/mL). The same was observed for the pure compounds, since **1**, **2**, **5** and **7** showed as well an increased G2 arrest at concentrations between 0.01 and 1 μM for **1**, and between 0.01 and 0.1 μM for **2**, **5**, and **7**. At higher concentrations the number of cells in the G2 phase decreased similarly as seen for the extract. Only **3** showed a continuous dose-dependent G2 phase arrest over the whole tested concentration range between 0.01 and 10 μM (Fig. 5).

3.4. Effect of H. brachiata EtOAc extract and isolated substances on the activation state and cytokine production of human T lymphocytes

During an immune response, T helper cells ($CD4^{+}$) and cytotoxic T cells ($CD8^{+}$) secrete various cytokines that promote the immune response. In addition, the activation marker CD69 is expressed on the cell surface of both cell types [12]. To evaluate a functional T lymphocyte suppression by the *H. brachiata* EtOAc extract and the pure compounds, the production of different cytokines and the expression of surface markers was investigated. A multifluorescence panel was used to examine the production of CD69, IFN-γ, IL-2, TNF-α, and IL-21 of $CD4^{+}$ lymphocytes, and CD69, IL-2, TNF-α, and MIP1-β of $CD8^{+}$ lymphocytes.

The extract decreased the secretion of IL-2 (Fig. 6A) and IL-21 (Fig. 6B) of $CD4^{+}$ lymphocytes, and of IL-2 (Fig. 6C), TNF-α (Fig. 6D), and MIP1-β (Fig. 6E) of $CD8^{+}$ lymphocytes in a concentration-dependent manner. The inhibition of IL-2 secretion of $CD4^{+}$ lymphocytes by the extract was significant between concentrations of 30 and 100 μg/mL (Fig. 6A). The remaining effects are merely trends, without statistical

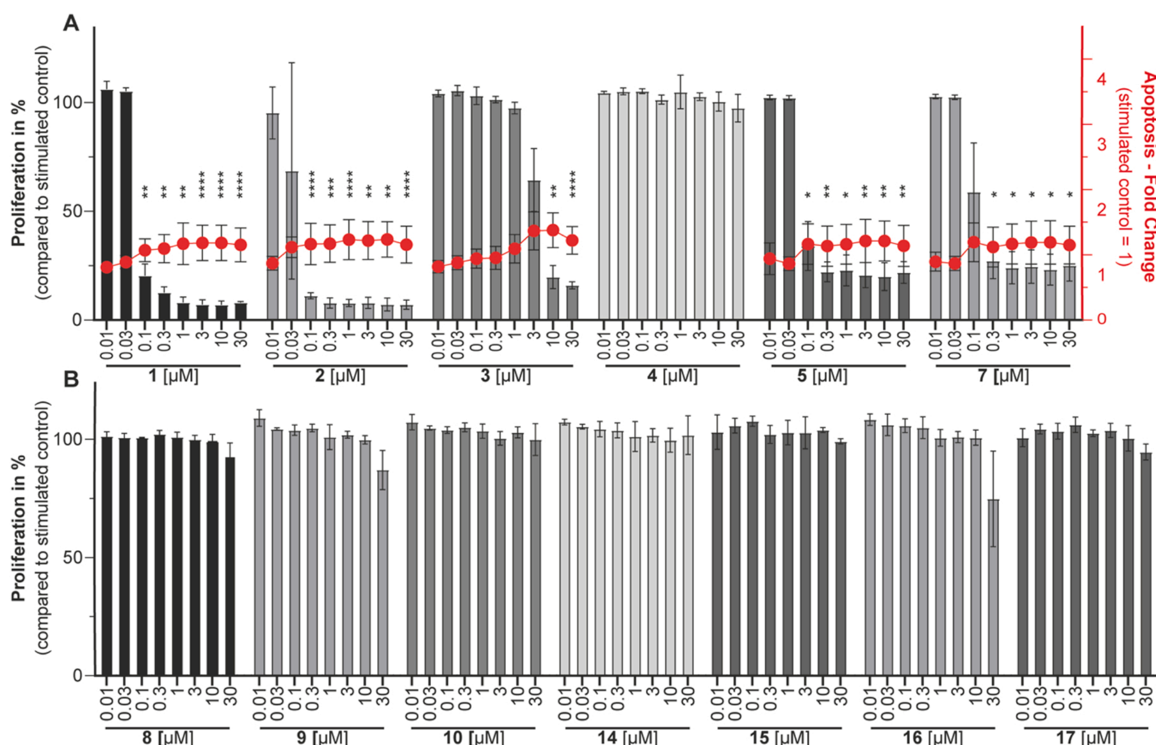

Fig. 4. Effects of compounds on T lymphocyte proliferation and apoptosis. Effects on proliferation is shown as black and gray bars (left y-axis), and apoptosis induction as red dots (right y-axis). Primary human lymphocytes were stained either with CFSE or Annexin V-FITC stainings in proliferation or apoptosis experiments, respectively. Lymphocytes were stimulated with anti-CD3 and anti-CD28 mAbs and incubated for 72 h in the presence of isolated compounds or commercial podophyllotoxin. Concentrations tested in μM. Both, proliferation and apoptosis induction were analyzed by flow cytometry. Only the proliferation results were normalized to stimulated control. Results are depicted as mean ± standard deviation. The controls are shown in Fig. 1. n = 3; *p < 0.05; **p < 0.01; ***p < 0.005; * ** *p < 0.001. The Shapiro-Wilk test was used to test for a normal distribution. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA, followed by Dunnet́s T3 post-hoc test.

