Reactivation of latent HIV-1 *in vitro* using an alcoholic extract from *Euphorbia umbellata* (Euphorbiaceae) latex.

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Abstract

Euphorbia umbellata (E. umbellata) belongs to Euphorbiaceae family popularly known in Brazil as Janauba and contains in its latex a combination of phorbol esters with a strong affinity to different PKC isoforms. In this work we could show the presence of 4 deoxi-phorbol esters able to reactivate HIV latency through nuclear interiorization of NFKb and activation of HIV LTR promoter. Our E. umbellata latex could activate the latent HIV in human PBMCs isolated from HIV+ patients under HAART therapy showing virological suppression. The same way our extract could reactivate latent SIV in non-human primate primary CD4 lymphocytes in in vitro assays. These results strongly indicate that the phorbol esters present in S grantii latex are good candidate compounds for future clinical trials for HIV eradication.

INTRODUCTION

The Acquired Immunodeficiency Syndrome (AIDS) is caused by the Human Immunodeficiency Virus (HIV), which was identified in 1983(1,2) from patients with AIDS and at risk for AIDS. In those 34 years of the pandemic, more than 20 million people died from AIDS-related causes, and by 2011 more than 34 million people were infected with HIV (UNAIDS, 2017 see in http://www.unaids.org/sites/default/files/media_asset/20170720_Data_book_2017_en.pdf).

The pandemic of HIV infection is an important socioeconomic burden and is considered one of the largest documented epidemics in history. The pathogenesis of HIV infection involves infection and replication in CD4 + T lymphocytes, macrophages and dendritic cells. Replication and destruction of CD4 + T cells, which are key effector cells in the host immune response, leads to a clinical picture of immunosuppression known as AIDS(3).

The current strategy used in the treatment of HIV infection is called Highly Active Antiretroviral Therapy (HAART), which is based on a combination of antiretroviral drugs, which target different stages of the HIV replicative cycle. The primary goal of HAART is to block viral replication and slow down the progression of immunodeficiency, restoring, immunity, to improve the quality of life of the infected person (8). Although HART allow these HIV+ individuals to live relatively normal lives, these patients continue to suffer from immunological, neurological, and metabolic co-morbidities associated with HIV infection (9).

Even when an undetectable viral load is achieved with effective HAART therapy, it is possible to detect latent viral reservoirs where HIV proviral DNA remains at rest on memory CD4 + T cells and are responsible for some degree of chronic activation of the immune system (10). These cells containing latent viral genomes become viral reservoirs inaccessible to HARTthat can only act on actively replicating viruses. Once HAART is stopped, viral load increases rapidly, and systemic infection reestablishes (11). Therefore, the complete eradication of HIV from an infected individual is one of the most challenging areas of HIV research today.

New latent virus reactivating compounds also called latency reactivating agents (LRA) when utilized in conjunction with HAART may be capable to eliminate transcriptionally inactive viruses, ultimately resulting in the eradication of HIV infection. This strategy is called *shock and kill* and it postulates that memory CD4 + lymphocytes treated with LRA along with HAART could purge totally or partially the viral reservoir while blocking replication of emergent latent virus, leading to a HIV eradication or at least a functional cure (12).

Different molecules with therapeutic potential have been reported as possible candidates for elimination of viral reservoirs. Among these agents we highlight the action of so-called Protein Kinase C (PKC) agonists (10). This class of molecules, we can mention Prostratin (13), Bryostatin (14), Ingenol (15) and Phorbol 12-Myristate 13-Acetate (PMA;16). These compounds can reactivate latent HIV in lymphoid and myeloid cells and also reactivate CD4 and CD8 lymphocytes. Although several transcriptional regulatory mechanisms for HIV latency have been described in the context of epigenetic silencing and "repression", recent work suggests that reactivation of latent HIV is positively

correlated with cellular and NF-κB activation, demonstrating the importance of this factor in latency reactivation (17).

Many natural compounds have been investigated for their antiviral and latency-reversing properties, with some even being indicated as candidates for clinical trials. Among these compounds, many are derived from the secondary metabolism of plants, such as terpenoids (18), polyphenols (19), alkaloids (20) and phorbol esters (21). Among these plants, the Euphorbiaceae family is found worldwide spread and has been used by traditional healers for the treatment of several diseases(22-26). *Euphorbia umbellate* belongs to Euphorbiaceae family and it is popularly known in Brazil as Janauba or *leiterinha* and is used, in traditional medicine, as a latex preparation (diluted in water) for the treatment of gastric disorders, such as peptic ulcer and gastritis, as well as neoplastic diseases, allergy, diabetes, influenza, and leprosy among other diseases (27). Several papers have reported the isolation of lectins, phorbols, terpenes, flavonoids and other phenolic compounds from *E. umbellata* latex (28, 29, 30).This plant contains phorbol esters in its latex similar to PMA isolated from the seeds of *Croton tiglium*, another representative of the Euphorbiaceae family. These compounds are efficient in inhibiting the cytopathic effect generated by HIV infection on lymphocyte cells, as well as activating protein kinase C (PKC), which together with nuclear translocation of NF-κB are important for the reactivation of HIV latency (31).

In this work we evaluated the ability of an alcoholic extract obtained from Janauba latex to reactivate latent HIV and investigated the molecular mechanisms involved in this process and its direct action on *ex vivo* human PBMC HIV- cell cultures as well CD4 Lymphocytes isolated from SIV infected non-human-primates.

MATERIAL AND METHODS

from *Euphorbia umbellata*. *Euphorbia umbellata* (Pax) Bruyns, the current accepted name for *Synadenium grantii* Hook.f., was collected and identified with the respective voucher specimen kept at the RFA Herbarium under the number Trovó, M.L.O 764. The crude latex was collected directly from incision mechanically made in the trunk of cultivated specimens in the Barretos, São Paulo state, Brazil. The latex was straightly diluted in 80% ethanol (1:10 v/v) to produce a white rubber-like precipitate that was filtered off by gravity in filter paper. The filtrate was dried in a rotary evaporator to afford a fine white powder, which was diluted in DMSO to attain the fix concentration of 20 mg/mL to provide the stock for cell testing. This extract material was labeled as **JALEx** (Janaúba Alcoholic Latex Extract). **JALEx** was chromatographed in a silica gel column using a solvent gradient (0-5%) of ethyl ether in chloroform to enrich the phorbol-containing fractions and favor their detection and characterization in the same GC conditions of **JALEx** analysis.

Chemical profile of JALEx. **JALEx** was analyzed by gas chromatography coupled to mass spectrometry (GC-MS) by using an Agilent 6890N chromatograph (Palo Alto, CA) equipped with a capillary column DB-H17HT (30 m × 0.25 mm × 0.15 µm film thickness). Helium was the carrier gas at 1.0 mL/min and inlet pressure of 3.14 psi, 10:1 split/splitless ratio. The oven temperature programmed from 150˚C to 340˚C at 4˚C/min and then held for 17 minutes. The injection volume was 1 µL of a 2 mg/mL chloroform solution. Data were processed using MSD Productivity Chem Station Software operating with ion source at 250˚C and electron impact ionization at 70 eV. Individual components were characterized by their fragmentation patterns in the mass spectra.

Analysis of the reactivation potential of latent HIV after treatment with JALEx. Human **l**ymphocyte cell line models for the latency study (J-Lat 8.4 and 10.6) were used. These cells are derived from Jurkat cells, which have integrated into their genome, the modified HIV genome, containing an open reading frame for encoding the GFP protein substituting the viral nef gene. These cells, J-Lat 8.4 and 10.6, have different levels of reactivation capacity, depending on the site of integration of the HIV genome. In this way, J-Lat 10.6 is a more responsive cell than J-Lat 8.4, always showing reactivation levels higher than those observed by strain 8.4. J-Lat cells are cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin / streptomycin antibiotics. J-Lat cells at density of 10^5 cells in wells of the 24-well plate were treated with different concentrations of **JALEx** dissolved in DMSO over a 24-hour period. After this interval, cells were analyzed for flow cytometric reading (BD Accuri C6) for GFP expression. Positive controls for reactivation of

latent virus were either ingenolB (0.32 µM), or PMA(1 µM). This assay was performed in three independent experiments.

Analysis of the cytotoxicity of natural extracts by viability dye. Evaluation of the cytotoxicity of the compounds was performed by incubating J-Lat cells (clones 8.4 and 10.6) with the dilutions of the compounds previously tested in the reactivation assays. The cell viability assessment was performed on 96-well plates in three independent experiments, each in six-fold, with each well containing 10^4 cells. After 5 days of culture, vital resazurin dye (CellTiter Blue, Promega) was added which, when metabolized by cells that were alive, would become another byproduct, resorufin, of pink color that emits fluorescence which is therefore measured in Fluorimeter (Glomax).

Analysis of the activation of PKCs. To assess the mechanism of action of HIV latency reactive by Janauba compounds, J-Lats cells at the density of 10^5 cells per well of the 24-well plate were treated with different specific inhibitors of PKCα, PKCδ and PKCγ isoforms Go6976, Go6983, Ro-31-8220, respectively for 24 hours (32-34) . After this time, cells were treated with the positive control of PKC activation (1µMPMA) and two concentrations of **JALEx** previously determined in the reactivation and cytotoxicity assays. Untreated cells were referred to as MOCK. The following concentrations of the alcoholic extract were tested: 0.01 µg / mL and 0.001 µg / mL and the percentage of GFP positive cells was assessed by flow cytometry, from the reactivation of HIV integrated into the J-Lats genome.

In addition, the PKC activation was evaluated by immunofluorescences to detect cell relocation of different PKC isoforms. For this, HeLa cells were plated at the density of 10^4 cells per well of 96-well black bottomed glass bottom plates. These cells were transfected with three different plasmids encoding three PKC isoforms fused to GFP (PKCα-GFP, PKCδ-GFP and PKCγ-GFP) for 24 hours and then treated with 1 µg / mL and 0.1 µg G / mL of the **JALEx**, for 10 minutes or 24 hours. Cells were visualized in a fluorescence confocal microscope (Molecular Devices, Inc) and the redistribution of PKC isoforms was evaluated.

