VITAE, REVISTA DE LA FACULTAD DE CIENCIAS FARMACÉUTICAS Y ALIMENTARIAS
ISSN 0121-4004 / ISSNe 2145-2660. Volumen 23 número 2, año 2016
Universidad de Antioquia, Medellín, Colombia. págs. 109-118
DOI: http://dx.doi.org/10.17533/udea.vitae.v23n2a03

IN VITRO ANTI-HIV-1 ACTIVITY OF THE ENZYMATIC EXTRACT ENRICHED WITH LACCASE PRODUCED BY THE FUNGI *GANODERMA SP*. AND *LENTINUS SP*.

ACTIVIDAD *ANTI-VIH-1 IN VITRO* DEL EXTRACTO ENZIMÁTICO ENRIQUECIDO CON LACASA PRODUCIDO POR LOS HONGOS *GANODERMA SP.* Y *LENTINUS SP.*

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Received: July 24, 2015 Approved: August 11, 2016

ABSTRACT

Background: Natural compounds are a good source for the development of antiretroviral drugs with low cytotoxicity. The laccase enzyme, produced by fungi of the genera *Ganoderma sp.* and *Lentinus sp.,* inhibits the reverse transcriptase (RT) of the human immunodeficiency virus 1 (HIV-1), in cell-free models *in vitro*. **Objetives***:* In this study we evaluated the anti-HIV-1 activity of the enzymatic extracts (EE) enriched with laccase, produced by two native species of fungi of the same genera in an *in vitro* cell culture model. **Methods:** The inhibition of viral replication was performed using the U373-MAGI cell line infected with recombinant viruses in the presence/absence of the EE and 48 hpi, the percentage of infected cells was evaluated by flow cytometry for green fluorescent protein –GFP– and ELISA for p24. The inhibition of the RT was determined by quantification of early and late products of reverse transcription using quantitative PCR. **Results**: The EEs from *Ganoderma sp.* and *Lentinus sp.* inhibited the replication of HIV-1 between 80 and 90% and decreased the production of early and late transcripts between 55,5%-91,3% and 82,1%-93,6% respectively. The EE from *Lentinus sp.* had the best selectivity index (SI: 8.3). **Conclusions:** These results suggest the potential anti-HIV-1 activity of the EE for the exploration of an alternative therapy against HIV-1 infection.

Keywords: Human immunodeficiency virus type 1 (HIV-1); *Ganoderma*; *Lentinus*; laccase; antiviral activity; natural products.

RESUMEN

Antecedentes: Los compuestos naturales son una buena fuente para el desarrollo de fármacos antirretrovirales con baja citotoxicidad. La enzima lacasa, producida por hongos del género *Ganoderma sp.* y *Lentinus sp.*, inhibe la transcriptasa reversa (TR) del virus de la inmunodeficiencia humana tipo 1 (VIH-1), en modelos *in vitro,* libres de células. **Objetivos:** En este estudio se evaluó la actividad anti-VIH-1 del extracto enzimático (EE) enriquecido con lacasa, producida por dos especies nativas de hongos de los mismos géneros en un modelo *in vitro* de cultivo celular. **Métodos**: La inhibición de la replicación viral se realizó usando la línea celular U373-MAGI infectada con virus recombinantes en la presencia/

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ausencia del EE y 48 hpi, el porcentaje de células infectadas se evaluó mediante citometría de flujo para GFP y ELISA para p24. La inhibición de la TR se determinó mediante la cuantificación de los productos tempranos y tardíos de la transcripción reversa utilizando una PCR cuantitativa. **Resultados**: El EE de *Ganoderma sp.* y *Lentinus sp.* inhibió la replicación del VIH-1 entre el 80 y 90% y disminuyó la producción de transcriptos tempranos y tardíos entre el 55,5% -91,3% y 82,1% -93,6%, respectivamente. El EE de *Lentinus sp.* mostró el mejor índice de selectividad (IS: 8.3). **Conclusiones**: Estos resultados sugieren el potencial anti-VIH-1 del EE para la exploración de una terapia alternativa contra la infección por el VIH-1.

Palabras clave: Virus de la Inmunodeficiencia Humana tipo 1 (VIH-1); *Ganoderma*; *Lentinus*; lacasa; actividad antiviral; productos naturales.

INTRODUCTION

The pandemic of human immunodeficiency virus (HIV) has become a major global public health problem, and a challenge in terms of its prevention and control. Globally, nearly 78 million individuals have been infected with HIV-1 and 35 million have died from causes related to this virus: by the end of 2015, 36.7 million people were living with HIV (1).

The antiretroviral therapy (ART), the standard treatment for HIV-1, is very efficient at reducing the viral load and the probability of new infections; nevertheless, its use has been associated with drug hypersensitivity (2), and collateral effects on the immune system and other systems of the host due to the high toxicity of its components (3).

