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

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30 **Abstract**

31 *Euphorbia umbellata* (*E. umbellata*) belongs to *Euphorbiaceae* family, popularly known
 32 as Janauba, and contains in its latex a combination of phorbol esters with biological
 33 activities described to different cellular protein kinase C (PKC) isoforms. Here, we
 34 identified deoxi-phorbol esters present in *E. umbellata* latex alcoholic extract able to
 35 increase HIV transcription and reactivate HIV from latency models. This activity was
 36 mediated by NF-κB activation followed by nuclear translocation and binding to HIV LTR
 37 promoter. In addition, *E. umbellata* latex extract induced the production of pro
 38 inflammatory cytokines together with IL21 in *in vitro* human PBMC cultures. Our latex
 39 extract activates latent HIV in human PBMCs isolated from HIV positive patients as
 40 well as latent SIV in non-human primate primary CD4⁺ T lymphocytes. These results
 41 strongly indicate that the phorbol esters present in *E. umbellata* latex are promising
 42 candidate compounds for future clinical trials for *shock and kill* therapy to promote HIV
 43 cure and eradication.

44

45 **Keywords:** HIV-1, latency, reactivation, PKC, NF-κB.

46 **Running Title :** New natural extract for reactivation of latent HIV-1

47 INTRODUCTION

48 The Acquired Immunodeficiency Syndrome (AIDS) is caused by the
49 Human Immunodeficiency Virus (HIV), which was identified in 1983 **(1,2)** from
50 patients with AIDS. In those 34 years of pandemic, more than 35 million people
51 have died from AIDS-related illnesses since the start of the epidemic until 2016
52 **(3).**

53 The pandemic of HIV infection is an important socioeconomic burden and
54 is considered one of the largest documented epidemics in history. HIV
55 pathogenesis involves infection and replication in CD4⁺ T lymphocytes,
56 macrophages and dendritic cells. Replication and destruction of CD4⁺ T cells,
57 which are key effector cells in the host immune response, leads to a clinical
58 outcome of immunosuppression known as AIDS **(4).**

59 The current strategy used in the treatment of HIV infection is named
60 Antiretroviral Therapy (ART), which is based on a combination of antiretroviral
61 drugs, which target different stages of HIV replicative cycle. The primary goal of
62 ART is to block viral replication and slow down the progression of
63 immunodeficiency, restoring immunity and improving the quality of life of the
64 infected patient **(5).** However, adverse effects have been reported as a
65 consequence of the antiretroviral therapy, in conjunction with immunological,
66 neurological and metabolic co-morbidities associated either with HIV infection or
67 drugs interaction **(6).** These facts triggered the search for a functional or
68 sterilizing cure for HIV infection.

69 Even when an undetectable viral load is achieved with effective
70 antiretroviral therapy, it is still possible to detect latent viral reservoirs where HIV
71 proviral DNA is integrated in resting memory CD4⁺ T cells **(7).** These cells
72 containing latent viral genomes become viral reservoirs inaccessible to ART
73 that can only act on actively replicating viruses. Once ART is stopped, viral load
74 increases rapidly and systemic infection reestablishes **(8).** Therefore, the
75 complete eradication of HIV from an infected individual is one of the most
76 challenging areas of HIV research today. The way to eliminate HIV from patient
77 is to activate the virus in the presence of ART and or activate the immune
78 response to destroy viral reservoir or make patient's immune response to keep
79 viral replication at the bay.

80 New latent virus reactivating compounds, also called latency reversing
 81 agents (LRA), when used conjointly with ART, are capable to eliminate
 82 transcriptionally inactive viruses, ultimately resulting in the eradication of HIV
 83 infection. Histone deacetylase (HDAC) inhibitors are promising classes of
 84 latency-reversing agents (LRAs) that are undergoing extensive testing in *in*
 85 *vitro* models and in initial pilot clinical trials to reactivate latent HIV-1 infection.
 86 HDAC inhibitors were developed as anticancer drugs as HDACs play important
 87 role in epigenetic and non-epigenetic transcriptional regulation, inducing
 88 apoptosis and cell cycle arrest **(9)**. In the context of HIV-1 reactivation, HDAC
 89 inhibitors induce transcription at the HIV-1 long terminal repeat (LTR) **(10-13)**.
 90 This strategy is called “shock and kill” and it postulates that memory CD4⁺ T
 91 lymphocytes treated with LRA along with ART could purge totally or partially the
 92 viral reservoir while blocking replication of emergent latent virus, leading to a
 93 HIV eradication or at least a functional cure **(14)**. Another promising class of
 94 molecules with therapeutic potential has been reported as possible candidates
 95 for elimination of viral reservoirs are the Protein Kinase C (PKC) agonists **(7)**.
 96 This class includes: Prostratin **(15)**, Bryostatin **(16)**, Ingenol **(17)** and Phorbol
 97 12-Myristate 13-Acetate (PMA) **(18)**. These compounds can reactivate latent
 98 HIV in lymphoid and myeloid cells. Although several transcriptional regulatory
 99 mechanisms for HIV latency have been described in the context of epigenetic
 100 silencing and transcription repression, recent work suggests that reactivation of
 101 latent HIV is positively correlated with NF-κB activation, demonstrating the
 102 importance of this factor in latency reactivation **(19, 20)**.

