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SYNTHESIS OF (-) - 6,6'-DINITROHINOKININ FROM HINOKININ NATURAL PRODUCT AND *in silico* AND *in vitro* TRYPANOCIDAL ACTIVITY ASSESSMENT

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Abstract

Chagas disease is a public health problem affecting approximately seven million people worldwide. Thus, there is a need to discover drugs for the adequate treatment of this disease because currently available drugs have serious side effects. Therefore, this study aimed to evaluate the *in vitro* trypanocidal activity of (-)-6,6'-dinitrohinokinin, obtained from the partial synthesis of (-)-hinokinin, on the trypomastigotes and amastigotes forms. For the trypomastigote assay, blood was collected from mice infected with *Trypanosoma cruzi* through cardiac puncture at the parasitemic peak. The results show that (-)-6,6'-dinitrohinokinin was effective against the trypomastigote forms, presenting an IC_{50} of 19.83 µM and lysis percentage values of 78.4% and 69.4% at concentrations of 200 and 100 µM, respectively. Molecular docking calculations indicate that (-)-6,6'-dinitrohinokinin favorably interacts with the amino acids present in the active site of the protein trypanothione reductase, a typical target for anti-trypanosomal drug development. According to the results, the (-)-6,6'-dinitrohinokinin showed more significant trypanocidal activity with IC_{50} of 1.83 µM than benzonidazole positive control with IC_{50} of 53.2 µM, showing to be a prototype molecule promising for the development of a new antiparasitic drug.

Keywords: Molecular docking. Natural products. Trypanocide. *Trypanosoma cruzi*.

1. Introduction

Although *Trypanosoma cruzi* has been known as the causative agent of Chagas disease for over 100 years, the disease is still considered a public health problem, especially in Latin America. The World Health Organization recognizes it as one of the 17 neglected tropical diseases worldwide (WHO 2010; Pérez-Molina and Molina 2018). Approximately seven million people worldwide are estimated to be infected

with *T. cruzi*, and 75 million people are at risk of contracting the disease (WHO 2021). However, despite this large number and its considerable implications for morbidity and mortality, researchers have largely neglected Chagas disease. The drugs currently available for the treatment of Chagas disease include benznidazole and nifurtimox (interrupted production), which are administered during the acute phase of the disease. In the chronic phase, these drugs have low efficacy and present significant toxicity and side effects in patients (Saraiva et al. 2010; WHO 2021).

In this context, there is a need to identify new drugs and chemotherapeutic agents that are more effective in the treatment of Chagas disease, to improve efficacy in both phases, accessibility to patients (low cost), the absence of teratogenic or secondary effects, and toxicity to different forms of parasites (Alviano et al. 2012). Thus, through screening processes and synthesis of derivatives, natural products often cooperate in developing new drugs (Ioca et al. 2016; Adamante et al. 2019; Luiza et al. 2020; Paula 2021). Therefore, *Piper cubeba*, which is used in folk medicine to treat asthma, abdominal pain, gonorrhea, dysentery, and syphilis, can be a source of natural antiparasitic compounds (Magalhães et al. 2012). Fractionation of dried *P. cubeba* seeds provides (-)-cubebin (CUB), a lignan derived from dibenzylbutyrolactone. (-)-Hinokinin (HNK) is obtained by oxidation of CUB (Esperandin et al. 2013). (−)-Hinokinin (HNK) is obtained through the partial synthesis of CUB and has anti-inflammatory and analgesic actions and *in vitro* and *in vivo* trypanocide (Da Silva et al. 2005). Finally, HNK subjected to nitration provides dinitrohinokinin (DNK), the substance used in this study (Da Silva et al. 2005).

DNK exhibits schistosomicidal activity (INPI Patent); however, few studies have investigated its trypanocidal activity. Thus, based on previous studies conducted by our group (Da Silva et al. 2005), this study aimed to synthesize DNK and evaluate its *in vitro* toxicity against trypomastigote and amastigote forms of *T. cruzi*. An in silico analysis was performed to elucidate the anti-trypanocidal activity of DNK.

