



Boswellia carteri extract and 3-O-acetyl-alpha-boswellic acid suppress T cell function

Amy M. Zimmermann-Klemd^{a,1}, Jakob K. Reinhardt^{b,1}, Thanasan Nilsu^c, Anna Morath^{d,e,f,g}, Chiara M. Falanga^a, Wolfgang W. Schamel^{d,e,g}, Roman Huber^a, Matthias Hamburger^b, Carsten Gründemann^{h,*}

^a Center for Complementary Medicine, Institute for Infection Prevention and Hospital Epidemiology, Faculty of Medicine, University of Freiburg, Freiburg, Germany

^b Pharmaceutical Biology, Pharmazentrum, University of Basel, Basel, Switzerland

^c Kamnoetvidya Science Academy, Wang Chan, Rayong, Thailand

^d Signalling Research Centres BIOS and CIBS, University of Freiburg, Freiburg, Germany

^e Institute of Biology III, Faculty of Biology, University of Freiburg, Freiburg, Germany

^f Spemann Graduate School of Biology and Medicine, University of Freiburg, Freiburg, Germany

^g Center for Chronic Immunodeficiency, Medical Center Freiburg and Faculty of Medicine, University of Freiburg, Freiburg, Germany

^h Translational Complementary Medicine, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland

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ABSTRACT

Resins from various *Boswellia* species have a long track record in different cultures as a treatment for inflammatory diseases. This study was designed to provide evidence for the anti-inflammatory capacity and medicinal use of *Boswellia carteri* (Burseraceae). A dichloromethane (DCM) extract of *B. carteri* gum resin and isolated compounds thereof were immunologically characterized. Flow cytometric-based analysis was performed to investigate the impact of *B. carteri* extract on proliferation, viability, and function of anti-CD3 and anti-CD28 activated human primary T cells. The secretion level of IL-2 and IFN- γ was determined by a bead array-based flow cytometric technique. HPLC-based activity profiling of the *B. carteri* extract identified active compounds. The impact of *B. carteri* extract and isolated compounds on the IL-2 transcription factor activity was addressed using specially designed Jurkat reporter cells. The extract of *B. carteri* suppressed the proliferation of human primary T lymphocytes in vitro in a concentration-dependent manner, without inducing cytotoxicity. Thereby, the *B. carteri* extract further reduced the degranulation capacity and cytokine secretion of stimulated human T cells. Transcription factor analysis showed that the immunosuppressive effects of the extract are based on specific NFAT-conditioned suppression within T cell signaling. Through HPLC-based activity profiling of the extract, 3-O-acetyl- α -boswellic acid was identified as the compound responsible for the NFAT-based mechanism. The recent study presents a scientific base for the immunosuppressive effects of *B. carteri* gum resin extract including a mode-of-action via the NFAT-conditioned suppression of T lymphocyte proliferation. The immunosuppressive effects of 3-O-acetyl- α -boswellic acid are depicted for the first time.

Abbreviations: AKBA, *o*-acetyl-11-keto- β -boswellic; AP-1, activator protein 1; APC, allophycocyanin; ASE, accelerated Solvent Extraction; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; CPT, camptothecin; CsA, cyclosporine A; DCM, dichloromethane; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; FA, formic acid; FACS, fluorescence-activated cell sorting; FCS, fetal bovine serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; HPLC, high-performance liquid chromatography; i.d., inner diameter; IFN-, interferon-; IL-, interleukin-; KBA, 1-keto- β -boswellic acid; mAb, monoclonal antibody; NFAT, nuclear factor of activated T-cells; NF- κ B, nuclear factor kappa-light-chain-enhancer of activated B cells; *p*, *p*-value; PBMC, peripheral blood mononuclear cell; PBS, phosphate buffered saline; PE, phycoerythrin; Pen/Strep, penicillin/streptomycin; PI, propidium iodide; PMA, phorbol-12-myristat-13-acetate; RPMI 1640, Roswell Park Memorial Institute medium; SD, standard deviation; TCM, Traditional Chinese Medicine; TCR, T cell receptor

* Corresponding author at: Translational Complementary Medicine, University of Basel, Department of Pharmaceutical Sciences, Klingelbergstrasse 50, CH-4056 Basel, Switzerland.

E-mail address: carsten.gruendemann@unibas.ch (C. Gründemann).

¹ Equally contributed to this work.

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1. Introduction

The medical use of the *Boswellia* species (Burseraceae) has a long track record in various cultures. The resin of the *Boswellia* species was known as frankincense, or olibanum, and was used for the treatment of various disorders, including inflammatory diseases [1]. In Ayurvedic medicine, the resin of *Boswellia serrata* Roxb. ex Colebr. is used, whereas the resin of *Boswellia sacra* Flueck. (*B. carteri* Birdw.) and other species are employed in traditional Chinese medicine (TCM). *B. serrata* and *B. carteri* resins are complex mixtures of triterpenoids; mono-, sesqui-, and diterpenoids; and polysaccharide gum [2,26].

The anti-inflammatory properties of *Boswellia* resins have already been investigated to a certain extent. For example, cultured, lipopolysaccharide (LPS)-stimulated, peripheral blood monocytes (PBMCs) showed a reduced secretion of tumor necrosis factor α (TNF α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and interleukin-8 (IL-8), following treatment with *B. serrata* extract [11,12]. Additionally, the downregulation of Th1 cytokines interferon- γ (IFN- γ) and interleukin-12 (IL-12) and an upregulation of Th2 cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) were observed upon treatment of PBMCs with *B. serrata* extract [11]. Further in vitro studies using polymorph mononuclear neutrophil leukocytes (PMNLs) confirmed an inhibitory effect of *B. serrata* on mediators of the arachidonic acid cascade [25]. *B. carteri* gum resin is studied less, but in mouse splenocytes a suppression of the Th1 cytokine production, and an enhanced Th2 cytokine production was observed [6].