103 Many natural compounds have been investigated for their antiviral and
 104 latency-reversing properties, being indicated as candidates for clinical trials.
 105 Several of these compounds are derived from secondary metabolics of plants,
 106 such as terpenoids **(21)**, polyphenols **(22)**, alkaloids **(23)** and phorbol esters
 107 **(24)**. *Euphorbiaceae* family is a source of potent phorbol esters, which
 108 specimens are found worldwide and have been used for the treatment of
 109 several diseases in the popular medicinal use **(25-29)**. *Euphorbia umbellata* is
 110 popularly known in Brazil as Janauba or *leiterinha* and is used, in traditional
 111 medicine, as a latex preparation (diluted in water) for the treatment of gastric
 112 disorders, such as peptic ulcer and gastritis, as well as neoplastic diseases,
 113 allergy, diabetes, influenza, leprosy, among other diseases **(30)**. Several papers

114 have reported the isolation of lectins, phorbols, terpenes, flavonoids and other
 115 phenolic compounds from *E. umbellata* latex **(31-33)**. This plant contains
 116 phorbol esters in its latex similar to PMA isolated from the seeds of *Croton*
 117 *tiglium*, another representative of the *Euphorbiaceae* family. These compounds
 118 are efficient in inhibiting the cytopathic effect generated by HIV infection on
 119 lymphocyte cells, as well as activating protein kinase C (PKC) that in turn
 120 activates NF- κ B, promoting its nuclear translocation and promoting HIVLTR
 121 transcriptional reactivation reverting viral latency **(34)**.

122 In this work we evaluated the ability of an alcoholic extract obtained from
 123 Janauba latex (JALEx) to reactivate latent HIV and investigated the molecular
 124 mechanisms involved in this activity. The anti-latency property of JALEx was
 125 demonstrated either in *ex vivo* HIV latent derived human PBMC cells or in CD4+
 126 T Lymphocytes isolated from SIV infected non-human-primates. All these
 127 properties makes JALEx a promising natural medicine to be used in HIV cure
 128 strategies.

129

130

131 MATERIAL AND METHODS

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

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

156 **Viruses and cells.** Analysis of HIV reactivation *in vitro* in latent infected cells
 157 after treatment with JALEx. Human HIV latent lymphocyte cell models (J-Lat 8.4
 158 and 10.6) were used (35). These cells are derived from Jurkat cells containing
 159 copies of HIV genome in fusion with GFP reporter gene integrated into the
 160 nuclear. These cells have different levels of reactivation capacity, depending on
 161 the site of HIV genome integration. The J-Lat 10.6 clone is a more responsive
 162 one showing reactivation levels higher than those observed by the J-Lat 8.4

clone. Both 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 per well of 24-well plates were treated with different concentrations of JALEx dissolved in DMSO for 24 hours. Positive controls for reactivation of HIV latent virus were ingenol B (0.32 μ M) and PMA (1 μ M). Cells were analyzed by flow cytometric (BD Accuri C6) to determine the level of GFP expression, in order to evaluate HIV transcription. A total of 10,000 gated live cells were collected and data represent the percentage of GFP-expressing cells in total gated events. All the experiments were performed in three independent replicates.

Cell viability assay. Evaluation of the cytotoxicity of the compounds was performed by incubating J-Lat cells with the serial dilutions of JALEx compounds previously tested in the reactivation assays. Cell viability assessment was performed in 10^4 cells plated in 96-well plates, in three independent experiments, each in sextuplicates. After 5 days of culture, vital resazurin dye (CellTiter Blue, Promega) was added to the cells and absorbance levels were evaluated by spectrophotometer at 595 nm.

Analysis of the activation of PKCs. To assess the mechanism of action of HIV latency reactivation by Janauba compounds, 10^5 J-Lat cells in 24-well plates were treated with different specific inhibitors of PKC α , PKC δ and PKC γ isoforms Go6976, Go6983, Ro-31-8220, respectively, for 24 hours **(36-38)**. After this time, cells were treated with the positive control of PKC activation (1 μ M PMA) and two concentrations of JALEx previously determined in the reactivation and cytotoxicity assays. Untreated cells were referred as MOCK. The following concentrations of the alcoholic extract were tested: 0.01 μ g/mL and 0.001 μ g/mL and the percentage of GFP positive cells was assessed by flow cytometry analysis.

In addition, PKC activation was evaluated by immunofluorescence to detect cytosolic location of different PKC isoforms. For this, 10^4 HeLa cells were plated in black 96-well glass bottom plates. These cells were transfected with three different plasmids encoding three PKC isoforms fused to GFP (PKC α -GFP, PKC δ -GFP and PKC γ -GFP) for 24 hours and then treated with 1 μ g/mL and 0.1

195 $\mu\text{g/mL}$ of the JALEx, for 10 minutes or 24 hours. Positive control was performed
196 using PMA ($1 \mu\text{M}$). Cells were washed with PBS and DAPI stained. Cellular
197 localization of PKC-GFP was evaluated by High Content Screening confocal
198 microscope (Molecular Devices, Inc). The redistribution of PKC isoforms was
199 quantified in ImageJ software.

