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Article

Synthesis, characterization and utilization of a new series of 1,2,3-triazole derivatives to neutralize some toxic activities of *Bothrops jararaca* snake venom

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Snake envenomation is a public health problem, and while serum therapy prevents death, the local effects of venoms can lead to amputations or morbidities. Thus, alternative treatments deserve attention. In this study, we tested eight derivatives of 1,2,3-triazole against some toxic activities of *Bothrops jararaca* venom. The derivatives were synthesized, and their structures analyzed by infrared and nuclear magnetic resonance. After that, the ability of compounds to inhibit hemolysis, coagulation, proteolysis, hemorrhaging, edema, and lethal activities of *B. jararaca* venom was investigated. The derivatives were incubated with *B. jararaca* venom (incubation protocol), administered before (prevention protocol) or after (treatment protocol) injecting venom into the mice. Then, hemorrhaging assay occurred. As a result, most of the derivatives inhibited the activities, even if they were incubated, injected before or after *B. jararaca* venom. However, the derivatives TRI 07 and TRI 18 seemed to be the most efficient in impairing hemorrhaging. The derivatives showed a low drug score of toxicity based on an *in silico* technique. Therefore, the derivatives fulfilled physicochemical and biological requirements to become drugs, and they may be a brand new initiative for designing antivenom molecules to complement antivenom therapy to efficiently block tissue necrosis or any other local effects.

Keywords: 1,2,3-triazole derivatives. Organic synthesis. *Bothrops jararaca*. Snake venom. Neutralization. Antivenom.

INTRODUCTION

According to the World Health Organization, after 2017, snakebite envenoming was classified as a neglected tropical disease (NTD), affecting at least 5 million people annually worldwide, with 140,000 deaths and 400,000 morbidities (Ren *et al.*, 2019; Williams *et al.*, 2019a; 2019b). However, it is known that this number of cases is underestimated. Brazil has been greatly affected by snake envenomation, with approximately 30,000 accidents and 150 deaths annually. The Bothrops genus is responsible for 90 % of the official registered cases, of which, *B. jararaca* is the most medically important snake species (Chippaux, 2017; Frare *et al.*, 2019). *B. jararaca* is found in regions from southern Bahia to northern Rio Grande do Sul, Brazil as well as nearby in Argentina or Paraguay. This snake can reach up to 1.5 m in length and displays some biological functions benefitting the environment, such as population control of rodents.[6] *B. jararaca* venom is composed of a variety of proteins that produce many toxic effects in victims, such as pain, inflammation, edema, hemorrhaging, skeletal muscle damage, intravascular

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coagulation, heart or renal failure, and death. *B. jararaca* venom is rich in serine proteinases (SVSPs), metalloproteinases (SVMPs), phospholipases A_2 (PLA_2s), C-type lectins (CTL), L-amino acid oxidases (LAAOs), and other biologically active and non-active peptides (de Farias *et al.*, 2018; Senise Yamashita, Santoro, 2015). These enzymes may produce one or more of the toxic effects observed in victims after envenoming.

The specific official treatment for snakebite envenoming is performed through an intravenous administration of monovalent or polyvalent antivenom, of which the manufacturing procedure has remained practically the same over a hundred years, with no significant changes. However, other procedures have been investigated to improve antivenom therapy, based on antibody technologies (Knudsen *et al.*, 2019; Campos *et al.*, 2020). Antivenom is effective in preventing death of victims, but, by contrast, it has some drawbacks: low capability to inhibit local effects of venoms, low stability in liquid form, thermolability, high cost of production, and in some cases, antivenom may produce fever or anaphylactic reactions (Williams *et al.*, 2019b; Gómez-Betancur *et al.*, 2019). Thus, the limited efficacy of antivenoms may justify alternative therapies, which include monoclonal antibodies, aptamers, molecules derived from sponges, seaweed, plants or organic synthesis (Williams *et al.*, 2019b; Campos *et al.*, 2020; Ascoët, Ward, 2020; Faioli *et al.*, 2013; da Silva *et al.*, 2017; de Oliveira *et al.*, 2016; Domingos *et al.*, 2013; Pucca *et al.*, 2019). Triazole is a five-membered heterocyclic compound containing three nitrogen atoms, with two isomeric forms (1,2,3-triazole or 1,2,4-triazole). Triazole derivatives can bind to biological targets through weak interactions such as hydrogen bonding, hydrophobic effects, and Van der Waals forces; and some pharmacological activities have been related to them, such as antifungal, anticancer, antituberculosis, anti-inflammatory, antiviral, analgesic, antiplatelet aggregation or anticoagulant effects (Zhou, Wang, 2012; Kumar, Kavitha, 2013; Moura *et al.*, 2016; Malik *et al.*, 2020). In addition, some molecules containing the triazole nucleus have been used as a core structural component of drugs (Kharb, Sharma, Yar, 2011). Our group has demonstrated the inhibitory potential of triazole derivatives against the toxic activities of the venom of *B. jararaca* and *L. muta* snakes (Domingos *et al.*, 2013). Therefore, we decided to evaluate the effect of eight derivatives of a new series of 1,2,3-triazole against some toxic activities of *B. jararaca* venom.

MATERIAL AND METHODS

Reagents

Dimethylsulfoxide (DMSO) and azocasein were purchased from Sigma Chemical Co (St. Louis, Missouri, USA), acetonitrile HPLC grade from Tedia (Fairfield, OH, USA), and water was purified by a Milli-Q system (Millipore). All other reagents or solvents were of the best grade available.

Snake venom and animals

Lyophilized *B. jararaca* crude venom was kindly supplied by the Ezequiel Dias Foundation (FUNED), Belo Horizonte, MG, Brazil and maintained at -20 °C and diluted in physiological saline prior to use in the biological assays. Venom collection was conducted under the authorization of the Brazilian National System for Genetic Heritage Management and Associated Traditional Knowledge (SISGEN) (Process number A39CD4E). BALB/C mice (18-20 g) were obtained from the Animal Laboratory (NAL) of the Federal Fluminense University (UFF). The animals were allowed *ad libitum* supply of water and food and were maintained under controlled luminosity and temperature. The UFF Institutional Committee for Ethics in Animal Experimentation approved all the experiments under protocol number 508, in accordance with the Brazilian Committee for Animal Experimentation (COBEA) guidelines.

Organic synthesis of compounds

The starting materials used for the synthesis of compounds were purchased from Sigma-Aldrich. The anilines used were purified by recrystallization prior to use. Column chromatography was performed

with F60 silica gel (Merck 40-65 μm). Analytical thin-layer chromatography was performed with silica gel plates (Merck, TLC silica gel 60F-254) and visualized under ultraviolet light or developed by immersion in an ethanolic solution of vanillin. Yields refer to chromatographically and spectroscopically homogeneous materials. Melting points were obtained on a Fisatom apparatus (430 D model). Infrared spectra data were recorded from KBr pellets on a Thermo Scientific model Nicolet 6700-FTIR spectrophotometer calibrated relative to the 1601.8 cm^{-1} absorbance of polystyrene. NMR spectra were recorded on a Bruker AVHD 9.40 T (400.13 MHz ^{1}H and 100.61 MHz ^{13}C) and AVIII 11.75 T (500.13 MHz ^{1}H and 125.76 MHz ^{13}C) system in $CDCl_3$ solutions using tetramethylsilane as the internal reference standard (0.0 ppm). Coupling constants (*J*) are reported in hertz and refer to apparent peak multiplicities. Elemental analysis was used to ascertain purity > (95%) of all compounds for which biological data were determined. The CHN elemental analyses were performed on a Perkin-Elmer 2400 CHN elemental analyzer.

