UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

Faculdade de Farmácia

Disciplina de Trabalho de Conclusão de Curso de Farmácia

Atividade *anti-Trichomonas vaginalis* de derivado dissubstituído de 2,4-diamino-quinazolina

Juliana Inês Weber

Porto Alegre, dezembro de 2017.

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Orientadora: Prof. Dra. Tiana Tasca

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Resumo:

Trichomonas vaginalis é o agente da tricomoníase humana, infecção sexualmente transmissível (IST) mais prevalente no mundo. Classificada como uma doença negligenciada e não sendo de notificação compulsória, seus números são subestimados, o que a torna um sério problema de saúde pública. Considerando as complicações decorrentes da tricomoníase e as falhas no tratamento, é imprescindível a busca por novas alternativas terapêuticas. Neste estudo foi avaliada a viabilidade de *T. vaginalis* frente a um derivado dissubstituído de 2,4-diamino-quinazolina, um derivado dissubstituído da quinazolina. Os resultados demonstraram promissora atividade anti-*T. vaginalis* do composto, com valores de MIC e IC_{50} iguais 90 e 50 µM, respectivamente. O composto apresentou citotoxicidade contra a linhagem celular de fibroblastos 3T3-C1 e efeito hemolítico. O derivado de 2,4-diamino-quinazolina não induziu a produção de espécies reativas de oxigênio nos trofozoítos. Este dado é importante pois sugere um mecanismo de ação distinto dos 5-nitroimidazois, metronidazol e tinidazol, utilizados no tratamento da tricomoníase, visto que já existem mecanismos de resistência para estes fármacos. O mecanismo de ação provável envolve o processo de morte celular por apoptose, sendo necessário mais experimentos específicos para confirmação.

Palavras-chave: *Trichomonas vaginalis*, atividade anti-*T.vaginalis*, quinazolina, morte celular, apoptose.

Anti-*Trichomonas vaginalis* activity of disubstituted 2,4-diamine-quinazoline

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Keywords: *Trichomonas vaginalis*, anti-*T. vaginalis* activity, quinazoline, cell death, apoptosis.

Short running title: Quinazoline against *T. vaginalis*

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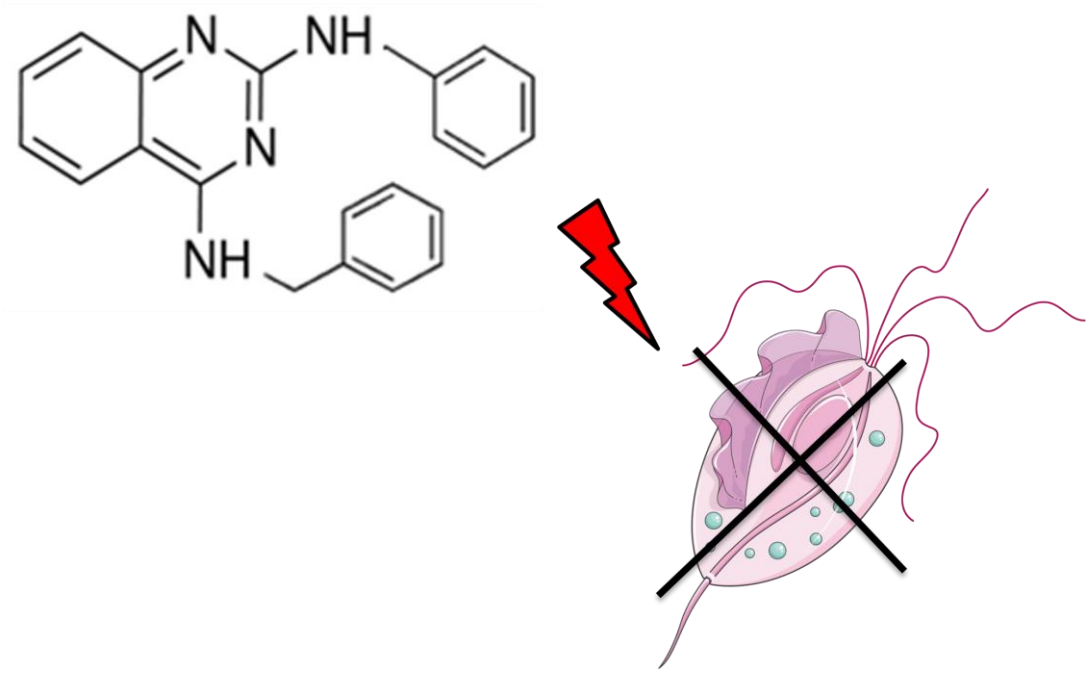
2,4-diamine-quinazoline derivative



Trichomonas vaginalis is the agent of human trichomoniasis. The 2,4-diamine-quinazoline derivative was active against this protozoan and it has presented hemolysis and cytotoxic effect against the fibroblast cell line. There was no ROS production indicating a distinct mechanism of action from that of metronidazole, that is known to generate drug resistance. Anexin V assay data revealed fluorescent units in characteristic regions, indicating apoptosis.