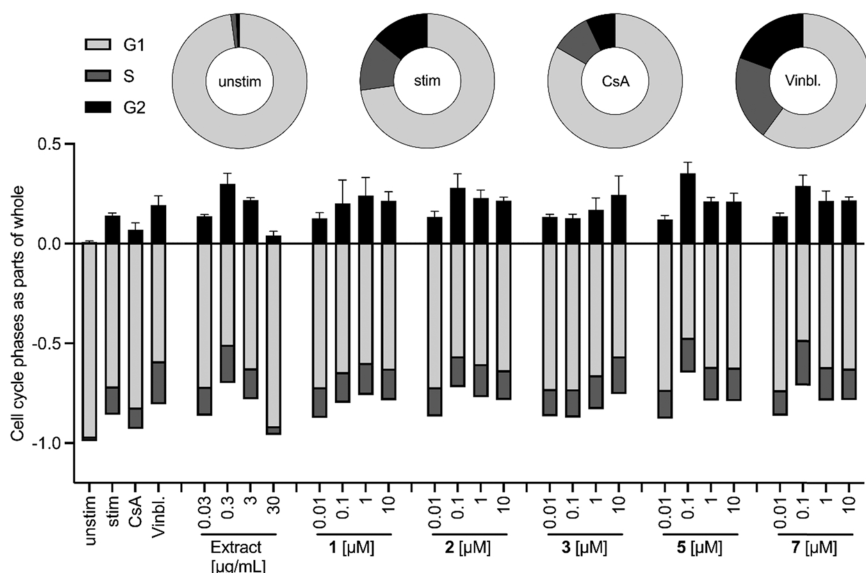

Fig. 5. Effects of *H. brachiata* EtOAc extract and aryltetralin lignans on the cell cycle of human T lymphocytes. Primary human lymphocytes were stimulated with anti-CD3 and anti-CD28 mAbs. Stimulated cells were incubated for 72 h in the presence of H. brachiata EtOAc extract, or isolated aryltetralin lignans. PI staining of the DNA was performed. The mean number of lymphocytes in G2 phase ± standard deviation is shown above the x-axis. The proportions of lymphocytes in G1 and S phase are depicted below the x-axis. Concentrations tested for the extract in μg/mL and for substances in μM. Controls (unstim, stim, cyclosporine (CsA; 4.16 μM), vinblastine (Vinbl.; 0.03 μM)) are shown as pie charts and indicate the proportions of lymphocytes in each phase. n = 3. The Shapiro-Wilk test was used to test for a normal distribution. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA, followed by Dunnet́s T3 post-hoc test.

significance.

Of the isolated substances only the triterpenes **14** and **15** weakly inhibited the secretion of IL-2 (Fig. 6A) and IL-21 (Fig. 6B) by $CD4^+$ lymphocytes, and of IL-2 (Fig. 6C), TNF-α (Fig. 6D), and MIP1-β (Fig. 6E) by $CD8^+$ lymphocytes in a concentration range between 0.3 and 30 μM. Expression of IL-21 by $CD4^+$ lymphocytes, and of TNF-α and MIP1-β by $CD8^+$ lymphocytes was concentration-dependent, but not significant. The effects on IL-2 secretion of $CD4^+$ and $CD8^+$ T lymphocytes were not concentration-dependent. The other activation markers and cytokines were neither affected by the extract nor by the triterpenes (data not shown). No significant effects on the secretion of cytokines were observed for aryltetralin and arylnaphthalene lignans, as well as for flavonoids (data not shown).

