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Potential antiviral and immunomodulatory activity of Amazonian medicinal plant compounds

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Abstract

Novel antiretroviral drugs are constantly needed for human immunodeficiency virus (HIV)/acquired immunodeficiency syndrome (AIDS) patients to confront the continuously emerging resistance to the commonly prescribed combination of anti-HIV synthetic agents and their side effects. Amazonian medicinal plants, *Licania macrophylla* (LM) (*Chrysobalanaceae*) and *Ouratea hexasperma* (OH) (*Ochnaceae*), were assayed for antiretroviral and immunomodulatory activity, by utilizing an established human leukocyte cell line and the simian immunodeficiency virus (SIV). Interleukin (IL)-4, IL-6, IL-8, IL-10, and interferon-gamma were quantified after leukocyte culture was stimulated with ethanolic plant extracts and subsequently challenged with lentivirus infection. Mitotic activity induced by OH extract was significantly more pronounced than that of LM extract. Cytokine modulation was observed in SIV-infected cells under independent treatment with OH and LM extracts. Betulinic acid, niruriflavone, (-)-epigallocatechin, (-)-gallocatechin, and 4′-O-methyl-epigallocatechin were isolated from LM. In summary, the tested extracts hold application potential in the therapy of HIV/AIDS pathology by regulating cellular proliferative activity and cytokine levels, as the isolated compounds from these plants have been reported to exhibit antiviral activity.

Keywords: Simian immunodeficiency virus; Human leukocytes; *Ouratea hexasperma*; *Licania macrophylla*

1. Introduction

Amazonian medicinal plants boast a major source of therapeutic agents and active pharmacological compounds. Despite the superiority of bioinformatic approaches in altering natural compounds and/or designing and synthesizing new drugs, materials sourced from biodiverse environments remain the most powerful and potential options for discovering new chemical compounds to be used as a large array of prophylactic, therapeutic, and cosmetic applications. Nevertheless, both strategies are applied with the common purposes of rationally exploring natural resources and putting them to beneficial use.[1,2] Infectious diseases, particularly those of viral etiology, have agents that are suitable for vaccine development; however, the high genetic variability of RNA viruses hinders the protective role of immunogens under investigation, as seen with the dengue virus, human immunodeficiency virus (HIV), and severe acute respiratory syndrome coronavirus 2, among other pathogens of epidemiological significance.[3] Chemical compounds that interact with viral receptors or function as ligands for cell receptors display varied activities. Therefore, possible mechanisms with virus, glycoproteins, and/or ligands, such as blocking their coupling to host cell receptors or acting as metabolites to induce cytokine expression, are under investigation.[4]

The arsenal of antiretroviral drugs must be continuously expanded, mainly due to the emergence of drug resistance and the development of side effects, which commonly occur in individuals receiving antiretroviral therapy for HIV infections and related diseases, such as acquired immunodeficiency syndrome (AIDS).[5,6] The host defense system against viral infections begins with the organism's physical, chemical, and biological barriers to invasive agents and can continue with the innate immune response, which may or may not progress to the adaptive immune response. The immune response process usually involves cytokine signaling to activate the effector mechanisms of both innate and adaptive immune responses.[7,8] Cytokines mediate both the inflammatory and anti-inflammatory host responses during infection, and in healthy individuals, morbidity is usually resolved.[9] Nowadays, mainly in urban areas, people are often deprived of a health environment, as well as adequate nutrition, mental well-being, and emotional balance.[10] Depression is prevalent in large metropolitan areas worldwide and is immunologically characterized by the secretion of proinflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factor-alpha, and IL-6.[11] In addition, chronic inflammation predisposes individuals to a large array of diseases, mainly linked to poor immune responses to infectious agents.[12] Amazonian biodiversity provides a bunch of natural resources for human health, including medicinal plants that are commonly adopted in traditional medicine to treat various ailments. However, the folklore and knowledge of ancient civilizations regarding these plants remain largely undocumented in scientific literature, with only the preparation methods and usages being preserved. To recover the chemical, biological, and medical knowledge of these plants, systematic documentation of their use, geographical distribution, and preliminary chemical and biological studies have been conducted.[13-17]

Licania macrophylla (LM) (Benth), which was later reclassified as *Hymenopus macrophyllus* (Benth) by Sothers and Prance[18] in the *Chrysobalanaceae* botanical family, is popularly known as "anauerá," a name derived from a native Amerind language in the Amazon. LM exhibits a typical bush morphology, reaching a height of 17 – 28 m and growing along the margins of inundated areas. In addition to its medicinal use in treating gastrointestinal disorders of infectious etiology, its timber is employed for construction purposes.[19] The chemical compounds of anauerá have been isolated and characterized, such as licanol, a flavanol identified as (-)-4'-O-methyl-epigallocatechin-3'-O-α-L-rhamnoside.[20] Previously, the inhibitory activity of LM ethanolic extract (bark) against multidrug-resistant *Staphylococcus aureus* and *Pseudomonas aeruginosa*[21] was reported.