General procedure for obtaining aromatic azides from aromatic amines (2a-c)

In a beaker containing 1 mmol of aromatic amine (1a-c) and 1 mL of 6 M hydrochloric acid solution (50%) in an ice bath (maintaining temperature between 0-5 °C), an aqueous solution of 1.5 mmol of sodium nitrite ($NaNO_2$) in 2.5 mL of distilled water was slowly added under vigorous stirring. Thereafter, stirring was continued at a low temperature for 30 min. Subsequently, a 4 mmol solution of sodium azide (NaN_3) in 5 mL of distilled water was slowly added while maintaining temperature between 0-5 °C. The reaction was maintained at room temperature for the necessary amount of time until it reached completion. Then, the mixture was extracted with ethyl acetate and the organic phase was washed with saturated sodium bicarbonate solution and water and dried with anhydrous sodium sulfate. The solvent was evaporated under reduced pressure to obtain the aromatic azides (2a-c). The residual crude product was used directly without purification.

1-azido-2-methoxybenzene (2a)

Compound 2a was obtained as a brown liquid with 88 % yield. Rf = 0.69 (hexane/ethyl acetate: 7/3). IR (KBr pellet) ν(cm^{-1}): 3066 (C-H sp^2), 2940 (C-H sp^3), 2113 (-N=N=N). ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ: 3.87 (s, 3H, OCH_3), 6.89 (d, 1H, *J* = 8.0 Hz, H-Ar), 6.9 (t, 1H, *J* = 7.8 Hz, H-Ar), 7.01 (d, 1H, *J* = 7.8 Hz, H-Ar), 7.10 (t, 1H, *J* = 7.9 Hz, H-Ar). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ: 56.07 (OCH_3), 112.25 (C-Ar), 120.46 (C-Ar), 121.47 (C-Ar), 125.84 (C-Ar), 128.49 (C-Ar), 152.05 (C-Ar).

1-azido-4-methoxybenzene (2b)

Compound 2b was obtained as a yellow solid with 92 % yield. Rf = 0.49 (hexane/ethyl acetate: 7/3). IR (KBr pellet) ν(cm^{-1}): 3056 (C-H sp^2), 2103 (-N=N=N), 1592 and 1492 (C=C r). ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ: 3.70 (s, 3H), 6.91-6.85 (m, 2H, H-Ar), 6.99-6.92 (m, 2H, H-Ar). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ: 55.77 (CH_3), 115.35 (C-Ar), 120.20 (C-Ar), 132.57 (C-Ar), 157.23 (C-Ar).

1-azido-4-chlorobenzene (2c)

Compound 2c was obtained as a brown liquid with 74 % yield. Rf = 0.61 (hexane/ethyl acetate: 7/3). IR (KBr pellet) ν(cm^{-1}): 3054 (C-H sp^2), 2130 (-N=N=N). ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ: 6.95 (d, 1H, *J* = 2.0 Hz, H-Ar), 7,30 (d, 2H, *J* = 2.1 Hz, H-Ar). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ: 120.48 (C-Ar), 130.05 (C-Ar), 130.44 (C-Ar), 138.88 (C-Ar).

Method for the preparation of 1H-1,2,3-triazol-4-substituted compounds from aromatic azides

5 mmol of terminal alkyne (3), 0.1 mmol of $CuSO_4.5H_2O$ and 0.2 mmol of sodium ascorbate were added to a solution containing 1 mmol of the appropriate aromatic azide (2a-c) in *t*-BuOH (1 mL) and water (1 mL). The resulting suspension was maintained at room temperature for 2 hr. After this time, the reaction was quenched with $NaHCO_3$ to neutralize the solution. The mixtures were diluted with 5 mL of dichloromethane

and 5 mL of water. The organic phases were separated, dried with anhydrous sodium sulfate, and concentrated at reduced pressure, furnishing the 1,2,3-triazole compounds, which were analyzed by ^{1}H and ^{13}C NMR spectroscopy and IR spectroscopy. When isolated as crude material, the compounds were subjected to purification prior to biological assays to ensure that no residual metals or other organic impurities were present. Filtration was performed in a flash-type column, using an elution gradient of hexane/ethyl acetate.

(1-(2-methoxyphenyl)-4-phenyl-1H-1,2,3-triazole (TRI 03)

Compound TRI03 was obtained as a yellowish oil with 80 % yield. Rf = 0.44 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3229, 3107, 1872, 1726, 1613, 968, 895. ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ 3.91 (s, 3H), 7.15-7.08 (m, 2H, H-Ar), 7.36-7.41 (m, 1H, H-Ar), 7.46–7.41 (m, 3 H, H-Ar), 7.93–7.82 (m, 3 H, H-Ar), 8.32 (s, 1H, triazole). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 55.50 (OCH_3), 113.31 (C-Ar), 114.43 (C-Ar), 1121.1 (C-Ar), 127.60 (C-Ar), 128.07 (C-Ar), 128.44 (C-Ar), 129.10 (C-Ar), 129.69 (C-Ar), 130.42 (CHtriazole), 131.22 (Cq-triazole), 148.00 (C-Ar). Anal. Calcd for $C_{15}H_{13}N_3O$: C, 71.70; H, 5.21; N, 16.72. Found: C, 71.72; H, 5.22; N, 16.70.

1-(1-(2-methoxyphenyl)-1H-1,2,3-triazol-4-yl) cyclohexan-1-ol (TRI 04)

Compound TRI04 was obtained as a yellowish powder with 82 % yield. mp 57-59 °C; Rf = 0.49 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3231, 3107, 1872, 1726, 1613, 968, 895. ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ 1.25-1.35 (m, 2H), 1.68 (s, 2H), 1.75-1.80 (t, 2H, *J*= 6.2 Hz), 1.90-1.96 (m, 2H), 2.00-2.05 (m, 2H), 2.30 (s, 1H, -OH), 3.90 (s, 3H), 7.00-7.03 (d, 2H, *J* = 9 Hz), 7.60-7.63 (d, 2H, *J* = 9 Hz), 7.83 (s, 1H, triazole). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 21.09 (CH_2), 26.50 (CH_2), 38.22 (CH_2), 55.63 (OCH_3), 76.70 (Cq), 113.78 (C-Ar), 114.32 (C-Ar), 119.20 (CH-triazole), 121.47 (C-Ar), 128.20 (C-Ar), 129.73 (C-Ar), 132.08 (Cqtriazole), 154.21 (Cq-OCH_3). Anal. Calcd for $C_{15}H_{19}N_3O_2$: C, 65.91; H, 7.01; N, 15.37. Found: C, 65.94; H, 7.00; N, 15.38.

1-(4-chlorophenyl)-4-phenyl-1H-1,2,3-triazole (TRI 07)

Compound TRI07 was obtained as a yellow powder with 66 % yield. mp 220-223 °C; Rf = 0.34 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3231, 3110, 1882, 1726, 1610, 964, 895. ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ 7.39 (d, *J* =7.2 Hz, 1H), 7.40 (t, *J* =7.4 Hz, 2H), 7.52 (d, *J* =8.8 Hz, 2H), 7.77 (d, *J* =8.8 Hz, 2H), 7.98 (d, *J*=7.2 Hz, 2H), 8.10 (s, 1H). ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ Anal. Calcd for $C_{14}H_{10}ClN_3$: C, 65.76; H, 3.94; N, 16.43. Found: C, 65.75; H, 3.96; N, 16.44.

