

Evaluation of triterpenes derivatives in the viability of *Leishmania amazonensis* and *Trichomonas vaginalis*

Simone Tasca Cargnin¹, Andressa Finkler Staudt¹, Camila Menezes¹, Ana Paula de Azevedo², Saara Neri Fialho², Tiana Tasca¹, Carolina Bioni Garcia Teles², Simone Baggio Gnoatto¹,*

¹School of Pharmacy, Federal University of Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil, ²Malaria and Leishmaniasis Bioassays Platform, Fiocruz, Porto Velho, RO, Brazil.

Trichomonas vaginalis and *Leishmania* spp. are protozoal species responsible for millions of cases of parasitic diseases worldwide. Considering the potential of natural products and the need for more effective and less toxic alternatives to treat trichomoniasis and leishmaniasis, this study aimed to evaluate the effect of two series of triterpenes derivatives with different modifications at C-3 and C-28 positions of the ursolic acid (UA) and betulinic acid (BA) against trophozoites of *Trichomonas vaginalis* and promastigotes forms of *Leishmania (L.) amazonensis*. The compounds modified just at C-3 were the most active. The 3 β -acetyl betulinic acid (1b) reduced the trophozoites viability of *T. vaginalis* at 74%, followed by the 3-oxo ursolic acid and 3-oxo betulinic acid (3a and 3b) compounds (55% of reduction). The compound 3 β -isobutyl ursolic acid (7a) inhibited the viability of *L. amazonensis* promastigotes by 55%. Therefore, analyzing the structure-activity relationship and the data of literature, it is possible to suppose that the inclusion of polar groups in the skeletons could improve the antiprotozoal activity. Overall, further studies are necessary to develop triterpenic derivatives with more powerful trichomonocidal and leishmanicidal properties.

Keywords: Betulinic acid. *Leishmania amazonensis*. Semisynthesis. *Trichomonas vaginalis*. Ursolic acid.

INTRODUCTION

Human pathogens, such as *Trichomonas vaginalis* and *Leishmania* spp., are unicellular eukaryotes (protists) representative of the supergroup Excavata that includes a few other parasites such as *Trypanosoma* and *Giardia*. These protozoa are responsible for a broad range of health diseases around the world, mainly in the developing countries (Kusdian, Gould, 2015).

Trichomonas vaginalis is a microaerophilic mucosal pathogen, which affects the human urogenital tract causing trichomoniasis, the most common non-viral sexually transmitted disease (STD) in the world (WHO, 2012). According to the World Health Organization (2012), there are about 276 million new cases of trichomoniasis annually worldwide. The parasite mainly affects the urogenital tract of both men and women, and it may cause asymptomatic infection or lead to urethritis or vaginitis.

Studies have indicated several complications related to this disease, including amplification of HIV transmission, risk of low birth weight, and preterm delivery (Schwebke, Burgess, 2004). The Food and Drug Administration (FDA, USA) recommends the treatment for trichomoniasis with a few drugs of choice belonging to 5-nitroimidazole class, with metronidazole and tinidazole - the only two approved drugs. Besides the side effects observed during treatment, another important limitation regarding the nitroimidazoles administration is the emergence of metronidazole-resistant isolates estimated in 2.5 to 10%. The reliance on a single therapeutic class is problematic and alternative treatments are urgently needed (Klebanoff *et al.*, 2001; Cudmore *et al.*, 2004).

Leishmaniasis are neglected infectious diseases caused by protozoan belonging to the genus *Leishmania*, and are transmitted via the bites of infected female phlebotomines. There are three main forms of the disease: visceral, cutaneous, and mucocutaneous (Silveira, Lainson, Corbett, 2004; WHO, 2018). An estimated 700,000 to 1 million new cases and 20,000 to 30,000 deaths occur annually around the world, still

