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Exploring Immune Modulatory Effects of Cyclotide-Enriched *Viola tricolor* Preparations

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ABSTRACT

Viola tricolor is a medicinal plant with documented application as an anti-inflammatory herb. The standard of care for the treatment of inflammatory bowel disease is immunosuppressive therapeutics or biologics, which often have undesired effects. We explored *V. tricolor* herbal preparations that are rich in an emerging class of phytochemicals with drug-like properties, so-called cyclotides. As an alternative to existing inflammatory bowel disease medications, cyclotides have immunomodulatory properties, and their intrinsic stability allows for application in the gastrointestinal tract, for instance, via oral administration. We optimized the isolation procedure to improve the yield of cyclotides and compared the cellular effects of violet-derived organic solvent-extracts, aqueous preparations, and an isolated cyclotide from this plant on primary human T lymphocytes and macrophages, i.e., cells that are crucial for the initiation and progression of inflammatory bowel disease. The hot water herbal decoctions have a stronger immunosuppressive activity towards proliferation, interferon-γ, and interleukin-21 secretion of primary human T cells than a DCM/MeOH cyclotide-enriched extract, and the isolated cyclotide kalata S appears as one of the active components responsible for the observed effects. This effect was increased by a longer boiling duration. In contrast, the DCM/MeOH cyclotide-enriched extract was more effective in reducing the levels of cytokines interleukin-6, interleukin-12, interleukin-23, tumor necrosis factor-α, and C–X-C motif chemokine ligand 10, secreted by human monocyte-derived macrophages. Defined cyclotide preparations of *V. tricolor* have promising pharmacological effects in modulating immune cell responses at the cytokine levels. This is important towards understanding the role of cyclotide-containing herbal drug preparations for future applications in immune disorders, such as inflammatory bowel disease.

* These authors contributed equally to this work.

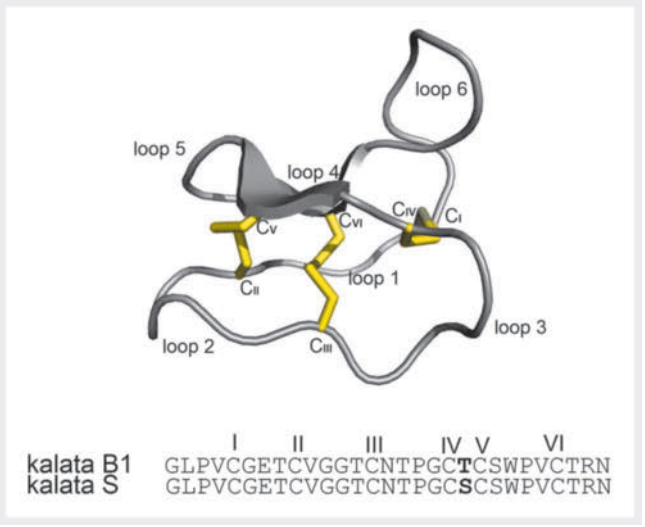
Introduction

Viola tricolor L. (Violaceae), also known as wild pansy or heartsease, is a medicinal plant documented in the Pharmacopoeia of Europe. Several biological activities have been attributed to *V. tricolor* extracts or isolated compounds, including antioxidant, antitumor, cytotoxic, antimicrobial, immunosuppressive, and anti-HIV activities [1, 2].

V. tricolor contains tannins, saponins, alkaloids, terpenoids, and flavonoids [3]. Additionally, it is rich in circular peptides termed cyclotides [4]. Cyclotides are head-to-tail cyclized plant peptides containing three disulfide bonds arranged in a knotted topology [5] (▶ **Fig. 1**). This unique tertiary structure makes them exceptionally stable towards enzymatic breakdown, acidic conditions, and heat [6]. Cyclotides have been identified in the plant families of Violaceae, Fabaceae, Rubiaceae, Solanaceae, and Curcurbitaceae [5]. Intriguingly, by using a peptidomics workflow, it was demonstrated that a single plant, namely, *V. tricolor*, can express at least 164 different cyclotides [4].

Several studies elucidated the immunomodulatory properties of cyclotides. A cyclotide-rich extract prepared from *Oldenlandia affinis* and the main cyclotide in this extract, termed kalata B1, were antiproliferative towards activated primary human lymphocytes [7]. Another study established structure-activity relationships of cyclotides on the proliferation of lymphocytes and T cells. Interestingly, cyclotides mutated in a hydrophilic inter-cysteine loop 1 and 2 region lost their activity, while the active ones acted antiproliferative by reducing the expression of the interleukin (IL)-2 surface receptor and IL-2 cytokine secretion [8]. Recently, an anti-inflammatory function of ipecac root extracts and ipecac cyclotides was reported [9]. The immunomodulatory functions of cyclotides were also shown *in vivo*, namely, in the experimental autoimmune encephalomyelitis (EAE) mouse model of multiple sclerosis [10]. In fact, the cyclotide (Lys^{20})-kalata B1 (acronym "T20K") reduced and diminished symptoms and is currently in a clinical trial for the treatment of multiple sclerosis [11]. Interestingly, the native cyclotide fold is important for the immunosuppressive properties of cyclotides [12]. Extracts from *V. tricolor* were antiproliferative on activated lymphocytes, but hitherto, no purified cyclotide from *V. tricolor* has been isolated and tested on immune cells [13]. So far, it has been difficult to obtain reasonable yields of purified peptides since there are many coeluting peaks, and *V. tricolor* is rich in mucilage, i.e., polar glycoproteins and exopolysaccharides. Hence, an optimized extraction procedure was needed to increase the yield of peptides.

Here, we modified the peptide extraction protocol from *V. tricolor* to optimize the relative yield of peptides in various extracts and semiquantified the cyclotide content. We compared the efficacy of these different *V. tricolor* extracts and preparations, as well as the main cyclotide, kalata S, on the proliferation and cytokine secretion of T cells. The most active cyclotide-enriched preparations were further analyzed by their effects toward the secretion of relevant cytokines from macrophages. Pathogenic T cells and macrophages, which secrete excessive proinflammatory cytokines, are significantly involved in the development and progression of inflammatory bowel diseases (IBDs) [14–17]. The incidence of IBD, including Crohn's disease (CD), is steadily increasing