1-(1-(4-chlorophenyl)-1H-1,2,3-triazol-4-yl) cyclohexan-1-ol (TRI 08)

Compound TRI08 was obtained as a yellow powder with 65 % yield. mp 138-140 °C; Rf = 0.30 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3300, 3234, 3105, 1882, 1730, 1615, 964, 867. ^{1}H NMR ($CDCl_3$, 400.13 MHz) δ 1.35-2.42 (m, 10H, cyclohexyl-CH_2), 5.3 (bs, 1H, OH), 7.44 (d, 2H, Ar-Hs, *J* = 7.7 Hz), 7.8 (d, 2H, Ar-Hs, *J* = 7.57 Hz), 8.20 (s, 1H, H-triazole) ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 20.00, 24.31, 39.63 (cyclohexyl-CH_2), 64.87 (C-OH), 122.90 (CH triazole), 128.76 (Ar-C), 130.23 (Ar-C), 45.71 (Ar-Cs), 150.00 (Cqtriazole). Anal. Calcd for $C_{15}H_{19}N_3O_2$: C, 65.91; H, 7.01; N, 15.37. Found: C, 65.90; H, 7.03; N, 15.40.

1-(4-chlorophenyl)-4-(cyclohex-1-en-1-yl)-1H-1,2,3-triazole (TRI 09)

Compound TRI15 was obtained as a yellow powder with 73 % yield. mp 83-86 °C; Rf = 0.44 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3227, 3115, 1885, 1720, 1614, 965, 890. ^{1}H NMR ($CDCl_3$, 500.13 MHz) δ 1.60-1.68 (m, 2H), 1.82-1.73 (m, 2H), 2.20-2.23 (m, 2H), 2.45-2.42 (m, 2H), 6.63-6.59 (m, 1H), 6.73 (d, *J*= 9.3 Hz, 2H), 7.61 (d, *J*= 9.3 Hz, 2H), 7.78 (s, H-triazole). ^{13}C NMR ($CDCl_3$, 125.76 MHz) δ 23.71 (CH_2), 24.60 (CH_2), 25.00 (CH_2), 30.65 (CH_2), 115.00 (C-Ar), 122.24 (C-Ar), 123.01 (CHtriazole), 124.64 (=CH), 129.60 (C-Ar), 134.97 (=CH), 144.90 (Cq-triazole), Anal. Calcd for $C_{14}H_{14}ClN_3$: C, 64.74; H, 5.43; N, 16.18. Found: C, 64.70; H, 5.45; N, 16.19.

1-(4-methoxyphenyl)-4-phenyl-1H-1,2,3-triazole (TRI 16)

Compound TRI16 was obtained as a yellow powder with 65 % yield. mp 114-116 °C. Rf = 0.26 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3220, 3110, 1879, 1730, 1614, 968, 890. ^{1}H NMR ($CDCl_3$, 400.13 MHz) δ 3.84 (s, 3H, OCH_3), 6.99 (d, *J* = 8.7 Hz, 2H), 7.43 (t, *J* = 7.2 Hz, 1H), 7.52 (t, *J* = 7.2 Hz, 2H), 7.81-7.79 (m, 2H), 7.83 (d, *J* = 9.0 Hz, 2H), 8.02 (s, 1H, CH-triazole). ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ Anal. Calcd for $C_{15}H_{13}N_3O$: C, 71.70; H, 5.21; N, 16.72. Found: C, 71.72; H, 5.23; N, 16.73.

1-(1-(4-methoxyphenyl)-1H-1,2,3-triazol-4-yl) cyclohexan-1-ol (TRI 17)

Compound TRI17 was obtained as a yellow powder with 54 % yield. mp 200-203 °C; Rf = 0.36 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): ^{1}H NMR ($CDCl_3$, 400.13 MHz) δ 1.36-1.46 (m, 2H), 1.59 (s, 2H), 1.81 (d, 2H, *J* = 3 Hz), 1.93-2.00 (m, 4H), 2.67 (s, 1H, -OH), 3.90 (s, 3H), 7.00 (d, 2H, *J*= 9 Hz), 7.61 (d, 2H, *J* = 9 Hz), 7.89 (s, 1H); ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 21.60 (CH_2), 25.62 (CH_2), 38.45 (CH_2), 54.61 (OCH_3), 77.54 (Cq), 114.30 (C-Ar), 119.90 (CHtriazole), 122.00 (C-Ar), 129.87 (Cq-Ar), 132.95 (Cq-triazole), 160.65 (OCH_3). Anal. Calcd for $C_{15}H_{19}N_3O_2$: C, 65.91; H, 7.01; N, 15.37. Found: C, 65.93; H, 7.05; N, 15.39.

4-(cyclohex-1-en-1-yl)-1-(4-methoxyphenyl)-1H-1,2,3-triazole (TRI 18)

Compound TRI18 was obtained as a yellowish powder with 76 % yield. mp 108-110 °C; Rf = 0.46 (hexane/ethyl acetate: 1/1). IR (KBr pellet) ν(cm^{-1}): 3158, 2932, 2836, 1523, 1257, 1235, 1035, 832. ^{1}H NMR ($CDCl_3$, 400.13 MHz) δ 1.70-1.68 (m, 2H), 1.80-1.77 (m, 2H), 2.25-2.20 (m, 2H), 2.45-2.42 (m, 2H), 3.90 (s, 3H, OCH_3), 6.61-6.59 (m, 1H), 6.71 (d, *J*= 9.1 Hz, 2H), 7.61 (d, *J*= 9.1 Hz, 2H), 7.73 (s, H-triazole). ^{13}C NMR ($CDCl_3$, 100.61 MHz) δ 22.7 (CH_2), 23.7 (CH_2), 25.7 (CH_2), 30.6 (CH_2), 55.8 (OCH_3), 114.00 (C-Ar), 122.3 (C-Ar), 123.00 (CHtriazole), 124.65 (=CH), 129.65 (C-Ar), 133.97 (=CH), 144.87 (Cq-triazole), 160.6 (OCH_3). Anal. Calcd for $C_{15}H_{17}N_3O$: C, 70.56; H, 6.71; N, 16.46. Found: C, 70.54; H, 6.70; N, 16.45.

The effect of *B. jararaca* venom on coagulation of human plasma

Plasma was obtained from a pool of healthy volunteer donors at the blood bank of Antônio Pedro Hospital of the Federal Fluminense University (HUAP), after centrifugation at 1,300 *g* for 15 min of the whole blood that was withdrawn in citrate (0.31 % v/v), as anticoagulant. 200 µL of plasma (previously diluted in an equal volume of physiological saline) was maintained at 37 °C for 1 min, and then, *B. jararaca* venom (50 µL) was added in different concentrations (10-60 µg/mL) to the medium in order to trigger plasma coagulation. The time of coagulation was monitored in seconds (s), using a digital multichannel coagulometer (Amelung KC4A, Labcom, Germany). The amount of *B. jararaca* venom (µg/mL) able to coagulate plasma in around 60 s was denoted as the minimal coagulation concentration (MCC). After that, one MCC of *B. jararaca* venom (30 µg/mL) was incubated with 150 µg/mL of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) or with the solvents DMSO or saline (positive controls) for 30 min at 25 °C. Each derivative was incubated with solvents in the absence of venom for the negative control. Then, an aliquot of each mixture (50 µL) was added to plasma and coagulation time was recorded, as described. Coagulation was monitored over a maximal period of 800 s, and after that, plasma was considered incoagulable. The total reaction volume was 250 µL.