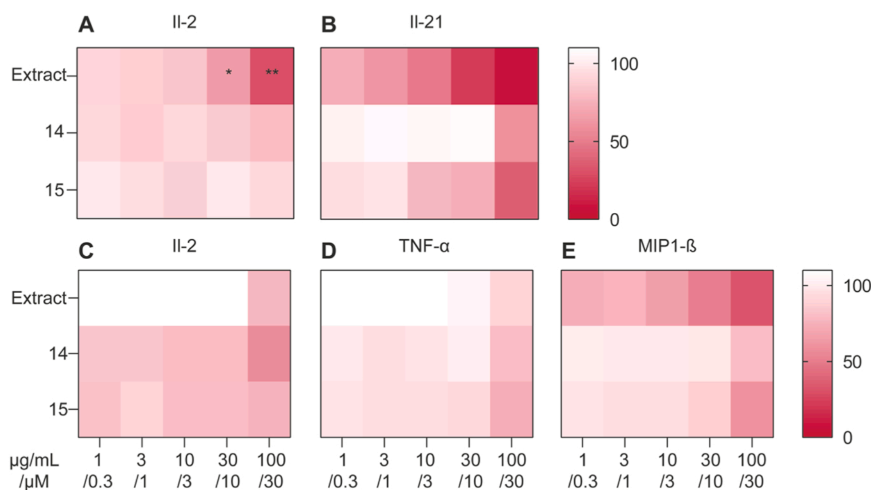

Fig. 6. Effects of *H. brachiata* EtOAc extract and triterpenes **14** and **15** on the activation state and cytokine production of primary human lymphocytes. After treatment with extract and triterpenes for 44 h $CD4^+$ lymphocytes and $CD8^+$ lymphocytes were separately analyzed by their functional status using two flow cytometric staining panels. The effects of the extract and triterpenes **14** and **15** on the secretion of IL-2 (A) and IL-21 (B) by $CD4^+$ lymphocytes and of IL2 (C), TNF-α (D), and MIP1-β (E) by $CD8^+$ lymphocytes are shown as heat maps. n = 3; $^*p < 0.05$; $^{**}p < 0.01$. The Shapiro-Wilk test was used to test for a normal distribution. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA, followed by Dunnet's T3 post-hoc test.

4. Discussion

Continued research into new therapies for the treatment of autoimmune diseases is of great importance to overcome current therapeutic limitations. Plants are a promising source for the discovery of novel immunosuppressive substances given the high structural diversity of their secondary metabolites. In the screening of a library of 600 plant extracts for T lymphocyte proliferation inhibition an EtOAc extract from the aerial parts of *H. brachiata* showed strong inhibitory effects (Fig. 1). Subsequent investigation resulted in the isolation of seven aryltetralin lignans, five arylnaphthalene lignans, three triterpenes, two flavonoids, and cinnamyl cinnamate (Figs. 2 and 3; Tables S1-S18). This study represents the first detailed report on the phytochemical composition of *H. brachiata*. However, most of the isolated lignans have already been identified in other species of the Hyptidinae subtribe, such as *Condea verticillata* (syn. *Hyptis verticillata*), *Mesosphaerum suaveolens* (syn. *Hyptis suaveolens*), or *Eriope latifolia* (syn. *Hyptis latifolia*) [13]. Lignans **6** and **12** are described for the first time in a species of the Hyptidinae subtribe, but were previously isolated from *Libocedrus chevalieri* (Cupressaceae) [14], *Dysosma versipellis* (Berberidaceae) [15], and *Sinopodophyllum hexandrum* (Berberidaceae) [16,17]. Most of the triterpenes, flavonoids, and cinnamic acid derivatives have also been previously reported in other Hyptidinae species [13], except the flavonoid **17**, which has been identified in various other Lamiaceae species [18,19] and the cinnamic acid derivative **18,** a metabolite known for example from *Cinnamomum cassia* (Lauraceae) [20].

Of the isolated substances only the aryltetralin lignans strongly suppressed T lymphocyte proliferation, whereas the other compounds were inactive. Both the EtOAc extract and the aryltetralin lignans slightly increased the induction of apoptosis (Figs. 1 and 4). However, compared to unstimulated T lymphocytes the effect on apoptosis induction was weak, and apoptosis as such could, therefore, not explain the observed proliferation inhibition.

Podophyllotoxin is a widely investigated substance that mainly gained attention because of its antimitotic effects, and therefore for its anti-tumor properties [21]. Even though podophyllotoxin and its analogues proved to be highly effective as antitumor agents they failed in clinical investigations mainly due to severe gastrointestinal side effects [22].

Aryltetralin lignans have also gained interest as immunosuppressive agents [23–25]. Proresid®, podophyllotoxin, and Reumacon® even reached the stage of clinical studies in the treatment of rheumatoid arthritis because they clearly improved the clinical manifestation of the disease by significant reduction of inflammatory activity through inhibitory effects on lymphocyte proliferation [23,26–36]. Additionally, podophyllotoxin was investigated in clinical studies for the treatment of psoriasis because it effectively reduced IL-1 and TNF-α serum levels, as well as lymphocyte activity [37]. All observed effects could be explained by aryltetralin lignans that bind to tubulin and inhibit microtubule formation [25,38,39].