Abstract:

Trichomonas vaginalis is the agent of human trichomoniasis, the most prevalent non-viral sexually transmitted infection (STI) in the world. This infection is classified as a neglected disease and compulsory notification is not required, leading to underestimated prevalence, which makes it a serious public health issue. Considering the complications due to trichomoniasis and the failures in the treatment, it is crucial to search for new therapeutic alternatives. In this study the anti-*T. vaginalis* activity of the 2,4-diamine-quinazoline derivative, was evaluated. The compound showed cytotoxicity against the 3T3-C1 lineage of fibroblasts and hemolytic effect. The compound 2,4-diamine-quinazoline derivative did not induce the production of reactive oxygen species by the trophozoites. This result is important since it suggests a mechanism of action distinct of 5-nitroimidazoles, metronidazole and tinidazole, used in the treatment of trichomoniasis, taking into account that resistance mechanisms for these drugs have been already described. The probable mechanism of action may involve the process of cell death of apoptosis, being required more specific experiments for this confirmation.

1. INTRODUCTION

The sexually transmitted infections (STIs) are public health problems, despite the incentive for protection, there are still many barriers for an efficient fighting line to be established. One of the contributing factors for the high number of cases is the lack of access to quick and accurate diagnosis of some STIs by low-income countries populations.[1] The number of cases of such diseases is in fact, underestimated, including that for trichomoniasis that is not of compulsory notifying[2], which contributes for an inaccurate number of cases.

Trichomoniasis is the most prevalent non-viral STI in the world. Estimates showed that in 2008 the numbers of new cases among adults, between 15 and 49 years old, infected by STIs including *C. trachomatis*, *N. gonorrhoeae*, syphilis and *T. vaginalis* reached 448 million, being the *T.vaginalis* responsible for 276 million of cases.[3] Studies showed that 80% of cases of trichomoniasis are asymptomatic among women and men,[2] a fact that potentiates the risk of transmission. Beyond the problems derived from trichomoniasis, the infection is associated with the potential risk of acquiring other STIs, such as HIV.[2] The *T. vaginalis* infection shows as main complications the increased risk of cervical cancer[4] and it's a possible factor for the development of aggressive prostate cancer,[5] and in addition, problems during pregnancy.[6]

There are only two drugs approved by the FDA (Food and Drug Administration, USA) for the treatment of trichomoniasis, metronidazole and tinidazole, that belong to the same class, the 5-nitroimidazoles.[7] The mechanism of action of metronidazole is based on the formation of a nitro-radical anion, which is toxic to the parasite, through transferring of an electron to the compound nitro group [7]. The treatment with metronidazole occasionally causes side-effects, such as nausea, vomiting, diarrhea, abdominal discomfort, and hypersensitivity [8]. The major issue in trichomoniasis treatment is that up to 10% of infected individuals are non-responsive and the resistance to

metronidazole has become very significant, being estimated in 2.5-9.6% of cases[9]. Therefore, research on new agents for the treatment of the trichomoniasis is required, taking into consideration parasite survival mechanisms for the finding of new therapeutic targets.

Quinazoline possesses two fused six-membered aromatic rings (benzene and pyrimidine).[10] The interest for quinazoline has been intensified by the discovery of the febrifugine, which presents potent antimalarial activity.[10] The FDA has approved several quinazoline derivatives as anticancer drugs, such as gefitinib, erlotinib, and lapatinib[10]. Besides that, studies have already shown that the derivatives of quinazoline present a variety of biological activities, such as anti-inflammatory,[11] anticancer,[12,13,14] against multi-resistant *Staphylococcus aureus*,[15] anti-*Leishmania*,[16,17] anti-*Trypanossoma,*[17] anti-malarial,[17-19] for Alzheimer treatment,[20] obesity and diabetes control,[21] antimicrobial activity,[22] anti-hyperglycemic,[23] anticonvulsant, antidepressant,[24] antioxidant,[25] anti-hypertensive[26] and antihistamine.[27]

Based on data of previously reported studies for quinazoline and on the growing need for the development and synthesis of new molecules that meet the lack of drugs for the treatment of trichomoniasis, this study aimed to evaluate the potential anti-*T. vaginalis* activity of 2,4-diamine-quinazoline derivative.