The effect of *B. jararaca* venom on proteolytic activity with azocasein as a substrate

The proteolytic activity of *B. jararaca* venom was determined using azocasein as a substrate, with modifications (Garcia, Guimarães, Prado, 1978). *B. jararaca* venom (8-40 µg/mL) was incubated with 0.4 mL of azocasein (0.2 % p/v) dissolved in 0.4 mL of Tris-HCl (200 mM), $CaCl_2$ (20 mM), pH 8.8, and the volume of reaction medium was adjusted to 1.2 mL by adding saline. Then, tubes were incubated at 37°C for 90 min, and the

reaction was stopped by adding 0.4 mL of trichloracetic acid (10 % p/v). Further, tubes were centrifuged at 12,000 rpm for 3 min, and 1 mL of supernatant was removed and mixed with 0.5 mL of NaOH (2 N). Finally, tubes were read at an absorbance of 420 nm, and the concentration of *B. jararaca* venom (µg/mL) able to produce reads of 0.2 in A 420 nm was considered 100 % proteolytic activity and referred to as the effective concentration (EC). After that, one EC of venom (15 µg/mL) was incubated with 150 µg/mL of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 °C, and an aliquot of the mixture was added to the reaction medium and proteolytic activity of *B. jararaca* venom was evaluated. As negative controls, each derivative or solvents were added to the reaction medium in the absence of venom.

Indirect hemolytic activity of *B. jararaca* venom

Hemolytic activity of *B. jararaca* venom was determined using the indirect hemolytic test with a suspension of washed red blood cells and an emulsion of hen's egg yolk, as a source of phospholipids to be the substrate for PLA_2 enzymes in the venom (Fuly *et al.*, 1997). After creating a concentration-response curve (20-70 µg/mL), the amount of *B. jararaca* venom (µg/mL) able to cause 100 % hemolysis was called the minimum indirect hemolytic concentration (MIHC). Then, one MIHC of *B. jararaca* venom (50 µg/mL) was incubated with solvents (DMSO – 0.9 % v/v or saline – positive control groups) or with 150 µg/mL of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 °C. Then, an aliquot was added to the reaction medium, and a hemolytic test was done. The negative control was performed by mixing each derivative with solvents in the absence of venom, followed by a hemolytic test. The hemolytic activity of *B. jararaca* venom was evaluated in the absence of hen's egg yolk to exclude direct hemolytic activity on red blood cells by the venom.

Hemorrhagic activity of *B. jararaca* venom

Hemorrhaging was measured according to Kondo *et al.* (1960), where *B. jararaca* venom was injected subcutaneously (s.c.) into the abdominal skin of mice. After 2 hours, the animals were euthanized, the abdominal skin was removed, stretched, and hemorrhagic lesions were quantified by measuring the halo, in millimeters (mm). One minimal hemorrhagic dose (MHD) was defined as the dose of venom (µg venom/mouse) able to produce a hemorrhagic halo of 10 mm, which was 50 µg/mouse. Two MHD of *B. jararaca* venom (100 µg/mouse) were incubated with 150 µg/mL of derivatives (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) or with DMSO or saline (positive controls) for 30 min at 25 °C. After incubation, an aliquot of the mixture was injected s.c. into mice and hemorrhagic activity was evaluated. Moreover, two additional sets of experiments were performed, in which the derivatives TRI 04, TRI 07, TRI 09, and TRI 18 were injected s.c. 30 min before (called prevention protocol) or after (called treatment protocol) injecting *B. jararaca* venom. The volume of each injection into the mice was 100 µL.

Edematogenic activity of *B. jararaca* venom

Edematogenic activity of *B. jararaca* venom was determined according to (Yamakawa, Nozani, Hokama, 1976) with some modifications. Samples containing *B. jararaca* venom (positive control group) or only solvents (negative control group) were injected s.c. into the right or left sub plantar paw of the mice, respectively. After 1 hr, the animals were euthanized and their paws were removed at the ankle joint and weighed. The increase in paw weight due to edema was calculated as the edema proportion, equal to edematous paw weight x 100/negative control paw weight. The minimal edematogenic dose (MED) was defined as the dose of venom (µg of venom/mouse) able to produce an increase of 120 % edema. The neutralizing effect was evaluated by incubating one MED of *B. jararaca* venom (50 µg/mouse) with 150 µg/mouse of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 °C. The derivatives were incubated with DMSO or saline (negative controls). Then, an aliquot of each sample mixture was injected into mice, and an edema test was performed. The volume of injection into the mice was 50 µL.

Theoretical toxicity study

Theoretical toxicity of the derivatives was determined using a free access program, Osiris® Property Explorer (http://www.organic-chemistry.org/prog/peo/). The chemical structure of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) was obtained and compared to some toxic substances and drugs already available in the program's database. The program evaluates some toxic (mutagenicity, tumorigenicity, irritability, and negative effects on reproduction) or physical-chemical parameters (lipophilicity, solubility, and molecular mass), druglikeness, and drug-score. All of these parameters allow us to predict whether derivatives have the potential or not to become medicinal drugs.

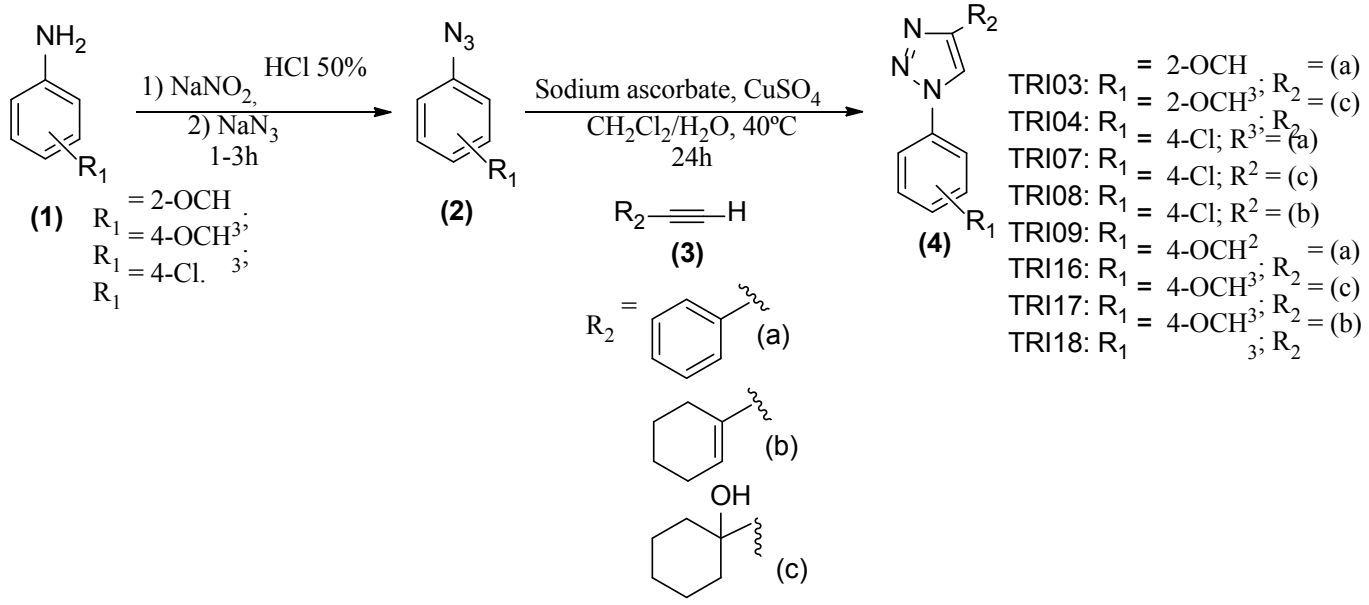
Statistical analysis

Results are expressed as mean + standard deviation (SD) obtained with the indicated number of animals or experiments performed and analyzed through analysis of variance (ANOVA) and Dunnett post-hoc tests, using GraphPad Prism. P values < 0.05 were considered significant.