Medicinal mushrooms such as *Tricholoma giganteum* (4), *Hericium erinaceum* (5), *Russula paludosa* (6), *Pleurotus eryngii* (7), *Ganoderma lucidum* (8) and *Lentinus edode*s (9) contain in their extracts, ribosome inactivating proteins, lectins, ubiquitin-like proteins and laccases with strong antiviral effects (6,10).

Among these fungi, members of the genera *Ganoderma* and *Lentinus* have been extensively studied because some species are considered medicinal mushrooms due to their great health benefits and apparent absence of side effects (11,12). Such species are found in both tropical and temperate geographical regions, including Colombia (13). They produce the enzyme laccase, which besides its diverse applications in biotechnology was shown in recent years by cell-free assays to possess anti-HIV-1 activity (4,14,15). These authors reported the *in vitro* inhibition of DNA synthesis by the HIV-1 reverse transcriptase, using purified laccase obtained from different species of fungi as shown in Table 1.

Table 1. Laccase with anti-HIV-1 RT activity produced from different fungi.

Species	Source	IC_{50} [a]	Reference
Tricholoma giganteum	Fruiting body	2.2 μM	(4)
Pleurotus eryngii	Fruiting body	2.2 μM	(7)
Hericium erinaceum	Fruiting body	9.5 μM	(5)
Ganoderma lucidum	Fruiting body	1.2 μM	(8)
Pleurotus cornucopiae	Fruiting body	22 μM	(16)
Tricholoma mongolicum	Mycelium	1.4 μM	(17)
Agrocybe cylindracea	Fruiting body	12.7 μM	(18)
Lentinus edodes	Fruiting body	7.5 μM	(9)
Lentinus tigrinus	Micelium	2.4 μM	(14)
Hericium coralloides	-	0.06 μM.	(19)
Agaricus placomyces	Mycorrhiza	1.25 μM	(15)

[a] IC_{50}: Enzyme concentration needed to reach 50% inhibition of RT activity.

So far, there are few reports on the presence of these genera and their therapeutic potential in Colombia, South America (13,20,21). Interestingly, taxonomic classification suggests that they are different species or strains from the ones previously reported, including native species such as *Ganoderma Amazonense* (21), *Lentinus swartzii* and *Lentinus crinitus* (13).

In the present study, we evaluate the anti-HIV-1 activity of enzymatic extracts (EE) obtained from native specimens of fungi belonging to the genera *Ganoderma* and *Lentinus*, in a cellular model, using an *in vitro* single-round, recombinant-based viral infectivity assay.

MATERIALS AND METHODS

Cells, vectors and reagents

U373-MAGI cells were obtained through the AIDS Research and Reference Reagent Program,

NIAID, NIH, from Drs. Michael Emerman and Adam Geballe (22,23). 293T cells were purchased from the American Tissue Culture Collection (ATCC). Cell lines were maintained in Dulbecco modified Eagle medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. The plasmids pNL4-3 delta env GFP (HIV-GFP), encoding full-length NL4-3 HIV-1 proviral DNA with a frameshift in the *env* gene and expressing GFP instead of *nef*, as well as pVSV-G that expresses the G protein of Vesicular stomatitis virus (VSV) were kindly provided by Dr. Johnny He, who developed these plasmids (24).

Virus production

HIV-1 pseudotyped with the VSV-G envelope were prepared using the following procedure: 293T cells were seeded at $4x10^5$ cells/well in 6-well plates; 24 h later they were co-transfected with 1.6 μg of HIV-GFP reporter plasmid and 0.4 μg of pVSV-G expression plasmid per well using the calcium phosphate precipitation method, as previously reported (25). Cell culture supernatants were collected 48 h after changing the transfection medium and centrifuged at 700 x g for 4 min, followed by filtration (0.22 μm pore) and precipitation with PEG 8000 (Sigma-Aldrich, St Louis, MO) for 48 h. Finally, the PEG fraction was centrifuged at 13000 x g for 30 min at 4°C, and the viral pellet was suspended in DMEM and stored as virus stock at -70°C.

Production of the EE

The enzymatic extracts were produced in a 5 L bioreactor (Braun Biotech Inc.) independently for each fungus, under the following operating conditions, temperature: 30°C, agitation: 200 rpm, aeration: 7.5 L/min, initial pH: 4.5, in a culture medium for ligninolytic fungi with an inducer for laccase enzyme, for 5 days. Samples were taken daily from the bioreactor to determine the enzyme activity, and axenic growth of each fungus. Subsequently, each EE was concentrated on an ultrafiltration unit (Millipore, Amicon) using a membrane with a cutoff of 10 kDa, filtered independently on a Whatman membrane and dialyzed for 16 h at 4°C in a dialysis membrane with a cutoff of 12 kDa to a final volume of 50 ml.