2. MATHERIALS AND METHODS

2.1. 2,4-diamine-quinazoline derivative synthesis

The compound was synthesized as previously described with some modifications,[28] by the research group coordinated by Professor Dr. Saulo Fernandes de Andrade, Faculdade de Farmácia, UFRGS, starting from

dichloroquinazoline substitutions and obtaining the desired compound 2,4-diamine-quinazoline derivative.

2.2. Culture *in vitro* of tricomonads

The *T. vaginalis* isolate ATCC 30236 was cultured *in vitro* at 37°C in trypticase-yeast extract-maltose (TYM) medium, supplemented with 10% (v/v) heat inactivated bovine serum.[29] Parasites in the logarithmic phase of growth were centrifuged and resuspended on new TYM medium for anti-*T. vaginalis* assays.

2.3. Susceptibility assay

The compound 2,4-diamine-quinazoline derivative was solubilized in dimethyl sulfoxide (DMSO) as vehicle at final concentration 100 µM. The assay was performed in 96-microtiter plates with TYM medium of a parasite suspension, resulting in final density of 1.0 x 10^5 trophozoites/mL. The microplates were incubated at 37 ºC, with 5% CO_2 atmosphere for 24 h. Two controls were used: negative control with parasites only and vehicle control (DMSO 0.6%). The viable trophozoites densities were estimated by counts in a hemocytometer, using trypan blue (0.2%, v/v) as exclusion dye. The results were expressed as the percentage of living organisms compared to negative control, considering motility and normal morphology.

2.4. Minimum inhibitory concentration (MIC) and IC_{50} determination

The MIC value, which is the minimum concentration able to kill 100% of parasites, and the IC_{50} value, the concentration needed to kill 50% of parasites, were determined on 96-well microtiter plates, where different volumes of TYM medium were added to each well. Thereafter, different volumes of 2,4-diamine-quinazoline were added to obtain a serial dilution (100, 90, 80, 70, 60, 50, 40 and 30 µM). Subsequently, 150 µL of solution containing the trichomonads were added, reaching 1.0 x 10^5 trophozoites/mL as the final density of the parasite. Two controls were prepared: negative control with parasites only and vehicle

control (DMSO 0.6%). After 24 h of incubation at 37°C, 5% CO_2, the parasites were counted using hemocytometer and analyzed for their motility and morphology by Trypan Blue exclusion dye (0.2%, v/v). The wells corresponding to MIC value and concentrations below and above, as well as controls were inoculated in fresh TYM medium at 37 °C. The parasites were analyzed every 24 hours for 120 h to confirm MIC. The results were expressed as the percentage of viable trophozoites compared to negative control. The IC_{50} value was assessed using software GraphPad Prism6.

2.5. Hemolytic assay

The rate of hemolysis was performed as described by Gauthier, et al. (2009)[30] with some modifications. Erythrocytes were obtained from the heparinized blood of healthy human donors. The Universidade Federal do Rio Grande do Sul Research Ethical Committee approved documents, procedures, and project under authorization CAAE 47423415.5.0000.5347. Erythrocytes obtained of fresh human blood were washed three times with PBS 1x (pH 7.0; 37°C) at 3000 rpm for 5 min and re-suspended to obtain a 1.0% (v/v) erythrocytic suspension. Then, erythrocytes (1.0%) were incubated with compound at IC_{50} concentration, at 37 °C for 1 h and 24 h. The absorbance of supernatant was measured at 540 nm. Two controls were prepared: negative control (erythrocytes and ethyleneglycol) and positive control (erythrocytes and Triton X-100 0.2%). Results were expressed as hemolysis percentage of compound, comparing to 100% hemolysis that was attributed to hemolytic action of the positive control Triton X-100 0.2%.

2.6. *In vitro* cytotoxicity

The fibroblasts lineage, 3T3-C1, was cultured at 37°C, in Dulbecco´s Modified Eagle´s Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and incubated at 5% CO_2. For the test, 1.0 x 10^4 cells/well were seeded in 96-well microtiter plates for 24 h. After, the medium was replaced with fresh medium containing 2,4-diamine-quinazoline derivative at the IC_{50} value. Three controls were prepared: negative control with no compound, vehicle control

(DMSO 0.6%) and positive control (Triton X-100 0.2%). The plates were incubated for 48 h. After this time, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/mL) was added and incubated for one hour at 37 °C. The MTT was removed and the insoluble purple formate was dissolved in DMSO. The amount of reduced MTT was measured at 570 nm.