Commonly known as "barbatimão do cerrado" among natives in northern Brazil, *Ouratea hexasperma* (OH) (A. St. -Hil.) Baill is taxonomically classified within the *Ochnaceae* botanical family. Its shrubby structure reaches a height of 5 m, featuring wrinkled bark and tortuous branches, which are typical of "cerrado" vegetation (Brazilian savanna).[22] Phytochemical analysis carried out by Daniel *et al*.[23] revealed the presence of 7"-O-methylagathisflavone, agathisflavone, epicatechin, and the mixture of 6-C-glycopyranosyl-luteolin and 3-O-glycopyranosyl-quercetin. In addition, Fidelis *et al*.[24] reported the isolation of trans-3-O-methyl-resveratrol-2-C-β-glucoside, litho spermoside, 2,5-dimethoxy-p-benzoquinone, lup-20(30)-ene-3β,28-diol, 7-O-methylgenistein, apigenin, luteolin, and amentoflavone. The above-characterized compounds and crude extracts of OH have exhibited various *in vitro* biological activities, such as antioxidant, anticarcinogenic, antidiabetic, antimicrobial, and anti-inflammatory properties, among others.[17,24,25]

2. Materials and methods

2.1. Plant extracts

Botanic samples of LM (*Chrysobalanaceae*) and OH (*Ochnaceae*) were collected from lowland areas surrounding the Amapari river at the geographic coordinates 0°46'14"N/51°55'54"W (Pedra Branca do

Amapari municipality) and in the Amapa "cerrado" (savanna) at 0°55'51"N/51°11'35"W (Ferreira Gomes municipality), respectively (Figure 1). One hundred grams of each dried and pulverized plant material, including the leaves of LM and the bark of OH, were macerated in 1 L of ethanol for 7 days at room temperature. This procedure was repeated 3 times. The extract was obtained using a rotary evaporator (Quimis, Brasil) at 40°C under low pressure and kept under refrigeration until use. Plant materials of OH and LM were deposited at the Institute of Scientific and Technological Research of Amapa State under registration numbers 16593 and 16594, respectively.

2.2. Ultra-high-performance liquid chromatography (UHPLC) analysis

The methanolic extract of LM was analyzed by ultra-high-performance liquid chromatography (Shimadzu Nexera, Japan), which composed of a controller (CBM 20-A), a degasser (DGU-20A), two binary pumps (Nexera X2 LC-30AD), an autoinjector (Nexera X2 SIL-30AC), a thermostable column compartment (CTO-20AC), a photodiode array detector (PDA)/ultraviolet detector (SPD-M20A), and a triple quadrupole mass spectrometer (LC-MS-8030) equipped with an electrospray ionization source. Separation was achieved on an LC18 column of 100 × 2.1 mm and 1.6 µm particle size (Luna Omega Polar, Phenomenex, United States). Data were processed using LabSolutions LCMS software version 5.96 (Shimadzu Corporation, Japan).[13,15]

2.3. Cell viability assay

Human leukocytes transformation was carried out as described elsewhere.[26,27] Briefly, leukocytes were obtained from total heparinized blood by centrifugation in a sucrose gradient (Histopaque-1077, Sigma, United States), and then maintained in RPMI medium supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin and streptomycin, Sigma, United States), and transformed by adding 10% of Hut-78[28] cell culture supernatant. Cells were cultivated in an incubator (Sanyo, Fisher Scientific, United States) at 37°C, 5% CO_2 atmosphere, and controlled humidity. The established human leukocyte culture (EHLC) was treated with multiple serial dilutions of each extract. Cell viability was assessed using Trypan blue staining (Sigma, United States) to identify and count damaged/dead cells,[29] as well as spectrophotometric measurement of mitochondrial succinate tetrazolium reductase enzyme activity in the cell culture supernatant, using a commercial cytotoxic assay kit (CytoSkan WST-1, Roche, Switzerland).[30]