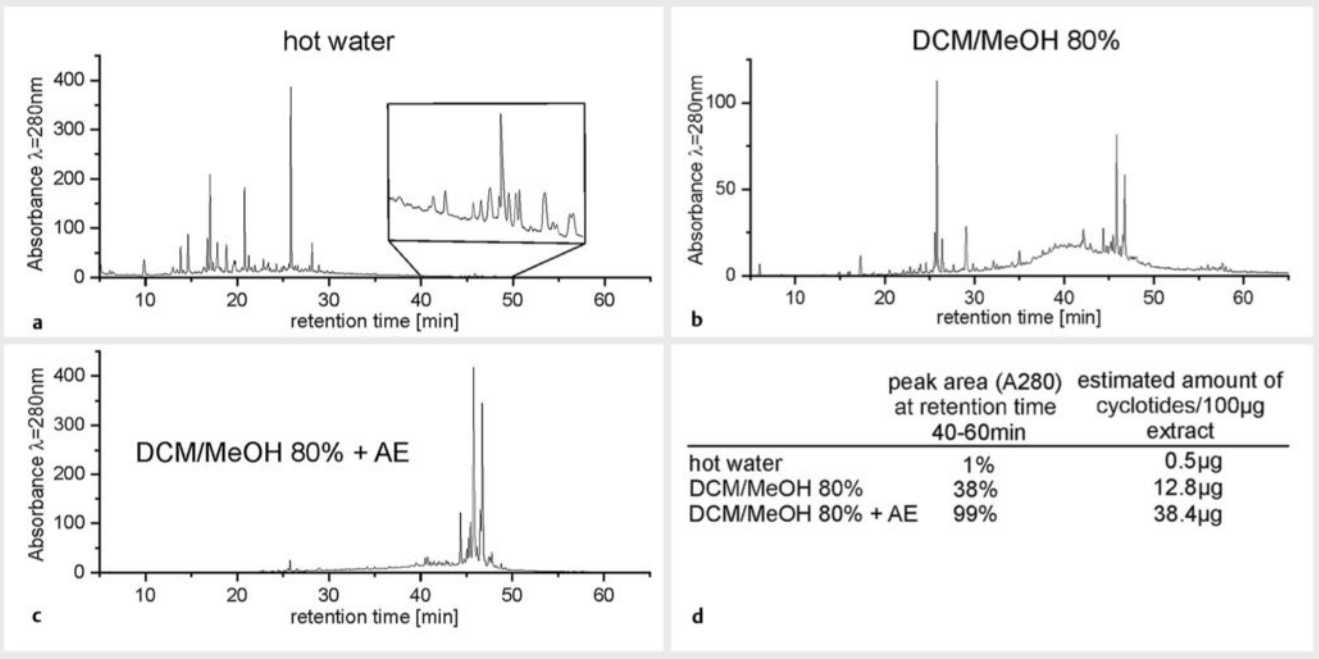
▶ **Fig. 1** Structure of cyclotides. Structural cartoon of the prototypic cyclotide kalata B1 (pdb: 1NB1) containing the cyclic cystine knot motif (left) and the sequences comparison of kalata B1 and kalata S (right), which differ by one residue (Thr vs. Ser, indicated in bold). Conserved cysteines C_I-C_{VI} are labelled with Roman numerals; six inter-cysteine loops are indicated from 1–6.

[18]. Although there are therapeutics on the market for the treatment of IBD, further research into the discovery of innovative drugs is crucial due to existing limitations, such as various side effects and non-response, as well as high treatment costs [19–23]. The present study contributes to the pharmacological exploration of defined Herbae Violae tricoloris preparations. Specifically, we focused on the analysis of plant-derived peptides and assessed their immune modulatory effects for developing novel and alternative treatment strategies for multifactorial autoimmune disorders in the future.

Results

In this study, we characterized extracts prepared from *V. tricolor* as well as the main purified peptide, kalata S. First, we optimized the extraction procedure and quantified the cyclotide content in all prepared extracts.

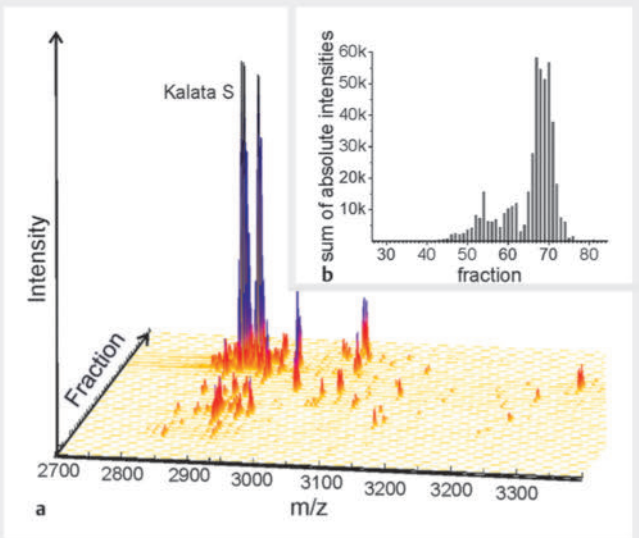
Three procedures were initially compared: (i) hot water extraction (decoction), (ii) DCM/MeOH chemical extraction followed by C_{18} SPE enrichment, and (iii) procedure ii plus additional anion exchange chromatography enrichment. The hot water decoction procedure had the lowest yield of cyclotides measured by the relative peak area (1 %) and based on analytical HPLC quantification (0.5 µg per 100 µg of extract) (▶ **Fig. 2 a, d**). Using DCM/MeOH solvent extraction, the peak area in the typical cyclotide elution range increased to 38 % and the yield was approximately 12.8 µg per 100 µg of extract (▶ **Fig. 2 b, d**). The highest relative peak area in the cyclotide elution range (99 %) as well as the highest yield of cyclotides (38.4 µg per 100 µg of extract) was obtained by subjecting the DCM/MeOH extract to anion exchange chromatography (referred to as "optimized DCM/MeOH" extract) (▶ **Fig. 2 c, d**).



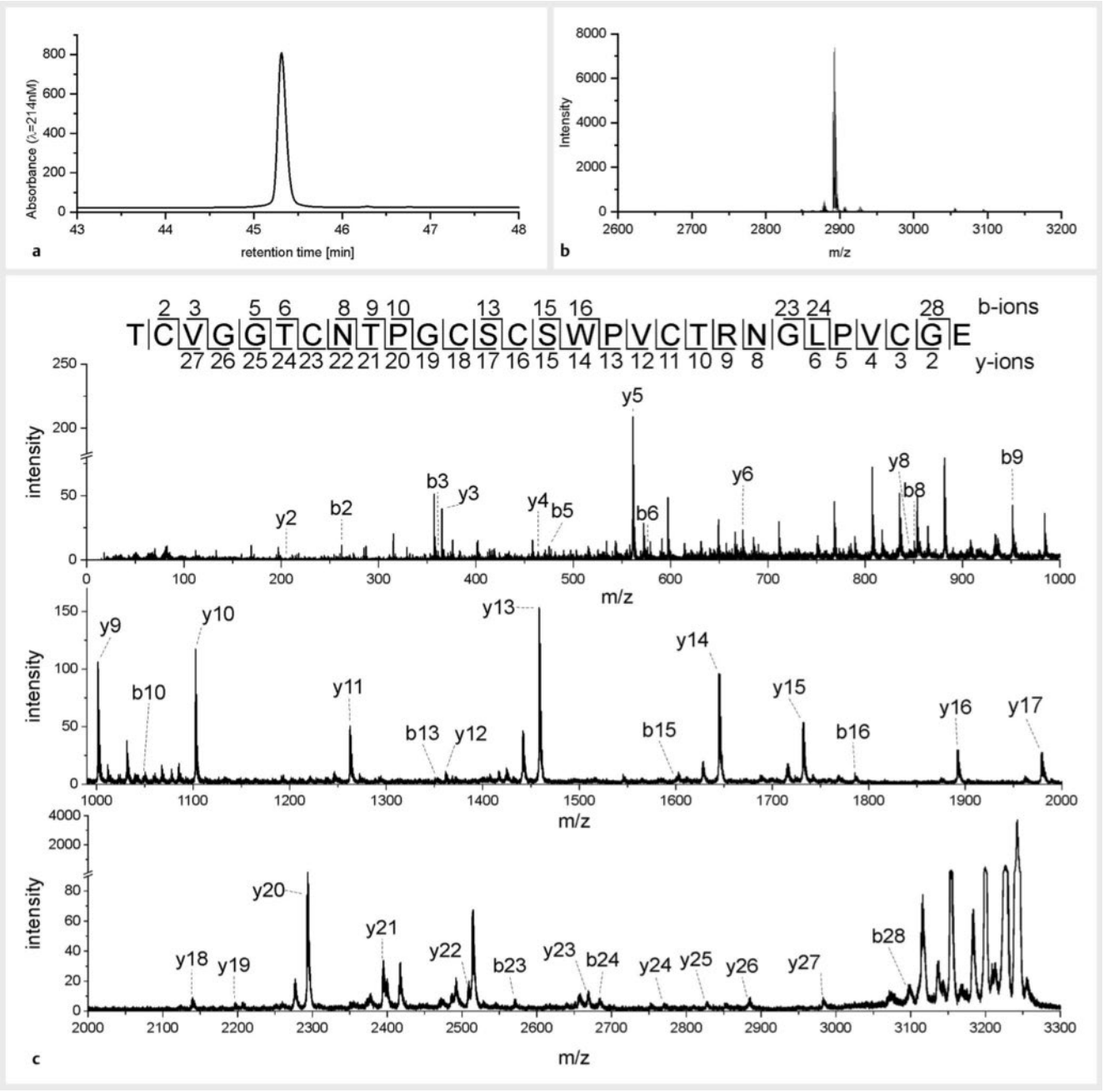
▶ **Fig. 2** Analysis of *V. tricolor* extracts. Analytical HPLC traces (280 nm) of various *V. tricolor* extracts are shown. **a** Hot water extract with magnification of the HPLC trace between retention times 40–50 min. **b** DCM/MeOH 80% extract. **c** DCM/MeOH 80% extract additionally subjected to anion exchange chromatography. The corresponding MS spectra of each extract is shown in **Fig. 1S**, Supporting Information. **d** Quantification of the cyclotide content in *V. tricolor* extracts. The peak area measured in the typical elution range of cyclotides > 40% solvent B (90% MeCN, 10% H_2O, 0.1% TFA) is stated. Additionally, the estimated amount of cyclotides/100 µg was calculated by quantifying the peak area (280 and/or 214 nm) of cyclotide peaks based on a standard curve prepared by analyzing known amounts of kalata S via analytical HPLC (**Fig. 2S**, Supporting Information).