Phosphorylation of some PKC isoforms was also investigated in order to better analyze its activation pathway. For this, Jurkat cells were treated with 0.01 µg / mL and 0.001 µg / mL of chloroform extract and with 1 µM PMA as a positive control of PKC phosphorylation for various time intervals (10, 30 minutes and 1, 6 and 24 hours). The cells were then removed from the plates and lysed in lysis buffer (50 mM Tris (pH 7.6–8), 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4, 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 and containing protease inhibitors cocktail(Sigma L2884) for 1h. Soluble proteins were quantified by the Bradford method (Sigma) and 30 µg total proteins of each condition was evaluated on 10% polyacrylamide gels by SDS-PAGE electrophoresis and

transferred onto a Hybond-C-supported nitrocellulose membrane (Amersham Biosciences, Little Chalfont, UK) using a mini Trans-Blot® Turbo Transfer System (BioRad) at 100 v for 30 min. After transfer of the proteins to nitrocellulose membrane, it was incubated with different primary antibodies: anti-PKC phosphorylated PKC (pan, δ, θ) (Cell Signaling) at the 1: 500 dilution and β-tubulin (Cell Signaling), atthe dilution of 1: 2000, the latter used as the gel loading control. Blot detection was achieved by chemiluminescence (ECL Western Blotting Detection Reagents, RPN2109, GE Healthcare), and acquired by ImageQuant™ LAS 4000 mini (GE Healthcare). The quantification was done using band densitometry analysis, performed with the Image J software (version 1.41; National Institutes of Health).

Analysis of nuclear translocation of NF-κB and dependence of NF-κB for reactivation of latent HIV. In order to verify whether **JALEx** induces the internalization of NF-κB, HeLa cells were plated at the density of $2x10^4$ cells per well in 96-well plates with black borders. Cells were treated with 1 µg / mL and 0.1 µg / mL of **JALEx** for 6 or 24 hours. As a positive control of internalization, 1 µM PMA was used and untreated cells were called MOCK. After this time, the immunofluorescence protocol for anti-p65 labeling, which detects the endogenous subunit of NF-κB in red, and labeling of the cell nucleus with DAPI, visualized in blue, was started. The photos were acquired using fluorescence confocal microscopy (Molecular Devices, Inc).

In order to evaluate the role of NF-κB in reactivation of latent HIV, Jurkat cells (10^6 cells) were transfected with two different plasmids: pNL4.3-LUC (NFkB WT), provided by Dr. Renato S. de Aguiar (UFRJ) which is derived from pNL4.3-Luc-ER- (AIDS Reagent Program) and had the env stop codon reverted to its original sequence; And the plasmid p-LTR-MUT (NFkB MUT) which was generated by site-directed mutagenesis by altering the two NF-κB binding sites, rendering that plasmid nonfunctional for NF-κB binding. Both constructs show the luciferase sequence under the control of the HIV viral promoter. Jurkat cells (10^6 cells) were transiently transfected with 8µg of each plasmid pNL4.3-LUC (NFkB WT) or p-LTR-MUT (NFkB MUT) with the aid of the Neon Transfection System kit (Thermo Fisher Scientific) According to the manufacturer's recommendations. After transfections, the cells were equally divided into two groups: treated or untreated with 1µg / mL of **JALEx**, for 24 hours. After this interval, cells were lysed in luciferase lysis buffer (Promega) and luciferase activity was assessed in a luminometer (Glomax, Promega).

Analysis of surface marker expression and cytokine production in primary cells treated with JALEx. In order to expand the results obtained in HIV latency model cells, we performed assays with **JALEx** in Peripheral blood mononuclear cells (PBMCs) from 4 healthy donors. After separation of buffy

coat by means of Ficoll centrifugation, CD4 + T cells were isolated using the Dynabeads CD4 Positive Isolation Kit (Thermo Fisher) kit, according to the manufacturer's recommendations. For isolation of 10^7 PBMCs from each of the 4 donors, for each of the conditions to be tested. After this step, the TCD4 + cells were treated with 1 µM PMA, 10 µg / mL (10^{-3}) or 1 µg / mL (10^{-4}) of Janauba **JALEx**, in addition to MOCK (untreated cells), for24 hours. After this period, the expression of some cell surface molecules was evaluated, such as expression of the CD4 receptor and CCR5 and CXCR4 co-receptors, as well as the expression of activation markers such as CD25, CD38, HLA-DR and CD69 by means of specificantibody labeling and flow cytometric analysis (BD Accuri C6). Comparatively, this assay was also performed on cells of MT4 lymphocyte line, maintaining the same number of cells treated, with the same concentrations of **JALEx**, in the same time interval.

All the cell supernatant from the TDC4 + cells was collected for analysis of the cytokine production by the cells treated with **JALEx**. This analysis was performed by means of a colorimetric multiplex immunoassay Bio-Plex Pro Human Cytokine 17-plex Assay (Bio-Rad) containing fluorescent microspheres conjugated to different monoclonal antibodies specific for each target cytokine. The following cytokines were measured: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL- IL-17, G-CSF, GM-CSF, MCP-1 / MCAF, MIP-1β, IFNγ and TNF-α.

QVOA assay using SIVmac239 in NHP CD4+ Lymphocytes. To compare the ability of the compounds to stimulate virus replication in latently infected cells we used a variation of the quantitative viral outgrowth assay (QVOA) described by Laird et al. (35). Cells for the QVOA were isolated from viremic SIVmac239-infected cynomolgus macques at the Wisconsin National Primate Research Center (WNPRC). PBMC isolated by density gradient centrifugation were enriched for CD4+ T cells using the CD4 T cell enrichment kit for non-human primates (Miltenyi Biotec, San Diego, CA). After enrichment cells were plated at $1x10^6$ cells per well. These cells were co-cultured with “stimulator” CEMx174 cells that were irradiated with 10,500 rads prior to co-culture. The ratio of stimulator cells to CD4+ T cells was 2:1. Purified CD4+ T cells were stimulated with JALEx, concanavalin A, ingenol, or no mitogen overnight. After stimulation cells were washed and re-plated in serial dilutions in triplicate. At this point additional, non-irradiated CEMx174 cells were added to each well to serve as targets for induced virus to infect. The target cells were added at a 2:1 ratio to the CD4+ T cells. Co-cultures were maintained for 1 week after addition of target cells. On the last day supernatant was collected and RNA was isolated to test for the presence of SIV. RNA was isolated using the Viral Total Nucleic Acid kit for the Maxwell 16 MDx instrument (Promega, Madison, WI). Viral RNA was quantified using a qRT-PCR assay targeting the gag gene as described previously (36) except that analysis was performed using the Lightcycler 480 rather than the Light Cycler 2.0.

Analysis of JALExeffect on both viability and cytokine production by T cells from AIDS patients. In order to analyze the ability of Janauba (JALEx) in regulating different T cell phenotypes in the context of HIV-1 infection, peripheral blood of 15 adult HAART-treated Aids patients (8 women and 9 men), with plasma viral load of 953 ± 1.924 copies of RNA/mL and $CD4^+$ (845 ± 345/mm^3) and $CD8^+$ (845 ± 345/mm^3) higher than 350 cells/mm^3,were collected and PBMC were obtained from Ficoll-Paque gradient. Viable PBMC were quantified by trypan blue exclusion and suspended in RPMI-1640 medium supplemented with 2 µM of L-glutamine (GIBCO, Carlsbad, CA, USA), 10% of fetal calf serum, 20 U/mL of penicillin, 20 µg/mL of streptomycin and 20 mM of HEPES buffer. Approximately 1 x 10^6 PBMC/mL were cultured for 24hours in 24-well plates with 1mL of complete medium in the presence or absence of different concentration of **JALEx** (1, 0.1 and 0.01 µg/mL). As positive control, PBMC cultures were stimulated with phorbol-12-myristate-13-acetate (PMA, 20 ng/mL) plus ionomycin (600 ng/mL). To optimize intracellular cytokine staining, brefeldin A (10 µg/mL) was added to the cultures in the last 4 hours of incubation time. For the fluorescence labeling, mouse anti-human fluorescent monoclonal antibodies (mAbs) directed against CD4-FITC, CD8-FITC were purchased from BD Bioscience (San Diego, CA, USA). Briefly, anti-CD4 and anti-CD8 mAbs were added to the PBMC (2 X 10^5/tube) and incubated for 30 min at room temperature in the dark. The cells were washed with PBS and then permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) at 4 °C for 20 min. After washing, the antibodies for intracellular staining (anti- IL-17-PECy7, anti-IFN-γ-PE-Cy5, anti-IL-10-PE-Cy7, anti-IL-21-PE), or the corresponding anti-IgG1 isotype control, were added in various combinations and incubated for 30 min at 4 °C. The cells were analyzed in the Attune ® (Thermo Fischer, Co) and FlowJo software. Isotype control antibodies and single-stained samples were periodically used to check the settings and gates on the flow cytometer. After acquisition of 100,000 to 200,000 events, lymphocytes were gated based on forward and side scatter properties after the exclusion of dead cells, by using propidium iodide, and doublets. The analysis of CD4+ and CD8+ T cells viability was determined using 7-aminoactinomycin D (7-AAD) (R&D systems). Further, the gated cells were negative for CD14 marker. The CD4 + T lymphocyte count (CD4) was performed on a flow cytometer (FACS Count, Becton Dickson®). Plasma HIV RNA quantification was done using the RT-PCR with Abbott M2000 Sq HIV VL technology

Statistical analysis. The statistical analysis was performed using Prism 5.0 software (GraphPad Software). The Student's t test was applied to compare the proportion of different AIDS-derived cytokine-secreting T cells in cell cultures maintained in the presence or absence of Janauba. The significance in all experiments was defined as $p<0.05$.

RESULTS

The **JALEx** soluble in water and ethanol was characterized mainly by a mixture of triterpenes and phorbol ester diterpenes. The GC profile and MS data indicated that the first group was predominant throughout the extract, with two main constituents standing out. (Figure 1). The triterpenes profile is in accordance with data from the literature that report the isolation of euphol, lanosterol, friedelin and 3β-friedelinol in the latex, leaves and stem of *E. umbellata* (37), whilst studies with the latex have led to the characterization of euphol and the steroid citrostadienol (38). A phorbol esters-rich fraction was obtained by chromatographing the crude **JALEx** in a silica gel open column using mixtures of chloroform-ether as eluent (Figure 1B). The class of phorbol ester has been represented by the 12-O-tigloyl-4-deoxyphorbol-13-isobutirate (39), 12-deoxyphorbol-13-(2-methylpropionate), phorbol 12,13,20-triacetate (40), 4-deoxyphorbol-12,13-ditiglate and 3,4,12,13-tetraacetylphorbol-20-phenylacetate (41) distributed in several plant tissues (Supplemental Figure 1).

Analysis of latent reactivation potential of latent cytotoxicity and extracts produced from JALEx.