From these findings we can conclude that the aryltetralin lignans from *H. brachiata* also inhibit T lymphocyte proliferation by binding to tubulin monomers and therefore inhibit the formation of the mitotic spindle. The most active lignans were **1** and **2**, followed by **5**, **7**, and **3**, while lignan **4** did not show any effect (Fig. 4A). The differences observed between the aryltetralin lignans on antimitotic activity can be explained in the light of reported structure-activity relationships. For tubulin polymerization inhibition, the almost co-planar orientation of a 7′-(*R*), 8′-(*R*), and 8-(*R*) configuration at the C-ring and the *trans*-fused γ-lactone D-ring is important (Fig. 3) [40,41]. The most active aryltetralin lignans **1**, **2**, and **5** show the 7′-(*R*), 8′-(*R*), and 8-(*R*) configuration. Compound **3** shows a 7′-(*R*), 8′-(*S*), and 8-(*R*) configuration, resulting in a *cis*-fused γ-lactone D-ring. This changes the quasi-axial orientation of the E-ring in trans-fused ring systems to a quasi-equatorial orientation of the E-ring, which in turn results in a decreased activity (Fig. 4) [22,40,42]. The 7′-(*S*), 8′-(*R*), 8-(*S*) configuration of lignan **4** leads to an inversed spatial orientation of the E-ring that prevents binding to the colchicine binding site (Fig. 4) [22,43,44]. Although lignan **7** possesses a double bond between C-8 and C-8′ the almost co-planar disposition remains, and the E-ring keeps a quasi-axial orientation. Therefore, it shows a comparable potency as the *trans*-lactone **5** (Fig. 4) [45]. Removal of the 4′-O-methyl group of the E-ring and of the 7-hydroxy group at the C-ring, as well as an additional 6-hydroxy group at the B-ring is known to increase potency [40,46]. Accordingly, the 4′-O-demethyl lignan **1** and the 6-hydroxy lignan **2** were the most active substances from *H. brachiata* (Fig. 4). Arylnaphthalene lignans show a rigid flat aromatic core structure resulting in a loss of activity [42,43], as seen with arylnaphthalenes **8**–**10** (Fig. 4).

Since podophyllotoxin (**5**) and similar aryltetralin lignans are known to cause G2/M arrest in dividing cells [22,23,32,39,47,48], the isolated lignans were tested in a cell cycle arrest assay to get insight in their mode of action (Fig. 5). The distribution of cells in each cell cycle phase was determined using a flow cytometric analysis. Vinblastine was used as positive control, and cyclosporine A was included to compare different mode of actions. As expected, the EtOAc extract as well as the aryltetralin lignans **1**, **2**, **3**, **7**, and podophyllotoxin (**5**) showed a dose dependent accumulation of cells in G2 phase. The same was observed for vinblastine, thereby suggesting an interaction with microtubule assembly in early stage of mitosis. For the extract as well as for **1**, **2**, **7**, and podophyllotoxin (**5**) this G2 arrest was observed at only lower test concentrations. At higher concentrations the G2 phase arrest declined again, presumably due to cytotoxicity, because dead cells cannot be

measured as G2 cells. Even though aryltetralin lignans increase dose-dependently apoptosis rate, apoptosis data of this study could not explain this decrease in G2 cells at higher test concentrations, because the change in apoptosis was not significantly different from that of unstimulated or stimulated cells (Figs. 1 and 4). Only lignan **3** showed a dose dependent increased G2 phase arrest without any decline over the investigated concentration range. Furthermore, the slight induction of apoptosis observed for **3** occurred only at higher concentrations compared to the other lignans. As expected, unstimulated, stimulated, and cells treated with cyclosporin A are accumulated mainly in G1 phase.

Beside antimitotic effects, the production and secretion of cytokines and the expression of surface activation markers of activated $CD4^+$ lymphocytes, like CD69, IFN-γ, IL-2, TNF-α, and IL-21, and of activated $CD8^+$ lymphocytes, like CD69, IL-2, TNF-α, and MIP1-β, was investigated. Podophyllotoxin and other aryltetralin lignans have been shown to influence the expression and secretion of different cytokines [29,37,49,50]. The EtOAc extract showed a weak concentration-dependent decrease in the secretion of IL-2 and IL-21 by $CD4^+$ lymphocytes, and a weak concentration-dependent decrease in the secretion of IL-2, TNF-α, and MIP1-β by $CD8^+$ lymphocytes. To trace back the observed effects to single substances, we tested all isolated compounds. Even though effects of aryltetralin lignans on cytokines have been reported in literature, we did not observe any effects on expression of activation markers and on cytokine release for both aryltetralin and arylnaphthalene lignans. No effects were also found for flavonoids. Although they did not show any effects on proliferation and apoptosis, the triterpenes **14** and **15** showed weak inhibitory effects on the secretion of IL-2 and IL21 in $CD4^+$ lymphocytes, and of IL-2, TNF-α, and MIP1-β in $CD8^+$ lymphocytes. From that we assume that the suppressive effects on T lymphocytes by the EtOAc extract from *H. brachiata* may be explained by synergistic effects between structurally different substance classes, the aryltetralin lignans inhibiting cell division and triterpenes reducing the secretion inflammatory cytokines. This is an interesting aspect since little is known about super additive and synergistic properties of different substance classes in this area. The groups of Onlamoon [51] and Gertsch [52] demonstrated that the flavonoids hispidulin and neptin as well as N-alkylamide combinations from different plants modulate immune cellular function of human peripheral lymphocytes in a synergistic and superadditive manner, respectively.

