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Evaluation of the biological activity of CH-derived compounds *in-vitro* in *L. mexicana* and murine macrophages RAW 264.7

Tesis en torno a una hipótesis o problema de investigación y su contrastación

Ronny Javier Pibaque Sanchez

Patricio Rojas Silva, M.D. Ph.D.
Director de Trabajo de Titulación

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Ronny Javier Pibaque Sánchez

Nombre del Director del Programa:	Patricio Rojas Silva
Título académico:	M.D. PhD.
Director del programa de:	Maestría en Microbiología

Nombre del Decano del colegio Académico:	Carlos Valle
Título académico:	Ph.D.
Decano del Colegio:	COCIBA

Nombre del Decano del Colegio de Posgrados:	Hugo Burgos
Título académico:	Ph.D.

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Nombre del estudiante:	Ronny Javier Pibaque Sanchez
Código de estudiante:	00325896
C.I.:	131288520-3
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RESUMEN

Leishmaniasis es una enfermedad desatendida que se encuentra en casi todas las provincias del Ecuador y esta se encuentra principalmente en áreas rurales que a menudo se caracterizan por una mala condición socioeconómica y una composición geográfica compleja. En aquellos países donde la leishmaniasis es altamente endémica, existe la necesidad de buscar activamente nuevos compuestos con actividad leishmanicida. Esto debido a que los tratamientos actuales poseen varias limitaciones, como son el alto costo, tu toxicidad, baja especificidad y el alto porcentaje de fracaso del tratamiento. Una posible explicación es que los tratamientos son específicos para cada especie de *Leishmania,* es decir que diferentes tratamientos deben ser generados para especies locales de *Leishmania* en un país donde la enfermedad sea altamente endémica. En esta investigación, exploramos la actividad leishmanicida de 19 compuestos derivados CH contra parásitos de *Leishmania (L.) mexicana* y la actividad citotóxica, de los compuestos como mayor actividad leishmanicida, contra macrófagos murinos RAW264.7.

La reducción de MTT a cristales de fromazán (bromuro de 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio) por parte de la actividad metabólica de células o de los parásitos se utilizó para determinar el efecto en la viabilidad celular de todos los compuestos. De los resultados que se obtuvieron se determinó que dos compuestos CH 02 y CH 09 fueron los más activos contra los parásitos de *Leishmania (L.) mexicana* (IC_{50} < 20.0 μM). Estos compuestos obtuvieron también un efecto evidente en las células de macrófago murino (RAW264.7) con lo cual se obtuvieron valores en el índice de selectividad (IS) menores a 1.11. Los cuales no son óptimos considerando que un IS para continuar con el desarrollo de estos compuestos debe ser mayor a 10.0. A pesar de esto, los compuesto CH 02 y CH 09 pueden ser parte de una línea de desarrollo de fármacos para futuros estudios. Por otro lado, se determinó que es posible

tener un modelo de nuestros parásitos en su formar intracelular para realizar un cribado de futuros compuestos.

Palabras clave: Leishmaniasis, *Leishmania mexicana*, endémico, falla terapéutica, CHs, ensayo MTT, actividad biológica, índice de selectividad.

ABSTRACT

Leishmaniasis is a Neglected Tropical Disease that is found in nearly all provinces in Ecuador, and it mainly affects rural areas which are often characterized by poor socioeconomic condition and complex geographical composition. In countries where leishmaniasis is highly endemic, there is a need to actively search for new leishmanicidal compounds as various are the limitations of current treatment options, such as high cost and toxicity, low specificity, and high percentage of treatment failure. One plausible explanation is that treatments are specie specific and countries where leishmaniasis is a problem health should be looking for treatments that target local species of *leishmania*. In this research, we explored the leishmanicidal activity of 19 CH-derived compounds against promastigotes parasites of *Leishmania (L.) mexicana* and their cytotoxic activity against murine macrophage RAW 264.7. Reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) into formazan crystal by metabolic activity was used to determine cell viability from all compounds in both *in-vitro* models. From these results, it was shown that only 9 compounds have leishmanicidal activity against the extracellular parasite with IC_{50} values ranging from 15.84. to 53.22 μM. These nine compounds also showed moderate to high cytotoxic activity with CC_{50} ranging from 1.95 to 73.96 μM. None of the compounds tested for both their leishmanicidal and cytotoxic activity displayed an optimal selectivity index (SI >10). Compounds CH 02 and CH 09 with the lowest IC_{50} have the potential to be part of a drug development pipeline for future studies. From our study, the potential to have an intracellular amastigote assay for screening potential leishmanicidal drug candidates in a more clinically relevant model.

