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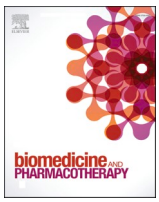

Ipecac root extracts and isolated circular peptides differentially suppress inflammatory immune response characterised by proliferation, activation and degranulation capacity of human lymphocytes in vitro

Chiara Madlen Falanga [a], Carmen Steinborn [a], Edin Muratspahić [b], Amy Marisa Zimmermann-Klemd [d], Moritz Winker [d], Liselotte Krenn [c], Roman Huber [a], Christian W. Gruber [b], Carsten Gründemann [d,*]

[a] Center for Complementary Medicine, Department of Internal Medicine II, Faculty of Medicine, University of Freiburg, Freiburg, Germany
[b] Center for Physiology and Pharmacology, Medical University of Vienna, Vienna, Austria
[c] Department of Pharmacognosy, University of Vienna, Vienna, Austria
[d] Translational Complementary Medicine, Department of Pharmaceutical Sciences, University of Basel, Basel, Switzerland

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ABSTRACT

Circular peptides are attractive lead compounds for drug development; this study investigates the immunomodulatory effects of defined root powder extracts and isolated peptides (called cyclotides) from *Carapichea ipecacuanha* (Brot.) L. Andersson ('ipecac'). Changes in the viability, proliferation and function of activated human primary T cells were analysed using flow cytometry-based assays. Three distinct peptide-enriched extracts of pulverised *ipecac* root material were prepared via C_{18} solid-phase extraction and analysed by reversed-phase HPLC and mass spectrometry. These extracts induced caspase 3/7 dependent apoptosis, thus leading to a suppressed proliferation of activated T cells and a reduction of the number of cells in the G2 phase. Furthermore, the stimulated T cells had a lower activation potential and a reduced degranulation capacity after treatment with ipecac extracts. Six different cyclotides were isolated from *C. ipecacuanha* and an T cell proliferation inhibiting effect was determined. Furthermore, the degranulation capacity of the T cells was diminished specifically by some cyclotides. In contrast to kalata B1 and its analog T20K, secretion of IL-2 and IFN-γ was not affected by any of the caripe cyclotides. The findings add to our increased understanding of the immunomodulating effects of cyclotides, and may provide a basis for the use of *ipecac* extracts for immunomodulation in conditions associated with an exessive immune responses.

1. Introduction

Cyclotides are ribosomally synthesised and post-translationally modified peptides derived from plants [1]. These peptides have a head-to-tail circular backbone which is stabilised by three interlocking disulphide bonds—a motif known as a cyclic cystine-knot [2]. This characteristic three-dimensional structure is responsible for the remarkable stability of these peptides against chemical, enzymatic and thermal degradation [3], making them valuable candidates for pharmaceutical applications. Cyclotides were first isolated from plants of the families Rubiaceae and Violaceae [4–6], and till date they have also been found in the families Cucurbitaceae [7,8], Fabaceae [9,10], Solanaceae [11] and Apocynaceae [12]. It is thus likely that numerous other cyclotides have yet to be discovered, which would make these peptides one of the most versatile class of compounds amongst plants [7, 13].

In previous studies, the cyclotide kalata B1 was found to be an active compound in *Oldenlandia affinis* of the family Rubiaceae [14]. Treatment of primary human lymphocytes with kalata B1—or its modification, T20K—in vitro induced an interleukin-2 (IL-2) dependent inhibition of T cell proliferation [15]. Using a mouse model of multiple sclerosis, the oral application of T20K led to a significant delay and reductions in

Abbreviations: CPT, camptothecin; CD, clusters of differentiation; CFSE, carboxyfluorescein diacetate succinimidyl ester; CsA, cyclosporine A; IL, interleukin; mAB, monoclonal antibody; PBMC, peripheral blood mononuclear cells.

* Correspondence to: Klingelbergstrasse 80, 4056 Basel, Switzerland.
E-mail address: carsten.gruendemann@unibas.ch (C. Gründemann).

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clinical scores [16]. Based on these promising findings and considering their remarkable stability, cyclotides are compelling drug candidates for the treatment of autoimmune diseases [16]. The prevalence of autoimmune diseases is increasing—especially in Western countries—with rates as high as 5% [17]. Hence, there is an unmet need for new and effective therapies that overcome limitations, such as side effects, nonresponse, bacterial or viral resistance, and the steep cost of available pharmaceuticals [18–22]. Because of the growing need for novel immunosuppressive drugs and the promising findings on kalata B1 and its mutants, this study focuses on the possible immunomodulatory effects of other plants containing cyclic peptides.

Therefore, we screened *Carapichea ipecacuanha* (Brot.) L. Andersson (syn. *Cephaelis ipecacuanha* (Brot.) Willd., syn. *Cephaelis acuminata* H. Karst.), another plant belonging to the Rubiaceae family, for its immunomodulatory effects. *C. ipecacuanha*, known as the wild ipecacuanha, is extensively distributed throughout South America [23]. The shrub has a long history of traditional use and is still one of the most important medicinal plants in South America. Presently, the ipecacuanha root is still used phytotherapeutically to treat gastrointestinal complaints and as an expectorant for bronchitis [24,25]. At high dosages, however, it causes vomiting and was thus utilised as an emetic following intoxication [26]. The most important compounds in *C. ipecacuanha* are isoquinoline alkaloids, especially emetine and cephaeline [27]. Different cyclotides, such as caripe, have been discovered in *C. ipecacuanha* [13]. To further analyse the therapeutic potential of this promising plant, we examined the immunomodulatory effects of defined fractions and isolated cyclotides from *C. ipecacuanha* using a reliable chemical analytical technology platform and flow cytometry based bioassays.

2. Methods

2.1. Ethics statement

Written informed consent was obtained from patients prior to blood donation for research purposes. All experiments conducted on human material were approved by the Ethics committee of the University Freiburg (472/19).

2.2. Plant extraction

The extracts of C. ipecacuanha (Brot.) L.Andersson roots (Alfred Galke GmbH, Germany) were prepared as previously described [28,29]. Briefly, 300 g of the dried plant material were treated with 1 L of methanol/dichloromethane (1:1, v/v) followed by continuous agitation overnight at room temperature. The next day, the plant material was removed by filtration, a 0.5 vol of ddH_2O was added to the filtrate and the aqueous phase containing cyclotides was separated from the organic phase in a separating funnel. Following evaporation and freeze-drying, the aqueous phase was subsequently applied to the C_{18} solid-phase extraction. The dried crude water-soluble part was dissolved in 5% solvent B (90% acetonitrile, 9.9% ddH_2O, 0.1% trifluoroacetic acid, v/v) and loaded onto the C_{18} material ZEOprep 60 Å, irregular 40–64 μm (Zeochem, Uetikon, Switzerland). After equilibration of the column with solvent A (99.9% ddH_2O/0.1% trifluoroacetic acid, v/v) and washing with either 10% of solvent B, the cyclotide-containing fractions were separated from hydrophilic components by eluting with 80% of solvent B. This 10–80% cyclotide-containing extract, referred to as C10, was then evaporated, lyophilized and split into two equal portions for further cyclotide enrichment. Whereas one portion of the C10 extract was washed with 30% of solvent B and eluted with 80% of solvent B (C30), the other portion was enriched by washing it with 50% of solvent B and eluting with 70% of solvent B (C50). The eluted fractions were analysed for the presence of cyclotides by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and reversed-phase (RP) HPLC.