The cyclotide content was analyzed by analytical HPLC and MS. For analytical HPLC-based quantification, the anion exchanged extract was fractionated into 12 fractions using semipreparative HPLC (**Fig. 3S**, Supporting Information) (1%/min gradient from 20 to 80% solvent B) and each fraction was analyzed by analytical HPLC (**Fig. 4S**, Supporting Information) and quantified by area under the curve (AUC) calculations (**Fig. 5S**, Supporting Information), which led to the estimation of the relative content of the two main cyclotides in the extracts, i.e., kalata S (21.3%) and kalata B1 (22.0%). In addition, we performed MS-based quantification of individual cyclotides from the anion exchange chromatography extract, which was separated by semipreparative HPLC (1%/min gradient from 20 to 80% solvent B). Fractions were collected every 30 s and analyzed by matrix-assisted laser desorption/ionization (MALDI)-MS (**Fig. 6S**, Supporting Information), which provided a slightly different estimated yield – explainable by the different methods used for quantification – for kalata S (41.5%) and kalata B1 (26.2%). Nevertheless, when comparing the absolute peak intensity of each fraction (▶ **Fig. 3a**) and the sum over all absolute intensities of the monoisotopic cyclotide peaks (▶ **Fig. 3b**), it is evident that we identified more than 70 different cyclotides in total, and that kalata S is one of the main cyclotides in these *Viola* preparations, which we therefore decided to isolate.

Kalata S was isolated by preparative and semipreparative HPLC (▶ **Fig. 4a**). Its purity was determined by analytical HPLC to > 95% (▶ **Fig. 4a**) and the final product has a monoisotopic molecular weight of 2875.74 Da (calculated: 2876.13 Da) (▶ **Fig. 4b**). Linear peptide suited for MS/MS sequencing was prepared by reduction



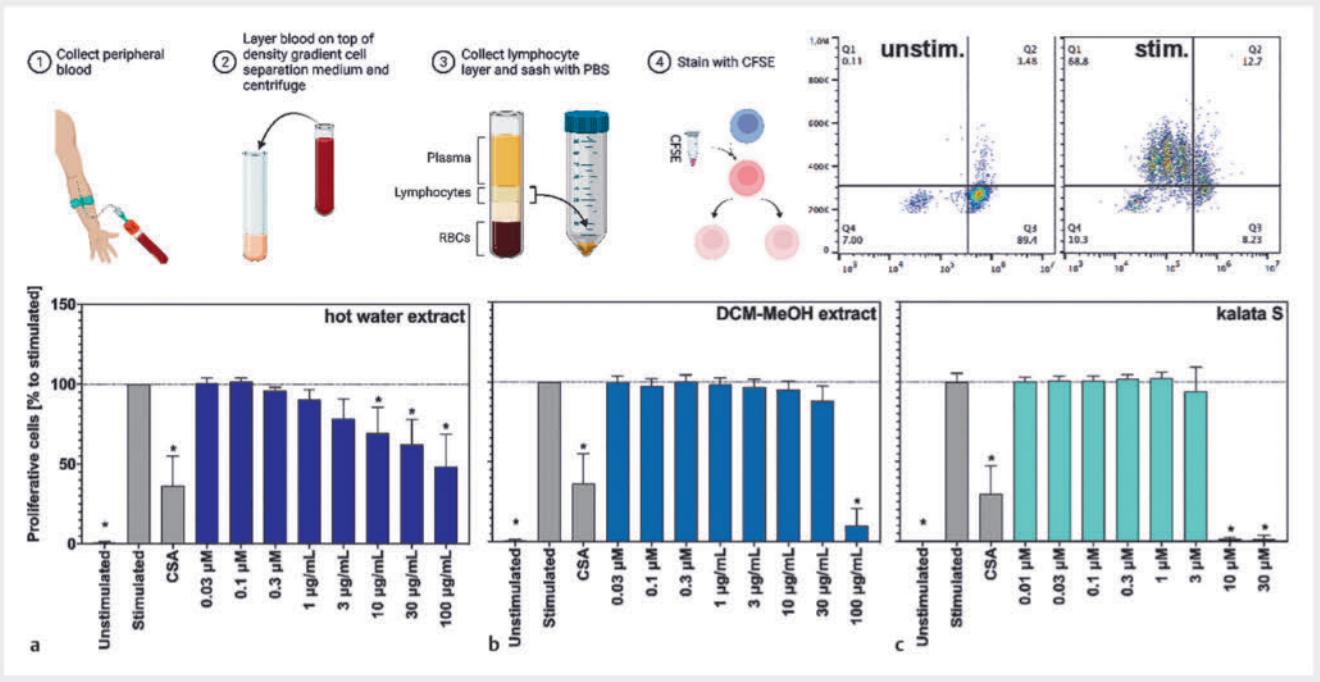
▶ **Fig. 3** Analysis of cyclotide content in *V. tricolor*. **a** 3D chromatogram obtained after separation of cyclotides by HPLC. Semipreparative HPLC was used to fractionize the anion exchange chromatography extract. Fractions were collected every 30 s and each fraction was analyzed by MALDI-MS. Individual MALDI spectra were merged using a custom Python script (see Materials and Methods) and visualized with TOPPview [46]. **b** MS-based quantification of peptide content in HPLC fractions (**Fig. 6S**, Supporting Information). The sum of all peptides signals is plotted against the fraction number.