200 Phosphorylation of PKC isoforms was also investigated. For this, Jurkat cells
201 were treated with $0.01 \mu\text{g/mL}$ and $0.001 \mu\text{g/mL}$ of JALEx and with $1 \mu\text{M}$ PMA as
202 a positive control for 10 and 30 minutes, 1, 6 and 24 hours. Cellular extractes
203 were prepared in lysis buffer (50 mM Tris pH 7.6, 150 mM NaCl, 5 mM EDTA, 1
204 mM Na_3VO_4 , 10 mM NaF, 10 mM NaPyrophosphate, 1% NP-40 supplemented
205 with protease inhibitors cocktail) for 1 hour. Lysates were subjected to 10%
206 SDS-PAGE gels and after transferred to nitrocellulose membranes the following
207 antibodies were used for immunoblotting: anti-PKC phosphorylated PKC (pan,
208 δ , θ) (Cell Signaling; 1:500) and β -tubulin (Cell Signaling; 1:2000). Proteins
209 quantification was evaluated using band densitometry analysis, performed with
210 the Image J software (version 1.41; National Institutes of Health).

211 **NF- κB activation assays.** In order to verify whether JALEx induces the
212 activation and downstream nuclear internalization of NF- κB , HeLa cells were
213 plated at the density of 2×10^4 cells per well in 96-well plates. Cells were treated
214 with $1 \mu\text{g/mL}$ and $0.1 \mu\text{g/mL}$ of JALEx for 6 or 24 hours. PMA ($1 \mu\text{M}$) was used
215 as positive control. The NF- κB subunit p65 was visualized by
216 immunofluorescence (anti-p65, 1:300, Santa Cruz Biotechnologies), and nuclear
217 (DAPI) translocation was evaluated by fluorescence confocal microscopy
218 (Molecular Devices, Inc).

219 In order to evaluate the role of NF- κB in reactivation of latent HIV, Jurkat cells
220 (10^6 cells) were transiently transfected with 8 μg of pBlue30LTR-Luc Wild Type
221 (NF κB WT) or p-LTR-MUT (NF κB MUT) using the Neon Transfection System kit
222 (Thermo Fisher Scientific) according to the manufacturer's recommendations.
223 The latter plasmid was generated by site-directed mutagenesis by altering the
224 two NF- κB binding sites, rendering that plasmid nonfunctional for NF- κB
225 response. Both plasmids were provided by Dr. Renato S. de Aguiar (UFRJ) and
226 those constructs have the luciferase sequence under the control of the HIV viral

promoter. In the case of plasmid carrying NF- κ B mutate sequence, the second guanine at both NF- κ B binding sites into LTR promoter was changed to a cytosine, resulting in the plasmid designed here as *pBlue3LTR NF- κ B MUT-Luc* (NF- κ B BS⁻). This plasmid carries a LTR sequence lacking functional NF- κ B binding sites. After transfections, cells were equally divided into two groups: treated or untreated with 1 μ g/mL of JALEx, for 24 hours. After this interval, cells were lysed and luciferase activity was assessed in a luminometer (Glomax, Promega).

Analysis of surface markers expression and cytokines 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 Ficoll centrifugation, CD4⁺ T cells were isolated using the Dynabeads CD4 Positive Isolation Kit (Thermo Fisher) kit to obtain 10⁷ PBMCs, according to the manufacturer's recommendations. TCD4⁺ cells were treated with 1 μ M PMA, 10 μ g/mL (10⁻³) or 1 μ g/mL (10⁻⁴) of JALEx, in addition to MOCK (untreated cells), for 24 hours. Expression of surface markers such as CD4, CCR5 and CXCR4, as well as the expression of activation markers CD25, CD38, HLA-DR and CD69 were evaluated by specific antibody labeling and flow cytometric analysis (BD Accuri C6). Comparatively, this assay was also performed on MT4 lymphocyte cells line at the same conditions.

Cell supernatants from the TDC4⁺ cells were collected for analysis of the cytokine production stimulated by JALEx treatment. 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 was chosen for this analysis. The following cytokines were measured: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-17, G-CSF, GM-CSF, MCP-1 / MCAF, MIP-1 β , IFN γ and TNF- α .

Quantitative viral outgrowth assay. 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. (39). 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 1x10⁶ 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. 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 (40) except that analysis was performed using the Lightcycler 480.

Effect of JALEx in T cell activation from HIV positive patients. In order to analyze the ability of JALEx in regulating different T cell phenotypes in the context of HIV-1 infection, peripheral blood of 15 adults ART-treated patients (8 women and 9 men), with a mean plasma viral load of 953 ± 1.924 copies of RNA/mL and CD4⁺ (845 ± 345/mm³) and CD8⁺ (845 ± 345/mm³) higher than 350 cells/mm³, 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 10⁶ PBMC/mL were cultured for 24 hours 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). Phorbol-12-myristate-13-acetate (PMA 20 ng/mL) plus ionomycin (600 ng/mL) were used as positive control. 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 (2×10^5 /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. 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 performed 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). Statistical evaluations were performed by Kruskal-Wallis Test, Mann–Whitney U test and Two-way ANOVA. The analysis are indicated in the figures.

RESULTS

JALEx isolation from *Euphorbia umbellata* and chemical analysis.

