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Maca *(Lepidium meyenii* Walp.) inhibits HIV-1 infection through the activity of thiadiazole alkaloids in viral integration

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ABSTRACT

Ethnopharmacology relevance: Lepidium meyenii Walp. (maca) has been traditionally used for centuries in the Central Andes region both as food and as medicine. In the last decades, its fertility enhancer properties have gained importance, with the majority of the scientific literature related to this topic. However, other traditional uses are less known as metabolic or infectious diseases.

Aim of the study: The main purpose of this study is to investigate the anti-infectious activity of *L. meyenii*, specifically in HIV-1 infection. There are previous reports of the transcriptional related activity of *L. meyenii* extracts in human T lymphocytes via transcription factors as NF-κB. Since T lymphocytes are the main target of HIV-1 infection and NF-κB is strongly involved in HIV-1 transcription, *L. meyenii* could display antiviral activity.

Material and methods: Chromatography and spectroscopy techniques were used to isolate and identify the compounds in the active extracts. An antiviral assay system based on recombinant viruses was used to evaluate the anti-HIV activity. Cell toxicity was tested for all the extracts and compounds. Viral entry was studied using VSV-HIV chimera viruses and reverse transcription and viral integration were studied by qPCR of viral DNA in infected cells. Finally, viral transcription was studied in primary lymphocytes transfected with HIV-1 or NF-κB luciferase reporter plasmids.

Results: n-Hexane extracts of purple maca displayed anti-HIV activity in an *in vitro* assay. A bioassay-guided fractionation led to the identification of three thiadiazole alkaloids with antiviral activity. All the compounds were able to inhibit HIV infection of MT-2 cell lines and primary lymphocytes (PBMCs) with IC_{50} values in the low micromolar range. The mechanism of action differs between the three compounds: one of them showed activity on viral entry, and all the three compounds inhibited viral integration at low concentrations. Remarkably, none of the compounds inhibited reverse transcription or viral transcription.

Conclusions: n-Hexane extracts of the purple ecotype of *L. meyenii* inhibit HIV-1 infection *in vitro* and three active thiadiazole alkaloids were isolated acting mainly on viral integration and viral entry.

1. Introduction

Lepidium meyenii Walp. (Maca) is an annual or biennial herb of the *Brassicaceae* family, cultivated for more than 2000 years in the central Andes of Peru, Bolivia and north-western Argentina (Meissner, 2017). *L. meyenii* has been traditionally used both as food and as medicine to treat stress and fatigue, as fertility enhancer for both humans and cattle and as pain reliever, although scientific evidence does not fully support these uses (Beharry and Heinrich, 2018). However, traditional medicine includes immune related conditions (Chang, 2014), and infectious diseases as *L.meyenii* therapeutic indications, as described in some South American Floras, where it is described as anti-infective for lung diseases and a disinfecting agent (De Lucca and Zalles, 1992) and for parasites infestations and diarrhoea (Pestalozzi and Torrez, 1998), highlighting

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Table 1
Anti-HIV activity and cytotoxicity of extracts and fractions obtained from *L. meyenii*. Results are presented as percentage (%) of infection and viability or IC_{50} (Inhibitory concentration 50%) and CC_{50} (Cytotoxic concentration 50%) values in μg/mL. CI95% Confidence Interval 95%.

		μg/mL	% Infection	% Viability	SD	SD
Maca mix Extracts	Hexanic extract (LM0)	50	35%	101%	5%	3%
		25	41%	98%	5%	3%
	Ethanolic extract (LM2)	50	74%	102%	7%	1%
		25	81%	103%	13%	2%
	Aqueous extract (LM3)	1200	22%	91%	3%	4%
		400	66%	91%	14%	15%
Fractions	F4	100	2%	120%	0%	7%
		10	66%	83%	6%	18%
	F7	100	1%	136%	0%	3%
		10	43%	102%	5%	6%
	F8	100	1%	113%	0%	9%
		10	6%	123%	1%	9%

	IC_{50} μg/mL	CC_{50} μg/mL	CI95% IC_{50}	CI95% CC_{50}	R^2 IC_{50}	R^2 CC_{50}
Purple Maca	24,73	123,8	12,14 to 50,39	62,29 to 246,2	0,6632	0,6694
Yellow Maca	71,90	158	42,96 to 120,3	84,84 to 294,3	0,9168	0,8566
Black Maca	67,56	109,1	29,19 to 156,4	72,34 to 164,4	0,8216	0,9108
Maca Mix	65,12	213,9	38,75 to 109,4	89,20 to 512,8	0,912	0,7333

the potential uses of this species as an anti-infective agent. *L. meyenii* contains important secondary metabolites, including macamides, macaenes, glucosinolates, isothiocyanates, beta-carboline alkaloids and imidazole alkaloids (Lepidiline A-D) (Huang, 2018), the latter being specific of *L. meyenii* (Cui, 2003). However, different ecotypes (colours) have been described and little is known about the differences in their phytochemical profile and clinical use (Minich, 2024).

During the past few decades, the interest in *L. meyenii* has increased due to uses other than the traditional ones, as energising, adaptogenic and as a treatment for osteoporosis, hyperlipidaemia, prostate hyperplasia and various dermatological issues (da Silva Leitão Peres, 2020). There are also some reports about its activity as immunomodulatory, antifatigue, antioxidant and antinflammatory activities through NF-κB inhibition (Zheng, 2018; He et al., 2022; Orhan, 2022; Yu et al., 2020). Since NF-κB is one of the most important cellular transcription factors involved in HIV-1 transcription, we have hypothesized that this effect could be associated with a potential anti-HIV activity.

In fact, there are some reports on the antineoplastic and anti-influenza activities of maca in human T lymphocytes (Alzamora, 2007; Del Valle Mendoza, 2014). However, there are limited literature about its antiviral activity and little is known about the compounds responsible for this activity.

HIV-1 infection is a relatively new infection, since it is believed to have arisen in Africa in the mid-20th century and thus, traditional uses to treat this infection have not been described. However, *L. meyenii* have been traditionally used for its anti-infective properties, although there is scarce scientific literature about this topic. On the other hand, antiretroviral therapy (ART) effectively controls HIV-1 infection, keeping the viral load undetectable. However, HIV-1 remains hidden in the viral reservoirs, and, when ART is discontinued, viral rebound occurs. Therefore, ART must be maintained for the patient's entire life, leading to viral resistances and drug-related adverse effects. Anti-HIV natural products with novel mechanisms of action could provide new tools to eliminate HIV-1 infection.

In this paper, we will study the antiviral effect of maca on HIV-1 infection, identifying the compounds responsible for this activity and their targets in the viral replication cycle.

2. Material and methods

2.1. Plant material

The hypocotyls of *L. meyenii* Walp. (plant name has been checked with https://www.worldfloraonline.org//) were collected from the Colchani community, Aroma province, La Paz, Bolivia (17°11′00.0″S 67°54′00.0″W), in June 2015, at an altitude of 3800 m. The botanical identification was confirmed by the National Herbarium of Bolivia (No. 10981).

2.2. General experimental procedures

Thin layer chromatography (TLC, Merck Silica gel 60-F_{254} plates) was used for compounds identification. TLC plates were visualised by UV light (Spectroline® E-Series UV lamp at 365 and 254 nm, 230V, New York, USA), or by impregnating the plate with the 2-aminoethyl diphenyl borate reagent (1% solution of ethanolamine diphenyl borate in methanol + 5% ethanolic solution of polyethylene glycol) or phosphomolybdic acid reagent (Phosphomolybdic acid 10% in absolute ethanol) followed by heat application. Column chromatography was used for fractionation and compound isolation and was performed with silica gel (20–45 μm, Merck). All organic solvents that were used for isolating the compounds were purchased from Sigma-Aldrich.

