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Identification of potential bi-triazole based antimalarial compounds and their effects against asexual stages of *Plasmodium* isolates

Identificação de compostos antimaláricos potenciais à base de bi-triazóis e seus efeitos contra estágios assexuados de isolados de *Plasmodium*

Identificación de potentes compuestos antipalúdicos a base de bi-triazoles y sus efectos contra las fases asexuales de cepas de *Plasmodium*

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ABSTRACT
Malaria is a parasitic infection that can become severe and lead to a patient's death, besides having records of resistance to treatment and only a few options to treat it, generating the need for more research on new antimalarial compounds. The present study aimed at evaluating the antiplasmodial activity of two synthetic compounds, through *in vitro* assays, and *ex vivo* assays. The *in vitro* studies demonstrated that the synthetic molecules presented inhibitory concentrations for 50% of the parasite population (IC_{50}) of 4.4 and 6.3 µM against sensitive and resistant strains, respectively. Compound 3RJ was observed to act in the first 8h against young trophozoites. Regarding the *ex vivo* study, 3RJ didn't behave similarly to the reference drug, but remained effective against the circulating

strains of *P. falciparum* in the state of Rondônia, Brazil. The results observed demonstrate that these triazole-based compounds are promising candidates in the development of antimalarial drugs.

Keywords: Triazoles. Bi-Triazoles. *P. falciparum*. Development.

RESUMO

A malária é uma infecção parasitária que pode se tornar grave e levar o paciente à morte, além de ter registros de resistência ao tratamento e poucas opções de tratamento, gerando a necessidade de mais pesquisas sobre novos compostos antimaláricos. O presente estudo teve como objetivo avaliar a atividade antiplasmódica de dois compostos sintéticos, por meio de ensaios *in vitro* e ensaios *ex vivo*. Os estudos *in vitro* demonstraram que as moléculas sintéticas apresentaram concentrações inibitórias para 50% da população de parasitas (IC50) de 4,4 e 6,3 µM contra cepas sensíveis e resistentes, respectivamente. Observou-se que o composto 3RJ agiu nas primeiras 8 horas contra trofozoítos jovens. Em relação ao estudo *ex vivo*, o 3RJ não se comportou de forma semelhante ao medicamento de referência, mas permaneceu eficaz contra as cepas circulantes de *P. falciparum* no estado de Rondônia, Brasil. Os resultados observados demonstram que esses compostos à base de triazol são candidatos promissores para o desenvolvimento de medicamentos antimaláricos.

Palavras-chave: Triazóis. Bi-Triazóis. *P. falciparum*. Desenvolvimento.

RESUMEN

La malaria es una infección parasitaria que puede llegar a ser grave y provocar la muerte del paciente, además de tener registros de resistencia al tratamiento y pocas opciones terapéuticas, lo que genera la necesidad de investigar más sobre nuevos compuestos antipalúdicos. El objetivo de este estudio fue evaluar la actividad antiplasmódica de dos compuestos sintéticos mediante ensayos *in vitro* y *ex vivo*. Los estudios *in vitro* mostraron que las moléculas sintéticas presentaban concentraciones inhibitorias para el 50% de la población parasitaria (IC50) de 4,4 y 6,3 µM frente a cepas sensibles y resistentes, respectivamente. Se observó que el compuesto 3RJ actuaba en las primeras 8 horas contra los trofozoítos jóvenes. En cuanto al estudio *ex vivo*, 3RJ no se comportó de forma similar al fármaco de referencia, pero siguió siendo eficaz contra las cepas circulantes de P. falciparum en el estado de Rondônia, Brasil. Los resultados observados demuestran que estos compuestos basados en triazoles son candidatos prometedores para el desarrollo de fármacos antimaláricos.

Palabras clave: Triazoles. Bi-Triazoles. *P. falciparum*. Desarrollo.