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 triterpenes was predominant throughout the whole extract, with two main constituents standing out, represented as T1 and T2 in figure 1A. The triterpenes profile is in accordance with data from the literature that report the isolation of euphol, lanosterol, friedelin and 3 β -friedelinol in the latex, leaves

and stem of *E. umbellata* (**41**), whilst studies with the latex have led to the characterization of euphol and the steroid citrostadienol (**42**). 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 (Ph1 and Ph 2 - Fig. 1). The class of phorbol ester has been represented by the 12-O-tigloyl-4-deoxyphorbol-13-isobutirate (**43**), 12-deoxyphorbol-13-(2-methylpropionate), phorbol 12,13,20-triacetate (**44**), 4-deoxyphorbol-12,13-ditiglate and 3,4,12,13-tetraacetylphorbol-20-phenylacetate (**45**) distributed in several plant tissues (Fig. S1).

333

334 **Reactivation of latent HIV by JALEx and its cytotoxicity effects**

In this work, we used the alcoholic extracts from JALEx resuspended in DMSO. The reactivation of latent HIV was evaluated using the lymphocytic lineage J-Lat cells (clones 8.4 and 10.6) harboring the HIV integrated genome. The cells were exposed to increasing concentrations of JALEx, ranging from 10 to 10^{-7} $\mu\text{g/mL}$. The reactivation of latent HIV was evaluated by GFP reporter gene expression cloned in fusion with the virus integrated genome. In parallel, cytotoxicity assay was performed to evaluate whether the concentrations of JALEx would be toxic to these cells. JALEx treatment at concentrations of 10 to 0.01 $\mu\text{g/mL}$ showed HIV activation percentage similar to positive controls (PMA 1 μM and ING-B 0.32 μM), dropping to less than 5% reactivation at dilutions of 0.001 to 0.0000001 $\mu\text{g/mL}$ in the J-Lat 8.4 clone (Fig. 2A). Our results showed cytotoxic effects of JALEx at 10 $\mu\text{g/mL}$, while the other concentrations were well tolerated, showing a good cell viability (Fig. 2B). As expected, HIV reactivation by JALEx was more expressive in J-Lat cells clone 10.6, reaching values of 75% compared to 12% reactivation in 8.4 cells (Fig. 2C). In relation to the cytotoxicity, JALEx at 10 $\mu\text{g/mL}$ was also toxic for J-Lat 10.6, whereas the other concentrations presented cells viability between 80% and 75% (Fig. 2D). These results demonstrated the ability of JALEx to stimulate HIV transcription and reactivate virus from latency models.

354

355 **JALEx reactivation of latent HIV is mediated by PKC**

356 In order to evaluate whether JALEx activation of HIV transcription is
 357 mediated by PKC pathway J-Lat 8.4 and 10.6 cells were treated for 24 hours
 358 with different PKC inhibitors (Gö6666, Gö6983 and RÖ31-8220) to prevent
 359 JALEx stimulation. After this period, the cells were incubated with non cytotoxic
 360 concentrations of JALEx (0.01 and 0.001 µg/mL). PMA is a phorbol ester that
 361 activates different isoforms of PKCs and was used as positive control (1 µM).
 362 HIV reactivation was evaluated by flow cytometry for quantification of GFP
 363 positive cells. The pre-exposition of both clones of J-Lat cells (8.4 and 10.6) to
 364 the PKC inhibitors reverse the activation promoted by the PKC agonist PMA
 365 compared with PMA only treated cells (Fig. 3). The same results were found
 366 when J-Lat clone 8.4 was pre-incubated with inhibitors Gö6976, Gö6983 and
 367 RÖ31-8220 before JALEx treatment either at concentration 0.01 or 0.001 µg/mL.
 368 This result suggests that JALEx is a pan activator of different isoforms of PKCs
 369 (PKCα, PKCδ and PKCγ) (Fig. 3 - left panel). The same profile was found with
 370 the J-Lat clone 10.6 indicating that PKC inhibitors block the reactivation of latent
 371 HIV by JALEx treatment. RÖ31-8220 was the only inhibitor that could not totally
 372 prevent JALEx stimulation reaching values around 20% of activation compared
 373 with 60% of non-treated cells (Fig. 3 - right panel). All together, these results
 374 suggests that JALEx is a broad range PKC agonist, since the treatment with
 375 PKC inhibitors Gö 6976 (specific for PKC-α and β), Gö 6983 (specific for PKC-
 376 α, PKC-β, PKC-γ, PKC-δ and PKC-ζ) and RÖ-31-8220 (specific for PKC-α,
 377 PKC-β, PKC-ε and PKC-γ) decreased the levels of HIV reactivation compared
 378 with non treated cells.

