UNIVERSIDAD DE INVESTIGACIÓN DE TECNOLOGÍA EXPERIMENTAL YACHAY

Escuela de Ciencias Biológicas e Ingeniería

TÍTULO: Experimental Evaluation of Different Compounds Like Thiadiazine and their Anti-malarial Effect in the Murine Model Infected with *Plasmodium berghei* ANKA and *Plasmodium yoelii* 17XL Strains

Trabajo de integración curricular presentado como requisito para la obtención del título de Bióloga

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Dedicatoria

Cuando un sueño nace, una nueva meta es parte de tus planes, te expones a un sin número de cambios y nuevas experiencias que al final del día se suelen reducir en alegría, tristeza o melancolía. Desde mi perspectiva son sentimientos que no son fáciles de lidiar, pero ¿saben? aprendí que las únicas personas capaces de transmitir entereza en los momentos de desconcierto, son tus padres y tu familia.

Son mis padres y mi familia quienes han tenido las palabras precisas ante cualquier circunstancia y a quienes hago una justa dedicatoria por este logro.

Gracias por su apoyo, serenidad, fortaleza e ímpetu.

Aquel que tiene un porqué para vivir,

se puede enfrentar a todos los “cómos”

Friedrich Nietzsche

Katherine Stefania Loachamin Gualotuña

Agradecimiento

Como olvidar la primera vez que llegue a Yachay, fue una mezcla de sentimientos y emociones que ahora mismo no sabría explicarlos, sin embargo, tenía un ingrávido palpitar de encontrar aquello que buscaba. Reconozco que no ha sido fácil alcanzar esta meta, pero vale más tener cicatrices por valiente que piel intacta por cobarde.

Durante el periodo universitario he sido partícipe de gratas experiencias académicas que han permitido desenvolverme exitosamente en el presente trabajo de investigación. Le doy un especial agradecimiento a mi tutora; Lilian Spencer, quien supo transmitirme sus conocimientos y experiencias, sin olvidar que, su paciencia y dedicación hicieron de esto, una investigación de calidad. A Hortensia Rodríguez por su desempeño como co-tutora y por la donación de los compuestos derivados de bis-THTT. Al Profesor José Manuel Lozano y Dra.Beatriz Pernía Santos, integrantes de las distintas instituciones colaboradoras, Muchas Gracias. Su participación mediante donaciones y análisis estadísticos, han contribuido al objetivo del tema planteado en mi trabajo de investigación.

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Katherine Stefania Loachamin Gualotuña

Resumen

La malaria es una enfermedad potencialmente mortal causada por parásitos protozoarios del género *Plasmodium* y se transmite a los humanos por la picadura de mosquitos infectados del género *Anopheles*. En el 2017 la OMS reportó 219 millones de casos de malaria y se estimaron 435,000 muertes. El tratamiento antipalúdico que se usa con mayor frecuencia se basa en la cloroquina, que se ha utilizado durante varias décadas. Esta aplicación prolongada ha provocado que el parásito desarrolle resistencia al uso del medicamento mencionado, por lo que se hace necesario buscar nuevos tratamientos. En el presente trabajo, seis compuestos derivados de bis-THTT (JH1, JH2, JH3, JH4, JH5 y JH6) se evaluaron como posibles fármacos antipalúdicos en ratones BALB/c infectados con las cepas *Plasmodium berghei* ANKA y *Plasmodium yoelii* 17XL. Además, evaluamos la respuesta humoral de cada compuesto experimental, mediante una prueba ELISA indirecta, utilizando cada compuesto como antígeno. Finalmente, evaluamos la relación que existe entre la estructura química y la actividad biológica de cada compuesto. Nuestros resultados mostraron que los compuestos JH2 y JH4 presentaban un control eficaz de la parasitemia en ratones infectados con *P. berghei*. Además, los compuestos JH5 y JH6, exhibieron resultados similares en el control de infección que la cloroquina en ratones infectados con *P. yoelii.*

Palabras Clave: *Plasmodium berghei, Plasmodium yoelii*, bis-THTT, fármacos antipalúdicos.

Abstract

Malaria is a life-threatening disease caused by protozoa parasites of the genus *Plasmodium* and it is transmitted to humans by the bite of infected mosquitoes of the genus *Anopheles*. WHO has reported 219 million cases of malaria and 435,000 deaths were estimated in 2017. The anti-malarial treatment more frequency used is based on chloroquine, which has been used for several decades. This prolonged application has caused that the parasite developed resistance to the use of mentioned drug, so it becomes necessary to search for new treatments. In the present work, six bis-THTT derivatives compounds (JH1, JH2, JH3, JH4, JH5 and JH6) were evaluated as potential anti-malarial drugs in BALB/c mice infected with *Plasmodium berghei* ANKA and *Plasmodium yoelii* 17XL strains. In additional, we evaluated the humoral response of each experimental compounds by indirect ELISA test using each compounds as antigen. Finally, we evaluate the relationship that are between chemical structure and biological activity of each compounds Our results showed that JH2 and JH4 compounds, presented effective parasitaemia control in mice infected with *P. berghei*. Besides, JH5 and JH6 compounds, exhibited similar results in the infection control as chloroquine in mice infected with *P. yoelii*.

Key words: *Plasmodium berghei*, *Plasmodium yoelii*, bis-THTT, anti-malarial drugs.

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°C: Degree Celsius

%: Percentage

ABTS: 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfuric acid)

ACT: Artemisinin combination therapy

AS+SP: Artesunate + sulfadoxine-pyrimethamine

Bis-THTT: Bis-tetrahydro-(2H)-1,3,5-thiadiazine-2-thione.

CM: Cerebral malaria

CQ: Chloroquine

DMSO: Dimethyl-sulfoxide

DNA: Deoxyribonucleic acid

ELISA: Enzyme-linked immunosorbent assay

G6PD: Glucose-6-phosphate dehydrogenase

IgA: Immunoglobulins A

IgG: Immunoglobulins G

IgM: Immunoglobulins M

IP: Intraperitoneal

P: Plasmodium

PAHO: Pan American Health Organization

PBS/T: Phosphate buffered saline and Tween 20

PBS: Phosphate buffered saline

PRBC: Parasitized red blood cells

RBC: Red blood cells

RPM: Revolutions per minute

SAR: Structure-activity relationship

spp: Specie

UNICEF: United Nations International Children´s Emergency Fun

WHO: World Health Organization

μg: microgram

μL: microlite

INTRODUCTION

Malaria is a global public health problem that in 2017 alone affected around 219 million people. Most cases of malaria occur in children, especially in rural areas with limited access to health care facilities. Malaria is one of the main causes of anemia in infants and pregnant women, low weight in newborns, premature births, and infant mortality (WHO, 2018). Institutions such as UNICEF recognize that malaria is one of the five leading causes of deaths in children under five years of age (Phillips, 2001). Malaria is caused by a protozoan of the genus *Plasmodium*, which can be found in 87 tropical and subtropical countries in Africa, Asia, and the Americas. The rate of transmission is especially high in endemic areas, where sanitary control is wanting or lacking. Malaria is difficult to control in areas where the parasite has developed chloroquine (CQ) resistance. Therefore, the development of a new treatment against malaria is an urgently need.

Treatment is a relevant aspect in the control of malaria infections. CQ has been used as anti-malarial since 1940 and is still used in most countries for the treatment of *Plasmodium vivax* and *Plasmodium falciparum*, which are the most common in humans. However, the *P. falciparum*, one of the most dangerous parasite strain, has developed slowly and progressively resistance to this drug (Abdi et al., 1995). For this reason, WHO currently recommends the use of Artemisinin to the treatment of *P. falciparum* in CQ resistance areas. In the present work, we have tested the efficacy of six bis-THTT derived compounds as anti-malarial drugs in BALB/c mice strain, which were infected with *Plasmodium berghei* ANKA and *Plasmodium yoelii* 17XL strains.

3.1 Malaria history

The name malaria, derived from ‘mal-aria’ meaning bad air in Medieval Italian, originated in Ancient Rom, whose inhabitants thought that malaria came from the fumes of swamps and, foul gasses released from soil, water and air (Bruni, 1974; Opeskin, 2009). The first evidence for the malaria parasite was discovered in mosquitoes preserved in amber from the Palaeogene period (approximately 30 million years ago) (Poinar, 2005). In 1897, Ronald Ross, after two years of continuous experiments in India, showed that malaria is transmitted

by mosquitoes. He also described the life cycle of the malaria parasite in the Anopheles mosquito (Ross, 1897).

Human malaria likely originated in Africa and has co-evolved with its intermediate and definite hosts, non-human primates and mosquitoes, respectively. Currently, malaria is distributed mainly in tropical and subtropical countries (Sanz et al., 2012). It has been postulated that malaria spread by human migration in the Neolithic period to Europe, the Middle East, Asia, India, and China. Malaria probably was introduced by slave trade into the Americas, between the 16th and the 19th century (Yalcindag et al., 2011; Rodrigues et al., 2018).

According to the last report issued by PAHO in the year 2017, malaria cases have increased in five countries: Brazil 174,522 cases, Ecuador 1,279 cases, Mexico 704 cases, Nicaragua 10,846 cases and Venezuela 319,765 cases. In Ecuador 72% of all cases were caused by *P. vivax* and 28% by *P. falciparum*. The four Ecuador provinces with the highest number of cases during 2017 were Morona Santiago (489 cases), followed by Orellana (240 cases), Pastaza (223 cases) and Esmeraldas (215 cases) (PAHO, 2018).

3.2 Human *Plasmodium*

The DNA of the malaria parasites of the genus *Plasmodium* encodes for at least 5,600 genes. According to taxonomic classification, *Plasmodium* belongs to the phylum *Apicomplexa* (characterized by the presence of an apical complex at some stages in the life cycle), class *Aconoidasida*, subclass *Coccidia* and order *Haemosporina*. The apical complex of the *Apicomplexa* consists of rhoptries, micronemes, secretory organelles and dense bodies, which allow the parasites to enter the host cell and protect the parasite from the host immune response (Cowman and Crabb, 2006).

There are five species within the genus *Plasmodium* that cause malaria in humans: *P. ovale, P. malariae, P. knowlesi, P. falciparum* and *P. vivax*, the last two species are responsible for the majority of malaria cases in the Americas. The species that causes the most serious symptoms and sometimes death is *P. falciparum.* The main cause of pathology and mortality is this species is the adherence of parasitized erythrocytes to the endothelium of cerebral and other capillaries, which can lead to vascular obstruction, frequently followed by coma and death (WHO, 2018; PAHO, 2018).

Plasmodium is a complex organism that is carried by female *Anopheles* mosquitoes and enters the bloodstream of humans (vertebrate vector) when the mosquito imbibes a blood meal. In humans, the parasite goes through many stages, eventually bursting open red blood cells (erythrocytes) and causing a disease typified by fever, muscle pain, nausea, vomiting and fatigue. These symptoms are usually recurrent every two or three days. As the disease progresses, the patient presents anemia and thickening of the spleen, in extreme cases, cerebral malaria (CM) occurs (Abolghasemi et al., 2012).

3.2.1 The life cycle of the human *Plasmodium*

The life cycle of the malaria parasite in the human host is complex. Humans act as a reservoir and intermediate host, because in them the parasite develops asexually. This is called the schizogonic phase. The female mosquito of the genus *Anopheles* is the definitive host, because sexual reproduction of the parasite takes place here.

The malarial life cycle can be divided mainly into four phases: first, fertilization (sexual phase), which takes place in the stomach of the mosquito. The second phase is, sporogony (first asexual phase), which occurs in the wall of the stomach of the mosquito. The third phase, hepatic schizogony (second asexual phase), progresses in the liver cells. Finally, the fourth phase erythrocytic schizogony (third asexual phase), takes place within the erythrocytes (Knell, 1991).

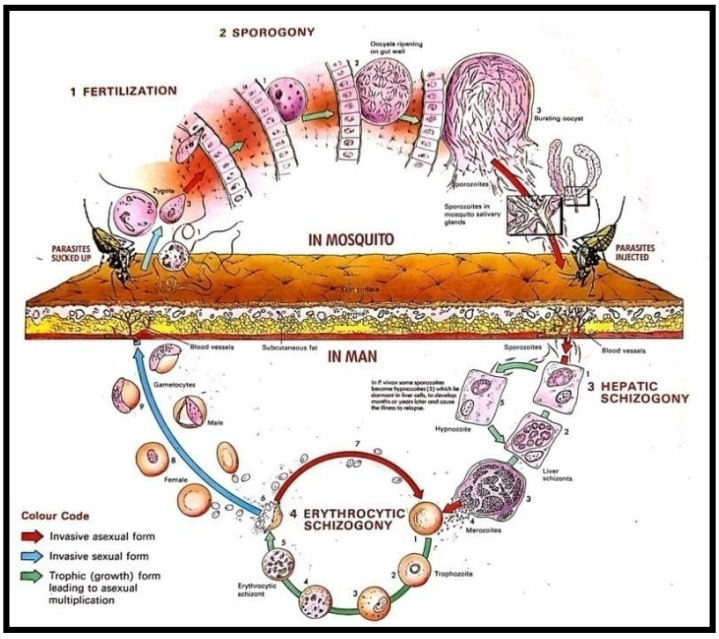

Figure 1. The life cycle of *Plasmodium* in humans (Recovered from Knell, 1991).

This cycle begins with the bite of an infected *Anopheles* mosquito with *Plasmodium*. When female *Anopheles* mosquito (infected) imbibes a blood meal from a human, the mosquito injects sporozoites into the blood through their epidermal capillaries. Later, sporozoites move through the bloodstream to invade liver cells, using gliding motility as the invasion mechanisms (Menard, 2001; Spencer et al., 2016). In the liver cells, sporozoites become hepatic trophozoites, which grow and undergo asexual multiplication to produce thousands of invasive merozoites (hepatic schizogony phase). Finally, the liver cells burst, releasing merozoites into the bloodstream. In a few seconds, merozoites invade, multiply, and rupture red blood cells to become erythrocytic trophozoites (Erythrocytic schizogony phase). These trophozoites multiply within the red blood cells (RBCs) and release from 8 to 16 new merozoites, such that fresh RBCs are rapidly reinvaded to repeat the erythrocytic cycle.

As the infection progresses, some merozoites develop into gametocytes to initiate the parasite's sexual stage. When the mosquito bites an infected human, it may ingest some gametocytes which produce male (microgamete) and female (macrogamete) gametes in the mosquito's stomach (sexual phase). Each gamete is haploid and after fertilization, the parasite becomes a diploid zygote. The zygote matures over the next few hours into a mobile ookinete which reaches the stomach wall and passes in between the epithelial cells to reach the basement membrane and form an oocyst. This oocyst eventually releases thousands of sporozoites (sporogony phase) which migrate to the mosquito's salivary glands to repeat the cycle (Knell, 1991).

3.3 Murine *Plasmodium* species

Murine malaria has been isolated from wild rats of five different countries: Cameroon, Central African Republic, Congo, Zaire, and Nigeria. In the middle of the 20th century, four species of murine *Plasmodium* were described: *Plasmodium berghei, P. chabaudi, P. vinckei* and *P. yoelii*, which include thirteen sub-species. Small differences exist in the biology of the rodent malaria parasites and this makes them particularly interesting models to investigate different aspects of human malaria (Killick-Kendrick, 1978). The usefulness of these parasites has been demonstrated in several investigations. *P. yoelii, P. chabaudi, P. berghei,* and *P.*

vinckei cause lethal infection in mice and are a model to investigate compounds with chemotherapeutic activity (Carter and Diggs, 1977).

3.3.1 *Plasmodium berghei*

This species was first described by Vincke and Lips in 1948 in the Belgian Congo (Carter and Diggs, 1977). Originally, this strain was isolated from thicket rats in Central Africa (Killick-Kendrick, 1978). *P. berghei* is analogous to *P. falciparum* that generate malaria in humans, they have a similar life cycle, structure, physiology, and pathology (Craig et al., 2012).

Within the *P. berghei* there are various strains that have been used experimentally, including *Plasmodium berghei* ANKA. The genome of *P. berghei* ANKA was sequenced in 2005, and given that transfection methods have also been developed for this strain, it has become an attractive choice for biologists that study malaria (Hall et al., 2005; Janse et al., 2006).

P. berghei ANKA model replicates many events seen during human CM and is accepted as the best available experimental model of CM (analogous to *P. falciparum*). The time to onset of clinical signs varies depending on the infection dose, the genetic background of the host and the specific clone of infecting parasites, but is typically between 5 and 10 days post-infection (De Souza and Riley, 2002).