1 INTRODUCTION

Malaria is a parasitic, febrile and acute disease, and half of the world's population is exposed to the risk of contracting this infection (Chu *et al*., 2019). According to the 2023 global malaria report, 2022 showed 249 million cases with 608 thousand deaths. Of the six species of *Plasmodium* that cause human malaria, the species *P. falciparum* is considered the most virulent, being the predominant species on the African and Asian continents where the highest numbers of cases are recorded globally. In the Americas, the species *P. vivax* represents 74% of cases, with the highest morbidity rates being found among the countries that belong to the Amazon (WHO, 2023). According to the global technical strategy for malaria control 2016-2030, one of the obstacles to the global elimination of this infection is drug resistance to a variety of antimalarial drugs, mostly recorded by the species *P. falciparum*, including partial resistance to artemisinin derivatives. This fact generated a call for countries to monitor the effectiveness of antimalarials, a feat that will significantly contribute to the process of eliminating this infection (WHO, 2015).

Despite all the concerns about the species *P. falciparum*, due to the characteristics mentioned above, isolates of the species *P. vivax* from different endemic regions have been presenting gene mutations (Cheong *et al*., 2020; Mbenda *et al*., 2020); synonymous with the emergence of resistant strains, triggering an alert for authorities seeking to eradicate malaria. Furthermore, the lack of funding and trained people who can carry out more detailed research, providing new developments in the fight against malaria, adds negative points in combating the aforementioned parasite (Ferreira *et al*., 2021).

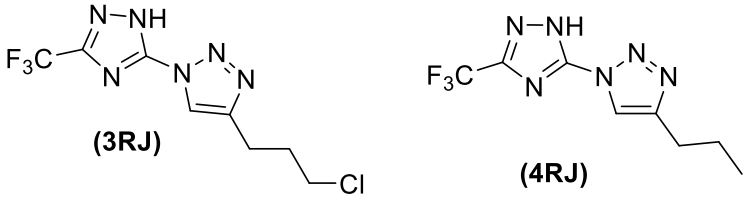
From this perspective, nitrogenous heterocyclic triazole derivatives, which have wide biological applicability, contribute significantly to medicinal chemistry in the search for new antimalarial drugs. This class of molecules was used in *in vitro* studies in the search for new antiplasmodial compounds, in addition to being the target of *in silico* studies against the lactate dehydrogenase enzyme from *Plasmodium berghei* and, in the *in vivo* study model for human malaria (Park *et al*, 2017; Brandão *et al*., 2017). Triazoles are important for medicinal chemistry,

as they can act as a pharmacophoric group and as a connection between two or more molecules, which can provide a variety of structural modifications. The addition of the second triazole ring has already been described in the literature in studies of the structure-activity relationship, enhancing bioactive molecules. The 1,2,3 triazole ring is very stable under physiological conditions, making the ring a promising ligand (Hou *et al*., 2012), in addition to being recognized as a bioisostere of amide and imidazole groups presenting similar physicochemical properties (Freitas *et al*., 2011; Agalave; Maujan; Pore, 2011). Bi-triazole derivatives, a group of molecules that constitute the presence of a second triazole ring, are in the introductory phase of research with biological activities. However, the bet in this direction is based on literary reports describing the class of bi-triazoles, a similar group, modified only with regard to the spacing between the carbons of the rings. In synthetic studies, the comparison between the two classes infers bi-triazoles to have promising applicability in medicinal chemistry, coordination chemistry, biochemistry and supramolecular chemistry (Zheng *et al*., 2015).