RESULTS AND DISCUSSION

The 1,2,3-triazoles 1,4 dissubstituted with different carbocycles in position 4 were synthesized using a methodology similar to that reported by (Boechat *et al.*, 2011) (Scheme 1). For this route, the first step involved the preparation of aromatic azides from 4-chlorine, 4-methoxy, 2 methoxyanilines, producing good yields, ranging from 88-90 %. The azides were properly characterized, mainly by the analysis of the crude product using FTIR spectroscopy which presented a strong absorption band around 2110 cm^{-1}, referring to the vibrations of N3 stretching in the IR spectrum of the azides. With the prepared azides, it was possible to obtain the desired triazoles through Huisgen's Cycloaddition with click-reflection conditions. The 1,3-dipolar cycloaddition reaction between alkyl, alkenyl and aromatic azide terminals catalyzed by copper sulfate ($CuSO_4$) and sodium ascorbate guided the selectivity of the region. For the formation of triazole, it is necessary that Cu (I) is present as a catalyst; however, the system uses Cu (II), since sodium ascorbate has the function of reducing this ion Cu (II) to Cu (I), generating these ions *in situ*. The use of tert-butanol at room temperature was the best condition to be most efficient in terms of reaction time and low formation of by-products. The triazole compounds were obtained as yellowish solids or oils with yields between 65-80 %.

The triazole structures were confirmed by analysis using FTIR, ^{1}H and ^{13}C NMR. The FTIR analysis revealed the absence of stretching vibrations in the azide group. In the ^{1}H NMR spectrum, the signals from the protons of the synthesized compounds themselves were verified based on their chemical shifts, multiplicities and coupling constants. The characteristic proton signal for identification of the triazole nucleus can be observed as a single one around 7.5-8.5 ppm. All data obtained were consistent with the data previously published in the literature for this family of compounds.



SCHEME 1 - Synthetic route of production of 1,2,3-triazoles 1,4-disubstitutes.

The pathogenesis of Bothropic envenomation is characterized by severe local pain, edema, local hemorrhaging, tissue necrosis, systemic hemorrhaging, hemolysis, renal or cardiac failure, and death. These clinical symptoms are due to the presence of some toxins in the venom of these animals, mainly serine proteinases, metalloproteases and phospholipases A_2 (Tasoulis, Isbister, 2017). Snake venoms are composed of different isoforms of enzymes and have different antigenic patterns; thus, because of these factors, antivenoms do not efficiently block all of the enzymes.

The official treatment used to neutralize the toxic effects induced by venomous snakebites is the administration of antivenom obtained through the hyperimmunization of animals, usually horses or sheep (Williams *et al.*, 2019b; Knudsen *et al.*, 2019; Campos *et al.*, 2020; Gómez-Betancur *et al.*, 2019; Preciado *et al.*, 2018). However, this therapy may induce mild adverse effects (nausea, fever, and chills) or may cause more serious complications, like bronchospasms and anaphylactic reactions (Clark *et al.*, 2002; Dart, Mcnally, 2011). The administration of incorrect antivenoms may enhance the incidence of adverse reactions. Furthermore, antivenoms poorly neutralize local effects of snakebites, causing an increase in cases of amputation of or disabilities in the affected limb. Thus, in order to improve and lower adverse effects of antivenoms, several authors have been searching for other therapies than conventional antivenoms using small molecules, peptides, and recombinant antivenoms (Williams *et al.*, 2019a; Campos *et al.*, 2020; Ascoët, Ward, 2020; Pucca *et al.*, 2019; Dart, Mcnally, 2011; Clark *et al.*, 2002). Moreover, the extracts or products of plants and seaweed have been tested as well (da Silva *et al.*, 2017; de Oliveira *et al.*, 2016; de Oliveira *et al.*, 2014; Souza *et al.*, 2020). On the other hand, molecules derived from organic synthesis are not being deeply explored. Triazoles and their derivatives are stable groups, with applications in many areas, such as in agriculture and to treat diseases (Kumar, Kavitha, 2013; Malik *et al.*, 2020). These structures have been reported as good bioisosteres, with a high capacity to interact with different biological targets, mainly by hydrogen bonding and dipole-dipole interactions (Kumar, Kavitha, 2013; Malik *et al.*, 2020). Therefore, in this study, a new series of triazole derivatives was tested as antivenom against some of the main toxic activities of *B. jararaca* venom. Overall, the derivatives inhibited the *in vitro* (proteolytic, hemolytic, and coagulant) and *in vivo* (hemorrhagic and edematogenic) activities of the venom of the *B. jararaca* snake.

Our group has previously tested triazoles with different chemical groups against the toxic activities of snake venoms. Domingos *et al.*, (2013) tested six triazole derivatives, and they inhibited the coagulant, proteolytic, hemolytic, hemorrhagic, and edematogenic activities of *B. jararaca* and *Lachesis muta* venom, regardless of the substituent attached to the triazole ring. Thus, these data corroborate the potential use of triazoles as antivenoms, since they are stable, have a simple and cheap route of synthesis, and are devoid of toxicity.

All of the derivatives inhibited coagulation caused by *B. jararaca* venom (Figure 1), with the derivatives TRI 07 and TRI 16 being the most efficient, since they delayed plasma coagulation approximately seven times more (400 and 450 s) than the positive group (*B. jararaca* + saline or *B. jararaca* + DMSO, 70 s). The derivatives TRI 09 and TRI 08 prolonged coagulation five and four times more than the positive control, respectively. The derivatives alone did not induce plasma coagulation during 800 s of monitoring (data not shown), and thus, they do not interfere with the hemostatic system. Plasma did not coagulate in the absence of *B. jararaca* venom over a maximal period of monitoring, which was chosen as 800 s (data not shown).

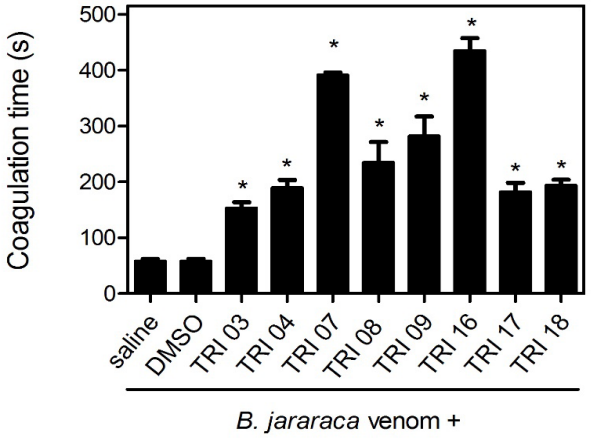


FIGURE 1 - Effect of the derivatives on coagulation time of *B. jararaca* venom.

B. jararaca venom (30 µg/mL) was incubated with saline, DMSO (0.9 % v/v, final concentration) or with 150 µg/mL of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 ºC. Then, an aliquot of the mixture was added to plasma, and coagulation time (s) was determined, as described in the method section. Results are expressed as means ± SD of two individual experiments (n=3). *, significance level ($p < 0.05$) when compared to *B. jararaca* venom + saline or *B. jararaca* + DMSO (positive control groups).