The enzymatic activity of each EE and a commercial laccase (Novozyme®, isolated from *Myceliophthora thermophila*) was determined using a UV-Vis spectrophotometer Cary 50 bio at an absorbance of 420 nm, using 2.2'-azino-di-(3-ethylbenzothiazozin-6-sulfonic acid) (ABTS) as mediator, and citrate buffer (pH 3). One unit (U) of laccase is defined as the amount of enzyme required to oxidize 1 mol of ABTS per minute. These EEs were filtered on a 0.22 μm membrane to avoid microbial contamination in cell cultures and were then aliquoted and stored at -70°C until used.

Cytotoxicity assay

To determine the cytotoxicity of each EE, the cell line U373-MAGI was used. After 24 h of culture, doubling dilutions of each EE, commercial laccase and production medium (vehicle control) were added in triplicate in a final volume of 200 μl. Forty-eight hours after treatment, the cytotoxic effect was evaluated by the MTT assay. This assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability, therefore we could determine at what concentration each treatment was able of killing 50% of cells (CC_{50}). We used that concentration and two consecutive lower dilutions to evaluate their antiviral effect. The cytotoxicity percentage was calculated as follows: $((\lambda_{cm} - \lambda_x) / \lambda_{cm}) \times 100$, in which λ_{cm} is the mean optical density of the control, and λ_x is the optical density obtained for each concentration of each treatment.

Inhibition of viral replication

U373-MAGI cells were plated at a density of 10^4 cells/well in a 96-well plate and allowed to grow for at least 24 h. They were treated or not for 1 h with the EE obtained from *Ganoderma sp.* or *Lentinus sp.*, at the three concentrations chosen by the MTT assay; AZT at 1 μg/ml was used as a positive control. The cells were then infected with 100 ng of gag p24 HIV-GFP-VSV-G reporter virus in the presence of 8 μg/ml polybrene. After 3 h, the virus was removed, the cells were extensively washed, and then fresh medium with or without the EE dilutions was added. Forty-eight hours post-treatment the levels of p24 were evaluated by ELISA from cell culture supernatant using Alliance HIV-1 p24 Antigen ELISA Kit (PerkinElmer); likewise, the percentage of infected cells was determined by flow cytometry for GFP expression, the data were acquired on a FACS-CANTO II (BD Pharmingen) and results were analyzed using the FACS-DIVA software. The percentage of inhibition was calculated as follows: 100 – (Infection percentage in

treated cells x 100 / percentage in control infection). The percentages of HIV-1 inhibition obtained from p24 ELISA assay were used to calculate the IC_{50}, which was determined by linear regression analysis.

Inhibition of reverse transcription

Inhibition of reverse transcription was evaluated by detecting and quantifying early and late products by qPCR. Briefly, cells were cultured, treated and infected as above. After 3 h, the virus was removed, and the cells were extensively washed and fresh medium devoid of, or containing EE was added. Forty-eight hours later, DNA was extracted using cell lysis solution and protein precipitation solution (Qiagen, GentraPuregen), following the manufacturer's protocol. Each 20 μL of qPCR mixture consisted of 2 μL of DNA, 1X of Maxima SYBR Green/qPCR Master Mix (Fermentas), and primers (0.4 μM each). Primer sequences for early and late reverse transcripts were previously reported (26). Early Fw: 5'GTGCCCGTCTGTTGTGTGAC3', Rv: 5'GGCGCCACTGCTAGAGATTT3'. Late Fw: 5'TGTGTGCCCGTCTGTTGTGT3', Rv: 5'GAGTCCTGCGTCGAGAGAGC3'. GAPDH DNA was used to normalize the DNA content in each preparation. The primer sequences for GAPDH were: Fw: 5'ACCATTGAGAACTCCAGGATTGTC3', Rv: 5'CTCATGCGCAGAGCCTGTT3'. The cycling profile was: 95ºC for 10 min followed by 45 cycles at 95ºC for 10 sec and 63ºC for 60 sec. A melting curve to confirm the specificity of the PCR products was included. All qPCR amplifications and data acquisition were performed using the CFX96 real-time system (Bio-Rad, Hercules, CA), and software CFX Manager Version: 1.5.534.0511 (Bio-Rad). Relative expression was calculated by the ΔCt method (27). The results are given as average relative expression units of triplicate assays.

Statistical analysis

IC_{50} and CC_{50} values were estimated by linear regression of concentration–response curves generated from the data using the statistical GraphPad Prism 5.0. (GraphPad Software, San Diego, CA). Statistical differences between AZT and each concentration of the EEs, and between the concentrations within each treatment were assessed with Mann-Whitney U test using GraphPad Prism version 5.0. All tests were two-sided, and a $p<0.05$ was considered statistically significant.