2.7. Effect of 2,4-diamine-quinazoline in *T. vaginalis* kinetic growth assay

The *T. vaginalis* ATCC 30236 isolate was treated or not with 2,4-diamine-quinazoline derivative at a density of 1.0 x 10^5 trophozoites/mL (including vehicle control and control with parasites only) at the IC_{50} value and incubated in TYM medium. The counting of viable trophozoites with hemocytometer was performed at 2, 4, 6, 12, 24, 48, 72, 96 and 120 h. The results were expressed as trophozoites/mL by comparing treated via viable trophozoites with untreated parasites.

2.8. Quantification of reactive oxygen species (ROS) production by trichomonads

Some modifications were carried out to adjust the experiment, previously described.[31] Trophozoites of ATCC 30236 isolate were washed with PBS 1x (pH 7,0; 37°C), resuspended at 5.0 x 10^6 trophozoites/mL density and were incubated for 1 h at 37 °C with 2′,7′-dichlorofluorescein diacetate (2′,7′-DCF-DA) in a final concentration of 10 µM. After, compound was added at the IC_{50} value and incubated for additional 1 h. Two controls were prepared: negative control (trophozoites with no treatment) and the positive control (parasites treated with 5mM of hydrogen peroxide). In order to evaluate the ROS production, fluorescence was measured by flow cytometry (FACSVerse, Becton Dickinson, CA) and 10,000 cells were gated and analyzed using FACSuite™ software (Becton Dickinson).

2.9. Anexin V

Trichomonads, that were treated with 2,4-diamine-quinazoline derivative at the IC_{50} for 24 h, were washed twice with PBS 1x (pH 7.0; 37°C), resuspended in binding buffer, resulting in a concentration of 1.0 x 10^6 trophozoites/mL. Then, 100 µL of parasite suspension, 5 µL of annexin V-FITC, and 5 µL of propidium iodide (PI) were added to the incubation media at 25°C for 15 minutes (protected from light) and after, 400 µL of binding buffer added. Apoptosis was measured by flow cytometry (FACSVerse, Becton Dickinson, CA) and 10,000 trophozoites were gated and analyzed using FACSuite™ software (Becton Dickinson).

2.10. Statistical analysis

All experiments were performed in triplicate and with at least three independent cultures (n=3). Data were expressed by mean ± standard deviation (S.D.). Statistical analysis was conducted using the Student's t test and a 5% level of significance was applied to the data. The GraphPad Prism software (San Diego, CA) was used for IC_{50} determination by non-linear regression.

3. RESULTS

3.1. Disubstituted 2,4-diamine-quinazoline has presented anti-*T. vaginalis* activities

In order to evaluate the anti-*T. vaginalis* activity of 2,4-diamine-quinazoline derivative, the MIC and IC_{50} values were determined. The compound revealed potential anti-*T. vaginalis* activity with MIC and IC_{50} values of 90 and 50 µM, respectively. Consequently, the effect of this compound at IC_{50} was investigated in the subsequent experiments.

3.2. Disubstituted 2,4-diamine-quinazoline has presented hemolysis

The hemolysis assay contributes to elucidate the mechanism of death exerted by the compound tested. As seen in Table 1, 50 μM quinazoline caused mildly hemolytic effect after 1 h and 24 h of incubation with erythrocyte suspension. These data suggest damage of the compound in eukaryotic plasma membranes.

Table 1: Hemolytic activity of 2,4-diamine-quinazoline derivative at IC_{50} value (50 μM). Results were expressed as percentage of erythrocytes lysis in comparison with positive control (0.1% Triton X-100).

Hemolysis (%)	**1 hour**	**24 hours**
Triton X-100 0.2%	100.00 ± 0.22	100.00 ± 0.10
Negative control	8.18 ± 0.01	15.32 ± 0.01
Vehicle 0.6% DMSO	8.00 ± 0.01	13.50 ± 0.01
2,4-diamine-quinazoline derivative (50μM)	11.82 ± 0.02*	42.18 ± 0.03*

Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p<0.05$) when compared to the negative control by the Student's t test.