In this work, we used the alcoholic extracts from **JALEx** resuspended in DMSO for carrying out specific cell based assays. The reactivation of latent HIV present in J-Lat cells, 8.4 and 10.6,from lymphocytic lineage latency models was performed with various concentrations of **JALEx**, ranging from 10 to 0.0000001 µg / mL. In parallel to the reactivation assay, the cytotoxicity assay was performed to evaluate whether the concentrations of **JALEx** would be toxic to these cell lines. Figure 2 presents the results for reactivation and cytotoxicity assays. Figure 2A shows the positive reactivation controls (PMA 1 µM and ING-B 0.32 µM) for the J-Lat line 8.4, showing a percentage of about 12% of green cells, positive for reactivation of latent HIV. Concentrations of 10 to 0.01 µg / mL of **JALEx** showed activation percentage similar to positive controls, dropping to less than 5% reactivation at dilutions of 0.001 to 0.0000001 µg / mL. In relation to the cytotoxicity of this compound in these cells (Figure 1B, in annex), we observed that the highest concentration of the tested extract, 10 µg / mL was toxic for this cell line, while the other concentrations were well tolerated, showing a good cell viability . Figure 2 B shows that for the J-Lat line 10.6, reactivation levels were more expressive when compared to line 8.4, as expected, with positive controls and extract dilutions of 10 to 0.01 µg / mL presented about 75% green cells, while at the lowest dilutions of 0.000001 to 0.0000001 µg / mL the tested extract we observed decay for up to 10% green positive cells. In relation to the cytotoxicity of **JALEx** for J-Lat 10.6, we also detected that the concentration of 10 µg / mL was toxic for this cell line,

whereas the other concentrations presented cells viability between 80% and 75%.

Analysis the action of PKC inhibitors in HIV reactivation by JALEx.

In order to evaluate whether **JALEx** is able to activate PKC pathway and identify whether this pathway is responsible for latent HIV transcriptional activation, J-Lat 8.4 and 10.6 cells were treated for 24h with different PKC inhibitors (Go 6666, Go 6983 and Ro-31-8220). After this period, the cells were incubated with PKC activation positive control(PMA 1 µM), in addition to the concentrations of **JALEx** of 0.01 and 0.001 µg/mL, which previously were not toxic inthe cytotoxicity assay and were able to reactivate latent HIV. The readout for this assay was flow cytometry for quantification of the percentage of GFP positive cells. In this assay we used PMA as a positive control. PMA is Phorbol esters and can replace diacylglycerol (DAG) in the activation of PKCs and unlike DAG, as phorbol esters are not rapidly metabolized, treatment of cells with these compounds results in a prolonged activation of PKCs (16). Figure 3 shows a similar pattern of latent HIV activation after PMA and **JALEx** treatment in both J-Lat lines when PKCs inhibitors wereadded to culture. With the pretreatment of the cells with the PKC inhibitors, we observed very low reactivation ratesfor J-Lat 8.4 cells which were incubated with the inhibitors G6666, G6983 and Ro-31-8220, respectively, and were then treated with**JALEx** at a concentration of 0.01 µg / mL. The percentage of reactivation of the J-Lat 8.4 cells incubated with Gö6976, Gö 6983 and Ro-31-8220 was 0.03, 0.02 and 0.06%, respectively, when later incubated with **JALEx** at a concentration of 0.001 µg/mL. The percent of reactivation of J-lat 10.6 after treatment with the inhibitors Gö6976, Gö6983 and Ro-31-8220 was 6.76; 1.41 and 11.89%, respectively, followed by treatment with **JALEx** at a concentration of 0.001 µg / mL when compared to cultures without PKC inhibitors.

Analysis of PKC isoforms activated by JALEx.

The PKC family is involved in control of several functions of cellular proteins by phosphorylation of hydroxyl groups in serine / threonine residues of these proteins. Moreover, the catalytic domain present in the structure of PKCs is essential for its autophosphorylation and, consequently, its cellular activation and relocation. PKCs are part of a large family of proteins that comprise different isoforms spanning different regulatory and catalytic domains. The isoforms are divided into three subgroups: the conventional isoforms α, βI, βII and γ; The new ones encompassing the δ, ε, η and θ isoforms and the atypical isoforms ι and λ isoforms. This division is based on the differences found in its structures and pathways of activation, which may or may not be Ca -dependent.

For these reasons, we investigated whether phosphorylation of different PKC isoforms was occurring in response to treatment of Jurkat cells with **JALEx**. In these experiments two concentrations of **JALEx** (0.01 µg / mL and 0.001 µg /

mL) was used and PMA was utilized as positive control of PKC phosphorylation. Cells were collected at different time intervals (10, 30 minutes and 1, 6 and 24 hours). Figure 4A shows western blottings for analysis of the phosphorylation pattern in PKCs of conventional and novel isoforms. We observed that the phosphorylation of conventional PKCs, identified by anti-pan-PKC-phosphorylated antibody labeling, occurs quite rapidly, after 10 minutes of treatment with JALEx, in the two concentrations tested. This activation is long lasting, remaining phosphorylated for up to 24 hours after the treatments. The same pattern was observed at the same time intervals for cells treated with PMA. For the novel PKC isoforms, PKC θ exhibited a rapid phosphorylation pattern that did not sustain over time. PKC δ showed an early phosphorylation pattern maintained for up to 24 h after treatment with **JALEx**, which in this time interval at the concentration of 0.001 µg / mL exhibited more pronounced phosphorylation. Figure 4B shows fold change in relation to the negative control (DMSO) the proportion of times we had the phosphorylation / degradation of PKC isoforms. Values above one show that there was phosphorylation of a certain PKC isoform, and values below one, the non-activation of a given PKC isoform, for each treatment, and interval. We observed that after 1 h of treatment with both **JALEx** and PMA concentrations, conventional isoforms exhibited their maximum phosphorylation. Whereas for new isoform PKC θ exhibited in 10 minutes at the concentration of 0.001 ug/mL of JALExfor maximum phosphorylation, whereas for PKC δ maximum phosphorylation was visualized after 24 h of treatment with 0.001 µg/mL of **JALEx**.

Because of its phosphorylation and activation, PKCs are translocated to the plasma membrane by the action of RACK proteins (41). To verify if **JALEx** would be able to promote the translocation of different PKC isoforms to the plasma membrane, HeLa cells were transfected with plasmids expressing three PKC isoforms fused to GFP (PKCα-GFP, PKCδ-GFP and PKCγ -GFP). After transfection, the cells were treated with 1 and 0.1 ug/mL of **JALEx** for 24 h, concentrations that had previously been tested for reactivation and cytotoxicity for 10 minutes or 24 hours. Figure 5 shows fluorescence confocal microscopy images showing a diffuse pattern of PKC isoform distribution in untreated cell cytoplasm (MOCK). The images of Figure 5 A refer to 10 minutes of treatment with PMA (positive control) and also 10 minutes of treatment with **JALEx** at concentrations of 1 and 0.1 µg / mL. These images evidenced a distribution pattern close to the plasma membrane, showing that this short period of treatment with **JALEx** was enough to cause the translocation of the PKC isoforms, especially PKCα and PKCδ.

Figure 5 B refers to the 24-hour treatment, both with PMA positive control and with **JALEx** at concentrations of 1 and 0.1 µg / mL. We observed a pattern of distribution of the three PKC isoforms near the plasma membrane of cells, a

fact that is not observed in untreated cells (MOCK), where the diffuse distribution pattern of the three isoforms remains unchanged.

Analysis of nuclear translocation of NF-κB and dependence of NF-κB for reactivation of latent HIV

As previously described in the literature, activation of PKC leads to nuclear translocation of the NF-κB transcription factor, thanks to proteasomal degradation of IKK (43-45). For this reason, we evaluated the effect of the treatment with **JALEx** on nuclear translocation of NF-κB by two methodologies. In the first one, from confocal fluorescence microscopy, we analyzed the occurrence of nuclear translocation of NF-κB after treatment of HeLa cells with 1 and 0.1 µg / mL of **JALEx** for 6 or 24 hours, and labeling with anti-p55 antibody, which detects the endogenous subunit of NF-κB , in red. Figure 6A shows that 6 h after treatments we observed no significant translocation of NF-κB to the nucleus of HeLa cells in any of the conditions tested, maintaining a pattern of NF-κB distribution similar to that observed in untreated cells (MOCK). Already 24 h after the treatments, we visualized the cells with their red nuclei, confirming higher nuclear translocation levels of NF-κB (Figure 6B). To better quantify the translocation of NF-κB, Figure 6C shows the graph where the mean values of the fluorescence intensity between the nucleus and the cytoplasm of 100 HeLa cells were calculated under all conditions at the time intervals of 6 and 24 h after the treatments. We observed that in 6 h (light gray bars) we had few cells with internalization pattern, exhibiting their levelslike those observed in untreated cells. As early as 24 h (dark gray bars) we visualized a higher proportion of cells with internalized NF-κB as compared to their fluorescence in the cytoplasm of the cells. For the treatment condition with **JALEx** at the concentration of 1 µg / mL, we observed the highest internalization pattern, higher than the PMA.

The second methodology to evaluate the recruitment of NF-κB by the **JALEx** involved an assay with LTR-luciferase reporter plasmids to quantify the nuclear translocation of NF-κB. HIV transcription is sensitive to the activation of its LTR promoter by the NF-κB transcription factor, since there are multiple NF-κB binding copies in its promoter (46, 47). In order to verify whether the transcriptional activation of HIV carried out by **JALEx** would be mediated by binding of NF-κB to the HIV LTR promoter, Jurkat cells were transfected with NFkB WT or NFkB MUT. Each of these constructs expresses a luciferase reporter gene under the control of the HIV viral LTR promoter, but NFkB WT contains intact NF-κB binding sites, while NFkB MUT contains mutations that prevent binding of this transcription factor to the viral promoter. Transfected cells were treated with 1 µg / mL of **JALEx** and luciferase activity was assessed by luminometer. Figure 7 shows that treatment with the extract

induced a 5-fold increase in luciferase expression in cells transfected with pNFkB WT. No luciferase activity was observed in cells transfected with pNFkB MUT, suggesting that the transcriptional activation promoted by the **JALEx** is dependent on the intact NF-κB binding sites in the LTR promoter of HIV.

Analysis of surface marker expression and cytokine production in primary human CD4+ lymphocytes treated *ex vivo*with JALEx.

To analyze whether the effects of treatment with **JALEx** would have an impact on primary human cells, we performed assays on CD4 + T cells isolated from PBMCs from 4 healthy donors. TCD4 + cells were treated with 1 µM PMA, 10 µg / mL (10^{-3}) or 1 µg / mL (10^{-4}) of **JALEx**, in addition to MOCK (untreated cells) for 24 hours. After this period, expression of the CD4 receptor and CCR5 and CXCR4 co-receptors, in addition to the expression of activation markers such as CD25, CD38, HLA-DR and CD69 were assessed by specific antibody labeling and flow cytometric analysis . Figure 8 shows the expression pattern of all such surface molecules.