*Correspondence: S. C. B. Gnoatto. Laboratório de Fitoquímica e Síntese Orgânica, Faculdade de Farmácia, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, Rio Grande do Sul (RS), Brasil. Tel.: (51) 3308-54.51; Fax: (51) 3308-53.13. E-mail: simone.gnoatto@ufrgs.br

being a serious disease in tropical and subtropical areas (WHO, 2018). There are more than 20 *Leishmania* species that are transmitted to humans, among them *Leishmania (L.) amazonensis* (Lainson, 2010). This species is the most widely distributed in Brazil and can cause mucocutaneous leishmaniasis, which ranges from small cutaneous nodules to gross mucosal tissue destruction (Silveira, 2009; Machado, Penna, 2012). The type of disease, parasite species, and the immunological status of the host defines the treatment of leishmaniasis. The pentavalent antimonials, pentamidine, amphotericin B, paromomycin, and miltefosine are the drugs available for disease treatment; however, they all have limitations such as high costs, specific toxicity, the emergence of resistance and the need for parenteral administration. In this scenario, the discovery and development of new effective drugs is imperative; nevertheless, leishmaniasis is one of the most neglected tropical diseases in terms of drug discovery (Singh *et al.*, 2014; Rajasekaran, Chen, 2015).

Taking into account the need for more effective and safer alternatives to treat trichomoniasis and leishmaniasis, and the rich structural diversity of natural products, research with focus on the investigation of natural products with activity against these protozoa have increased. A recent review referring to the potential of natural and synthetic products with anti-trichomonal activity, demonstrated that terpenes, phenolic compounds, and alkaloids are promising potential compounds against *T. vaginalis* (Vieira *et al.*, 2015). Likewise, it has been demonstrated for *Leishmania*, that quinones, alkaloids, terpenes, saponins, phenolic and their derivatives have shown antiparasitic properties and selective pharmacological properties modes of action (Singh *et al.*, 2014).

Considering the potential of the natural products and that research of therapeutic alternatives for parasitic diseases are needed, this study aimed to evaluate the activity of two series of semisynthetic derivatives with different modifications at C-3 and C-28 positions of the triterpenes ursolic acid and betulinic acid against trophozoites of *T. vaginalis* and promastigotes forms of *Leishmania (L.) amazonensis*.

MATERIAL AND METHODS

Ursolic and betulinic acids extraction

Betulinic acid (BA) was obtained from barks of *Platanus acerifolia*, collected in Bento Gonçalves, RS, Brazil (29°10'40.43"S 51°34'2.2"W). Ursolic acid (UA) was isolated from apple pomace (*Malus domestica*); a

by-product of juice manufacture Tecnovin Ltd., Bento Gonçalves, RS, Brazil (Cargnin, Gnoatto, 2017). The triterpenes identities were confirmed by spectroscopic comparison with analytical standard and related literature (Tkachev *et al.*, 1994).

Semisynthesis of UA and BA series

Briefly, compounds modified at C-3 position were prepared by the reaction of UA or BA with the appropriate anhydride or oxidizing agent, as previously described (Gnoatto *et al.*, 2008b; Silva *et al.*, 2013). Derivatives with ester or ketone substituents at C-3 were then submitted to another reaction for modifications at C-28, and methyl and imidazole ring at C-28 were incorporated (Scheme 1). The derivatives identities were confirmed by analysis of spectroscopic data (IR, ¹H-NMR and ¹³C-NMR spectra and Mass spectra – LC-MS), and compared with data of literature (Santos *et al.*, 2009; Leal *et al.*, 2012; Silva *et al.*, 2013).