As shown in Figure 2A, *B. jararaca* venom (15 µg/mL) incubated with saline or DMSO caused 100 % of proteolytic activity on azocasein. Then, each derivative (150 µg/mL) was incubated with *B. jararaca* venom, followed by the determination of the proteolytic activity. As seen in Figure 2A, the derivative TRI 18 inhibited 80 % of proteolysis caused by *B. jararaca* venom. The derivatives TRI 03, TRI 07, TRI 08, and TRI 17 inhibited proteolytic activity by around 30 %, while the derivatives TRI 04, TRI 09 and TRI 16 did not inhibit this activity caused by *B. jararaca* venom (Figure 2A). The derivatives alone did not hydrolyze azocasein; thus, they were considered devoid of proteolytic activity (data not shown). The effect of the derivatives on the hemolytic activity of *B. jararaca* venom was tested (Figure 2B). Incubation of *B. jararaca* venom (50 µg/mL) with saline or 0.9 % (v/v, final concentration) of DMSO (positive control groups) lysed 100 % of red blood cells, and all of the derivatives inhibited around 80 and 90 % of *B. jararaca* venom-induced hemolysis (Figure 2B). The derivatives or solvents alone did not lyse cells. *B. jararaca* venom in the absence of hen's egg yolk did not lyse red blood cells, and, thus, it did not show direct hemolytic activity (data not shown). Hemolysis is caused by the action of lysophatidylcholine (lyso-pc), a product of a PLA_2-catalyzed reaction upon the hen's egg yolk which is rich in phospholipids. Thus, hemolysis occurred through indirect activity of *B. jararaca* venom.

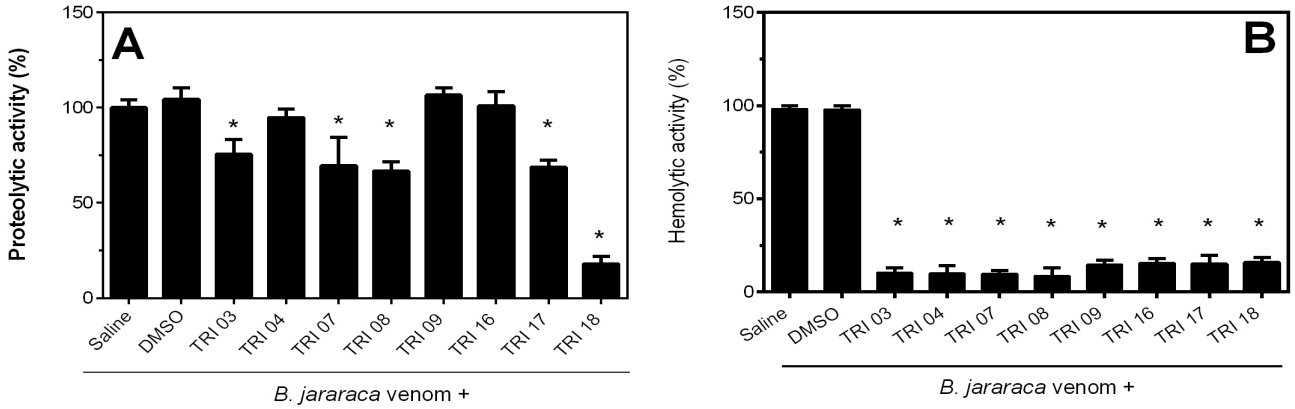


FIGURE 2 - Effect of the derivatives on proteolytic or hemolytic activity of *B. jararaca* venom.

(A), *B. jararaca* venom (15 µg/mL) was incubated with saline, DMSO or with 150 µg/mL of the derivatives (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 ºC, and proteolytic activity was subsequently evaluated, as previously described. (B), *B. jararaca* venom (50 µg/mL) was incubated with saline, DMSO (0.9 % v/v, final concentration) or with 150 µg/mL of the derivatives (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 ºC, and then the hemolytic activity was evaluated, as described. Results are expressed as means ± SD of two individual experiments (n = 3). *, significance level ($p < 0.05$) when compared to *B. jararaca* venom + saline or *B. jararaca* venom + DMSO (positive control groups).

Injection of *B. jararaca* venom (100 µg venom/mouse) incubated with saline or DMSO (0.9 % v/v, final concentration) produced a hemorrhagic halo of 24 millimeters which was considered to be 100 % of hemorrhagic activity (Figure 3). As seen in Figure 3A, in the incubation protocol, the derivative TRI 07 inhibited 70 % of *B. jararaca* venom-induced hemorrhaging. The derivatives TRI 03, TRI 04, TRI 08, TRI 09, and TRI 18 inhibited hemorrhaging by around 35 %, and the derivatives TRI 16 and TRI 17 did not prevent hemorrhaging caused by *B. jararaca* venom. Moreover, two additional sets of experiments were performed for the derivatives TRI 04, TRI 07, TRI 09, and TRI 18, named the treatment protocol (Figure 3B) and prevention protocol (Figure 3C). In the treatment protocol, each of the derivatives (TRI 04, TRI 07, TRI 09, and TRI 18) was injected s.c. 30 min after injecting *B. jararaca* venom, while in the prevention protocol, these derivatives were injected s.c. 30 min prior to injecting *B. jararaca* venom. Hemorrhaging caused by *B. jararaca* venom was inhibited by the derivatives by around 20 or 70 % in the treatment protocol (Figure 3B) or prevention protocol (Figure 3C), respectively. Injection of the derivatives or solvents did not induce hemorrhaging (data not shown). Thus, regardless of whether the derivatives were incubated with venom and injected after or before injecting the venom, inhibition of hemorrhaging caused by *B. jararaca* venom was achieved, though with different inhibitory percentages.

In most studies with molecules that are candidates for neutralizing toxic activities of snake venoms, the experimental design is usually performed by incubating them with the venom, and then a toxic activity assay is performed. However, this protocol does not simulate a real envenomation situation, since the snake first bites the victim, injecting the venom into the victim, then the victim goes to a hospital, and finally, the victim receives antivenom. The time between envenomation and receiving antivenom is crucial to prevent death, amputations or deformity of the affected limb. However, we understand the importance of testing protocols other than just pre-incubating molecules with the venom. Therefore, in addition to this incubation protocol, in this study, we performed the treatment or prevention protocols

using the hemorrhagic activity assay. It was observed that the derivatives protected better against hemorrhaging caused by *B. jararaca* venom if they were injected prior to injecting the venom.

Preciado *et al.* (2018) tested a flavonoid, myricetin, which inhibited hemorrhaging caused by crude venom and by an SVMP (Batx-I) isolated from *B. atrox*; edema was not affected. The results of the *in situ* test showed lower inhibition of hemorrhagic activity in the treatment protocol when compared to the incubation protocol. The authors suggest that this reduction might be due to the rapid hemorrhagic action of SVMPs from *B. atrox*. In our study, the prevention protocol protects animals from hemorrhaging induced by *B. jararaca* venom; however, further investigations need to be carried out to better understand the mechanism of action.