5. Conclusion

In conclusion, the EtOAc extract of *H. brachiata* exhibited strong inhibitory effects on T lymphocyte proliferation. The observed effect could be traced back to a synergistic interplay between different aryltetralin lignans and triterpenes. The aryltetralin lignans showed an antimitotic effect, while the triterpenes weakly lowered the production of inflammatory cytokines such as IL-2 and TNF-α by activated T lymphocytes. This makes the extract worthy of further investigation in the treatment of inflammatory and autoimmune diseases. Especially the aryltetralin lignans, which have already been investigated as antimitotic substances and as T lymphocyte proliferation inhibitors seem to be interesting candidates for further development in the field of T lymphocyte driven autoimmune diseases. In this context, research is ongoing on the development of new aryltetralin-like lignans, which combine the strong antimitotic effects with a decreased development of drug resistance and lower toxicity [53]. Beside single compounds, extracts of *H. brachiata* which contain both lignans and triterpenes with complementary modes of actions may also have a potential for future developments.

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CRediT authorship contribution statement

Matthias Hamburger, Olivier Potterat, Carsten Gründemann: Conceptualization, Validation. **Morris Keller, Moritz Winker**: Data curation. **Morris Keller, Moritz Winker, Nino Sperisen**: Formal analysis, Investigation. **Morris Keller, Moritz Winker, Mahabir P. Gupta, Matthias Hamburger, Olivier Potterat, Carsten Gründemann**: Methodology. **Amy Marisa Zimmermann-Klemd, Olivier Potterat, Carsten Gründemann**: Project administration. **Mahabir P. Gupta, Matthias Hamburger, Olivier Potterat, Carsten Gründemann**: Resources. **Morris Keller, Moritz Winker, Amy Marisa Zimmermann-Klemd, Nino Sperisen, Mahabir P. Gupta, Pablo N. Solis, Matthias Hamburger, Olivier Potterat, Carsten Gründemann**: Software. **Amy Marisa Zimmermann-Klemd, Matthias Hamburger, Olivier Potterat, Carsten Gründemann**: Supervision. **Morris Keller, Moritz Winker, Amy Marisa Zimmermann-Klemd, Olivier Potterat, Carsten Gründemann**: Visualization. **Morris Keller, Moritz Winker, Amy Marisa Zimmermann-Klemd**: Writing – original draft. **Morris Keller, Moritz Winker, Amy Marisa Zimmermann-Klemd, Olivier Potterat, Carsten Gründemann**: Writing – review & editing.

Conflict of interest statement

The authors declare no competing interests.

Data availability

The datasets generated for this study are available on request to the corresponding author.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2023.114328.

References

[1] U. Khan, H. Ghazanfar, T Lymphocytes and autoimmunity, Int. Rev. Cell Mol. Biol. 341 (2018) 125–168, https://doi.org/10.1016/bs.ircmb.2018.05.008.
[2] M. Her, A. Kavanaugh, Alterations in immune function with biologic therapies for autoimmune disease, J. Allergy Clin. Immunol. 137 (2016) 19–27, https://doi.org/10.1016/j.jaci.2015.10.023.
[3] A.C. Allison, Immunosuppressive drugs: the first 50 years and a glance forward, Immunopharmacology 47 (2000) 63–83, https://doi.org/10.1016/S0162-3109(00)00186-7.
[4] S. Mathur, C. Hoskins, Drug development: lessons from nature (review), Biomed. Rep. 6 (2017) 612–614, https://doi.org/10.3892/br.2017.909.
[5] J. Río, M. Comabella, X. Montalban, Predicting responders to therapies for multiple sclerosis, Nat. Rev. Neurol. 5 (2009) 553–560, https://doi.org/10.1038/nrneurol.2009.139.
[6] C. Daubert, N. Behar, R.P. Martins, P. Mabo, C. Leclercq, Avoiding non-responders to cardiac resynchronization therapy: a practical guide, Eur. Heart J. 38 (2017) 1463–1472, https://doi.org/10.1093/eurheartj/ehw270.
[7] D.D. Chaplin, Overview of the immune response, J. Allergy Clin. Immunol. 125 (2010) S3–S23, https://doi.org/10.1016/j.jaci.2009.12.980.
[8] M.D. Rosenblum, K.A. Remedios, A.K. Abbas, Mechanisms of human autoimmunity, J. Clin. Invest. 125 (2015) 2228–2233, https://doi.org/10.1172/JCI78088.
[9] D. Velázquez, E. de Arrioja, S. Tillett, Usos populares de Lamiaceae en Venezuela, Acta Botánica Venezuélica 18 (1995) 5–20.
[10] T. Hell, A. Rutz, L. Dürr, M. Dobrzyński, J.K. Reinhardt, T. Lehner, M. Keller, A. John, M. Gupta, O. Pertz, M. Hamburger, J.L. Wolfender, E. Garo, Combining activity profiling with advanced annotation to accelerate the discovery of natural