Nuclear magnetic resonance (NMR) experiments were performed on Bruker Advance DRX 250 and 700 spectrometers operating at 250 MHz, 700 MHz (^{1}H) or 60 MHz, and 176 MHz (^{13}C) with tetramethylsilane (TMS) as the reference solvent (δ_H 0 ppm). Spectra were calibrated by assignment of the residual solvent peak to δ_H 7.26, δ_H 3.31 and δ_C 77.16, δ_C 49.00, for $CDCl_3$ and MeOD, respectively. The complete assignment of protons and carbons was done through ^{1}H–^{1}H COSY, HSQC and HMBC spectra analysis.

HREIMS analyses were performed using a mass spectrometer with a hybrid quadrupole time-of-flight (QTOF) analyser model MAXIS II from the commercial house Bruker, S.A. Samples were analysed using the electrospray ionisation technique, by direct infusion at a flow of 3 μL/min, using methanol with 0.1% formic acid as the ionising phase. The source parameters were the following: End Plate Offset: 500 V; Capillary: 3500; Nebuliser: 0.2 bar; Dry Gas: 2.0 L/min; Dry Temp.: 250 °C; and Mass range of 50–3000 Da.

2.3. Extraction and isolation

The hypocotyls of *L. meyenii* were dried and pulverised (250 g) and extracted with *n*-hexane (LM0), ethanol (LM2) or water (LM3), obtaining 20.40 g (LM0), 90.12 g (LM2) and 36.36 g (LM3). In the same way, black, purple and yellow hypocotyls (250 g for each) were extracted with *n*-hexane (LM0), obtaining *n*-hexane extracts from black hypocotyls (29.65 g), purple hypocotyls (23.63 g) and yellow hypocotyls (17.92 g). The *n*-hexane extract of purple maca hypocotyls was fractionated using a chromatographic column (2 × 50 cm), employing Si-60 silica gel (40–63 μm, Merck) as the stationary phase, and using a gradual gradient of *n*-hexane/Ethyl acetate (AcOEt) (3:1–1:1 v/v) as eluent obtaining twelve fractions (F1–F12).

Subsequently, based on the antiviral and cell viability data (Table 1), separations of **F4**, **F7** and **F8** fractions were carried out using column chromatography with Si-60 Silica gel (40–63 μm, Merck). Fraction **F4** was purified obtaining compound **1** (14.80 mg), fraction **F7** (1.53 g) was purified obtaining compound **2** (13.93 mg) and fraction **F8** was purified obtaining compound **3** (40.10 mg) (For a detailed fractionation process see methods in Supplementary Material (Material and Methods, a and c).

2.4. Cell culture reagents

MT-2 cells are lymphoblastic cells expressing the receptors CD4 and CXCR4 needed to be permissive to HIV-1 infection. These cells were

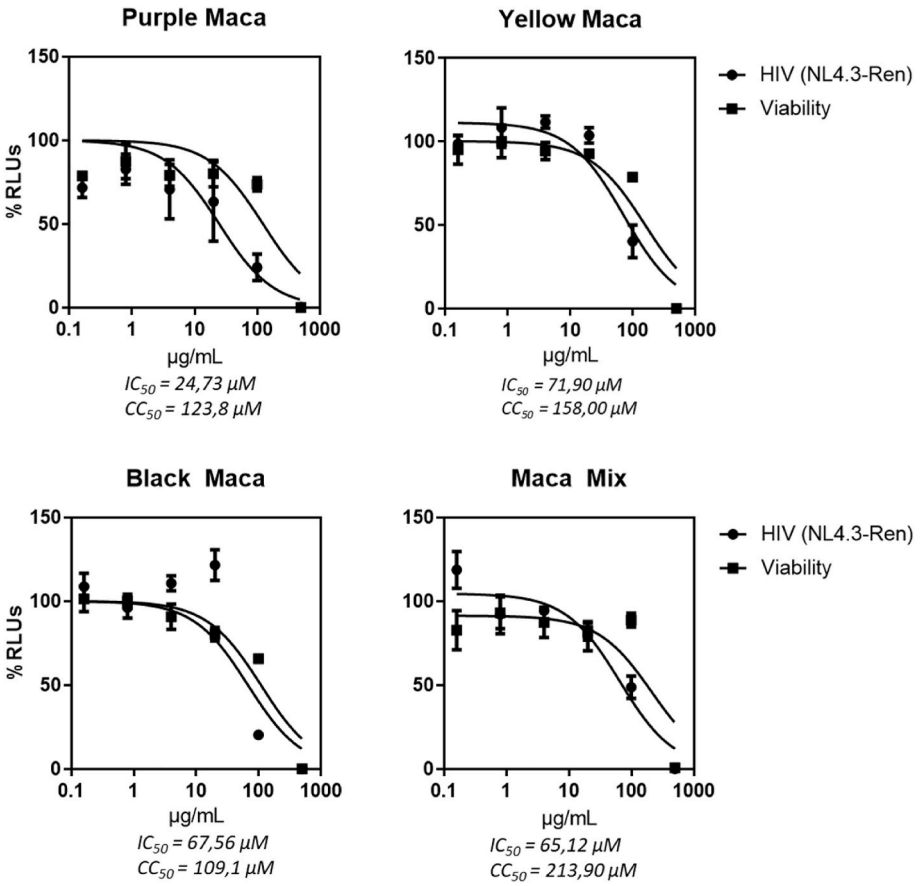

Fig. 1. Anti-HIV-1 activity and toxicity of *L. meyenii* (maca). MT-2 cells were pre-treated with different concentrations of purple, yellow, black or mix *n*-hexane extracts (base 2 serial dilutions in DMSO. Cmax 500 µg/mL) and infected with a recombinant HIV-1 (NL4.3-Ren. 100.000 RLUs per well). Renilla activity (RLUs) in cell lysates was measured 48 h later. Cell viability was measured in mock infected treated cells and detected by CellTiter Glo luminescence viability assay. Results are expressed as a percentage of relative luminescence units (RLUs), where 100% is the level of viral replication (HIV) or cell viability (Viability) obtained in the presence of the vehicle used to dissolve the compounds.

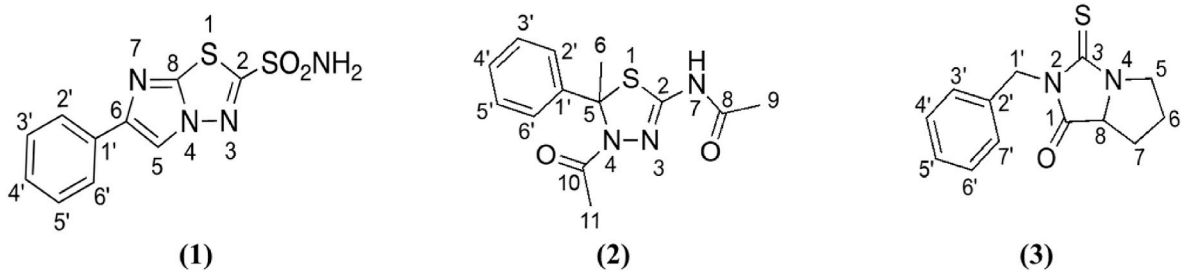

Fig. 2. Chemical structures of compounds **1**–**3** isolated from the purple hypocotyls of *L. meyenii*.

cultured in RPMI-1640 medium and 293T cells in DMEM medium, both containing 10% (v/v) fetal bovine serum, 2 mM L-glutamine, penicillin (50 IU/mL) and streptomycin (50 µg/mL) (all Whittaker M.A. Bio-Products. Walkerville, MD, USA). Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere and split twice a week. MT-2 cell line was established by co-cultivation of cells from a 45 years old female T-cell leukaemia patient with cord leukocytes from a male infant (Harada, 1985; Miyoshi, 1982). Thus, sex gender bias would not apply. The following reagents were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: Enfuvirtide (T-20), tenofovir (TFV), efavirenz (EFV), Etravirine (ETV) and raltegravir (RAL). Phorbol 12-Myristate 13-Acetate (PMA) was obtained from Merck (Lyon, France). Buffy Coats from anonymous healthy blood donors were obtained from the Centro de Transfusiones de la Comunidad de Madrid. Proper informed consent was obtained from each subject in accordance with the Spanish legislation on blood donor regulations. Confidentiality and privacy were assured. Sex and gender-based analyses were not performed since the gender of the donor was not disclosed. However, several buffy coats from different donors were used in all the experiments and thus, gender bias would not apply to our methodology. Peripheral blood mononuclear cells (PBMCs), including $CD4^+$ lymphocytes of non-infected patients, were then isolated from buffy coats by centrifugation through a Ficoll-Hypaque gradient (Pharmacia Corporation, North Peapack, NJ) and were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and antibiotics (100 mg/L streptomycin and 100 U/mL penicillin) (all Whittaker M.A. Bio-Products, Walkerville, MD, USA) before culture at a concentration of $2{\cdot}10^6$ cells/mL. PBMCs were maintained in culture (resting) or