In the few literature records with biological activities, bi-triazoles are suggested as a class for new attempts to screen new antiviral candidates (Xia *et al*., 2006). Also, antibacterial activity of moderate to good intensity is already recorded, still acting as fungicides and anthelmintics (Arafa; Mohamed, 2011; Dawood; Abdel-Wahab and Raslan, 2018). An unprecedented study previously carried out by our group of researchers demonstrated the ease of triazole synthesis and their bi-triazole derivatives, as well as the potential antimalarial activity and cytotoxicity of some of the compounds produced (Addla *et al*., 2023) highlighted the molecules 14b and 14a, which presented the following values: IC_{50} values of 4.5 ± 0.30 and 8.9 ± 0.38 µM (*P. falciparum* W2), respectively, and CC_{50} values greater than 500 µM (HepG2 cell line), as well as selectivity index values greater than 56.2 and 111.1, respectively (Figure 1). Our previous *in silico* analysis also showed that compounds 14a and 14b had the best physical chemical features, and this is the reason why they were selected for the study. Such results aroused interest in continuing to survey the antimalarial activity in more detail of these two compounds, which will be shown in this manuscript.

Thus, the objective of the study was to evaluate the antimalarial activity of two compounds, called here 3RJ, and 4RJ, *in vitro*, and *ex vivo* against circulating strains of *P. falciparum*, since these experiments would bring great contributions for the knowledge on bi-triazole-based compounds against *Plasmodium* species, and potentially lead to the development of an antimalarial compound;

Figure 1. Compounds 14b and 14a (Addla *et al*., 2023).



Source: authors of the study

2 MATERIALS AND METHODS

2.1 DATA ON OBTAINING AND INITIAL EVALUATION OF THE MOLECULE

The bi-triazole compounds 4-(3-chloropropyl)-1-(5-(trifluoromethyl)-4H-1,2,4-triazol-3-yl)-1H-1,2,3-triazole (coded as 3RJ) and 4-propyl-1-(5-(trifluoromethyl)-4H-1,2,4-triazol-3-yl)-1H-1,2,3-triazole (coded as 4RJ), were synthesized as described by our group Addla *et al*. (2023). In this case study (Addla *et al*., 2023), these involved the molecules coded as 3RJ (14b) and 14a.

2.2 PARASITE CULTURE AND SYNCHRONIZATION

The strains of *P. falciparum* W2 - Chloroquine resistant and NF-54 - Chloroquine sensitive were cultivated in human red blood cells (O+) and RPMI-1640 medium (Sigma-Aldrich), supplemented according to Trager and Jensen (1976), incubated at 37 ºC with a gasogenic mixture (5% O_2, 5% CO_2 and 90% N_2), and parasitemia was monitored daily. The cultures, with a predominance of young trophozoites, were synchronized with D-sorbitol (5%) glycosylated (0.5%), as described by Lambros and Vanderberg (1979).

2.3 ANTIMALARIAL ACTIVITY BY USING THE *SYBR GREEN I* METHOD

To evaluate the antimalarial activity of compounds 3RJ and 4RJ at an initial concentration of 50 µM, against the W2 strain of *P. falciparum*, cultures were used that showed a minimum parasitemia of 8%, with a predominance of young trophozoites, these being synchronized later (item 2.6). The test was then carried out as described by Addla *et al*. (2023), with an adjustment in the incubation time for treatment, which was 72 h. After this interval, the assay was carried out by fluorescence using the SYBR Green I technique, according to Smilkstein *et al*. (2004). The resulting fluorescence values were converted into percentage growth inhibition (IC) and evaluated as a ratio of serial concentrations. Data analysis was performed using the Origin software (Origin Lab Corporation, Northampton, MA, USA) which determined the inhibitory concentration for 50% of parasitic growth (IC_{50}). Compounds were classified according to the following criteria: active IC_{50} values < 25 µM; partially active IC_{50} values < 50 µM (Boechat *et al*., 2014: Cos *et al*., 2006).