products targeting oncogenic signaling in melanoma, J. Nat. Prod. 2022 (2022) 1540–1554, https://doi.org/10.1021/acs.jnatprod.2c00146.
[11] G. Tafurt-Garcia, A. Munoz-Acevedo, A.M. Calvo, L.F. Jimenez, W.A. Delgado, Volatile compounds of analysis of *Eriope crassipes*, *Hyptis conferta*, *H. dilatata*, *H. brachiata*, *H. suaveolens* and *H. mutabilis* (Lamiaceae), Bol. Latinoam. Y. Del. Caribe Plantas Med. Y. Aromát 13 (2014) 254–269.
[12] S.F. Ziegler, F. Ramsdell, M.R. Alderson, The activation antigen CD69, Stem Cells 12 (1994) 456–465, https://doi.org/10.1002/stem.5530120502.
[13] H. Bridi, G. de Carvalho Meirelles, G. Lino von Poser, Subtribe Hyptidinae (Lamiaceae): a promising source of bioactive metabolites, J. Ethnopharmacol. 264 (2021), 113225, https://doi.org/10.1016/j.jep.2020.113225.
[14] Y.J. Zhang, M. Litaudon, H. Bousserouel, M.T. Martin, O. Thoison, S. Léonce, V. Dumontet, T. Sévenet, F. Guéritte, Sesquiterpenoids and cytotoxic lignans from the bark of *Libocedrus chevalieri*, J. Nat. Prod. 70 (2007) 1368–1370, https://doi.org/10.1021/np070124q.
[15] Z. Yang, P. Guo, R. Han, J.M. Gao, Preparative separation of flavone dimers from *Dysosma versipellis* by counter-current chromatography: trifluoroacetic acid as a solvent system modifier, J. Sep. Sci. 41 (2018) 3631–3643, https://doi.org/10.1002/jssc.201800530.
[16] M. Atta-ur-Rahman, M.Iqbal Ashraf, Choudhary, M.H. Habib-ur-Rehman, Kazmi, Antifungal aryltetralin lignans from leaves of *Podophyllum hexandrum*, Phytochemistry 40 (1995) 427–431, https://doi.org/10.1016/0031-9422(95)00195-D.
[17] Y.J. Sun, Z.L. Li, H. Chen, X.Q. Liu, W. Zhou, H.M. Hua, Three new cytotoxic aryltetralin lignans from *Sinopodophyllum emodi*, Bioorg. Med. Chem. Lett. 21 (2011) 3794–3797, https://doi.org/10.1016/j.bmcl.2011.04.036.
[18] C.O. Van Den Broucke, R.A. Dommisse, E.L. Esmans, J.A. Lemli, Three methylated flavones from *Thymus vulgaris*, Phytochemistry 21 (1982) 2581–2583, https://doi.org/10.1016/0031-9422(82)85261-8.
[19] M.A. El-Ansari, D. Barron, M.F. Abdalla, N.A.M. Saleh, J.L.Le Quéré, Flavonoid constituents of *Stachys aegyptiaca*, Phytochemistry 30 (1991) 1169–1173, https://doi.org/10.1016/S0031-9422(00)95197-5.
[20] X. Liu, J. Fu, X.J. Yao, J. Yang, L. Liu, T.G. Xie, P.C. Jiang, Z.H. Jiang, G.Y. Zhu, Phenolic constituents isolated from the twigs of *Cinnamomum cassia* and their potential neuroprotective effects, J. Nat. Prod. 81 (2018) 1333–1342, https://doi.org/10.1021/acs.jnatprod.7b00924.
[21] H.N. Brandão, H.H.S. Medrado, J.P. David, J.M. David, J.F.B. Pastore, M. Meira, Determination of podophyllotoxin and related aryltetralin lignans by HPLC/DAD/MS from Lamiaceae species, Microchem. J. 130 (2017) 179–184, https://doi.org/10.1016/j.microc.2016.09.002.
[22] M. Antúnez-Mojica, J. Rodríguez-Salarichs, M. Redondo-Horcajo, A. León, I. Barasoain, Á. Canales, F.J. Cañada, J. Jiménez-Barbero, L. Alvarez, J.F. Díaz, Structural and biochemical characterization of the interaction of tubulin with potent natural analogues of podophyllotoxin, J. Nat. Prod. 79 (2016) 2113–2121, https://doi.org/10.1021/acs.jnatprod.6b00428.
[23] S.R. Dahlqvist, B. Norberg, K. Sondell, I. Nordenson, G. Holmgren, The effect of CPH 82 on the growth of human lymphocytes in vitro. Definition of cytobiological action, Rheumatology 28 (1989) 418–421, https://doi.org/10.1093/rheumatology/28.5.418.
[24] T. Hirano, A. Wakasugi, M. Oohara, K. Oka, Y. Sashida, Suppression of mitogen-induced proliferation of human peripheral blood lymphocytes by plant lignans, Planta Med 57 (1991) 331–334, https://doi.org/10.1055/s-2006-960110.