379

380 **JALEx induces activation of different isoforms of PKC**

381 PKC family is a cytoplasmatic sensor of several signal transduction
 382 pathways by phosphorylation of hydroxyl groups in serine/threonine residues of
 383 other proteins. Moreover, the catalytic domain present in the structure of PKCs
 384 is essential for its auto-phosphorylation and, consequently, its cellular activation
 385 and relocation to the inner face of plasma membrane. PKC family comprises
 386 different isoforms spanning different regulatory and catalytic domains. The

isoforms are divided into three subgroups: the conventional isoforms α , β I, β II and γ that are Ca^{2+} and DAG dependent for activation; the novel class encompassing the δ , ϵ , η that require DAG, but not Ca^{2+} for activation and the atypical isoforms ι and λ that require neither Ca^{2+} nor diacylglycerol for activation. For these reasons, we investigated whether phosphorylation of different PKC isoforms was occurring in response to JALEx treatment. In these experiments, two concentrations of JALEx (0.01 $\mu\text{g/mL}$ and 0.001 $\mu\text{g/mL}$) were used and PMA was utilized as positive control of PKC phosphorylation. Cells were collected at different time points (10 and 30 minutes; 1, 6 and 24 hours). 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 (Fig. S2A, upper panels). This activation is long lasting, remaining phosphorylated for up to 24 hours after the treatments (Fig. S2A, upper panels). The same pattern was observed at the same time points for cells treated with PMA (Fig. S2A, upper panels). For the novel PKC isoforms, PKC θ exhibited a rapid phosphorylation pattern (10 min) that was not sustained over time (1 to 24h) (Fig. S2A, bottom panels). PKC δ showed an early phosphorylation pattern maintained for up to 6 hours with JALEx treatment in both concentrations (0.01 $\mu\text{g/mL}$ and 0.001 $\mu\text{g/mL}$). However, only JALEx at 0.001 $\mu\text{g/mL}$ induces PKC δ phosphorylation after 24 hours (Fig. S2A, bottom panels). Densitometry analyses compared with loading controls (α -tubulin) and normalized by DMSO treated cells showed that, in general, JALEx at concentrations of 0.01 $\mu\text{g/mL}$ and 0.001 $\mu\text{g/mL}$ induces the phosphorylation of conventional and novel isoforms of PKC that remains at least up to 6 hours of stimulation (Fig. S2B). However, as suggested by the WB analysis, the phosphorylation of PKC- δ is sustained until 24 hours post JALEx treatment. All these results are in agreement with the previous observations that JALEx is a pan activator of PKC isoforms.

activation, PKCs are translocated to the plasma membrane by RACK proteins (membrane-bound receptor for activated protein kinase C proteins) (46). To verify if JALEx promotes the translocation of PKC isoforms to the plasma membrane, HeLa cells were transfected with plasmids expressing three PKC isoforms fused to GFP (PKC α -GFP, PKC δ -GFP and PKC γ -GFP). After

transfection, cells were treated with 1 and 0.1 µg/mL of JALEx for 10 minutes or 24 hours and the cytoplasmatic location of PKC were evidenced by fluorescence confocal microscopy images. We observed a diffuse pattern of PKC distribution in untreated cells (mock). However, PMA or JALEx treatment at both concentrations (1 and 0.1 µg/mL) induced the periplasmatic distribution of all PKC isoforms (α, δ and γ) (Fig. 4A). The same results were obtained with longer treatment scheme (up to 24h) (Fig. 4B). Finally, these results showed that JALEx promotes activation of different classes of PKC through phosphorylation followed by downstream relocation to the plasma membrane.

JALEx promotes nuclear translocation of NF-κB and interaction with HIV promoter.

Phosphorylation of NF-κB inhibitor IκB by PKC leads to its ubiquitination and proteasomal degradation, releasing NF-κB complexes in the cytoplasm to translocate to the nucleus where they bind to HIV promoter (LTR region) and induce virus gene expression (47-49). For this reason, we evaluated the effect of JALEx treatment on nuclear translocation of NF-κB by means of two strategies. In the first one, 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, through immunofluorescence and confocal fluorescence microscopy using anti-p55 antibody. We observed no significant translocation of NF-κB to the nucleus of HeLa cells 6 hours post JALEx treatment with the same cytoplasmic distribution as observed in untreated cells (mock - Fig. 5A). However, we confirmed the NF-κB translocation to the nucleus after 24 hours of JALEx treatment, once NF-κB staining was located in the nuclei (Fig. 5B). To better quantify the translocation of NF-κB, we quantified the mean values of fluorescence intensity between the nucleus and the cytoplasm of 100 cells, in each experimental condition. We observed an increasing ratio of NF-κB fluorescence into the nucleus 24 hours after JALEx treatment, and the same was observed for the positive control (PMA - Fig. 5C). JALEx treatment at the concentration of 1 µg/mL showed the highest rates of NF-κB internalization (Fig. 5C).

We also evaluated the recruitment of NF- κ B to the HIV promoter by JALEx treatment. For that, we transfected Jurkat T CD4⁺ cells with LTR-luciferase reporter plasmids containing either intact NF- κ B binding sites (NF- κ B WT) or harboring mutations that prevent NF- κ B interaction (17, 50, 51). Transfected cells were treated with 1 μ g/mL of JALEx and luciferase activity was assessed 24 hours later. Our results showed that JALEx treatment induced a 5-fold increase in luciferase expression in cells transfected with pNF- κ B WT. However, no luciferase activity was observed in cells transfected with pNF- κ B MUT (Fig. 5D). Together, our results demonstrated that JALEx induces HIV transcription through NF- κ B release of its inhibitors, followed by nuclear translocation and binding to HIV promoter.