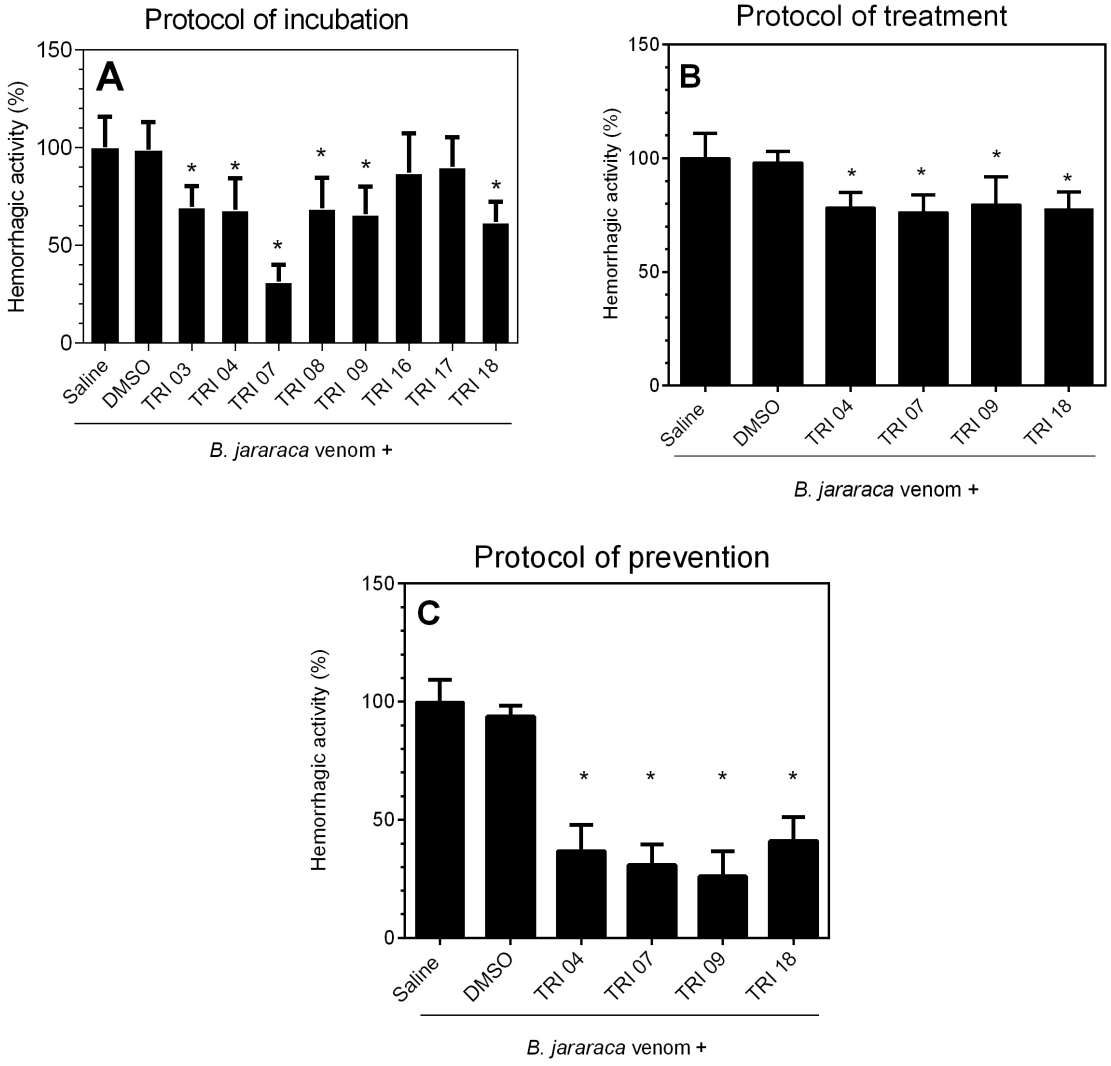


FIGURE 3 - Effect of derivatives on hemorrhaging caused by *B. jararaca* venom.

B. jararaca venom (100 µg/mL) was incubated with saline, DMSO or with 150 µg/mL of derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 ºC, and then the mixture was injected s.c. into mice (Panel A, incubation protocol). The derivatives (TRI 04, TRI 07, TRI 09, and TRI 18) were injected s.c. into mice 30 min after (Panel B, treatment protocol) or before (Panel C, prevention protocol) injecting s.c. *B. jararaca* venom. After the injections, the hemorrhagic halo was measured, and expressed as % of hemorrhage activity. Results are expressed as means ± SD of two individual experiments (n = 3). *, significance level ($p < 0.05$) when compared to the positive control groups (*B. jararaca* venom + saline or *B. jararaca* venom + DMSO).

B. jararaca venom (50 µg venom/mouse) incubated with saline or DMSO (positive controls) was injected into mice, their paws were weighed, and values were recorded as 100 % edematogenic activity (Figure 4). The derivatives TRI 03 and TRI 18 inhibited 100% of the edematogenic activity caused by *B. jararaca* venom. The derivatives TRI 08 and TRI 16 inhibited approximately 75 % of the edematogenic activity caused by *B. jararaca* venom, while the derivatives TRI 09 and TRI 17 inhibited 52 % of edema. The derivatives TRI 04 or TRI 07 did not significantly inhibit edema when compared to *B. jararaca* venom incubated with saline or DMSO (Figure 4). Injection of each derivative or solvents did not cause edema (data not shown).

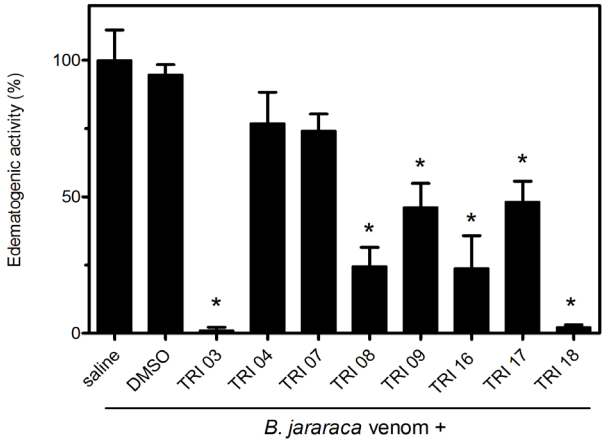


FIGURE 4 - Effect of derivatives on edema caused by *B. jararaca* venom.

B. jararaca venom (50 µg/mL) was incubated with saline, DMSO (0.9 %, final concentration) or with 150 µg/mL of each derivative (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) for 30 min at 25 ºC, and then the edematogenic activity was evaluated. Results are expressed as means ± SD of two individual experiments (n = 3). *, significance level ($p < 0.05$) when compared to the positive control groups, *B. jararaca* venom + saline or *B. jararaca* venom + DMSO.

Strauch *et al*., (2019) showed evidence that lapachol, a naphthoquinone isolated from a variety of plants inhibited proteolytic, hemorrhagic, and edematogenic activities of *B. atrox* venom and the hemorrhagic activity induced by *B. jararaca* venom, but failed to inhibit the phospholipase A_2, myotoxic, and coagulant activities of *B. atrox* venom. The authors postulated that laphacol and its derivatives may be inhibitors of SVMPs from these venoms.

Da Silva *et al*., (2008) tested some derivatives of ellagic acid from the plant *Casearia sylvestris* against a PLA_2 isolated from *B. jararacussu* venom, named Bothropstoxin-II (BthTX-II). They showed that ellagic acid inhibited the PLA_2 myotoxic and edematogenic activities of BthTX-II more efficiently than the crude venom, probably because the venom of this species contains isoforms of PLA_2. On the other hand, Villar *et al*. (2008) showed that derivatives of nitrostyrene inhibited edematogenic and myotoxic activities of BthTX-II. The authors observed that in a structure-activity relationship study, the interaction of derivatives with BthTX-II occurred in a different region of the active site of this enzyme. Here, in our study, we observed that all of the derivatives were able to inhibit hemolytic and edematogenic activity of *B. jararaca* through an interaction with PLA_2. However, this hypothesis should be verified by evaluating the binding of these derivatives to the isolated enzyme.