3.3. Citotoxicity was presented

The cytotoxicity of 2,4-diamine-quinazoline at IC_{50} value against fibroblast is shown in Figure 2. There was high reduction in cell viability. As expected, the positive control, Triton X-100 reduced the cell viability whereas the vehicle control caused no damage. Considering these results, the compound 2,4-diamine-quinazoline showed low selectivity for fibroblast with selectivity index of

0.7 (ratio between the CC_{50} - concentration needed to kill 50% of cells - and IC_{50} values).

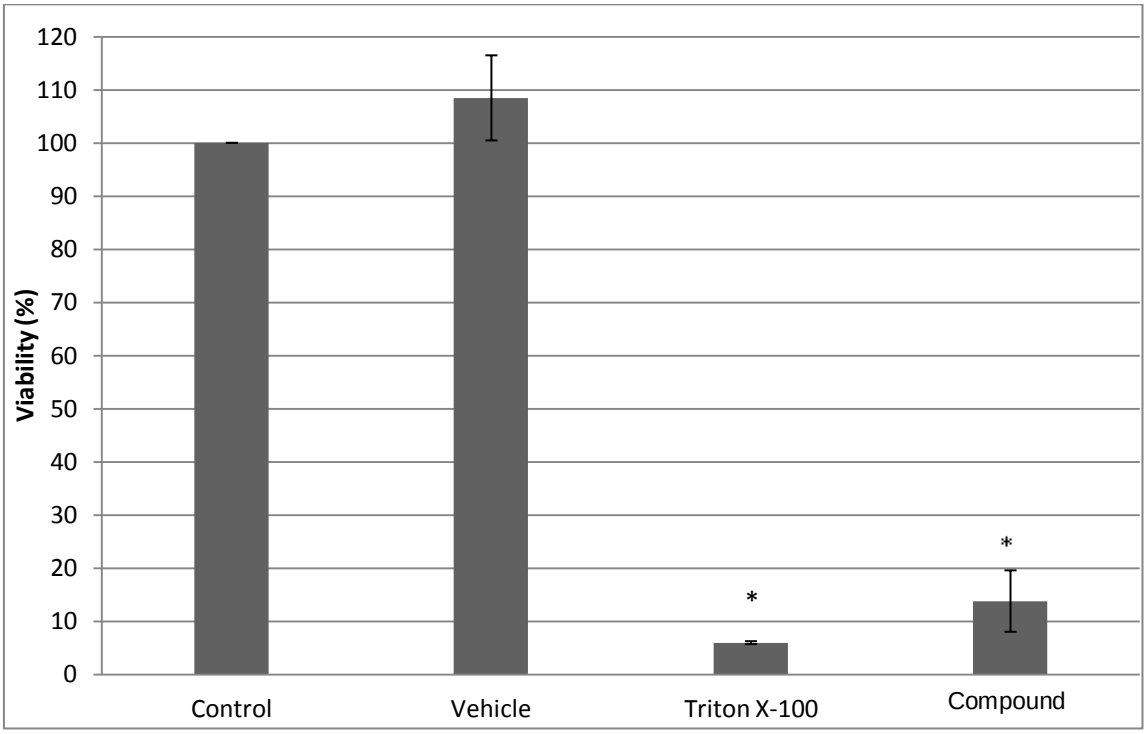


Figure 1: Effect of 50 µM 2,4-diamine-quinazoline derivative on the viability of fibroblast cells exposed for 48 h. Triton X-100 0.2% was used as positive control. Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p < 0.05$) when compared to the negative control by the Student's t test.

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3.4. Disubstituted 2,4-diamine-quinazoline has inhibited *T. vaginalis* growth

To analyze the influence of 2,4-diamine-quinazoline on *T. vaginalis* proliferation, kinetic growth experiments were performed. An initial inoculum of 1.0×10^5 trophozoites/mL was incubated in the presence of compound at IC_{50} values and the results show the expected curve of viable untreated trichomonads. The parasite growth was inhibited by 2,4-diamine-quinazoline derivative after 24 h of incubation.