3.3.2 *Plasmodium yoelii*

The specie of *P. yoelii* was isolated from a thicket rat (*Thamnomys rutilans*), trapped in the Central African Republic (Landau and Chabaud, 1965). The original isolate of *P. yoelii* from wild rat was obtained in laboratory mice. The subspecies of *P. yoelii* are distinguishable by morphological characteristics such as their forms in erythrocytic schizogony, and their geographical distribution. *Plasmodium yoelii* 17XL is a lethal strain cloned from *P. yoelii* 17XNL (Yoelii et al., 1975). This strain induces experimental CM that is associated with sequestration of infected red blood cells in the microvasculature of the mouse brain. Also, the *P. yoelii* 17XL strain has been used to investigate the role of chemokines (Sarfo et al., 2005; Otsuki et al., 2009).

3.5 Anti-malarial drugs

Malaria is a preventable and treatable infectious disease. The aim of malaria treatment is to achieve the complete and rapid cure and total elimination of the *Plasmodium* parasite from the patient's blood. In order to prevent chronic infection that leads to malaria-related anemia or death. Before anti-malarial treatment the patients with suspected malaria should have a parasitological diagnosis with either microscopy or rapid diagnostic test. Prompt treatment should be within 24 hours of fever onset, this is necessary for an effective cure and prevent complications.

Artemisinin combination therapy (ACT) is suggested as first-line therapy for uncomplicated infections with *P. falciparum* (WHO, 2018). ACT combines 2 active ingredients with different mechanisms of action. It consists of an artemisinin component (artesunate, artemether, or dihydroartemisinin), which rapidly reduces parasitemia and a second partner anti-malarial drug that slowly eliminates the residual parasites. The oral ACT treatments have few adverse effects and are available as fixed-dose combinations (artemether-lumefantrine, dihydroartemisinin-piperaquine, artesunate-sulfadoxine/pyrimethamine artesunate-amodiaquine, artesunate-mefloquine, and recently added artesunate-pyronaridine) (Plewes et al., 2019).

P. vivax infections should be treated with an ACT or CQ. In areas where *P. vivax* has been found to be chloroquine-resistant, infections should be treated with an ACT, preferably one in which the partner medicine has a long half-life. With the exception of artesunate + sulfadoxine-pyrimethamine (AS+SP) combination, all ACTs are effective against the blood stage infections of *P. vivax*. To avoid relapses, primaquine should be added to the treatment; dose and frequency of the administration should be guided by the patient's glucose-6-phosphate dehydrogenase (G6PD) enzyme activity (Galappaththy et al., 2007; WHO, 2018).

3.5.1 Chloroquine

This drug has been used as an anti-malarial drug for more than 50 years, and it has various uses in medicine. It is used as an anti-inflammatory drug to treat systemic lupus erythematosus and rheumatoid arthritis (Lu et al., 2017). CQ has been widely used as an anti-

malarial drug since 1940, so resistance has developed slowly and progressively (Abdi et al., 1995).

The mechanism of action of CQ is *Plasmodium* is not exactly known, but it is thought inbibit the activity of the heme polymerase. Normally, when *Plasmodium* spp. are in merozoite or trophozoite stage, inside to RBCs, they degrade hemoglobin to acquire essential amino acids, which the parasites require as proteins source and for their own energy metabolism. Degradation produces high quantities of free heme, which is toxic to cells and the parasite polymerizes the heme group to form hemozoin (malaria pigment), a non-toxic molecule (Wishart et al., 2017).

The cell membrane of the *Plasmodium* has a permeable quality that allows the CQ to enter inside the parasite and then by simple diffusion CQ enters into the digestive vacuole of the parasite. In the vacuole, the CQ becomes protonated (CQ^{2+}). Then, CQ cannot leave by diffusion and it caps hemozoin molecules, which prevent bio-polymerization of heme (Yayon et al., 1984). CQ binds to heme to form what is known as the FP-CQ complex; this complex is highly toxic to the cell and disrupts membrane function and results in cell lysis and ultimately parasite cell auto-digestion (Slater et al., 1991).

CQ sensitive parasites accumulate large amounts of heme in their digestive vacuoles, as opposed to CQ resistant parasites. Previous studies conducted in *P. falciparum* have proposed that this difference may be due to a point mutation in the gene that codes for the protein PfCRT, which is found in the membrane of the digestive vacuole (Bray et al., 1999; Chinappi et al., 2010).

3.5.2 Drug resistance

The development of parasite resistance to drugs is one of the greatest threats to malaria control. Resistance to anti-malarial drugs, has been confirmed in two of the five human malaria parasite species. *P. falciparum has* developed CQ resistance in some areas from Southeast Asia, Oceania, and South America in the late 1950s and early 1960s. In addition CQ resistance in *P. vivax* was identified in 1989 in Australians, Southeast Asia, Ethiopia, and Madagascar. Additionally, *P. falciparum* has developed resistance to nearly all currently available anti-malarial drugs, such as sulfadoxine/pyrimethamine, mefloquine, halofantrine,

and quinine; however, resistance to these drugs tends to be much less widespread geographically (Plowe et al., 2007).

A strategy that has received much attention recently to counter drug resistance is the combination of anti-malarial drugs, such as mefloquine, sulfadoxine/pyrimethamine, or amodiaquine, with an artemisinin derivative (White et al., 1999). Artemisinin drugs are highly efficacious, rapidly active, and have an action against different parasite stages.

3.6 BALB/c murine model

BALB/c is a strain of albino mice and weigh around 25g. The name BALB is a concatenation of Bagg and Albino mice. The founding animals of the strain were obtained by Halsey J. Bagg of Memorial Hospital, New York, from a mouse dealer in Ohio in 1913. Later in 1920 BALB/c mice were distributed globally from New York. Currently, these mice are among the most widely used inbred strains animal experimentation (Potter, 1985; Jackson Laboratory, 2011).

Figure 2. BALB/c murine model.

BALB/c mice are a popular strain and are used in different research disciplines, such as aging and pharmacological essays.

3.7 Compounds derived from bis-tetrahydro-(2H)-1,3,5-thiadiazine-2-thione (bis-THTT)

The tetrahydro-(2H)-1,3,5-thiadiazine-2-thione (THTT) derivatives have great application in the drug research as a biolabile prodrug, due to its high lipid solubility and, the

enzymatic rate of hydrolysis and stability in simulated gastric fluid. In addition, several *in vitro* and *in vivo* tests have shown that THTT derivatives have antibacterial, antifungal, anthelmintic, and tuberculostatic properties. For these excellent physicochemical properties, THTT derivatives have become in a key compound of an integral project for the development of new antiparasitic agents (Coro et al., 2005).

Following the interest in anti-protozoal drugs, the synthesis and biological evaluation of two new series of alkyl-linked bis-tetrahydro-(2H)-1,3,5-thiadiazine-2-thione (bis-THTT) derivatives was reported in 2005. In this process, two THTT rings were incorporated into the same molecular structure (see Figure 3.), with the aim they enhance the anti-protozoal effect. (Coro et al, 2005).

Since 2005, different series of the bis-THTT derivatives have been studied. In the first study, compounds derived from alkyl-linked bis-(2-thioxo-[1,3,5]thiadiazinan-3-yl) carboxylic acid (Figure 3.), were evaluated for their activity *in vitro* against *Trypanosoma cruzi* strain CL (clone CLB5) and *Trichomonas vaginalis* strain JH31A. The preliminary biological evaluation demonstrated that some of the new compounds possess notable activity against *T. cruzi* and *T. vaginalis* (Coro, 2005).

In 2006, a second study of *in vitro* antiprotozoal activity of the compounds derived from alkyl-linked bis-(2-thioxo-[1,3,5]thiadiazinan-3-yl) carboxylic acids was done (see Figure 4.). They were evaluated against *Leishmania donovani, Trypanosoma brucei rhodesiense STIB900*, and *Plasmodium falciparum 3D7*. The results were satisfactory only in *T. b. rhodesiense* (Coro, 2006).

Finally, in 2008 an *in vitro* antiprotozoal evaluation of N4-(benzyl)spermidyl-linked to bis-THTT derivatives from N4-(benzyl)spermidine was done. These novel bis-THTTs were evaluated in-vitro, against *L. donovani, T. cruzi, T. b. rhodesiense STIB900, and P. falciparum 3D7*. The preliminary results showed a potent protozoocidal activity against *T. cruzy* and *L. donovani* (Coro, 2008).

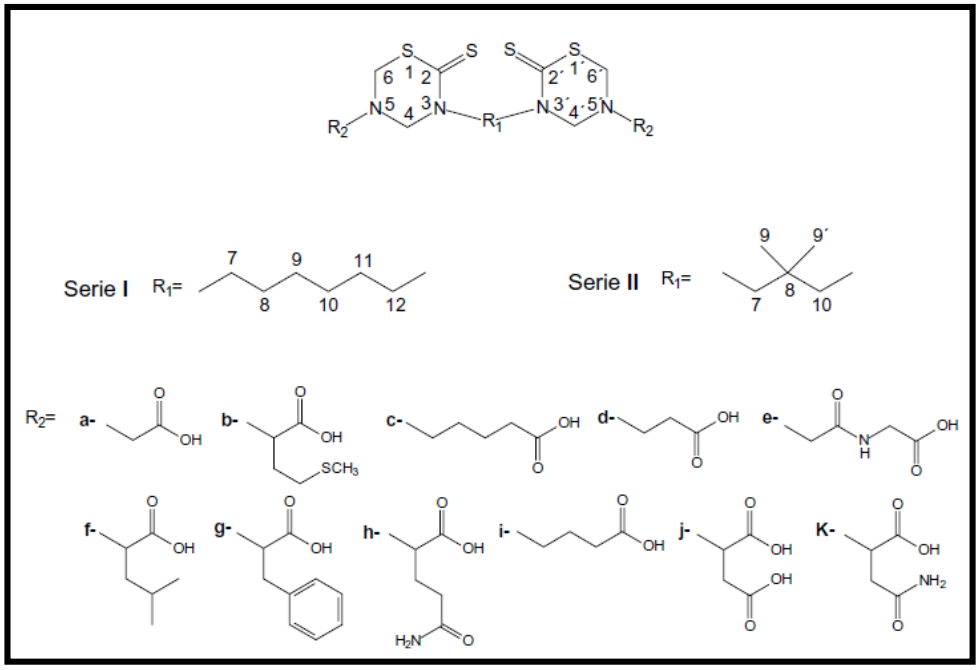

Figure 3. Chemical structure of the compounds derived from alkyl-linked bis-(2-thioxo-[1,3,5]thiadiazinan-3-yl) carboxylic acids. Recovered from: doi:10.1016/j.bmc.2005.03.009.

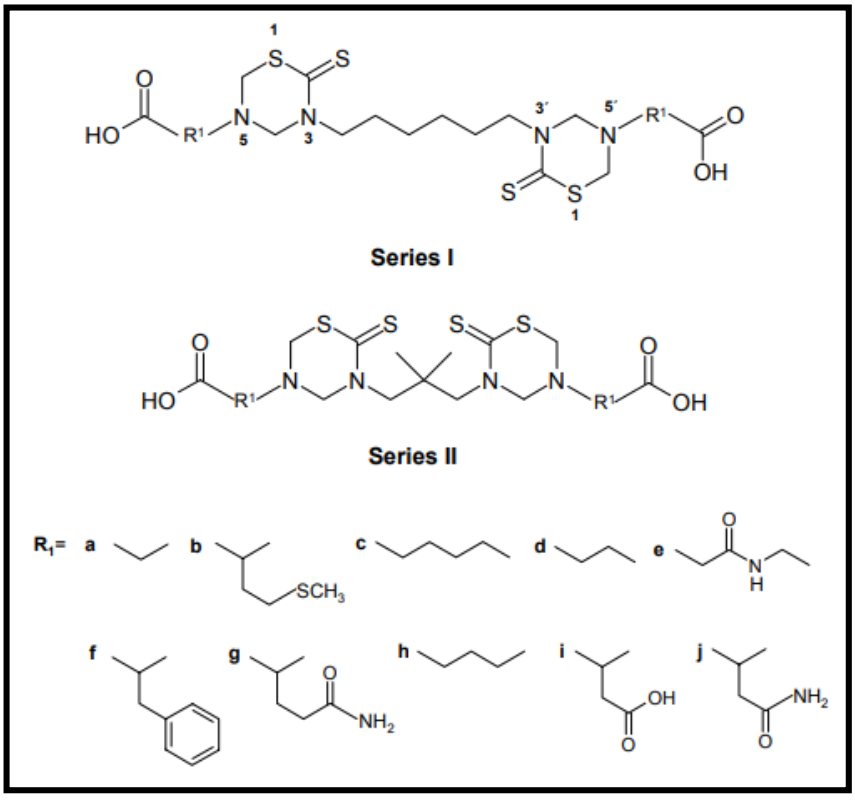

Figure 4. Chemical structure of compounds derived from alkyl-linked bis-(2-thioxo-[1,3,5]thiadiazinan-3-yl) carboxylic acids. Recovered from: doi:10.1016/j.bmcl.2005.11.060.

3.8 Chemical structure of bis-THTT and chloroquine

In this *in vivo* assay, we evaluated the behavior of six compounds derived from bis-THTT as anti-malarial drugs in BALB/c mice infected with *P. berghei* and *P. yoelii*. Chemical structure of bis-THTT consists basically of two THTT rings, connected to each other via their N-3 atom by a linear aliphatic backbone and bearing carboxyl residues at N-5. Also, we used 7-chloro-N-[5-(diethylamino)pentan-2-yl]quinolin-4-amine (Chloroquine) as a positive control of malaria cure. CQ is a 4-aminoquinoline with schizontocidal activity against the blood forms of the parasites *P. ovale* and *P. malariae*, and against the susceptible strains of *P. vivax* and *P. falciparum* (Wishart et al., 2017).

4. PROBLEM STATEMENT

Malaria is one of the infectious diseases with the highest mortality in the world; therefore it has become a global health problem. According to the last report emitted by the WHO, 219 million cases and 435,000 deaths were reported in 2017 (WHO, 2018). Currently, there is no effective vaccine against malaria, so classical anti-malarial drugs, such as, CQ and its derivatives are used for clinical manifestations. However, in some areas *P. falciparum* and *P. vivax*, which are responsible for the majority of malaria cases in humans, have developed resistance to anti-malarial drugs (PAHO, 2017). Therefore, research is necessary to evaluate new treatments to eradicate the malaria disease.

5. HYPOTHESIS

Previous investigations of the bis-THTT derived compounds show satisfactory results in the treatment against protozoan parasites such as *Trypanosoma cruzi, T. vaginalis, T. b. rhodesiense* and *Leishmania donovani*. Therefore, hypothesize that compounds derived from bis-THTT are effective in other protozoan parasites such as *Plasmo*

6. OBJECTIVES

6.1 General Objective

Biological evaluation *in vivo* of six compounds derived from bis-tetrahydro-(2H)-1,3,5-thiadiazine-2-thione: JH1, JH2, JH3, JH4, JH5, and JH6 as possible anti-malaria drugs, in BALB/c mice infected with *Plasmodium berghei* ANKA and *Plasmodium yoelii* 17XL strains and evaluation of the humoral response of each compound by indirect ELISA test.

6.2 Specific Objectives

- The evaluation of compounds derived from bis-THTT as an anti-malarial treatment in BALB/c mice experimentally infected with the strains *P. berghei* ANKA and *P. yoelii* 17XL, will determine their curative effects through the determination of the parasitemia percentage.
- The evaluation of the humoral response of the compounds derived from bis-THTT in BALB/c mice, by means of an indirect ELISA test using said compounds as antigens.
- Analyze the relationship between the chemical structure and the biological activity of compounds derived from bis-THTT that have favorable anti-malarial activity.

7. METHODS AND MATERIALS

7.1 Malaria parasite

Strains of *Plasmodium yoelii* 17XL and *Plasmodium berghei* ANKA were used *in vivo* assays. The parasites were kept cryopreserved (20% glycerol at -80 °C) and activated by intraperitoneal (IP) inoculation of 200 µL in approximately 10 weeks old adult BALB/c mice. For each strain, parasitemia ([the number of parasite-infected cells counted the/total number of red blood cells counted] x 100%) was monitored and determined daily by microscopic examination of blood smears stained with Giemsa reagent until reaching a value equal to or greater than 40% of parasitemia.

7.2 Obtaining parasitized red blood cells (PRBC) with *P. berghei* and *P. yoelii*to inoculate different groups of experimental mice.