▶ **Fig. 4** Analysis and sequencing of kalata S. **a** Analytical HPLC trace of kalata S. **b** MALDI-MS spectrum of kalata S. **c** Annotated MS/MS spectrum of the *m/z* 3243.14 precursor obtained after proteolytic cleavage with endoproteinase Glu-C in the range *m/z* 0–1000 (top), *m/z* 1000–2000 (middle), and *m/z* 2000–3300 (bottom). Additionally, the sequence of kalata S is shown on top and identified b- and y-ions are indicated. A list of all identified fragment ions is given in **Table 1S**, Supporting Information.

of the disulfide bonds followed by carbamidomethylation and a ring opening with endoproteinase Glu-C (**Fig. 7S**, Supporting Information). The sequence was confirmed by assigning 13 of 29 b-ions and 25 of 29 y-ions (▶ **Fig. 4c** and **Table 1S**, Supporting Information) and validated using amino acid analysis (**Table 2S**, Supporting Information). Given the similarity of the kalata S sequence to the prototypic cyclotide kalata B1 (they differ by only one residue) (▶ **Fig. 1**), it is safe to assume that kalata S has comparable stability to kalata B1 [6].

Next, we measured the effects of *Viola* extracts and kalata S on the proliferation of activated human T lymphocytes. Increased proliferation of human T lymphocytes is known to play a role in the pathogenesis of many autoimmune diseases, such as IBD [17]. Hence, the immunosuppressive efficacies of *Viola* extracts and kalata S peptide were analyzed by an *in vitro* proliferation assay using primary human T cells. Cyclosporin A, a cyclic peptide isolated from the Norwegian tubular fungi *Tolypocladium inflatum* W. Gams and *Cylindrocarpon lucidum* Booth and used in the clinic

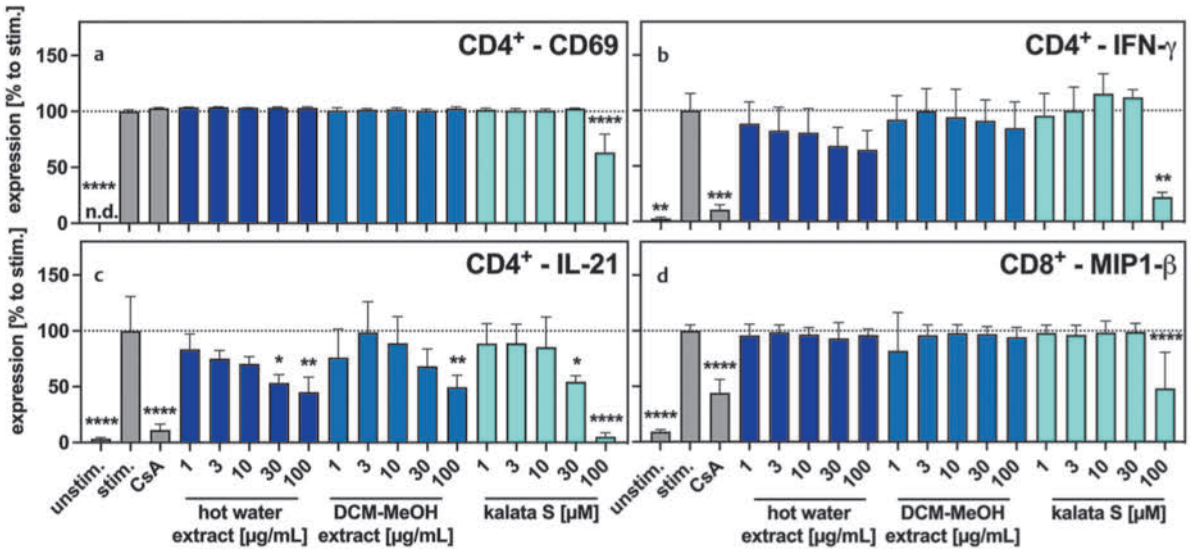


▶ **Fig. 5** Effects of *Viola* extracts and kalata S on the proliferation capacity of stimulated T cells. **a–c** Human PBMCs were stained with CFSE and stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each) (except the NC), as shown in the workflow in the upper panel of the figure. Stained and stimulated cells were treated with medium (unstimulated, stimulated), cyclosporin A (CsA; 4.16 µM), *Viola* hot water extract (**a**), *Viola* optimized DCM/MeOH extract (**b**), or *Viola* peptide (**c**) for 72 h. The proliferation capacity was determined by flow cytometric analysis. The percentage of proliferating cells was compared and normalized to the stimulated control and is depicted as the mean ± standard deviation (n = 3; $^{*}p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$).

as an immunosuppressant in IBDs, was used as a control for proliferation inhibition. Both *Viola* extracts and the kalata S peptide exhibited concentration-dependent inhibition of human T lymphocyte proliferation, with significant inhibition of cell division starting at 10 µg/mL for the hot water extract (▶ **Fig. 5a**), 100 µg/mL for the optimized DCM/MeOH extract (▶ **Fig. 5b**), and 10 µM for kalata S (▶ **Fig. 5c**). However, forward and side scatter analyses indicated toxicity of the DCM/MeOH extract at concentrations above 30 µg/mL and toxicity of the kalata S peptide at concentrations above 30 µM (**Fig. 8S**, Supporting Information). No toxic effects were observed for the hot water extract (**Fig. 8S**, Supporting Information).

Since T cells promote the pathogenesis of IBD by secreting inflammatory cytokines [16], we analyzed the effects of *Viola* extracts and kalata S on the activation status and cytokine production of human T lymphocytes. A distinction was made here between T helper cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells). The influence of *Viola* extracts and the peptide kalata S on the activation status and cytokine secretion of primary human T helper cells and cytotoxic T cells was evaluated by a multi-fluorescence panel measurement. Cyclosporin A is a calcineurin inhibitor that can inhibit the production of a variety of cytokines and cell surface receptors by inhibiting the binding of the transcription factor nuclear factor of activated T cells (NFAT). Cyclosporin A was therefore used as an inhibition control. Surface expression of CD69, a C-type lectin present on activated T helper cells, was significantly inhibited only by the highest concentration of kalata S peptide (▶ **Fig. 6a**). IFN-γ secretion by T helper cells was also significantly reduced only at the highest kalata S concentration (▶ **Fig. 6b**). However, a concentration-dependent nonsignificant inhibition of IFN-γ secretion was found for *Viola* hot water extract (▶ **Fig. 6b**). The secretion of IL-21 was inhibited in a concentration-dependent manner by both *Viola* extracts and the kalata S peptide (▶ **Fig. 6c**). Significant inhibition was observed for 30 and 100 µg/mL of the hot water extract and 30 and 100 µM for the kalata S peptide, respectively, and 100 µg/mL of the optimized DCM/MeOH extract (▶ **Fig. 6c**). For the IL-2, IL-17, and TNF-α secretion of the T helper cells, neither an influence of the *Viola* extracts nor of the kalata S peptide could be detected (**Fig. 9S**, Supporting Information). Activated cytotoxic T cells also express CD69 on their surface. The expression of CD69, as well as the release of the cytokines IL-2 and TNF-α, was not affected by the *Viola* extracts nor by the kalata S peptide (**Fig. 9S**, Supporting Information). Only MIP1-β secretion was significantly inhibited by treatment with 100 µM of kalata S (▶ **Fig. 6d**). Since concentrations of > 30 µM of kalata S, as well as the DCM/MeOH extract, may already be cytotoxic, we continued to explore the hot water cyclotide-containing preparations (in comparison to the chemical extract) given their relevance as an herbal medicinal product.