We observed in Figure 8A that the treatment with 1 µg / mL (10^{-4} - slashed bars) of **JALEx** was responsible for the negative regulation of CD4 and CXCR4 levels and did not alter the CCR5 co-receptor expression. The same pattern was observed for cells treated with PMA (black bars). Figure 8 B shows CD25, CD38 and HLA-DR activation markers, all expressed at higher levels when compared to control cells after treatment with 1 µg / mL extract (10^{-4}-hatched bars). The values are expressed as fold change over the control values (Mock - untreated). The same activation pattern is observed in PMA-treated cells (Fig. 8B, black bars). Figure 8 C shows that CD69 activation marker expression is 75-fold higher relative to control cells in both treatments. Figure 8 D shows expression of CD4, CCR5, CXCR4 in lymphocyte line MT4 cells after treatment with positive control (1M PMA - black bar) 10 µg / mL of **JALEx** (hatched bar) and1 µg / mL extract (striped bar). The pattern observed in CD4 + T cells was the same observed in lineage cells, with abrupt negative regulation of CD4 and CXCR4. However, we did not notice a slight negative regulation, about 0.25 times in relation to the untreated control in relation to CCR5 expression. These suggest that **JALEx** could block HIV infection of cells since there is a negative regulation of the receptor and one of the co-receptors used by the virus during its cell infection.

To evaluate if there was a change in cytokine production by cells treated with the **JALEx**, all cell supernatant from human CD4 + T cells was collected. This analysis was performed by means of a multiplex immunoassay to quantify the production of 17 different cytokines. Figure 9 shows that there were increased production of the following cytokines: IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL- 17, G-CSF, GM-CSF, MCP-1 / MCAF, IFNγ and TNF-α. The highest levels of production in relation to the untreated cells were observed for IFN γ

and TNF-α, with a quantification of 5,000 pg / mL and 1,800 pg / mL in the condition of 1 µg / mL of the **JALEx**. The high expression pattern of the cytokines followed the pattern exhibited by the treatment with PMA, being the concentration of 1 µg / mL of the extract that produced the most changes, when compared to the concentration of 10 µg / mL of the extract.

Ability of JALEx on cytokines-secreting T cells from Aids patients

Previous findings showed the ability of Janauba in up-regulating pro-inflammatory cytokines by PBMC cultures from healthy subjects. In HIV-1 infection, progression for AIDS is mainly characterized by $CD4^+$ T cell depletion of some Th phenotypes, such as Th1 and Th17 cells, which impacts negatively on immune protection against different pathogens. The first event analyzed was T cell viability in PBMC cultures from Aids patients when maintained in the presence of different **JALEx** concentrations by cytometry. As demonstrated in Figure 10 A, highest **JALEx** dose (1 µg/mL) induced significant $CD4^+$ T cells death (ranging 43% to 82%), and this should be linked to extension of virus replication detected in this cell cultures (Figure 10 B). At same dose, **JALEx** also induced lower, but significant, CD8+ T cells death (ranging from 5.2 to 19.8%) (Figure 10 B). Take into account these findings, our assays aiming to evaluate the *in vitro* T cells cytokine profile those patients were stimulated with 0.01 and 0.1 µg/mL of **JALEx**. As observed in the figure 10A, after 24 h of culturing, 0.1 µg/mL of JALEx was more efficient with regard to elevate the frequency of $CD4^+$ and $CD8^+$ T cells positives for IL-17, IFN-γ, and IL-21 cytokines. Moreover, when compared with PMA/IO, **JALEx** at 0.1µg/mL was more efficient at elevating the frequency of IL-21-secreting ($CD4^+$ and $CD8^+$) T cells (Figure 11 A and B). In addition, **JALEx** (0.1 µg/mL) enhanced not only the percentage of single IL-21-secreting ($CD4^+$ and $CD8^+$) T cells, but also Th1 and Th17-like subsets positives for IL-21. In contrast, no difference was observed regarding either IFN-γ- or IL-17-secreting CD4+ and CD8+ T cells from PBMC cultures from Aids patients following addition of **JALEx** at 0.1 µg/mL (Figure 11C). This result is very interesting due to protective roles played by IL-21 cytokine against different chronic virus infections, such as HCV and HIV (48,49)

Study of JALEx effects in latently SIV-infected macaque cells.

Finally, we explored the capacity of JALEx to induce latent viruses present in CD4 + lymphocytes from SIVmac239-infected cynomolgus macaques. We obtained a large population of purified resting memory CD4+ T cells from a single viremic macaque and prepared replicate serial dilutions of these cells to seed into quantitative viral outgrowth assays (QVOA). Cells in these assays were stimulated in triplicate at each cell dilution with JALEx or other compounds known to reactivate latent HIV/SIV through PKC activation (ingenol and

concanavalin A). To compare the ability of these compounds to reactivate latent SIV, we measured the frequency of infected cells (quantified as infectious units per million cells; IUPM) across treatments; a higher frequency of infected cells after stimulation with a given compound would suggest that this compound was more efficient at reversing latency. The highest frequency of infected cells was observed in cultures treated with JALEx, although this frequency was not significantly different from those observed in other cultures (Supplemental Table 1). Notably, however, JALEx was the only compound capable of reactivating SIV in each replicate culture at each dilution of input cells tested in this experiment.

DISCUSSION

In this work we addressed the ability of an ethanolic extract obtained from *Euphorbia umbellata* latex (**JALEx**) to reactivate HIV/SIV latency in several *in vitro* experiments. The traditional medical use of this Euphorbiaceae are well known in South America and this plant is vastly used diluting a small volume of crude latex in drinkable water and consuming this solution regularly (21). **JALEx** contains a series of bioactive phorbol esters (see Supplemental Figure 1). These class of molecules such as PMA are known PKC agonist implicated in lymphocyte activation as well HIV reactivation in well conducted *ex vivo* studies and these activities requires both PKCα and PKCθ isoforms activation (50). Modifications in phorbol ring can lead molecules able to induce carcinogenesis and others such as a deoxy in ring position 4 (4-deoxyphorbol) can lead to molecules unable to induce carcinogenesis but retaining its PKC activation capacity (51). The presence of this modified phorbol esters was previously shown in *Euphorbia umbellata* latex (40). Therefore, our **JALEx** is not likely to induce cell carcinogenesis retaining the capacity to reactivate HIV/SIV latency. Many LRA fit in this class such as prostatin (13), bryostatin (14), ingenol B (15), and phorbols such as PMA (16). Prostratin and bryostratin, are strong PKC agonist and they are very toxic when injected intravenously. In fact, bryostatin was used in a phase II study with 17 patients with progressive non-Hodgkin's lymphoma of indolent type (NHL), previously treated with chemotherapy. These patients received a median of 6 intravenous infusions of 25 μg/m^{2}bryostatin 1 given once weekly over 24 h and the principal toxicities were myalgia and phlebitis (52). Ingenol B a semisynthetic compound isolated from *Euphorbia tirucalli* was able to reactivate HIV *ex vivo* with great potency using the PKC/NFkB pathway. It was also clear that the use ofingenol B in human CD4+ lymphocytes increased cellular levels of CycT1 and CDK9 in ex vivo experiments. (15). Similar mechanism of action can be found in PMA in *in vivo* study. In our study we used **JALEx** to explore the capability of 4-deoxyphorbol esterspresent in its latex to reactivate HIV latency taking the advantage of the use in traditional medicine without serious side effects whenever used orally. First we used JALEx in J-Lat 8.4 and 10.6 cells and we could show the potency of this extract reactivates latent HIV transcription present in J-Lat 8.4 and 10.6 cells in low concentrations (0.01 μg / mL).We also demonstrate that **JALEx** act through PKC by inhibiting J-Lat activation with three different PKC inhibitors (G6666, G6983 and Ro-31-8220). In our assays Gö6983 was more powerful inhibitor totally blocking HIV reactivation in both J-Lat cell lines. Go 6983 is a fast pan-PKC inhibitor against PKCα, PKCβ, PKCγ and PKCδ with IC_{50} of 7 nM, 7 nM, 6 nM and 10 nM, respectively; less potent to PKCζ and inactive to PKCμ.Additionally, we showed that **JALEx** activates the PKC pathway by promoting NF-κB internalization into the nucleus and increasing HIV transcription, which is dependent on NF-κB binding to LTR promoter. Using image and western blot analysis we showed that **JALEx** was able to activate classical and novel PKC isoforms such as: the conventional isoforms α, βI, βII and γ as well the new ones encompassing the δ, ε, η and θ isoforms in *in vitro* experiments using JURKAT cell lines.

We also evaluate the capacity of **JALEx** to induce cytokines production in human PBMC cells *in vitro*. This pattern of alteration in the production of cytokines from treatment with reactivating compounds is quite common in the case of the shock and kills strategy, which aims to activate the cells and promote the exit of HIV latency that is integrated into the cells T. This phenomenon is known as "cytokine storm" and needs

to be further studied in animal models to define a dose that does not induce this toxic reaction. Although **JALEx** induced a conjunction of pro inflammatory cytokines we could observed a noticeable level of induction of IL-21bydifferent CD4+ and CD8+T lymphocyte subsets from PBMC cultures from Aids patients, a noble cytokine enrolled in the HIV virus load control in HIV+ individuals (5). For this reason, the search for new, more selective compounds that target only a few PKC isoforms or that have their mechanism of action in other stages of the activation pathways to reduce these risks is necessary. Of note, leaf and latex ethanolic extract of E. umbellata latex had low toxicity in adults Rat when dose orally and acute doses of 2000mg/kg given orally was well tolerated (53).

In our experiments **JALEx** was efficient in reactivating HIV production directly in resting T CD4+ cells in HIV+ individuals fully virally suppressed by HAART as well primary non-human primate T CD4+ cells counterpart in SIV251 latency model using a QVOA assay in low concentrations. The anti-HIV action of **JALEx** can occur three synergistic effects: a) reactivation of HIV from latency and its destruction by HAART; b) blocking infection of new cells through the down-modulation of CD4 on the lymphocyte surface; and c) by inducing the secretion of antiviral cytokines such as IL21. All these *in vivo* properties of **JALEx** in non-human primate cell models added to the molecular mechanisms of reactivation presented here as well the traditional use of *E. umbellata* latex, strongly recommends JALEx or other *E. umbellata* latex preparations as a great candidate for future clinical trials for HIV eradication.