2.3. Peptide purification and analysis using liquid chromatography and mass spectrometry

The dried C50 fraction was dissolved in 5% of solvent B and subjected to preparative and semi-preparative RP-HPLC to purify cyclotides. The automatic preparative and manual semi-preparative fractionations were performed on Phenomenex Jupiter C_{18} column (250 mm × 21.2 mm, 10 μm, 300 Å; Phenomenex, Aschaffenburg, Germany) and a Kromasil C_{18} column (250 mm × 10 mm, 5 μm, 100 Å) using the flow rates of 8 and 3 mL/min, respectively and linear gradients of solvent B between 5% and 65% at 1%/min. Analytical RP-HPLC was conducted on a Kromasil C_{18} column (250 mm × 4.6 mm, 5 μm, 100 Å; dichrom GmbH, Marl, Germany) at a flow rate of 1 mL/min, and peptides were separated with linear gradients of solvent B between 5% and 65% at 1%/min. The elution profile of cyclotides was monitored via UV absorbance at 214, 254, and 280 nm. Mass spectrometry-based analysis of cyclotide-enriched fractions and isolated cyclotides was carried out using a MALDI-TOF/TOF 4800 analyser (AB Sciex, Framingham, MA, USA) in a reflector positive ion mode with 2000–10 000 total shots per spectrum and a laser intensity of 3500–3700. Samples were prepared by mixing 0.5 μL with 3 μL of matrix solution (α-cyano-hydroxyl-cinnamic acid (α-CHCA, Sigma–Aldrich, St. Louis, MO, USA)), dissolved in ddH_2O/acetonitrile/trifluoroacetic acid, 50/49.9/0.1% (v/v/v) (final concentration 5 mg/mL). Samples were spotted directly onto the MALDI target plate and spectra were acquired, processed, and analysed using the Data Explorer Software (AB Sciex).

2.4. Preparation and cultivation of human immunocompetent cells

Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of adult donors obtained from the Blood Transfusion Centre (University Medical Center Freiburg). Venous blood was centrifuged on a LymphoPrep™ gradient (density: 1.077 g/cm^3, 20 min, 500g, 20 °C; Progen). Afterwards cells were washed three times with PBS and cell viability and concentration was determined using the trypan blue exclusion test. Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (PAA), 2 mM L-glutamine, 100 U/mL penicillin and 100 U/mL streptomycin (Invitrogen) at 37 °C in a humidified incubator with a 5% CO_2/95% air atmosphere. PBMC were additionally stimulated with anti-human CD3 (clone HIT3) and anti-human CD28 (clone 28.6) mAb (100 ng/mL; both from eBioscience). Incubation was carried out as indicated in the figure captions in the presence of medium alone, different control substances, *C. ipecacuanha* fractions, or isolated cyclotides.

2.5. Cell division tracking using CFSE

PBMC were harvested and washed twice in cold PBS (Invitrogen) and resuspended in PBS at a concentration of 5×10^6 cells/mL. CFSE (carboxyfluorescein diacetate succinimidyl ester, 5 mM; Sigma) was diluted 1/1000 and incubated for 10 min at 37 °C. The staining reaction was stopped by washing twice with complete medium. PBMC were activated as described above and cultured with *C. ipecacuanha* fractions (C10, C30, C50), or cycloides (caripe 7, 8, 9, 10, 11, 12, 13) for 72 hrs. Cyclosporine A (CsA, 5 μg/mL, Sandimmun™ 50 mg/mL, Novartis) served as a control substance for proliferation inhibition. Cell division progress was analysed from three independent experiments with a BD FACSCalibur flow cytometer using BD CellQuest Pro Software.

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

The level of apoptosis and necrosis of PBMC was determined in three independent experiments using the annexin V-FITC apoptosis detection kit (eBioscience) according to the manufacturer's instructions. Cells