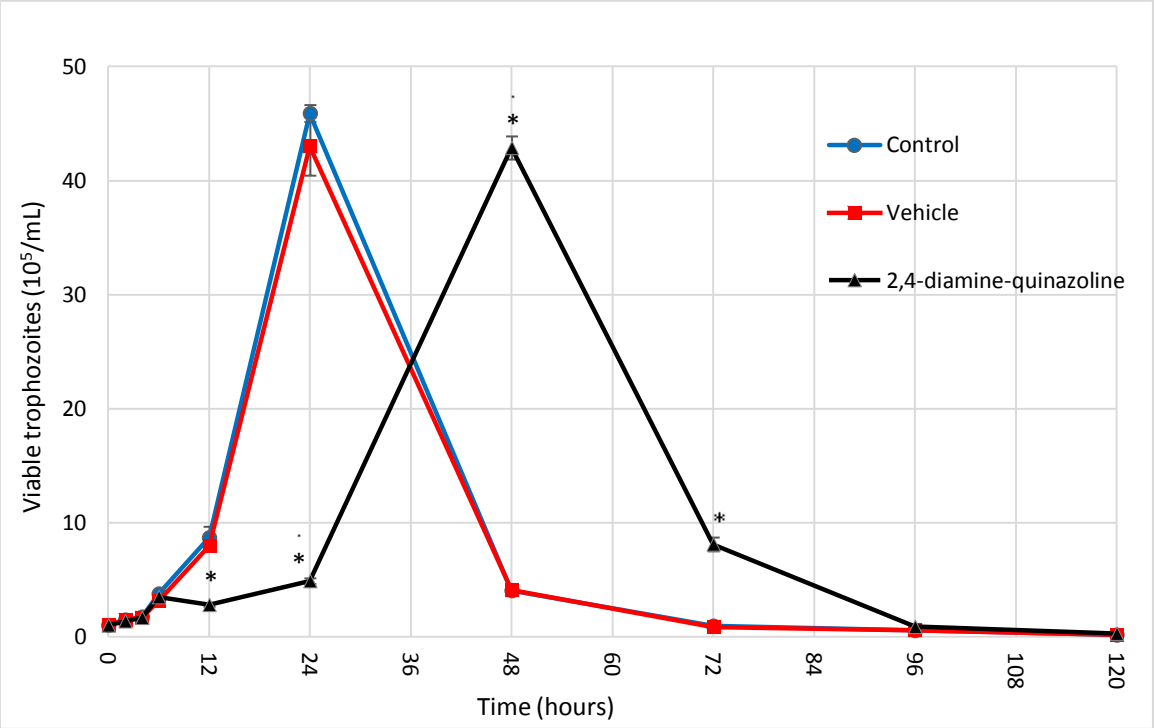

Figure 2: Effect of 2,4-diamine-quinazoline derivative on *T. vaginalis* kinetic growth at IC_{50} in comparison to untreated parasites (control) and parasites with 0.6 % DMSO (vehicle). Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p<0.05$) when compared to the negative control by the Student's t test.

3.5. The effect of 2,4-diamine-quinazoline derivative on ROS production by tricomonads was not sgnificant

Addition of 2,4-diamine-quinazoline derivative did not significantly affect intracellular ROS production when compared to hydrogen peroxide (positive control) which showed high fluorescence units.