Previous investigations identified boswellic acids, especially 11-keto- β -boswellic acid (KBA) and *O*-acetyl-11-keto- β -boswellic acid (AKBA), as compounds responsible for the anti-inflammatory properties of *Boswellia* resin [1]. There is evidence that boswellic acids suppress the immune response via inhibition of the NF- κ B activity in LPS

activated monocytes (Syrovets et al., 2005a), and the immune suppressive effects were confirmed in an autoimmune model for psoriasis in rodents [30].

In autoimmune diseases, the immune cells lose their ability to distinguish between self and nonself structures [31]. This in turn leads to an overreaction of the immune system, including an enhanced proliferation, activation and mediator secretion of T lymphocytes.

We here focus on other, not yet investigated, aspects of *B. carteri* extract with relation to an overwhelming immune system, such as the proliferation, activation, and function of T cells. The results indicate a mode-of-action of *B. carteri* gum resin extract and boswellic acids via NFAT-conditioned immune suppression. 3-*O*-acetyl- α -boswellic acid was found to be at least partly responsible for the observed effects on the NFAT activity.

2. Materials and methods

2.1. Ethics statement

All experiments conducted with human material were approved by the Ethics Committee of the University of Freiburg (55/14; 11.02.2014), and all methods used were compliant with the regulations of the Ethics Committee.

2.2. Preparation of *B. carteri* dichloromethane (DCM) extract

B. carteri gum resin extract was prepared using Accelerated Solvent Extraction (ASE). For this, the ground gum resin of *B. carteri* (Lian Chinaherb AG, Wollerau, Switzerland, Article Nr. 2174, charge M08201004A; a voucher specimen with the number 00969 has been

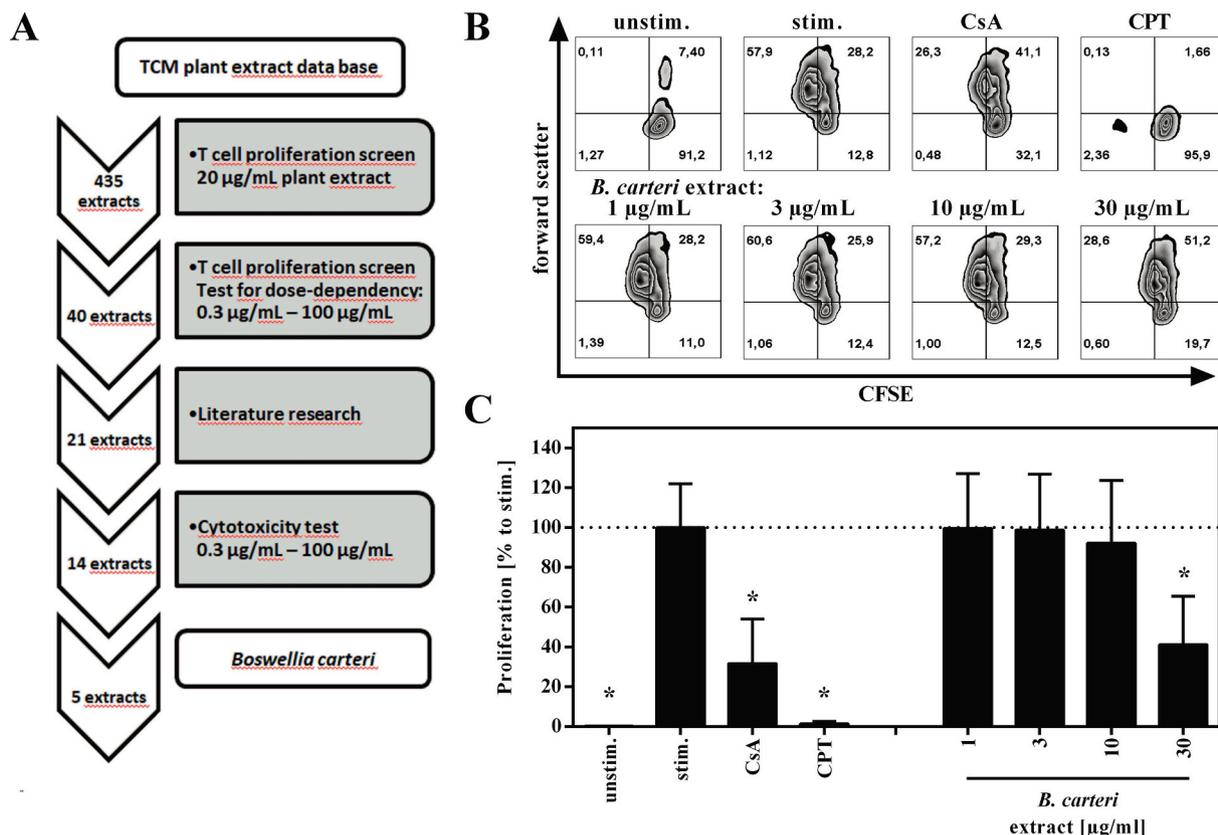


Fig. 1. Inhibitory effects of *B. carteri* extract on the proliferation of T lymphocytes. (A) Flowchart of the screening process. (B, C) Human PBMCs (2×10^5) were stained with CFSE and stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). Unstimulated cells (unstim.) served as a control. Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 72 h in the presence of medium (stim.), cyclosporine A (CsA; 4.16 μ M), camptothecin (CPT; 300 μ M), or the *B. carteri* gum resin extract. Cell division was analyzed by flow cytometry. Data are depicted as zebra plots (B). Numbers indicate the percentage of quadrants. The percentage of proliferating cells was compared and normalized to the stimulated control and depicted as mean \pm standard deviation (C). $n = 3$; * $p < .05$.