RESULTS

Cytotoxic concentrations of the EEs and other treatments

The cytotoxicity of the EEs was determined by the MTT assay, using the cell line U373-MAGI. In all treatments there was a lower cytotoxicity as the concentrations decreased, in a dose-dependent manner (Fig. 1); likewise, the production medium was less cytotoxic at higher dilutions. For the antiviral experiments, three concentrations lower than CC_{50} were used. The CC_{50} values of each treatment are shown in Table 2.

Table 2. Biological activity of the Enzymatic extracts and Commercial laccase

Treatment	CC_{50}[a] (U/L)	IC_{50}[b] (U/L)	SI (CC_{50}/ IC_{50})[c]
EE from *Ganoderma sp.* batch 1	5557	1042.127	5.3
EE from *Ganoderma sp.* batch 2	8450	2034.835	4.1
EE from *Lentinus sp.*	4308	516.871	8.3
Commercial Laccase	178	30.90	5.7

[a] **CC_{50}:** EE concentration needed to reach 50% cytotoxicity of the cellular population (three independent experiments in triplicate).

[b] **IC_{50}:** EE concentration needed to reach 50% inhibition of HIV-1 replication (three independent experiments in triplicate).

[c] **SI:** ratio of CC_{50}/ IC_{50}.

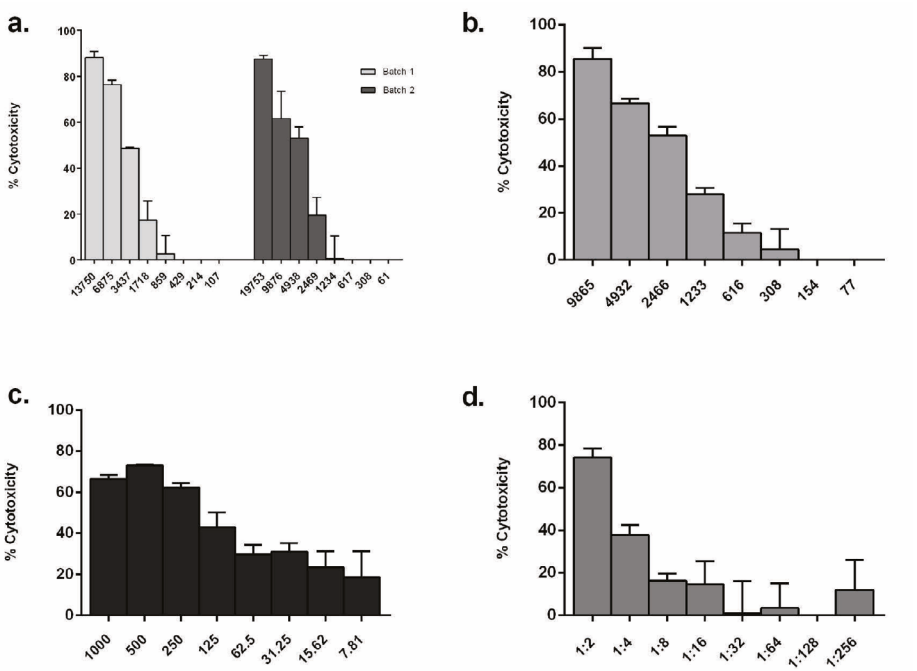

Figure 1. Cytotoxic effect of the different treatments. The U373-MAGI cell line was treated with doubling dilutions of the EEs of *Ganoderma sp.* batches 1 and 2 (1a) and *Lentinus* sp. (1b). A commercial laccase (Novozyme®) (1c), and the production medium (1d) were used as controls. Forty-eight hours post-treatment, the cytotoxic effect was determined by the MTT assay. The figures represent the median of three independent experiments performed in triplicate. The values from the X axis on a, b, and c are given as U/L and dilutions for the last panel (d).

The EEs inhibit HIV-1 replication

The percentage of inhibition of HIV-1 replication based on data obtained from flow cytometry is shown in Figure 2. Most concentrations of the EEs extracted from the fungi *Ganoderma sp.* and *Lentinus sp.*, as well as those of commercial laccase, inhibited over 50% of virus replication. The minimal inhibition percentages obtained were 45.4% and 44.8% for batch 1 of EE from *Ganoderma sp.* at a concentration of 3437 U/L and commercial laccase at a concentration of 62.5 U/L, respectively. The maximum inhibition percentage 86.4% was obtained with EE from *Lentinus sp.* at a concentration of 2466 U/L, The production medium had no relevant inhibitory effect on replication, whose highest percentage was 20.4%, compared to AZT that inhibited 97.7% viral replication.