JALEx modulates surface markers expression and cytokines release in primary human CD4⁺ lymphocytes

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. Isolated TCD4⁺ cells were treated with PMA (1 μ M) or JALEx 10 μ g/mL or 1 μ g/mL for 24 hours. We evaluated the expression of HIV receptor and co-receptors (CD4, CCR5 and CXCR4) and activation markers (CD25, CD38, HLA-DR and CD69) in response to JALEx treatment. We observed that JALEx treatment (1 μ g/mL) downregulates the surface receptors CD4 and CXCR4 expression levels without any alteration in CCR5 expression levels (Fig. 6A). The same pattern was observed for cells treated with PMA (Fig. 6A). Indeed, JALEx treatment also induced the expression of surface activation markers CD25, CD38 and HLA-DR when compared to non-treated cells, and with PMA control (Fig. 6B). Figure 6C shows that CD69 activation marker expression is 75-fold higher relative to control cells in both treatments. These results suggest that JALEx not only reactivate latent HIV, but also could prevent new cycles of virus infection down modulating the virus receptors and co-receptors.

In the light of the previous results of JALEx inducing PBMC activation, we further analyzed cytokines release directly from the supernatant of human CD4⁺ T cells. This analysis was performed using a multiplex immunoassay to quantify

the production of 17 pro-inflammatory cytokines. JALEx treatment induced the production of the following cytokines relative to DMSO treated cells: IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 (p70), IL-13, IL-17, G-CSF, GM-CSF, MCP-1/MCAF, IFN γ and TNF- α (Fig. 6D). Overall, the highest concentration of JALEx (10 μ g/mL) induced the same levels of cytokines as did PMA positive control. IFN- γ and TNF- α were highly induced under JALEx treatment, 5,000 and 1,800 pg/mL, respectively (Fig. 6D). Those results showed that JALEx activate T CD4⁺ cells in order to secrete important pro-inflammatory cytokines involved in macrophage recruitment (MCP-1) and T CD8 response (INF- γ) to block HIV replication. MIP- β , IL-7 e IL-8 levels were undetectable.

494

495 **JALEx increases viral load in PBMC from HIV-1 positive patients and** 496 **induces Th1 and Th17 immune response**

Progression for AIDS in HIV-1 positive patients is mainly characterized by CD4⁺ T cell depletion specifically Th1 and Th17 cells, which impact negatively on immune protection against different opportunistic pathogens. We first analyzed the virus activation on PBMC isolated from HIV-1 positive patients under ART treatment. We found that JALEx at 1 μ g/mL increases HIV viral load up to 1.5 log compared with non treated PBMC cells (Fig. 7A). This data was significant in all PBMC generated from the 15 HIV positive patients. This increase in HIV viral load was followed by T CD4⁺ cells depletion (up to 82%) with no correspondence in T CD8⁺ cells (Fig. 7B). In order to investigate the immune response in those patients we evaluate cytokine production in T CD4 and T CD8 positive cells. JALEx treatment (0.1 μ g/mL) increased the frequency of CD4⁺ and CD8⁺ T cells secreting IL-17, IFN- γ and IL-21 cytokines. This induction was higher compared to PMA positive controls (Fig. 7C). Moreover, JALEx (0.1 μ g/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 (Fig. 7D). In contrast, no difference was observed regarding either IFN- γ or IL-17-secreting CD4⁺ and CD8⁺ T cells from PBMC cultures of HIV-1 positive patients following JALEx treatment (0.1 μ g/mL, Fig. 7D). These results suggested that JALEx treatment could reactivate HIV from reservoirs in patients

516 with ART suppressive therapy and induces CD4 and CD8 positive cells
517 secreting protective levels of IL-21.

518 JALEx reactivation effects in latently SIV-infected macaque cells

519 Finally, we explored the capacity of JALEx to reactivate latent viruses in
520 CD4⁺ lymphocytes from SIVmac239-infected *cynomolgus* macaques. We
521 purified resting memory CD4⁺ T cells from a single viremic macaque and
522 prepared serial dilution replicates of these cells to seed into quantitative viral
523 outgrowth assays (QVOA). Cells in these assays were stimulated in triplicate at
524 each cell dilution with JALEx or other compounds known to reactivate latent
525 HIV/SIV through PKC pathway (ingenol and concanavalin A). In order to
526 compare the ability of these compounds to reactivate latent SIV, we measured
527 the frequency of infected cells (quantified as infectious units per million cells;
528 IUPM) across treatments. A higher frequency of infected cells after stimulation
529 with a given compound suggests its efficiency to reverse latency. The highest
530 frequency of infected cells was observed in cultures treated with JALEx,
531 although this frequency was not significantly different from those observed in
532 other cultures (Table S1). However, JALEx was the only compound capable of
533 reactivating SIV in all replicates tested showing its superiority compared with
534 others PKC agonists, such as Ingenol and Concanavalin A.