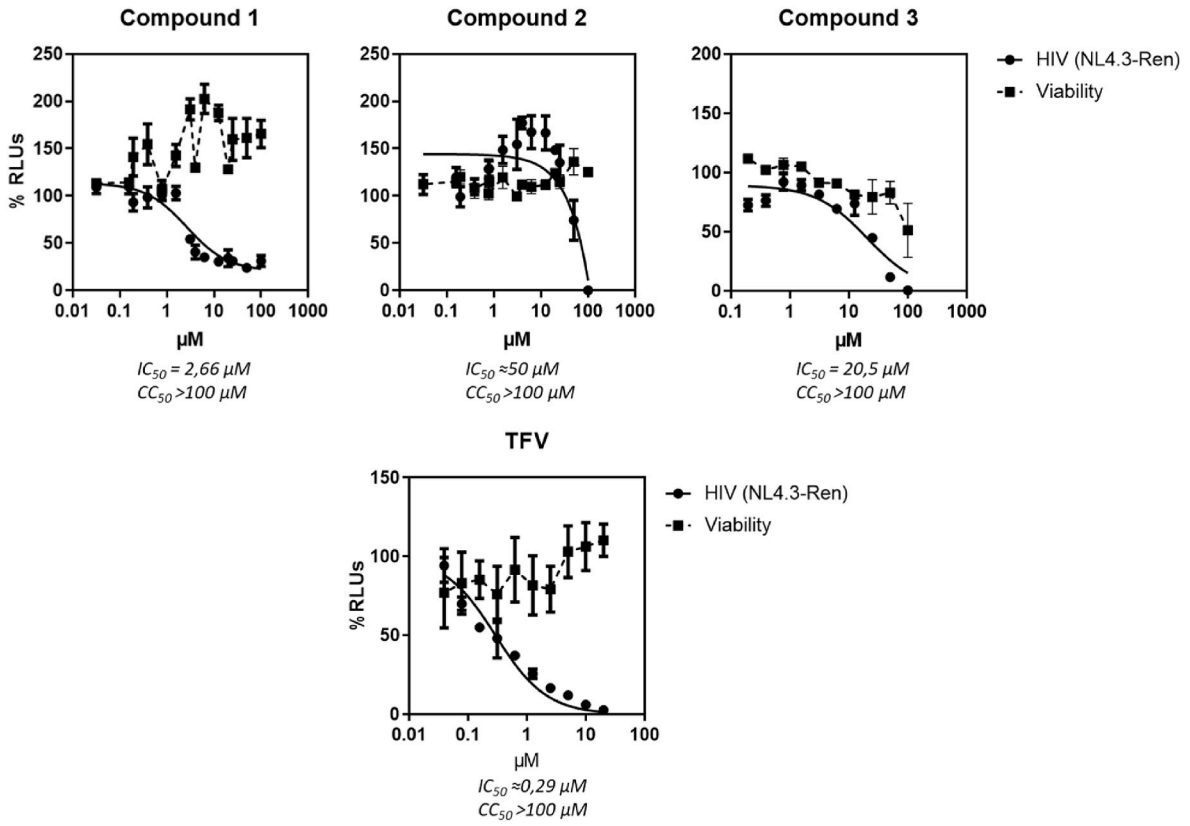

Fig. 3. Anti-HIV-1 activity and toxicity of the alkaloids isolated from *L. meyenii* (compounds **1**, **2** and **3**) in MT-2 cells. Cell cultures were pre-treated with different concentrations of compounds (base 2 serial dilutions in DMSO. Cmax 100 μM) and infected with a recombinant HIV-1 (NL4.3-Ren. 100.000 RLUs per well). Renilla activity (RLUs) in cell lysates was measured 48 h later. Cell viability was measured in mock infected treated cells and detected by CellTiter Glo luminescence viability assay. Tenofovir (TFV) was used as inhibitor control. Results are expressed as a percentage of relative luminescence units (RLUs), where 100% is the level of viral replication (HIV) or cell viability (Viability) obtained in the presence of the vehicle used to dissolve the compounds. IC_{50} (Inhibitory concentration 50%) and CC_{50} (Cytotoxic concentration 50%).

activated by the addition of 300 IU/mL Interleukin-2 (IL-2) (Emeryville, CA. USA) every 48 h.

2.5. Plasmids and viral supernatants

NL4.3 viral supernatants were obtained by several passages in MT-2 cells and titrated using p24-gag protein ELISA (Innotest HIV antigen mAb, Fujirebio, Zwijnaarde, Belgium). The plasmids pNL4.3-luc and pNL4.3-Ren have been previously described (Garcia-Perez, 2007). Plasmid pNL4-3.Luc.R^-.E^- was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH from Dr. Nathaniel Landau, and plasmid pcDNA-VSV, encoding the G protein of VSV, from Dr. Arenzana-Seisdedos from Pasteur Institute (Oberlin, 1996). The 3-enh-κB-ConA-luc plasmid (pNF-κB Luc) carries a luciferase gene under the control of three synthetic copies of the κB consensus of the immunoglobulin k-chain promoter cloned into the *Bam*HI site located upstream from the conalbumin transcription start site (Arenzana-Seisdedos, 1993).

Plasmids were amplified transforming *E. coli* DH5α cells and cultured in LB (Merck, Schnelldorf, Germany) supplemented with ampicillin (Merck, Lyon, France) at 37 °C for 18 h. All plasmids were purified using Qiagen Plasmid Maxi Kit (Qiagen, Hilden, Germany) following the manufacturer instructions. DNA concentration was determined in a Nanodrop (Thermo Scientific).

Infectious supernatants were obtained by calcium phosphate transfection of 293T cells of plasmids pNL4.3 Ren (wild type HIV) or by co-transfection of pNL4-3.Luc.R-E and pcDNA-VSV (HIV-VSV). All the infectious supernatants obtained were titrated in the target cells by measuring the Relative Luminescence Units (RLUs) and p24-gag protein in the supernatants and stored at −80 °C until use.

2.6. Evaluation of anti-HIV activity

Extracts, fractions and isolated compounds were dissolved in ultrapure water or in dimethyl sulfoxide (DMSO Merck, Lyon, France), at 10 mg/mL or 10 mM and aliquoted and stored at −20 °C. NL4.3-Ren infectious supernatants (100.000 RLUs per well or 20 ng gag-p24 per well) were used to infect MT-2 cells or IL-2 activated PBMCs in the presence or absence of serial diluted concentrations of the compounds and extracts. Infected cultures were maintained in 5% CO_2 humidified atmosphere for 48 h. Afterwards, anti-HIV activity was quantified by measuring renilla-luciferase activity following the manufacturer instructions of the Renilla Assay System (Promega Madison, WI, USA) and RLUs were obtained in a luminometer (Berthold Detection Systems, Pforzheim, Germany). All the experiments were controlled with cells treated with the same solvent (water or DMSO) concentration of extracts or compounds (100%). IC_{50} was calculated with a non-linear regression analysis using GraphPad Prism 8 software.

2.7. Evaluation of cell viability

Cell viability was measured in treated mock-infected cells in parallel in the same conditions as in the antiviral assays by the CellTiter Glo Assay System (Promega, Madison, WI, USA). Cell viability is expressed as percentage of viable cells compared to a non-treated (Water or DMSO at the same concentration as extracts or compound) control (100%). CC_{50} was calculated with a non-linear regression analysis using GraphPad Prism 8 software.