Keywords: Leishmaniasis, *Leishmania mexicana*, endemic, treatment failure, CHs, MTT assay, biological activity, selectivity index.

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INTRODUCTION

Leishmaniasis is recognized as a group of diverse pathologies caused by the protozoan parasites known as *Leishmania* that belong to the genus with the same name and to the class *Kinetoplastida* which is often used for order; it belongs to the Family of Trypanosomatidae. There are three subgenera in the section of Euleishmania: *L. (Leishmania), L. (Viannia)* and *L. (Sauroleishmania).* Nearly 53 *Leishmania* species have been identified; however, conflicting literature is often found because of unrecognized or informally named species (Maurício, 2018). One of the key aspects to this problem is the definition of species for unicellular organisms such as *Leishmania* (Maurício, 2018). Due to this, the number of *Leishmania* species is thought to be smaller as newer technologies including genomic-based phylogeny approaches re-organize the above-mentioned number of species (Schönian et al., 2018).

Despite of the controversy around the classification of *Leishmania* parasites, more than 20 species distributed globally (Table 1) are known to be etiological agents for the different forms of the disease in humans (World Health Organization, 2023). These parasites can be transmitted by infected female phlebotomies of the genera *Phlebotomus* in what is denominated as the Old World (OW) and *Lutzomyia* in the New World (NW). These are the *Leishmania* vectors that tend to feed on mammals' blood as part of their reproductive cycle (Claborn, 2010). There is a worldwide distribution of these phlebotomies, also known as sandflies, with more than 90 species being responsible for parasitic transmission in humans (World Health Organization, 2023). The worldwide distribution of these sandflies has become an increasing concern due to the encounter of potential vectors and parasites beyond endemic areas which can lead to the spread of the disease to places where it has never been reported before. Some estimates suggest that there are 350 million people at risk of getting infected by the parasite (Tamiru et al., 2019).

Table 1: Overview of the global distribution of the most relevant *Leishmania* parasites, their associated form of leishmaniasis and their taxonomic classification. Adapted from (A. Kumar, 2013)

Subgenus	Species	Associated form of the disease	Geographical area	Reference
L. (Leishmania)	*L. donovani*	Visceral leishmaniasis	Old world	
	L. infantum	Visceral leishmaniasis	Old world	
	L. tropica	Localized cutaneous leishmaniasis	Old world	
	L. mexicana	Diffuse cutaneous leishmaniasis	New world	
	L. chagasi	Visceral leishmaniasis		
	L. major	Localized cutaneous leishmaniasis, Mucosal leishmaniasis	Old world	
	L. aethiopica	Localized and diffuse cutaneous leishmaniasis	Old world	
	L. amazonensis	Localized and diffuse cutaneous leishmaniasis, Mucosal leishmaniasis	New world	
L. (Viannia)	*L. braziliensis*	Disseminated cutaneous leishmaniasis, Mucosal leishmaniasis	New world	
	L. guyanensis	Mucosal leishmaniasis	New world	
	L. peruviana	Mucosal leishmaniasis	New world	
	L. panamensis	Mucosal leishmaniasis	New world	
	L. naiffi	Localized cutaneous leishmaniasis and mucocutaneus leishmaniasis	New world	(Cantanhêde & Cupolillo, 2023)

Leishmaniasis can have diverse clinical manifestations in humans that mainly depends on the *Leishmania* species and the host immunity (Cecílio et al., 2022; Rossi & Fasel, 2018). In some cases, the infection combined with exogenous or endogenous agents can also affect the disease outcome (Telleria et al., 2018; Van Bockstal et al., 2020). These factors have contributed to the appearance of a wide range of pathologies; however, three main categories have been predominantly used to classify leishmaniasis: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniasis (VL). According to the World Health Organization (WHO), CL and MCL are the most predominant clinical forms with more than 1 million leishmaniasis cases occurring annually (World Health Organization, 2023). For VL, the number of cases has ranged from 100.000 to 400.000 annually in endemic regions (CDC, 2020). Most of the affected people are from the lowest socioeconomic status and often suffer from malnutrition, weakened immune systems and insufficient financial resources to face a possible infection (CDC, 2020; World Health Organization, 2023).

Leishmaniasis has been categorized as a neglected tropical disease (NTD) meaning that it is highly prevalent in tropical countries, and it has not received as much attention as other diseases (NIAID, 2016). As it is common for an NTD, current treatments for leishmaniasis remain ineffective, toxic, expensive, painful, and impractical for sufferers. Efforts to develop safer treatments or vaccine candidates are insufficient and they do not generate an impact on society. Even with organizations focused on *Leishmania* research, a great challenge to overcome is the fact that the great genomic diversity of the *Leishmania* parasites worldwide renders potential treatments specific for only one species of *Leishmania* (Garza-Tovar et al., 2020). Another problem is the lack of complete epidemiological data that can aid in implementing strategies to eradicate the disease. Because of the lack of research and pharmacological development on this NTD, there is a need in endemic countries to start

gathering epidemiological data and developing potential drug candidates to effectively treat local cases of leishmaniasis (Reithinger et al., 2007).