535

536

537 DISCUSSION

538 In this work we addressed the ability of an ethanolic extract obtained
 539 from *Euphorbia umbellata* latex (JALEx) to reactivate HIV/SIV latency in several
 540 *in vitro* experiments. The traditional medical uses of this Euphorbiaceae are well
 541 known in South America and this plant is vastly used diluting a small volume of
 542 crude latex in drinkable water and consuming this solution regularly (24). JALEx
 543 contains a series of bioactive phorbol esters (see Supplemental Figure 1).
 544 These class of molecules such as PMA are known PKC agonist implicated in
 545 lymphocyte activation as well HIV reactivation in well conducted *ex vivo* studies
 546 and these activities requires both PKC α and PKC θ isoforms activation (52).
 547 Modifications in phorbol ring can lead molecules able to induce carcinogenesis
 548 and others such as a deoxy in ring position 4 (4-deoxyphorbol) can lead to
 549 molecules unable to induce carcinogenesis but retaining its PKC activation
 550 capacity (53). The presence of this modified phorbol ester was identified in our
 551 JALEx in our chemical analysis and in a previous study done with *Euphorbia*
 552 *umbellata* latex (45). Therefore, our JALEx is not likely to induce cell
 553 carcinogenesis retaining the capacity to reactivate HIV/SIV latency. Many LRA
 554 act activating PKC and several compounds such as prostatin (15), bryostatin
 555 (16), ingenol B (17), and phorbols such as PMA (18). Fit in this class of
 556 molecules Prostratin and bryostatin, are strong PKC agonist and they are very
 557 toxic when injected intravenously. In fact, bryostatin was used in a phase II
 558 study with 17 patients with progressive non-Hodgkin's lymphoma of indolent
 559 type (NHL), previously treated with chemotherapy. These patients received a
 560 median of 6 intravenous infusions of 25 $\mu\text{g}/\text{m}^2$ bryostatin 1 given once weekly
 561 over 24 h and the principal toxicities were myalgia and phlebitis (54). Ingenol B
 562 a semisynthetic compound isolated from *Euphorbia tirucalli* was able to
 563 reactivate HIV *ex vivo* with great potency using the PKC/NF- κ B pathway
 564 inducing increased cellular levels of CycT1 and CDK9 in human
 565 CD4+lymphocytes in *ex vivo* experiments (17).

566 In our study we used JALEx to explore its capability to reactivate HIV
 567 latency taking the advantage of the use of this plant in traditional medicine
 568 without serious side effects. First we used JALEx in J-Lat 8.4 and 10.6 cells and
 569 we could show the potency of this extract reactivates latent HIV transcription

570 present in J-Lat 8.4 and 10.6 cells in low concentrations (0.01 μ g / mL). We also
 571 demonstrate that JALEx act through PKC by inhibiting J-Lat activation with
 572 three different PKC inhibitors (G6666, G6983 and Ro-31-8220). In our assays
 573 Gö6983 was more powerful inhibitor totally blocking HIV reactivation in both J-
 574 Lat cell lines. Go 6983 is a fast pan-PKC inhibitor against PKC α , PKC β , PKC γ
 575 and PKC δ with IC₅₀ in low nM range then JALEx could be reactivating latent
 576 HIV through the activation of different PKC isoforms. Additionally, we showed
 577 that JALEx activates the PKC pathway by promoting NF- κ B internalization into
 578 the nucleus and increasing HIV transcription, which is dependent on NF- κ B
 579 binding to LTR promoter. Using image and western blot analysis we showed
 580 that JALEx was able to activate classical and novel PKC isoforms such as: the
 581 conventional isoforms α , β I, β II and γ as well the new ones encompassing the δ ,
 582 ϵ , η and θ isoforms in *in vitro* experiments using JURKAT cell lines. JALEx
 583 could induce θ isoform, in high levels. The transcription factors NF- κ B and AP-1
 584 are the primary physiological targets of PKC θ , and efficient activation of these
 585 transcription factors by PKC θ requires integration of TCR and CD28
 586 costimulatory signals. PKC θ cooperates with the protein Ser/Thr phosphatase,
 587 calcineurin, in transducing signals leading to activation of JNK, NFAT, and
 588 the *IL-2* gene. PKC θ also promotes T cell cycle progression (57).

589 We also evaluate the capacity of JALEx to induce cytokines production in
 590 human PBMC cells *in vitro*. This pattern of alteration in the production of
 591 cytokines from treatment with reactivating compounds is quite common in the
 592 case of the shock and kills strategy, which aims to activate the cells and
 593 promote the exit of HIV latency that is integrated into the cells T. This
 594 phenomenon is known as "cytokine storm" and needs to be further studied in
 595 animal models to define a dose that does not induce this toxic reaction.
 596 Although JALEx induced a conjunction of pro inflammatory cytokines we could
 597 observed a noticeable level of induction of IL-21 by different CD4+ and CD8+T
 598 lymphocyte subsets from PBMC cultures from Aids patients, a noble cytokine
 599 enrolled in the HIV virus load control in HIV+ individuals (55, 58, 59). For this
 600 reason, the search for new, more selective compounds that target only a few
 601 PKC isoforms or that have their mechanism of action in other stages of the
 602 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 (56).