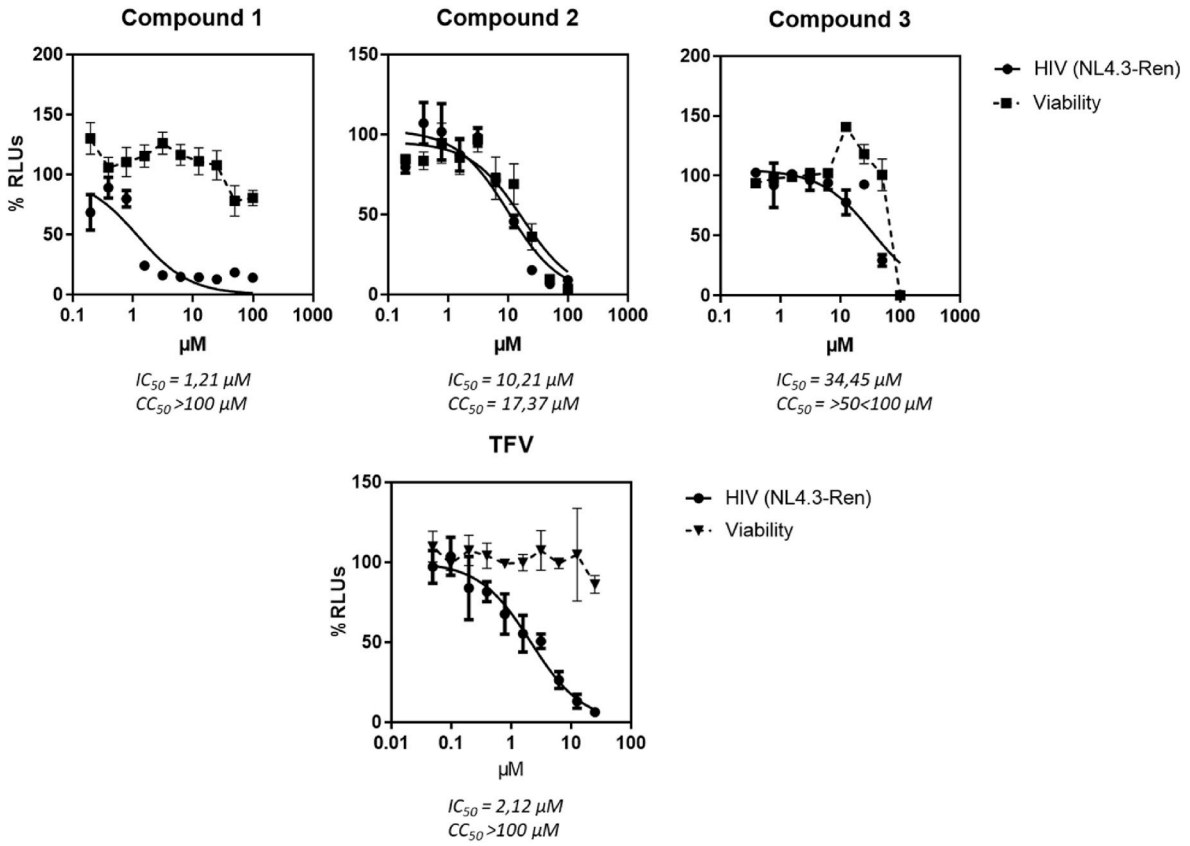

Fig. 4. Anti-HIV-1 activity and toxicity of the alkaloids isolated from *L. meyenii* (compounds **1**, **2** and **3**) in PBMCs. Cell were isolated from Buffy Coats obtained from healthy donors and treated with IL-2 for at least 48 h. Afterwards, cell cultures were pre-treated with different concentrations of compounds (base 2 serial dilutions in DMSO. Cmax 100 µM) and infected with a recombinant HIV-1 (NL4.3-Ren. 100.000 RLUs per well). Renilla activity (RLUs) in cell lysates was measured 48 h later. Cell viability was measured in mock infected treated cells and detected by CellTiter Glo luminescence viability assay. Tenofovir (TFV) was used as inhibitor control. Results are expressed as a percentage of relative luminescence units (RLUs), where 100% is the level of viral replication (HIV) or cell viability (Viability) obtained in the presence of the vehicle used to dissolve the compounds. IC_{50} (Inhibitory concentration 50%) and CC_{50} (Cytotoxic concentration 50%).

2.8. Viral entry inhibition

To evaluate the effect of the isolated compounds in HIV-1 entry we have compared MT-2 infections performed with a recombinant wild type HIV-1 (NL4.3-Ren) (100.000 RLUs or 20 ng gag-p24 per well) and a VSV-pseudotyped recombinant HIV (HIV-VSV) (100.000 RLUs or 20 ng gag-p24 per well), which enters the target cells by a receptor independent mechanism. The methodology is similar to the antiviral activity evaluation. Infections were performed with both virus (HIV and VSV-HIV) in parallel and, after 48 h, RLUs obtained in a luminometer (Berthold Detection Systems Pforzheim, Germany). Enfuvirtide (ENF) was used as control of viral entry inhibition. IC_{50} was calculated with a non-linear regression analysis and ANOVA analysis was performed to calculate the significant differences (*p* value) between HIV IC_{50} and VSV-HIV IC_{50}, both using GraphPad Prism 8 software.

2.9. Quantification of early and late reverse transcription

MT-2 cells were pre-treated with different concentrations of the isolated compounds and infected with a NL4.3 wild type HIV-1 (2 ng gag p24 per well). Infected cultures were then thoroughly washed with PBS 1x and maintained at 37 °C and 5% CO_2 for 5 h. Compound concentrations were chosen based on their previously observed EC_{50} and CC_{50} values (10 and 20 µM). Total genomic DNA was isolated with a QIAamp DNA blood mini kit, Qiagen (Qiagen, Hilden, Germany) and quantified with a Nanodrop-1000 spectrophotometer (ThermoFisher, Wilmington USA).

Early and late viral DNAs were quantified by qPCR as previously described (Bermejo et 2015). Briefly, 50–100 ng DNA were mixed with 1 µM forward and reverse primers (MA pr-243 and MA pr-244, and MA pr-275 probe for early RT and primers MH531 and MH532 and LRT-P probe for late RT) (Table 1S) and 1x GoTaq Probe Universal Master Mix (Promega, Madison, WI, USA) in a final volume of 16 µL. qPCR was performed in triplicates in a StepOne Real-Time PCR system (Life Technologies) using standard cycling conditions (Table 1S). Serial dilutions of genomic DNA from 8E5 cell line, which contain a single integrated copy of HIV-1 (Folks, 1986) were used as standard curve. The CCR5 gene was used as endogenous control (primers CCR5_F and CCR5_R and CCR5 probe in Table 1S).

2.10. Quantification of proviral integration

Integrated proviral DNA was quantified within whole genomic DNA from 5-day post-infection cell cultures, with the same infection conditions as in reverse transcription evaluation and also treated with two concentrations of the isolated compounds (10 and 20 µM). Whole genomic DNA was isolated with a QIAamp DNA blood mini kit, Qiagen (Qiagen, Hilden, Germany) and quantified with a Nanodrop-1000 spectrophotometer (ThermoFisher, Wilmington USA) and submitted to a nested Alu-LTR PCR, as previously described (Brussel and Sonigo, 2003) using a StepOne Real-Time PCR System (Life Technologies). In brief, a first conventional PCR was performed using oligonucleotides against Alu sequence and HIV-1 LTR (Alu 1 and Alu 2, and reverse primer L-M667 in Table 1S). Then, a second qPCR was performed using the HIV-1 LTR primers Lambda T and AA55M and the Taqman probe MH603 (Table 1S) (Integrated DNA Technologies) and 1x GoTaq Probe

Table 2
Anti-HIV activity and cytotoxicity of the isolated compounds from *L. meyenii* in MT-2 cells and PBMCs infected with HIV-1 (NL4.3-Ren) or VSV-HIV. Results are presented as IC_{50} (Inhibitory concentration 50%) and CC_{50} (Cytotoxic concentration 50%) values in μM. CI95% Confidence Interval 95%.

	IC_{50} μM	CC_{50} μM	CI95% IC_{50}	CI95% CC_{50}	R^2 IC_{50}	R^2 CC_{50}
MT-2 HIV-1 INFECTION AND CYTOTOXICITY						
Compound 1	2,66	>100	1,49 to 4,75	–	0,7601	–
Compound 2	≈50	>100		–		–
Compound 3	20,5	≈100	13,93 to 30,16	–	0,8095	–
Tenofovir (TFV)	0,29	>100	0,24 to 0,35	–	0,9294	–
Enfuvirtide (ENF)	0,007	>100	0,005 to 0,010	–	0,9066	–
MT-2 HIV-VSV INFECTION						
Compound 1	3,33	–	1,47 to 7,44		0,7572	
Compound 2	>100	–	–		–	
Compound 3	34,07	–	18,93 to 65,71		0,6396	
Enfuvirtide (ENF)	>100	–	–		–	
PBMCs HIV-1 INFECTION AND CYTOTOXICITY						
Compound 1	1,21	>100	0,53 to 2,74	–	0,7189	–
Compound 2	10,21	17,37	5,95 to 17,52	10,07 to 29,98	0,838	0,8183
Compound 3	34,45	>50 < 100	18,25 to 65,04	–	0,7184	–
Tenofovir (TFV)	2,12	>100	1,77 to 2,54	–	0,9008	–

Universal Master Mix (Promega, Madison, WI, USA). Serial dilutions of genomic DNA from 8E5 cell line were used as standard curve. The CCR5 gene was used as endogenous control (primers CCR5_F and CCR5_R and CCR5 probe in Table 1S).

2.11. Transcriptional activity evaluation

Resting PBMCs were maintained for one day in culture without stimuli. Afterwards, cells were suspended in 350 μL of RPMI without supplements in 4 mm cuvettes (Equibio, Middlesex, UK) and transfected/pulsed at 320V, 1500 μF and maximum resistance using an Easyject plus Electroporator (Biorad) with 1 μg/10^6 cells of plasmid DNA (pNL4.3-Luc or pNF-κB Luc). After transfection, cells were immediately seeded in 24 well microplates ($5{\cdot}10^6$ for PBMCs) and treated with different concentrations (5, 10 and 20 μM) of the isolated compounds, using PMA (0.1 μM) as a control of HIV-1 transactivation, and cultured in complete RPMI at 37 °C. 48 h later, cell cultures were harvested and lysed with luciferase buffer (Luciferase assay system buffer, Promega, Madison, WI, USA). Luciferase activity (RLUs) was then measured in a luminometer (Berthold Detection Systems, Pforzheim, Germany).

2.12. Statistical analysis

Calculations of 50% inhibitory concentrations (IC_{50}) and 50% cytotoxic concentrations (CC_{50}), T-test and one-way ANOVA statistical analysis (post-test Bonferroni multiple comparison test, $*p < 0.05$; $**p < 0.01$; $***p < 0.001$; $****p < 0.0001$) were performed using GraphPad Prism 8 software.

3. Results

3.1. Isolation and fractionation of compounds

Previous results of a screening of several medicinal plants as HIV-1 inhibitors *in vitro* led us to identify the anti-HIV activity of *L. meyenii* hot water extract. However, its activity was only detected at very high concentrations (>400 μg/mL. Table 1). Thus, we have performed an extraction with solvents of increasing polarity such as *n*-hexane (LM0), absolute ethanol (LM2) and hot water (LM3). Results showed that *n*-hexane extract (LM0) inhibited HIV-1 replication at lower concentrations (35% infection with 50 μg/mL) than the ethanol extract (LM2) (Table 1).

To further explore the anti-HIV activity of maca, *n*-hexane extracts of different colours (black, yellow, purple and mix) were prepared and IC_{50} and CC_{50} values were calculated for each one (Fig. 1 and Table 1). The purple ecotype showed the most potent activity at non-toxic concentrations, with an IC_{50} value of 24.73 μg/mL, while the other ecotypes displayed cell toxicity along with antiviral activity. Although, maca mixture displayed less toxicity than the yellow or black ecotypes, it showed a higher IC_{50} (65.12 μg/mL) than purple ecotype (Table 1), and, therefore, its antiviral activity could be consequence of the presence of purple ecotype in the preparation.

Analysis of the NMR spectra of the *n*-hexane extracts from the three ecotypes revealed a number of differences between them (Fig. 2S). Aromatic signals were observed only in purple maca, at δ_H 8.5–7.5 ppm, indicating the possible presence of aromatic rings such as polyphenolic compounds or alkaloids. Likewise, the signals between δ_H 5.5–5.0 ppm could be assigned to CH-type protons close to carbonyl groups and tertiary amines. Finally, signals between δ_H 4.5–4.0 ppm correspond to $–CH_2$-type protons close to tertiary amines. On the other hand, all ^1H NMR spectra of *n*-hexane extracts showed a pattern of aliphatic compounds (saturated and unsaturated fatty acids) present between δ_H 3.0–0.0 ppm (Zhao, 2012).

The *n*-hexane extract of purple maca (LM0) was selected for fractionation and chemical characterization due to its higher antiviral activity. During this process, antiviral activity of the different fractions was used as a guide for fraction selection, choosingthe most active ones, fractions 4, 7 and 8 (Table 1 and material and methods C, supplementary material), finally obtaining three pure compounds: compound **1:** 6-Phenylimidazo[2,1-b][1,3,4]thiadiazole-2-sulfonamide, compound **2:** *N*-(4-Acetyl-5-methyl-5-phenyl-4,5-dihydro-1,3,4-thiadiazol-2-yl) acetamide and compound **3:** 2-Benzyl-3-thioxohexahydropyrrolo[1,2-c]imidazole-1-one with anti-HIV activity (Fig. 2. Structural data in Supplementary Material, Material and methods, B). These compounds have never been isolated from other natural sources, including *L. meyenii*.

3.2. Antiviral activity evaluation of maca alkaloids

Compounds **1**, **2** and **3** were tested in lymphocytes cell cultures (MT-2-cells and PBMCs) infected with a recombinant HIV (NL4.3-Ren) or in mock infected cells (Viability) (Figs. 3 and 4, Table 2).

All the three compounds were able to inhibit HIV-1 replication, although compound **1** was the most potent one, with an IC_{50} of 2.66 μM in MT-2 cells, 10-fold higher than a well-known nucleotide reverse transcriptase inhibitor (NtRTI), tenofovir (IC_{50} 0.29 μM), without showing toxicity at the concentrations tested. Moreover, when PBMCs were used as targets of HIV infection, compound **1** IC_{50} was 2-fold lower than that of tenofovir (1.21 μM vs 2.12 μM, respectively), showing a higher potency as HIV inhibitor in PBMCs than the widely used NtRTI.

Compounds **2** and **3** showed mixed results, although antiviral activity was confirmed in both, MT-2 cells and PBCMs. Compound **2** was the less potent compound in MT-2 cells infections, with an IC_{50} of around 50 μM, while compound **3** showed an IC_{50} of 20.5 μM. However, compound **2** was more potent in PBMCs infections (IC_{50} 10.21 μM) than compound **3** (IC_{50} 34.45 μM) (Table 2). Finally, compound **2** showed higher toxicity in PBCMs, with a CC_{50} value very close to the IC_{50} (17.31 μM), pointing out that its antiviral activity could be related to cell toxicity.