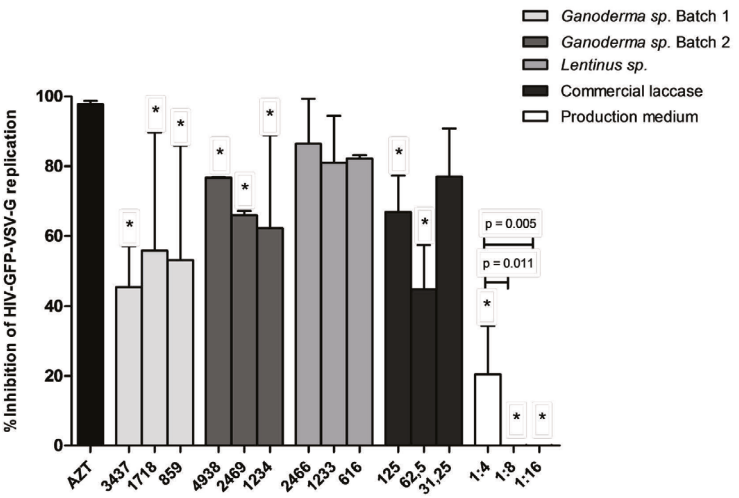

Figure 2. Anti- HIV-1 effect of the different treatments, evaluated by flow cytometry. The U373-MAGI cell line was infected with HIV-1-GFP, with or without the EE of *Ganoderma sp.* batches 1 and 2 and *Lentinus sp.* A commercial laccase (Novozyme®), the production medium, and AZT were used as controls. Forty-eight hours post-treatment the percentage of infected cells was determined by flow cytometry for GFP. The values from the X axis are given as U/L and dilutions for production medium. The graphics represent the median of three independent experiments in triplicate. The Mann-Whitney U test was used to evaluate statistical differences between AZT and each concentration of the EEs (Asterisk (*) means $p<0.05$), and between the concentrations within each treatment.