Previously inoculated mice that reached at least 40% parasitemia were sacrificed and blood was collected using heparinized Krebs solution (0.9% NaCl + 4.2% sorbitol in a 1:20 dilution of Heparin). The parasites in schizont and trophozoite stage were isolated through a cellulose column (CF-11 (Sigma)with a NaCl buffer 0.9%), the white blood cells were retained in the cellulose column. The sample eluted from the cellulose column was centrifuged at 2,000 rpm for 5 minutes at a temperature of 4 °C. Subsequently, the supernatant was discarded and the pellet (the portion containing the PRBC) was resuspended at twice the volume with the Krebs solution. The percentage of parasitized red blood cells (PRBCs) was determined using a Neubauer chamber, to find the concentration of $5x10^3$ PRBCs for each inoculation.

7.3. Experimental inoculation with *P. berghei* and *P. yoelii* to BALB/c mice.

Once the concentration of PRBCs was determined in the Neubauer chamber, the inoculum with parasites of *P.berghei* and *P. yoelii* was prepared, in such a way that in 200 µL of inoculum there were 5000 PRBCs. The inoculum was administered intraperitoneally to female BALB/c mice about 10 weeks old.

The following table shows the distribution of each experimental group of mice, which were evaluated with six compounds derived from bis-THTT.

Table 1. Distribution of the BALB/c mice infected with *P. berghei* and *P. yoelii* to the treatment with compounds derived from bis-THTT and chloroquine.

Grup 1: *P. berghei*			Grup 2: *P. yoelii*		
Mice box	Compounds derived from bis-THTT	Number of mice	Mice box	Compounds derived from bis-THTT	Number of mice
1	CQ	3	9	CQ	3
2	JH1	3	10	JH1	3
3	JH2	3	11	JH2	3
4	JH3	3	12	JH3	3
5	JH4	3	13	JH4	3
6	JH5	3	14	JH5	3
7	JH6	3	15	JH6	3
8	Parasitemia control	3	16	Parasitemia control	3
Total number of mice		24	Total number of mice		24

7.4. Preparation and administration of treatments with compounds derived from bis-THTT

The experimental compounds evaluated in this study were six derivatives of bis-tetrahydro-(2H)-1,3,5-thiadiazine-2-thione as anti-malarial drugs: JH1, JH2, JH3, JH4, JH5, JH6 and the CQ as a positive control (cure). In figures: 5, 6, 7, 8, 9, 10, and 11 show chemical structures of compounds derived from bis-THTT and CQ.

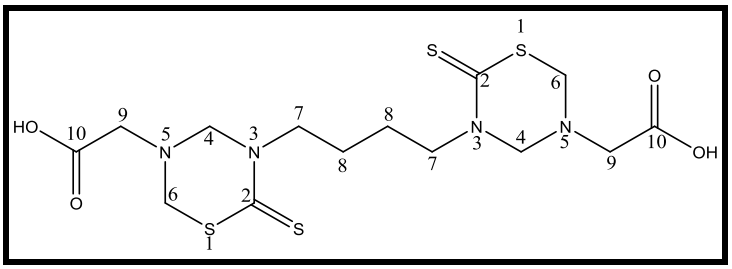

Figure 5. Chemical structure of JH1 compound, 2-(5-{6-[5-(Carboxymethyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}-6-thioxo-1,3,5-thiadiazinan-3-yl)-ethanico acid.

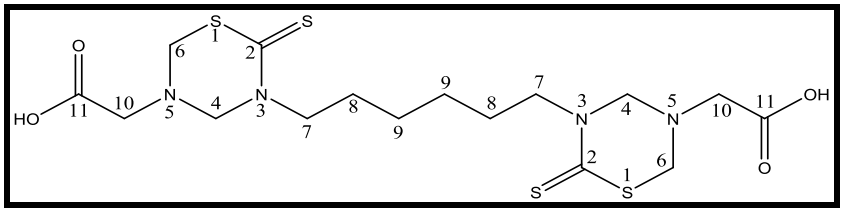

Figure 6. Chemical structure of JH2 compound, 2- (5- {6- [5- (carboxymethyl) -2-thioxo-1,3,5-thiadiazinan-3-yl] hexyl} -6-thioxo 1,3,5-thiadiazinan-3-yl) ethanoic acid.

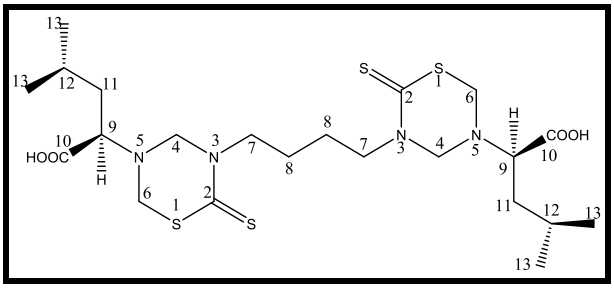

Figure 7. Chemical structure of JH3 compound, (2S)-2-[5-(6-{5-[(1S)-1-carboxy-3-methylbutyl]-2-thioxo-1,3,5 thiadiazin-3-yl}butyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]-4-methylpentanoic acid.

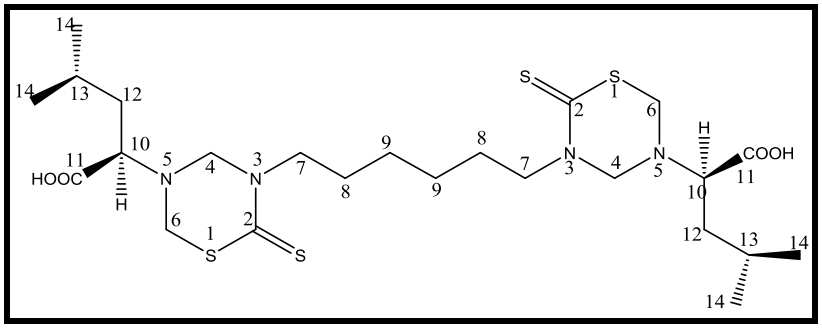

Figure 8. Chemical structure of JH4 compound, (2S) -2- [5- (6- {5 - [(1S) -1-carboxy-3-methylbutyl] -2-thioxo-1,3,5-thiadiazinan-3-yl}hexyl)-6-thioxo-1,3,5-thiadiazinan-3-yl]-4-methylpentanoic acid.

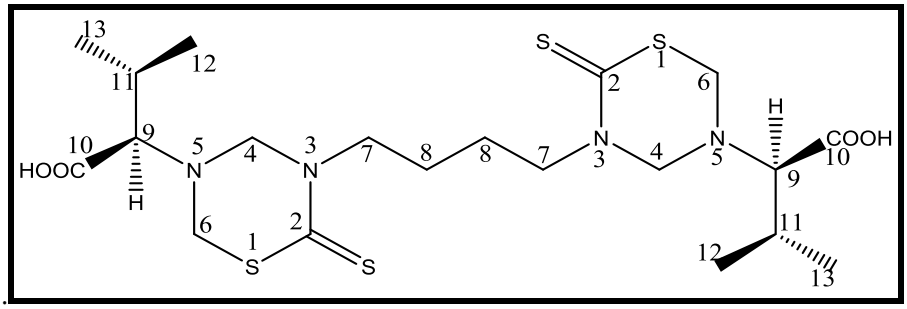

Figure 9. Chemical structure of JH5 compound, 2-(5-{6-[5-(1-carboxy-1-methylpropyl)-2-thioxo-1,3,5-thiadiazinan-3-yl]butyl}-6-thioxo-1,3,5-thiadiazin-3-yl)-3-methylbutanoic acid.

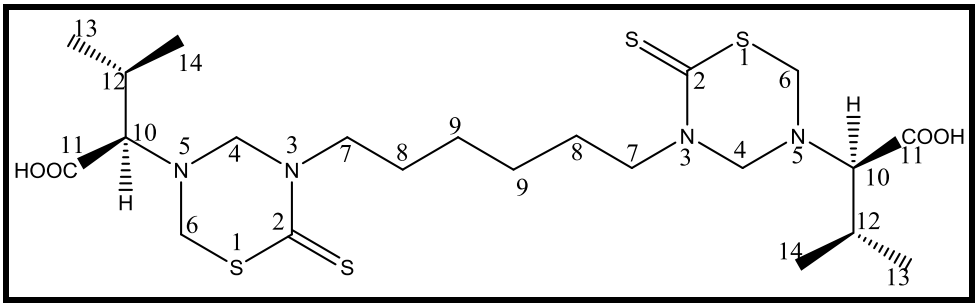

Figure 10. Chemical structure of JH6 compound, 2-(5-{6-[5-(1-carboxy-1-methylpropyl)-2-thioxo-1,3,5-thiadiazinan-3-yl] hexyl}-6-thioxo-1,3,5-thiadiazin-3-yl)-3-methylbutanoic acid.

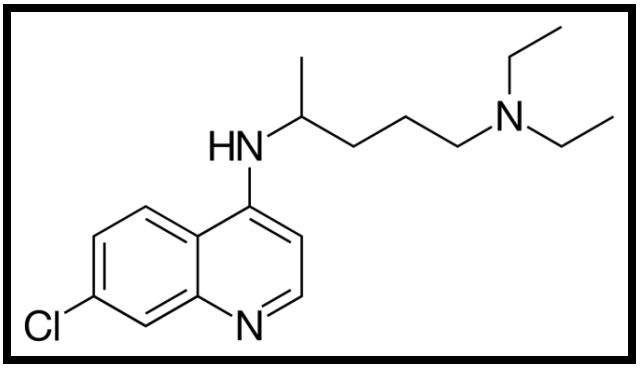

Figure 11. Chemical structure of chloroquine.

The six compounds derived from bis-THTT were synthesized and kindly donated by Dr. Hortensia Rodriguez, Department of Chemistry from Yachay Tech University.

7.4.1. Experimental Treatment assay with compounds derived from bis-THTT to BALB/c mice infected with *P. berghei* and *P. yoelii.*

The administration scheme of compounds derived from bis-THTT was carried out by four consecutive days (after of 3 days of the infection with *Plasmodium* strains). The compounds were administered orally using a solution of cellulose 1% and Tween 80 0.5% as a vehicle solution (protocol of Moore et al., 2011). The concentration used for the six compounds derived from bis-THTT and CQ was 20 mg/kg, calculated according to the average weight of the mice, in a final volume of 200 μL of treatment per mouse. In order to dissolve the experimental compounds, we used dimethyl-sulfoxide (DMSO).

DMSO is a solvent used experimentally for its power to dissolve hydrophobic substances and its excellent ability to transport active principles. Notwithstanding, the concentration of DMSO, to be administered, must not exceed 10%, because, high concentrations of DMSO can be toxic or affect locomotor activity to the experimental animals (mice) (Castro et al., 1995; Colucci et al., 2008).

7.5. Obtaining pre-immune and hyperimmune sera of each compounds derived from bis-THTT and chloroquine.

In order to obtain the sera, 8 groups of female BALB/c mice were used, one group for each bis-THTT experimental compound, a group for CQ and one control group, three mice for each experimental group. The tail end of each mouse was cut to obtain pre-immune sera and blood was collected without anticoagulant, the obtained sera were stored at -20 °C until later use (Spencer et al., 1998; Moll et al., 2003).

The obtaining of hyperimmune sera were carried out after immunizing the female BALB/c mice intraperitoneally, administering 200 μL of the experimental compound and Freund's Adjuvant. Immunizations were performed 4 times, the first immunization was performed with complete Freund's Adjuvant, while the remaining 3 immunizations were with incomplete Freund's Adjuvant. After the last immunization, the mice were sacrificed and the blood was collected. After the formation of the blood clot, the sample was centrifuged for 15 minutes at 2,500 rpm, the serum was extracted and stored at -20 °C (Moll et al., 2003; Spencer et al., 1998).

7.6. Indirect ELISA test using the experimental compounds as antigens.

To establish the optimal conditions for the indirect ELISA test, the protocol described by Voller (1976, 1980) was followed in order to obtain optimal conditions.

1. ELISA plates (96-well Nunc) were sensitized, with 100 µL of the soluble compounds by each well at increasing concentrations of 5 to 10 µg/mL diluted in carbonate-bicarbonate buffer at pH 9.6 (0.5 M Na2CO3 and 0.35 M NaHCO3). The plates were incubated in a humid chamber at a temperature of 4 °C, for 16 hours and then washed (3 times for 3 minutes each time) using phosphate buffered saline and Tween 20 at 0.005% (PBS/T).
2. The wells were blocked with 100 µL of human albumin (solution at 3%) diluted in PBS buffer; then, the plates were incubated in a humid chamber at 37 °C for one hour. After this time the plates were washed (3 times for 3 minutes each time) with PBS/T solution.
3. In the wells were added 100 µL of PBS (negative control), pre-immiune blood sera (from healthy BALB/c mice), and hyperimmune blood sera (from mice immunized with the compounds JH1, JH2, JH3, JH4, JH5, JH6, and CQ). See distribution in the tables 2 and 3. Later, the plates were incubated in a humid chamber at 37 °C for one hour. Subsequently, the plates were washed (3 times for 3 minutes each time) with PBS/T solution.
4. In each well 100 µL of the conjugate, rabbit anti-mouse polyvalent immunoglobulins (IgG, IgA, and IgM) conjugated to the peroxidase enzyme (HRP-peroxidase Horseradish; Sigma) was added (1:1000 dilutions in PBS). After, the plates were incubated in a humid chamber at 37 °C for 1 hour. Then, the washing of the plates was carried out quickly (3 times for 3 min each time) with PBS/T solution.
5. Finally, for the revelation of the chromogenic enzyme reaction, 100 µL of the chromogen ABTS (2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfuric acid) (Sigma)) and 0.05% of H_2O_2 were added in each well. Then, plates remained in the dark for 15 minutes at room temperature. The reactivity was read at 405 nm, at 15 and 30 minutes after the addition of the substrate.

Table 2. Distribution of antigens and sera in the ELISA plate 1

				Pre-immune sera		Hiperimmune sera					Pre-immune Sera		Hiperimmune sera		
			PBS	1:100	1:200	1:100	1:200			PBS	1:100	1:200	1:100	1:200	
			1	2	3	4	5	6	7	8	9	10	11	12	
CLQ	5 mg/mL	A													JH2
		B													
	10 mg/mL	C													
		D													
JH1	5 mg/mL	E													JH3
		F													
	10 mg/mL	G													
		H													

Table 3. Distribution of antigens and sera in the ELISA plate 2.

				Pre-immune sera		Hiperimmune sera					Pre-immune Sera		Hiperimmune sera		
			PBS	1:100	1:200	1:100	1:200			PBS	1:100	1:200	1:100	1:200	
			1	2	3	4	5	6	7	8	9	10	11	12	
JH4	5 mg/mL	A													JH6
		B													
	10 mg/mL	C													
		D													
JH5	5 mg/mL	E													
		F													
	10 mg/mL	G													
		H													

7.7. Statistical analysis

Each experimental value is presented as the mean of three replicates ± standard deviation. Data normality was verified using the Anderson-Darling test and homoscedasticity using the Levene test. We performed a comparison of means, using a one-way ANOVA test, to determine if there were statistically significant differences in the parasitemia for the different experimental compounds ($p < 0.05$) and a posteriori Tukey test was used for pairwise comparisons. The program used to analyze the data was MINITAB version 17.

8. RESULTS, INTERPRETATION, AND DISCUSSION

8.1. Determination of dilution of experimental compounds using DMSO

The six experimental compounds and CQ were solubilized with different amounts of DMSO in distilled water because they are not completely soluble in water. Table 4 shows the concentration and percentage of DMSO used in the dilution of the compounds, which did not exceed 10% of the final volume of the solution (12 ml). If the percentage of DMSO was higher than 10%, the solution would have been toxic for the mice.

Table 4. Amount of DMSO to dissolve 24 mg of each experimental compound.

Experimental compounds	DMSO Volume (µL)	DMSO Percentage (%)
Chloroquine	100	0,8
JH1	100	0,8
JH2	125	1
JH3	100	0,8
JH4	100	0,8
JH5	150	1,2
JH6	100	0,8

According to Table 4, the experimental compounds most soluble in DMSO (in increasing order) were: CQ, JH1, JH3, JH4, and JH6 at 0.8% of DMSO; JH2 at 1% of DMSO and JH5 at 1.2% of DMSO. Therefore, the least soluble compound in DMSO was JH5 with 1.2%. Nevertheless, it does not even reach half the tolerance of DMSO by the oral administration in experimental animals (Colucci et al., 2008).

8.2. Evaluation of the percentage of parasitemia due to the effect of the experimental compounds in the BALB/c mice model.

After the inoculation with $5x10^3$ PRBC of both strains, the percentage of parasitemia was daily monitored by blood smears and Giemsa-stain until day 15 post-infection. In each case, the percentage parasitemia ([the number of parasite-infected cells counted /total number

of red blood cells counted] x 100) was determined. The animals were considered to be clear of infection if no parasites could be detected on a blood smear.