Structural activity analysis, showed lower activities for compounds **2** and **3**, although compound **2** includes also the thiadiazole moiety present in compound **1** (Fig. 2). However, compound **2** lacks the imidazole

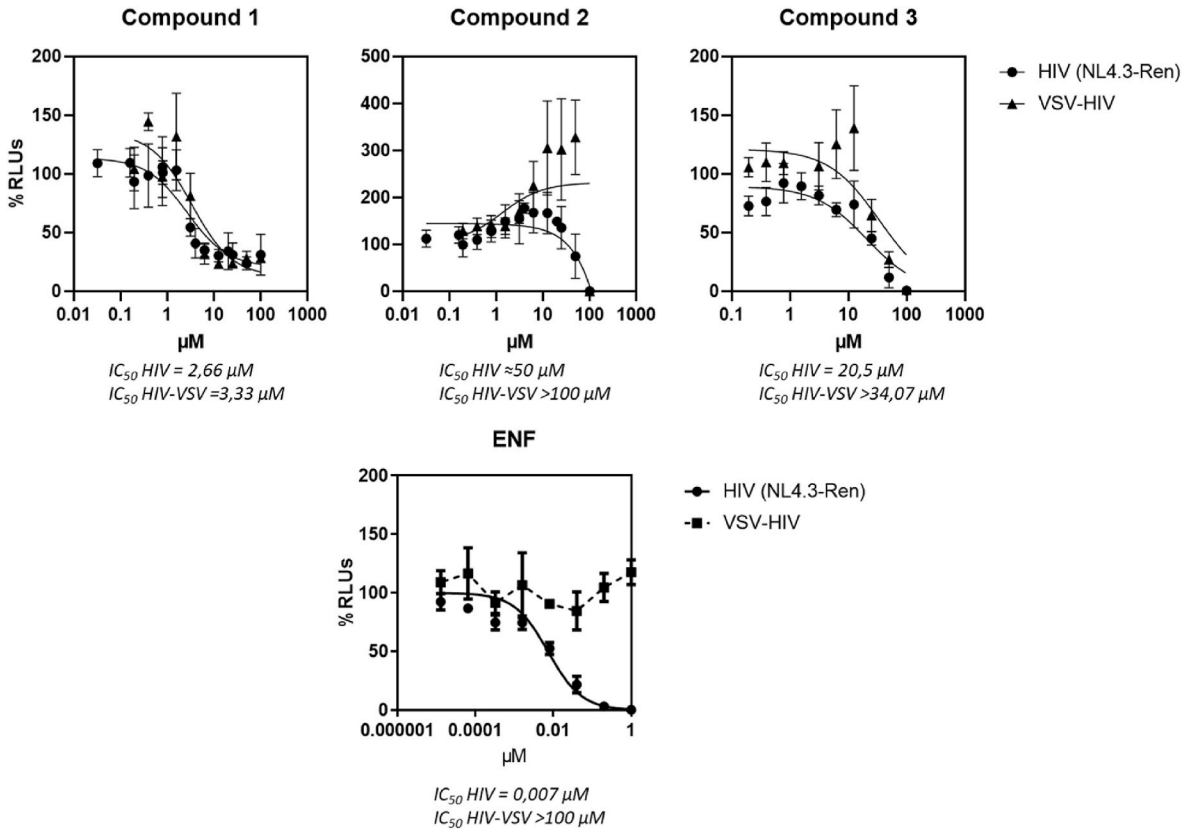

Fig. 5. Evaluation of the effect of *L. meyenii* alkaloids (compounds **1**, **2** and **3**) in viral entry. MT-2 cells were pre-treated with different concentrations of compounds (base 2 serial dilutions in DMSO. Cmax 100 μM) and infected with a recombinant HIV-1 or with a VSV-pseudotyped HIV-1 (VSV–HIV) (Both 100.000 RLUs per well). Renilla or Luciferase activity (RLUs) in cell lysates was measured 48 h later. Enfuvirtide (ENF) was used as viral entry inhibitor control. Results are expressed as a percentage of relative luminescence units (RLUs), where 100% is the level of viral replication (HIV or VSV-HIV) obtained in the presence of the vehicle used to dissolve the compounds. IC_{50} HIV (Inhibitory concentration 50% for HIV-1 infections) and IC_{50} HIV-VSV (Inhibitory concentration 50% for HIV-VSV infections).

heterocycle and the sulphonamide group present in compound **1** which could be important for its activity. Finally, the exocyclic, double bonded sulphur atom in compound **3**, differently to the sulphur heterocycle atom of compound **1**, offers a particularly high density of polar interaction sites and hydrogen bonds, leading theoretically to diverse biological interactions.

3.3. Viral entry

Since the main barrier to cure HIV-1 infection is the establishment of the viral reservoirs, new drugs with anti-HIV activity should act in the previous steps of viral integration to avoid their formation. The first step of the viral replication cycle is viral entry, and thus, we have compared the alkaloids effect on viral infections performed with a wild type HIV-1 and a VSV enveloped HIV-1 (VSV–HIV), that enter the target cells through a CD4, CXCR4 or CCR5 independent mechanism. The inhibition of both infections with similar IC_{50}s strongly suggest an HIV-1 envelope independent effect. As shown in Fig. 5 and Table 2, compound **1** and **3** displayed inhibition curves that almost overlapped, with IC_{50} values very similar between both infections (2.66 μM vs 3.33 μM for compound **1** and 20.5 μM and 34.07 μM for compound **3** in HIV and VSV-HIV infections, respectively) ruling out an entry dependent inhibition. However, compound **2** inhibition of VSV-HIV was not reached even at the higher concentration (100 μM), suggesting that viral entry is, at least, one of its targets in the HIV-1 replication cycle. This effect is independent of the toxicity showed by compound **2** in PBMCs, since it is not toxic at these concentrations in MT-2 cells infections (Table 2).

3.4. Reverse transcription and viral integration

After viral entry, viral RNA must be reverse transcribed to proviral DNA by the reverse transcriptase (RT) encoded by HIV-1 before viral integration can occur. We have tested RT activity infecting MT-2 cells with a wild type HIV-1 (NL4.3) in the presence of two non-toxic concentrations of the isolated compounds (10 and 20 μM) for 5 h. Subsequently, DNA have been extracted and early and late transcripts quantity measured by qPCR.

As shown in Fig. 6A, the effect of the three compounds in early reverse transcription (early RT) is highly variable among compounds and concentrations. The only significant inhibitory activity was observed for compound **1** at 20 μM and compound 2 at 10 μM, though not concentration dependent. While the activity on early transcripts should be more difficult to detect, since it is easier for the viral RT to produce short LTR DNA transcripts, late transcripts theoretically would be inhibited more easily. However, late reverse transcription (Late RT) results, though less variable, shows that none of the compounds was able to reduce the quantity of viral DNA, suggesting that reverse transcription is not the main target of maca compounds.

On the other side, when viral integration DNA was analysed, all the three compounds were able to decrease the quantity of integrated DNA in cell genome below 5% levels as compared to a non-treated control (NL4.3, 100%), with results statistically significant with *p*-values <0.0001, similar to those obtained with raltegravir, a well-known integrase inhibitor. This effect is very interesting, since it is the last viral step before viral DNA integration, and thus, the last chance to block viral reservoirs formation. Besides, the integrase effect of all the three compounds displaying similar chemical structures (thiadiazole ring)