2. Material and Methods

Obtaining the DNHK

For the first stage, *P. cubeba* seeds were purchased from Suraji Bala Exports (New Delhi, India). The description of the isolation and characterization of CUB and its oxidation synthesis to obtain HNK is consistent with that of Esperandim et al. (2013). Second, DNK, a nitrated derivative, was prepared from HNK. First, 1.0 g of HNK was reacted with fuming nitric acid (7.9 EqM), stirring at 0°C for 2 h. Subsequently, a sodium bicarbonate solution was added to terminate the reaction (Figure 2). Partitions were performed with chloroform (3 × 30 mL), and the organic phase was separated by adding sodium sulfate as a desiccant. The mixture was filtered, and the solvent was rotated under vacuum. The obtained product was recrystallized from methanol. The purity was determined using high-performance liquid chromatography and subjected to ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. As this is the compound studied by our research group, the criteria were followed according to a methodology previously proposed (Da Silva et al. 2005; De Souza et al. 2005).

In vitro trypanocidal assay against the trypomastigote form of *T. cruzi*

The assay used blood from infected albino mice obtained by cardiac puncture at the peak (7th day of infection). The infected blood was diluted in saline to a final concentration of 10^6 trypomastigotes/mL. The DNK sample was diluted in dimethyl sulfoxide (DMSO), and an aliquot of this stock solution was added to the infected blood in a microtiter plate (96 wells), totaling 200 µL. The trypanocidal assay evaluated the sample in triplicate at 12.5, 25, 50, 100, and 200 µM. In addition, 25% DMSO was used as a positive control, and 0.5% DMSO was used as a negative control. The microplate was incubated at 4°C for 24 h, after which the activity was quantitatively verified by counting the trypomastigotes according to Brener, 1962. The percentage of parasite lysis was determined by comparison with that of the negative control group.

In vitro trypanocidal assay against the amastigote form of *T. cruzi*

Assays on the amastigote forms were performed in LLCMK2 cell culture, prepared according to Giorgio (1998), being grown in RPMI 1640 medium, supplemented with 2 mM L-glutamine, 10 mM $NaHCO_3$, 100 U/mL penicillin, 100 µg/mL of streptomycin, and 5% inactivated fetal bovine serum. The medium containing the cells was aliquoted into 24-well microplates (1 x 10^6cells/well, counted using a Neubauer chamber), and this system was incubated for 24 h at 37°C in a 5% CO_2 environment with 95% humidity. The culture medium containing the trypomastigote forms was removed from the cell cultures and subjected to centrifugation at 760 rpm for 8 min at 12°C. Thus, trypomastigotes remained in the supernatant and cells in the pellet. Then, 1 x 10^6 trypomastigote forms were added to the microplates and incubated for 48 h. Stock solutions were prepared by dissolving the substances in DMSO. This stock solution was added to the infected medium to obtain final concentrations of 200, 100, 50, 25, and 12.5 µM. Subsequently, the samples were added and incubated for 48 h, followed by Giemsa staining. The activity was quantitatively verified by counting the number of infected cells and determining the percentage of parasite reduction compared to the control. Negative (DMSO 0.5%) and positive (benzonidazole) controls were used in parallel. The assay was performed in triplicates.

Cytotoxicity assay

LLCMK2 strain fibroblasts were cultured in RPMI 1640 medium supplemented with 10% inactivated fetal bovine serum (100 mL of serum) and 5 mL of antibiotic in culture bottles at 37°C in a 5% CO_2 environment, with a humidity of 95%. On the day of the experiment, the bottle was scratched with a tip (5-mL pipette), the surface was rinsed, and the medium was transferred to a sterile falcon tube. The mixture was centrifuged at 1500 rpm at 4°C for 15 min. After centrifugation, the tube was inverted to remove the supernatant, and 1 mL of complete RPMI was added. Then, 10 µL was taken from the cell solution, and 990 µL of TURK solution was added. The dye in this solution can stain viable cells, that is, cells that must be counted. From this new solution, 10 µL was taken to conduct the counting in a Neubauer chamber. The values obtained were summed and divided by four to obtain the average value. After adjusting the number of cells to 10^6/mL, 100 µL was taken from this solution, which was then added to the microplate wells. The cytotoxicity assay evaluated the samples at 400, 200, 100, 50, 25, 12.5, and 6.25 µM, and the following controls were used: (1) Positive control: 25% DMSO; (2) Negative control: 0.5% DMSO. The plates were then incubated in a CO_2 oven for 24 h. At the end of this period, the plate was read on an enzyme-linked immunosorbent assay reader using the colorimetric MTT assay.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed using the GraphPad Prism software (version 5.0). Data were statistically analyzed using one-way analysis of variance (ANOVA), followed by Dunnett's comparison test.