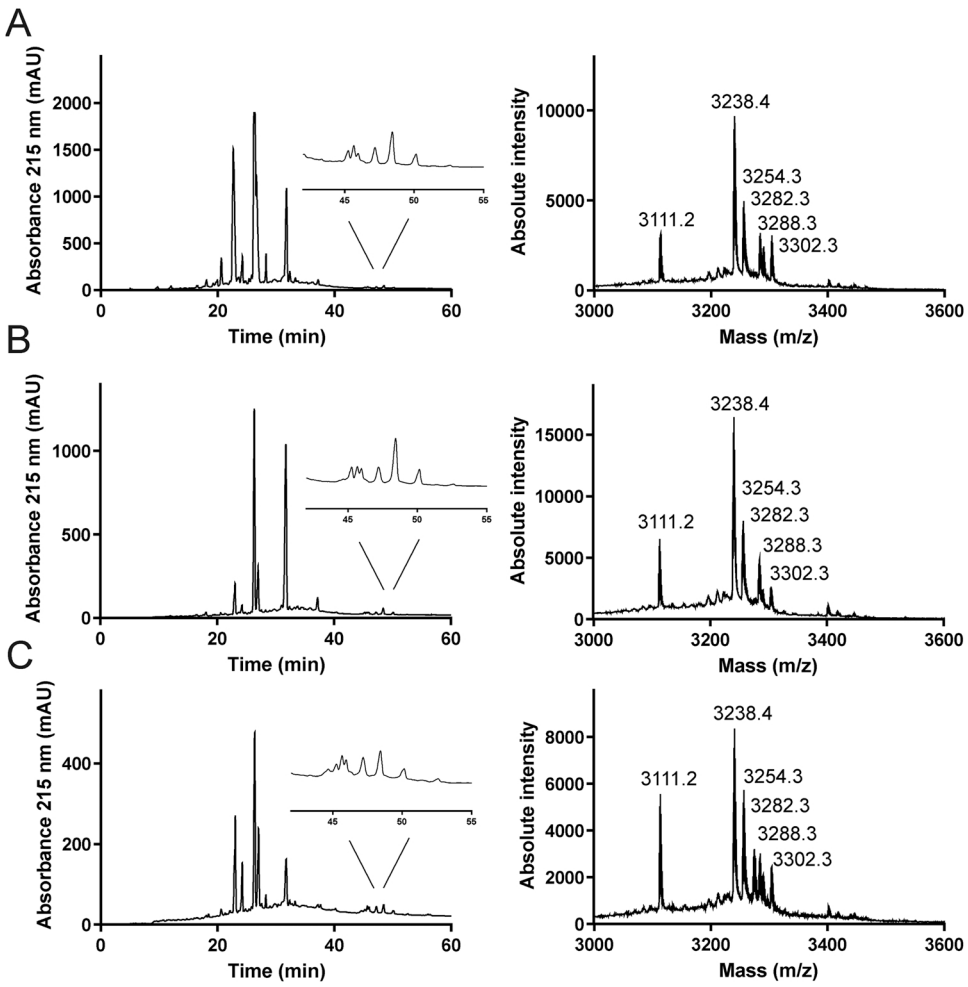


Fig. 1. Generation of cyclotide-rich *C. ipecacuanha* fractions. Powdered plant material was extracted with a mixture of dichloromethane/methanol (1:1, v/v) overnight and subjected to liquid/liquid-phase separation and C_{18} solid-phase extraction. RP-HPLC (left) and MALDI-MS (right) profiles of cyclotide-enriched ipecac fractions washed and eluted with (A) 10% and 80%, (B) 30% and 80% and (C) 50% and 70% of solvent B, respectively. The elution profile and monoisotopic masses of *C. ipecacuanha* cyclotides are shown as inset and $[M+H]^+$, respectively.

were activated and treated for 24 hrs before they were stained with annexin V and propidium iodide (PI; eBioscience). As control substances, camptothecin (CPT; 30 μg/mL; Tocris) was used for the induction of apoptosis, triton-X 100 (0.5%; Carl Roth) for the induction of necrosis. The amount of apoptosis and necrosis was analysed using flow cytometry.

2.7. CD25 and CD69 activation marker detection

T-cell activation was assessed by analysing the expression of surface antigens CD69 and CD25 using APC-conjugated anti-human CD4, PE-conjugated anti-human CD25 and FITC-conjugated anti-human CD69 mAbs (all from eBiosciense) from three different experiments by flow cytometry. Cells were activated and treated for 24 hrs before the surface marker staining was carried out. CsA and CPT served as control substances.

2.8. Analysis of cell cycle progression by determining the DNA content

Isolated PBMCs were activated and treated with different *C. ipecacuanha* fractions, cyclic peptides, or CsA as a control for 72 hrs. After washing, cells were fixed and permeabilized using 70% ice-cold ethanol. Staining was carried out using PI and RNase. Flow cytometric analysis was carried out using three independent experiments.

2.9. Quantification of intracellular cytokine production

For determination of IL-2 and IFN-γ production by PBMC, cells were activated and treated with *C. ipecacuanha* fractions, cyclic peptides, or CsA for 48 hrs. PBMC were then re-stimulated using phorbol-12-myristate-13-acetate (PMA; 50 ng/mL) and ionomycin (500 ng/mL; both from Sigma) and incubated with BD Golgi Plug™ (BD Biosciences) for 4 hrs. After washing, cells were fixed with 4% paraformaldehyde (PFA; Morphisto) and staining of intracellular cytokines was carried out using PE-conjugated anti-human IL-2 or PE-conjugated anti-human IFN-γ mAbs (both from Affymetrix). Levels of cytokine production were determined using flow cytometric analysis of three independent experiments.

2.10. Measurement of $CD8^+$ T cell degranulation

PBMC were activated and treated with *C. ipecacuanha* fractions, cyclic peptides, or CsA and CPT as control substances for 24 hrs. Cells were then re-stimulated using PMA and ionomycin for 4 hrs. Staining of the degranulation-specific membrane protein CD107a was carried out using PE-conjugated anti-human CD107a mAbs (Affymetrix), followed by treatment with BD GolgiStop™ (BD Biosciences). Levels of degranulation were analysed by flow cytometry in three independent experiments.