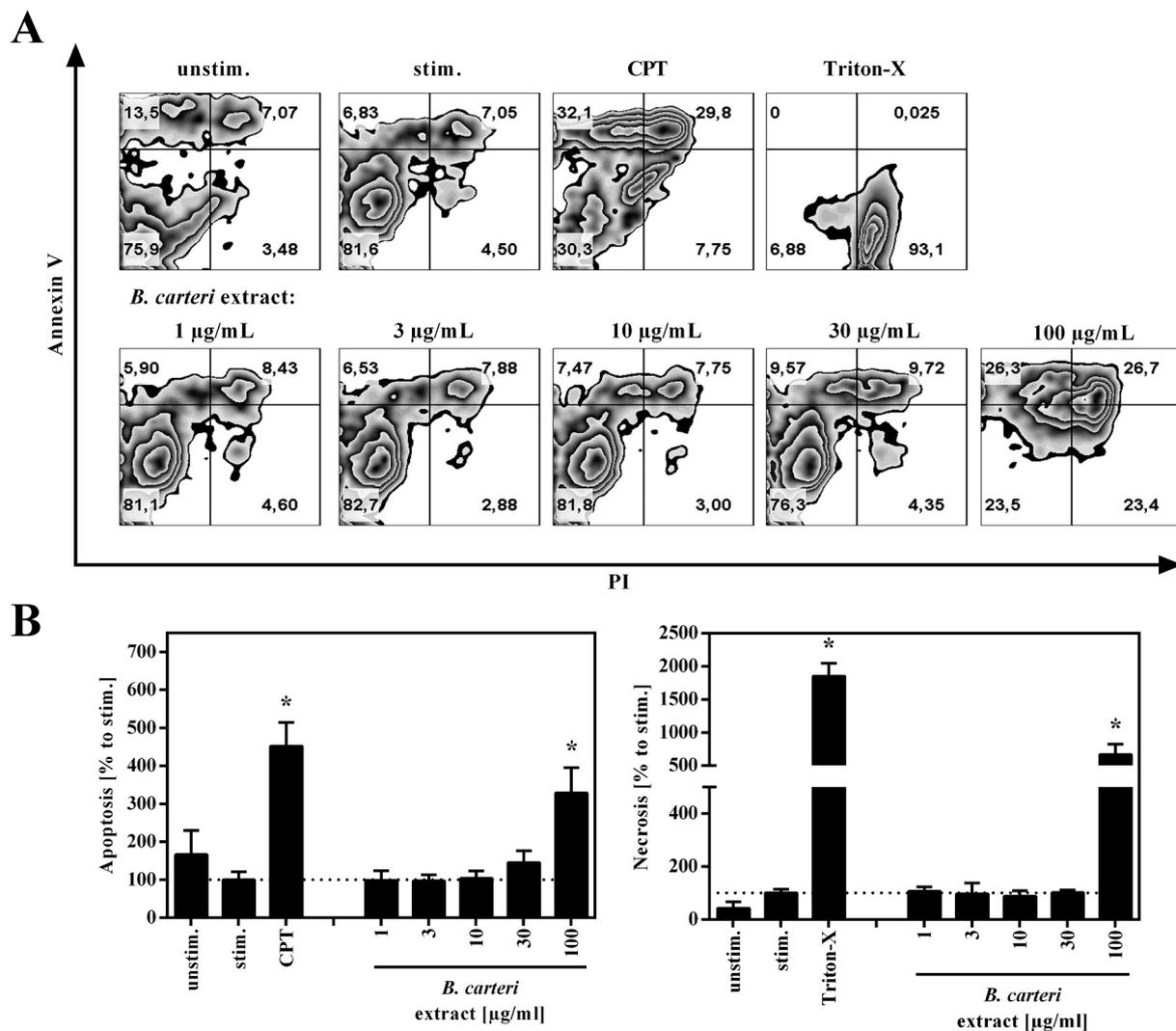


Fig. 2. Effect of *B. carteri* extract on the viability of T lymphocytes. Human PBMCs (2×10^5) were stimulated with anti-CD3 and anti-CD28 mAbs antibodies (100 ng/mL each). Unstimulated cells served as a control (unstim.). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 48 h with medium (stim.), camptothecin (CPT; 300 μ M), Triton-X 100 (0.5%) or *B. carteri* gum resin extract. Annexin V-FITC and PI double staining was performed. The proportions of viable, necrotic and apoptotic cells were analyzed via flow cytometry. Data are depicted as zebra plots (A). Numbers indicate the percentage of quadrants. The amount of apoptotic/necrotic lymphocytes compared to the stimulated control was determined and depicted as mean \pm standard deviation (B). $n = 3$; $*p < .05$.

deposited at the Division of Pharmaceutical Biology, University of Basel, Switzerland), was extracted with redistilled, technical grade, dichloromethane (DCM) for three cycles with an ASE 200 extraction system with solvent module (Dionex) at 70 $^{\circ}$ C and 120 bar. The HPLC profile of the extract is shown in Fig. S1. The extracts from all cycles were combined and dried in vacuo yielding a crude extract with approx. 19% yield.

2.3. Preparation and cultivation of human peripheral lymphocyte

Preparation and cultivation of human peripheral lymphocytes was performed as indicated in [32]. Briefly, lymphocytes were isolated from the blood of healthy adult donors obtained from the Blood Transfusion Centre (Medical Centre – University of Freiburg) via sugar gradient separation and were finally cultured in RPMI 1640 full medium. Lymphocytes were stimulated with anti-CD3- and anti-CD28 mAbs and treated as described in the figure legends and already by our group [32]. After cultivation, the cells were assessed in biological tests as indicated.