Molecular docking calculations

The target in the molecular docking calculation was *T. cruzi* trypanothione reductase (TR). TR is an enzyme that converts trypanothione disulfide to its dithiol form, dihydrotrypanothione, in *T. cruzi* (Bond et al. 1999). This is important because this metabolite is related to scavenging free radicals and oxygen-reactive species produced by metabolic processes or when parasites are exposed to oxidative stress by the host immune response (Fairlamb; Cerami 1992.). Thus, TR represents a target for anti-trypanosomal drug development (Bond et al. 1999). The structural coordinates of this protein were obtained from Protein Data Bank ID 1BZL (Bond et al. 1999). The binding site was determined using Discovery Studio 2020 (Dassault Systèmes BIOVIA 2020) software, considering a 10 Å docking sphere from the ligand's centroid associated with the protein obtained from the trypanothione protein data bank: trypanothione (Bond et al. 1999). The protein and ligand were prepared, and docking simulations were performed using GOLD

2020.2.0 software (Jones et al. 1995). The best poses were ranked using the GoldScore fitness function. Pose selection is accompanied by a scoring function that incorporates the following components: the hydrogen bond energy of the complex, internal energy of the ligand, and torsional energy (Jones et al. 1997; Verdonk et al. 2003). The rescore was realized using the ChemScore fitness function to obtain the term dG, representing the total free energy change upon ligand binding (Jones, Willett, and Glen 1995). While maintaining the conformations of the protein fixed, the conformations of the compounds were allowed to change during docking. The best poses and their corresponding molecular interactions were analyzed using the Discovery Studio 2020 software (Dassault Systèmes BIOVIA 2020).

3. Results

Synthesis of DNK

DNK was obtained by the nitration of HNK. The product obtained, DNK (Figures 1 and 2), was subjected to successive recrystallization with methanol, resulting in a yellowish powder; this reaction yielded 97.6%, determined using high-performance liquid chromatography and identified by nuclear magnetic resonance (Da Silva et al. 2005; De Souza et al. 2005). The position of the nitro groups in the aromatic rings was confirmed by the absence of coupling between aromatic protons (Figures S1–S6); thus, the nitro groups are linked in positions 6 and 6’ of the aromatic rings (Da Silva et al. 2005).

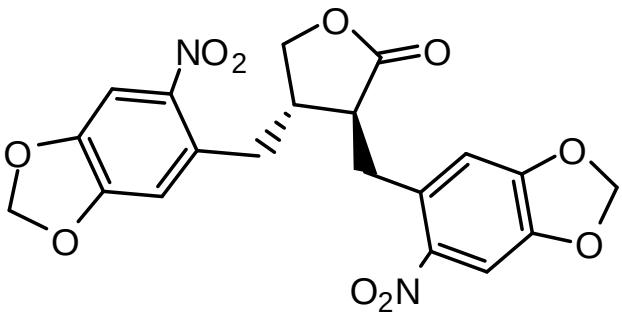

Figure 1. Structure of (-)-6,6'-dinitrohinokinin.

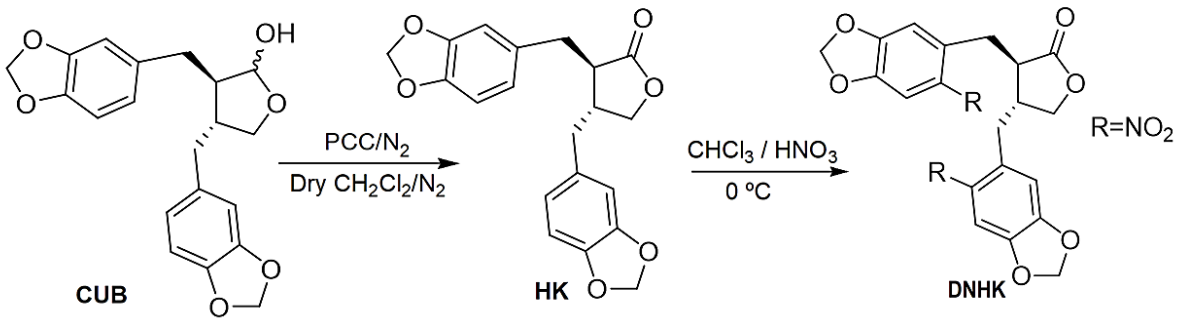

Figure 2. Steps to obtain HK and DNHK from CUB.