2.4 PHENOTYPIC PROFILING

In order to determine the time of action of the 3RJ compound, the specificity, the possible morphological changes in the different asexual forms of the parasite and the classification of the compound as parasiticidal or parasitostatic, the study used a culture with a predominance of young trophozoites (rings) (80%) from the W2 strain of *P. falciparum*, according to Lambros and Vanderberg (1979). Therefore, parasitemia was adjusted to 5% and hematocrit to 2%, and the parasites were placed in the 96-well culture plate (U bottom). The concentration of the reference drug (artemisinin) and the test compound were adjusted to 10x those of the IC_{50} value (Hofer *et al*., 2008). To evaluate the time of action, intervals of 8, 16 and 24 h were determined based on the variations recorded between the stages (ring, trophozoites and schizonts) previously observed in the culture used, as adapted from Morita *et al*. (2015). For each proposed interval, two plates were prepared, A and B, both containing

untreated parasites as positive controls. Plate A was revealed by optical microscopy and plate B was revealed using the SYBR Green I technique (Smilkstein *et al*., 2004). The action of the compound began on plates A, after the proposed interval where triplicates of blood smears (2 µL) stained with a panoptic kit and identified as pre-removal were taken. After this process, the compound was removed by washing plate A (Morita *et al*., 2015) and the culture was incubated again until the positive control showed parasitemia ≥ 50% of schizonts. Upon reaching this maturation, the test was completed by taking new smears in triplicates and these were identified as post-removal. The prepared slides were read using optical microscopy (100X), totaling 100 forms, which were classified into ring, trophozoite, schizont and altered forms (parasites with altered morphology).

For plate B, in addition to the above-mentioned positive control, a negative control (uninfected red blood cells) was added. After each interval proposed to interrupt the treatment, the compound was removed by washing, then plate B was incubated again until completing 72 h of total culture, counting from the beginning of the experiment. After this process, the assay was developed using the SYBR Green I technique, to evaluate total parasitemia (Wein *et al*., 2010; Sonoiki *et al*., 2017). The data was plotted using the Graph Pad Prism 6.01 software, considering the predominant morphology at pre- and post-removal times, for the blood smear results. For the results revealed using the SYBR Green I technique, the difference between the reading values of the control, reference drug and test compound was considered, represented in RFU (relative fluorescence unit). The data were validated using one-way analysis of variance (ANOVA), and Tukey's post-test with significance ≤ 0.05.

2.5 *EX VIVO* STUDY WITH LOCAL ISOLATES OF *P. FALCIPARUM* AND *P. VIVAX*

In this study, after approval from the research ethics committee (5,832,189), patients over 18 years old infected with *P. vivax* or *P. falciparum*, presented with parasitemia above two crosses (++) (2,000 parasites/mm^3),

uncomplicated malaria or lack of treatment with antimalarials in the last 20 days, were recruited at CEPEM (Center for Research in Tropical Medicine), Porto Velho, RO and invited to participate in the aforementioned research, through the Informed Consent Form (TCLE). After accepting the patients, blood was collected and a slide was taken to check the current stage of the parasites; In this assay, the parasites needed to be in the young trophozoite (ring) stage, so that maturation could be monitored. Leukocytes were isolated using a CF11 cellulose column (SRIPRAWAT *et al*., 2009), then the experiment was performed following Renapurkar *et al*. (1989), with some adjustments. On this occasion, the medium used to maintain the cultivation was McCoy's for the species *P. vivax* and RPMI for the species *P. falciparum*. This medium was supplemented with 20% human plasma, compatible with the patient's blood ABO system. The compounds involved were the reference drug Dihydroartemisinin [5µM], indicated as a killing control, and the test compound 3RJ [100 µM], both at the initial concentration of a triplicate involving 8 points. Following the distribution of the compounds on the plate, the hematocrit was adjusted to 20% with complete medium, and the parasites were incubated at 37 ºC, where maturation was monitored for an interval that varied between 24 – 48 h. After a minimum maturation of 40% of the parasites in a total of 200 forms, the assay was revealed by optical microscopy (1000x). The results were plotted using the Origin 9.1 and Graph Pad Prism 6.01 software. This study did not include patients with severe or complicated malaria as defined by the WHO (WHO, 2016): elderly people, comorbidities, pregnant women, indigenous people, and people with mental disabilities.