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FIGURES AND LEGENDS

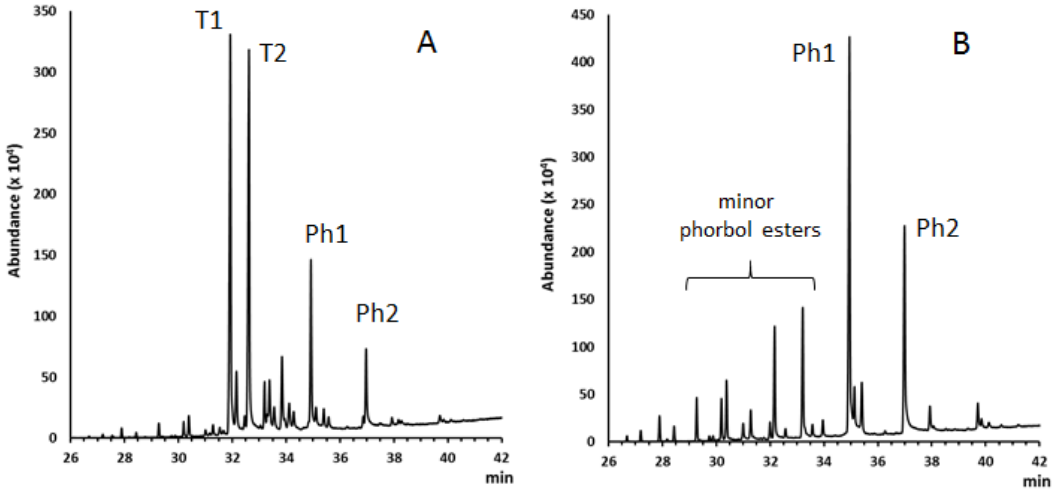


Figure 1. Gas chromatograms of (A) whole extract **JALEx** and (B) Porbol esters-enriched JALEx fraction. T1 and T2 = major triterpenes constituents; Ph1 and Ph2 = major phorbol esters constituents. The chemical classes were indicated by the characteristic mass fragmentation in the GC-MS analysis and data from literature.

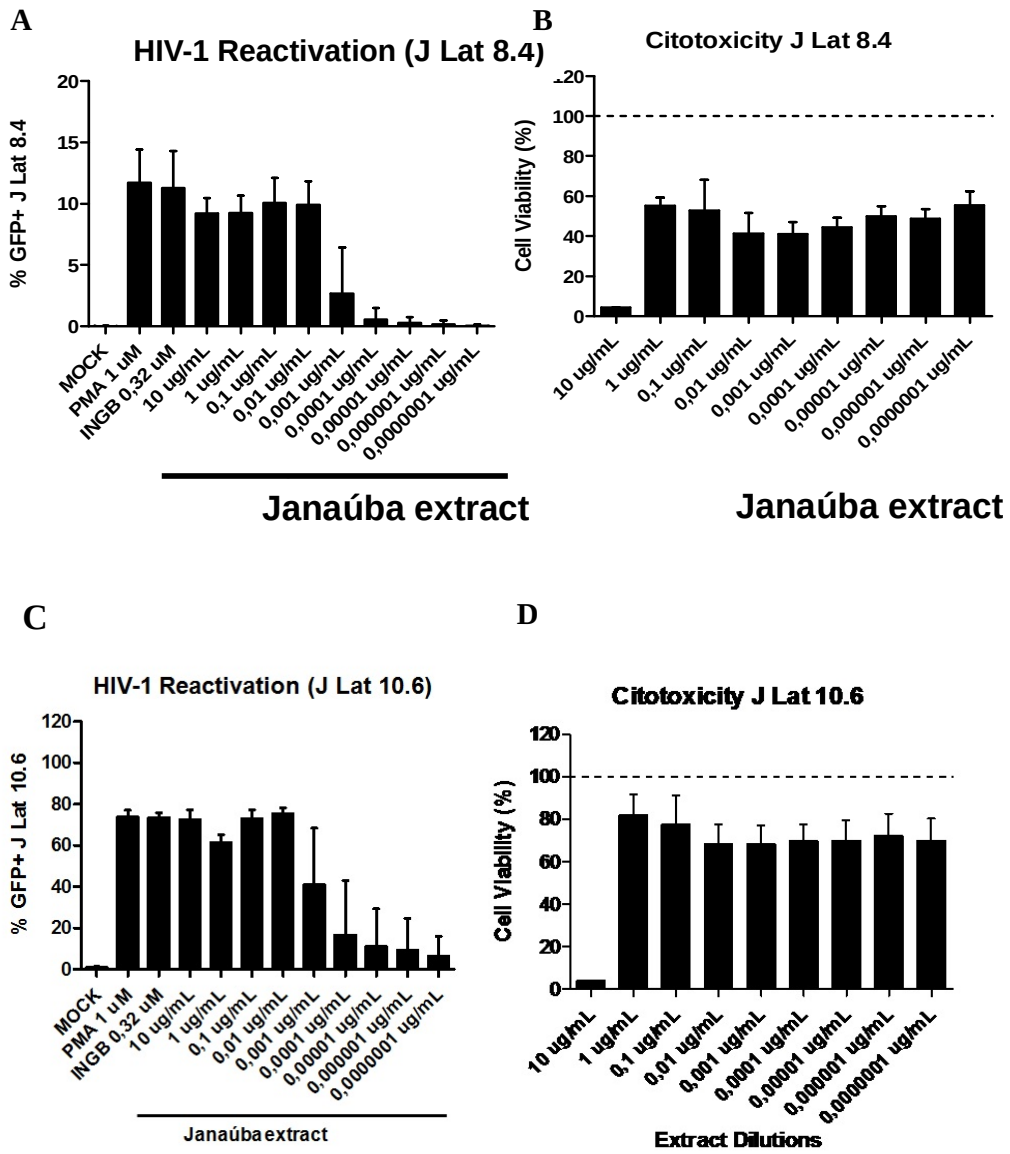


Figure 2: JALEx promotes HIV transcription. JALEx reacts latent HIV-1 transcription in J-Lat 8.4 (A) and J-Lat 10.6 (C) cells. Cells were submitted to increasing concentrations of **JALEx** and activation was assessed by GFP expression by flow cytometry 24 hours after treatment of the cells. MOCK cells were not treated with the compound, whereas the positive controls were performed with two potent reactors already known: PMA (1 µM) and INGB (0,32 µM). The cytotoxicity of this compound was evaluated by CellTiter Blue vital stain 5 days after treatment with the compound. In (C) we observed the percentage of viable J-Lat 8.4 cells after treatment and in (D), the percentage of J-lat 10.6 cells.

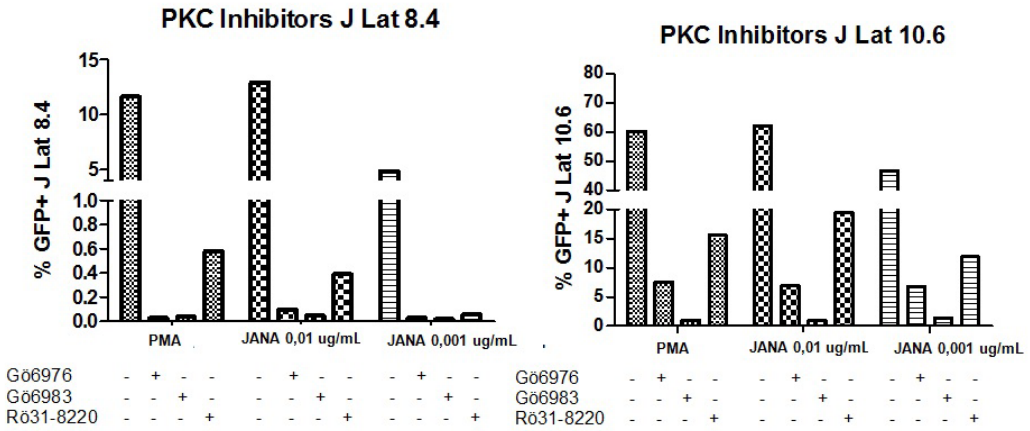


Figure 3: JALEx -mediated HIV-1 reactivation occurs via PKC activation. J-Lat cells 8.4 (left panel) and J-Lat 10.6 (right panel) were pretreated for 24 h with three different PKC inhibitors (G6666, G6363and Ro-31-8220) at the concentration of 1 µM each. The cells were then incubated with different concentrations of **JALEx** for an additional 24 hours and GFP expression was assessed by flow cytometry. PMA (1 µM) was used as a positive control for activation of PKC-dependent HIV-1.