Trypanocidal activity assessment

Although *in vitro* assays are not considered fully adequate for elucidating all aspects of the activities of the compounds to be tested, *in vitro* studies on new trypanocidal drugs provide the first evidence and essential information to advance studies focusing on the least possible damage to organisms (Croft 1999; Holtfreter et al. 2011). For example, after obtaining the DNK, the *in vitro* trypanocidal assay was started under the amastigote forms at 12.5, 25, 50, 100, and 200 µM. The results obtained were compared with those of controls to calculate the percentage of lysis. As shown in Table 1, DNK showed an IC_{50} value of 145.6 µM and lysis percentage values of 55.6% and 45.4% at concentrations of 200 and 100 µM, respectively.

Table 1. Results of the evaluation of trypanocidal activity in amastigotes *in vitro.*

Compound	% lyses ± S.D./concentration (µM)					
	200	100	50	25	12.5	IC_{50}(µM)
1	55.6 ± 2.4	45.4 ± 9.1	34.5 ± 5.5	23.1 ± 6.1	26.8 ± 0	145.6

1. (-)-6,6'-dinitrohinokinin; Negative control: DMSO 0,5%; Positive control: DMSO 25% and anfotericina B with %lyses ± S.D./concentration (µM) of 100% ± 0,00.

Table 2. Results of the evaluation of trypanocidal activity *in vitro.*

Compounds	% lyses ± S.D./concentration (µM)					
	200	100	50	25	12.5	IC_{50} (µM)
1	78.4 ± 1.2	69.4 ± 3.1	65.2 ± 1.2	52.7 ± 2.4	43.0 ± 1.2	1.83
Benznidazole	78.3 ± 1.8	66.3 ± 1.0	47.7± 4.6	23.8 ± 5.7	16.4 ± 5.9	53.2

1. (-)-6,6'-dinitrohinokin; Negative control: DMSO 0,5%; Positive control: DMSO 25%.

Assays that verify cytotoxicity *in vitro* are essential to guide the experimental design of a study, with information on the concentrations and treatment time of the compound under investigation. Natural plant products often exhibit low toxicity (Gosh et al. 2020). Therefore, the cytotoxic evaluation of the DNK compound was conducted, which proved to be devoid of cytotoxic effect since the percentage of viable cells reaches 19.99% at the highest concentrations of 200 and 400 µM and has an IC_{50} value of 583.3 µM.

Analysis of the anti-trypanocidal activity via molecular docking

TR is an enzyme that converts trypanothione disulfide to its dithiol form, dihydrotrypanothione (Bond et al. 1999). This is essential because this metabolite is related to the scavenging of free radicals and oxygen-reactive species produced by metabolic processes or when parasites are exposed to oxidative stress by the host immune response (Fairlamb; Cerami 1992). Thus, TR protein represents a target for anti-trypanosomal drug development (Bond et al. 1999). The main interactions between the benzonidazole or DNK and TR were investigated (Figure 3). The substrate trypanothione disulfide asymmetrically interacts with the protein TR through the amino acids: Ser15, Leu18, Glu19, Trp22, Val54, Val59, Lys62, Ile107, Ser110, Tyr111, Thr335, Pro336, Ile339, Phe396, Gly459', His461', Pro462', Thr463', Ser464', Glu466', and Glu 467'. The superscript (') indicates that the residue is related to the partner subunit (Bond et al. 1999). Similarly, the compounds: i) benzonidazole; and ii) DNK also interact with the amino acids: i) Thr463' and Glu467'; and ii) Ser15, Glu19, Ile107, Ser110, Tyr111, and His461', respectively. The total free energy change that occurs upon DNK binding to the TR active site (−26.19 kcal mol^{-1}) is more attractive for the binding of the molecule benzonidazole to the TR active site ((−17.71 kcal mol^{-1}).