2.4. Determination of apoptosis and necrosis of T cells

Cells were treated for 48 h, as described in 2.3. Cultured cells were washed with PBS and stained with Annexin V-FITC and propidium

iodide using the Apoptosis Detection kit (eBioscience, Frankfurt, Germany) according to manufacturer's instructions. Apoptosis and necrosis rates were determined by flow cytometric analysis using a FACS analysis. Cell populations were described as follows: viable (annexinV⁻/PI⁻), early (annexinV⁺/PI⁻) and late apoptotic (annexinV⁺/PI⁺) and necrotic (annexinV⁻/PI⁺). In graphs, early and late apoptotic cells were summarized as apoptotic cells.

2.5. Determination of T cell proliferation

The proliferation of T lymphocytes was determined via carboxyfluorescein diacetate succinimidyl ester (CFSE) staining, as described earlier [13,21].

2.6. Analysis of activation marker of T cells

The activation state of T lymphocytes was determined via cell surface analysis of CD25 and CD69, as as previously reported [14].

2.7. Determination of cytokines

Cells were treated for 20 h, as described in 2.3, and restimulated

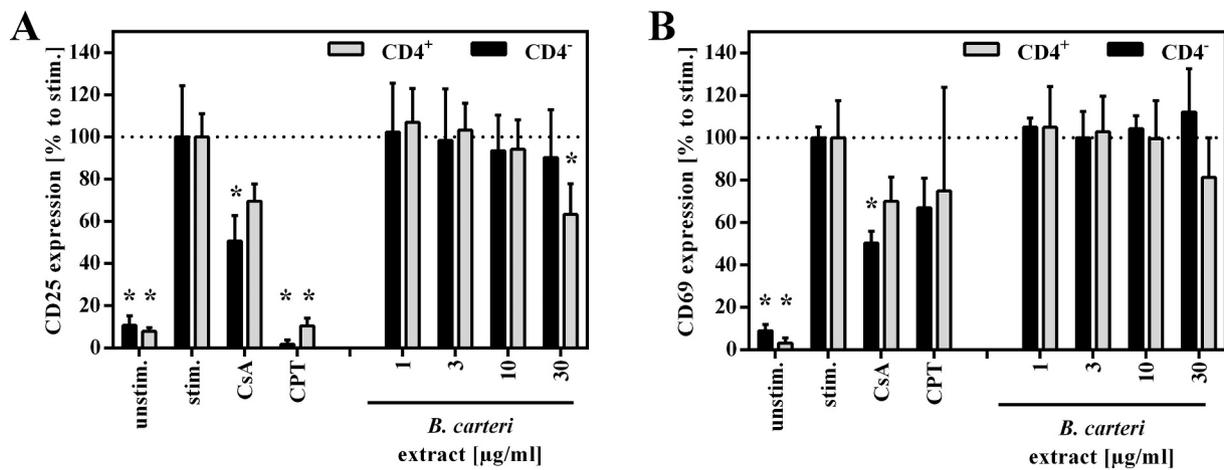


Fig. 3. Effects of *B. carteri* extract on the activation of T cells. Human PBMCs (2×10^5) were left unstimulated (unstim.), or stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 24 h with or without (stim.) the addition of cyclosporine A (CsA; 4.16 μ M), camptothecin (CPT; 300 μ M), or the *B. carteri* gum resin extract. Cells were stained with anti-CD25-PE (A) or anti-CD69-FITC (B) and anti-CD4-APC and analyzed by flow cytometry. Bar diagrams depict the percentage of CD4⁺ and CD4⁻ T lymphocytes that express CD25 or CD69 in relation to the untreated, stimulated control \pm standard deviation. n = 3; *p < .05.