2.4. Antiretroviral assay and cytokine quantification

EHLC, cultivated in 25 cm^2 flasks with 5×10^5 cells/mL, was treated with a low cytotoxic concentration (1:1024) of plant extracts. The cell viability was subsequently determined using Trypan blue staining and spectrophotometric quantification of mitochondrial succinate tetrazolium reductase enzyme activity.[29,30] After 24 h of treatment, cells were infected with 100 µL of SIVmac251 (NIH AIDS Research Reagents, United States), containing an SIVp27 concentration of 599.64 pg/mL, as a measure of viral load.[27] Cell supernatant samples were harvested at 48, 144, and 240 h post-infection, centrifuged, and kept at −20°C for later use in antiretroviral activity assessment by quantifying SIVp27 antigen using the SIVp27 antigen capture assay (Advanced Bioscience Laboratory, Inc, United States). In addition, samples were used for quantification of interleukin (IL)-4, IL-6, IL-8, IL-10, and interferon-gamma (IFN-γ) using the DuoSet ELISA Development System (R&D Systems, United States). After each cell supernatant collection, an equal amount of 3 mL of fresh medium was added. SIVmac251 was previously produced in Hut-78 cells, and the SIVp27viral antigen was purified from the culture supernatant. For viral antigen quantification, 25 µL of disruption buffer was added to each microtiter well pre-coated with monoclonal antibodies against SIVp27 antigen, followed by the addition of 100 µL of cell supernatant to each well. A serial dilution of SIVp27 standard antigen (2,000 – 62.5 pg/mL) was prepared, including the negative control containing a complete RPMI medium. After 60-min incubation at 37°C, the wells were washed and 100 µL of horseradish peroxidase-labeled mouse monoclonal antibody against SIVp27 was added, followed by another 60-min incubation. The wells were then washed, and 100 µL of peroxidase substrate was added. After 30 min at room temperature, the chromogenic reaction was stopped by adding 2N sulfuric acid solution. The absorbance was measured at 450 nm using a microplate reader (Thermoplate, Italy), with results expressed in optical density.

Figure 1. Sites of plant material collection in Amapa state, northern Brazil. Notes: Black pin: Pedra Branca do Amapari municipality; Red pin: Ferreira Gomes municipality.

2.5. Cytokine quantification

EHLC was treated with an optimal, low-toxicity concentration of plant extract and infected with SIVmac251 after 24 h. Supernatant samples were harvested at 48, 144, and 240 h post-infection. The control groups included supernatant from uninfected cells and simian immunodeficiency virus (SIV)-infected cells, SIV-uninfected and plant extract-untreated cells, as well as SIV-uninfected and plant extract-treated cells. Plate wells were sensitized overnight at 4°C with capture antibodies for each IL-4, IL-6, IL-8, IL-10, and IFN-γ, diluted in PBS according to the kit instructions (DuoSet ELISA Development System, R&D Systems, United States). After 4 times washes with buffer, the blocking solution (PBS-BSA 1 – 0.05% NaN_3) was added. Following a 1-h incubation at room temperature, the wells were washed again, and reference ILs and leukocyte cell culture supernatant were added to each well in triplicate. Samples were incubated at room temperature for 2 h. Subsequently, diluted detection antibody solution was added to each washed well. After 2-h incubation, streptavidin-peroxidase solution was introduced, followed by a 20 min incubation and subsequent washing. Chromogenic substrate solution (TMB) was then added, protected from light exposure, and incubated for 30 min. Sequentially, the reaction was stopped with sulfuric acid solution, and absorbance was measured at 450 nm using a multiplate spectrophotometer (Thermoplate, Italy), with results expressed in optical density. These results were further confirmed by immunoassays for the same cytokines by utilizing ELISA kits kindly donated by ImmunoTools GmbH.[27]

2.6. Statistical analysis

The obtained data were analyzed by utilizing GraphPad Prism software (version 10.4, US) and subjected to two-way ANOVA, followed by Tukey's multiple comparison tests, where appropriate.

3. Results

The UHPLC-PDA-MS analysis revealed betulinic acid and niruriflavone as the most abundant compounds in the leaf extract of LM. Additional compounds identified by comparison with the literature data included (-)-gallocatechin, (-)-epigallocatechin, and (-)-4'-O-methyl-epigallocatechin.

Previously, cytokine production was not detected in the Hut-78 cell line supernatant after stimulation with plant extracts and SIV infection. Therefore, EHLC was generated to carry out experiments to detect cytokine production.

The optimal plant extract dilution was determined based on cytotoxic assay results and phenotypic analysis (cell counting/Trypan blue staining). An optimal dilution of 1:1024, corresponding to 0.34 µg/mL for both botanical species, was utilized to treat the EHLC in culture. Cells were counted at time intervals ranging from 48 to 288 h post-treatment with plant extracts. Regarding LM extract treatment, at the initial and final time intervals of 48 and 288 h, treated cells had 1.5 times more cells than in the control (1.5×10^5 treated cells vs. 1.0×10^5 untreated cells) and 2.3 times more cells than in the control (1.6×10^5 treated cells vs. 0.7×10^5 untreated cells), respectively, with statistically significant differences (p=0.0133 and p=0.0122, respectively). At the 96-h time point, there was no significant difference in cell density between treated and untreated cells (p=0.0842). At intermediate intervals of 144, 192, and 240 h, LM extract-treated cells exhibited reduced growth than in the control, with statistically significant differences (p=0.0069, p=0.0177, and p=0.0039, respectively). Cells treated with OH extract had exceptional cell growth at 48 and 96 h post-treatment compared to the control, with increases of about 3.3 times and 1.4 times (p=0.0182 and p=0.0087, respectively). At 192 h, the growth of treated cells dropped from 4.6×10^5 to 2.8×10^5 cells/mL, while untreated cells remained at 4.4×10^5 cells/mL (p=0.0087). At the final time interval of 288 h, OH extract-treated cells continued growing, reaching 1.5×10^5 cells/mL, whereas the untreated cell growth declined to 0.5×10^5 cells/mL (p=0.0033). Comparing the mitotic activity between LM and OH extract treatments, OH extract-treated cells generally showed higher cell density than in LM extract-treated cells. From 48 to 192 h, the mitotic activity difference between OH and LM extract-treated cells reduced from 2.4 times to 1.5 times in favor of OH extract treatment (p=0.018, p=0.0074, and p=0.0170, respectively). At the final intervals of 192 – 288 h, the difference in cell proliferation between the treatments was minimal (p=0.1001 and p=0.0125) (Figure 2).