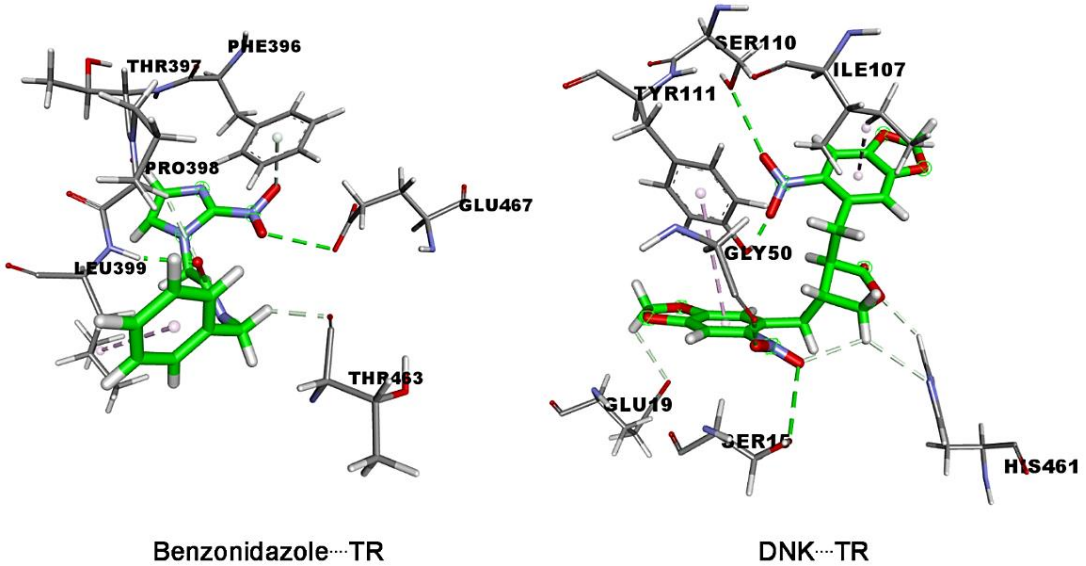

Figure 3. Main interactions between the compounds: i) benzonidazole (left) or ii) DNK (right), and the amino acids present in the protein TR.

Table 3. Results of the evaluation of the *in vitro* cytotoxic activity

Compounds	% Viable cells ± S.D./concentration (μM)							
	6.25	12.5	25	50	100	200	400	IC_{50} (μM)
(-)-6,6'-dinitrohinokinin	99.7 ± 0.7	99.1 ± 0.9	98.6 ± 1.1	98.6 ± 0.1	90.9 ± 6.8	81.0 ± 2.5	72.8 ± 1.4	583.3
Benznidazole	71.3 ± 0.4	67.6 ± 0.3	67.4 ± 1.2	65.8 ± 0.3	61.8 ± 2.2	56.1 ±1 .1	37.2 ± 0.3	246.1

Negative control: DMSO 0,5%; Positive control: DMSO 25%.

Table 4. Main interactions between the molecules benzonidazole or DNK and the amino acids present in the protein TR.[a] dG represents the total free energy change on ligand binding.

Amino Acid	Benzonidazole····TR (dG = -17.71 kcal mol^{-1})
Phe396'	Hydrogen Bond (C=O····Aromatic Ring)
Thr397'	Hydrogen Bond (C-H····O=C)
Pro398'	Hydrogen Bond (C=O····H-C)
Leu399'	Hydrophobic Interaction (HC····Aromatic Ring)
Thr463'	Hydrogen Bond (HC-H····O=C)
Glu467'	Hydrogen Bond (N^+-O^-····O^--C)
Amino Acid	**DNK····TR (dG = -26.19 kcal mol^{-1})**
Ser15	Hydrogen Bond (N^+-O^-····OH)
Glu19	Hydrogen Bond (HC-H····O^--C)
Gly50	Hydrogen Bond (N^+-O^-····O=C)
Ile107	Hydrophobic Interaction (Aromatic Ring····CH)
Ser110	Hydrogen Bond (N^+-O^-····OH)
Tyr111	Hydrophobic Interaction (Aromatic Ring····Aromatic Ring)
His461'	2x Hydrogen Bonds (O····H-C and HC-H····N)

[a] The superscript prime (') indicates that the residue is related to the partner subunit.

4. Discussion

The available treatments for Chagas disease lack efficacy and specificity and cause several side effects. Thus, many efforts have been made to develop suitable drug therapies (Pérez-Molina and Molina 2018). Many studies have focused on evaluating plant extracts and synthetic derivatives and reporting their potential effects against *T. cruzi* in different parasite forms (García-Huertas and Cardona-Castro 2021). Therefore, DNK has proven biological activities such as anti-inflammatory (Da Silva et al. 2005), leishmanicidal (Pereira et al. 2015), antibacterial, antifungal (Silva et al. 2007), and trypanocidal activities. The active derivative HNK was obtained through CUB derived from *P. cubeba*, which has more significant activity than benznidazole *in vivo* and *in vitro* studies and is considered to be of great interest in the development of new drugs for the treatment of Chagas disease (Saraiva et al. 2007). In addition, many studies have focused on modifying the chemical structures of active compounds to improve their biological activities and reduce their side effects (Wermuth 1996).