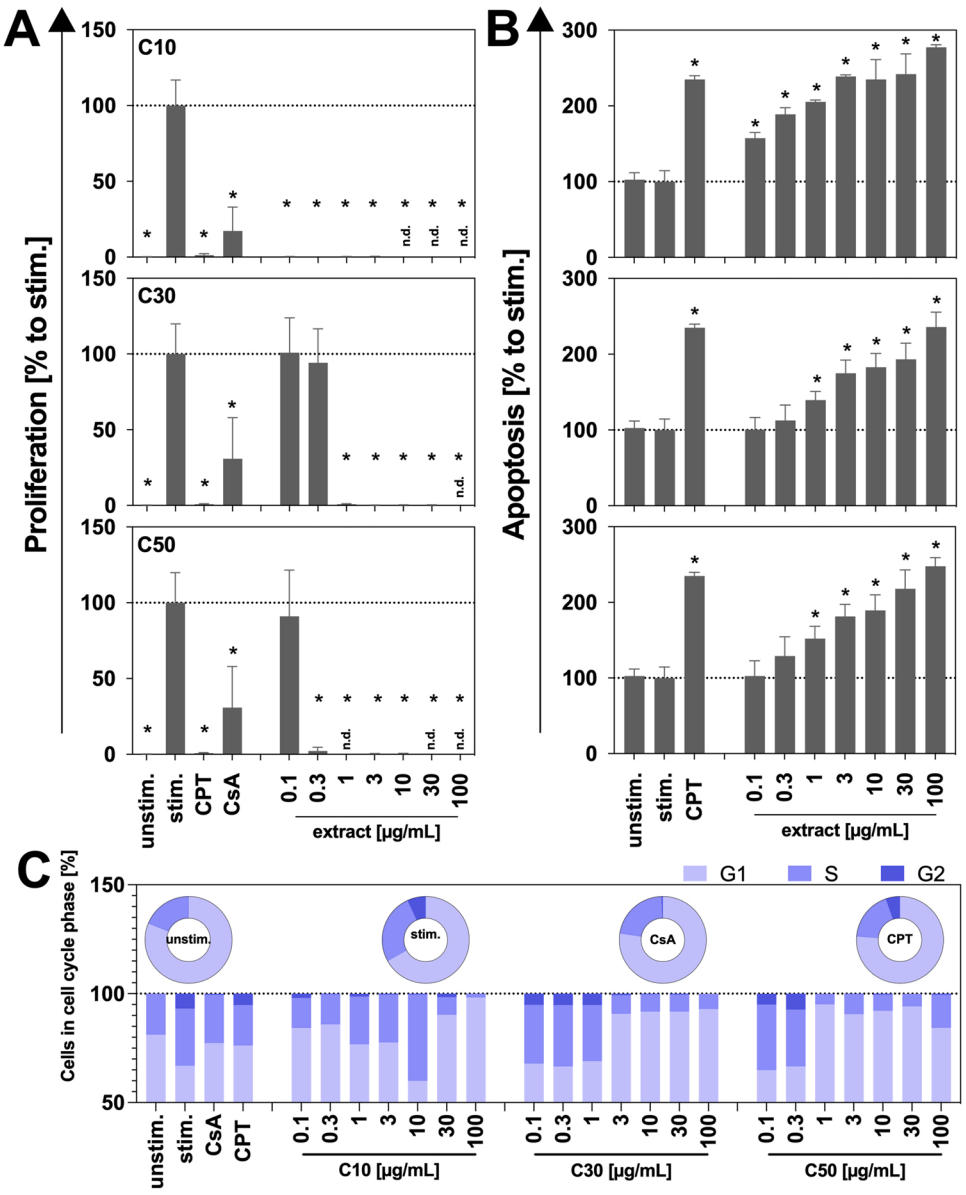


Fig. 2. : Inhibitory effects of *C. ipecacuanha* extracts (C10, C30, C50) on proliferation, apoptosis induction and cell cycle of T lymphocytes. (A, B) Human PBMCs (2 *10^5) were stained with CFSE (A) and stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 72 hrs in the presence of medium (stim., unstim.), cyclosporine A (CsA; 4.16 µM), camptothecin (CPT; 300 µM), or the extracts. (A) The number of proliferating cells was compared and normalised to the stimulated control and depicted as mean ± standard deviation. n = 3; *$p < 0.05$. (B) Annexin V-FITC and PI double staining was performed. The number of apoptotic/necrotic lymphocytes compared to the stimulated control was determined and depicted as mean ± standard deviation. n = 3; *$p < 0.05$. (C, D) PI staining of the DNA was performed and the number of lymphocytes in each phase compared to all living lymphocytes was determined and depicted as mean. n = 3.

2.11. Analysis of autophagy

PBMC were treated with rapamycin (30 nM) and chloroquine (3 µM; both from Enzo Life Sciences) and different concentrations of *C. ipecacuanha* fractions for 16–18 hrs. Staining of accumulated autophagosomes was done using CYTO-ID® Green Detection Reagent 2 (Enzo Life Sciences) and levels of autophagy were determined by flow cytometric analysis using three independent experiments.

2.12. Measurement of caspase 3/7 activity

PBMC were activated and incubated with *C. ipecacuanha* fractions, cyclic peptides, or staurosporine as a control (STS, 1 µM; Sigma) for 24 hrs. Cells were then incubated with Caspase-Glo® 3/7 (Promega) for one hour. Luminescence was measured using a microplate reader in three independent experiments.

2.13. Data analysis

For statistical analysis, data were processed with Microsoft Excel and SPSS software (IBM, Version 22.0, Armonk). Data were adjusted in relation to untreated control cells (= 100% ± SD) and values are presented as mean ± SD. Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc pairwise comparisons. P values < 0.05 were considered as statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

3. Results

3.1. Preparation and immunomodulatory characterisation of the extract

Based on the knowledge that cyclotides isolated from *Oldenlandia affinis* DC. [14,15] and *Viola tricolor* L. [30] are immunosuppressive molecules, the primary aim of this study was to analyse the T cell modulating effects of another cyclotide plant, i.e., *C. ipecacuanha*. First, we prepared three distinct fractions from an extract of pulverised *C. ipecacuanha* roots via C_{18} solid-phase extraction. Because *C. ipecacuanha* is rich in alkaloids, the initial ipecac extract was purified by washing and elution with 10% and 80% solvent B (labelled C10). Then this portion was subjected to a second cycle of solid-phase extraction to obtain cyclotide-enriched ipecac fractions (Fig. 1). For that purpose, the 10–80% ipecac fraction was washed with either 30%