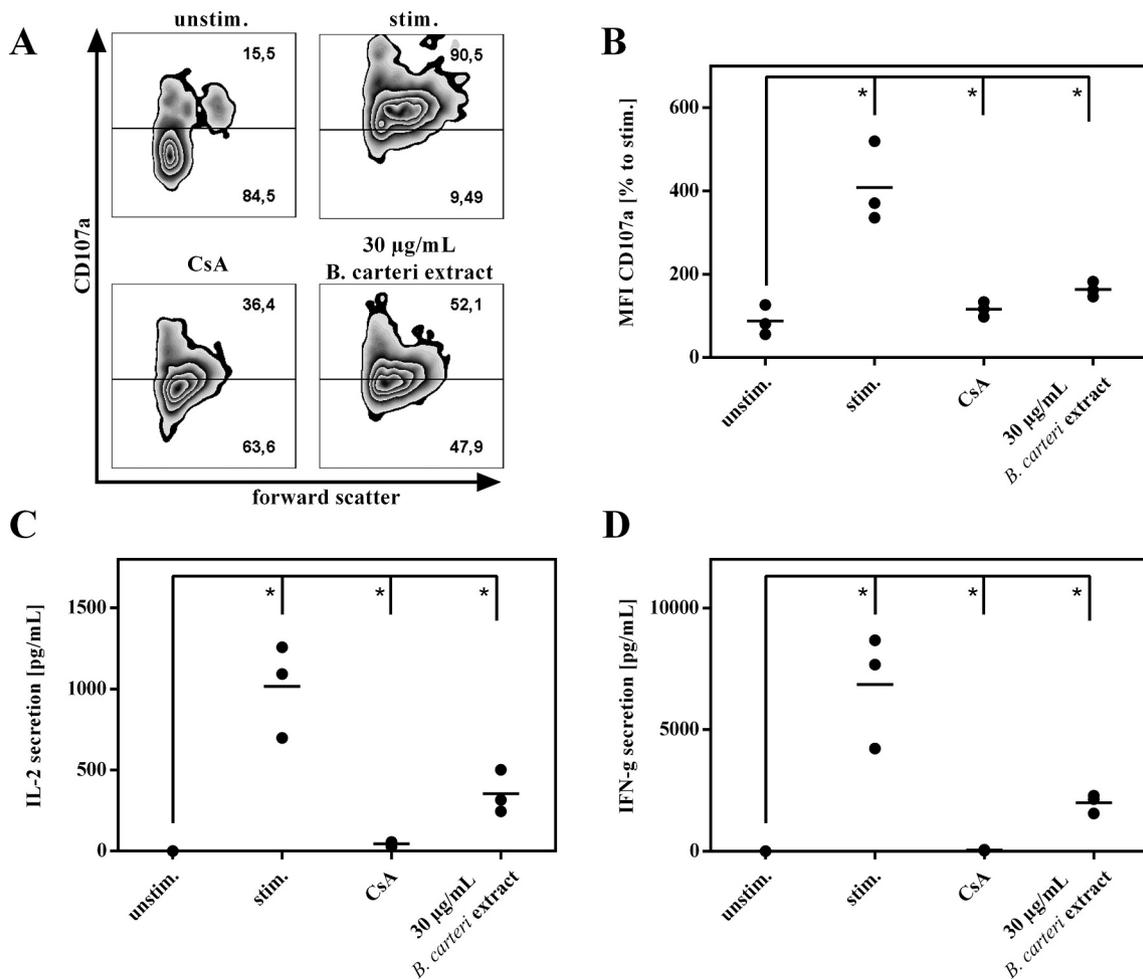


Fig. 4. Effects of *B. carteri* extract on the degranulation capacity of T lymphocytes and on IL-2 and IFN- γ secretion. Human PBMCs (2×10^5) were stimulated with anti-human CD3 and anti-human CD28 mAbs (100 ng/mL each) or left unstimulated (unstim.). Anti-CD3 and anti-CD28 activated cells were incubated for 20 h with medium (stim.), cyclosporine A (CsA; 4.16 μ M) or *B. carteri* gum resin extract. Cells (except the unstim. control) were re-stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) for 4 h. (A, B) Cells were stained with CD107a-PE, and CD107a surface expression was determined via flow cytometry. Data are depicted in zebra plots (A). The mean fluorescence intensity (MFI) of CD107a was determined and is depicted in relation to the stimulated control (B). n = 3, *p < .05. (C, D) The amounts of IL-2 (C) and IFN- γ (D) were determined in the supernatant by LEGENDplex™. Results are depicted as mean \pm standard deviation in relation to the untreated, stimulated control. n = 3; *p < .05.

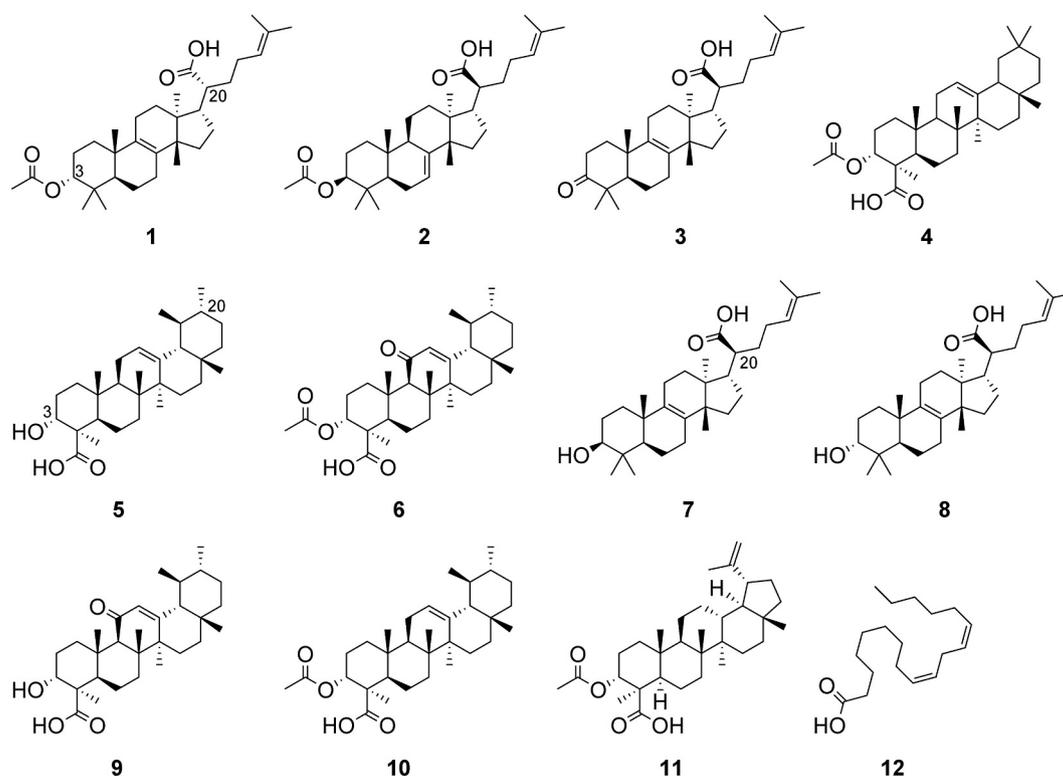


Fig. 5. Isolated compounds 1–12.

with PMA (50 ng/mL; Sigma-Aldrich, Taufkirchen, Germany) and ionomycin (500 ng/mL; Sigma-Aldrich, Taufkirchen, Germany) for additional 4 h at 37 °C. Supernatants were harvested by centrifugation and stored at –20 °C. The amount of cytokines was determined using LEGENDplex™ according to the manufacturer's instructions (BioLegend, San Diego, CA).