[25] C. Brigati, B. Sander, CPH-86, a highly purified podophyllotoxin, efficiently suppresses in vivo and in vitro immune responses, Immunopharmacol. Immunotoxicol. 7 (1985) 285–302, https://doi.org/10.3109/08923978509026477.
[26] J. Lysholm, T. Weitoft, Proresid in the long-term treatment of rheumatoid arthritis, Scand. J. Rheumatol. 17 (1988) 465–468, https://doi.org/10.3109/03009748809098808.
[27] L. Truedsson, P. Geborek, G. Sturfelt, Antiproliferative effects on human peripheral blood mononuclear cells and inhibition of in vitro immunoglobulin synthesis by podophyllotoxin (CPH86) and by semisynthetic lignan glycosides (CPH82), Clin. Exp. Rheumatol. 11 (1993) 179–182.
[28] S. Rantapää-Dahlqvist, E. Löfvenbert, B. Norberg, Effect of CPH82 in rheumatoid arthritis. Accumulation of bone marrow cells in mitosis and clinical response, Clin. Rheumatol. 12 (1993) 541–542, https://doi.org/10.1007/BF02231790.
[29] K. Carlström, P.J. Hedin, L. Jönsson, T. Lerndal, J. Lien, T. Weitoft, M. Axelson, Endocrine effects of the podophyllotoxine derivative drug CPH 82 (Reumacon®) in patients with rheumatoid arthritis, Scand. J. Rheumatol. 29 (2000) 89–94, https://doi.org/10.1080/030097400750001888.
[30] E.S. Gudmundsdóttir, H. Jónsson, CPH 82 (Reumacon®) in refractory inflammatory arthritis, Scand. J. Rheumatol. 29 (2000) 323–325, https://doi.org/10.1080/030097400447723.
[31] T. Lerndal, B. Svensson, A clinical study of CPH 82 vs methotrexate in early rheumatoid arthritis, Rheumatology 39 (2000) 316–320, https://doi.org/10.1093/rheumatology/39.3.316.
[32] S.R. Dahlqvist, G. Landberg, G. Roos, B. Norberg, Cell cycle effects of the anti-rheumatic agent CPH82, Rheumatology 33 (1994) 327–331, https://doi.org/10.1093/rheumatology/33.4.327.
[33] A. Larsen, I. Petersson, B. Svensson, Podophyllum derivatives (CPH 82) compared with placebo in the treatment of rheumatoid arthritis, Rheumatology 28 (1989) 124–127, https://doi.org/10.1093/rheumatology/28.2.124.
[34] B. Svensson, K. Herlyn, B. Rosén, CPH 82 (Reumacon®): A new and promising basic therapy in rheumatoid arthritis, Aktuelle Rheumatol. 24 (1999) 58–61, https://doi.org/10.1055/s-2008-1043548.
[35] B. Mansson, P. Geborek, T. Hallberg, G. Sturfelt, Inhibition of ConA-induced proliferate response in human lymphocytes by podophyllotoxin and a detergent, Tween80, Clin. Exp. Rheumatol. 6 (1988) 405–407.
[36] S. Meske, H.H. Peter, Podophyllotoxin therapy in rheumatoid arthritis: a clinical open dose-response study, Aktuelle Rheumatol. 15 (1990) 154–155, https://doi.org/10.1055/s-2008-1047442.
[37] L. Bohlin, B. Rosén, Podophyllotoxin derivatives: drug discovery and development, Drug Discov. Today 1 (1996) 343–351, https://doi.org/10.1016/1359-6446(96)10028-3.
[38] M. Antúnez-Mojica, A. León, A.M. Rojas-Sepúlveda, S. Marquina, M.A. Mendieta-Serrano, E. Salas-Vidal, M.L. Villarreal, L. Alvarez, Aryldihydronaphthalene-type lignans from *Bursera fagaroides* var. *fagaroides* and their antimitotic mechanism of action, RSC Adv. 6 (2016) 4950–4959, https://doi.org/10.1039/c5ra23516b.
[39] M. Antúnez-Mojica, A.M. Rojas-Sepúlveda, M.A. Mendieta-Serrano, L. Gonzalez-Maya, S. Marquina, E. Salas-Vidal, L. Alvarez, Lignans from *Bursera fagaroides* affect in vivo cell behavior by disturbing the tubulin cytoskeleton in zebrafish embryos, Molecules 24 (2019) 8, https://doi.org/10.3390/molecules24010008.