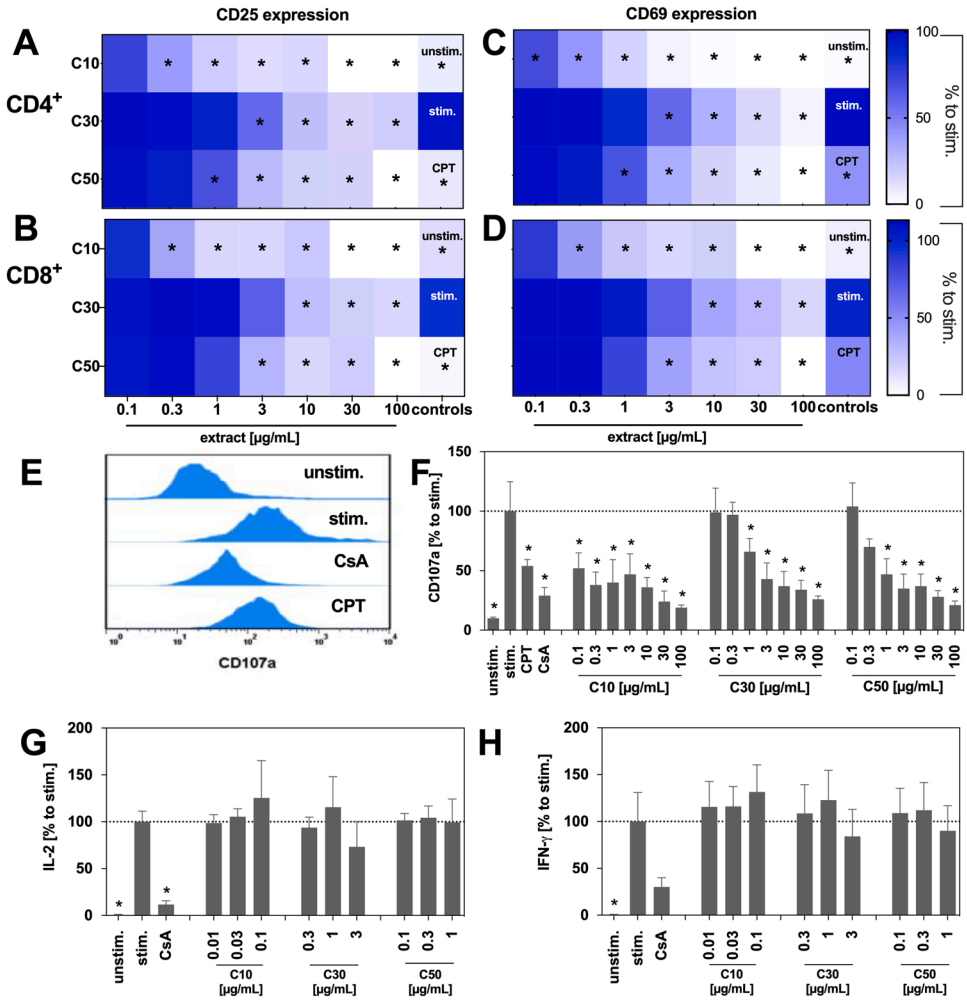


Fig. 3. Effects of *C. ipecacuanha* fractions (C10, C30, C50) on the activation, degranulation capacity and cytokine expression of T cells. Human PBMCs ($2 * 10^5$) were left unstimulated (unstim.) or stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 24 hrs (A, B, C, D, E, F) or 48 hrs (G, H) in the presence of medium (stim., unstim.), cyclosporine A (CsA; 4.16 µM), camptothecin (CPT; 300 µM), or the fractions. Cells were stained with anti-CD25-PE (A, B) or anti-CD69-FITC (C, D) and anti-CD4-APC. Heat maps depict the percentage of $CD4^+$ (A, C) and $CD4^-$ (B, D) T lymphocytes that express CD25 or CD69 in relation to the untreated, stimulated control. n = 3; $*p < 0.05$. (E, F, G, H) Cells (except the unstim. control) were re-stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) and BD Golgi Plug™ was added (G, H) for 4 hrs. (E, F) Cells were stained with CD107a-PE and treated with GolgiStop. Data are depicted in histogram (E). The MFI of CD107a was determined and is depicted in relation to the stimulated control (F). n = 3, $*p < 0.05$. (G, H) Cells were stained with anti-IL-2-PE or anti-IFN-γ-PE. The number of IL-2 (G) or IFN-γ (H) expressing cells was compared and normalised to the stimulated control and depicted as mean ± standard deviation. n = 3; $*p < 0.05$.

or 50% solvent B and eluted with either 80% (C30) or 70% (C50) solvent B.

3.2. Anti-proliferative effects of C. ipecacuanha extracts on T Lymphocytes

Subsequently, the three different extracts obtained via solid-phase extraction on a C_{18} stationary phase were screened for inhibitory effects on T cell proliferation using primary human lymphocytes from different blood donors. All three fractions (C10, C30 and C50) suppressed the proliferation of activated lymphocytes, and significant effects were observed at concentrations as low as 0.01 µg/mL for C10, 1 µg/mL for C30, and 0.3 µg/mL for C50, with IC_{50} values of 0.019 µg/mL, 0.455 µg/mL and 0.021 µg/mL, respectively (Fig. 2A).

To determine whether these effects were caused by cytotoxicity, the numbers of apoptotic and necrotic cells were determined and analysed after incubation with the extracts. The data indicates that the levels of apoptosis were significantly increased by 0.1 µg/mL for C10 and 1 µg/mL for C30 and C50 (Fig. 2B). Necrosis was not induced by any of the fractions (data not presented). To investigate the mechanism of T cell proliferation inhibition, cell cycle progression was analysed (Fig. 2C, Supplementary Information Table S1). A significantly larger number of cells in the G1-phase were observed only at 100 µg/mL for C10 (Fig. 2 C, Supplementary Information Table S1). Although there was a trend towards an increasing number of cells remaining in the G1-phase after incubation with C30 or C50 ($p = 0.091$ for 100 µg/mL of C30 and for 100 µg/mL of C50), these results were not statistically significant (Fig. 2C, Supplemtentary Information Table S1). Furthermore, changes in the number of S phase cells were only descriptive and not statistically significant (Supplementary Information Table S1); in particular, the C30 and C50 fractions lowered the number of cells in the S phase (Fig. 2C). Incubation of lymphocytes with any of the *C. ipecacuanha* fractions reduced the proportion of cells in the G2-phase. This effect was significant at 0.1 µg/mL of C10, 3 µg/mL of C30, and 1 µg/mL of C50 (Fig. 2C, Supplementary Information Table S1).

3.3. Effect of the C. ipecacuanha fractions on the activation and function of human T lymphocytes

Stimulation of the T cell receptor (TCR) induces the expression of surface activation markers. Hence, the impact of *C. ipecacuanha* extracts on the activation of T cells was studied by measuring changes in CD25 and CD69 expression levels. All three fractions induced a concentration-dependent reduction in these marker expressions on $CD4^+$ and $CD8^+$ T cells (Fig. 3A–D). Significant results were obtained at concentrations as low as 0.1 µg/mL of C10 for CD25 expression (Fig. 3A, B), 0.3 µg/mL of C10 for CD69 expression (Fig. 3C, D), and 3 µg/mL of C30 and 1 µg/mL C50 for both activation markers. The effect of *C. ipecacuanha* extracts on the function of $CD8^+$ T cells was examined further by measuring the expression of degranulation capacity using the CD107a surface marker (Fig. 3E, F). All of the three *C. ipecacuanha* extracts yielded a reduction in the number of CD107a expressing cells with a concentration-dependency for C30 and C50. Significant results were obtained at concentrations as low as 0.1 µg/mL of C10 and 1 µg/mL of C30 and C50

Table 1
Sequences and quality control of isolated caripe cyclotides.

Peptide	Sequence	Calculated mass (*m/z*)	Observed mass (*m/z*)
caripe 7	cyclo-CGESCVFIPCTVTALLGCSCKNKVCYRNGIP	3254.5	3254.3
caripe 8	c-CGESCVFIPCITAAIGCSCKKKVCYRNGVIP	3238.6	3238.4
caripe 10	c-CGESCVFIPCFSTVIGCSCKNKVCYRNGVIP	3302.5	3302.3
caripe 11	c-CGESCVFIPCISTVIGCSCKKKVCYRNGVIP	3282.6	3282.3
caripe 12	c-CGESCVFIPCFSSVIGCSCKNKVCYRNGVIP	3288.5	3288.3
caripe 13	c-CGESCVFIPCFTSVFGCSCKDKVCYRNGIP	3238.4	3238.2

Purity of caripe cyclotides was calculated by manual peak integration from 40 to 60 min using RP-HPLC Kromasil C18 column (250 mm × 4.6 mm, 5 μm, 100 Å) at a flow rate of 1 mL/min and linear gradients of solvent B between 5% and 65% at 1%/min. Monoisotopic masses of caripe cyclotides measured by MALDI-MS are shown.

(Fig. 3F).

Upon activation, T cells produce IL-2 and interferon-γ (IFN-γ). Both cytokines are crucial for promoting cellular immune response, but in the case of over-reactivity, they likely contribute to the development of autoimmune diseases. Intracellular staining of both mediators (IL-2 and IFN-γ) was performed to determine the influence of the three *C. ipecacuanha* extracts. However, none of the three extracts induced a reduction in cytokine levels at concentrations lower than those at which significant apoptosis rates were induced (Fig. 3G, H).