2.8. Analysis of T cell degranulation

A CD107a surface staining was performed, as described [15], to determine the T cell degranulation capacity.

2.9. Reporter cell experiments for the determination of NFAT-, NF-κB- and AP-1 activity

A 96 well F-bottom cell culture plate was coated with anti-human CD3 monoclonal antibodies (mAbs) (clone OKT3, 1 µg/mL, 50 µL/well) or phosphate buffered saline (unstim.). Here we used individual designed human Jurkat T cell reporter cells, where the response elements for NF-κB, NFAT and AP-1 drive the expression of each fluorescent proteins (CFP, eGFP and mCherry, respectively). Reporter cells [17] were seeded in 5% FCS RPMI 1640 cell culture medium (0.15×10^6 cells in 200 µL/ well) and treated with inhibitors (1 nM SP100030 for AP-1, 5 µg/mL cyclosporine A for NFAT and 20 µM parthenolide for NF-κB), gum resin extract or isolated compounds from *B. carteri* or remained untreated (unstim., stim.). Cells were incubated at 37 °C for 8 h (AP-1) or 24 h (NFAT and NF-κB). The expression of eGFP was determined by FACS analysis.

2.10. Isolation and identification of compounds 1–12

Extraction and isolation of compounds 1–12 from *B. carteri* gum resin DCM extract is described in detail in the supplementary information. NMR data were recorded on a Bruker Avance II NMR spectrometer operating at 500.13 MHz for ¹H and 125.77 MHz for ¹³C nuclei. ¹H NMR data and COSY, HSQC, HMBC, and ROESY spectra were

measured at 18 °C in a 1 mm TXI probe with a z-gradient. ¹³C NMR/DEPTQ spectra were recorded at 23 °C in 3 mm tubes with a 5 mm BBI probe. Spectra were analyzed by Bruker TopSpin 3.0 and ACDLabs Spectrus Processor. Samples were measured in CDCl₃ (Sigma-Aldrich).

2.11. Analysis of data

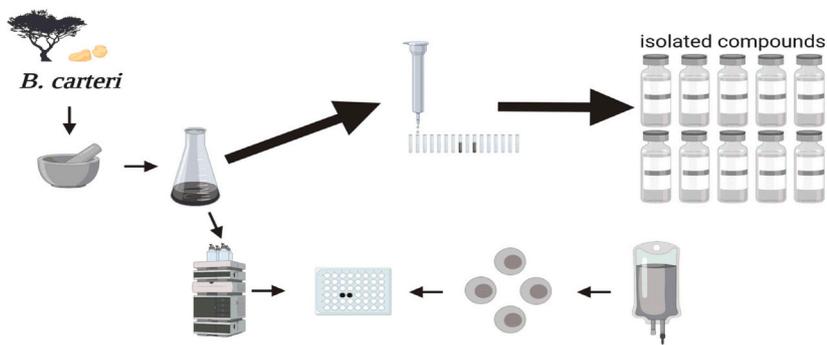
For statistical analysis, data was processed with Microsoft Excel and SPSS software (Version 22.0, IBM, Armonk, USA). Statistical significance was determined with the SPSS software by a one-way ANOVA followed by Dunnett's post hoc pairwise comparisons. Values are presented as mean ± standard deviation (SD) for the indicated number of independent experiments. The asterisks represent significant differences from controls (**p* < .05).

3. Results and discussion

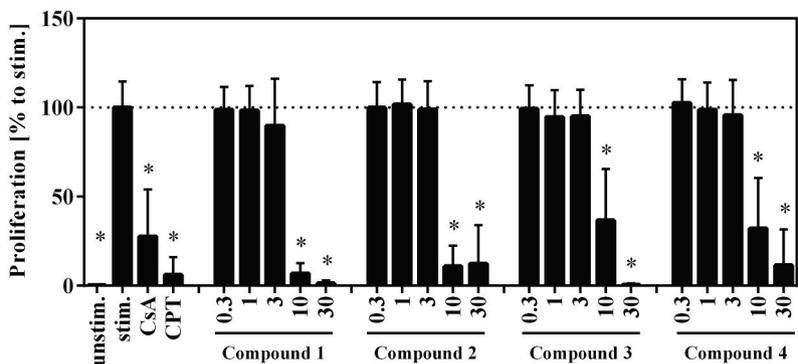
3.1. Effects of *B. carteri* extract on the proliferation capacity of activated human T lymphocytes

Autoimmune diseases are characterized by an overwhelming immune system with an enhanced proliferation of T cells. In a search for extracts and natural products that might suppress the proliferation of physiological (anti-CD3 and anti-CD28) activated human T lymphocytes, we screened a library of 435 extracts from plants used in TCM (Fig. 1A). An initial screening of extracts was performed at a concentration of 20 µg/mL, and active extracts were then tested in a concentration range of 0.3–100 µg/mL using cell division analysis. A shortlist of promising candidate extracts was established based on a literature search and the testing of extracts for the absence of cytotoxicity. A DCM extract of *B. carteri* gum resin inhibited the proliferation of activated T lymphocytes in a concentration-dependent manner (IC₅₀ of 27 µg/mL) (Fig. 1B and C), without inducing cytotoxicity. These results are in line with previous findings where a concentration-dependent proliferation inhibition of stimulated murine spleen cells from non-immunized mice was reported [27].