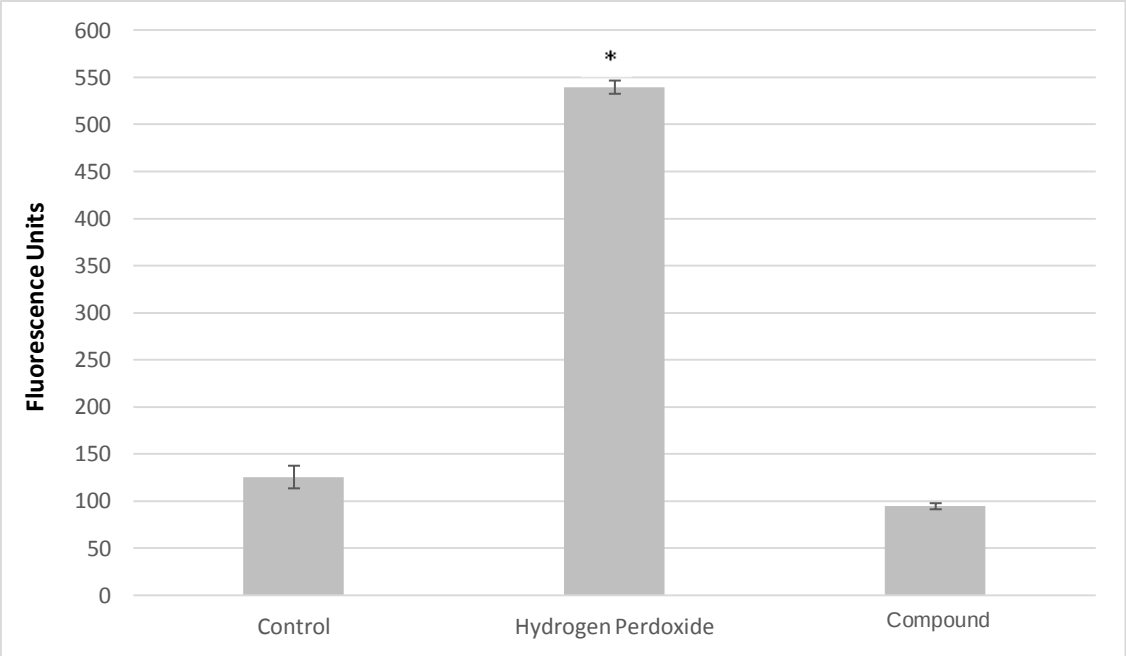

Figure 3: Effect of 50 µM 2,4-diamine-quinazoline derivative on ROS production by T. vaginalis. Hydrogen peroxide was used as positive control. Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p<0.05$) when compared to the negative control by the Student's t test.

3.6. Apoptosis by binding of Annexin V to phosphatidylserine

According to cytometer assay, the interaction of trophozoites with 2,4-diamine-quinazoline derivative possibly induced apoptosis, revealing fluorescent units in characteristic regions.

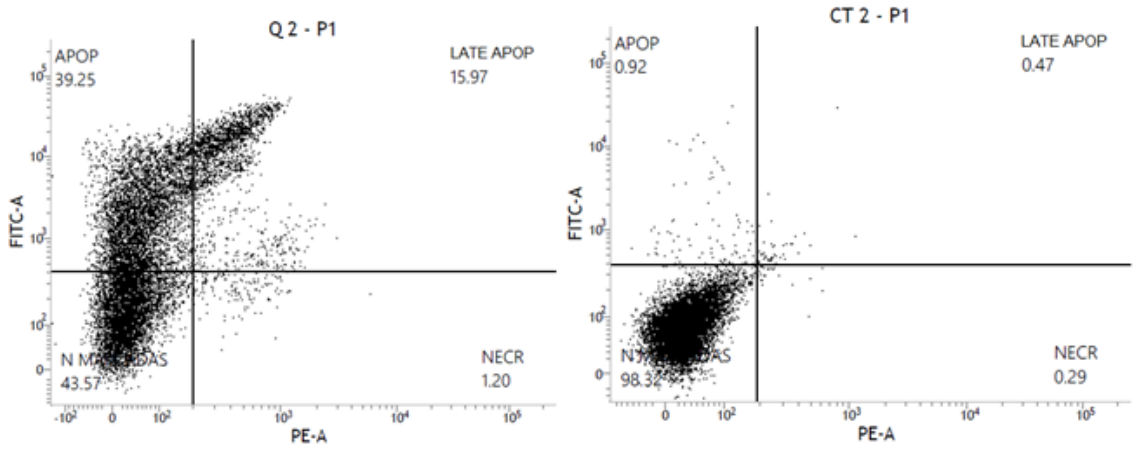

Figure 4: Effect of 50 µM 2,4-diamine-quinazoline derivative on trichomonads, suggested apoptosis. CT2: negative control (only trichomonads in TYM medium), Q2:

trichomonads treated with 2,4-diamine-quinazoline derivative. Data are representative of four different experiments (n=4).

Table 2: Comparison of cell death events including, apoptosis, late apoptosis, necrosis and normal morphology between control (only trichomonads in TYM medium) and compound (trichomonads treated with 2,4-diamine-quinazoline derivative).

%	Control	2,4-diamine-quinazoline
Normal Morphology	96.78 ± 0.44	38.38 ± 2.08
Apoptosis	2.00 ± 0.17	52.20 ± 7.00*
Late Apoptosis	0.94 ± 0.08	8.60 ± 1.90
Necrosis	0.33 ± 0.12	0.83 ± 0.50

Data represent mean ± standard deviation of three experiments in triplicate. (*) Statistically significant difference ($p < 0.05$) when compared to the negative control by the Student's t test.

4. DISCUSSION

Trichomoniasis is the most common non-viral STI in the world, with incidence numbers much higher than chlamydia infections, gonorrhea, and syphilis combined.[32] In spite of being a prevalent disease and cause of relevant health complications, trichomoniasis is a neglected disease, being a contributing factor that it mainly affects low-income populations.[32] The difficulties in treatment and the lack of therapeutic alternatives encourage the research of new compounds.