EHLC protection against lentivirus infection (SIVmac251) and/or viral protein synthesis, following treatment with LM and OH extracts, was evaluated based on cell survival and cytopathic effects. LM extract treatment had significant protective effects at 144 h and 192 h (p=0.0127 and p=0.0476, respectively), while OH extract treatment reduced the viral cytopathic effect at 96 h (p=0.0144). However, at the final time points of 240 h and 288 h, neither extract provided significant protection to the cells (Figure 3).

Cytokine expression by cells after treated with plant extracts and challenged with lentivirus infection (SIVmac251), along with controls represented by untreated and SIV-infected cells, showed the following patterns. Regarding cells culture treated with OH extract

and infected with SIV, IL-4 expression (Figure 4) was significantly higher at the 48-h interval in comparison to all other treatments (p-value ranging from 0.0021 to 0.0210), except in LM extract treatment (p=0.1057). At the 144-h interval, IL-4 expression remained significantly higher (p-value ranging from 0.0004 to 0.0022) than in all other treatments, including LM extract treatment combined with SIV infection and LM extract treatment alone (p-value ranging from 0.2736 to 0.3056). At 240 h, IL-4 expression was significantly higher compared to all other treatments, including SIV infection alone (p-value ranging from 0.0197 to 0.0205). Cells treated with LM extract and infected with SIV did not display significant differences in IL-4 expression compared to all other treatments at any time interval. In the initial stage of the experiment, differences were not significant between cells treated with either extract alone or when infected by SIV (p>0.9999). However, in all other conditions, comparing extract-treated cells to extract-treated and SIV- infected cells, there were significant statistical differences (p=0.0040).

IL-6 expression (Figure 5) by EHLC at the 48-h interval showed a significant difference (p=0.0006), with higher secretion in LM extract-treated cells. Comparing SIV-infected cells, including those previously and independently treated with LM and OH extracts, it was observed that OH extract-treated cells had significantly higher IL-6 secretion (p=0.0030). Cells treated with LM extract alone produced significantly more IL-6 than in OH extract-treated and SIV-infected cells (p=0.0033). Similarly, LM extract-treated cells had significantly higher IL-6 production than in OH extract-treated cells (p=0.0049). Other treatment conditions, with or without SIV infection, as well as controls, did not show statistically significant differences (p-value ranging from 0.0001 to 0.1467). At the 144-h interval, the highest IL-6 secretion (p<0.0001) was observed in cells treated with OH extract alone compared to LM extract-treated and SIV-infected cells, as well as in comparison to OH extract-treated and SIV-infected cells and controls (p=0.011). No statistically significant differences were observed in other conditions.

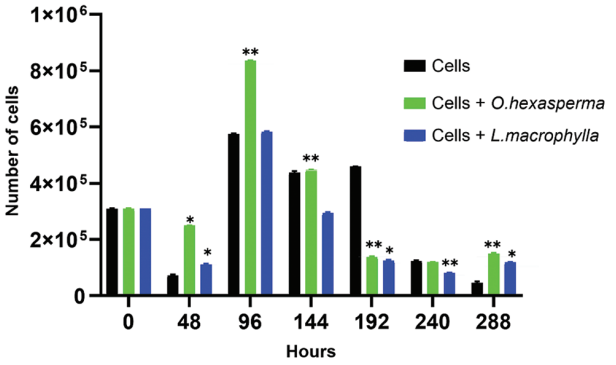

Figure 2. Growth of the established human leukocyte cell line population under treatment with *Licania macrophylla* and *Ouratea hexasperma* extracts over a 288-h culture period. At the initial time point of 0 h, all cells, both treated and untreated had the same population density. Note: *indicates significant at p<0.05.