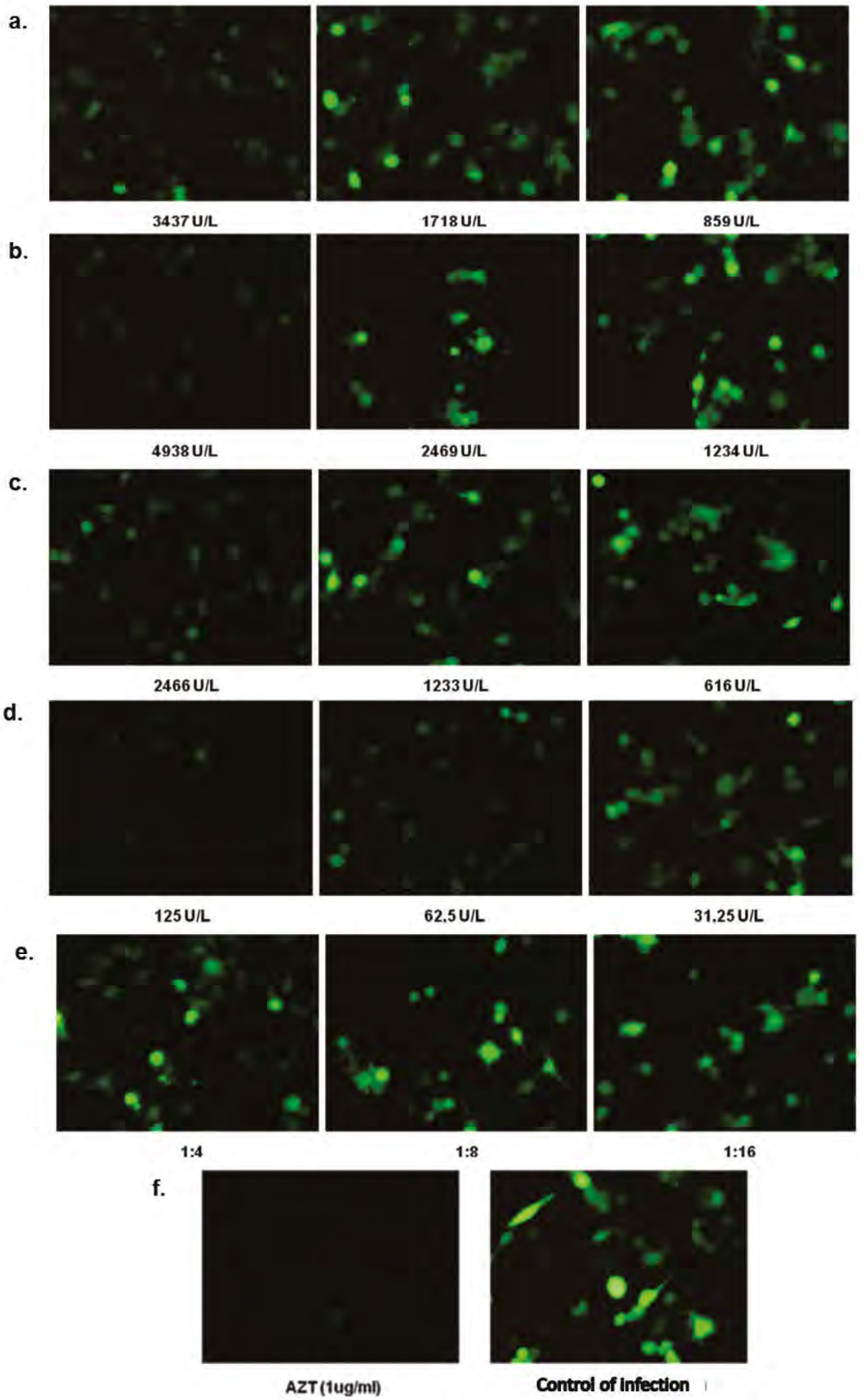

Figure 3. GFP expression in U373-MAGI cells infected with HIV-1-GFP in the presence of increased concentrations of the EE. GFP expression was determined by fluorescence microscopy in U373-MAGI cells infected with HIV-1-GFP, and treated for 48h with different concentrations of the EE of *Ganoderma sp.* batches 1 (a) and 2 (b), *Lentinus sp.* (c), commercial laccase (d) and production medium (e) and 1μg/ml of AZT (positive control of inhibition) (f) and untreated cells were used as control of infection (f).

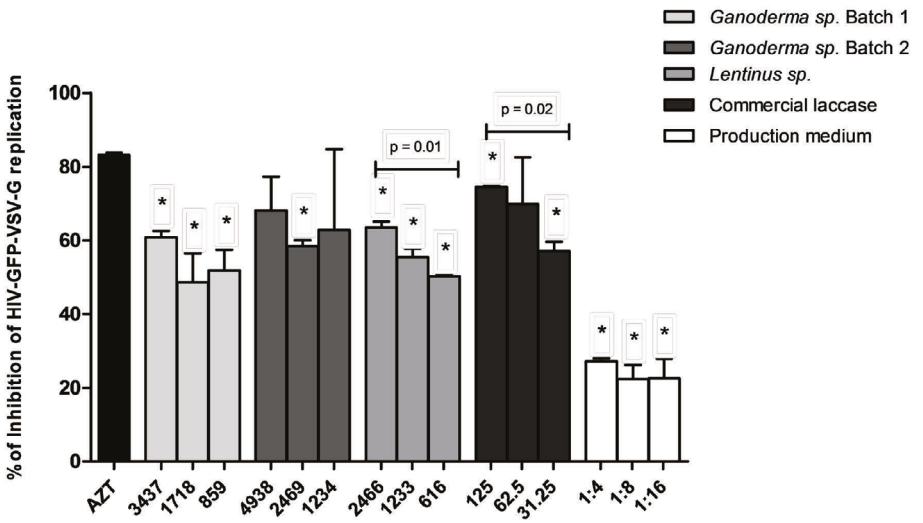

Figure 4. Anti-HIV-1 effect of the EEs, evaluated by quantification of p24 levels. The supernatant of U373-MAGI cells infected with HIV-1-GFP, and treated with or without the EE of *Ganoderma sp.* batches 1 and 2 and *Lentinus sp.* for 48 h, were evaluated by ELISA to determine the amount of p24 produced. Commercial laccase (Novozyme®), the production medium, and AZT were used as controls. The values from the X axis are given as U/L, except for the production medium. The figure represents the median of three independent experiments performed in triplicate. The Mann-Whitney U test was used to evaluate statistical differences between AZT and each concentration of the EEs (Asterisk (*) means $p<0.05$), and between the concentrations within each treatment.

Viral inhibition was also demonstrated by decreasing GFP expression in the infected cells in the presence of increased concentrations of the EE (Figure 3). Figure 4 shows the percentage of viral inhibition based on data obtained from ELISA for p24 for each treatment. The percentages of maximum inhibition obtained with each treatment were: 74.49% with commercial laccase at a concentration of 125 U/L, 68.1% with batch 2 of the EE from *Ganoderma sp.* at a concentration of 4938 U/L, 63.49% with EE from *Lentinus sp.* at a concentration of 2466 U/L, and 60.8% with batch 1 of the EE from *Ganoderma sp.* at a concentration of 3437 U/L. Although there were statistically significant differences between the inhibition obtained by AZT and certain concentrations of the EEs, the last also achieved inhibition percentages of over 50%. The production medium achieved 27.2% inhibition of virus replication at a dilution of 1:4; this percentage is very low in comparison to AZT, so that the inhibitory effect is not relevant. Using this technique, AZT was able to inhibit replication of the virus by 83.2%. Linear regression analyses, using the data obtained from ELISA for p24 were performed to determine the IC_{50}, and to calculate the SI of each EE. These values are also shown in Table 2. The best SI found corresponds to the EE from *Lentinus sp.*, followed by commercial laccase, then, batch 1 of the EE from *Ganoderma sp.* and last, batch 2 from the same fungus.