3 RESULTS AND DISCUSSION

3.1 EVALUATION OF THE ANTIMALARIAL ACTIVITY THROUGH THE *SYBR GREEN* METHOD

The bi-triazole compounds 3RJ and 4RJ showed promising IC_{50} values, being considered active, according to literary reports that discuss the potential of antimalarial candidates (Boechat, 2014; Cos, 2006), while the reference drug

artemisinin (Art) had an IC_{50} of 0.018 µM. The IC_{50} results of the test compounds are described in (Table 1) of this study.

Table 1. Antiplasmodial activity of the bi-triazole compounds 3RJ and 4RJ against the *P. falciparum* strain, the W2 strain

Compounds	$IC_{50\ \mu M}$
	W2
3RJ	4.4 ± 0.30
4RJ	6.3 ±0.71

IC_{50} values are represented by the mean of the three repetitions; (±) standard deviation relative to the values. Source: authors of the study

3.2 PHENOTYPIC PROFILING

Through this specific stage assay, we sought to investigate the performance of the 3RJ compound in relation to the time of action, possible morphological changes and stage specificity during the asexual phase of the parasite's life cycle. It is noteworthy that the culture incubation progressed as expected, as it is possible to monitor the maturation of the parasites (Figure 2A), where the positive control showed predominant forms of mature trophozoites at 8 h of cultivation and a predominance of schizonts in periods of 16 h and 24 hours. Regarding the time of action of compound 3RJ, it is notable, after reading the pre-removal blood smears, that maturation was prevented from the first 8 h, as the other forms (subsequent to the young trophozoites) are not visualized, still with a significant percentage of parasites with altered morphology (Figure 2A), as well as that recorded by the reference drug. In more detail (Figure 2B), in the positive control (C+) after 8 hours, the parasite is observed in the form of a mature trophozoite and its maturation into a schizont is observed in the following periods of 16 and 24 hours. The parasites treated with 3RJ presented: altered morphology, with cytoplasmic vacuolization (8 h and 16 h) and condensed nucleus (24 h), not evolving into the mature stage (schizont).

Figure 2A and 2B. Pre-removal evaluation of the compound: duration of action, possible morphological alterations and specificity of the asexual stage of P. falciparum - W2

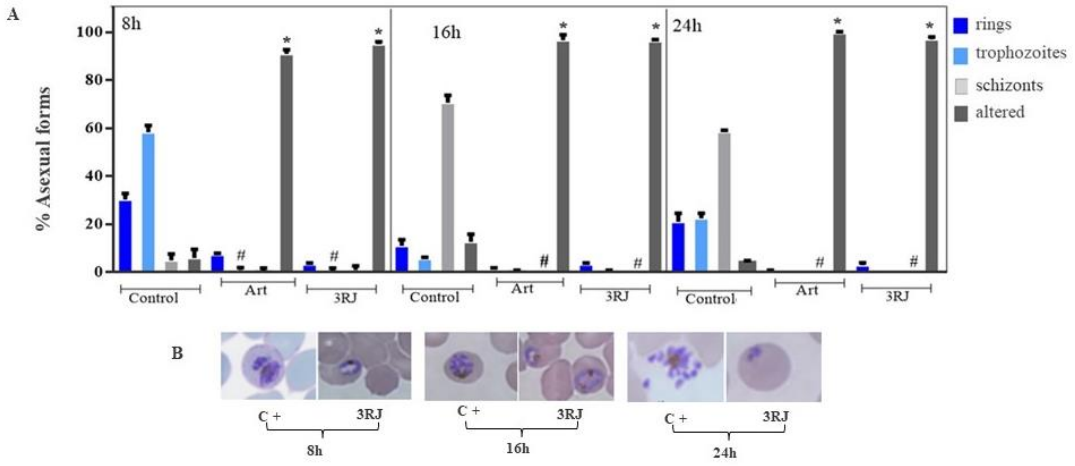


Figure 2A: Columns marked with hash sign (#) represent statistically significant difference in relation to the predominant stage in the positive control, just as columns marked with (*) represent parasites with altered morphology. Figure 2B: Optical microscopy (100X) of erythrocytes infected with *P. falciparum* W2 treated or not for 8, 16 and 24 h. C+ (Positive control). Source: authors of the study