Figures 12 and 13 show the average percentage of parasitemia from female BALB/c mice, which were infected with *Plasmodium berghei* and *Plasmodium yoelii*, respectively. In these figures, we presented the effect of each compound as possible anti-malarial drugs and CQ as control.

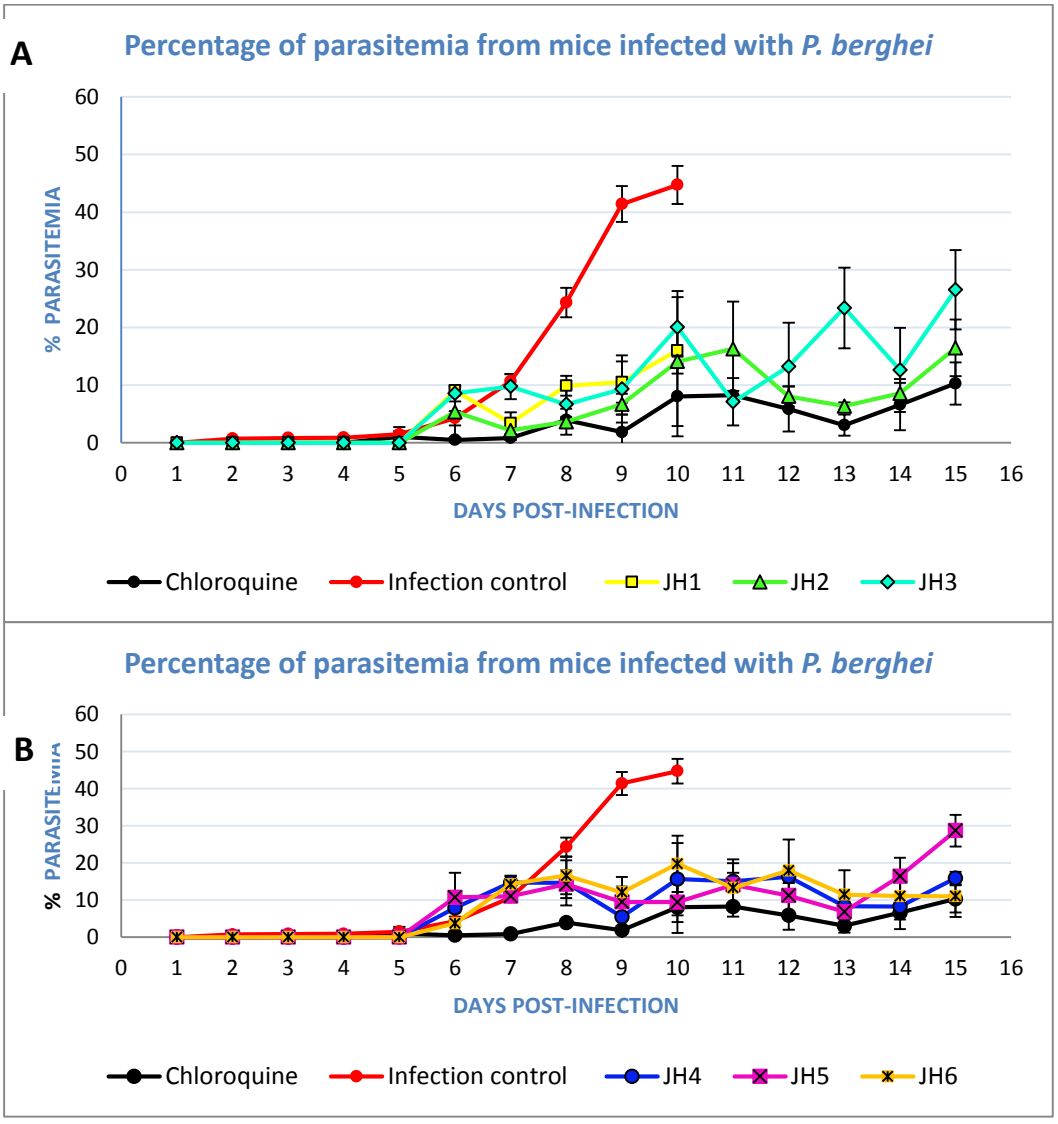

Figure 12. Parasitemia-Time profile using female BALB/c mice following the administration of a 4 dose regimen of 20 mg/kg Chloroquine (n = 3), JH1 (n = 3), JH2 (n=3), JH3 (n=3), JH4 (n=3), JH5 (n=3) and JH6 (n = 3); administered 72 hours after IP inoculation with $5X10^3$ *P. berghei* parasitized-erythrocytes. Data are shown as total parasitemia (mean percentage of infected red blood cells/total erythrocytes ± SD). Parasitemia was monitored daily by blood smears fixed with absolute methanol and stained with Giemsa.

From day 5 post-infection, we can observe that the percentage of parasitemia, from mice infected with *P. berghei* and without treatment (infection control), increases exponentially until day 10 post-infection, where all mice died (44.7% of parasitemia). On the other hand, the mice treated with CQ (control of cure) did not exceed 11% of parasitemia.

All compounds show a tendency similar to the CQ, eventhough, the mice evaluated with JH1 on day 10 post-infection died with an average of 16% parasitemia. If compared with the infection control group, the mice died on the same day but with 44.7% parasitemia. It suggests that JH1 is highly toxic and the mice evaluated with JH1 died due to the toxicity of the drug and not due to the parasitic infection.

The two compounds with activity very similar to CQ were JH2 and JH4, their curves of parasitemia percentage did not exceed 16.5% until day 15 post-infection, which suggest that JH2 and JH4 could have anti-malarial activity. Nevertheless, these experimental compounds are not as efficient as CQ, since the mice evaluated with CQ presented 9.8% of parasitemia on day 15 post-infection, which resulted in significantly lower level of parasitemia than JH2 and JH4 (See figure 12, panel A and B respectively).

In this Figure also, Panel A and B show that percentage of parasitemia of JH3 and JH5 experimental compounds fluctuated consistently between 10% and 30% of parasitemia, suggesting that these experimental compounds did not control the infection. All mice died on day 15 post-infection with 26.5% and 28.7% of parasitemia for JH3 and JH5 respectively.

Finally, panel B of Figure 12 shows the curve of the percentage of parasitemia from mice evaluated with the JH6 compound. If we compare the curves of the percentages of parasitemia for JH6 (11%) and CQ (9.8%) we can see that both curves are similar on the last 3 days post-infection. However, parasitemia from day 7 to day 13 post-infection constantly fluctuated between 10 and 20 percent, suggesting that compound JH6 did not completely control the infection. This suggestion is confirmed with the statistical analysis curve presented in Annex 1, where it is observed that the survival for JH6 was from 1 mouse.

The percentage of parasitemia, from mice infected with *P. yoelii*, is represented in Figure 13.

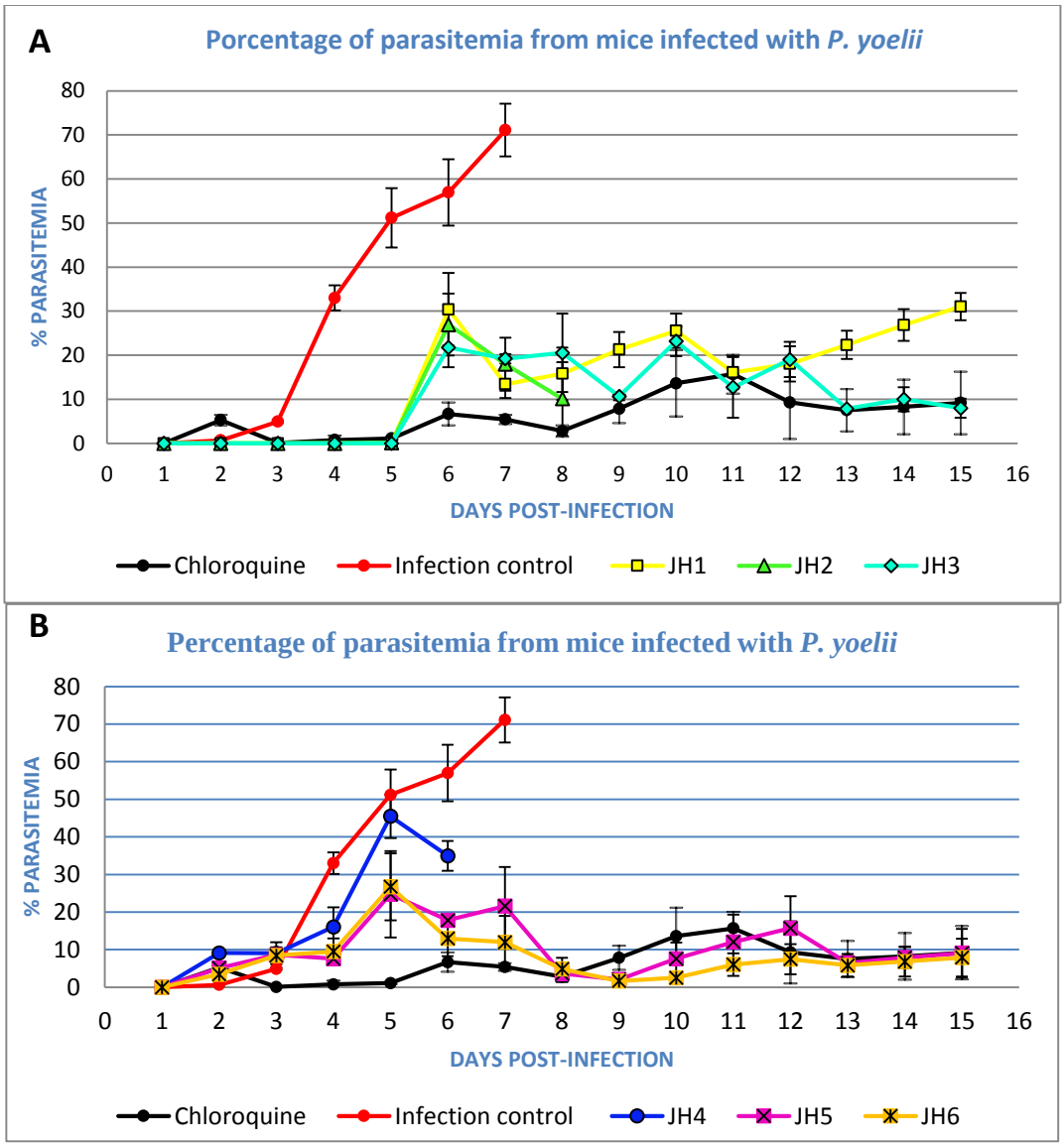

Figure 13. Parasitemia-Time profile using female BALB/c mice following the administration of a 4 dose regimen of 20 mg/kg Chloroquine (n = 3), JH1 (n = 3), JH2 (n=3), JH3 (n=3), JH4 (n=3), JH5 (n=3) and JH6 (n = 3); administered 72 hours after IP inoculation with 5X103 *P. yoelii* parasitized-erythrocytes. Data are shown as total parasitemia (mean percentage of infected red blood cells/total erythrocytes ± SD). Parasitemia was monitored daily by blood smears fixed with absolute methanol and stained with Giemsa..

In panel A from day 5 post-infection there is an evident increase in the percentage of parasitemia from mice treated with JH1 and JH3 was observed, but with fluctuations up to day 13. From day 13 post-infection the group treated with JH3 decrease the percentage of parasitemia equal to the group treated with CQ, reaching a parasitemia percentage 10%, and for JH1 the parasitemia increases up to 30% on day 15 post-infection.

In panel B, mice treated with JH4, JH5 and JH6 increase their parasitemia from day 2 to day 7 post-infection. The group JH4 reached a maximum parasitemia of 46% on day 5 and on day 6 all mice died, this suggests a low efficiency in infection control for *P. yoelii* with this compound. For compound JH5 and JH6, the maximum parasitemia was 23% and 27% respectively on day 5 post-infection. However, for these last two groups, there was a decrease in parasitemia, with 5% of parasitemia on day 8 post-infection, similar to the group treated with CQ.

The percentage of parasitemia from day 9 to day 15 post-infection was very similar for groups JH5, JH6 and CQ, presenting an average parasitemia of 8.5% for all these groups. However, only one mouse survived from the group of mice treated with compound JH6, which suggests high toxicity of the compound. On the other hand, in the group of mice treated with compound JH5 only one mouse died. However, in the group of mice treated with CQ, the survival was 100%. This suggests that the toxicity of compounds JH5 and JH6 is greater than CQ (Annex 6).

In summary, for mice infected with *P. berghei* the compounds that have control over the malaria infection are JH2 and JH4, and for mice infected with *P. yoelii* the compounds are JH5 and JH6. They show lower survival than the control group of CQ, however, suggesting greater toxicity of the compounds derived from bis-THTT than CQ.

Figure 14 shows pictures of the blood smears stained with Giemsa reagent, which was used to determine the percentage of parasitemia on day 7 post-infection. In the panel A, the pictures correspond to blood smears of mice infected with *P. berghei* and the Panel B with *P. yoelii*, with the compounds JH2, JH4, JH5, and JH6, which presented some anti-malarial activity.

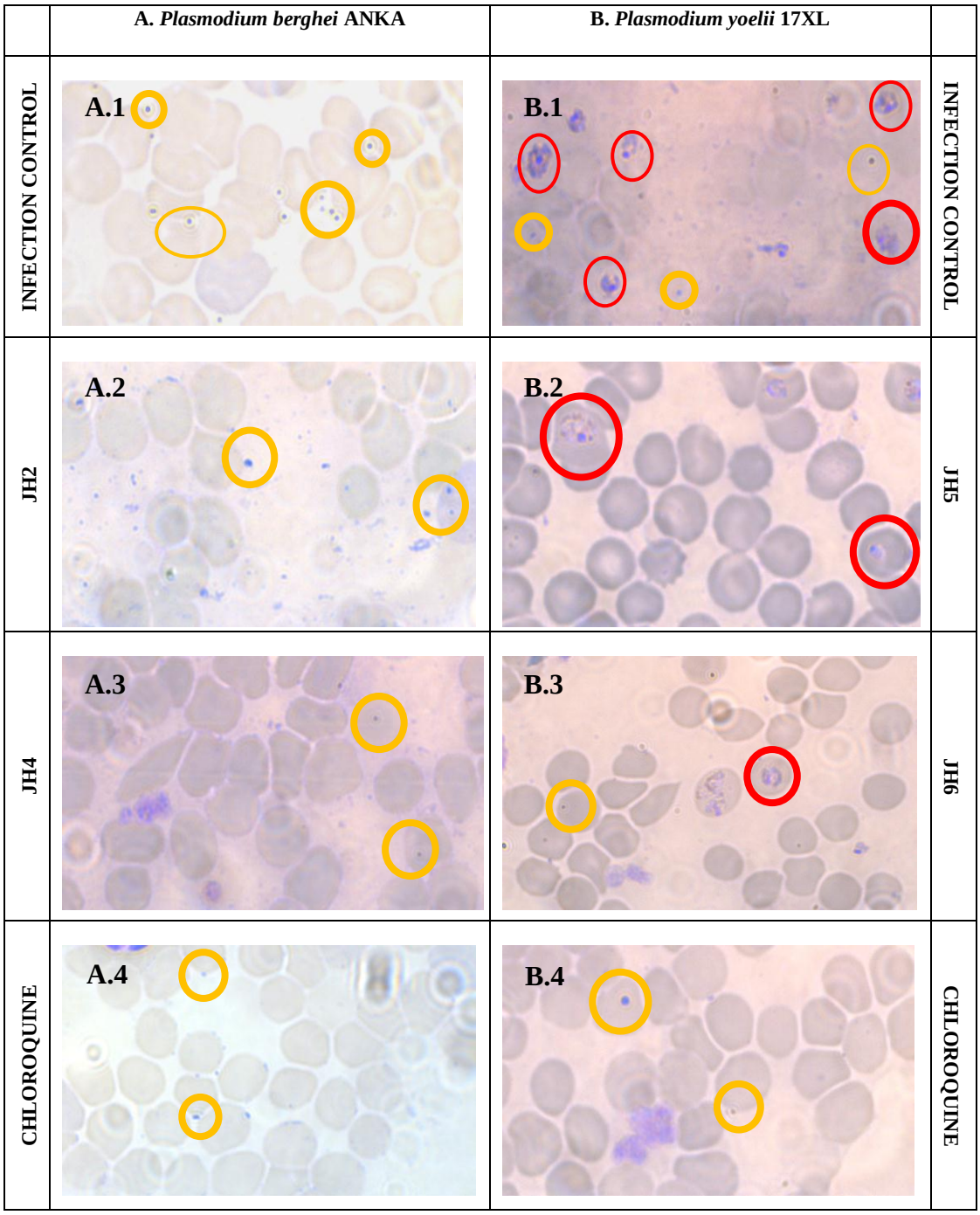

Figure 14. Blood smear of BALB/c mice infected with *P. berghei* (Panel A) *and P. yoelii* (Panel B), treated with the JH2, JH4, JH5 y JH6 compounds and Chloroquine as cure control. The orange circles show red blood cells with parasites in the merozoite and trophozoite stage and the red circles point out red blood cells with parasites in mature schizonte stage.