A similar effect was demonstrated in Souza et al. (2005) study, where the DNK tested under the amastigote forms also did not show relevant trypanocidal activity, with an IC_{50} value of 95.3 μM (De Souza et al. 2005). According to Saraiva et al. (2007), the presence of the nitro group at C-6 and C-6' and the carbonyl group at C-9 may have led to a reduction in trypanocidal activity; however, Saraiva et al. (2007) analyzed the activity of the DNK in the amastigote and epimastigote forms. Furthermore, Saraiva et al. (2007) proposed that the low trypanocidal activity of DNK in amastigote forms may be related to the low permeability of the compound through cell membranes. However, the trypanocidal assay on trypomastigote forms at concentrations of 12.5, 25, 50, 100, and 200 μM for 24 h (Table 2) showed that DNK presents *in vitro* trypanocidal activity with a value of IC_{50} of 19.83 μM and lysis percentage values of 78.4% and 69.4% at concentrations of 200 and 100 μM, respectively. These results showed that DNK reduced the cell viability of the trypomastigote forms since a great trypanocidal activity is related to a lower IC_{50} value, indicating that this value could lyse 50% of the parasites at the appropriate concentrations. The trypomastigote form of *T. cruzi* is the most common morphological form of the parasite in humans, increasing the relevance of this finding (Rocha et al. 2020).

Another study showed a similar reduction in schistosomicidal activity. Pereira et al. (2015) reported that DNK could not kill *Schistosoma mansoni* but minimized its movements at 100 and 200 µM (Pereira et al.2015). The parasites responsible for leishmaniasis and Chagas disease have several similarities, including their biochemical and cellular characteristics (Pereira et al. 2015). This similarity allowed us to infer that these parasites were vulnerable to the same substances. Considering the evaluation of cytotoxicity, benzonidazole was used for comparison, showing lower percentages of viable cells than DNK, with an IC_{50} of 46.1 µM (Table 3). Therefore, cytotoxicity evaluation is essential, as a compound is considered promising when it shows selectivity over target cells, such as, in this case, parasites, without harming host cells (Adan, Kiraz, and Baran 2016).

The promising biological activity presented by DNK can be explained by the *in silico* study, which shows that DNK has a more significant number of favorable interactions with the amino acids of TR (6× hydrogen bonds and 2× hydrophobic interactions) than benzonidazole (5× hydrogen bonds and 1× hydrophobic interaction) (Table 4). These data aided in elucidating the experimental results, in which DNK demonstrated significant anti-trypanocidal activity concerning the structure of benzonidazole.

5. Conclusions

In recent years, the search for substances of plant origin has increased because of their promising results, and, in most cases, they show low toxicity and low cost. According to the results of this study, DNK showed satisfactory trypanocidal activity against trypomastigote forms in the acute phase of the disease and showed more significant trypanocidal activity than benznidazole at the concentrations evaluated. In addition, the compound did not exhibit cytotoxic activity. Molecular docking calculations suggested that DNK preferably interacts with the protein TR (reference target to anti-trypanocidal action) regarding the compound benznidazole, supported by many non-covalent interactions. Our study provided new insights into understanding the trypanocidal activity of DNK.

Authors' Contributions: CARDOSO, S.C.S.: conception and design, acquisition of data, analysis and interpretation of data, drafting the article, and critical review of important intellectual content; CUNHA, W. R.: conception and design; CAVALLARI, P.S.S.R.: interpretation of data and drafting the article; DE ALMEIDA, S.G.: interpretation of data and drafting the article; JANUÁRIO, A. H.: conception and design and analysis and interpretation of data; PAULETTI, P.M.: conception and design and analysis and interpretation of data; SANTOS, F.A.: analysis and interpretation of data and drafting the article; LAURENTIZ, R.S.: conception and design and analysis and interpretation of data; MIRANDA, G.S.: analysis and interpretation of data and drafting the article; ANDRADE, J.V.: analysis and interpretation of data and drafting the article; ORENHA, R. P.: analysis and interpretation of data and drafting the article; PARREIRA, R.LT.: analysis and interpretation of data and drafting the article; ANDRADE E SILVA M.L.: conception and design and analysis and interpretation of data; SANTOS, M.F.C.: conception and design and analysis and interpretation of data; ESPERANDIM, V.: conception and design and analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

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