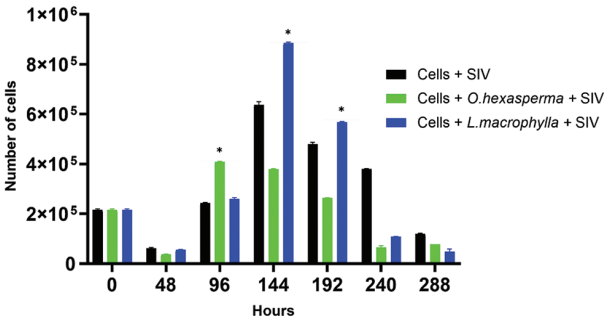

Figure 3. Established human leukocyte cell line treated for 24 h with *Licania macrophylla* and *Ouratea hexasperma* extracts, independently, and subsequently infected with SIVmac251. Cell viability was assessed up to 288 h of culture. At the initial time point of 0 h, all cells had the same population density. Note: *indicates significant at p<0.05.
Abbreviation: SIV: Simian immunodeficiency virus.

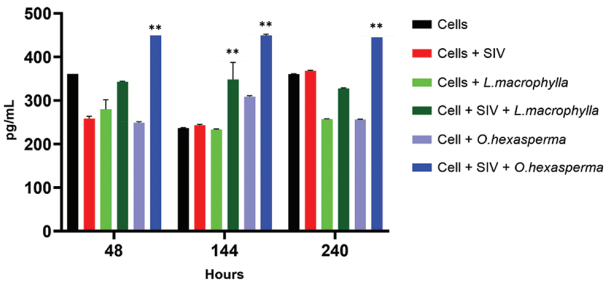

Figure 4. Interleukin-4 expression in an established human leukocyte cell line treated with *Licania macrophylla* and *Ouratea hexasperma*, followed by SIV infection, and in control cells treated with extracts but not infected with SIV. Note: *indicates significant at p<0.05.
Abbreviation: SIV: Simian immunodeficiency virus.

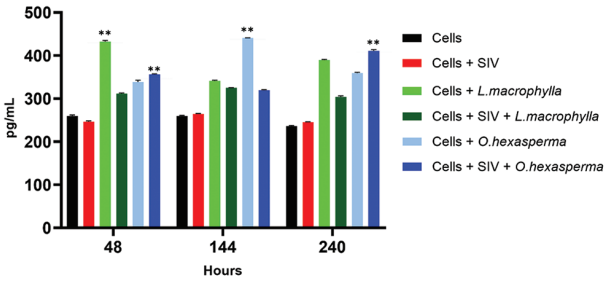

Figure 5. Interleukin-6 expression in an established human leukocyte cell line treated with *Licania macrophylla* and *Ouratea hexasperma*, followed by SIV infection, and in control cells treated with extracts but not infected with SIV. Note: *indicates significant at p<0.05.
Abbreviation: Simian immunodeficiency virus.

At the final 240-h interval, OH extract-treated and SIV-infected cells produced significantly more IL-6 than in LM extract-treated and SIV-infected cells (p=0.0013), as well as in comparison to cells treated with OH extract alone and controls (p=0.0177). Cells treated with LM extract alone had significantly higher IL-6 production than in LM extract-treated and SIV-infected cells (p=0.0017), as well as in comparison to cells treated with OH extract alone and controls (p=0.0096). However, there was no statistically significant difference between LM extract-treated and OH extract-treated and SIV-infected cells (p=0.0907).

At the 48-h time interval, EHLC treated with LM extract and infected with SIV produced significantly more IL-8 (Figure 6) than in cells treated only with OH extract (p=0.0241), as well as cells treated with OH extract and SIV-infected, and the controls (p=0.0113). Cells treated with LM extract alone also secreted significantly more IL-8 than in cells treated with OH extract alone (p=0.0248). The other treatments did not show any statistically significant differences (p-value ranging from 0.0530 to 0.1031). At the 144-h time interval, cells treated with OH extract alone secreted more IL-8 than in cells treated with LM extract alone (p=0.0058), as well as cells treated with OH extract and infected with SIV (p=0.0447). Cells treated with LM extract and SIV-infected expressed significantly more IL-8 than in cells treated with LM extract alone (p=0.0195), as well as cells treated with OH extract and infected with SIV (p=0.0386). Comparisons among cells treated with OH extract, with or without SIV infection, and cells treated with LM extract, with or without SIV infection, along with controls, did not show any statistically significant differences (p-value ranging from 0.0376 to 0.1541). Finally, at the 240-h time interval, leukocytes treated with OH extract and infected with SIV secreted significantly more IL-8 than in cells treated with OH extract alone (p=0.0036), as well as cells treated with LM extract alone (p=0.0016) and the controls (p=0.0015). The other conditions had no statistically significant differences in IL-8 expression (p-value ranging from 0.0058 to 0.2367).

At the 48-h time interval, only cells with LM extract secreted IL-10 (Figure 7), while in all other conditions, the cytokine was not detected. At the 144-h time interval, cells treated with LM extract alone produced significantly more IL-10 than in cells treated with OH extract (p=0.0156), and cells treated with LM extracted and infected with SIV (p=0.0209), while cytokine production remained negligible in the other treatments. At the final time interval of 240 h, cells treated with LM extract continued to produce more IL-10 than in other treatments, including cells treated with OH extract, whether infected with SIV or not (p-value ranging from 0.0131 to 0.0334).