In silico toxicity of the derivatives was determined using Osiris Property Explorer®, analyzing the parameters of mutagenicity, tumorigenicity, irritability, and reproductive negativity. As shown in Figure 5, the derivatives showed low risk of toxicity (score of 1), regardless of the parameter analyzed. As known from the literature, predicting risk of toxicity using the Osiris® program is not fully reliable. However, the low-toxicity profiles observed for the derivatives reinforce their potential as antivenom with no undesirable effects.

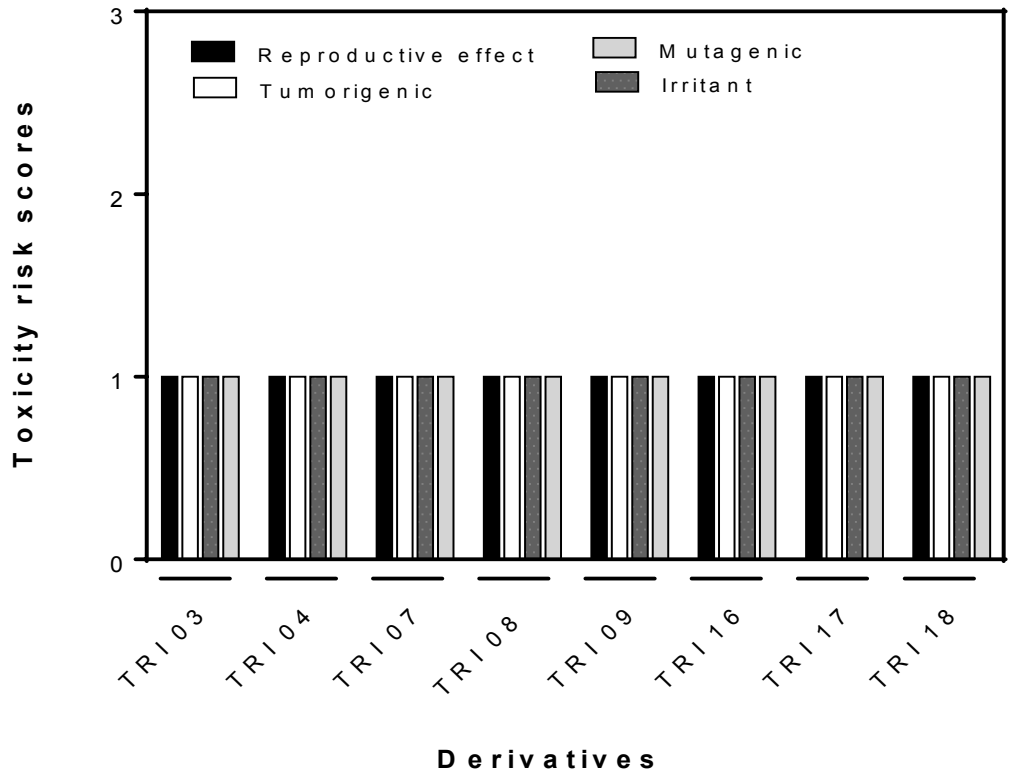


FIGURE 5 - Toxicity risk of the derivatives

The derivatives (TRI 03, TRI 04, TRI 07, TRI 08, TRI 09, TRI 16, TRI 17, and TRI 18) were evaluated on their theoretical risk of toxicity, negative reproductive effect, tumorigenic, mutagenic, and irritant aspects using the software Osiris Property Explorer®. Toxicity risk scores are: 1 = low toxicity, 2 = moderate toxicity, 3 = high toxicity.

Moreover, the theoretical values of druglikeness and drug-score of the derivatives were calculated. As seen in Table I, all of the derivatives presented negative values of druglikeness and, thus, their chemical structures do not match that of any commercial drugs, or at least those provided by the databank of this software. The drug-score parameter (from 0 to 1) predicts the potential for a molecule to become a drug, where a value of 1 means the molecule is devoid of potential risk to human health, and a value of zero reflects extremely high risk. The drug-score values of the derivatives varied from 0.43 to 0.48 (Table I). Therefore, the derivatives do not have any negative potential to human health. The derivatives TRI 04, TRI 17, and TRI 18 have the best LogS values, -2.65, -2.65 and -2.64, respectively. This value indicates that the derivatives are readily soluble, allowing them to cross the intestinal brush border in order to reach the target; however, LogS values below -4 are also acceptable for molecules to become drugs. Indeed, the low molecular weights (MW of 251 to 277) clearly indicate that these derivatives have the potential to become drugs, because they are easily absorbed through the gastrointestinal tract. As seen in Table I, all of the derivatives fulfilled the requirements to become orally active drugs for humans.

The results of the *in silico* theoretical toxic studies of all the derivatives showed low indices of toxicity and irritability, suggesting little risk of adverse effects (Table I). These data are in accordance with the values obtained through drug score analysis, confirming that these molecules do not present potential theoretical risks to human health. In addition, the druglikeness parameter of all the derivatives showed novelty in their structures, good absorption and solubility, and all of these parameters are important for the development of new drugs.

TABLE I - Prediction of theoretical toxicity of the 1,2,3-triazole derivatives

DERIVATIVES	cLogP	LogS	MW	Druglikeness	Drug-score
TRI 03	2.15	-3.14	251.0	-2.33	0.48
TRI 04	1.58	-2.65	273.0	-6.00	0.46
TRI 07	2.83	-3.85	255.0	-1.95	0.46
TRI 08	2.26	-3.36	277.0	-5.74	0.43
TRI 09	2.51	-3.36	259.0	-7.94	0.43
TRI 16	2.15	-3.14	251.0	-4.09	0.46
TRI 17	1.58	-2.65	273.0	-7.71	0.46
TRI 18	1.83	-2.64	255.0	-10.03	0.46

The physicochemical parameters evaluated were: lipophilicity (cLogP), solubility (LogS), molecular weight (MW), druglikeness, and drug-score of derivatives, predicted using the software Osiris® Property Explorer and Molinspiration.

Triazolic synthetic derivatives could be useful as a complementary treatment to antivenom therapy to more efficiently treat envenomation caused by *B. jararaca* venom. However, there is a need for additional experiments to elucidate the mechanisms by which the derivatives inhibit the enzymes present in the venom.

CONCLUSIONS

The search for molecules able to inhibit the main toxic effects of snake venoms is essential. Overall, the molecules derived from triazole synthesized in this study displayed promising antivenom potential with low theoretical risk of toxicity, suggesting safety and efficacy in treating envenoming by the snake *B. jararaca*.

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AUTHOR CONTRIBUTIONS

Nayanna de Mello Amorim (NMA): Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing-original draft, Writing-review & editing. **Luiz Carlos Simas Pereira Junior (LCSPJ):** Writing-review & editing, Methodology, Investigation, Writing-review & editing, Visualization. **Eladio Flores Sanchez (EFS):** Data curation, Formal analysis, Visualization, Resources, Writing-review & editing. **Gabriel Alves de Aquino (GASA):** Data curation, Resources, Methodology, Visualization, Writing-review & editing. **Vitor Francisco Ferreira (VFF):** Data curation, Visualization, Methodology, Writing-review & editing. **Sabrina Baptista Ferreira (SBF):** Data curation, Formal analysis, Funding acquisition, Resources, Investigation, Visualization, Writing original draft, Writing-review & editing. **André Lopes Fuly (ALF):** Conceptualization, Data curation, Formal analysis, funding acquisition, Investigation, Project administration, Resources, Software, Validation, Visualization, Writing-original draft, Writing-review & editing. **Eduardo Coriolano de Oliveira (ECO):** Conceptualization, Data curation,

Formal analysis, Investigation, Validation, Visualization, Writing-original draft, Writing-review & editing.

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