In our experiments JALEx was efficient in reactivating HIV production directly in resting T CD4+ cells in HIV+ individuals in ex vivo assays measuring viral RNA in cell supernatant 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 could occur by three synergistic effects: a) reactivation of HIV from latency and its destruction by ART; 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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868 **Acknowledgments**

869 We thanks Dr. Thomas Friedrich from Virology Services Unit at the Wisconsin National
870 Primate Research Center, Wisconsin, USA for QVOA assay using SIVmac239 in NHP
871 CD4⁺ Lymphocytes. We are also grateful to Mr. Wilson Camargo Barros Filho and Mr.
872 Luis Antônio Nogueira for providing *E. umbellata*'s latex used in this work. This work
873 was sponsored by CNPq, FAPERJ, CAPES and FINEP.

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876 FIGURES AND LEGENDS

Figure 1. Isolation and chemical structures of JALEx. Gas chromatograms of (A) whole extract JALEx and (B) Phorbol esters-enriched JALEx fractions. 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.

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Figure 2: JALEx reactivates virus transcription in HIV-1 latent models. J-Lat 8.4 (A) and 10.6 (C) cells were exposed to increasing concentrations of JALEx and HIV-1 activation was assessed 24 hours post-treatment by GFP positive cells screening by flow cytometry. Mock stands for not treated. PMA (1 μ M) and INGB (0.32 μ M) were used as positive induction controls. The cytotoxicity of JALEx was evaluated using CellTiter Blue vital stain 5 days after treatment in J-Lat 8.4 (B) and 10.6 cells (D). GFP positivity and cytotoxicity were expressed as percentage of cells normalized by mock non-treated cells (n=3). The dashed lines in Figure 2 B and D represents 100% of viability measures in MOCK. The bars in the figure represents 1 SD around the mean.

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 (n=1).

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Figure 4: 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 JALEx. (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 JALEx. As a positive control, PMA (1 μM) was used (n=3).

Figure 5: . JALEx -mediated transcriptional activation is dependent on NF-κB 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 NF-κB, 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 (n=3; Two-way ANOVA and (***) indicate p<0,0001). (D) Jurkat cells were transfected with pBlue30LTR-Luc (NF-κB WT) and the mutant construct, pBlue30LTR NF-κB MUT-Luc (NF-κB MUT), absent from the NF-κB binding site, preventing its interaction with NFκB. After transfections, the cells were treated with 1μg / 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 (n=4, Mann-Whitney test and (*) indicate p<0,05). The bars in Figure 5 C and D represents 1 SD around the mean.

Figure 6: JALEx modulates the expression of cell surface receptors, activation markers and cytokine secretion in primary CD4 + T cells from HIV- individuals. 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 (n=4). (B) Expression of CD25, CD38 and HLA-DR activation markers in CD4 + T cells isolated after treatment with JALEx (n=4). (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 μ M) and the gray bar represents the treatment with 10 μ g / mL of JALEx (n=4). (D) CD4+ T cells were treated with 1 μ M PMA, 10 μ g/mL (-3) or 1 μ g/mL (-4) of JALEx for 24 hours. After, supernatants from the CD4+ T cells were collected and cytokine analysis were performed by Bio-plex Pro Human Cytokines 17-plex platform (n=5, Kruskal-Wallis test and (*) indicate p<0.05). The bars in the figure represents 1 SD around the mean.

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887 **Figure 7. Effect of JALEx on T cell viability, HIV-1 replication in PBMC cultures and**
 888 **cytokine secretion in T cells from AIDS patients.** Different concentrations of JALEx (0.01, 0.1
 889 and 1 μg/mL) were added to PBMC cultures (1 x 10⁶/mL) obtained from 15 AIDS patients, and
 890 24h after, the (A) the number of HIV-1 into supernatants was determined through RT-qPCR, (B)
 891 CD4⁺ and CD8⁺ T cells viability was analyzed by FACS after 7-AAD staining. AIDS-derived
 892 PBMC cultures (1 x 10⁶/mL, n=15) were maintained for 24h in the presence of medium alone or
 893 with different JALEx (0.01 and 0.1 μg/mL). As positive control, some well receiving PMA plus
 894 ionomycine (PMA/IO). The percentage of CD4+ and CD8+ T cells producers of IFN-γ, IL-17, IL-
 895 21 and IL-10 was determined by cytometry (C). The mean values were compared and the p
 896 values are shown in the figures. In (D), the proportion of different (CD4+ and CD8+) T cell
 897 subsets of secreting different combinations of IL-17, IFN-γ and IL-21 in response to JALEx (0.1
 898 μg/mL) was also evaluated by cytometry. In the figure, (*), (**), and (***) indicate p<0.05,
 899 p<0.001 and p<0.0001, respectively. The bars in the figure represents 1 SD around the mean.

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907 **SUPPLEMENTAL MATERIAL**

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909 **Supplemental Figure 1**

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911 **Figure S1:** Phorbol esters isolated from *Euphorbia umbellata*. I = 12-O-Tigloyl-4-deoxyphorbol-
912 13-isobutyrate; II = Phorbol-12,13,20-triacetate; III = 12-Deoxyphorbol-13-(12-
913 methylpropionate); IV = 3,4,12,13-Tetraacetylphorbol-20-phenylacetate (synagranol A); V =
914 Deoxyphorbol-12,13-ditiglate (synagranol B).

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Supplemental Figure 2

935 **Supplemental Table 1**

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937 **Table S1- QVOA results using macaques CD4+ T Lymphocytes extracted from**
 938 **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}

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