EEs decrease early and late transcripts of HIV-1

Given previous studies showing laccase's inhibitory effect on the RT, the inhibitory effect of the EEs over the levels of early and late transcripts was evaluated, to associate it with the effect on the HIV-1 reverse transcription. The relative units of early and late transcripts were evaluated by qPCR and based on these results, the percentage of inhibition of transcript expression was calculated. As seen in Figure 5, batch 1 from *Ganoderma sp.* and the EE from *Lentinus sp.* inhibited both early and late transcripts with percentages over 55%, with no statistically significant differences between them and the inhibitory percentages achieved with AZT. The percentages of maximum inhibition for early transcripts were 86.6% for batch 1 from *Ganoderma sp.* at a concentration of 859 U/L and 91.3% for the EE from *Lentinus sp*, at a concentration of 1233 U/L. The percentages of maximum inhibition for late transcripts were 93.6% for batch 1 from *Ganoderma sp.*

at a concentration of 3437 U/L and 90.5% the EE from *Lentinus sp*, at a concentration of 2466 U/L. The control AZT had an inhibition corresponding to 93.8% for early transcripts and 95.1% for late transcripts. The percentages of inhibition obtained with batch 2 of *Ganoderma sp.* were similar to those obtained with batch 1 from the same fungus (data not shown). The inhibition seems to be more efficient for late than early transcripts using the batch 1 from *Ganoderma sp.* and the EE from *Lentinus sp*, and although there is not a strong dose-dependent effect in any of the treatments, the medians of the percentages of inhibition of late transcripts decrease in a dose-dependent manner: 93.6, 89.9, and 89.2 for *Ganoderma sp.* and 90.6, 87.97, and 82.17 for *Lentinus sp.*

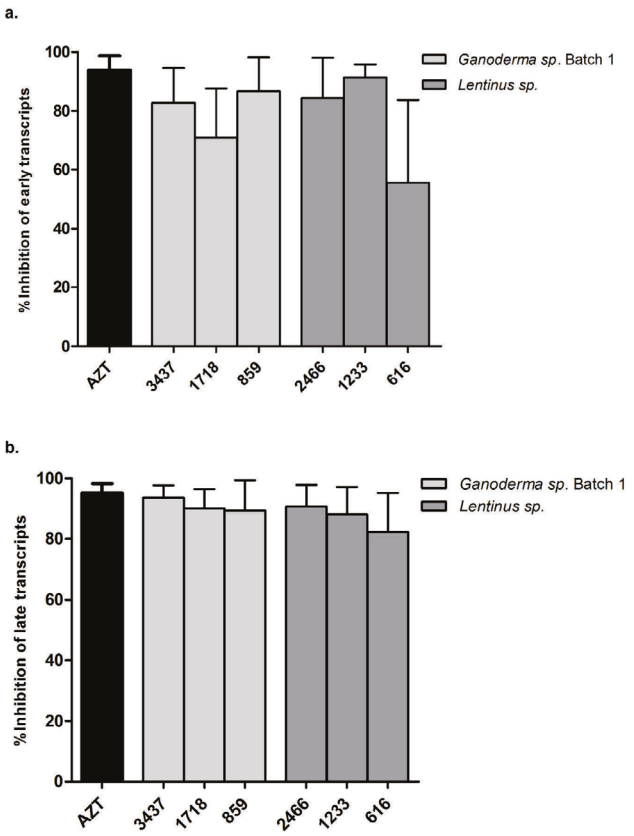

Figure 5. Anti-HIV-1 RT of the EEs. The DNA of U373-MAGI cells infected with HIV-1-GFP, with or without the EEs, was isolated 48 h post-treatment. The DNA was analyzed by qPCR for the early and late transcripts. The values from the X axis are given as U/L. The graphics represent the median of three independent experiments performed in triplicate. AZT was used as control.

DISCUSSION

There are many reports on the potential of natural products found in mushrooms to inhibit HIV infection. Nevertheless, despite the broad biodiversity found in Colombia and around the world, the compounds that can be obtained from these mushroom species have not been well explored. In this study, we tested the EEs obtained from the fungi *Ganoderma sp.* and *Lentinus sp.* for their anti-HIV-1 activity. This is the first time that the antiviral activity of these products has been evaluated in Colombia, using autochtonous mushrooms.