Panels A.2 and A.3 of Figure 14, correspond to blood smears of mice infected with *P. berghei* evaluated with JH2 and JH4, respectively. These pictures indicate that there are parasites in the merozoite and trophozoite stage (yellow circles) similar to CQ control (panel

A.4). In addition, we can observe that in the blood smear corresponding to infection control (panel A.1) there are more parasitized red blood cells, in merozoite and trophozoite stage, which suggests that JH2 and JH4 compounds control the infection.

On the other hand, panels B.2 and B.3 of Figure 14 correspond to the blood smears of mice infected with *P. yoelii* and treated with JH5 and JH6, respectively. We can observe that the amount of parasitized red blood cells in B.2 and B.3 panels are similar to B.4 panel, which is cure control (blood smear from mice treated with CQ), this indicates that JH5 and JH6 compounds have control on the infection.

Also, if we compare the blood smear of mice infected with *P. yoelii* and *P. berghei* we can observe that the parasites in blood smears of mice infected with *P. yoelii* reach the mature schizont stage (red circles), that is a more advanced in infection than the merozoite and trophozoite stage. The population of schizonts in mice infected with *P. yoelii* is more representative than mice infected with *P. berghei*, which suggests that the *P. yoelii* strain is much more virulent.

Taking into account that the lethality of *P. berghei* and *P. yoelii* in experimental mice is very similar (Carter and Diggs, 1997), we can suggest that the strain of *P. berghei* used in this research has lost virulence. Apparently, the parasite lost its virulence, because *P. berghei* strain used in this essay is a product of several replications in the vertebrate host (mice). Adequate conditions are that parasites pass through the host and the vector (mosquito), since, the saliva of the blood-sucking arthropods act against the homeostasis and immune responses of the host (mice), which would explain the decrease in virulence (Andrade et al., 2005; Song, 2018).

8.3 Evaluation of the experimental compounds immunogenicity by indirect ELISA test

To determine the immunogenicity of the bis-THTT-derived compounds, BALB/c mice were inoculated with these compounds. Later, their pre-immune and hyperimmune sera were used to determine humoral response by indirect ELISA test, using each experimental compound as antigen.

Figures 15 and 16 show results of the indirect ELISA test of each experimental compounds. To standardize this test, we used two different dilutions of sera (1:100 and 1:200) and two different concentration of the antigens (5 µg/mL and 10 µg/mL).

The bars represent hyper-immune serum and the dashed lines represent the cut-off point, which was obtained with the data of pre-immune sera (the mean + three standard deviations of the data). The cut-off point helps us to determine the immunogenicity of the antigens, which were used in this assay. If the absorbance value of the hyperimmune serum is similar to the value of the cut-off point, then there is low or zero humoral response. Subsequently, if the absorbance value of the hyperimmune serum is greater than cut-off point then there is humoral response.

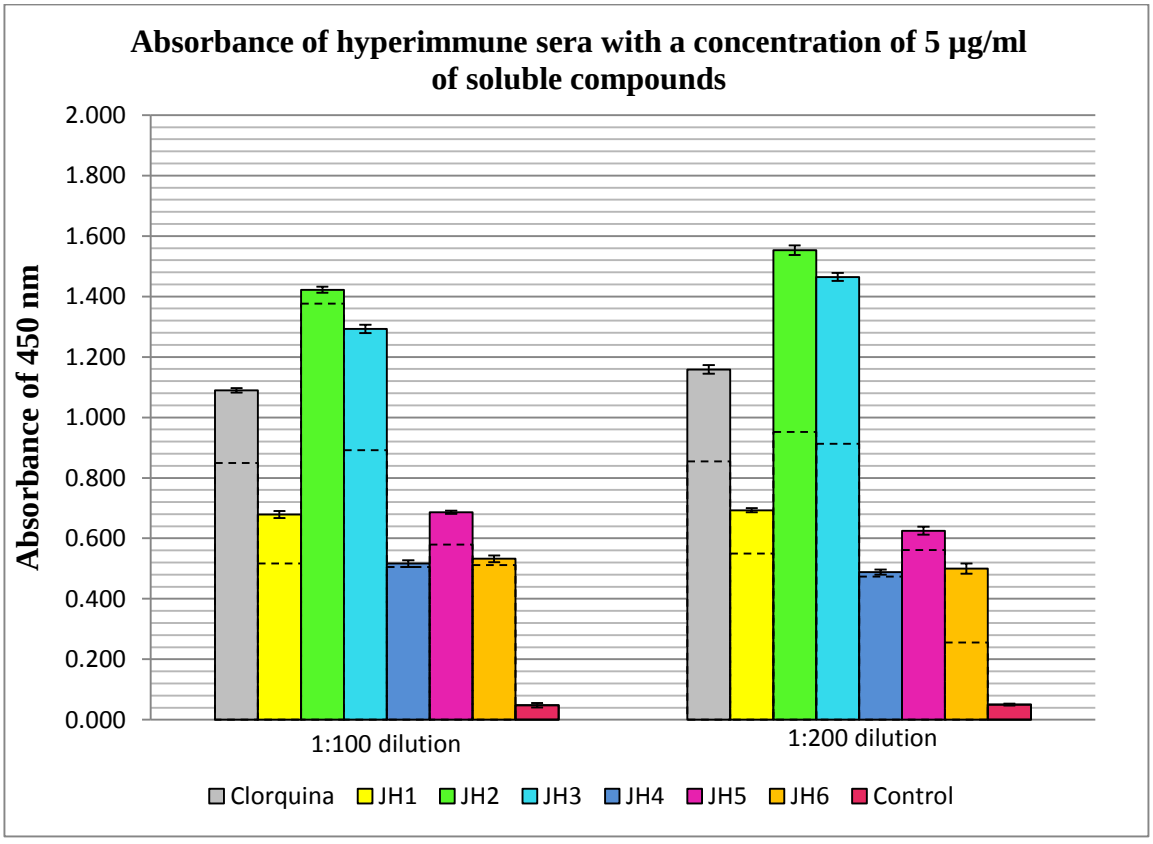

Figure 15. Results of indirect ELISA test, with 5 µg/mL concentration of soluble compounds as antigen. On the X-axis are represented the 1:100 and 1:200 dilutions of the hyperimmune sera vs the absorbance on the Y-axis. Each bar represents the immunogenicity of the six experimental compounds: JH1, JH2, JH3, JH4, JH5, JH6, and chloroquine. The dashed line represent the cut-off point of the hyperimmune sera. Additionally, the standard error for each compound is represented on the bars.

Figure 15 shows results of an indirect ELISA test with an antigen concentration of 5µg/mL. If we compared the results of the humoral response in the dilution 1:100 and 1:200 of the serum, we can see that the humoral response is more evident with the dilution 1:200 of the serum, because the difference between the absorbance of the cut-off point and hyperimmune serum is greater.

However, if we compare these results with the results of antigen concentration of 10 µg/ml (see Figure 16), we can see that the most optimal conditions to evaluate the humoral response is with the dilution 1: 200 of the serum and with a concentration of 10 µg/ml of the antigen.

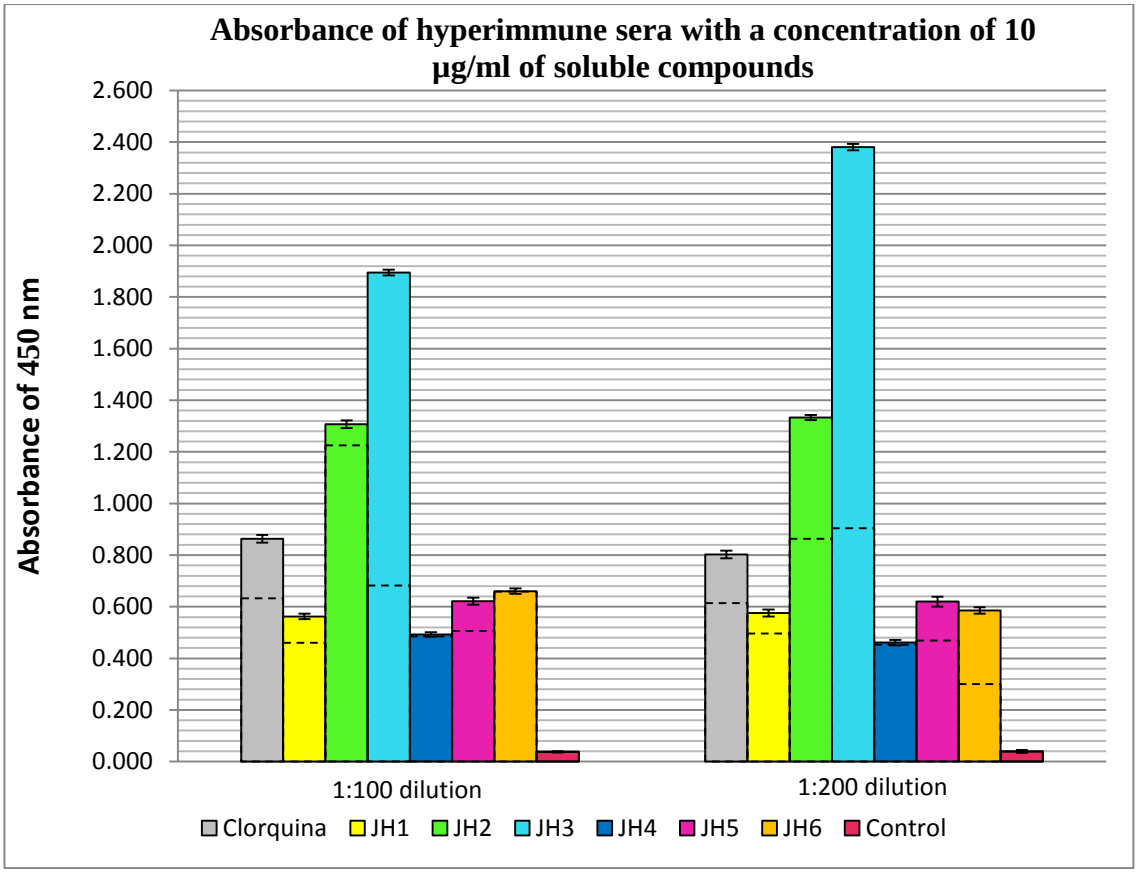

Figure 16. Results of indirect ELISA test, with 10 µg/mL concentration of soluble compounds. On the X-axis are represented the 1:100 and 1:200 dilutions of the hyperimmune sera vs the absorbance on the Y-axis. Each bar represents the antigenicity of the seven experimental compounds: JH1, JH2, JH3, JH4, JH5, JH6, and chloroquine. The dashed line represent the cut-off point of the hyperimmune sera. Additionally, the standard error for each compound is represented on the bars.

Figure 16 shows the best conditions for evaluating the humoral response of each experimental compound in BALB/c mice, with the concentration of 10 µg/mL of antigen and 1:200 dilution

of serum. Here, we can see that the humoral response of JH2 and JH3 is high compared to CQ. The humoral response of JH2 is 1.6 times greater than the humoral response of CQ, and JH3 humoral response is 3 times greater than CQ.

Furthermore, the humoral response of JH4 and JH5 is low compared to the humoral response of the CQ. The humoral response of JH4 is 2 times lower than CQ, and the humoral response of JH5 is 1.3 times lower than CQ. Taking into consideration that CQ is cure control of malaria and that JH2, JH4, JH5, and JH6 compounds show control of malaria infection, we could suggest that the anti-malarial activity of the compounds in mice infected with *P. berghei* and *P. yoelii* are independent of the humoral response.

8.4. Structure-activity relationship (SAR) analysis

The SAR allows us to tie together both, chemical (or tridimentional) molecular structure and its biological activity. Although the present anti-malarian study is preliminary, and only some compounds derived from bis-THTT have been analyzed, some conclusions related to mentioned SAR might be established. A 3D optimization for all structures was carried out in order to facilitate structural analysis (Figure 15).

In general, our results showed a greater anti-malarial effect for the compounds JH2 and JH4, when the infection with *P. berghei* was carried out. Both compounds have an aliphatic chain of six carbons linked the two N,N heterocycles, and glycine and leucine (neutral aminoacids) as substituents at N-5 position, respectively. Subsequently, compounds JH5 and JH6 showed higher anti-malarial activity for the infection with *P. yoelii*. In this case, the most active structures have different carbon chain linked to both N,N heterocycles, but the same substituent (valine residue) at N-5 position.

The analysis of optimized structures showed that JH1, JH3 and JH5 have N3-N3´ length close to the distance between the nitrogenous atoms of chloroquine, but, except JH5 in reference of *P. yoelii* infection, the other two compounds did not show good anti-malarial activity. In reference to *P. berghei* infection seems to be most important to the carbon chain and the distance between both heterocycles present in the same structure. JH2 and JH4 showed higher N3-N3´lengths in comparison with Chloroquine. On the contrary, when the infection is produced by *P. yoelii*, the carbon chain length was not important, and the best

results were obtained using valine (a neutral and nonpolar aminoacid) as a substituent at N-5 position. Future works would be focused on these preliminary results in the search for structures with best anti-malarial activity.

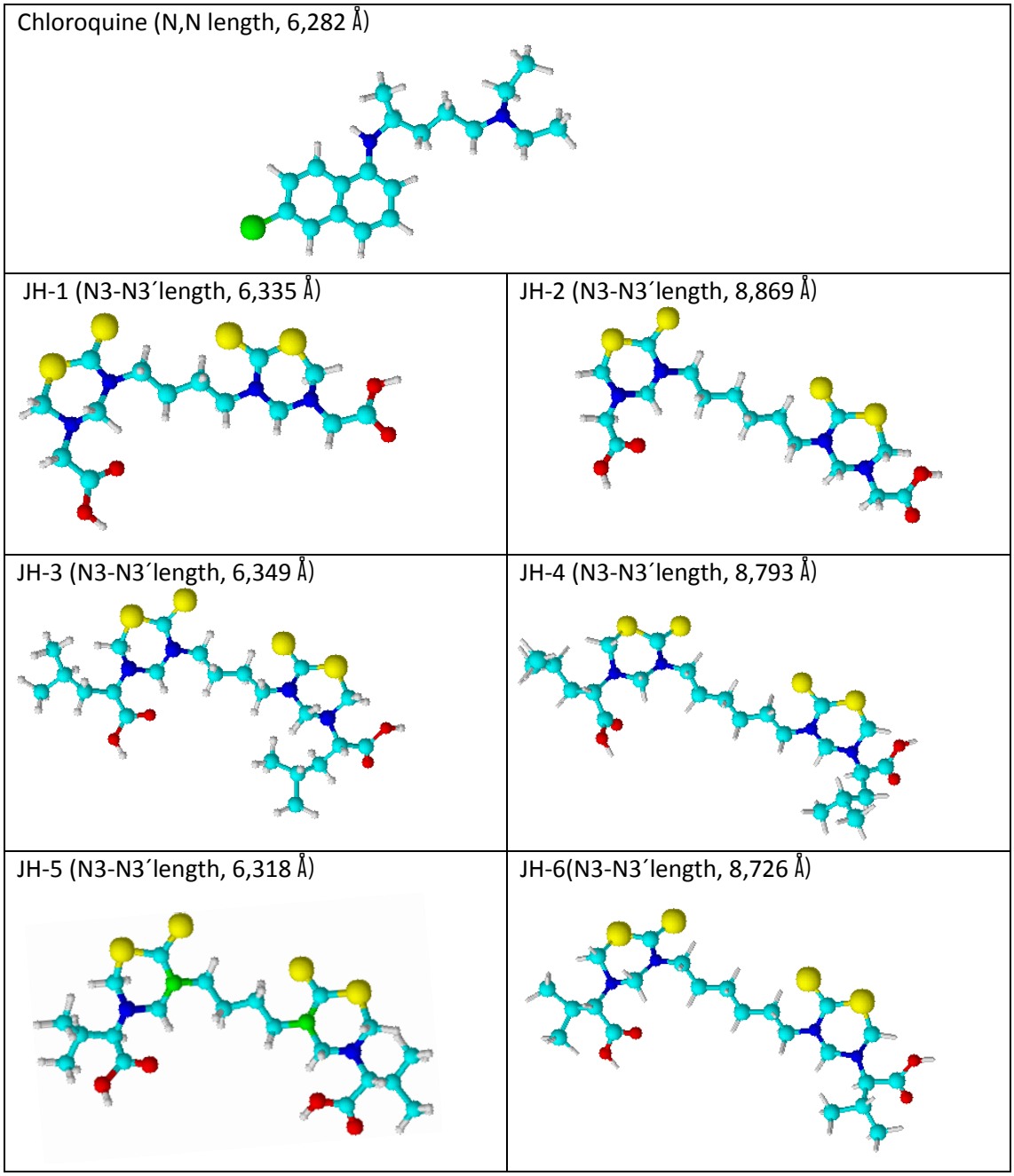

Figure 15. 3D structural optimization for cloroquine, and for all bis-THTT tested using ACD labs software.