After a 48-h incubation period, cells treated with LM extract produced significantly more IFN-γ (Figure 8) than in cells treated with LM extract and infected with SIV (p=0.0028). Other treatments did not show any significant statistical differences (p-value ranging from 0.0530 to 0.7585) among the following comparisons: OH extract treatment versus OH extract treatment and SIV infection; OH extract treatment versus LM extract treatment; OH

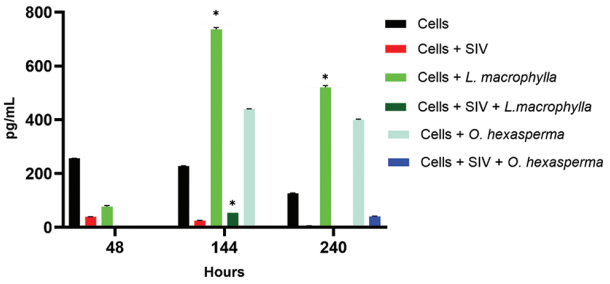

Figure 7. Interleukin-10 expression in an established human leukocyte cell line treated with *Licania macrophylla* and *Ouratea hexasperma*, followed by SIV infection, and in control cells treated with extracts but not infected with SIV. Note: *indicates significant at p<0.05.
Abbreviation: SIV: Simian immunodeficiency virus.

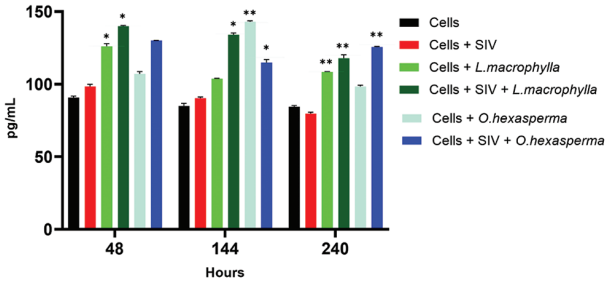

Figure 6. Interleukin-8 expression in an established human leukocyte cell line treated with *Licania macrophylla* and *Ouratea hexasperma*, followed by SIV infection, and in control cells treated with extracts but not infected with SIV. Note: *indicates significant at p<0.05.
Abbreviation: SIV: Simian immunodeficiency virus.

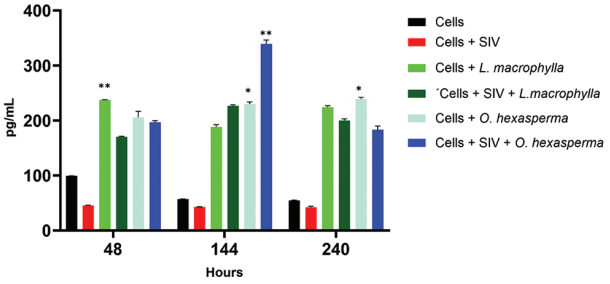

Figure 8. Interferon-gamma expression in an established human leukocyte cell line treated with *Licania macrophylla* and *Ouratea hexasperma*, followed by SIV infection, and in control cells treated with extracts but not infected with SIV. Note: *indicates significant at p<0.05.
Abbreviation: SIV: Simian immunodeficiency virus.

extract treatment versus LM extract treatment and SIV infection; OH extract treatment and SIV infection versus LM extract treatment; OH extract treatment and SIV infection versus LM extract treatment and SIV infection. At the next time interval of 144 h, cells treated with OH extract and infected with SIV expressed significantly more IFN-γ than in cells treated with LM extract, whether infected with SIV or not (p-value ranging from 0.0073 to 0.0370), as well as cells treated with OH extract or LM extract alone (p-value ranging from 0.0177 to 0.0252). Other comparisons did not show any statistical differences (p-value ranging from 0.0557 to 0.7666) among the following conditions: OH extract treatment versus LM extract treatment and SIV infection; LM extract treatment versus LM extract treatment and SIV infection. At the final 240-h incubation period, cells treated with OH extract expressed significantly more IFN-γ than in cells treated with OH extract and infected with SIV, as well as cells treated with LM extract and infected with SIV (p-value ranging from 0.0159 to 0.0475). In addition, cells treated with LM extract produced significantly more IFN-γ than in cells treated with LM extract and infected with SIV (p=0.0338). Other comparisons (OH extract treatment and SIV infection versus LM extract treatment and SIV infection; OH extract treatment and SIV infection versus LM extract treatment; OH extract treatment versus LM extract treatment) did not show any significant statistical differences (p-value ranging from 0.0887 to 0.2624).