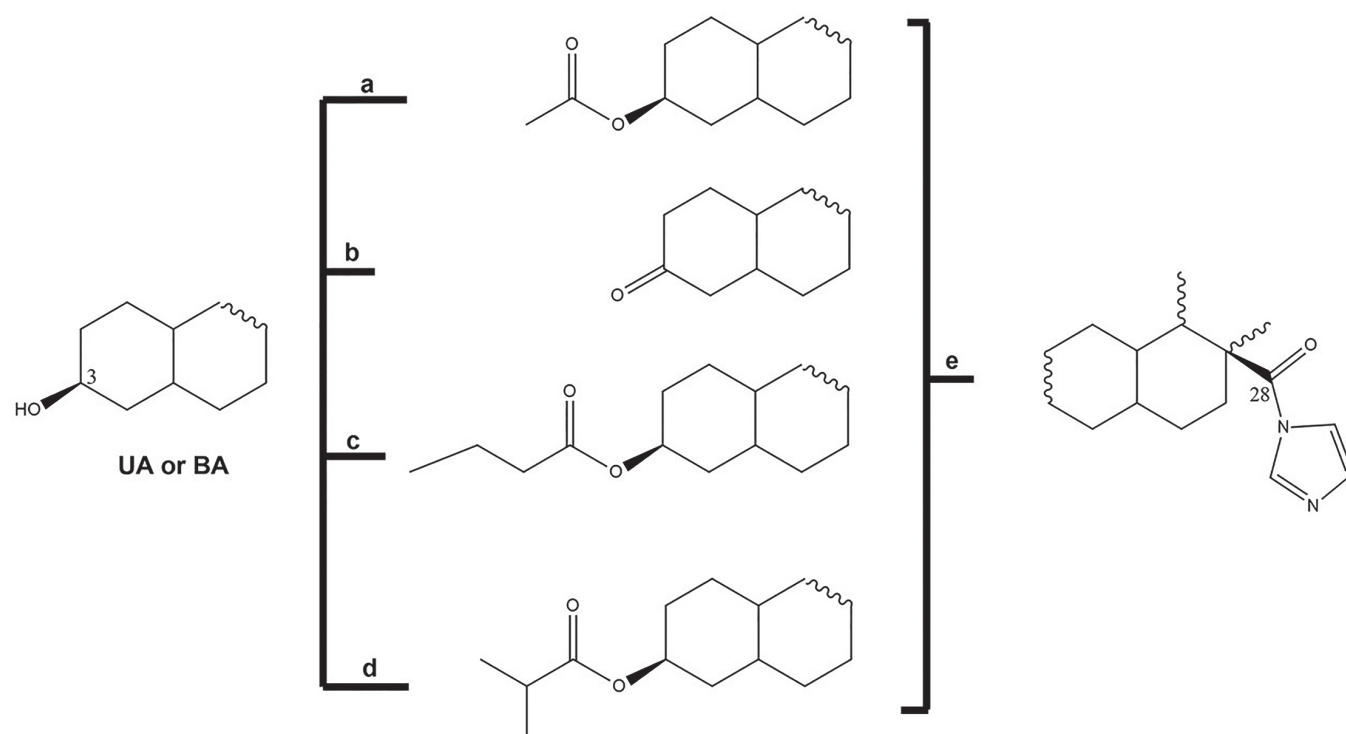
Biological evaluation

In vitro anti-Trichomonas vaginalis activity

Trichomonas vaginalis isolate (ATCC 30236) were cultured axenically *in vitro* in trypticase-yeast extract-maltose (TYM) medium (pH 6.0), supplemented with 10% heat-inactivated bovine serum (HIBS), and incubated at 37 °C (Diamond, 1957). Organisms in the logarithmic phase of growth and exhibiting more than 95% viability and normal morphology were harvested, centrifuged, washed and resuspended in fresh TYM medium. To perform the screening assay, the compounds were solubilized in dimethyl sulfoxide (DMSO) (0.6%) at final concentrations of 100 µM. The trophozoites were incubated at a density of 2.0×10⁵ trophozoites/mL, at 37 °C for 24 h. The *T. vaginalis* viability was determined by counting in hemocytometer using trypan blue as exclusion dye. A negative control with trophozoites maintained in TYM medium, a control of the vehicle, and a positive control (metronidazole 8.0 µM) were carried out. The results were expressed as the percentage of living parasites after 24 h of the incubation period considering motility and normal morphology (percentage of living organisms compared to negative control).

In vitro anti-Leishmania amazonensis activity

Promastigotes *Leishmania (Leishmania) amazonensis* (IFLA/67/BR/PH8) were cultured *in vitro* at 23 °C in RPMI medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 20 mM Hepes



SCHEME 1 - Synthesis of derivatives 1a-8b. For the compounds 1a,1b; 3a,3b; 5a,5b; 7a,7b, the UA and BA were submitted separately at followed reactions: (a) dichloromethane, acetic anhydride, pyridine, rt., 24h; (b) acetone, Jones Reagent at 0 °C, rt., 3h; (c) butyric anhydride, DMAP, rt., 24h; (d) isobutyric anhydride, DMAP, rt., 24h. For the compounds 2a, 2b; 4a, 4b; 6a, 6b; 8a, 8b, the synthetic route followed this condition: (e) dichloromethane, oxalyl chloride, N₂ atmosphere, 0 °C, 3h. - trimethylamine, 0 °C - imidazole, rt., 24h.