Based on the traditional use of Herba Violae tricoloris (European Pharmacopoeia), two different hot water extracts, which also contained the major cyclotide ingredients (e.g., kalata S) (**Fig. 10S**, Supporting Information), were prepared and tested. A 15-min hot water extract ("tea infusion") was compared to the



▶ **Fig. 6** Effects of *Viola* extracts and kalata S on the activation status and cytokine production of T helper cells ($CD4^+$ T cells) and cytotoxic T cells ($CD8^+$ T cells). **a–d** Human PBMCs were stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each) (except the unstim. control). Stimulated cells were incubated for 44 h in the presence of medium (NC, PC), cyclosporin A (CsA; 4.16 µM), extracts, or kalata S. T helper cells were analyzed for their expression of CD69 (**a**), IFN-γ (**b**), IL-21 (**c**), and cytotoxic T cells for MIP1-β (**d**) using flow cytometric panels. Results of activation markers and cytokine measurements were normalized to the positive control and are presented as the mean ± standard deviation (n = 3; $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$).

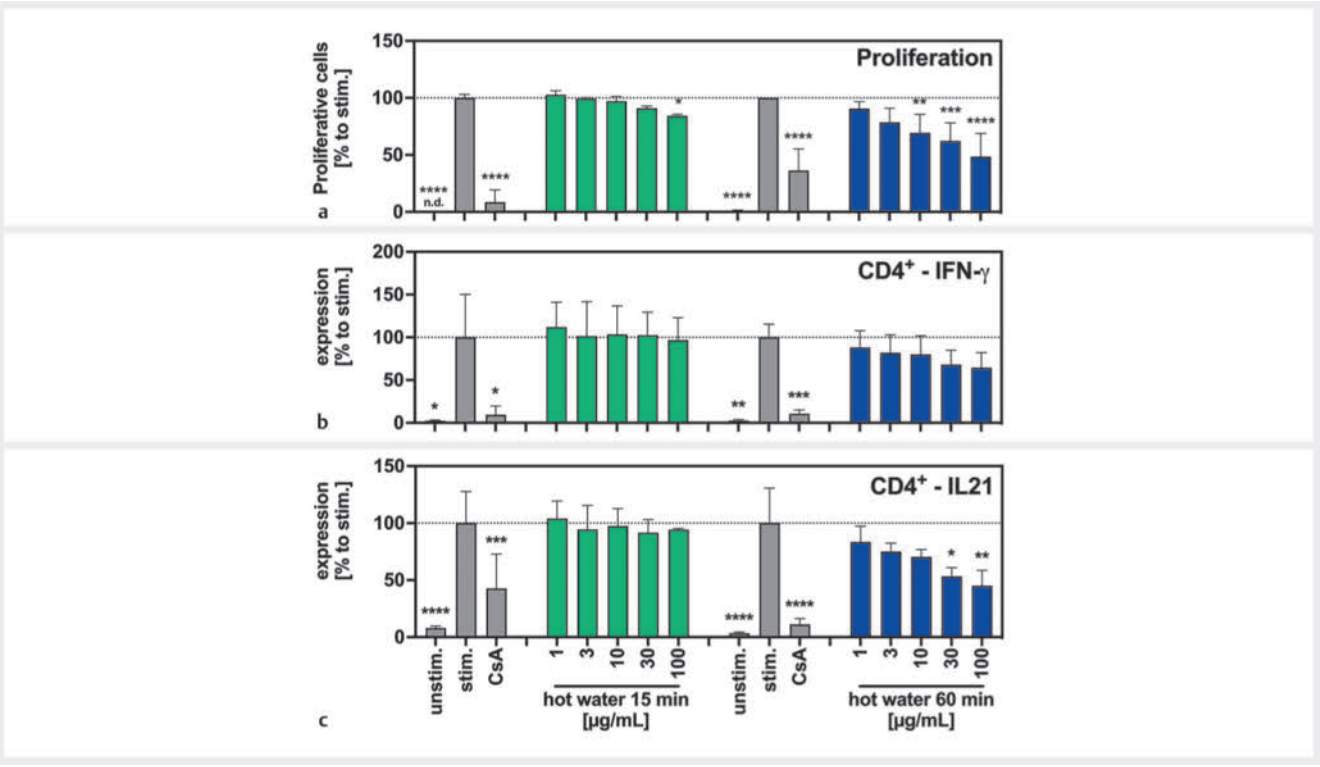
60-min hot water extract (decoction) and assayed for T lymphocyte inhibitory activity (▶ **Fig. 7**). The 60-min hot water extract showed a clear proliferation inhibitory effect, with significant results from a concentration of 10 µg/mL. In contrast, the 15-min hot water extract inhibited proliferation only slightly and significantly only at a concentration of 100 µg/mL (▶ **Fig. 7a**). The secretion of IFN-γ from $CD4^+$ T lymphocytes was only slightly and not significantly inhibited by the 60-min hot water extract (▶ **Fig. 7b**). The secretion of IL-21 by $CD4^+$ T lymphocytes was significantly inhibited by the 60-min hot water extract in a concentration-dependent manner from a concentration of 30 µg/mL (▶ **Fig. 7c**). The 15-min hot water extract did not inhibit IFN-γ production or IL-21 production by $CD4^+$ T lymphocytes (▶ **Fig. 7b, c**).

Macrophages are critical for the occurrence and development of IBD [15]. Therefore, the influence of *Viola* 60-min hot water extract and optimized DCM/MeOH extract on the release of pro-inflammatory cytokines by primary human macrophages was investigated *in vitro* in the following: both extracts (at concentrations of 30 µg/mL) significantly inhibited the secretion of IL12p70 (▶ **Fig. 8a**), TNF-α (▶ **Fig. 8b**), IL-6 (▶ **Fig. 8c**), IL12p40 (▶ **Fig. 8d**), IL-23 (▶ **Fig. 8e**), and C–X-C motif chemokine ligand 10 (CXCL10; IP10) (▶ **Fig. 8f**). The inhibitory effects of the optimized DCM/MeOH extract were generally stronger.

Discussion and Conclusion

Autoimmune diseases are a serious health problem. In 2019, about 5% of the EU population and 7% of the U. S. population suffered from an autoimmune disease, and the incidence is increasing [24, 25]. In Germany, the relative increase in prevalence has been greatest for CD, which has increased 25% over the last years [18]. Treatment of autoimmune diseases is based on symptomatic therapy with immunosuppressants, such as glucocorticoids, drugs that modulate T cell signaling, or modern biologics. However, problems with these therapies also occur, including nonresponse, various side effects, and the high cost of available drugs [19–23]. Therefore, despite the achievements of modern medicine in developing innovative drugs, continued research into novel drugs or herbal medicinal products is critical to overcoming current and future limitations in treating autoimmune diseases. Hence, we explored *V. tricolor* cyclotide-containing hot water preparations, an optimized DCM/MeOH extract, and the isolated cyclotide kalata S for effects towards primary T cells and macrophages, which are reportedly an important factor for the initiation of IBD.