The evaluation of post-removal blood smears (Figure 3A) aimed to investigate the action of the compound 3RJ as to whether it was parasiticidal or parasitostatic. There was a permanent difference between the predominant form of the positive control (untreated), the 3RJ compound and the reference drug, demonstrating that there was no rehabilitation on the part of the treated parasites. After removing the compounds, there was a prevalence of parasites with altered morphology, related to compound 3RJ and the reference drug, suggesting parasiticidal action to the test compound. Figure 3B complements these reports (Figure 2A) through the morphological comparison of the parasite treated with compound 3RJ and the positive control (untreated). The recovery interval determined for the parasites was set to the amount of time needed for the positive growth control to reach a parasitemia ≥ 50% schizonts.

Figures 3A and 3B. Post-removal evaluation of the compound: determination of the predominant forms and evaluation of the morphology of the parasites.

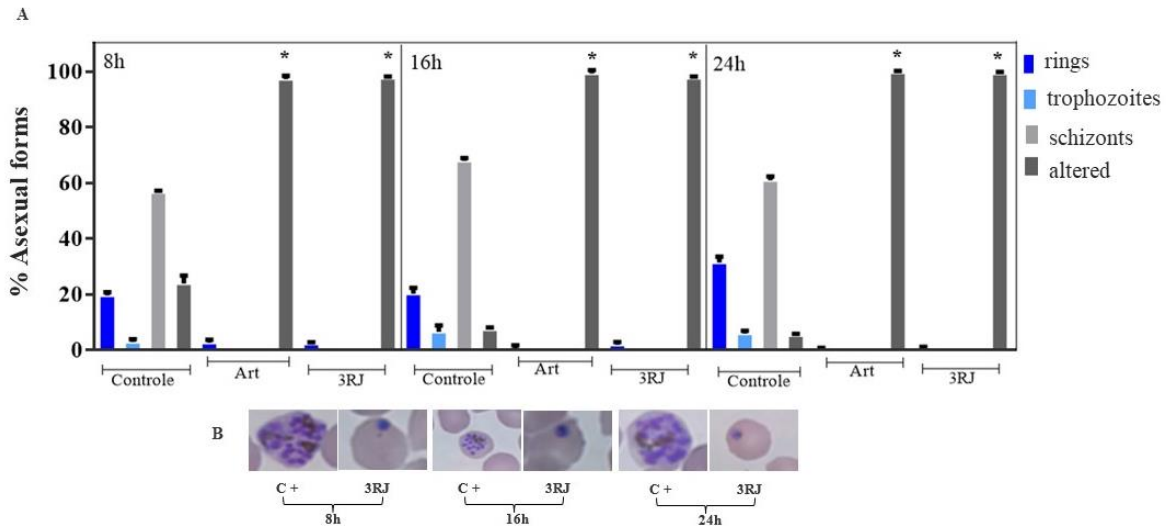
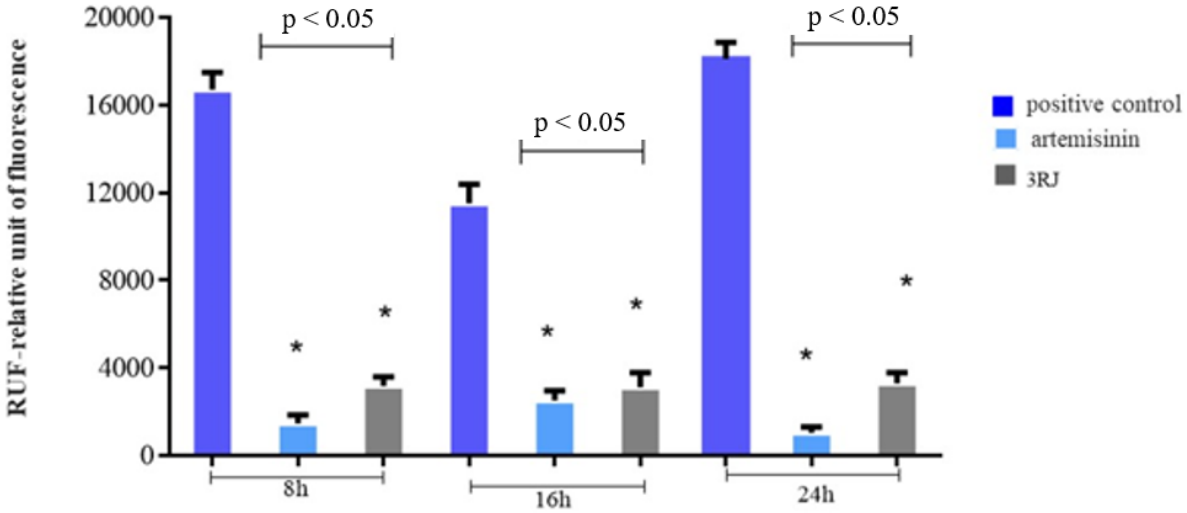