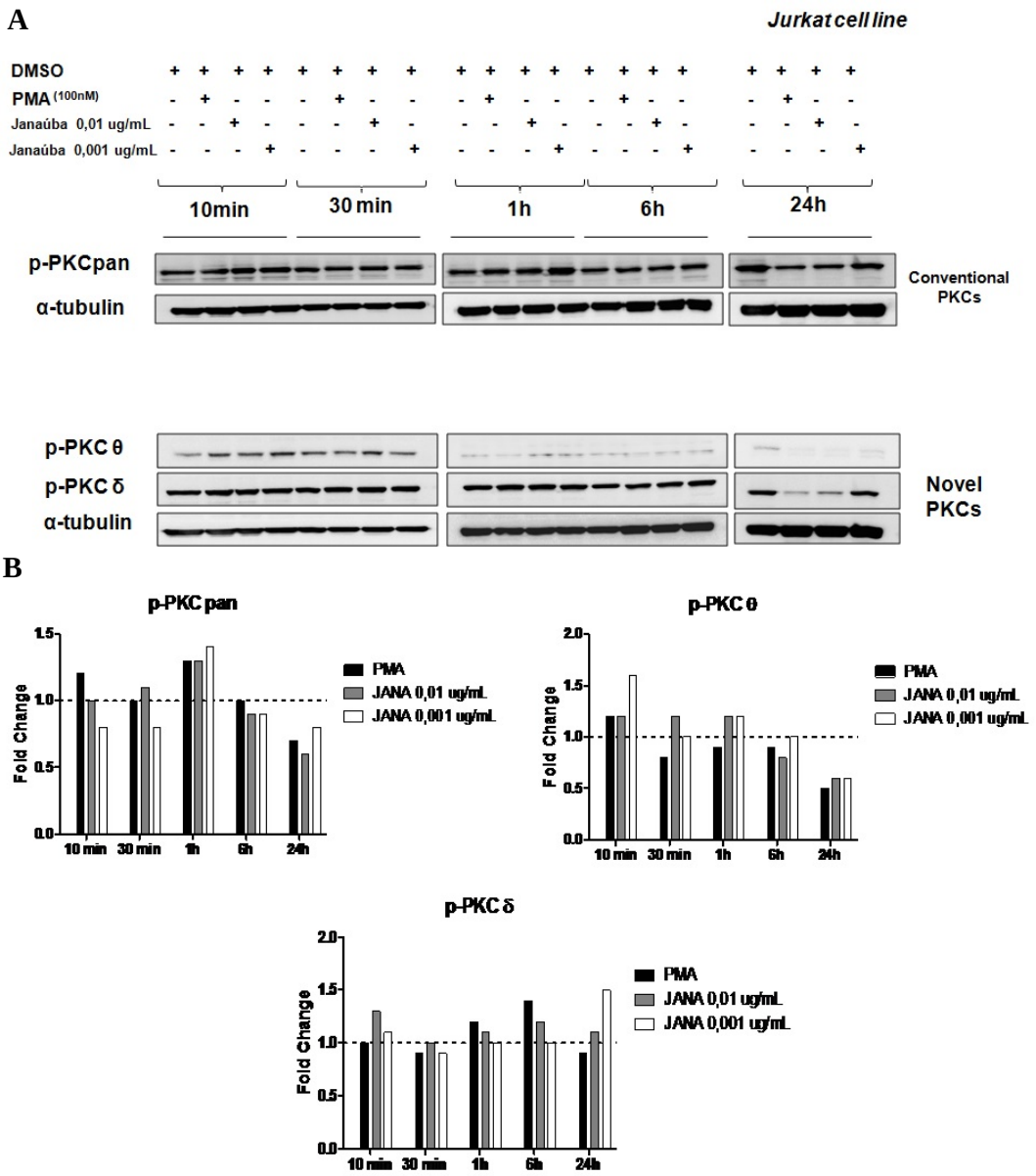


Figure 4. Panel of phosphorylation of different PKC isoforms after treatment with JALEx. (A) Jurkat cells were treated with two different concentrations of **JALEx** (0.01 µg / mL and 0.001 µg / mL), PMA (1 µM) as positive control, at different time intervals (10, 30 minutes, 1, 6 and 24 hours). Then the cells were lysed for western blotting with phosphorylated anti-PKC antibodies (pan, δ, θ) and anti-tubulin as the loading control. (B) The intensity of the western blotting bands corresponding to the Jurkat cells in (A) that were quantified by densitometry with the aid of the Image J program.

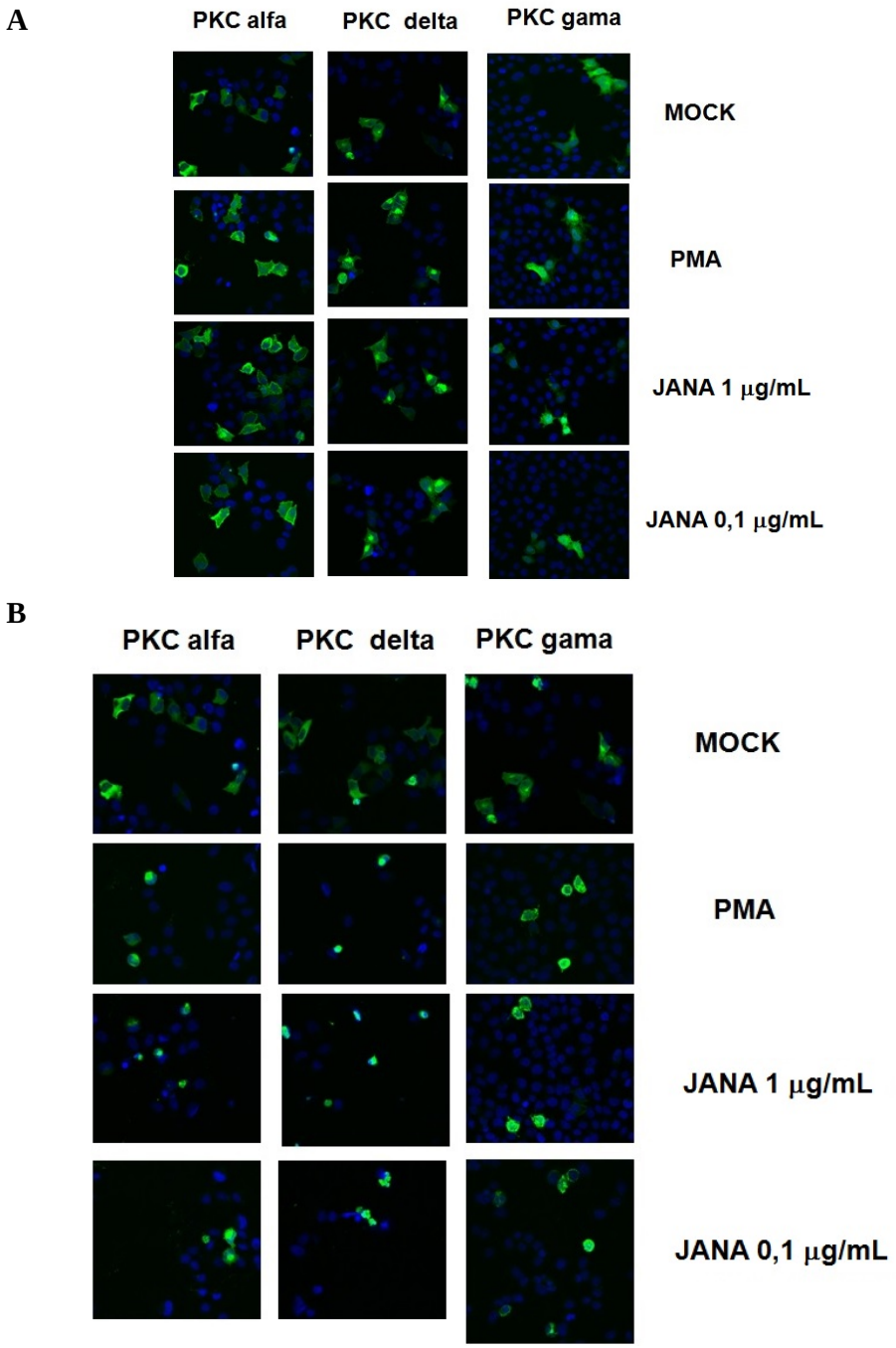
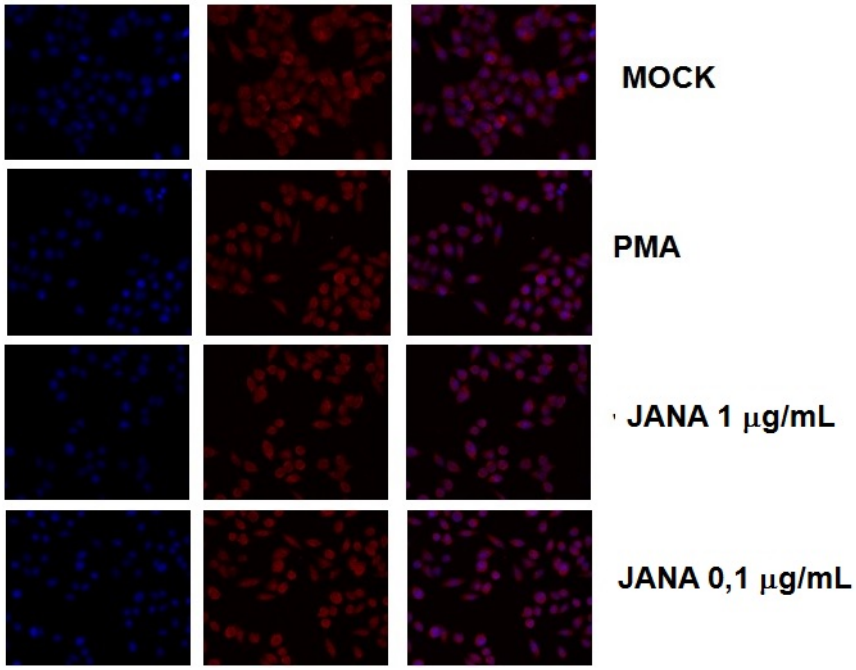
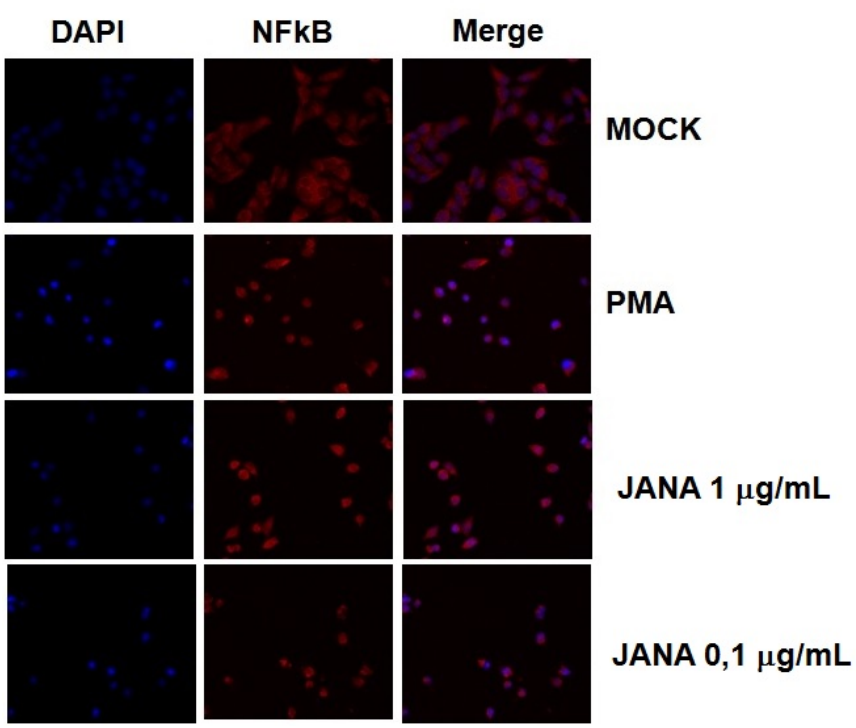


Figure 5: JALEx is responsible for the cellular relocation of PKC isoforms. Cellular location analysis of different PKC (alpha, delta and gamma) isoforms after treatment of HeLa cells with two concentrations of **JALEx**: 1 µg / mL and 0.1 µg / mL. (A) HeLa cells were transfected with 3 different PKC isoforms for 24 h. After this time the cells were treated for 10 minutes with the two concentrations of Janauba. (B) HeLa cells were transfected with 3 different PKC isoforms for 24 h. After this time the cells were treated for additional 24 hours with the two concentrations of Janauba. As a positive control, PMA (1 µM) was used.