Several compounds, such as derivatives of triterpenes,[33] derivatives of betulinic acid,[34] saponins,[35] and alkaloids,[36] have been studied for their anti-

T. vaginalis activity, exhibiting diverse mechanisms of action, for example the effect on the metabolism of polyamines,[37] alterations of the cytoplasmic membrane,[38] hydrogenosome related action,[39] and cellular death by paraptosis.[40,41] On the other hand, apoptosis or cell programmed death is a mechanism less studied in *T. vaginalis,* in which the cellular content is compacted to be phagocytosed by the immune system. Chose et al. (2002)[42] have described a form of programmed cellular death with characteristics of nuclear fragmentation and presence of apoptotic bodies, typical characteristics of apoptosis. However, the presence of cytoplasmic vacuoles have also been observed, characteristic of paraptosis.

Studies have shown that derivatives of quinazoline show activity against protozoa, such as inhibition of dihydrofolate reductase in *Trypanosoma cruzi*, *Leishmania major* and *Plasmodium vivax*,[17] inhibition of the plasmepsins class in *Plasmodium falciparum*,[41] and modifying of the *Toxoplasma gondii* structure preventing its entry in host cells.[44]

The present study has tested the anti-*T.vaginalis* activity of 2,4-diamine-quinazoline derivative, which exhibited MIC and IC_{50} values of 90 and 50 μM, respectively. To evaluate the compound effect on the proliferation of the parasite, a kinetic growth curve experiment has been conducted, and the trophozoites were incubated with the compound for up to 120 h, in the IC_{50} concentration, when growth has been reduced in 24 h. This result, together with the anti-*T. vaginalis* activity test, shows promise, leading to new experiments to evaluate its potential.

Subsequently the hemolytic effect of 2,4-diamine-quinazoline derivative was evaluated. The compound demonstrated to be hemolytic, result that indicates toxic effects to plasmatic membranes of eukaryotes. Afterward, the cytotoxic effect was evaluated in fibroblasts through incubation with the compound, in the IC_{50}. In this assay, 2,4-diamine-quinazoline derivative exerted high cytotoxic effect *in vitro* and low selectivity, requiring further tests to evaluate the toxicity *in vivo,* as the model of *Galleria mellonella* due to the similarities of its immune system to that of the mammals[45]. Besides, an alternative is to synthesize the quinazoline derivatives with molecular changes that allow for a reduction of the cytotoxic effect.

The redox balance is an important survival mechanism of *T. vaginalis*, because it avoids oxidative stress.[46] The 5-nitroimidazoles interfere in this balance, leading to the death of the parasite.[7] This pathway shows resistance mechanisms,[9] stressing the need to develop further research in this area. In this context, the obtained results were promising, taking into account that there was no increase in the production of ROS by the parasites, possibly indicating another course of action of the tested compound.

Based on the obtained result, the assay on the Anexin V was conducted, with the aim to investigate the type of cellular death derivate from the compound action. The outcome of this assay showed fluorescent unities in characteristic regions, suggesting that the probable type of cellular death caused by the action of 2,4-diamine-quinazoline derivative is apoptosis. Another mechanism that could be considered from these results is paraptosis, a programmed cell death distinct from apoptosis[40,41] which distinguishes morphologically from the apoptosis for presenting large cytoplasmic vacuoles and intact chromatin,[41] absence of nuclear fragmentation and apoptotic bodies.[40] Apoptosis markers have been identified in some protozoa, such as *Leishmania* and *Plasmodium berghei*.[47] Apoptosis is triggered in response to some stress, as oxidative damage, and causes the activation of the family of caspases,[47] so the apoptotic bodies are formed, which are then phagocytosed by the immune system, different from paraptosis that is independent of caspase activation.[40] Paraptosis is a different form of non-apoptotic programmed cell death, it does not show all criteria for apoptosis. In this context, new studies to ascertain the mechanism of action of 2,4-diamine-quinazoline derivative are essential.

5. CONCLUSION

The sexually transmitted infections are a great challenge for public health agencies, especially due to the lack of symptoms, a fact that makes for a high transmission rate. In addition to the fact that current drugs failure in the treatment, studies of compounds such as 2,4-diamine-quinazoline derivative, with alternative combat mechanisms against *T. vaginalis*, are emerging as promising therapeutic sources.

6. ACKNOWLEDGEMENTS

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7. CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Anexo

CHEMICAL BIOLOGY & DRUG DESIGN

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- Small-molecule diversity and synthetic methods important to investigate chemical space relationships to drug discovery
- Structure-based drug design, molecular modeling.
- Virtual screening of drug targets and related in silico chemoinformatics and bioinformatics technologies M
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