We prepared different *V. tricolor* extracts, and the main cyclotide kalata S was isolated. The extracts and purified peptide were initially tested in proliferation assays of activated T lymphocytes. The peptide kalata S inhibited the proliferation of human T lymphocytes at concentrations higher than 10 µM. Nevertheless, toxic effects were also observed in concentrations above 30 µM. The hot water extract was significantly antiproliferative at concentrations greater than 10 µg/mL, whereas the optimized DCM/MeOH extract was significantly antiproliferative at 100 µg/mL. However, forward and side scatter analyses indicated that the proliferation inhibition of the MeOH extract was probably due to toxic effects. The hot water extract, which contained overall lower amounts of cyclotides, was more active and less toxic compared to the optimized DCM/MeOH extract. This suggests that



▶ **Fig. 7** Effects of 15-min and 60-min *Viola* hot water extracts on the proliferation capacity of stimulated T cells and the cytokine production of T helper cells ($CD4^+$ T cells). **a** Human PBMCs were stained with CFSE and stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each) (except the unstim. control). Stained and stimulated cells were treated with medium (unstimulated, stimulated), cyclosporin A (CsA; 4.16 µM), or 15-min and 60-min *Viola* hot water extract for 72 h. The proliferation capacity was determined by flow cytometric analysis. **b, c** Human PBMCs were stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each) (except the NC). Stimulated cells were incubated for 44 h in the presence of medium (NC, PC), cyclosporin A (CsA; 4.16 µM), or 15-min and 60-min *Viola* hot water extract. T helper cells were analyzed for their expression of IFN-γ (**b**) and IL-21 (**c**) using flow cytometric panels. Results of proliferation (**a**) and cytokine (**b, c**) measurements were normalized to the positive control and are presented as the mean ± standard deviation (n = 3; $^*p \le 0.05$, $^{**}p \le 0.01$, $^{***}p \le 0.001$, $^{****}p \le 0.0001$).

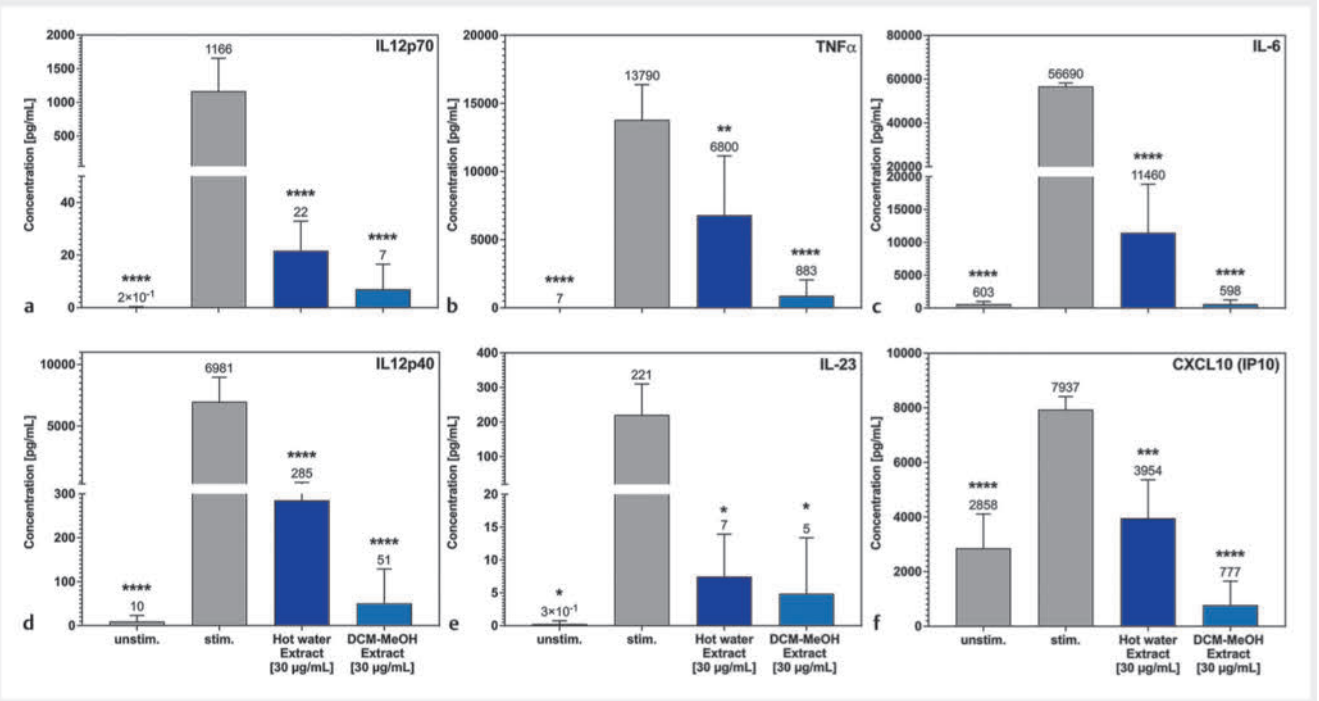
V. tricolor might contain molecules other than cyclotides that act antiproliferatively on activated T lymphocytes through a different mechanism of action. On the other hand, certain cyclotides that counteract antiproliferation might be enriched by the optimized chemical extraction procedure.

$CD4^+$ T cells play an essential role in the development and progression of IBD. Studies have shown that blocking or depletion of $CD4^+$ T cells is of therapeutic value for IBD [14]. Disruption of the cytokine balance of $CD4^+$ T cells contributes significantly to IBD pathogenesis [26]. Therefore, we continued by measuring the possible influences of *V. tricolor* extracts and kalata S on the secretion of CD69, IFN-γ, and IL-21 from $CD4^+$ T cells. Activation of $CD4^+$ T cells was significantly inhibited by 100 µM of kalata S, as shown by the inhibition of the C-type lectin CD69 and IFN-γ. However, this concentration is toxic and slightly higher than the proposed 25-µM IC_{50} threshold value for pure herbal compounds recommended by Butterweck and Nahrstedt, and therefore not primarily interesting for further therapeutic consideration [27].

In IBD patients, IL-21 is usually overexpressed by IFN-γ-producing $CD4^+$ T cells [28]. IL-21, in turn, promotes the expression of the IL-23 receptor (IL-23R), thereby enabling the cellular response to IL-23 [29]. IL-21 also induces the expression of the transcription factor retinoic acid receptor-related orphan receptor (RORγt) [29]. RORγt further promotes IL-23R expression in Th17 cells [30]. This leads to the initial release of IL-23, IL-1, IL-6, and IL-1β and finally to the differentiation of pathogenic Th17 cells in the submucosa and muscularis propria of CD patients [30]. The results of the present study show a significant suppressive effect of both *V. tricolor* extracts and kalata S on the secretion of IL-21 by $CD4^+$ T cells.

Since *V. tricolor* is an established medicinal herb, it was intriguing to determine the effects of a *Viola* hot water extract that was prepared according to the Pharmacopoeia (15-min tea infusion) and compare it to the longer (60-min) decoction. The data clearly demonstrated that a longer boiling time leads to stronger suppressive effects as determined by the secretion of IFN-γ and IL-21 by $CD4^+$ T cells. Nevertheless, in both water extracts, the effects were concentration dependent. Consequently, the cyclotide-containing hot water extracts might offer an intriguing opportunity for preparation of an herbal medicinal product to support treatment of colitis, driven by $CD4^+$ T cells.