CONCLUSIONS

- The *in vivo* evaluation of the compounds derived from bis-THTT in a model of BALB/c mice infected with *P. berghei* and *P. yoelii* strains suggest that some compounds have an anti-malarial effect, which depends on the *Plasmodium* strain used for its evaluation as an anti-malarial drug. Among the six experimental compounds evaluated, a greater anti-malarial effect of the compounds JH2 and JH4 were evidenced in the infection with *P. berghei* and the compounds JH5 and JH6 to the infection with *P. yoelii*. However, these compounds did not exceed the efficiency of CQ.

- The results of the indirect ELISA test for the evaluation of the humoral or antibody-mediated response in mice inoculated with compounds derived from bis-THTT, suggests that all experimental compounds have humoral response in BALB/c mice and also that there is not direct relationship between the immunogenicity of the compounds and their anti-malarial activity.

- The analyze of structure-activity relationship, show that the relevant in the chemical structure of the compounds JH2 and JH4, which have anti-malarial activity in mice infected with *P. berghei*, is the length of the aliphatic chain of 6 carbons attached to two N-N heterocycles, in addition to leucine and glycine as substituents at N-5 position. However, the relevance of the chemical structure of JH5 and JH6, which have anti-malarial activity in mice infected with *P. yoelii*, is the valine substituent at N-5 position and not the distance between the two N-N heterocycles.

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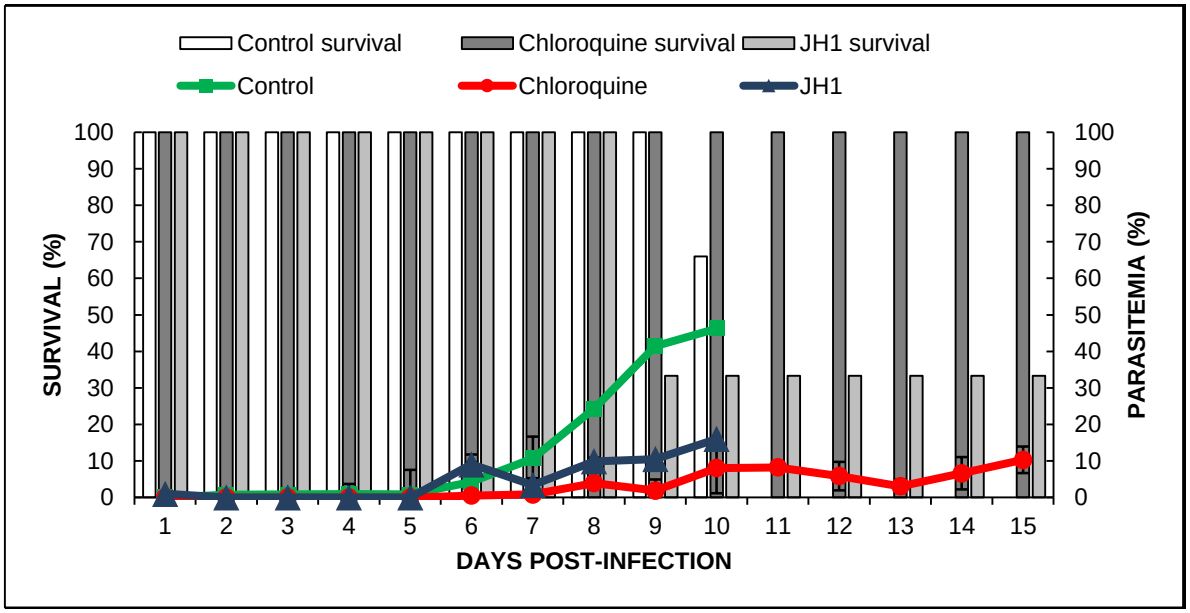
ANNEXES

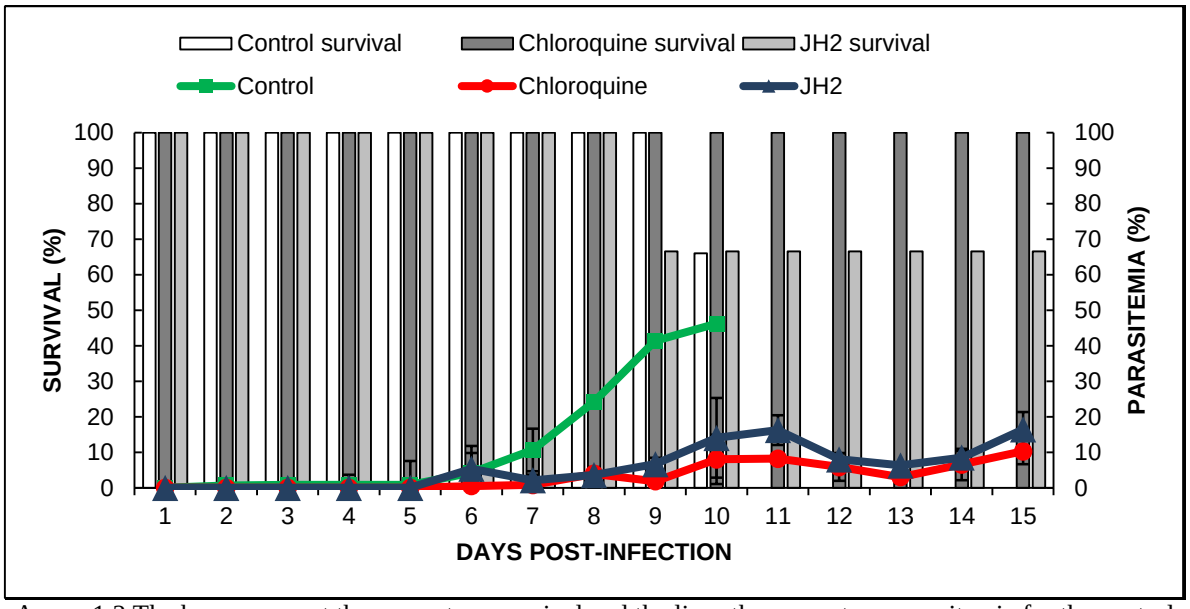
Statistical analysis of the percentage of parasitemia from mice infected with *P. berghei* and *P. yoelii* treated with compounds derived from bis-THTT and chloroquine as a cure control.

Results are shown as means ± standard deviation (SD). Data normality was verified using the Anderson-Darling test and homoscedasticity using the Levene test. We performed a comparison of means, using a one-way ANOVA test, to determine if there were statistically significant differences in the parasitemia for the different experimental compounds (taking $p<0.05$ as a significant value and a posteriori Tukey test).

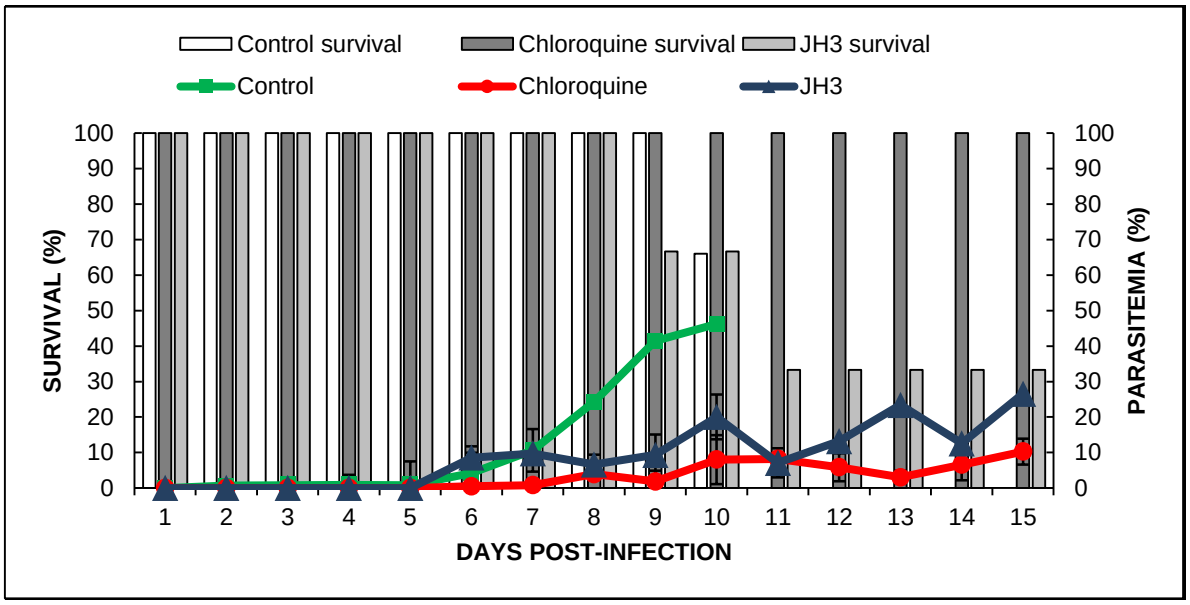
Annex 1. Show the percentage of parasitemia and survival from mice infected with *P. berghei* and treated with JH1, JH2, JH3, JH4, JH5, and JH6 compounds.



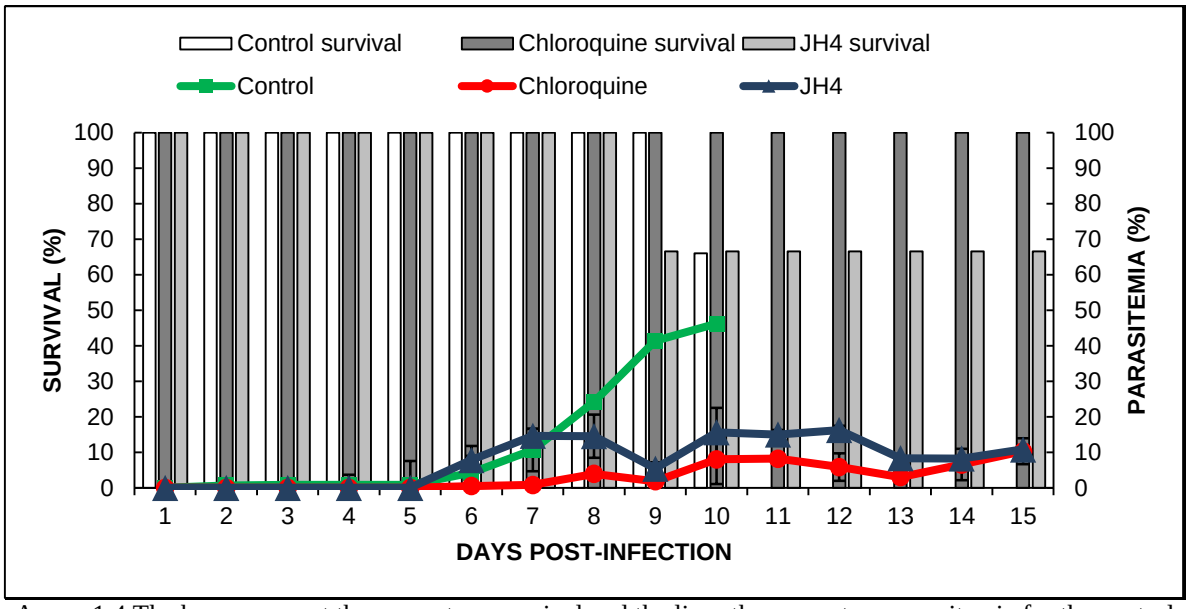
Annex 1.1 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH1 group.



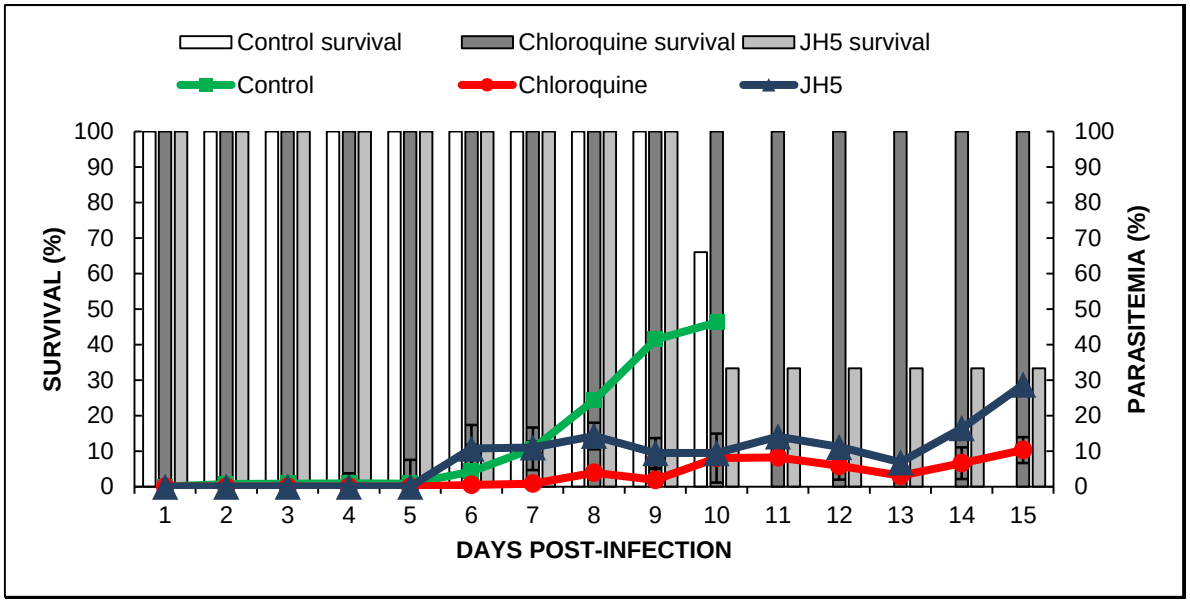
Annex 1.2 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH2 group.



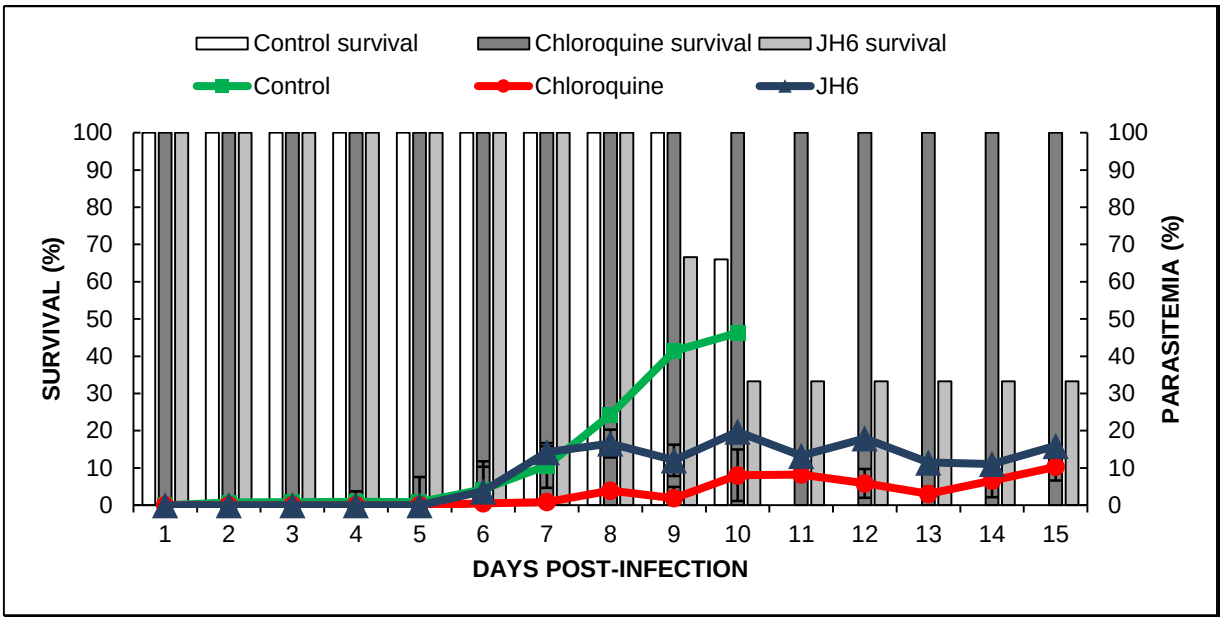
Annex 1.3 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH3 group.



Annex 1.4 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH4 group.

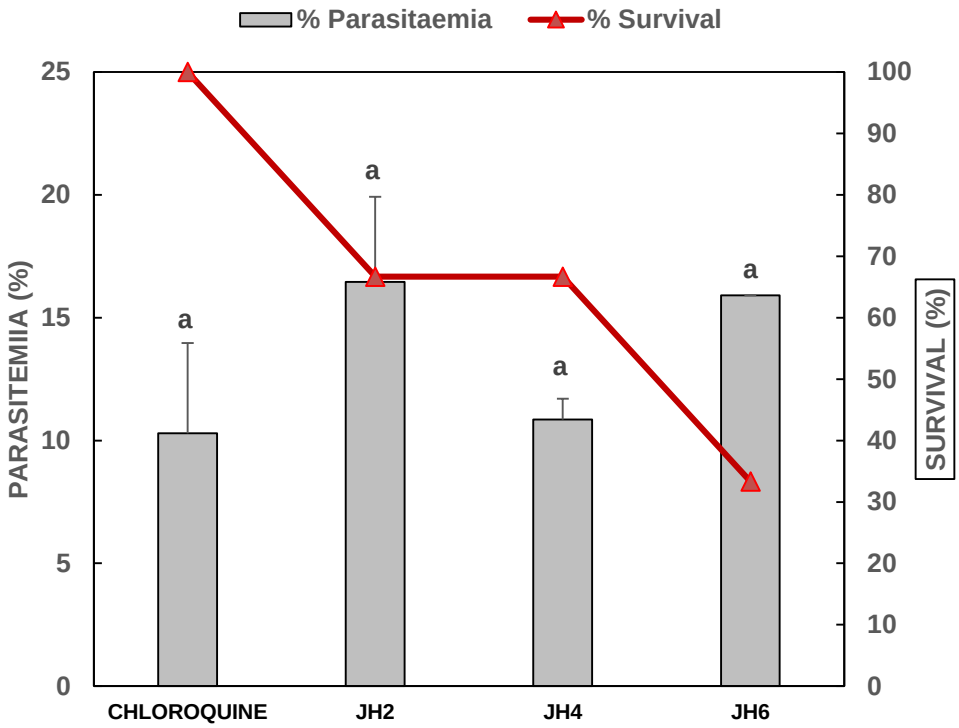


Annex 1.5The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH5 group.



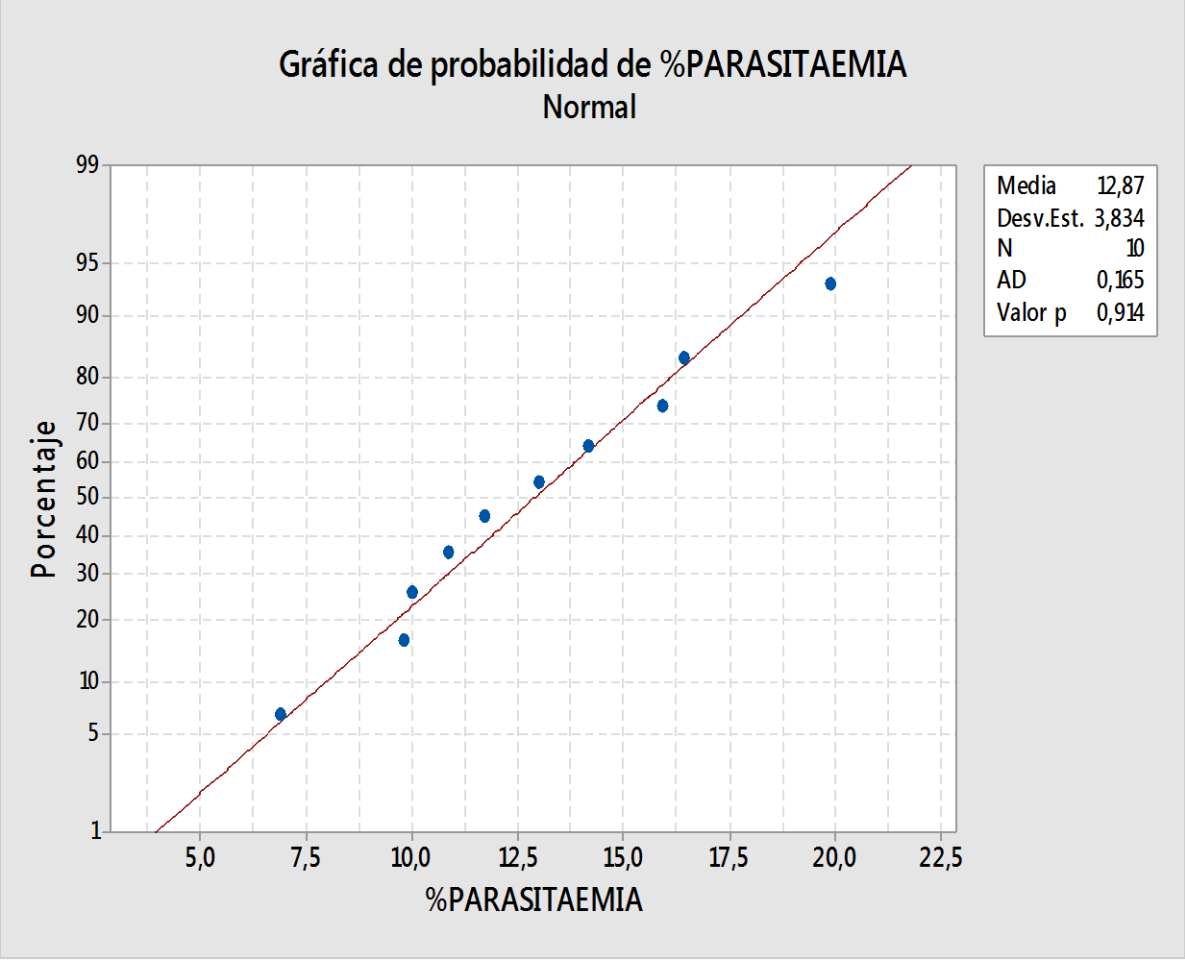
Annex 1.6 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH6 group.

Annex 2. Sensitivity of *P. berghei* for compounds derived from bis-THTT

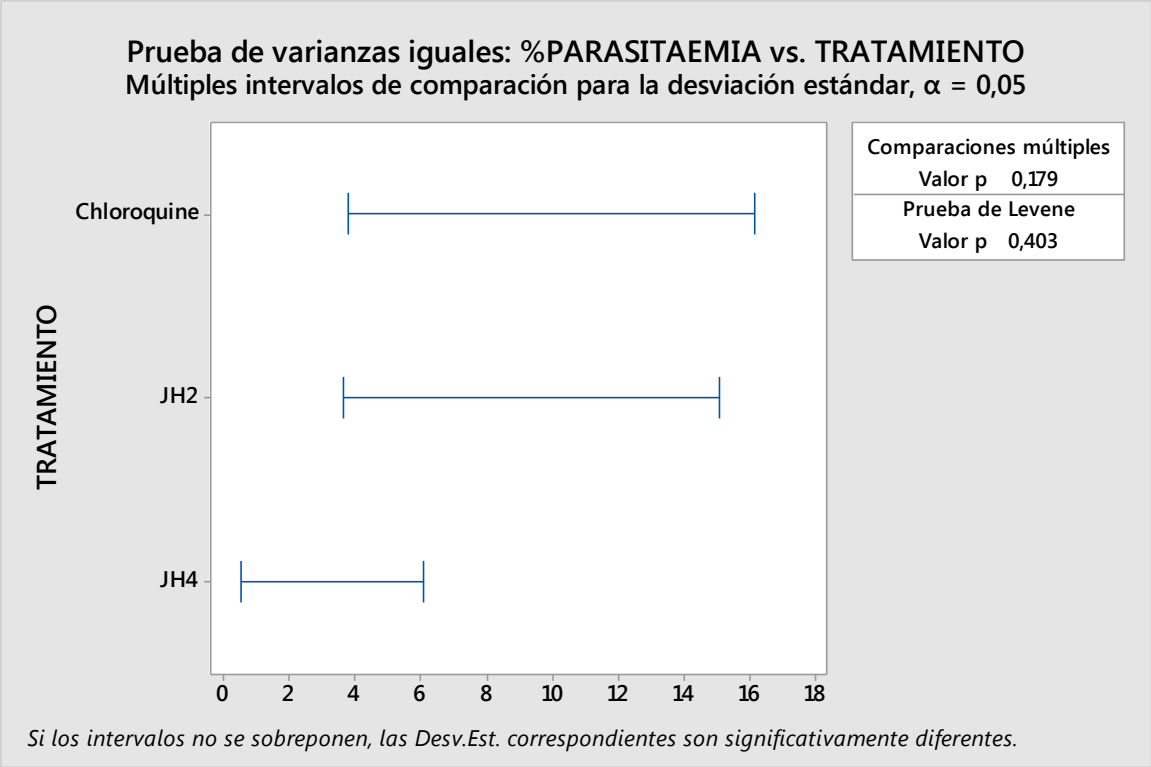


The species *P. berghei* was more sensitive to the compounds JH2, JH4 and JH6. On day 15 post-infection, no significant differences were observed in the percentage of parasitemia between the compounds JH2 (16.45%), JH4 (10.85%), JH6 (15.91%) and chloroquine (10.29%) (p> 0, 05), demonstrating the efficiency of the treatments. However, the survival percentagewas 100% for mice exposed to chloroquine, decreasing to 66% for JH2 and JH4 and 33% for JH6.

Annex 3. Anderson-Darling test to prove that the data set (percentage of parasitaemia from mice infected with *P. berghei*) adequately describes a normal distribution.



Annex 4. Statistical test of Levene to evaluate the equality of variances of the percentage of parasitaemia from mice infected with *P. berghei* (on day 15 post-infection).



Annex 5. One-way ANOVA test: percentage of parasitemia (from mice infected with *P. berghei)* vs Treatment (JH2, JH4, JH6, and chloroquine). Using MINITAB version 17.

Método

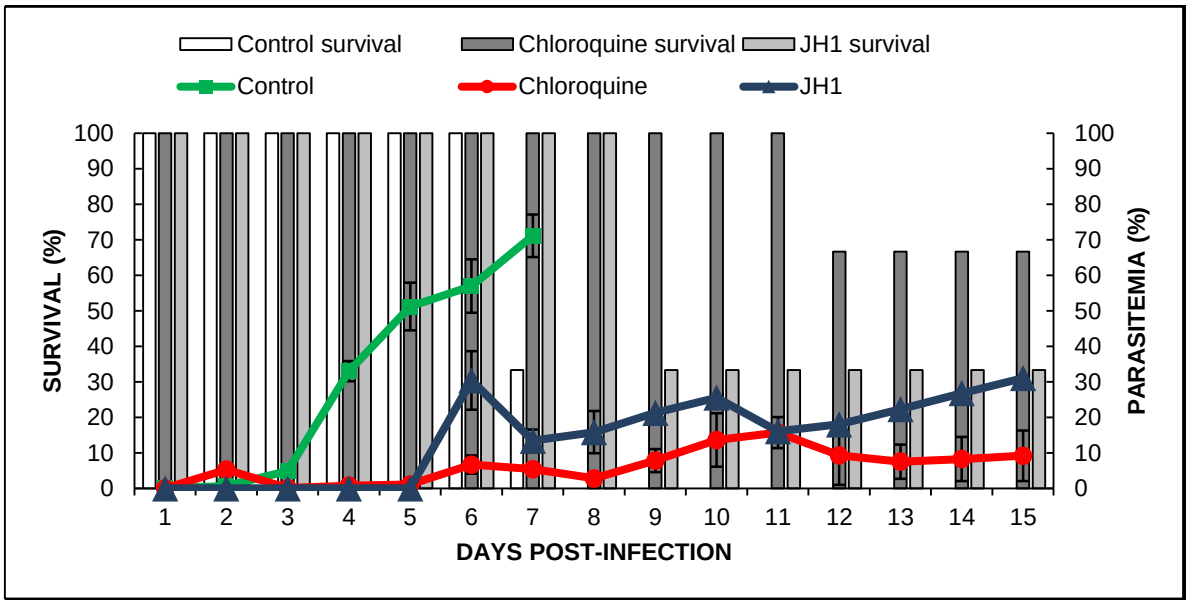
```
Hipótesis nula          Todas las medias son iguales
Hipótesis alterna       Por lo menos una media es diferente
Nivel de significancia  α = 0,05

Se presupuso igualdad de varianzas para el análisis.


Información del factor

Factor       Niveles  Valores
TRATAMIENTO        4  Chloroquine; JH2; JH4; JH6


Análisis de Varianza

Fuente       GL  SC Ajust.  MC Ajust.  Valor F  Valor p
TRATAMIENTO   3      79,91     26,636     3,05    0,114
Error         6      52,42      8,736
Total         9     132,32


Resumen del modelo

                    R-cuad.  R-cuad.
      S  R-cuad.  (ajustado)   (pred)
2,95568   60,39%      40,58%        *
```

Medias

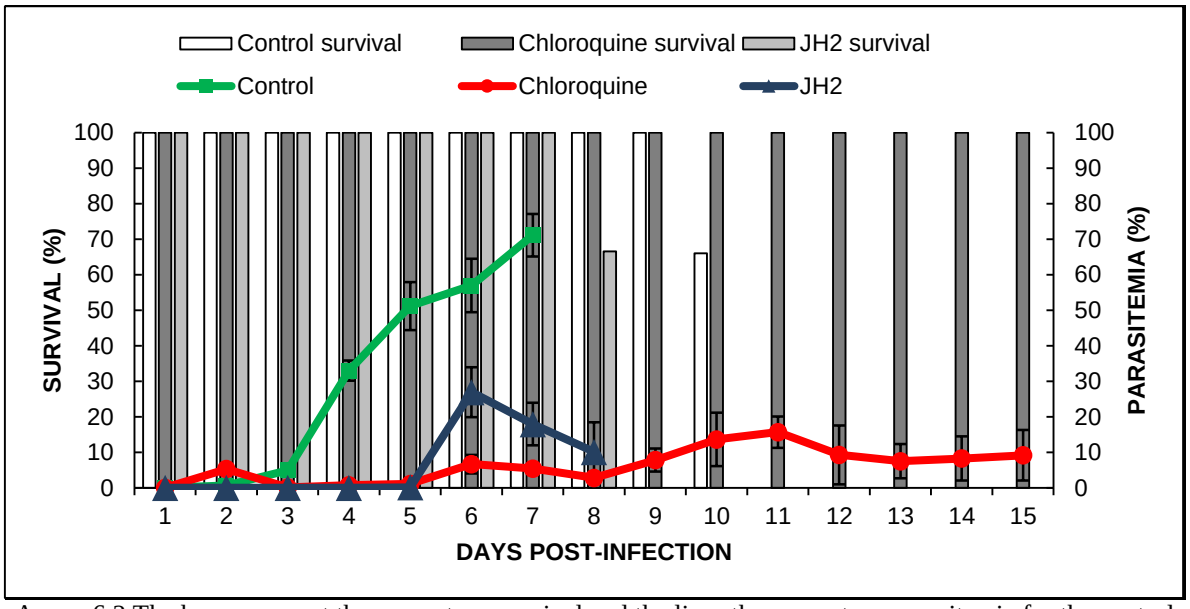
TRATAMIENTO	N	Media	Desv.Est.	IC de 95%
Chloroquine	3	10,29	3,67	(6,11; 14,47)
JH2	3	16,45	3,47	(12,28; 20,63)
JH4	3	10,854	0,854	(6,678; 15,029)
JH6	1	15,91	*	(8,68; 23,14)

Desv.Est. agrupada = 2,95568

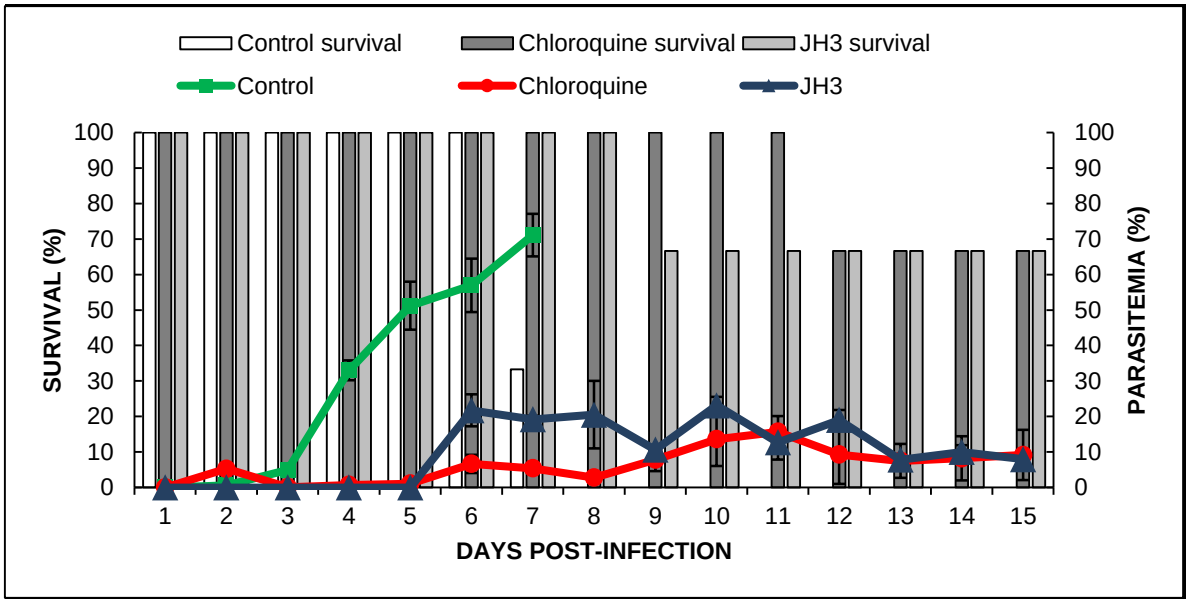
Annex 6. Show the percentage of parasitemia and survival from mice infected with *P. yoelii* and treated with JH1, JH2, JH3, JH4, JH5, and JH6 compounds.



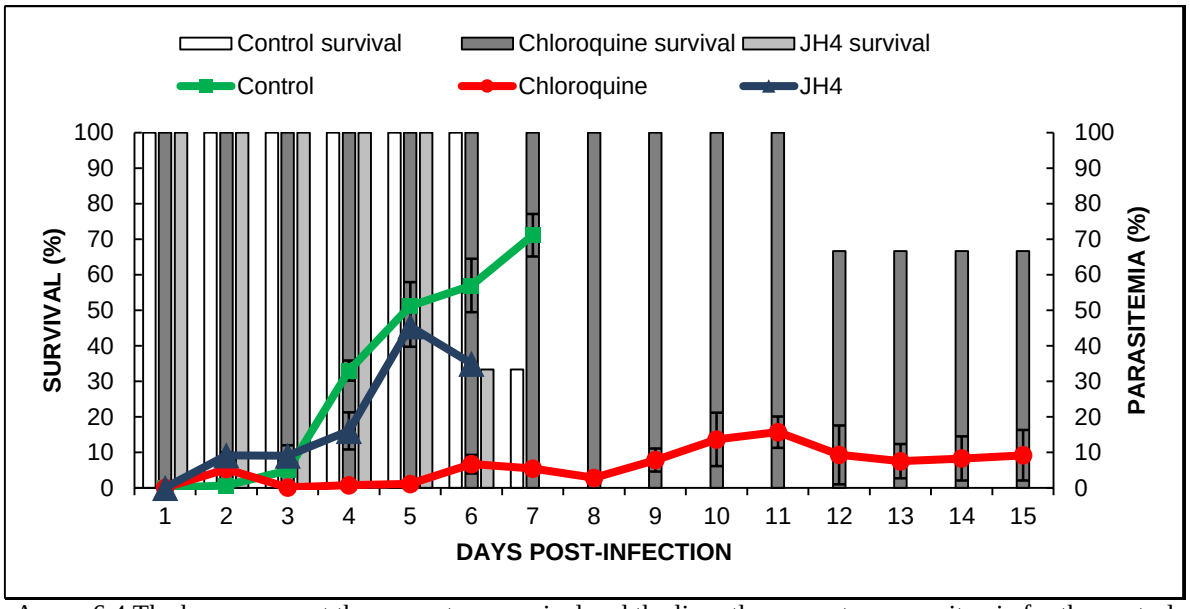
Annex 6.1 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH1 group.



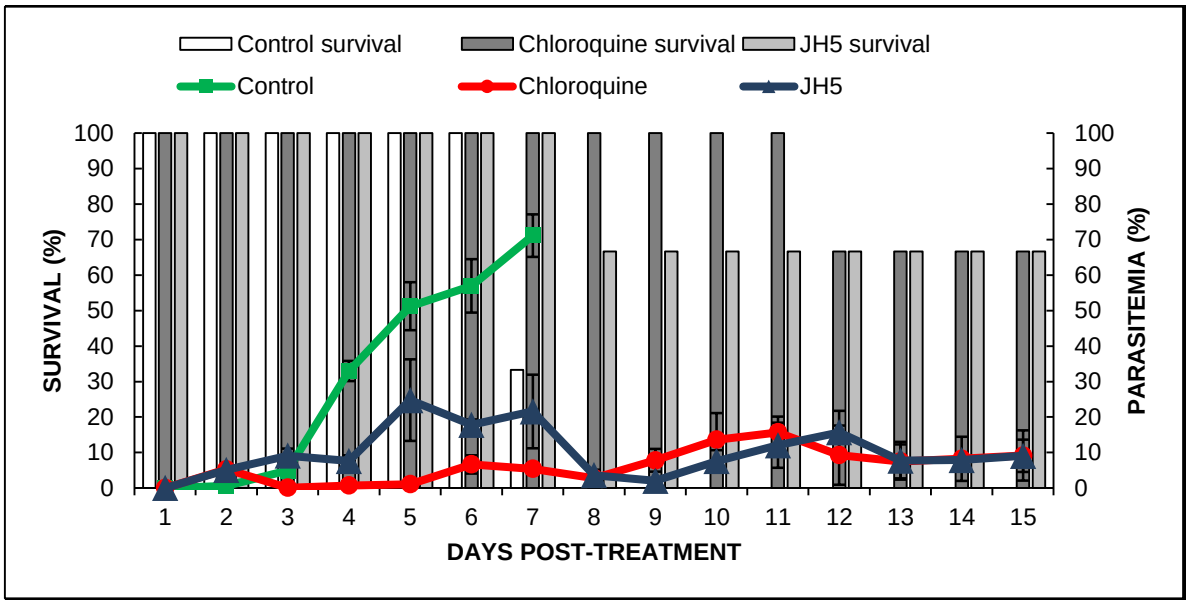
Annex 6.2 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH2 group.



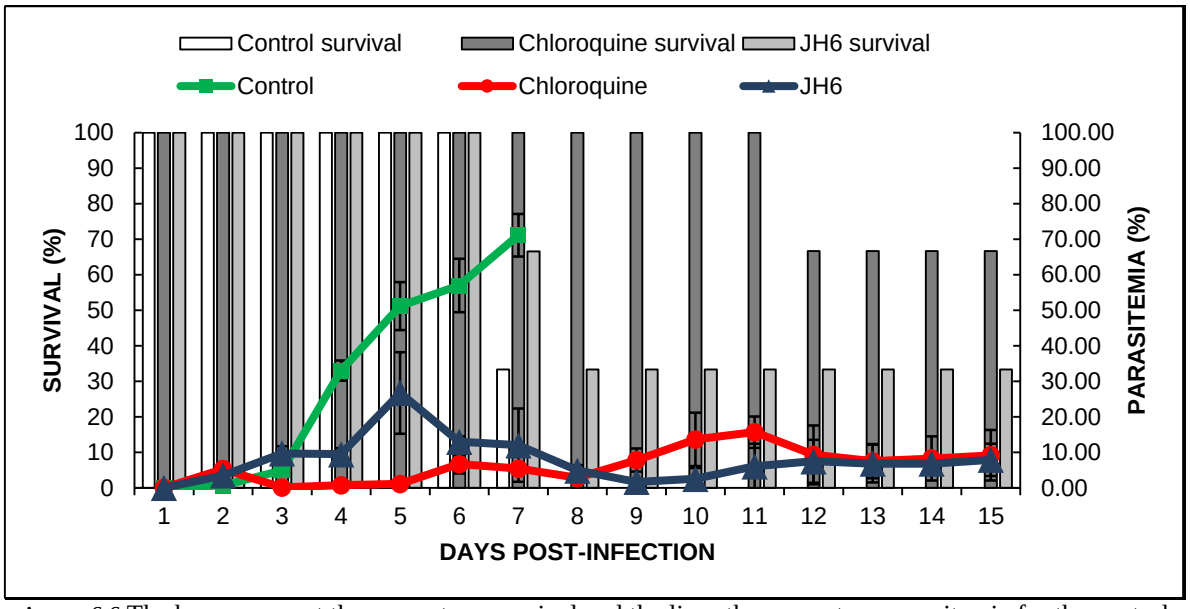
Annex 6.3 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH3 group.



Annex 6.4 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH4 group.

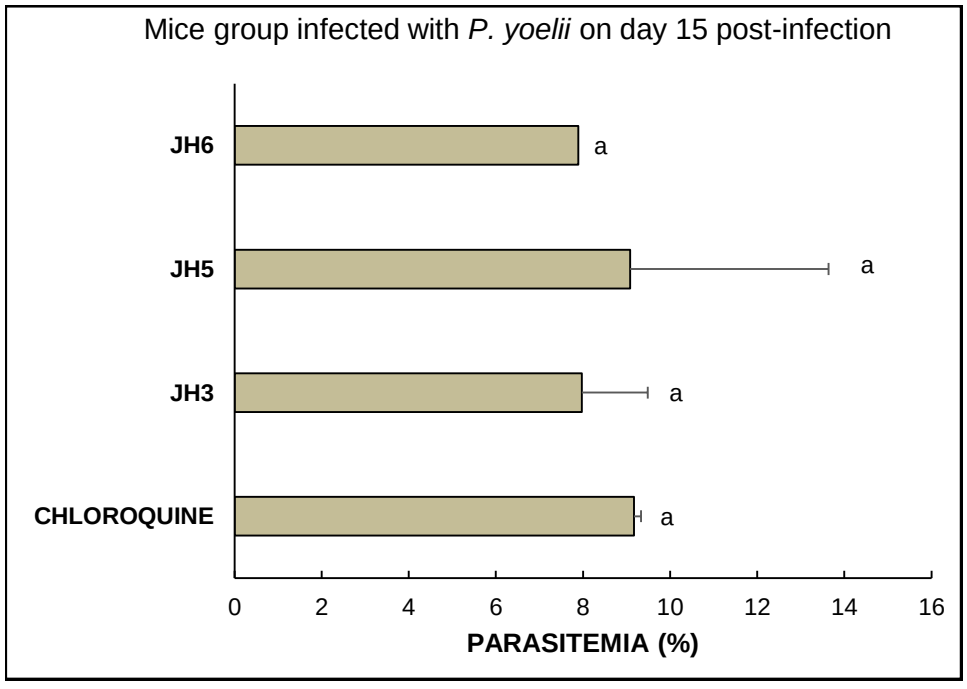


Annex 6. 5 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH5 group.



Annex 6.6 The bars represent the percentage survival and the lines the percentage parasitemia for the control group (Controls parasitemia), chloroquine group (cure control) and the JH6 group.

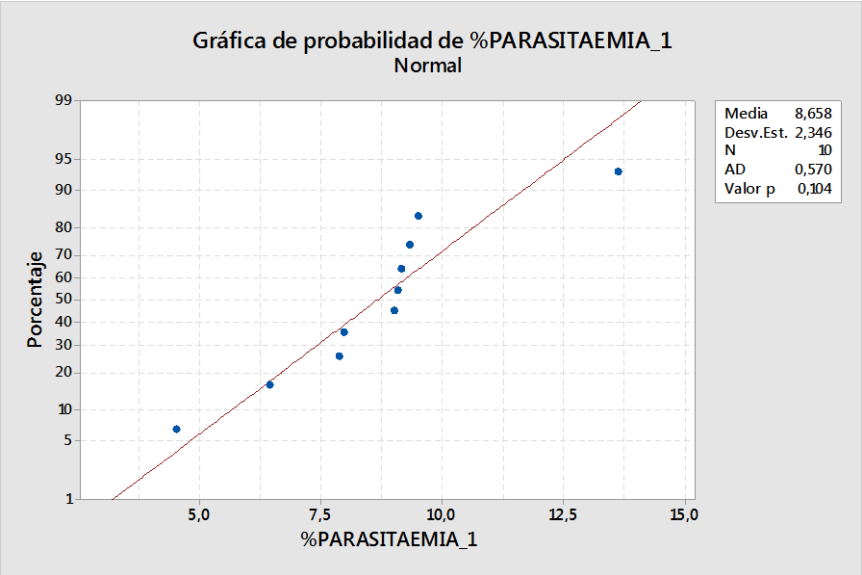
Annex 7. Analysis of percentage of parasitemia from mice infected with *P. yoelii* on day 15 post-infection



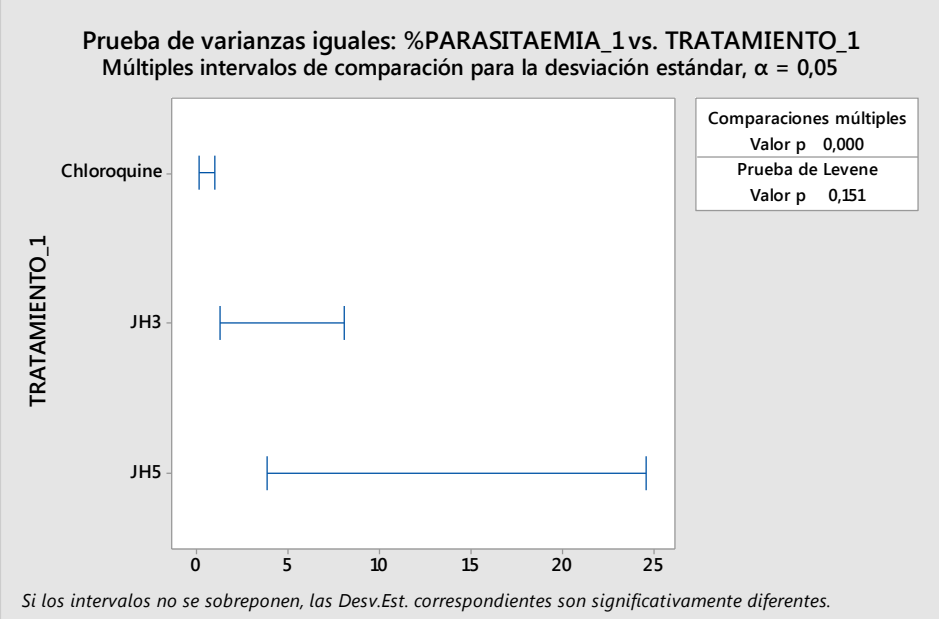
The group mice JH3, JH5, JH6 and chloroquine survived to 15 days post-infection,; therefore, a statistical analysis was performed to compare the percentage of parasitemia of the different experimental compounds. Also, It was observed that according to the Tukey test there were no significant differences between the treatments JH3, JH5 and JH6 and chloroquine, demonstrating the efficiency of these products. In addition, it

is shown that the mice treated with these drugs survived until the end of the experiment (day 15) with percentages of parasitemia similar to chloroquine (F = 0.14, p = 0.930).

Annex 8. Anderson-Darling test to prove that the data set (percentage of parasitaemia from mice infected with *P. yoelii*) adequately describes a normal distribution.



Annex 9. Statistical test of Levene to evaluate the equality of variances of the percentage of parasitaemia from mice infected with *P. berghei* (on day 15 post-infection).



Annex 10. One-way ANOVA test: percentage of parasitemia (from mice infected with *P. yoelii)* vs Treatment (JH3, JH5, JH6, and chloroquine). Using MINITAB version 17.

Método

Hipótesis nula	Todas las medias son iguales
Hipótesis alterna	Por lo menos una media es diferente
Nivel de significancia	α = 0,05

Se presupuso igualdad de varianzas para el análisis.

Información del factor

Factor	Niveles	Valores
TRATAMIENTO_1	4	Chloroquine; JH3; JH5; JH6

Análisis de Varianza

Fuente	GL	SC Ajust.	MC Ajust.	Valor F	Valor p
TRATAMIENTO_1	3	3,310	1,103	0,14	0,930
Error	6	46,235	7,706		
Total	9	49,545			

Resumen del modelo

S	R-cuad.	R-cuad. (ajustado)	R-cuad. (pred)
2,77593	6,68%	0,00%	*

Medias

TRATAMIENTO_1	N	Media	Desv.Est.	IC de 95%
Chloroquine	3	9,1731	0,1602	(5,2515; 13,0947)
JH3	3	7,977	1,525	(4,055; 11,899)
JH5	3	9,08	4,56	(5,16; 13,00)
JH6	1	7,890	*	(1,098; 14,682)

Desv.Est. agrupada = 2,77593