Figure 3A: Columns marked with a pound sign (#) represent a statistically significant difference when compared to the predominant stage in the positive control, just as the columns marked with an asterisk (*) represent the parasites with altered morphology. Figure 3B: Optical microscopy (100X) of erythrocytes infected with *P. falciparum* W2 treated or not for 8, 16 and 24 h. C+ (Positive control). Source: authors of the study

Assuming that the interval for rehabilitation of treated parasites determined in the post-removal evaluation was short, we proposed an evaluation of the stage-specific assay using the SYBR Green I technique, after 72 h of total culture. The results indicate that there was a significant difference in parasitemia values between the positive control, compound 3RJ and artemisinin (Figure 4). Correlating this Figure with Figures 2A and 3A, it is suggestive that the parasites with altered morphology were destined to die, with no recovery of these parasites after the removal of the compound and reincubation of the culture. The result of this test demonstrates that the action of the 3RJ compound is parasiticidal. The correlation between the cited Figures reveals that the 3RJ compound behaves in a similar way to artemisinin. Thus, after a period of 72 hours of total cultivation, it was verified that the few remaining parasites had not matured (Figure 4).

Figure 4. Evaluation of parasitemia and classification of compound action: post removal, 72h of total culture revealed by SYBR Green I.



Phenotypic profiling assay, evaluation 72 h of total cultivation; difference in parasitemia between the positive control (untreated) and the compounds. Results refer to the mean reading in normalized RFU (minus negative control values) and standard deviation (±) of triplicates. Source: authors of the study

In summary, compound 3RJ was added to the *P. falciparum* W2 culture with predominant forms of young trophozoites (rings) and monitored in the first 24 hours. The results indicated a parasiticidal effect (irreversible, cytotoxic) in the first 8 hours of treatment. Figure 2A shows the percentage (≅100%) of parasites with altered morphology, already in the first 8 h of incubation in relation to the untreated control and this result was confirmed after the removal of the compound 3RJ, (Figure 3A) and with the evaluation of parasitemia after 72 h of total incubation (Figure 4). The compound caused morphological changes in the parasite, noted from the 8-h period of treatment (Figure 2B), and no progression of the cell cycle to schizont formation was detected even after the removal of 3RJ (Figure 3B). These data suggest that the 3RJ compound is fast-acting, expressing its lethality during the young trophozoite stage, thus preventing the development of the parasite's life cycle. This ability to irreversibly alter the viability of the parasite can be interpreted as a source of more detailed information about its antimalarial potential.