Using an MTT assay and determining the CC_{50} of each EE we ruled-out the cytotoxic effect as a potential mechanism to explain viral inhibition.

The EEs obtained from the fungi *Ganoderma sp.* and *Lentinus sp.*, and the commercial laccase exhibited an important inhibitory activity against HIV-1, which increased by more than 50%; however they did not achieve the levels of viral inhibition reached with the antiretroviral AZT, which was 98%. Two different production batches from *Ganoderma sp.* were evaluated to test the consistency and reproducibility of the results. The differences in the levels of inhibition between the two batches can be explained by the difficulty in inducing the same type of laccase, among all possible variants. Besides the different laccase genes that have been reported, this enzyme is also present in different fungal parts, as isoenzymes that differ from one another by small changes in their sequence, or in their level of glycosylation. Because of these variables, the enzymatic activity of different laccases that may be obtained from the same species may vary and therefore also their biological potential (28). Consequently, rather than a disadvantage, this indicates the need there is to test the potential of different laccases to determine which one inhibits HIV-1 most efficiently, in order to establish a stable form for the production of the enzyme to guarantee a reproducible antiviral activity.

The commercial laccase showed inhibition percentages above 50%, comparable to those obtained with the EEs from both fungi. Therefore, these EEs can compete with compounds that are already available on the market, and it is expected that the inhibitory effect of the purified laccase from the EEs will increase significantly.

The antiviral activity observed with the production medium by ELISA, the highest percentage being 27.27%, could be explained by the existence of other components in the production medium that might have affected any point in the cycle between the translation of viral proteins and the output of new virions, thereby altering the concentration of p24 in the supernatant, but not GFP expression within the cell.

In general, there were some differences between the percentages of inhibition obtained by ELISA and by flow cytometry, which were expected considering that both techniques measure different products produced during the viral cycle. However, most of the treatments evaluated with both techniques showed a similar inhibitory effect when compared to controls.

Previous reports of laccases from different fungal species, including fungi belonging to the same genera of fungi as those evaluated here, showed that the enzyme inhibits the RT of the virus, in a cell-free assay. In the qPCR experiments, the EEs containing the laccase, almost completely decreased the early and late transcripts with inhibition percentages very similar to those obtained with AZT. Given that the early and late transcripts are result from an efficient reverse transcription process; these results suggest that the EEs have an effect on this step of the virus replication cycle.

Finally, considering that a therapeutic compound must have a high SI, the best SI corresponded to the EE from *Lentinus sp.* (SI= 8.3), followed by Commercial Laccase (SI= 5.7), batch 1 of the EE from *Ganoderma sp.* (SI= 5.3) and then batch 2 for the same fungus (SI= 4.1). Therefore with its great potential, it would be important to include the laccase from *Lentinus sp.* in further studies leading to the development of an alternative treatment for HIV-1, rather than the laccase from *Ganoderma sp.* Nevertheless, it should be noted that here we evaluated the effect of EEs that contains additional compounds besides the enzyme of interest, and further studies must be performed with the purified laccase of each fungus. Here, the SIs are very low compared the SI from AZT, which is above 10000 (31), but according to Chattopadhyay et al. (29) an SI >3 indicates potential anti-viral activity of the tested compound. Nevertheless, it is expected that purifying the enzyme will increase the SI value, as shown in the report from Notka et al. (30). One limitation of this study included low number of concentrations tested for antiviral activity, which was due to the availability of the EE, but it was compensate by R^2 higher than 0.8 and p values <0.05 in the linear regression analysis.

Previous reports on other fungal laccases, including the genus *Ganoderma* are rather limited since no cell models were evaluated, no controls of inhibition of infection were used, and the SI was not determined. Our study considered all these aspects, including a cellular model that is closer to the actual cellular microenvironment in which the infection develops, pointing to the relevance of the results obtained.

In conclusion, the EEs rich in laccase obtained from the fungi *Lentinus sp.* and *Ganoderma sp,* inhibit HIV-1 replication with percentages comparable to those of AZT. Although the effect of these EEs was not explored at all stages of the viral cycle, the results suggest that they may act at the level of reverse transcription, supporting previous results (Table 1). These results provide the basis for further research, in which the mechanism of action of the purified enzyme, its biochemical properties and other potential applications in pharmacology should be explored. Given that not only in Colombia, but also around the world the raw material is available for the production of these EEs, the potential is great for the development of new therapeutic strategies to combat HIV-1, based on this enzyme.

ACKNOWLEDGMENT

This work was supported by the Universidad de Antioquia, UdeA, Colombia. We thank the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH as well as Dr. Johnny He from the Indiana University School of Medicine for all the reagents donated. Finally, we thank Anne-Lise Haenni for all her constructive comments.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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