In addition, we determined the effects of extracts on T-cell autophagy. Autophagy is an essential mechanism through which immune cells maintain intracellular balance, eliminate pathogens and remove dead cells. Rapamycin and chloroquine can be used to induce autophagy in vitro. However, *C. ipecacuanha* extracts do not induce any changes in autophagy levels (Supplementary Information Fig. S2).

3.4. Isolation and immunomodulatory characterisation of C. ipecacuanha cyclotides

Because the C10, C30 and C50 extracts have immunomodulatory properties and are rich in cyclotides, we isolated purified cyclotides from *C. ipecacuanha* via liquid chromatography to investigate whether these peptides were responsible for the effects measured in the preceding experimental observations (Table 1; Supplementary Information, Fig. S3). The purified cyclotides induced a reduction in T cell proliferation, with significant effects at 10 μM for caripe 7, 8 and 10, and at 30 μM for caripe 11, 12 and 13. A 10 μM mixture of all the peptides also reduced T cell proliferation (Fig. 4A). A significant increase in the number of apoptotic cells was observed after incubation with 10 μM of caripe 10 or caripe 11 and 30 μM of caripe 12 (Fig. 4A), while the other peptides reduced cell viability via the induction of necrosis. Significant results were obtained after treatment with 3 μM of caripe 7, 10 μM of caripe 8, 30 μM of caripe 13, and 10 μM of the entire peptide mix (Fig. 4A).

To further characterise the inhibition of proliferation by the cyclic peptides, an analysis of the cell cycle was conducted (Fig. 4B). There was a considerable reduction in the number of cells in the G2-phase, which was induced by the cyclotides. These results were significant for 10 μM of caripe 7 and caripe 10, and 30 μM of caripe 8, 11, and 12 (Fig. 4B, Supplementary Information Table S4).

Neither the *C. ipecacuanha* extracts nor the isolated cyclotides induced a specific effect via the functional signalling pathways of lymphocytes, but rather induced apoptosis or necrosis. We therefore investigated whether the apoptosis was dependent on caspase activation. A significant induction of caspase 3/7 activity was detected for 3 μg/mL of C10. The C30 and C50 *C. ipecacuanha* extracts induced a descriptive yet non-significant increase in caspase activity (Fig. 4C), while none of the cyclotides affected caspase 3/7 (Fig. 4C).

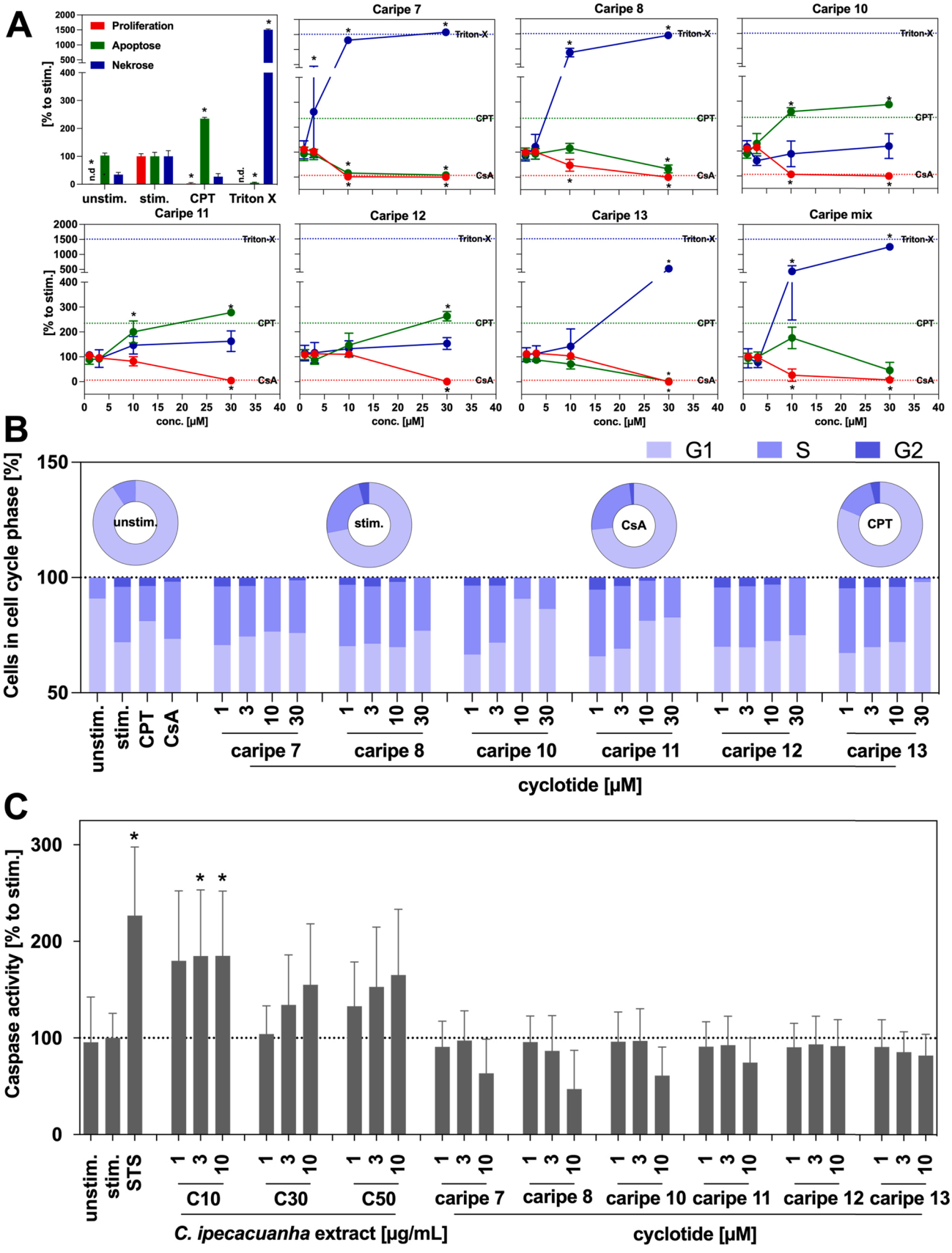
3.5. Effect of the C. ipecacuanha cyclotides on the activation and function of human T lymphocytes

Analogous to *C. ipecacuanha* extracts, the effect of the purified cyclotides on the activation and function of human T lymphocytes was assessed. The expression of the CD25 activation marker was significantly reduced by peptides caripe 7, 8, and 10 at a concentration of 10 μM (Fig. 5A, B). No significant effect could be determined for CD69 (Fig. 5C, D). An increase in the level of intracellular IL-2 at sub-toxic concentrations was observed for 10 μM of caripe 13 (Fig. 5E). None of the other cyclotides induced any changes in the IL-2 production of the lymphocytes (Fig. 5E). The effector function of cytotoxic T cells was impacted by all the cyclic peptides at high concentrations. Significant reduction in CD107a levels were observed for 10 μM of caripe 7, 8, and 10 (Fig. 5F). Interestingly, some of the cyclotides induce the opposite effect. A significant rise in degranulation levels was observed for 3 μM of caripe 8, 11, and 12 (Fig. 5F).