Macrophages, on the other hand, secrete proinflammatory cytokines, which can directly or indirectly damage the epithelial cells of the intestine [15]. These processes contribute significantly



▶ **Fig. 8** Effects of *Viola* extracts on cytokines of human macrophages. Monocytes were isolated from PBMCs and differentiated into macrophages. Macrophages were treated with 30 µg/mL *Viola* hot water extract or *Viola* optimized DCM/MeOH extract for 8 d and stimulated with LPS on day 7. The amounts of IL12p70 (**a**), TNF-α (**b**), IL-6 (**c**), IL12p40 (**d**), IL23 (**e**), and CXCL10 (IP10) (**f**) were determined in the supernatant by LEGENDplex. Results are presented as the mean ± standard deviation [n = 4; *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001 compared to the stimulated control (stim.)].

to the occurrence and development of IBDs. Macrophages act through pathogenesis by overexpression of the IL-12/IL-23 axis [31]. IL-12 is responsible for the induction of IFN-γ and the differentiation of Th cells [31–33], while IL-23 promotes the differentiation of pathogenic Th17 cells [31, 34, 35]. Secretion of the proinflammatory cytokines IL-6 and TNF-α further promotes inflammation, for example, by activating the nuclear factor "kappa-light-chain-enhancer" of activated B-cells (NFκB) pathway and initiating the infiltration of neutrophil cells [15]. CXCL10 is also secreted by macrophages and is one of the most abundant proteins found in the circulation of patients with autoimmune diseases [36]. The main function of CXCL10 is to recruit immune cells to the sites of inflammation [37, 38]. Another non-chemotactic function of CXCL10 is to promote the production of proinflammatory cytokines, such as IL-12p70, in activated monocytes [37]. This is enabled by the early production of CXCL10 and its particularly potent stimulatory effect on the production of proinflammatory cytokines (i.e., IL-12) [37]. The results of this study demonstrate that both *V. tricolor* extracts significantly inhibit the secretion of IL12p70, TNF-α, IL-6, IL12p40, IL-23, and CXCL10 by macrophages. Thus, the extracts could counteract the inflammatory response in IBD. These promising cell-based data need to be verified in animal models of the disease in future studies.

Current strategies for IBD treatment include the use of antibodies against disease-relevant cytokines. However, there is a significant proportion of nonresponders, for instance, to treatment with TNF-α antibodies [39]. Because the human immune system mostly has excellent compensatory mechanisms, the emergence of pathological conditions often requires multiple (genetic and/or environmental) factors [40, 41]. Therefore, an effective alternative approach to treating multifactorial diseases, such as IBD, could consist of therapy with multitarget drugs [42–44], which enable action at several key targets simultaneously and may also produce synergistic effects. In addition, taking a multidrug tablet has a positive effect on patient compliance compared to therapy with several single-target drugs [44]. This study provides proof-of-concept in that cyclotide-containing *V. tricolor* herbal extracts modulate the proliferation and IL-21 secretion of T cells and the secretion of relevant cytokines by macrophages. Since macrophages and T cells are resident in the intestine, one could envisage oral administration of the herbal extract/tea to yield the desired systemic immune response. Therefore, *Viola* preparations warrant further investigations, including *in vivo* studies to further assess their potential for the development of multitarget drug preparations to support treatment of IBD.

To sum up, in the present study, different methods for extraction and enrichment of cyclotides from *V. tricolor* were optimized, compared for cyclotide yields, and explored in cell-based assays for efficacy in inflammatory conditions, specifically those found in IBDs. The hot water preparations had superior activity against T cells and the strength of the effect was increased by a longer boiling duration. In contrast, the optimized cyclotide-enriched DCM/MeOH extract was more effective against the secretion of relevant cytokines by macrophages. The isolated cyclotide, kalata

S, appears to be at least partly responsible for the observed effects against T cells. Overall, cyclotides and cyclotide-enriched herbal preparations offer intriguing possibilities for drug development in the context of a multitarget approach to support treatment of IBD.

Material and Methods

General experimental procedures

MALDI-TOF-MS and MS/MS spectra were recorded with a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems) in the positive ion reflector mode. Per spectrum, ~ 5000–10 000 shots were acquired. The mass spectrometer was calibrated before data acquisition with the calibration standard PepMix 4 from Laser Biolabs. MS data were analyzed with Data Explorer Software, version 4.9 (Applied Biosystems) or converted from the. t2 d to the. txt format by a Python script and analyzed with the software mMass [45] version 5.5.0, OriginPro 2022b (OriginLab Corporation), or "OpenMS TOPPview" [46]. For sample preparation, 3 µL of matrix solution consisting of 10 mg of α-cyano-4-hydroxycinnamic acid (Sigma Aldrich) per 1 mL of 50 % acetonitrile (MeCN) and 0.1 % trifluoroacetic acid (TFA) (v/v) in ddH_2O was mixed with 0.5 µL of sample and spotted on a standard 384 target plate and dried in the dark. Reversed-phase HPLC (RP-HPLC) was run on a Dionex Ultimate 3000 system equipped with an HPG-3200SD standard binary pump, a TCC-3000SD column compartment, a WPS-3000TSL analytical autosampler, an AFC-3000 fraction collector, and a UV detector at variable wavelengths of 214, 254, and 280 nm. For preparative, semipreparative, and analytical RP-HPLC C_{18} columns with a pore size of 300 Å, particle size of 10/5 µm, and dimensions of 250 × 21.2 mm, 250 × 10 and 250 × 4.6 mm were used. The column oven was set to 30 °C for analytical runs. The composition of the polar and apolar solvent was ddH_2O with 0.1 % TFA (v/v) and 90 % MeCN, 10 % ddH_2O, 0.1 % TFA (v/v), respectively. For preparative/semipreparative/analytical HPLC runs, the flow rate was set to 8 mL/3 mL/0.3 mL, respectively, and a 60-min 1 %/min gradient was used.

Hot water extraction

For the preparation of the crude hot water extract (decoction), 100 g of *V. tricolor* plant (Herba Violae tricoloris, charge no. P13301077, i.e., whole or cut plants or parts of plants including leaves, roots, flowers, seeds, bark; Mag. Kottas, Vienna, Austria) was boiled with 1 L of water for 20 min and stirred for an additional 40 min without heating. Then, the extract was centrifuged (6000 rcf), filtered, and lyophilized. The tea infusion was brewed by incubation of *V. tricolor* powder for 15 min in 100 °C hot water.

Chemical extraction of *Viola tricolor*

An amount of 300 g of dried *V. tricolor* plant (as specified above) was extracted overnight in 1 L of a 1 : 1 mixture of dichloromethane (DCM) and methanol (MeOH) under constant agitation at room temperature. On the next day, the extract was filtered, a 0.5 volume of water was added, and the extract was mixed using a separatory funnel. After phase separation, the DCM phase was discarded, and MeOH was removed from the aqueous phase with a rotatory evaporator. The resulting extract was lyophilized and is hereto referred to as the crude extract.

Preparation of peptide-enriched extract

The crude extract was dissolved in solvent A (ddH_2O containing 0.1 % TFA) and loaded onto activated and equilibrated 40–63 µm ZEOprep C_{18} material (Zeochem) for solid-phase extraction (SPE). The column was washed with 20 % solvent B (90 % MeCN, 10 % H_2O, 0.1 % TFA, v/v) and the cyclotide-rich fraction was eluted with 80 % solvent B and lyophilized.