3.3 *EX VIVO* STUDY WITH ISOLATES OF *P. FALCIPARUM* AND *P. VIVAX*

The results found regarding the *ex vivo* susceptibility of the circulating strains of local isolates recorded the following medians of IC_{50}: 17.3 µM for the species *P. falciparum*, and 7.5 µM for *P. vivax*, a result compatible with that of the W2-resistant strain. DHA, used as a positive control, exhibited the following median IC_{50}'s of 0.0005 and 0.001 µM for *P. falciparum* and *P. vivax*, respectively (Figure 5).

Figure 5. Evaluation of the compound 3RJ against the asexual forms of *P. falciparum* and *P. vivax*, from local isolates.

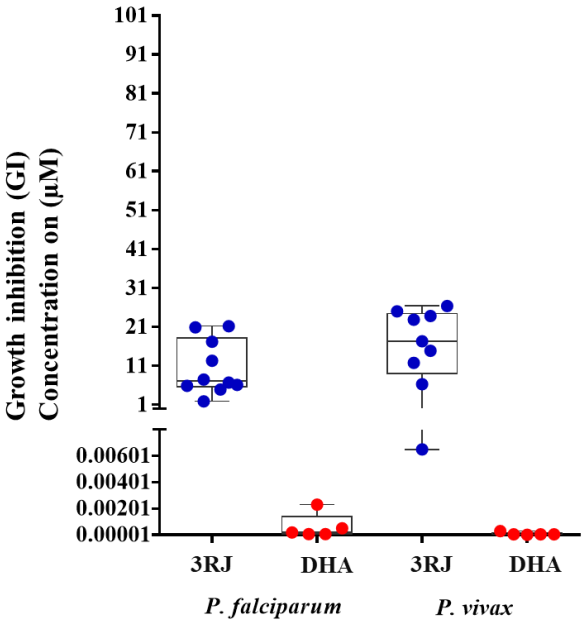


Image referring to the dispersion graph of inhibition of *P. falciparum* and *P. vivax* in samples of local isolates of the referred species. Treated with the death control DHA – Dihydroartemisinin (0.008µM) and the compound 3RJ (100 µM). Results referring to mean and standard deviation (±). Source: authors of the study

The results found for 3RJ, compared to local isolates of *P. falciparum* and *P. vivax*, are promising for this class of molecule, for which antimalarial studies are scarce. The IC_{50} values described here are higher than those of the death control drug. Studies that evaluate the criteria for the development of new medicines mention that a low IC_{50} value is highly desirable, however, other factors such as physicochemical properties and pharmacokinetic characteristics should be considered. In relation to antimalarial studies, the activity of this compound

must be detailed in terms of activity at the various stages of the parasite's life cycle (KATSUNO *et al*., 2015). It is reinforced that, as this is a class of synthetic molecules with a good history of structural changes, the results guide the study of the development of new drugs in this sense.

4 CONCLUSION

We evaluated the *in vitro* and *ex vivo* activity of two triazole-based compounds agaisnt the malaria parasites. This study demonstrated that the bi-triazole compounds are promising for targeting multiple stages of the parasite's life cycle, offering valuable insights for optimizing the antimalarial potential of triazole derivatives through structural modifications. Despite these findings, one main limitation exists: to better understand the toxicity and pharmacokinetics of these compounds in a living organism, further in vivo studies are necessary. This limitation should be addressed in future research to fully assess the safety and efficacy of these compounds as potential treatments. Nevertheless, the results provide important contributions to scientific knowledge on triazole-based compounds against the malaria parasite. These findings could ultimately aid in developing a new class of antimalarial drugs, helping to address a significant global public health challenge.

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