4. Discussion

Especially in Western countries there is a growing prevalence of autoimmune diseases with recorded rates as high as 5% [17]. Hence, there is a need for new and effective therapies [31]. A promising class of natural products with potential application for the treatment of autoimmune diseases are plant-derived cyclic peptides, the so-called cyclotides. Cyclotides have been identified as potent immunosuppressive substances and analogues of kalata B1, isolated from *Oldenlandia affinis,* family Rubiaceae, has recently been clinically tested for the treatment of multiple sclerosis [16]. In this study, we determined immunomodulatory effects of *C. ipecacuanha*, as well as of isolated cyclotides from this plant belonging to the Rubiaceae family.

Proliferation assays were performed to assess the immunomodulatory effects of defined *C. ipecacuanha* extracts. All extracts inhibited the proliferation of human lymphocytes in a concentration-dependent manner. The proliferation inhibiting effect of the extracts is characterised by a reduced number of cells in the G2 cell cycle phase due to a caspase 3/7 dependent induction of apoptosis. Overall, the activation status and effector functions were decreased, reflected by measurement of CD25 and CD69 as well as degranulation capacity, while none of the tested extract concentrations lowered IL-2 or IFN-γ production. A differential effect strength within the three Ipecac root extracts C10, C30 and C50 could also be observed, whereas C10 has the highest antiproliferative effect. The observed immunomodulatory effects of the Carapichea extracts may be due to impurities of other secondary plant compounds in the extract. Ipeac root powder is known to contain alkaloids—primarily emetine and cephaeline [27]. The structure and biological activity of emetine has already been described previously [32], along with a published report that emetine induces inhibition of cell prolieferation in many different cell lines [33,34], by inhibiting protein synthesis and interacting with DNA [32]. In general, the observed immunomodulatory effects are different to that of other cyclotide containing plant extracts, were one could find a additional



(caption on next page)

Fig. 4. Inhibitory effects of *C. ipecacuanha* extracts and cyclotides (7, 8, 10, 11, 12, 13) on proliferation, apoptosis and necrosis induction as well as the cell cycle and the caspase 3/7 activity of T lymphocytes. Human PBMCs ($2 * 10^5$) were stained with CFSE (just for the proliferation experiments) and 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 (C) or 72 h (A, B) in the presence of medium (stim.), cyclosporine A (CsA; 4.16 μM), camptothecin (CPT; 300 μM), Triton-X 100 (0.5%), staurosporine (STS; 1 μM) or fractions/cyclotides. (**A**) Annexin V-FITC and PI double staining was performed to determine apoptosis and necrosis induction. The percentage of proliferating, apoptotic and necrotic cells were compared and normalised to the stimulated control and depicted as mean ± standard deviation. n = 3; *$p < 0.05$. in a column diagram or in dot diagrams. (B) PI staining of the DNA was performed. The number of lymphocytes in each phase compared to all living lymphocytes was determined and depicted as mean. n = 3. (C) Luminescence was measured 1 hr after Caspase-Glo® 3/7 reagent addition. The caspase activity of each sample compared to the stimulated control was determined and depicted as mean ± standard deviation. n = 3; *$p < 0.05$.