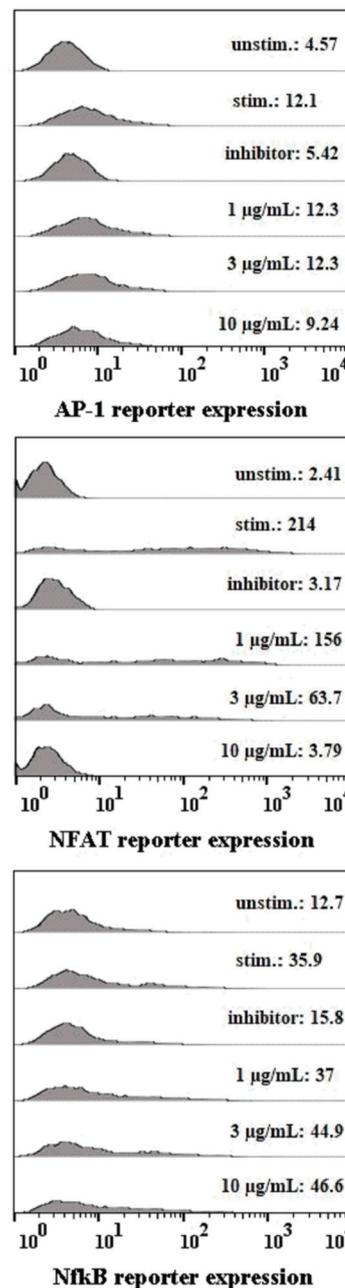
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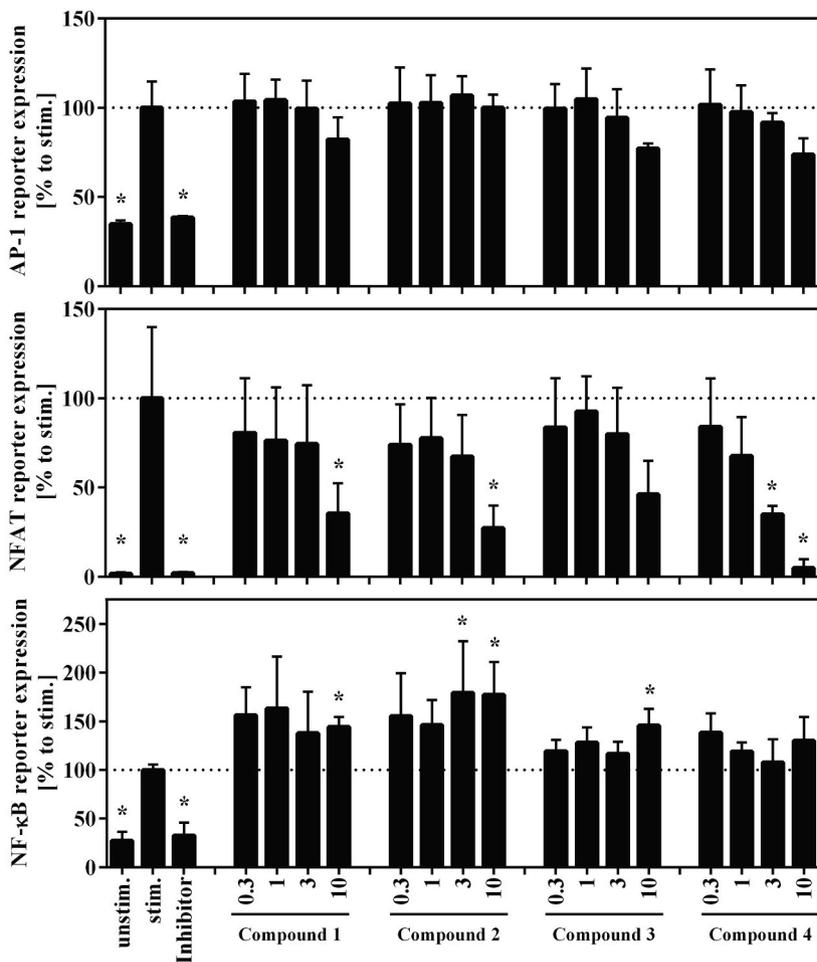
B



D



C



(caption on next page)

Fig. 6. Effects of *B. carteri* boswellic acids on T cell proliferation and *il-2* gene transcription factor induction. (A) Schematic overview of the HPLC-based activity profiling process. (B) Human PBMCs (2×10^5) were stained with CFSE, and stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). One control remained unstimulated (unstim.). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 72 h with medium (stim.), cyclosporine A (CsA; 4.16 μ M), camptothecin (CPT; 300 μ M), or compounds isolated from *B. carteri*. Cell division rate was determined via flow cytometric analysis. Data are depicted as mean \pm standard deviation. $n = 3$, * $p < .05$. (C, D) Jurkat reporter cells (0.15×10^6) were seeded on an anti-CD3-coated cell culture plate and incubated with medium, inhibitors (AP-1: 1 nM SP100030, NFAT: 4.16 μ M CsA, NF- κ B: 20 μ M parthenolide) or *B. carteri* compounds for 8 (AP-1) or 24 h (NFAT, NF- κ B). After incubation GFP expression was quantified by flow cytometry. The percentage of cells was compared and normalized to the stimulated control and depicted as mean \pm standard deviation (C). $n = 3$, * $p < .05$. Data are depicted as histogram plots of controls and indicated concentrations of 4 (D). Numbers indicate the MFI of the reporter expression.

3.2. Effects of the *B. carteri* extract on the induction of apoptosis and necrosis in human T lymphocytes

To evaluate whether the observed immunosuppressive activity of the extract was not due to general cytotoxicity, we determined the non-toxic concentration. Annexin V and PI double stainings were performed to detect the apoptosis and necrosis rates of cells. Only at the highest extract concentration of 100 μ g/mL were apoptosis and necrosis observed, while concentrations of 30 μ g/mL and lower were not harmful (Fig. 2A and B). For further mechanistic studies concentrations non-toxic concentrations were used.