A


DAPI
NFkB
Merge
MOCK
PMA
JANA 1 μg/mL
JANA 0,1 μg/mL

B


DAPI
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JANA 1 μg/mL
JANA 0,1 μg/mL

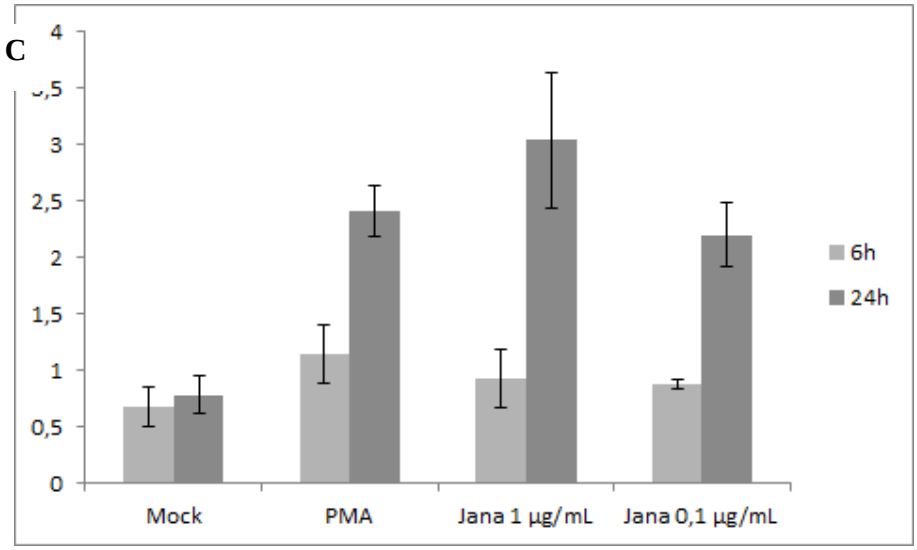


Figure 6: . JALEx -mediated transcriptional activation is dependent on NFκB activation. HeLa cells were treated with two concentrations of the **JALEx** (1 and 0.1 µg / mL) for 6 hours (A) and 24 hours (B). After these time intervals, the cells were subjected to immunofluorescence for labeling of NFκB, in red and cell nucleus, in blue. As a positive control, PMA (1 µM) was used. (C) Graph of the quantification of cells showing NF-κB translocation. An average value of the fluorescence intensity between the nucleus and the cytoplasm of 100 HeLa cells was calculated under all conditions, at the time intervals of 6 and 24 h after the treatments.

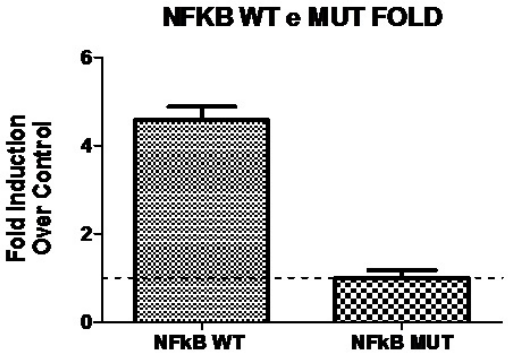


Figure 7.: The transcriptional activation of NFκB by JALEx is dependent on the NFκB binding site. Jurkat cells were transfected with pBlue30LTR-Luc (NF-κBWT) and the mutant construct, pBlue30LTRNF-κB MUT-Luc (NF-κB MUT), absent from the NFκB binding site, preventing its interaction with NFκB. After transfections, the cells were treated with 1æg / mL of the **JALEx** for 24 hours and luciferase expression was measured after lysis of the cells. The fold-change of the luciferase expression between the wild-type plasmid and the mutant is shown in the graph.

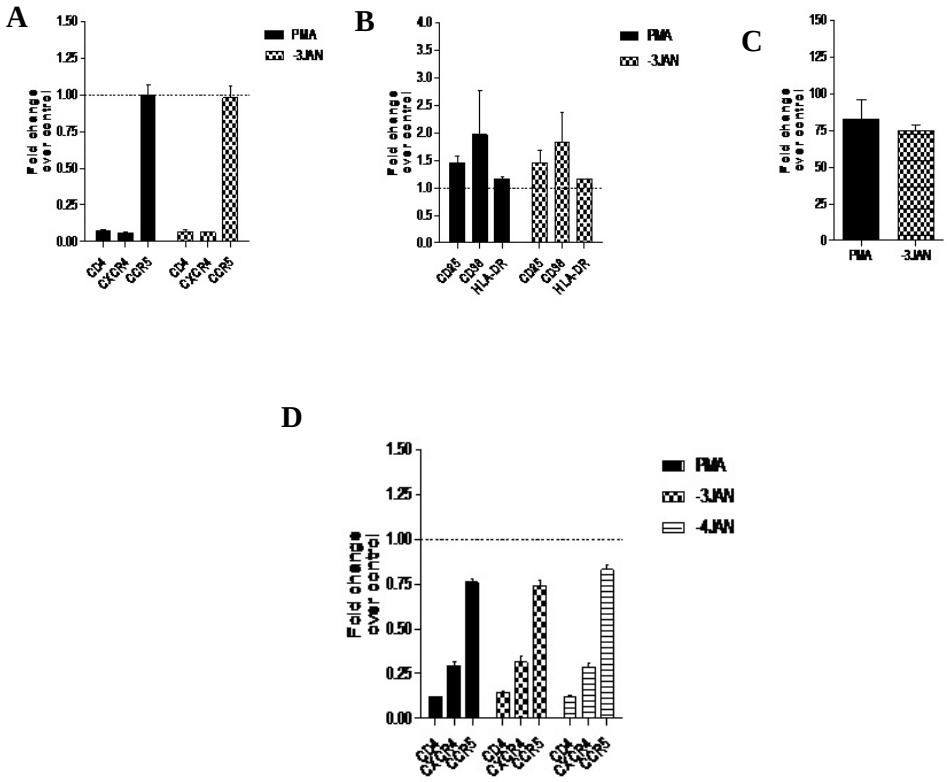


Figure 8: JALEx modulates the expression of cell surface receptors and activation markers in CD4 + T cells and lymphocytic lineage. TCD4 + cells were isolated from PBMCs from 4 healthy donors and treated for 24 hours with **JALEx**. After this period, the expression of some surface molecules was evaluated. (A) Expression of the CD4 receptor and CCR5 and CXCR4 co-receptors in CD4 + T cells isolated after treatment with **JALEx**. (B) Expression of CD25, CD38 and HLA-DR activation markers in CD4 + T cells isolated after treatment with **JALEx**. (C) Expression of the CD69 activation marker on CD4 + T cells isolated after treatment with janauba extract. The black bar represents the positive control (PMA 1 µM) and the hatched bar represents the treatment with 10 µg / mL of **JALEx**. (D) Expression of receptor and co-receptor molecules - CD4, CCR5, CXCR4 in lymphocyte MT4 cells. The black bar represents the positive control (PMA 1 µM) and the hatched bar represents the treatment with 10 µg / mL of **JALEx** and the striped bar represents the treatment with 1 µg / mL of **JALEx (n=4).**

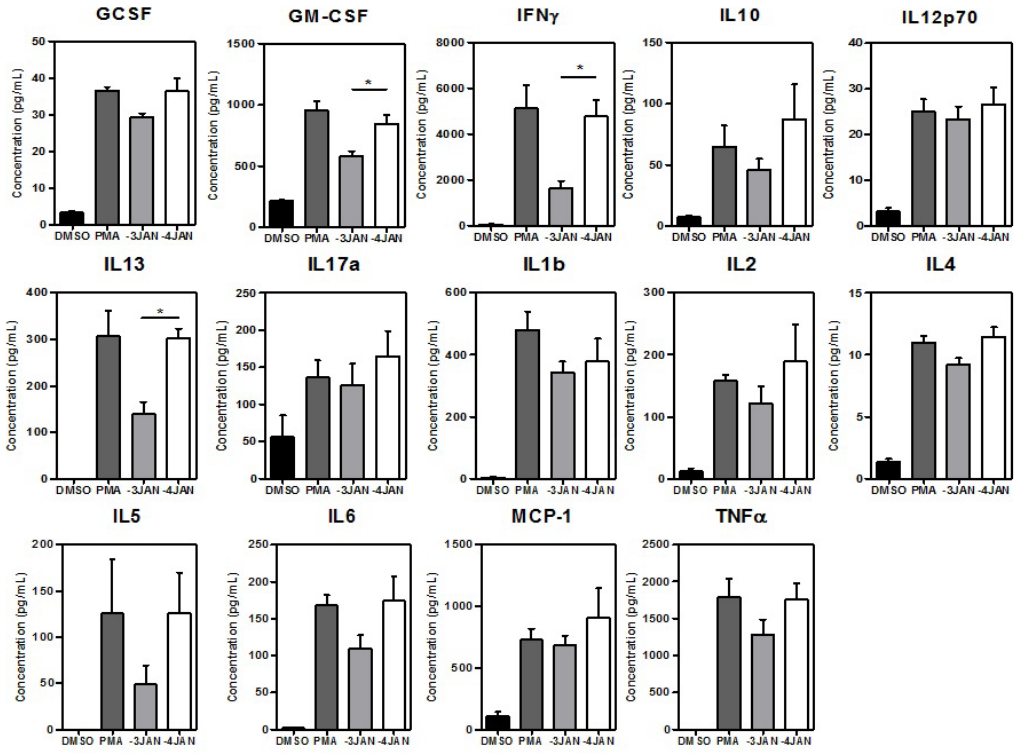


Figure 9: . Cytokine profile produced by peripheral blood primary cells after treatment with JALEx.