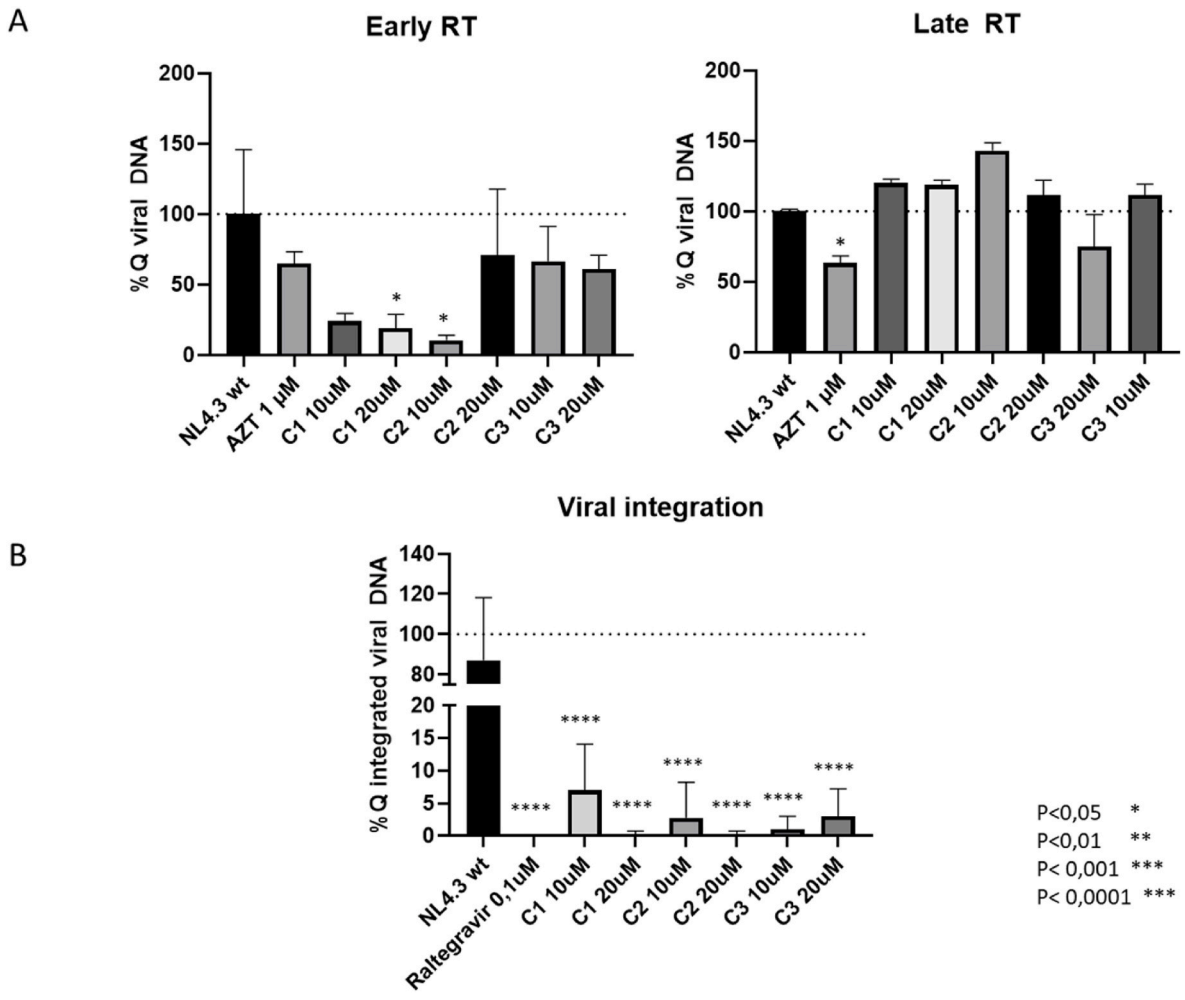

Fig. 6. Evaluation of the *L. meyenii* alkaloids (Compounds **1**, **2** and **3: C1, C2** and **C3**) activity on HIV-1 reverse transcription and viral integration. **A**: Analysis by qPCR of early and late reverse transcripts (RT) in MT-2 cells treated with alkaloids (5, 10, and 20 µM) and infected with NL4.3 wild type HIV-1 (NL4.3 wt). Results are shown as percentage (%) of the quantity (Q) of viral DNA in relation to an infected control (NL4.3 wt), which represents the HIV-1 copies detected in non-treated cultures (100%). 8E5 cells were used as a reference of the number of HIV-1 copies in cell culture. Azidotimidine (AZT 1 µM) was used as reverse transcription inhibitor control. ANOVA analysis was performed to determine the value of p (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

would point out that this moiety would target specifically this viral step in HIV-1 replication.

B: Analysis by qPCR of HIV integration in MT-2 cells treated with *L. meyenii* alkaloids (10, and 20 µM) and infected with NL4.3 wild type HIV-1 (NL4.3 wt). Results are shown as percentage (%) of quantity (Q) of integrated viral DNA in relation to an infected control (NL4.3 wt) which represents the quantity of integrated HIV-1 DNA detected in non-treated cultures (100%). Raltegravir (1 µM) was used as integrase inhibitor control and CCR5 was used as endogenous control. 8E5 cells were used as a reference of the number of HIV-1 copies in cell culture. ANOVA analysis was performed to determine the value of p (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3.5. Viral transcription

To evaluate the hypothesis of a transcriptional dependent effect, IL-2 preactivated PBMCs were transfected with a luciferase plasmid under the control of a whole recombinant HIV-1 genome (HIV transcription. Fig. 7A) in the presence of different concentrations of the three isolated compounds. None of them inhibited viral transcription at the concentrations tested. In fact, compound **1** and **2** showed a very slight tendency to increase viral transcription instead, although results were non-significant. To corroborate these results, a luciferase plasmid under the control of NF-κB, one of the main transcription factors involved in HIV-1 transcription reactivation and latency, were transfected in IL-2 activated PBMCs (Fig. 7B). As expected, results showed a lack of activity for all the three compounds. Compound **2** showed a slight tendency to decrease NF-κB transcription at the highest concentration (20 µM), but this effect was again not significant.

4. Discussion

Natural products are one of the most important sources of drugs with new targets. In fact, the activity of natural products is usually related to activity through several targets, that it is only detected by network pharmacology (Xie, 2021). In this sense, some species with nutritional value have previously shown activity on viral infections (Nurtay, 2021). Among them, maca (*Lepidium meyenii* Walp.) have been used for centuries as a nutrient supplement, as a stimulant and as immunomodulatory (Chang, 2014), but its exact mechanism of action is not fully described. As we have mentioned before, there are some reports about its activity through NF-κB inhibition and, since this transcription factor is strongly involved in HIV-1 transcription, we have hypothesized a potential HIV-1 inhibitory activity.

Therefore, we have studied the anti-HIV activity of maca extracts and we found a slight activity for one ecotype, the purple maca (Fig. 1). NMR spectra of purple maca *n*-hexane extracts showed the presence of aromatic signals not present in the other ecotypes. In fact, we found here

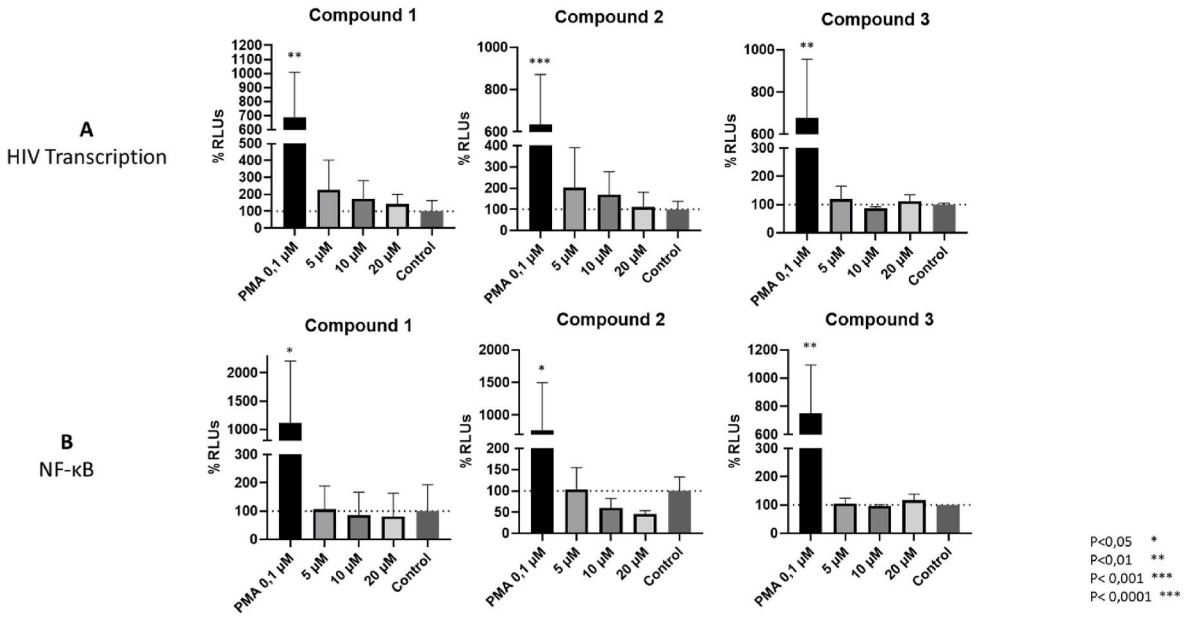

Fig. 7. Effect of L. *meyenii* alkaloids (compounds **1**, **2** and **3**) on HIV-1 transcription and NF-κB transcription factor. IL-2 preactivated PBMCs were transfected with plasmids encoding a luciferase reporter gene under the control of HIV-1 (HIV transcription) or NF-κB transcription factor (NF-κB). Afterwards cells were treated with compounds 1, 2 or 3 at three different concentrations (5, 10 and 20 μM) or PMA at 0.1 μM and cultured at 37 °C during 48 h. 100% represents the background level of expression without treatment. ANOVA analysis was performed to determine the value of p (*p < 0.05, **p < 0.01, ***p < 0.001).