3.3. Effects of the *B. carteri* extract on the upregulation of activation markers by activated human T lymphocytes

Following TCR stimulation, T cells become activated and thereby express the activation markers CD25 and CD69 on their surface. Hence, phenotyping of the cell surface using fluorescence-coupled antibodies against CD25 and CD69 allows quantifying their activation state via flow cytometry. The results did not point to an influence of *B. carteri* gum resin extract on the activation state of T cells (Fig. 3A and B). Solely the CD25 expression of CD4⁺ T cells was slightly reduced after treatment with 30 μ g/mL of extract (Fig. 3A).

3.4. Effects of the *B. carteri* extract on the degranulation and cytokine production of activated human T lymphocytes

Next, we analyzed whether the extract affected T lymphocyte function, in particular the release of perforin and granzymes. Therefore, analysis of the lysosomal-associated membrane protein 1 (LAMP-1, CD107a) showed a significantly reduced degranulation of activated T cells after treatment with 30 μ g/mL of extract (Fig. 4A and B). The secretion of the pro-inflammatory cytokines, interleukin-2 (IL-2), and IFN- γ upon T cell receptor (TCR) stimulation is an important function of T cells. The treatment of stimulated T lymphocytes with 30 μ g/mL of extract significantly lowered the secretion of IL-2 and IFN- γ (Fig. 4C and D). Our data corroborate earlier findings of a concentration-dependent inhibition of IL-2 and IFN- γ production of murine splenocytes by a *B. carteri* gum resin extract [6]. The inhibition of IL-2 production in turn leads to an inhibition of the proliferation and differentiation of T lymphocytes [24].

3.5. Isolated compounds

Plant extracts consist of a complex mixture of compounds. Compounds 1–12 (Fig. 5) were identified by fractionation of the extract of *B. carteri* gum resin guided by activity testing (Fig. 6A) [22]. The extraction of the compounds is described in the supplementary information. The triterpenes were identified by comparison with reported NMR data as 3-O-acetyl-8,24-dienetirucallic acid (1) [18], 3-O-acetyl-7,24-dienetirucallic acid (2) [29], 3-oxo-8,24-dienetirucallic acid (3) [29], 3-O-acetyl- α -boswellic acid (4) [4], β -boswellic acid (5) [8], 3-O-acetyl-11-keto- β -boswellic acid (6) [7], 3- β -8,24-dienetirucallic acid (7) [33], 3- α -8,24-dienetirucallic acid (8) [34], 11-keto- β -boswellic acid (9) [16], 3-O-acetyl- β -boswellic acid (10) [4], 3-acetyl-20(29)-lupene-24-oic acid (11) [3], and linoleic acid (12) [19]. Of these,

compounds 1–4 suppressed the proliferation of stimulated T lymphocytes in a defined concentration range of 10–30 μ g/mL (Fig. 6B). In case of compound 2, the activity was not certain as ca. 30% of unknown impurities were present. With respect to antiproliferative activity, 9 (KBA) and 6 (AKBA) have been predominantly investigated previously [1]. Interestingly, 6 was inactive in this assay at the test concentrations used, and 9 inhibited T cell proliferation via an induction of apoptosis (Fig. S2).

3.6. Impact of *B. carteri* compounds on the transcription factors AP-1, NFAT, and NF- κ B

To better understand the mode of action by which the triterpenoids exert their immunosuppressive activity, their effect on T cell signaling was investigated, specifically on the transcription factors of the *il-2* gene, activator protein 1 (AP-1), the nuclear factor of activated T cells (NFAT), and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B). These transcription factors bind to the *il-2* gene and enable the transcription and secretion of IL-2 [20]. IL-2 is crucial for the immune response and can be linked to immune overreactions because it autocrinely stimulates T cell proliferation. We used reporter cell lines [17,23] to investigate the effects on the *il-2* transcription factors. No inhibitory effect of the compounds on AP-1 or NF- κ B activity was observed (Fig. 6C and D). However, the NFAT activity was significantly and concentration-dependently suppressed by compound 4, with an IC₅₀ of 5.6 μ M (2.8 μ g/mL). The compound also showed a comparable IC₅₀ of 17.9 μ M (8.9 μ g/mL) for T cell proliferation inhibition (Fig. 6B) and is thus of interest for further investigation. The inhibition of NFAT activity by a boswellic acid is here reported for the first time, but has been described for other triterpenes [9,10].

Compound 6 has been previously shown to reduce the NF- κ B activity in activated monocytes via inhibition of I κ B kinase activity [28]. In vivo studies using a psoriasis mouse model confirmed the suppression by 6 of NF- κ B in macrophages [30]. In contrast to 6, 4 did not inhibit the NF- κ B activity. The major structural difference between 6 and 4 is the presence of an 11-keto group in 6 which constitutes a polar moiety in an otherwise rather lipophilic structure, and enables hydrogen bond formation. This may play a role for a differential interaction with proteins leading to different bioactivity.

4. Conclusion

Our study demonstrates that a lipophilic extract of *B. carteri* gum resin inhibited the proliferation, degranulation capacity and secretion of inflammatory mediators of physiologically relevant anti-CD3 and anti-CD28 activated human T lymphocytes in a non-toxic concentration range. In an activity-guided isolation approach eleven triterpenoids and linoleic acid were isolated, whereby 3-O-acetyl- α -boswellic acid (4) suppressed NFAT activity in a significant and concentration-dependent manner.

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Declaration of Competing Interest

The authors declare no competing interests.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.fitote.2020.104694>.

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