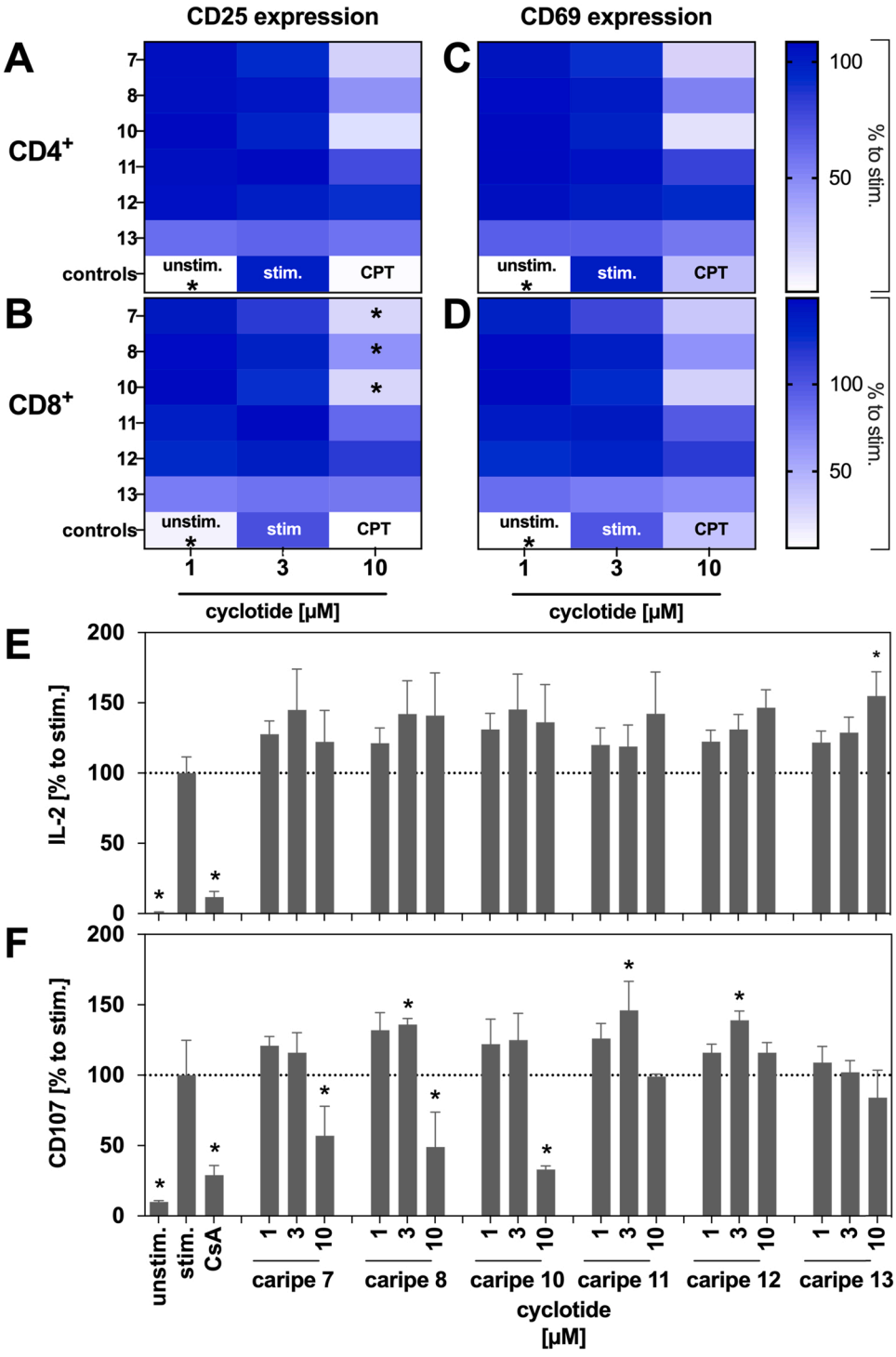


Fig. 5. Effects of *C. ipecacuanha* cyclotides (caripe 7, 8, 10, 11, 12 and 13) on the activation, degranulation capacity and cytokine expression of T cells. Human PBMCs ($2 * 10^5$) were left unstimulated (unstim.) or stimulated with anti-CD3 and anti-CD28 mAbs (100 ng/mL each). Afterwards, anti-CD3 and anti-CD28 activated cells were incubated for 24 h (A, B, C, D, F) or 48 h (E) with or without (stim.) the addition of cyclosporine A (CsA; 4.16 μM), camptothecin (CPT; 300 μM), or the cyclotides. Cells were stained with anti-CD25-PE (A, B) or anti-CD69-FITC (C, D) and anti-CD4-APC and analysed by flow cytometry. Heat maps depict the percentage of $CD4^+$ (A, C) and $CD4^-$ (B, D) T lymphocytes that express CD25 or CD69 in relation to the untreated, stimulated control. n = 3; *$p < 0.05$. (E, F) Cells (except the unstim. control) were re-stimulated with PMA (50 ng/mL) and ionomycin (500 ng/mL) and treated with BD Golgi Plug™ (E) for 4 h. (F) Cells were stained with CD107a-PE and treated with GolgiStop. The MFI of CD107a was determined and is depicted in relation to the stimulated control. n = 3, *$p < 0.05$. (E) Cells were stained with anti-IL-2-PE. The number of IL-2 expressing cells was compared and normalised to the stimulated control and depicted as mean ± standard deviation. n = 3; *$p < 0.05$.

inhibition on IL-2 and IFN-γ production [15,16,30].

The *C. ipecacuanha* cyclotides were further isolated and examined using the same experiments as for the fractions. The experiments demonstrate, that the caripe peptides has a defined inhibitory impact on T cell proliferation and hereby reflects the effect of the whole *C. ipecacuanha* extract. Both, the whole extract and the isolated cyclotides are characterised by a reduced number of cells in the cell cycle phase G2. In a further step, the effect of the caripe peptides were examined on activation markers, degranulation capacity and cytokine secretion to get an idea about the overall immunomodulatory potential. Inhibitory effects on activation and degranulation were found for distinct caripe cyclotides. Whereas, secretion of IL-2 and IFN- γ was not affected by any of the cyclotides or the caripe cyclotide mix which thus differentiates the mechanism of action from other immunosuppressive cyclotides.

An IL-2 dependent immunosuppression is described for kalata B1 from *O. affinis* [15]. Likewise, a plant extract from *Viola tricolor* inhibited the proliferation of human lymphocytes by suppressing IL-2 secretion. Bioassay-guided fractionations yielded two semipure cyclotide fractions as anti-proliferative active [30]. Beside from proliferation, effector function were thereby also diminished in the presence of pure isolated cyclotides and demonstrate a decreased production in IFN- γ and degranulation capacity. A general anti-proliferative effect has already been demonstrated for other cyclotides [35,36].

Kalata B1 a prototypic cyclotide member belongs to the Möbius subclass, characterised by a cis-Pro residue in loop 5 (Supporting Information Fig. S5), whereas the caripe cyclotides belong to the bracelet subclass [28,37]. Möbius cyclotides are generally considered to be potent to interact with membrane components as compared to the bracelet subclass [38], which could explain both the observed higher apoptosis capacity of the caripe cyclotides and the different mechanisms-of-action of immunosuppression. Furthermore there are several additional differences of kalata B1 and caripe cyclotides, for instance, loop 2 contains a hydrophobic patch in caripe cyclotides, which is not present in kalata B1; in loop 5 kalata B1 has the subclass typical Trp-Pro-Val motif, whereas caripe contains mainly positive charges Lys residues. Loop 3 exhibits diversity in a subclass independent manner (Supporting Information, Fig. S5). These sequence alterations may explain the subtle but distinct immunomodulatory differences amongst the caripe peptides, and different mechanism between caripe and kalata-type cyclotides.

In general, anti-proliferative substances are of great therapeutic importance. Hence, the induction of programmed cell death also makes use of the cyclotides in therapy conceivable. Aside from glucocorticoids, which prevent the expression of pro-inflammatory transcription factors [39], and drugs such as cyclosporine, which functionally inhibit the activation and proliferation of lymphocytes [40], proliferation inhibiting drugs, e.g. cyclophosphamide, are currently used to treat autoimmune diseases [41]. In this sense, the caripe cyclotide extracts investigated here also have the potential to be used in the case of excessive immune reactions.

5. Conclusion

This study exemplifies that screening members of the Rubiaceae family utilizing cell-based immunological assays is a powerful strategy for identifying natural products with immunomodulating properties. In particular, *C. ipecacuanha* extracts and isolated cyclic peptides differentially inhibit the proliferation and function of human T lymphocytes. The use of *C. ipecacuanha* extracts containing cyclotides might have potential applications in conditions associated with an overreactive immune system and should be explored in more detail in the future.

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CRediT authorship contribution statement

Chiara Madlen Falanga: Data curation, Formal analysis, Investigation, Software, Writing – review & editing. **Carmen Steinborn:** Data curation, Formal analysis, Software, Writing, original draft, Writing – review & editing. **Edin Muratspahić:** Data curation, Formal analysis, Investigation, Software, Visualization, Writing – review & editing. **Amy Marisa Zimmermann-Klemd:** Data curation, Formal analysis, Project administration, Software, Supervision, Validation, Visualization, Writing, original draft, Writing – review & editing. **Moritz Winker:** Formal analysis, Investigation. **Liselotte Krenn:** Investigation, Visualization, Writing – review & editing. **Roman Huber:** Resources, Writing – review & editing. **Christian W. Gruber:** Conceptualization, Funding acquisition, Methodolog, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing, Writing – review & editing. **Carsten Gründemann:** Conceptualization, Funding acquisition, Methodolog, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing, original draft, Writing – review & editing..

Conflict of interest statement

CWG is shareholder and scientific advisor of Cyxone AB. All other authors declare no conflict of interest. The funding sponsors had no role in the design, writing and publishing strategy of the study, as well as in collection, analysis, or interpretation of the data.

Data Availability

Data will be made available on request.

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Data statement

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

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.113120.

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