Summarizing the collective effects of plant extract treatments on both uninfected and SIV-infected cells, the compounds from both plants positively modulate cytokine expression, highlighting their potential therapeutic applications. Considering the integration of the lentiviral genome, the behavior of proviral forms within the cell metabolism remains complex, influenced by a plethora of factors, including the metabolic stimuli imposed by the plant compounds. IL-4 production is mostly triggered by OH extract (Figure 4), while IL-6 (Figure 5) and IL-8 (Figure 6) expression are induced by both extracts. The synthesis of IL-10 (Figure 7) is mainly stimulated by LM extract, whereas IFN-γ expression (Figure 8) is much more modulated but strongly stimulated by both extracts.

In all experiments, cytokine production is proportional to the cell density (Figures 4-8). While manual cell counting on the microscope using Trypan blue staining offers advantages over flow cytometry, such as the ability to visualize syncytia formation, which indicates the level of protection conferred by extract treatment. Moreover, the cell proliferation and mitotic activity induced by plant extracts (Figures 2 and 3) are clear, in contrast to the syncytium formation induced by lentivirus infection.

4. Discussion

Certainly, *in vitro* experiments have inherent limitations. In natural lentivirus infections, the virus surpasses the initial physical and biochemical barriers of the skin and mucosae, leading to virion phagocytosis and infection of $CD4^+$ T cells. Phagocytes, mainly macrophages and dendritic cells, work as Trojan horses, disseminating virions throughout the body, including hard-to-reach tissues. The failure of innate immune defenses triggers a battle between the adaptative immune response and the lentivirus's multiple evasion strategies, represented by the coordinated action of T and B lymphocytes and other associated immune cells. The generation of proviral forms in different infected cell populations and their migration to immune-privileged sites, such as gut-associated lymphoid tissue, the central nervous system, lymph nodes, and myeloid cells, makes it difficult to get rid of lentiviral infection.[31,32] In this *in vitro* system, transiently transformed human leukocytes serve as cell targets for SIV infection, along with established leukocyte cell lines such as Hut-78 and Molt-4,[33] which are very suitable for lentivirus infection but exhibit a limited immune response.[26,27] Therefore, this cell system represents a valuable model to evaluate cellular immune responses.[27] A collection of medicinal plants has been methodically tested for antiretroviral activity,[17,33] with additional research focusing on immune responses triggered by natural products.[27] In the folklore medicine, particularly in the Amazon region of Brazil, the therapeutic properties of medicinal plants, along with beliefs in their spiritual healing abilities, are typically based on the symptoms and signs observed in affected individuals. This knowledge has been passed down through generations among different Amerindian ethnic groups.[34] Therefore, analysis is mainly based by the indications of traditional medicine for the treatment of cancer, diarrhea, renal and cardiovascular diseases, diabetes, and other conditions. Among species of the *Chrysobalanaceae* family, some are traditionally used to treat infectious diseases, cancer, and diabetes. The *Ochnaceae* family, on the other hand, has a much broader range of therapeutic applications, including antiretroviral activity.[33]

Lentivirus infection in both humans and non-human primates increases the expression of inflammatory cytokines, prominently IL-8, alongside elevated SIV/HIV viral proteins synthesis during the virus replication cycle.[35] The consistently high IL-8 expression observed all time intervals, up to 240 h (Figure 6), indicates persistent SIVmac251 replication and chronic infection (Figure 3). This naturally occurs in lentivirus-infected hosts, mainly in untreated individuals, as well as in *in vivo* experiments reported by other authors. Notably, the HIV/SIV *vpr*

gene product is responsible for the elevated expression of IL-6, IL-8, and IL-10, among other cytokines in primate leukocyte cells used in these experiments.[35] Moreover, IL-8 and IL-6 expression are positively correlated to clinical symptoms and disease progression in HIV/AIDS patients.[36,37] The results obtained in this study corroborate with those of Dwivedi *et al*.,[37] who analyzed a cohort of HIV reservoir patients and found that HIV unspliced RNA was significantly associated with IL-10 signaling, as well as other factors such as toll-like receptor 4/microbial translocation and IL-1/NLR family pyrin domain containing 3 inflammasome. In the experiments, IL-10 was poorly expressed in SIV-infected cells (Figure 7), regardless of prior treatment with plants extracts, but it was abundantly secreted by uninfected cells, whether treated with plant extracts or not. This suggests that in this *in vitro* system, HIV unspliced RNA is rare or absent. If reduced IL-10 production by EHLC treated with LM and OH extracts, and subsequently challenged with SIV infection triggers lentiviral RNA splicing, the compounds from these plants could be potentially candidates for antiretroviral therapy, stimulating HIV replication in latently infected cells in drug-resistant patients.[39]