(*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), and 50 mg/mL of gentamycin and subcultured every 96 h. The anti-*L. amazonensis* activity of compounds was determined by measure of viability of promastigotes forms by MTT assay (Pal *et al.*, 2011), with modifications. For these experiments, promastigotes in stationary phase were seeded at 1.0×10^6 parasite/100 μ L/well in 96-well plates in complete RPMI medium with compounds at final concentration of 100 μ M, solubilized in DMSO (0.6%). Pentamidine (9.0 μ M) was used as reference anti-leishmanial agent. Promastigotes were incubated at 23 °C for 72 h. Afterwards, 10 μ L of a MTT solution (5 mg/mL in phosphate buffered saline - PBS) was added to each well and incubated for further 4 h at 23 °C. Subsequently, 100 μ L of DMSO was added to each well and was incubated for 1 h at room temperature. The optical density (OD) was measured at 540 nm. The results are expressed as percentage of viable promastigotes, compared with controls.

In vitro cytotoxicity assay

Cell viability was determined by a colorimetric method (Mosmann, 1983), using VERO (African Green Monkey Kidney, ATCC CCL-81) cells and MTT reagent (Sigma-Aldrich, USA). Briefly, VERO cells were

cultured in RPMI 1640 (Sigma) supplemented with 10% FBS, 20 mmol/L Hepes, and 50 mg/mL of gentamycin. Experiments were performed in 96-well microtiter plates, where a suspension of 1.0×10^4 cells per well was incubated in a humidified atmosphere with 5% CO₂ at 37 °C. After 24 h of cell adhesion, the cells were treated with test compounds at concentrations ranging from 400 to 6.25 μ M, dissolved in DMSO (0.5%). Next 24, 48 and 72 h of incubation, stock MTT solution (5 mg/mL in PBS) was added to each well and were incubated for more 2 h at 37 °C. Subsequently, 100 μ L of DMSO was added to dissolve the insoluble purple formazan, and the OD of each well was measured at 570 nm. Three wells per dose were analyzed for each sample in three different experiments, and the results were expressed as the percentage of viable cells in comparison to negative control (untreated cells). The cytotoxicity of each test compound was expressed as CC₅₀, the cytotoxic concentration of sample that inhibited cell growth by 50%.

Statistical analysis

All analyses were accomplished in triplicate and results were expressed as mean \pm standard deviation (SD).

The data were subjected to one-way analysis of variance (ANOVA) and P-values below 0.05 were regarded as significant. Dunnett's multiple comparisons test was used to identify significant differences between means among the different treatments (GraphPad Prism Software).

RESULTS AND DISCUSSION

The worldwide incidence rates of infection by *Leishmania* spp. and *T. vaginalis* are startling. Leishmaniasis and trichomoniasis are not notifiable diseases in all the countries where these diseases are endemic and any surveillance system is available to detect resistance. Therefore, a substantial number of cases of these diseases are underestimated, leading to neglected parasitic infection status (Choffnes, Relman, 2011).

Previously, our research group evaluated the potential of some semisynthetic derivatives of pentacyclic triterpenes ursolic acid (UA) and betulinic acid (BA) against *Leishmania* spp. and *Trichomonas vaginalis* species. Seven UA derivatives were evaluated against the promastigote forms of *L. amazonensis* and the N-{3-[4-(3-(Bis(4-hydroxybenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide (Figure 1) was the most active compound with $EC_{50} = 10 \mu\text{M}$ (Gnoatto *et al.*, 2008a). Against *T. vaginalis*, among the six derivatives tested, the N-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-acetylbetulinamide (Figure 1) was active, with a MIC value of $91.2 \mu\text{M}$ (Innocente *et al.*, 2014). Both active compounds have an acetyl group at C-3 and a piperazinyl at C-28 positions. Hence, in this study, we have evaluated the activity of different derivatives of UA and BA against these important neglected protozoa species.

Two series of ursolic acid and betulinic acid derivatives have been designed and semisynthesized

by modifications at C-3 and C-28 positions, generating sixteen derivatives (Figure 2). When they were evaluated against *T. vaginalis* (Figure 3), at $100 \mu\text{M}$, it was observed that the compound 1b, which is acetylated at C-3, reduces the parasite viability at 74%, followed by the compounds oxidized at C-3 (3a and 3b) (55% of viability reduction). The modifications at C-28 (2a, 2b; 4a, 4b; 6a, 6b; 8a, 8b) were especially not favorable for the anti-*T. vaginalis* activity. In the analysis of EC_{50} of some active compounds, betulin presented an EC_{50} value of the $60 \mu\text{M}$, and the compounds 1b and 5b, $30 \mu\text{M}$ and $50 \mu\text{M}$, respectively.

Among the derivatives tested against *L. amazonensis*, at concentration of the $100 \mu\text{M}$, three compounds presented reductions of viability below 50% (7a, 7b, betulin) (Figure 4). For these compounds, the EC_{50} values were evaluated; betulin presented an EC_{50} value of the $72.2 \mu\text{M}$, and the compounds 7a and 7b, $85.7 \mu\text{M}$ and $119.4 \mu\text{M}$, respectively. Differently from what was reported by Gnoatto *et al.* (2008b), who found a significant UA anti-promastigote activity ($EC_{50} = 20 \mu\text{M}$) in the tested conditions, UA was not active against *L. amazonensis* promastigotes. Considering the concentration tested, data is in agreement with Peixoto *et al.* (2011), which showed anti-*L. amazonensis* activity for UA in concentration higher than $100 \mu\text{M}$ ($EC_{50} = 360.3 \mu\text{M}$). Although the extraction of UA is simple and inexpensive, since the raw material is obtained from residue of apple juice manufacture (Cargnin, Gnoatto, 2017), the EC_{50} value is outlying of the range considered acceptable in the development of new semisynthetic drug.

The triterpene BA presented a carboxylic acid at C-28 and betulin, another triterpene with a lupane skeleton, presented an alcohol group at position C-28 (R_2). The leishmanicidal activity of betulin was evaluated and it was more active than compounds of BA series, with reduction

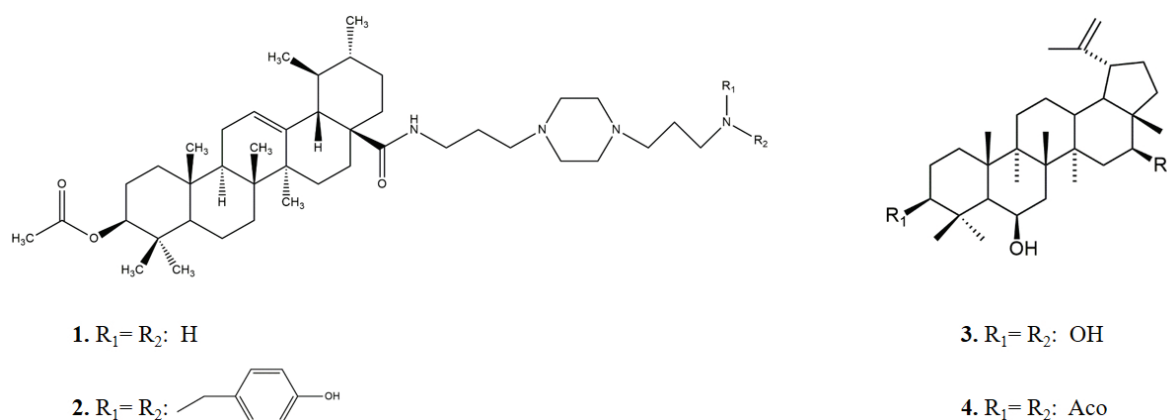


FIGURE 1 – Compounds previously tested: **1.** N-{3-[4-(3-(Bis(4-hydroxybenzyl)amino)propyl)piperazinyl]propyl}-3-O-acetylursolamide; **2.** N-{3-[4-(3-aminopropyl)piperazinyl]propyl}-3-acetylbetulinamide; **3.** 3 β ,6 β ,16 β -trihydroxylup-20(29)-ene; **4.** 3 β ,6 β ,16 β -trihydroxylup-20(29)-ene derivative, with 2 acetyl groups.

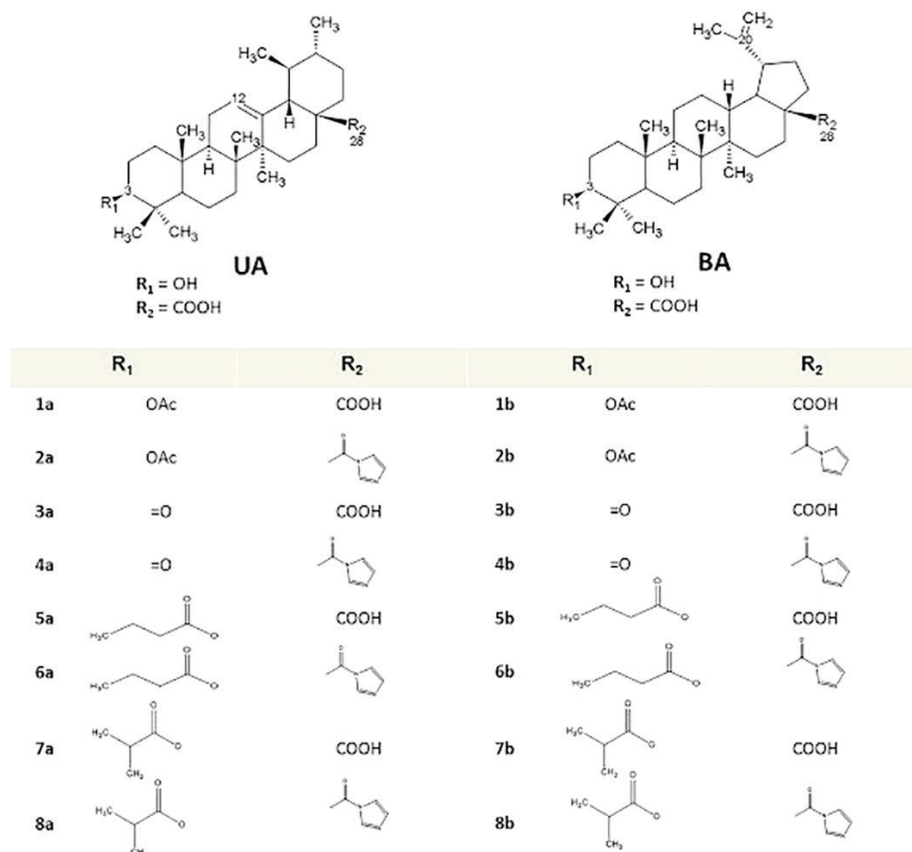


FIGURE 2 – Semisynthetic derivatives of UA and BA series.

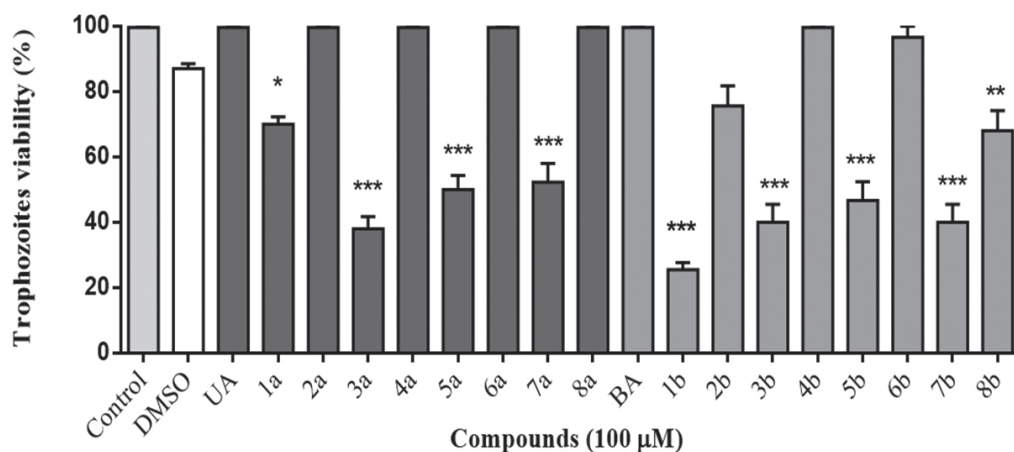


FIGURE 3 – Anti-*T. vaginalis* activity of UA and BA derivatives. Each bar represents the mean \pm standard deviation of at least three independent experiments. Statistically significant differences ($p \leq 0.05$) between treated and non-treated groups are indicated by (*).

of promastigote forms of 68.7% (Figure 4). The activity was more than 20% higher than the most active compound of BA series. Therefore, taking into account the structure-activity relationship, the hydroxyl groups in triterpenes skeleton seem to be important for anti-leishmanial activity. Corroborating with this hypothesis, previous studies revealed that lupane-triterpene ($3\beta,6\beta,16\beta$ -trihydroxylup-20(29)-ene) (Figure 1) isolated from *Combretum leprosum*

fruit extracts, exhibited a significant anti-leishmanial activity against *L. amazonensis* promastigotes, with an EC_{50} of $7.2 \mu\text{M}$, and is also effective in eliminating the *L. (L.) amazonensis* intracellular amastigotes at $109 \mu\text{M}$ (Teles *et al.*, 2011; Teles *et al.*, 2015). Moreover, a synthetic derivative with two hydroxyl groups replaced by acetyl (Figure 4), became inactive (Teles *et al.*, 2011).

Considering the evaluation of the *in vitro* cytotoxicity

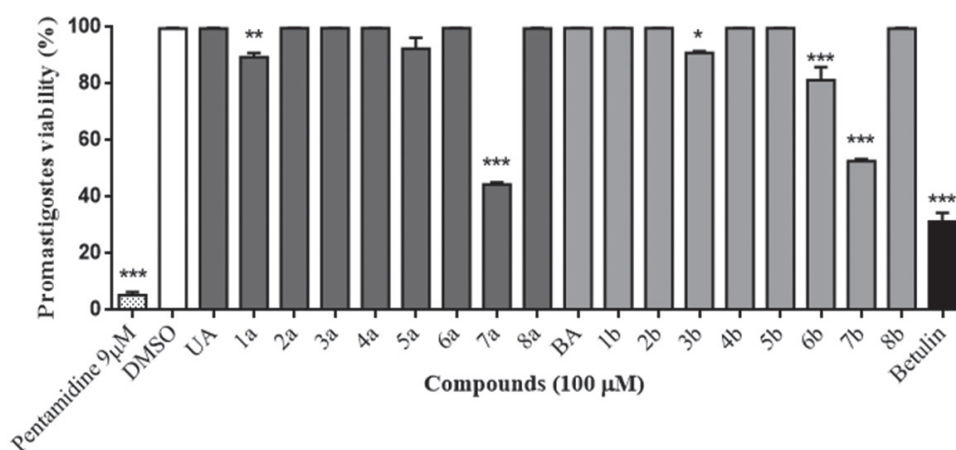


FIGURE 4 – Anti-*L. amazonensis* activity of UA and BA derivatives. Each bar represents the mean \pm standard deviation of at least three independent experiments. Statistically significant differences ($p \leq 0.05$) between treated and non-treated groups are indicated by (*).

of these compounds on mammalian cells (VERO cells) at 24, 48 and 72 h, the UA presented cytotoxic effect of CC_{50} 200, CC_{50} 101.64, and CC_{50} 99.91 μ M, respectively. The other compounds tested were not cytotoxic ($CC_{50} > 400 \mu$ M). Even though the compounds were not cytotoxic in this cellular model, some structure modifications are necessary to improve the activity profile towards parasites.

Therefore, further studies are required to develop UA and BA derivatives with more potent leishmanicidal and trichomonocidal properties. Nevertheless, in the test conditions, the derivatives investigated did not exhibit pronounced activity against *T. vaginalis* and *L. amazonensis*, these preliminary results showed that, based on structure-activity relationship, triterpenes could be a source of novel anti-leishmanial and anti-trichomonas agents. Hence, the delineation of additional semisynthetic modifications, especially including polar groups, such as hydroxyl groups, in the skeletons could improve the activity of these compounds.

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