that *n*-hexane extracts of purple maca were able to inhibit HIV-1 infection with an IC_{50} of 24.73 μg/mL at non-toxic concentrations (Fig. 1 and Table 1), and thus, we further explore this activity.

First, we have tried to identify the compounds responsible of the antiviral activity. Using a chemical-bioassay guided fractionation three compounds were isolated from the *n*-hexane extract of purple maca with anti-HIV activity. The three compounds showed antiviral activity in the low to middle micromolar range, with compound **1** as the most potent inhibitor, with an IC_{50} in cell lines (MT-2 cells) of 2.66 μM and 1.21 μM in primary lymphocytes (PBMCs) with no toxicity at the higher concentration tested (100 μM). These IC_{50} values are close to the ones displayed by a nucleotide reverse transcriptase inhibitor (NtRTI), tenofovir, widely used in the clinical setting, with IC_{50}s of 0.29 μM in MT-2 cells and 2.12 μM in PBMCs. Intriguingly, compound **2** was less potent in MT-2 cells (IC_{50} of around 50 μM) but more potent in PBMCs (10.21 μM) while compound **3** showed an inverse pattern (IC_{50} MT-2 20.5 μM and IC_{50} PBMCs 34.45 μM. Table 2). These differences between MT-2 cells and primary lymphocytes could be due to the different pathways involved in cellular activation, since PBMCs were only activated with IL-2 to maintain cell survival, while MT-2 cells are a cell line immortalised with HTLV-1 showing some characteristics of $CD4^+$ T-regs cells (Hamano, 2015). However, the consistent and more potent effect of compound **1** in HIV-1 infection in both, MT-2 and PBMCs, make this compound the most interesting one as HIV-1 inhibitor.

To further characterise the antiviral effect of these compounds, we have studied their mechanism of action. First, viral entry was tested by infecting cells with an HIV-1 pseudotyped with the VSV envelope. It is expected that the inhibition of this VSV-HIV chimera would rule out an HIV-1 enveloped dependent effect. Compound **1** and **3** results showed an antiviral effect independent of the viral entry, while compound **2** showed a strong HIV envelope dependent effect. The chemical structure of compound **2** is somewhat diverse, since it lacks the bicycle present in compounds **1** of **3**, suggesting that a thiadiazole monocycle could be a requisite for the activity on viral entry.

We could not find activity for any of the isolated compounds in reverse transcription. On the other hand, viral integration was strongly inhibited by the three compounds highlighting integration as the main target of the three compounds. However, the effect of compound **2** in viral integration could be a result of viral entry inhibition, and, thus, its target would not be clearly defined. However, total viral DNA levels (Late RT) were not decreased in MT-2 cells treated with compound **2**, suggesting that a viral escape occurred in the viral entry inhibition, probably due to the low concentrations tested. In this case, we could assume that compound **2** was able to inhibit viral integration at lower concentrations than viral entry, pointing to a multi-target behaviour.

These results suggest that the thiadiazole scaffold could be used as a pharmacophore targeting viral integration, blocking the viral replication and the formation of new viral reservoirs. Known HIV-1 integrase inhibitors as raltegravir, dolutegravir, elvitegravir or cabotegravir have chemical structures that includes a diketo acid and a halogenated benzyl moieties as structural requisites for activity (Smith, 2021). The diketoacid moiety acts as a pharmacophore with electronegative atoms positioned to engage the Mg^{2+} cofactors in the integrase active site, while the halogenated benzyl moiety allows π–π interactions with the Y143 side chain in the penultimate cytosine at the 3′ end of viral DNA. Maca compounds lack of these moieties, but raltegravir and cabotegravir comprise also a methyl oxadiazole and a methyl oxaimidazole groups that could resemble the thiadiazole moiety present in maca compounds, highlighting the importance of these moieties in the integrase activity. Besides, maca compounds could be reflective to common integrase mutations that confers resistance to integrase inhibitors, as Y143, involved in the methyl-oxadiazol π–π interactions or Q148 and N155, involved in drug to integrase binding (Jóźwik, 2020), since maca compounds do not display the chemical moieties involved in the development of these resistances.

Finally, we have tested the activity of the three compounds in viral transcription in IL-2 preactivated PBMCs (Fig. 7), but we could not find any activity in all the three compounds. Results with NF-κB confirm this lack of activity, ruling out an activity related to a potential anti-inflammatory or immunosuppressive effects, as stated before.

In summary, *Leppidium meyenii* (purple maca) *n*-hexane extracts inhibit HIV-1 replication. Three compounds have been isolated displaying this activity, and all the three are thiadiazole derivatives. These compounds show IC_{50} values in the low to middle micromolar range, acting through viral entry and, mainly, viral integration inhibition. Our results with HIV-1 and NF-κB transcription factor in human

lymphocytes, rule out the involvement of a potential anti-inflammatory or immunosuppressive effect in their mechanism of action. Therefore, in this paper we describe the discovery of new compounds from *L. meyenii* with a thiadiazole scaffold structure that could lead to new integrase inhibitors non-susceptible to current resistances. Further research is needed to scale up these isolated compounds to the clinical setting, including *in vivo* experiments and activity testing in infections with integrase inhibitors resistant HIV strains. Nevertheless, this work highlights the importance of the natural kingdom as a source of new antiviral drugs with low toxicity and novel mechanisms of action. This is particularly important in HIV-1 infection, since the current antiretroviral therapy cannot eliminate the infection, leading to lifelong treatments and a high risk of drug failure.

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CRediT authorship contribution statement

Luis Apaza-Ticona: Writing – review & editing, Writing – original draft, Methodology, Investigation, Data curation. **Manuela Beltrán:** Validation, Methodology, Investigation. **Elisa Moraga:** Validation, Investigation. **David Cossio:** Methodology, Investigation. **Paulina Bermejo:** Supervision, Project administration, Investigation, Funding acquisition, Conceptualization. **José A. Guerra:** Writing – review & editing, Visualization, Software, Methodology, Formal analysis, Data curation. **José Alcamí:** Resources, Project administration, Investigation, Funding acquisition, Conceptualization. **Luis M. Bedoya:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:L. A.T., J.A.G., J.A. P.B and L.M.B. participate as inventors in a patent application involving the antiviral activity of maca and thiadiazole compounds.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2024.118613.

Glossary

ART	Antiretroviral Therapy
ETV	Etravirine
HIV-1	Human Immunodeficiency Virus Type 1
L. meyenii	*Lepidium meyenii* Walp.
NMR	Nuclear Magnetic Resonance
NtRTI	Nucleotide Reverse Transcriptase Inhibitor
PBMCs	Peripheral Blood Mononuclear Cells
PMA	Phorbol 12-Myristate 13-Acetate
RAL	Raltegravir
EFV	Efavirenz
RLUs	Relative Luminescence Units
RT	Reverse Transcriptase
T-20	Enfuvirtide
TFV	Tenofovir
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
VSV	Vesicular Stomatitis Virus

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