As a point of reference, despite in an *in vivo* context, the clinical research conducted by Ka'e *et al*.[39] on HIV-1 infected adolescents in Cameroon undergoing antiretroviral therapy, quantified a high expression of pro-inflammatory cytokines, including IFN-γ and IL-6, as well as anti-inflammatory cytokines IL-4 and IL-10. Furthermore, when considering additional parameters, including data from uninfected subjects, the study identified inflammatory cytokines (IFN-γ and IL-12), anti-inflammatory cytokines (IL-4 and IL-10), and inflammation-related cytokines (IL-6 and IL-1β) as markers indicative of successful viral suppression in comparison to viral persistence. In this study, it was observed that extracts from OH and LM, regardless of SIVmac251 infection status, induced IFN-γ production. Notably, EHLC treated with OH and infected with SIV exhibited significantly higher IFN-γ expression (p=0.0073) at the 144-h time point compared to other treatment conditions. Regarding IL-6, both plant extracts induced its expression under different treatments. However, uninfected EHLC treated with OH extract demonstrated significantly higher IL-6 expression compared to other treatments, with or without SIV infection. Overall, SIV-infected leukocytes produced more IL-6 when treated with OH extract than in those treated with LM extract. With respect to anti-inflammatory cytokines, SIV-infected leukocytes treated with OH extract exhibited significantly higher IL-4 expression than other treatments across all analyzed time intervals (p-value ranging from 0.0208 to 0.0040). Secretion of IL-10 by EHLC was restricted in cells treated with LM extract at all-time points (p-value ranging from 0.0156 to 0.0131). SIV-infected leukocytes, even when treated with plant extracts, expressed very low levels of IL-10. IL-10 expression was detected only at the 144-h and 240-h time points in cells treated with OH extract. At the initial stage of the experiment, cells treated with plant extracts produced more cytokines than in SIV-infected cells alone (data not shown), except for IL-6, for which no statistically significant difference was observed. These *in vitro* results align with the data obtained from the clinical study on HIV-1-infected adolescents.

Whereas betulinic acid has been identified in several *Lycania* species, such as *Licania tomentosa*, *Licania heteromorpha*, *Licania carii*, *Licania pyrifolia*, *Licania cruegeriana*, and *Licania licaniaeflora*,[41-45] niruriflavone has thus far been isolated only from *Licania arianeae*.[46]

Many authors found that derivatives of betulinic acid inhibit the *in vitro* cytopathic effects of HIV-induced cell syncytia formation, suggesting that its anti-HIV activity occurs at the level of virus glycoprotein-cell receptor interactions.[47-49] This finding was later corroborated by Aiken and Chen,[50] who reported that betulinic acid inhibits the *in vitro* fusion of the HIV glycoprotein with the host cell receptor and also hampers virion maturation. Betulinic acid, which has been isolated from LM, exerts both mechanisms. In contrast, niruriflavone is known for its antioxidant activity and has not been associated with antiretroviral activity.[51] The other compounds obtained, (-)-gallocatechin and 4'-O-methyl-epigallocatechin, have been shown to inhibit the HIV-1 reverse transcriptase.[52-55]

Despite the crude extract of OH was not fractionated in this study, many reports have documented its isolated compounds.[23,24] Among them, the bioflavonoids, agathisflavone, epicatechin, 6-C-glycopyranosyl-luteolin, and amentoflavone, despite being derived from different plants, have exhibited *in vitro* anti-HIV activity through distinct mechanisms.[54,55] In addition, luteolin and apigenin have been shown to inhibit HIV enzymes, such as the protease, RNase H, and integrase.[55,56]

5. Conclusion

The experiments preliminarily demonstrate the mitotic, immune-modulating, and SIV-suppressive properties of Amazonian medicinal extracts from the plants LM and OH. For the most part, OH extract had the best mitotic activity, while LM extract provided the most protection against SIV cytopathicity. Regarding cytokines secretion in SIV-infected EHLC, OH extract was generally the most effective in inducing expression, except for IL-8, where no significant difference was observed between the two plant extracts. IL-10 expression in infected cells

was poorly stimulated by both plant extracts. The SIV-suppressive activity of the plant compounds may result from direct or indirect mechanisms, either by interfering with the lentivirus infection cycle and/or by the action of proinflammatory cytokines. Furthermore, restriction factors expressed by leukocytes may antagonize viral pathogenesis. Therefore, both the cell and the lentivirus models serve as to useful tools for screening antiviral and immune-modulating cytokines, offering potential therapeutic applications not only for retroviruses but also for other viral agents.

The immunomodulatory activity exerted by the plant extracts and the immune cellular response to SIV infection aligns with previously reported data from *in vivo* systems. However, the therapeutic use of the phytocompounds assessed in this study remains premature. Further analyses are required to thoroughly evaluate the potential toxicity of these plant extracts. In addition, the use of animal models has been circumvented by ethical reasons. Of potential application, RNA sequencing could be employed to analyze cell expression in these models, providing a comprehensive view of cell behavior under the experimental conditions.

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Conflict of interest

The authors declare that they have no competing interests.

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Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data

Data supporting the findings of this study are available from the corresponding author on reasonable request.

Further disclosure

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