[40] M. Gordaliza, M.A. Castro, M.D. García-Grávalos, P. Ruiz, J.M.M. Del Corral, A.S. Feliciano, Antineoplastic and antiviral activities of podophyllotoxin related lignans, Arch. Pharm. (Weinh.). 327 (1994) 175–179, https://doi.org/10.1002/ardp.19943270309.
[41] B. Botta, G. Monache, D. Misiti, A. Vitali, G. Zappia, Aryltetralin lignans: chemistry, pharmacology and biotransformations, Curr. Med. Chem. 8 (2001) 1363–1381, https://doi.org/10.2174/0929867013372292.
[42] A. San Feliciano, M. Gordaliza, J.M. Miguel Del Corral, M.A. Castro, M.D. Garcia-Gravalos, P. Ruiz-Lazaro, Antineoplastic and antiviral activities of some cyclolignans, Planta Med 59 (1993) 246–249, https://doi.org/10.1055/s-2006-959660.
[43] M. Novelo, J.G. Cruz, L. Hernández, R. Pereda-Miranda, H. Chai, W. Mar, J. M. Pezzuto, Cytotoxic constituents from *Hyptis verticillata*, J. Nat. Prod. 56 (1993) 1728–1736, https://doi.org/10.1021/np50100a011.
[44] F. Zavala, D. Guenard, J.P. Robin, E. Brown, Structure-antitubulin activity relationships in steganacin congeners and analogs. Inhibition of tubulin polymerization in vitro by (±)-isodeoxypodophyllotoxin, J. Med. Chem. 23 (1980) 546–549, https://doi.org/10.1021/jm00179a014.
[45] M. Gordaliza, G.T. Faircloth, M.A. Castro, J.M. Miguel Del Corral, M.L. López-Vázquez, A. San Feliciano, Immunosuppressive cyclolignans, J. Med. Chem. 39 (1996) 2865–2868, https://doi.org/10.1021/jm960023h.
[46] E. Ter Haar, H.S. Rosenkranz, E. Hamel, B.W. Day, Computational and molecular modeling evaluation of the structural basis for tubulin polymerization inhibition by colchicine site agents, Bioorg. Med. Chem. 4 (1996) 1659–1671, https://doi.org/10.1016/0968-0896(96)00158-7.
[47] M. Ángeles Castro, J.M. Miguel Del Corral, P.A. García, M. Victoria Rojo, A. C. Bento, F. Mollinedo, A.M. Francesch, A. San Feliciano, Lignopurines: a new family of hybrids between cyclolignans and purines. Synthesis and biological evaluation, Eur. J. Med. Chem. 58 (2012) 377–389, https://doi.org/10.1016/j.ejmech.2012.10.026.
[48] Y. Yong, S.Y. Shin, Y.H. Lee, Y. Lim, Antitumor activity of deoxypodophyllotoxin isolated from *Anthriscus sylvestris*: induction of G2/M cell cycle arrest and caspase-dependent apoptosis, Bioorg. Med. Chem. Lett. 19 (2009) 4367–4371, https://doi.org/10.1016/j.bmcl.2009.05.093.
[49] N. Pugh, I.A. Khan, R.M. Moraes, D.S. Pasco, Podophyllotoxin lignans enhance IL-1β but suppress TNF-α mRNA expression in LPS-treated monocytes, Immunopharmacol. Immunotoxicol. 23 (2001) 83–95, https://doi.org/10.1081/IPH-100102570.
[50] X.-H. Gan, J.P. Robin, J.M.M. Huerta, P. Braquet, B. Bonavida, Inhibition of tumor necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β) secretion but not IL-6 from activated human peripheral blood monocytes by a new synthetic demethylpodophyllotoxin derivative, J. Clin. Immunol. 14 (1994) 280–288, https://doi.org/10.1007/BF01540981.
[51] P. Thitilertdecha, V. Tantithavorn, P. Poungpairoj, N. Onlamoon, Synergistic immunosuppressive effect of hispidulin and nepetin mixtures on human T lymphocytes, Immunopharmacol. Immunotoxicol. 44 (2022) 693–703, https://doi.org/10.1080/08923973.2022.2077216.
[52] A. Chicca, S. Raduner, F. Pellati, T. Strompen, K.H. Altmann, R. Schoop, J. Gertsch, Synergistic immunomopharmacological effects of N-alkylamides in *Echinacea purpurea* herbal extracts, Int. Immunopharmacol. 9 (2009) 850–858, https://doi.org/10.1016/j.intimp.2009.03.006.
[53] S. Shen, Y. Tong, Y. Luo, L. Huang, W. Gao, Biosynthesis, total synthesis, and pharmacological activities of aryltetralin-type lignan podophyllotoxin and its derivatives, Nat. Prod. Rep. 39 (2022) 1856–1875, https://doi.org/10.1039/d2np00028h.