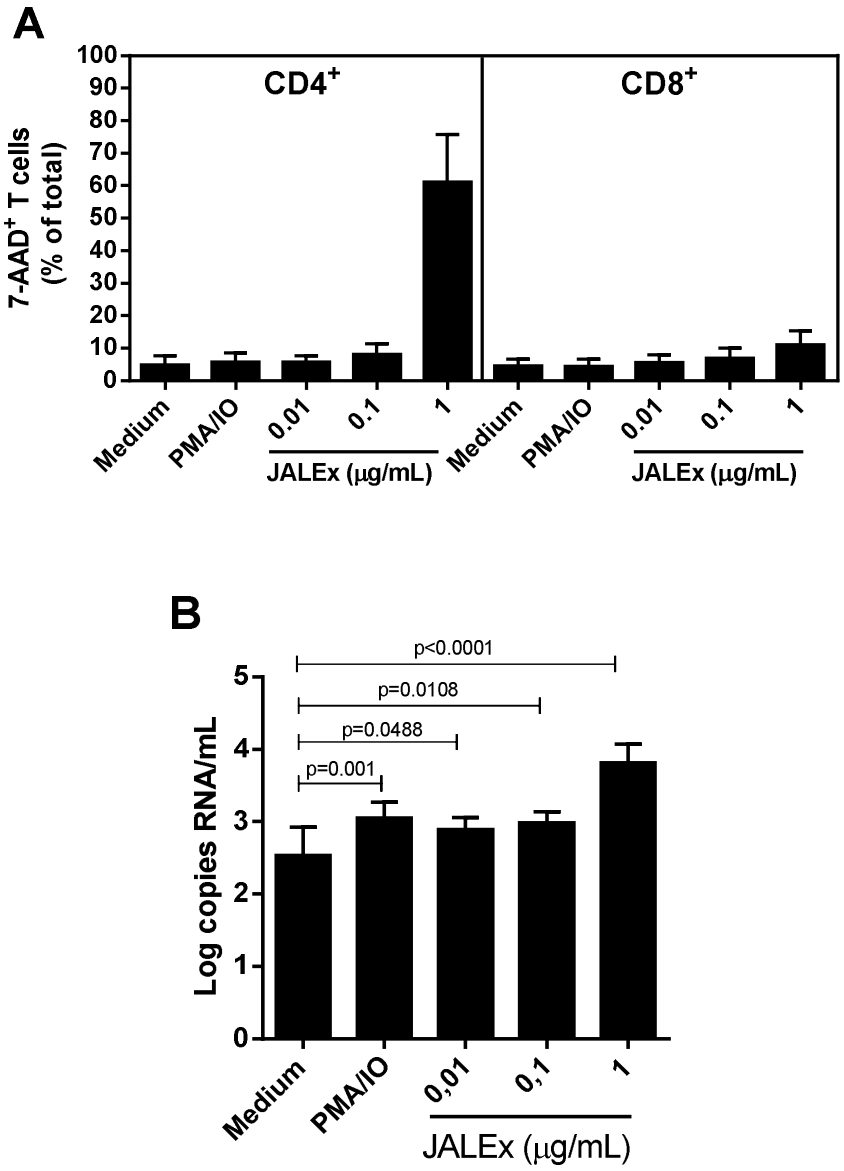


Figure 10. Effect of JALEx on T cell viability and HIV-1 replication in PBMC cultures from AIDS patients. Different concentrations of **JALEx** (0.01, 0.1 and 1 μg/mL) were added to PBMC cultures (1 x 10^6/mL) obtained from 15 AIDS patients, and 24h after, the (**A**) $CD4^+$ and $CD8^+$ T cells viability was analyzed by FACS after 7-AAD staining, and (**B**), the number of HIV-1 into supernatants was determined through RT-PCR.

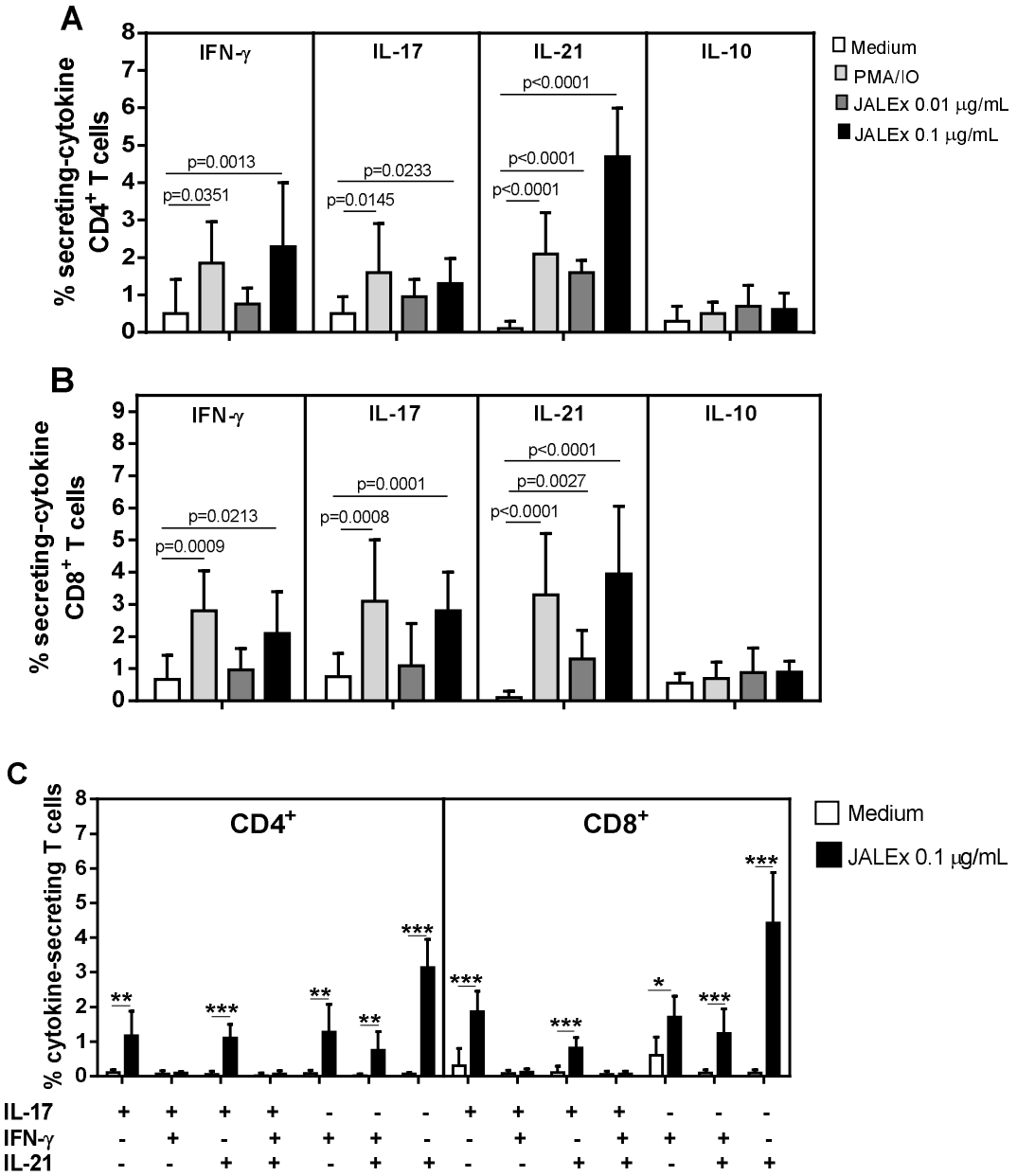
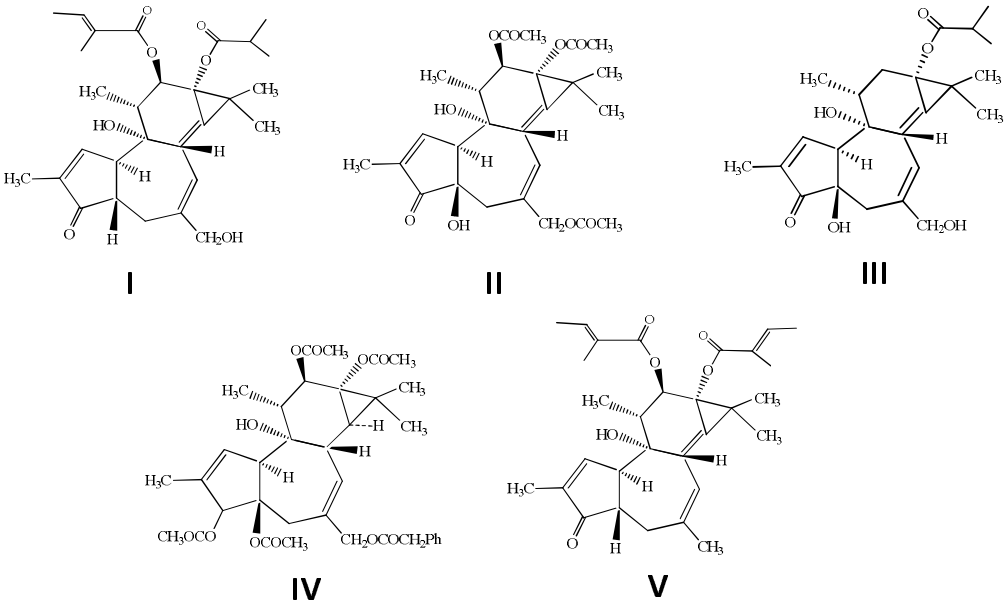


Figure 11. Ability of JALEx in modulating the frequency of cytokine-secreting T cells form AIDS patients. AIDS-derived PBMC cultures (1 x 10^6/mL, n=15) were maintained for 24h in the presence of medium alone or with different **JALEx** (0.01 and 0.1 μg/mL). As positive control, some well receiving PMA plus ionomycine (PMA/IO). The percentage of $CD4^+$ (**A**) and $CD8^+$ (**B**) T cells producers of IFN-γ, IL-17, IL-21 and IL-10 was determined by cytometry. In (**A**) and (**B**), the mean values were compared and the p values are shown in the figures. In (**C**), the proportion of different ($CD4^+$ and $CD8^+$) T cell subsets of secreting different combinations of IL-17, IFN-γ and IL-21 in response to JALEx (0.1 μg/mL) was also evaluated by cytometry. In the figure, (*), (**), and (***) indicate p<0.05, p<0.001 and p<0.0001, respectively.

Supllemental data

Supplemental Figure 1



Supplemental Figure 1-Phorbol esters isolated from *Euphorbia umbellata*. I = 12-*O*-Tigloyl-4-deoxyphorbol-13-isobutyrate; II = Phorbol-12,13,20-triacetate; III = 12-Deoxyphorbol-13-(12-methylpropionate); IV = 3,4,12,13-Tetraacetylphorbol-20-phenylacetate (synagrantol A); V = Deoxyphorbol-12,13-ditiglate (synagrantol B).

Supplemental Table 1

Table 1- QVOA results using macaques CD4+ T Lymphocytes extracted from SIV 251 infected Macaques treated with HAART therapy.

Cells/well[a]	No mitogen	Con A	Ingenol B	**JALEx**
1.25e5	+ / - / -[b]	- / - / -	+ / - / +	+ / + / +
6.25e4	- / - / +	- / + / +	+ / + / -	+ / + / +
3.1e4	+ / - / +	- / - / +	- / + / +	+ / + / +
Maximum likelihood (IUPM)	7.735675	5.229369	15.525127	**34.986678**
95% CI	{2.866090, 20.878852}	{1.680200, 16.275622}	{6.619641, 36.411279}	{12.619641, 66.411279}

[a]- Number of CD4+ T Lymphocytes extracted from SIV 251 infected Macaques treated with HAART therapy inoculated in each well. [b]- Number of well with p27 antigen positive. Con A: