Evaluation of the *in vitro* trypanocidal activity of triterpenes uvaol, betulinic acid and its semi-synthetic derivatives against the Y strain of *Trypanosoma cruzi*

Avaliação da atividade tripanocida *in vitro* dos triterpenos uvaol, ácido betulínico e seus derivados semissintéticos contra a cepa Y de *Trypanosoma cruzi*

DOI:10.34117/bjdv7n9-284

Recebimento dos originais: 07/08/2021
Aceitação para publicação: 17/09/2021

Moisés Soares Moreira Júnior
Mestre em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: moisessoress@hotmail.com

Lúzio Gabriel Bocalon Flauzino
Doutor em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: luzio_g_bocalon@hotmail.com

Maria Gabriela Marçal
Graduada em Biomedicina
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: mgmarçal@hotmail.com

Daniele da Silva Ferreira
Doutora em Parasitologia
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: danieleferreira06@yahoo.com.br

Patrícia Mendonça Pauletti
Doutora em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: patricia.pauletti@unifran.edu.br

Ana Helena Januário
Doutora em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: ana.januario@unifran.edu.br

Márcio Luís Andrade e Silva
Doutor em Ciências
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: marcio.silva@unifran.edu.br

Lizandra Guidi Magalhães
Doutora em Parasitologia
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: lizandra.magalhães@unifran.edu.br

Wilson Roberto Cunha
Doutor em Química
Universidade de Franca
Avenida Dr. Armando Salles de Oliveira, 201, CEP 14404-600, Franca, SP
E-mail: wilson.cunha@unifran.edu.br

ABSTRACT
Chagas disease is a neglected tropical disease that affects millions of people worldwide. Trypanosoma cruzi (T. cruzi) is the causative agent of Chagas disease and its transmission occurs through blood meal by triatomine bugs, being oral transmission the most common form. More than 100 years after the disease´s discovery, benzonidazole is the only efficient drug against T. cruzi; however, this drug has numerous serious side effects and is only efficient in the acute phase of the disease. Natural products, such as triterpenes, have been an important source of new substances to combat human parasitology. In this study, two triterpenes, uvaol and betulinic acid, were tested against the parasite T. cruzi. The best results of in vitro tests were observed for uvaol with an IC50 value of 70.3 µM against the trypomastigost forms and an IC50 value of 90.6 µM against the amastigost forms. Three semi-synthetic derivatives of betulinic acid were obtained; the acetylated derivative showed excellent results against trypomastigotes forms (IC50 = 15.67 µM), but was not active against the amastigotes forms. The cytotoxic MTT test was also performed on LLCMK2 cells (Macaca mullata kidney epithelial cells) and betulinic acid showed the highest selectivity index (SI) with a value of 1.3.

Keywords: betulinic acid, neglected tropical diseases, Trypanosoma cruzi, uvaol

RESUMO
A doença de Chagas é uma doença tropical negligenciada que afeta milhões de pessoas em todo o mundo. O Trypanosoma cruzi (T. cruzi) é o agente causador da doença de Chagas e sua transmissão ocorre através da farinha de sangue por insetos triatominos, sendo a transmissão oral a forma mais comum. Mais de 100 anos após a descoberta da doença, o benzonidazol é a única droga eficiente contra o T. cruzi; entretanto, esta droga tem inúmeros efeitos colaterais graves e só é eficiente na fase aguda da doença. Produtos naturais, tais como triterpenos, têm sido uma importante fonte de novas substâncias para combater a parasitologia humana. Neste estudo, dois triterpenos, o uvaol e o ácido betulínico, foram testados contra o parasita T. cruzi. Os melhores resultados dos testes in vitro foram observados para o uvaol com um valor de IC50 de 70,3 µM contra as formas trypomastigost e um valor de IC50 de 90,6 µM contra as formas amastigost. Três derivados semi-sintéticos de ácido betulínico foram obtidos; o derivado acetilado mostrou

excelentes resultados contra as formas tripomastigotas (IC50 = 15,67 µM), mas não foi ativo contra as formas amastigotas. O teste MTT citotóxico também foi realizado em células LLCMK2 (células epiteliais renais Macaca mullata) e o ácido betulínico mostrou o maior índice de seletividade (SI) com um valor de 1,3.

Palavras-chave: ácido betulínico, doenças tropicais negligenciadas, Trypanosoma cruzi, uvaol

1 INTRODUCTION

Neglected diseases are a diverse group of infectious diseases prevalent in tropical and subtropical areas, disproportionately affecting billions of people worldwide, almost exclusively the poorest populations and those living in remote, rural or conflict areas in developing countries.[1]

According to the WHO (2019),[2] twenty diseases are considered neglected tropical diseases (NTDs) and affect 1 billion people worldwide that live in poverty while more than 2 billion people are at risk of contracting some NTD. NTDs proliferate in underdeveloped areas in low-income countries, environments in which many people have little or no access to adequate health care, clean water, sanitation, housing, education and information.[3] Although neglected diseases are responsible for about 11% of the global disease burden, the development of therapeutic products for these diseases is disproportionately low. A survey published in 2013 showed that, of the 850 therapeutic products that reached the market between 2000 and 2011, only 37 (4.3%) were developed for neglected diseases, among which 336 were new chemical entities.[1]

Chagas disease, also known as “American trypanosomiasis”, is a potentially fatal disease caused by the protozoan parasite *Trypanosoma cruzi*.[2] Despite being endemic in the region of the Americas, predominantly in Latin America, in the last years Chagas disease has been detected in other regions of the globe as well, such as Canada and the United States, and in some European countries, due to high population mobility and the large amount of blood transfusions between these countries.[4]

Although more than one hundred years have passed since the discovery of Chagas disease, currently only two drugs are available for treatment, namely nifurtimox and benzonidazole. According to LANA et al. (2016),[5] benzonidazole is the only drug used in Brazil to treat Chagas disease. The discovery of new drugs that could be candidates to treat Chagas disease remains challenging. In the past 40 years, many synthetic and natural

compounds have been tested against *T. cruzi*; however, only a few compounds have advanced to clinical trials.[6]

Historically, natural products are a good strategy in the search for new bioactive compounds, as they provide a basis for both the design and the synthesis of derived compounds that can optimize biological activity and minimize side effects.[7]

Pentacyclic triterpenes are natural compounds of great interest due to their various biological activities, serving as candidates or prototypes for new drugs.[8,9] Previous studies carried out in our laboratories have demonstrated the antiparasitic activity of triterpenes.[9]

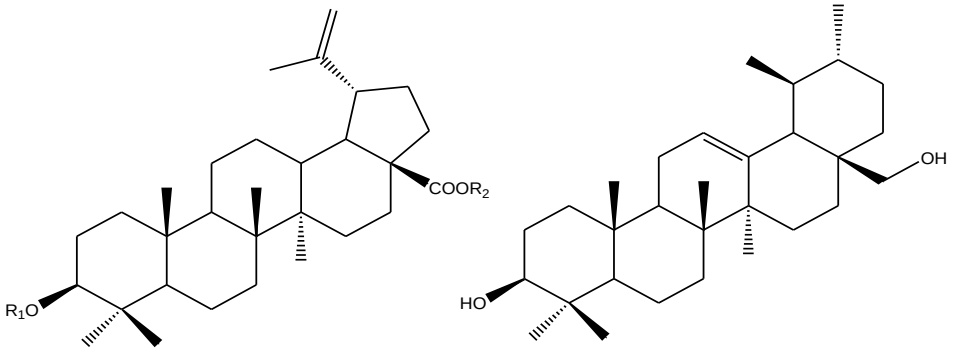
The aim of this work was to evaluate the *in vitro* trypanocidal activity of triterpenes uvaol, betulinic acid and some semi-synthetic derivatives against the Y strain of *T. cruzi*.

2 RESULTS AND DISCUSSION

2.1 ISOLATION OF THE COMPOUNDS

A previous study on crude extracts of the plant species *Davilla eliptica* St. Hill (Dilleniaceae) and *Styrax camporum* Pohl (Styracaceae) revealed the presence of the triterpenes betulinic acid (**1**) and uvaol (**2**) (Figure 1), respectively.[10,11] In this study, these compounds were reisolated from these plant species and their identification and purification were performed using HPLC. The triterpenes were identified by comparison with authentic standards (Sigma-Aldrich Co., St. Louis, MO, USA).

Figure 1: Chemical structures of betulinic acid (1), its semi-synthetic derivatives (1a, 1b and 1c) and uvaol (2)



1 R_1= H R_2= H
1a R_1= $COCH_3$ R_2= H
1b R_1= H R_2= CH_3
1c R_1= H R_2= K

2

In vitro trypanocidal activity against trypomatigost and amastigote forms

The in vitro anti-trypanosoma cruzi activity of betulinic acid, its semi-synthetic derivatives and uvaol were evaluated at concentrations of 6.25; 12.5; 25; 50; 100; 200 and 400 µM for 24 hours. The results obtained in the evaluation of the trypanocidal activity against the trypomastigote forms of T. cruzi are shown in Table 1.

Table 1. *In vitro* trypanocidal activity of triterpenes betulinic acid (1) and uvaol (2) against trypomastigote forms of strain Y of *Trypanosoma cruzi*.

Sample	**% lyse ± S.D./concentration (µM)**							
	400	**200**	**100**	**50**	**25**	**12.5**	**6.25**	**IC_{50} (µM)**
(**1**)	52.0±1.9	46.0±8.0	31.7±4.3	15.6±3.0	0.0±0.0	0.0±0.0	0.0±0.0	285.4
(**2**)	71.6±1.6	69.3±3.5	66.5±2.1	39.9±3.6	37.7±5.9	14.5±0.9	4.5±0.7	70.3

Positive Control: Benzonidazole (IC_{50} 29.4 µM); negative Control: DMSO 0.5% in NaCl solution 0.9%.

When analyzing these results, it is possible to observe that the triterpene uvaol was more active with an IC_{50} value of 70.3 µM and parasitic lysis values around 71.6% and 69.3% in the highest concentrations evaluated (400 and 200 µM, respectively).

For betulinic acid, the IC_{50} value found was 285.4 µM and the values referring to parasitic lysis were lower than those obtained for uvaol, being approximately 52.0% and 46.0% at concentrations of 400 and 200 µM, respectively. The IC_{50} value for benzonidazole, the reference drug for the treatment of Chagas disease, was 29.4 µM.

Triterpenes are chemically characterized by the presence of six isoprene units.[12] Lupanes (like betulinic acid) differ from ursanes (like uvaol) as the stereochemistry between D/E rings is trans, and that the E ring has five carbons, instead of six.[13] It is also possible to observe that uvaol has a hydroxyl radical at the C-3 position in its chemical structure and an OH function at the C-28 position, while betulinic acid has a carbon double bond at C-20, a hydroxyl at C-3 and a carboxyl group at position C-28. These structural differences may explain the different activities against *T. cruzi*.

Carmona *et al*. (2010)[14] isolated betulinic acid from the leaves of *Pentalinon andrieuxii* (Apocynaceae) and observed a moderate trypanocidal activity (IC_{50} = 50 µM) against the Tulahuen strain of *T. cruzi* and a good anitiplasmodial activity against the F32 strain of *Plasmodium falciparum* (IC_{50} = 22.5 µM). Meira *et al*. (2016)[15] extracted betulinic acid from the bark of *Ziziphus joazeiro* Mart (Rhamnaceae) and confirmed the antiparasitic activity of this compound against the trypomastigote forms of the Y strain of *T. cruzi*.

Studies involving uvaol and its tripanocidal activity against *T.cruzi* are still very scarce, but their biological antiparasitic activity has already been described on other parasites in which this triterpene demonstrated trypanocidal activity against *Trypanosoma brucei* (IC_{50} = 27.8 µM)[16]. Filho and collaborators (2009)[17] isolated uvaol from *Baccharis dracunculifolia* (Asteraceae) and identified antileishmanial and antiplasmodial activities for this triterpene.

The results obtained from the semi-synthetic derivatives of betulinic acid against the trypomastigote forms of *T. cruzi* are summarized in **Table 2**.

Table 2. In vitro trypanocidal activity of the semi-synthetic derivatives (1a, 1b, 1c) obtained from betulinic acid (1) against trypomastigote forms of strain Y of Trypanosoma cruzi.

Samples	% Viable cells±S.D./concentration (µM)							
	400	200	100	50	25	12,5	6,25	CC50 (µM)
(1a)	79.0±1.2	88.1±6.7	78.8±5.5	81.7±5.2	69.3±7.1	62.1±2.8	0.0±0.0	15.6
(1b)	83.4±0.3	81.7±5.9	71.0±4.7	51.9±5.6	34.5±6.5	9.7±4.3	1.0±1.7	51.2
(1c)	8.0±5.9	79.5±2.4	44.5±9.0	29.1±1.2	24.6±2.8	8.4±4.0	1.2±2.1	93.8

Positive Control: Benzonidazole (IC_{50} 29.4 µM); negative Control: DMSO 0.5% in NaCl solution 0.9%.

It was possible to observe that all semi-synthetic derivatives of betulinic acid (**Figure 1**) showed better results when compared to the authentic betulinic acid. An excellent result against trypomastigotes was obtained when inserting an acetyl group in the C-3 position. The acetylated derivative (**1a**, IC_{50} = 15.6 µM) reached an IC_{50} several times lower than that of betulinic acid (**1**, IC_{50} = 285.4 µM) and significantly lower than benzonidazole (IC_{50} = 29.4 µM). Changing the C-3 position of betulinic acid proved to be an interesting tool to potentiate its trypanocidal activity against free forms of *T. cruzi*. According to Carmona *et al*. (2010)[14], inserting an ester group to the C-3 position enhances the leishmanicidal and plasmodicidal activity of betulinic acid, while Carginin *et al*. (2019)[18] observed that acetylation at the C-3 position potentiated the triterpene acid against *T. vaginalis*.

The methylated semi-synthetic derivative (**1b**) showed an IC_{50} = 51.2µM and was also more active than betulinic acid, while the derived salt (**1c**) with an IC_{50} value of 93.8µM obtained the highest IC_{50} among the semi-synthetic derivatives, but with an IC_{50} even lower than that of betulinic acid. Changes in position C-28 were also promising. The methyl group showed better results than potassium salt. Several studies describe those structural changes in C-28 carbon enhance the antiparasitic, antiviral and antitumor activities of betulinic acid.[19,20,21] It was also found that the trypanocidal activity of

betulinic acid, acetylated, methylated and saline derivatives, presented a dose dependent characteristic.

Triterpenes **1** and **2** were also evaluated on the amastigote forms of *T. cruzi* and the results referring to this evaluation are shown in **Table 3**.

Due to the promising results of the acetylated derivative of betulinic acid (**1a**) against the trypomastigost forms of *T. cruzi* obtained in our studies, an assay was carried out involving this semi-synthetic derivative against the amastigote forms (**Table 3**).

Table 3. *In vitro* trypanocidal activity of the semi-synthetic derivatives obtained from the triterpene betulinic acid against amastigote forms of strain Y of *Trypanosoma cruzi*.

Sample	% lyse ± S.D./concentration (µM)							
	400	200	100	50	25	12.5	6.25	IC50 (µM)
(1)	64.4.±1.1	31.1±10.1	24.4±3.8	20.0±6.6	13.3±6.6	6.6±5.6	2.2±1.8	297.7
(2)	77.7±3.8	73.3±6.6	51.1±10.1	44.4±16.7	13.3±6.6	2.2±1.8	0.0±0.0	90.6
(1a)	37.9±2.2	36.3±4.0	32.1±2.2	29.8±1.1	23.7±2.8	19.5±1.1	11.4±1.1	>400

Positive Control: Benzonidazole (IC_{50} 53.2 µM); negative Control: DMSO 0.5% in NaCl solution 0.9%.

It can be verified that the most expressive parasitic lysis values were obtained for triterpene **2** at concentrations of 400 and 200 µM (77.7% and 73.3%, respectively) and the IC_{50} value found was 90.6 µM. Regarding triterpene **2**, the IC_{50} value was 297.7 µM and the values referring to parasitic lysis were lower than those obtained for **2**, approximately 64% at 400 µM and 31% at 200 µM. The positive control benzonidazole revealed an IC_{50} of 53.2 µM.

Analyzing these results, it can be verified that the acetylated derivative of betulinic acid (**1a**) did not show promising activity on the amastigote forms of *T. cruzi*, since the IC_{50} value found was greater than 400 µM and the parasitic lysis values were approximately 38% at 400 µM and 36% at 200 µM.

2.2 CYTOTOXICITY ACTIVITY

An MTT test was performed on LLCMK2 cells to verify the cytotoxic effect of the substances under study (uvaol, betulinic acid and acetylated derivative of betulinic acid). The concentration range was chosen based on the trypanocidal activity assay. The cultures were treated with the substances at concentrations of 6.25, 12.5, 25, 50, 100, 200 and 400 mM for 96 hours. The viability of the cultures was determined from the absorbance values obtained for each of the substances in relation to the control groups.

The results obtained regarding cytotoxic activity of triterpenes **1**, **2** and the acetylated derivative of the triterpene betulinic acid (**1a**) are shown in **Table 4**.

Table 4: *In vitro* evaluation of the cytotoxic activity of triterpenes betulinic acid (**1**) uvaol (**2**), and the acetylated derivative of the triterpene betulinic acid (**1a**) and benzonidazole (**4**)

Samples	% Viable cells±S.D./concentration (µM)							
	400	**200**	**100**	**50**	**25**	**12,5**	**6,25**	**CC_{50} (µM)**
(**1**)	58.5±3.9	63.1±1.6	64.8±1.2	76.0±0.9	74.8±1.0	87.0±0.1	90.0±0,6	>400
(**2**)	0.0±0.0	48.4±0.9	54.9±0.6	65.3±2.3	71.9±0.4	82.1±1.0	84.5±2.3	93.0
(**1a**)	2.9±1.5	52.6±3.6	61.4±0.3	67.1±1.0	70.9±0.8	82.5±0.6	95.8±1.0	112.6
(**4**)	46.0±1.4	48.0±2.1	54.7±3.2	75.6±1.7	83.0±0.9	100.0±0.0	100.0±0.0	210.9

Negative control: DMSO 0.5%; Positive control: DMSO 25%.

These results indicate triterpene betulinic acid (**1**) as the least toxic substance for LLCMK2 cells when compared to the other substances evaluated, since the CC_{50} value obtained was greater than 400 µM.

The low cytotoxicity of uvaol (**2**) was identified by Filho *et al*. (2009)[17] in mammalian cells of the Vero lineage (kidney of the African green monkey). On the other hand, ZOFOU and collaborators (2011)[21] identified high toxicity of betulinic acid (**1**) against LLCMK2 cells.

The selectivity index (SI) was calculated between the cytotoxicity ratio and the biological activity. This parameter reflects the amount of compound that is active against the parasite but not toxic to the host cell. [22]

The SI can indicate the selectivity of a compound between a parasitic and a normal strain, indicating the potential use of this compound in clinical tests. According to a study by Lenta and collaborators (2007)[23] and Weiss and collaborators (2011),[24] selectivity index values greater than 10 may suggest better product safety and selectivity for use in mammals.

The SI values were determined to verify the selectivity of the three samples in the amastigote stage of *T. cruzi*. According to the results presented in **Table 5**, the betulinic acid showed the highest selectivity index (SI) with a value of 1,3. For acetylated derivative (**1a**) and uvaol (**2**), the calculated values were 0.3 and 1.0 respectively. The reference drug, benzonidazole, presented a SI value of 3.9. Meira *et al*. (2016)[15] identified a SI value for betulinic acid of 0.0 against the Y strain of *T. cruzi*, very close to that obtained in our tests.

Table 5: Determination of the selectivity index (SI) of the compounds tested in the amastigote stage of *T. cruzi*.

Time	**1**		**2**		**1a**		**Benzonidazole**	
96 h	CC_{50}	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}	CC_{50}	IC_{50}

400	297.7	93.0	90.6	112.6	400	210.9	53.2
SI = 1.3		SI = 1.0		SI = 0.3		SI = 3.9	

SI: selectivity index (CC_{50}/IC_{50})

3 CONCLUSIONS

In conclusion, the undertaken study provided biological evidence that triterpenes and their derivatives are promising compounds that could be used as lead compounds for the development of new antitrypanossoma agents. The precise mechanism of action of pentacyclic triterpenes has not yet been clarified, however, its inhibitory effects on protein kinase C are known, and this property may be related to the antiparasitic activity presented by these substances and their semi-synthetic derivatives.[25]

It is also important to emphasize that, considering the results obtained, future studies are necessary to verify the effects *in vivo* of these triterpenes and to further uncover the possible mechanisms presented on treatment of the experimental Chagas disease.

4 EXPERIMENTAL SECTION

4.1 PLANT MATERIAL, EXTRACTS AND ISOLATION OF THE TRITERPENES

The aerial parts of *Davilla elliptica* were collected in the reserve of Jataí (June, 2015, SISGEN code AFAE3CE), in the city of Luís Antônio (SP). The identification was carried out by Prof. Dr. Milton Groppo from the Botanical Institute of FFCLRP-USP and an exsicata was deposited in the herbarium of the same Institute (SPFR 13702).[10]

The collected plant was stabilized and dried in a circulating air oven (40°C) and pulverized in a knife mill. The resulting powder (0.77 kg) was subsequently subjected to extraction with n-hexane, ethyl acetate and ethanol. Part of the ethyl acetate extract (2mg) was analyzed by high performance liquid chromatography (HPLC) in the isocratic condition methanol/H_2O (85:15v/v), with a flow rate of 1ml/min. The extract chromatogram was compared with that of the authentic betulinic acid standard (Sigma-Aldrich®), making it possible to compare the retention time and the UV-Visible spectrum. A preparative CLAE method was also carried out to isolate betulinic acid, obtaining 80 mg of betulinic acid from 500 mg of ethyl acetate extract (16% yield).

The extract of dichloromethane (7.85 g) of *Styrax camporum* (collected in February 2013, SISGEN Code A1484F8) was provided by Dr Patricia M. Pauletti for the isolation of triterpene uvaol.[11] The extract was previously purified in a column containing

celite and active carbon (3:1 w/w) as a stationary phase and chloroform as a mobile phase. Part of this extract in chloroform (2mg) was analyzed by high performance liquid chromatography (HPLC) in the isocratic condition methanol/H_2O (90:10 v/v), with a flow rate of 1ml/min. The extract chromatogram was compared with that of the authentic uvaol standard (Sigma-Aldrich®), making it possible to compare the retention time and the UV-Visible spectrum. A preparative CLAE method to isolate uvaol was also carried out, obtaining 32 mg of uvaol from 500 mg of dichloromethane extract (6.4% yield).

Both analytical and semi-preparative HPLC separation analyses were carried out on a Shimadzu SCL-10 AVP liquid chromatography system equipped with a SPD-M10AVP Shimadzu UV-DAD detector (the channel was set at 210 nm) and Shimadzu columns (ODS column, 250 x 4.6 mm, 5 μm for analytical analyses and ODS, 250 x 20 mm, 15 μm for semi-preparative separations).

4.2 PREPARATION OF THE TRITERPENES SEMI-SYNTHETIC DERIVATIVES.

The betulinic acid semi-synthetic derivatives (1a, 1b and 1c) were obtained according to the previously described procedure.[26,27] Betulinic acid (20 mg) (**1**) was treated with excess acetic anhydride in pyridine and resulted in C-3 acetoxyl derivative (15 mg) (**1a**)**.** In another preparation, **1** (about 20 mg) was treated with CH_2N_2 in Et_2O, yielding the respective C-28 methyl ester derivative (17 mg) (**1b**). To obtain the potassium salt derivative (**1c**), a solution of **1** was treated with 2% KOH in Me_2CO-H_2O (1:1) at room temperature for 30 min. After removal of Me_2CO by evaporation, the resulting material was chromatographed over Sephadex LH-20; elution with MeOH resulted in 15 mg of potassium salt of betulinic acid (**1c**) as a white powder.

4.3 ANTI-TRYPOMASTIGOTE ASSAY

In the search for potential trypanocidal agents, the compounds were tested *in vitro* against trypomastigote forms of the Y strain of *T. cruzi*. The *in vitro* trypanocidal assay was undertaken using trypomastigote forms of *T. cruzi*, which were obtained by culture of the LLCMK2 cell lineage, according to the

protocol established by Cunha et al. (2006).[28]. Benzonidazole and 0.5% DMSO (dimethylsulfoxide) were used as positive and negative controls respectively. The tests were carried out in triplicate.

4.4 ANTI-AMASTIGOTE ASSAY

The assays on amastigote forms were performed in LLCMK2 cell culture, according to the protocol established by De Souza et al. (2005).[29]

For this, a stock solution was prepared by dissolving the substances in 100% DMSO, and aliquots of these stock solutions were added to the infected medium to obtain final concentrations of 6.25; 12.5; 25; 50; 100 and 200 µM.

Determination of the activity was verified quantitatively counting the infected cells and determining the percentage of parasitic reduction, comparing with the control. Benzonidazole and DMSO 0.5% were used as positive and negative controls, respectively. The tests were carried out in triplicate.

4.5 CYTOTOXICITY AND SELECTIVITY INDEX

Cell toxicity assays were performed with the LLCMK2 strain. The cells were maintained in RPMI 1640 medium, supplemented with 10% fetal bovine serum, and 1% streptomycin buffered with sodium bicarbonate until pH 7.4. The culture was then incubated in plastic bottles at 37 ºC and 5% CO_2 in a humidified oven to obtain the cells for testing.

Approximately $1x10^6$ cells (LLCMK2) were added per well in a 96-well plate and the substances under analysis were added at concentrations of 400, 200, 100, 50, 25, 12.5 and 6.25 µM and the biological activity was evaluated using the MTT reduction method after 96 hours of incubation. DMSO 25% and DMSO 0.5% were used as positive and negative controls, respectively.

The concentration of substances capable of reducing the optical density of the treated cells by 50%, compared to the control, was determined calculating the CC_{50}. Three independent experiments were carried out in triplicate for each trial.

The ratio between the CC_{50} values (cytotoxic concentration for 50% of the cells) and the IC_{50} (inhibitory concentration of 50% of the parasites) values of the samples on *T. cruzi* trypomastigote forms were used to calculate the selectivity index (SI).

FUNDING

The authors are grateful to São Paulo Research Foundation (FAPESP) for financial support (grant number 2016/22228-1), and CNPq for fellowships. This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

REFERENCES

[1] L.G. Ferreira, A.D. Andricopulo, 'Drugs and vaccines in the 21st century for neglected diseases', *Lancet Infect. Dis*. **2019**, *9*, 125-127.

[2] World Health Organization (WHO), 'Chagas disease (American trypanosomiasis)' **2019**. https://www.who.int/chagas/disease/en

[3] World Health Organization (WHO), 'Integrating neglected tropical diseases into global health and development: fourth WHO report on neglected tropical diseases', Geneva, Switzerland, **2017**. http://apps.who.int/iris/bitstream/10665/255011/1/9789241565448-eng.pdf

[4] M.V.C. Feres, L.A. Silva, 'Doença de Chagas e patentes farmacêuticas: pesquisa exploratória sobre a efetividade do sistema de patentes', *REI* **2018**, *4*, 297-307.

[5] M. Lana, W.L. Tafuri, D.C Bartholomeu, '*Trypanosoma cruzi* e doença de Chagas', In: D.P. NEVES, 'Parasitologia Humana', ed.13. São Paulo: Atheneu, **2016**, 91-116.

[6] G. B. Santos, R. Krogh, L.G. Magalhães, A.D. Andricopolo, M.T. Pupo, F. S. Emery, 'Semisynthesis of new Aphidicolin derivatives with high activity against *Trypanosoma cruzi', Bioorg. Med. Chem. Lett*. **2016**, *26*,1205-1208.

[7] M.T. Scotti, L. Scotti, H. Ishiki, F.F. Ribeiro, R. M.D. da Cruz, M.P. de Oliveira, F.J.B. Mendonça, 'Natural products as a source for antileishmanial and antitrypanosomal agents', *Comb. Chem. High Throughput Screening*, **2016**, *19*, 537-553.

[8] A. A. Ragozoni, M. A. L. Vasconcelos, D. F. Galvão, N. L. Cunha, H. L. Nicolella, A. H. Januário, P. M. Pauletti, M. L. A. Silva, D. C. Tavares, C. H. G. Martins, W. R. Cunha, 'Antimicrobial activity of triterpene acids against phytopathogens. Brazilian Journal of Development, 2021, 7(3), 27870-27881.

[9] D.S. Ferreira, V.R. Esperandim, M.G, Marçal, N.B.R. Neres, N.L. Cunha, M.L Andrade e Silva, W.R. Cunha, 'Natural Product and Chagas's Disease; the action of triterpenes acids isolated from *Miconia* species', *Univ. Sc*., **2013**, *18*, 243-256.

[10] N.O. Acésio, P.F. de Oliveira, D.F.P. Mastrocola, I.M.S. Lima, C.C. Munari, V.L.F.L. Sato, A.A.S. Souza, L.G.B. Flauzino, W.R. Cunha, D.C. Tavares, 'Modulatory Effect of Betulinic Acid on the Genotoxicity Induced by Different Mutagens in V79 Cells', *Evid.-Based Complementary Altern. Med.*, **2016**, *2016*, Article ID 8942730.

[11] P.M. Pauletti, A.R. Araújo, V.S. Bolzani, 'Triterpenos de *Styrax camporum* (Styracaceae)', *Quím. Nova 2002*, *25*, 349-352.

[12] F.C.O. SILVA, M.K.A. Ferreira, W.A. da Silva, M.G.C. Matos, F.E.A. Magalhães, P. T. da Silva, P.N. Bandeira, J.E.S.A. de Menezes, H.S. dos Santos, 'Bioativities of plants-solated triterpenes: a brief review', *Rev. Virtual Quim.* **2020**, *12*, 234-247.

[13] E.P. Schenkel, G. Gosmann, M.L. Athayde, Saponinas. In: C. M. Simões. *et al*. *Farmacognosia: Da Planta ao Medicamento.* 5ª ed. Porto Alegre/Florianópolis: Editora da UFRGS/Editora da UFSC, **2004**. *27*, 711-740.

[14] B. A. CARMONA, F. Escalante-Erosa, K. Garcia, G. Ruiz-Pinell, D. Gutierrez-Yapu, M. Chan, A. Giménez-Turba, L.M. Peña-Rodriguez, 'Antiprotozoal activity of Betulinic acid derivatives', *Phytomedicine*, **2010**, *17*, 379-382.

[15] C.S. Meira, J.M. Barbosa Filho, A.L. Rangel, E.T. Guimarães, D.R.M. Moreira, M.B.P. Soares, 'Antiparasitic evaluation of betulinic acid derivatives reveals effective and selective anti-*Trypanosoma cruzi* inhibitors', *Exp. Parasitol.* **2016**, *166*, 108-15.

[16] S.Hoet, L. Pieters, G.G. Muccioli, J.L. Habib-Jiwan, F.R. Opperdoes, J. Quein-Lequercq, 'Antitrypanosomal activity of triterpenoids and sterols from the leaves of *Strychnos spinosa* and related compounds', *J. Nat. Prod.* **2007**, *70*, 1360-1363.

[17] A.A.S. FILHO, D.O. Resende, M.J. Fukui, F.F. Santos, P.M. Pauletti, W.R. Cunha, M.L.A. Silva, L.E. Gregório, J.K. Bastos, N.P.D. Nanayakkara, 'In vitro antileishmanial, antiplasmodial and cytotoxic activities of phenolics and triterpenoids from *Baccharis dracunculifolia* D. C. (Asteraceae)', *Fitoterapia* **2009**, *80*, 478-482.

[18] S.T. Cargnin, A.F. Staudt, C. Menezes, A.P. de Azevedo, S.N. Fialho, T. Tasca, C.B.G. Teles, C.B. Gnoatto, 'Evaluation of Triterpenes derivatives in the viability of *Leishmania amazonensis* and *Trichomonas vaginalis*', *Braz. J. Pharm. Sci.* **2019**, *55*, 1-7.

[19] H.J. Jeong, H.B. Chai, SY Park, D.S.H.L. Kim, 'Preparation of amino acid conjugates of betulinic acid with activity against human melanoma', *Bioorg. Med. Chem. Lett*. **1999**, *9*, 1201-1204.

[20] L.A. Baltina, B. Flekhter, L.R. Nigmatullina, E.I. Boreko, N.I. Pavlova, S.N. Nikolaeva, O.V. Savinova, G.A. Tolstikov, 'Lupane triterpenes and derivatives with antiviral activity', *Bioorg. Med. Chem. Lett*. **2003**, *13*, 3549-3552.

[21] D. Zofou, T.K. Kowa, H.K. Wabo, M.N. Ngemenya, P. Tane, V.P.K. Titanji, '*Hypericum lanceolatum* (Hypericaceae) as a potential source of new anti-malarial agents: a bioassay-guided fractionation of the stem bark', *Malar. J.* **2011**, *10*, article number 167.

[22] C.F. Silva, P.B. da Silva, M.M. Batista, A. Daliry, R.R. Tidwell, M.N.C. Soeiro, 'The biological in vitro effect and selectivity of aromatic dicationic compounds on *Trypanosoma cruzi'*, *Mem. Inst. Oswaldo Cruz*. **2010**, *105*, 239-245.
[23] B. N. Lenta, C.V. *Senecheau, R. F. Soh, R.; F, Tantangmo, S. Ngouela, M.Kaiser, E. Tsamo, R. Anton, B Weniger,* '*In vitro* antiprotozoal activities and cytotoxicity of some selected Cameroonian medicinal plants', *J. Ethnopharmacol.* **2007**, *111*, 8-12.

[24] L.M. Weiss, H.B.Tanowitz, L.V. Kirchhoff, 'Chagas Disease: Part A', Advances in Parasitology, London: Elsevier, **2011**. 367.

[25] J.D. MAYA, B.K. Cassels, P.I. Vasquéz, J. Ferreira, M. Faúndez, N. Galanti, A. Ferreira, A. Morello, 'Mode of action of natural and synthetic drugs against *Trypanosoma cruzi* and their interaction with the mammalian host', *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2007**, *146*, 601-620.

[26] J.A. Peixoto, M.L. Andrade e Silva, A.E.M. Crotti, R.C.S. Veneziani, V.M.M. Gimenez, A.H. Januário, M. Groppo, L.G. Magalhães, F.F. dos Santos, S. Albuquerque, A.A. da Silva Filho, W.R. Cunha, 'Antileishmanial activity of the hydroalcoholic extract of *Miconia langsdorffii*, isolated compounds, and semi-synthetic derivatives', *Molecules* ***2011***, *16*, 1825-1833.

[27] W.R. Cunha, G.X. de Matos, M.G.M. de Souza, M.G. Tozatti, M.L. Andrade e Silva, C.H.G. Martins, 'Evaluation of the antibacterial activity of the methylene chloride extract of *Miconia ligustroides*, isolated triterpene acids, and ursolic acid derivatives', *Pharm. Biol*. **2010**, *48*, 166-169.

[28] W.R. CUNHA, E.J. Crevelin, G.M. Arantes, A.E.M. Crotti, M.L. Andrade e Silva, N.A.J.C. Furtado, S. Albuquerque, D.S. Ferreira, 'A study of trypanocidal activity of triterpene acids isolated from *Miconia* species', *Phytother, Res.* **2006**, *20*, p.474-478.

[29] V.A. Souza, R. da Silva, A.C. Pereira, V.A. Royo, J. Saraiva, M. Montanheiro, G.H.B. de Souza, A.A. da Silva Filho, M.D. Grando, P.M. Donate, J. K. Bastos, S. Albuquerque, M.L. Andrade e Silva, 'Trypanocidal activity of (−)-cubebin derivatives against free amastigote forms of *Trypanosoma cruzi', Bioorg. Med. Chem. Lett*. **2005**, *15*, 303-307.