Anion exchange chromatography

For anion exchange chromatography using Dowex 22 (Sigma-Aldrich) self-packed columns, the sample was dissolved in solvent C (20 mM Tris, 100 mM ammoniumbicarbonate, pH: 8.2) at a concentration of 10 mg/mL. Then, the column was conditioned with 500 mL solvent C, and 250 mg of sample were loaded. Ten fractions à 25 mL were eluted and the column was washed with solvent D (20 mM Tris, 100 mM ammoniumbicarbonate, 1 M NaCl, pH 8.2). Fractions were analyzed by MALDI-MS and those with strong peptide signals were merged, acidified with TFA, and used for the isolation of kalata S.

Quantification of cyclotides

The amount of cyclotides in each extract was quantified using analytical HPLC. In each run, 10 µg of the extract was injected. For quantification of the cyclotides, the peak area in the typical elution range of cyclotides (40–60 %) was measured at 214 and/or 280 nm and quantified relative to the peak area of different defined amounts (0.5–8 µg) of kalata S via preparation of a standard curve using a linear curve fit.

Isolation and sequencing of kalata S

Several runs of preparative and semipreparative HPLC were used for the purification of kalata S from the anion exchange chromatography-enriched peptide extract. Disulfide bonds were reduced with 10 mM dithiothreitol (Sigma-Aldrich) for 3 h at 37 °C under moderate agitation and carbamidomethylated with 50 mM iodoacetamide (Sigma-Aldrich) for 10 min at 25 °C while shaking at 300 rpm. The reaction was quenched by the addition of dithiothreitol. To produce linear kalata S, the peptide was proteolytically cleaved by endoproteinase GluC (Sigma-Aldrich). Kalata S was sequenced by manual assignment of y- and b-ions as well as diagnostic neutral loss ions in the fragmentation spectra of the linearized peptide, similar to a previously established protocol [47]. The sequence was verified by comparison with a theoretical fragmentation spectrum calculated with the software Data Explorer. The sequence was verified by high sensitivity amino acid analysis carried out at the Australian Proteome Analysis Facility (APAF).

Ethics approval statement

All subjects gave written informed consent for blood collection. The blood samples were obtained in an anonymized and coded form from the central blood donation of the University Hospital in Basel. No ID number of the samples is visible, so that any assignment is impossible. The work does not fall within the scope of the Swiss Human Research Act.

Preparation and cultivation of human peripheral lymphocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the blood of healthy adult donors, which was provided by the blood transfusion center (University Hospital, Basel, Switzerland). Venous blood was centrifuged on a LymphoPrep gradient (1.077 g/cm^3, 20 min, 500 × g, 20 °C; Progen). After centrifugation, cells were washed with PBS and cultured in RPMI-1640 medium supplemented with 10 % heat inactivated fetal calf serum (Bioconcept AG), 2 mM L-glutamine, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Sigma-Aldrich). Cells were cultured at 37 °C in a humidified incubator with a 5 % CO_2/95 % air atmosphere.

Analysis of peripheral blood mononuclear cell proliferation

The proliferation of PBMCs was analyzed by carboxyfluorescein diacetate succinimidyl ester (CFSE; Sigma-Aldrich) staining, as described previously [7,48]. Lymphocytes were isolated, washed twice in cold PBS, and resuspended in PBS at a concentration of 5×10^6 cells/mL. Cells were stained for 10 min at 37 °C with CFSE (0.5 µM; Sigma-Aldrich). Complete medium was added to stop the staining reaction. Stained cells were stimulated with anti-CD3 (clone OKT3) and anti-CD28 (clone 28.2) mAbs (100 ng/mL each; both from eBioscience) (except the NC) treated with medium (unstimulated, stimulated), cyclosporin A (CsA; 4.16 µM; Sandimmun 50 mg/mL; Novartis Pharma), *V. tricolor* hot water extract, *V. tricolor* DCM/MeOH extract, or kalata S peptide for 72 h. The proliferation capacity was determined by flow cytometric analysis (CytoFLEX S Flow Cytometer; Beckman Coulter).

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

PBMCs (5×10^6 cells/mL) were stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each) (except the NC) and treated with medium (NC, PC), cyclosporin A (CsA; 4.16 µM), extracts, or kalata S for 40 h. Cells were restimulated with phorbol-12-myristat-13-acetate (PMA, 50 ng/mL; Sigma-Aldrich) and ionomycin (1 µg/mL; Sigma-Aldrich) (except the NC). The Golgi apparatus was blocked with GolgiPlug (1 µL/mL; BD Biosciences) and GolgiStop (0.65 µL/mL; BD Biosciences) and cells were incubated for another 4 h. A surface staining mix was prepared for 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)]. Cells were stained for 30 min at room temperature in the dark and resuspended in 100 µL Cytofix/Cytoperm solution for 15 min at 4 °C. The intracellular staining mix was prepared [Panel 1: IFN-γ fluorescein isothiocyanate (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)]. Cells were stained for 30 min at 4 °C. Afterwards, the cells were fixed with 2 % paraformaldehyde (Electron Microscopy Sciences) for 10 min at 4 °C. Fluorescence intensity of the cells was measured with a CytoflexS flow cytometer (Beckman Coulter). Analysis of the data was performed using FlowJo software.

Differentiation of macrophages from peripheral blood mononuclear cells/monocytes

PBMCs were plated at a concentration of 2×10^6 cells/cm^2 in cell culture multiwell plates (CELLSTAR). Cells were incubated at 37 °C for at least 1 h to allow the monocytes to adhere to the plastic of the cell culture plates. The other cells were then washed away with PBS. To generate macrophages, cells were treated with 40 ng/mL GM-CSF (Peprotech) and 50 ng/mL IL-4 (Biolegend) alone or with 30 µg/mL of *Viola* hot water or DCM/MeOH extract for 8 days. For stimulation, on day 7, 100 ng/mL LPS (Sigma-Aldrich) were added. To harvest the macrophages, the medium was removed, and the cells were washed. Cold PBS including 2 mM EDTA was added to the cells and incubated on ice for 15 min. Macrophages were then detached from the cell culture plate.

Analysis of macrophage cytokines

The supernatants of macrophages were harvested and frozen at − 20 °C. The amount of the described cytokines were determined using LEGENDplex according to the manufacturer's instructions (BioLegend).

Statistical analysis

Statistical data analysis was performed using PRISM (version 9.3.1; GraphPad Software). Normality was tested using the Shapiro-Wilk test. A multiple group comparison was performed with the Brown-Forsythe and Welch ANOVA for not normally distributed data and standard ANOVA for normally distributed data. Dunnet's T3 test was used as a post hoc test. Statistical significance was considered for $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Data repository

Python script used for analysis of MALDI data for quantification has been shared via GitHub (https://github.com/BernhardRetzl/viola-tricolor).

Supporting information

A description of the analytical and quantitative characterization of the extracts and peptides as well as additional *in vitro* assays are available as Supporting Information.

Contributors' Statement

C. W. G. and C. G. initiated the project. B. R. performed plant extraction, peptide isolation, sequencing and quantification. A. M. Z.-K., M. W., and S. N. isolated immune cells and conducted *in vitro* assays. All authors analyzed the data. B. R., A. M. Z.-K., C. G., and C. W. drafted the manuscript. All authors approved the final manuscript.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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