Ecuador is a highly endemic country with CL and MCL being the only forms of leishmaniasis reported (Ministerio de Salud Pública, 2023). These are mainly caused by *L. (Viannia) guyanensis* and *L. (V.) braziliensis* in coastal and amazon regions, while *L. (Leishmania) mexicana* is the most diagnosed pathogen in the highlands; however, however, other species have also been reported such as *L. (V.) naiffi* and *L. (V.) lainsoni* (Kato et al., 2016). CL and MCL can often be resolved with appropriate treatment and a medical follow-up; however, various limitations do not allow patients to undergo complete treatment, including the limited number of effective treatment options on the market, their expensiveness, and their highly undesirable side-effects (Surur et al., 2020). In addition to this, MCL, a disfiguring condition, creates a social stigma towards suffering patients (Reithinger et al., 2007). In other cases, like localized CL caused by *L. (L.) mexicana*, healing can occur spontaneously after 6 – 12 months; however, more than one-third of the patients suffer from relapses or even develop MCL or diffused cutaneous leishmaniasis (Torres-Guerrero et al., 2017). These problems with current treatments and complicated cases of leishmaniasis create an urgent need for new and safer alternatives to treat CL and MCL.

Accessing effective treatments for either CL or MCL has remained elusive for many of the patients suffering from the disease; these conditions are one of the major health problems mostly in endemic regions of Ecuador. Numerous strategies have been used to find leishmanicidal compounds, among them, the usage of small molecules seems to be a promising strategy. Seminal and recent reviews pointed out the advantages of drug development based on small-molecule candidates and how they can be combined with high-throughput technologies for improving the output of potential drug candidates (Bevan et al., 1995; Schneider, 2018). One group of compounds with potential leishmanicidal activity are CH- derivatives, which

have been shown to possess high biological activity (Elkanzi et al., 2022; Jung et al., 2017; Rozmer & Perjési, 2016). The biological activity of CH-s is attributed primarily to their derivation from natural sources, which have a long-standing history of providing us with compounds for resolving major health problems (Atanasov et al., 2021).

In this study, the biological activity of 19 CH-derived compounds, organically synthesized, was tested in the free promastigote form of *L. mexicana* and a murine macrophage model RAW 264.7. Both, half maximum inhibitory concentration (IC_{50}) for the parasites and the half maximum cytotoxic concentration (CC_{50}) for a murine macrophage cell lineage RAW 264.7, were determined to obtain the selectivity index (SI) of the compounds. In addition to the SI, an infection model was performed by aiming to infect the RAW264.7 cell line with the *L. mexicana* parasite.

LITERATURE REVIEW

The *Leishmania* parasite: Epidemiology and transmission

The occurrence of the vector-borne disease known as leishmaniasis takes place almost worldwide with a high percentage of the cases being reported in over 90 countries across the tropics, subtropics, and southern Europe (CDC, 2020; Tamiru et al., 2019). This leaves Antarctica and Australia as the only two regions in which endemic leishmaniasis cases have not been reported (CDC, 2020). Different factors have contributed to the global presence of leishmaniasis, mainly, high genetic diversity among *Leishmania* species (Herwaldt, 1999) as well as the availability and worldwide distribution of competent vectors known as phlebotomine sand flies (Cecílio et al., 2022; Oryan & Akbari, 2016). Other factors, such as globalization, climate change, reduction of natural habitats and global conflicts, are known to contribute to the spread of the disease beyond endemic regions (Reithinger et al., 2007). Even more concerning is the fact that certain species of phlebotomies have been reported to adapt and persist in urban and semi-urban environments which can lead to even further spread of the disease (Lisi et al., 2014; Souza et al., 2014; Tarallo et al., 2010) with estimates suggesting that more than 350 million people are at risk of infection (Torres-Guerrero et al., 2017). Leishmaniasis transmission and epidemiology are complex, thus, the literature review in this section will be presented on the most important aspects of the dynamics of leishmaniasis.

The heteroxenous life cycle of *Leishmania* parasites presents a significant challenge, requiring remarkable adaptive capabilities that ultimately determine the parasite's survival or death; these adaptations have severe implications in the dynamics of leishmaniasis transmission as well as disease outcome (El Baidouri et al., 2013; Santi & Murta, 2022; Sundar & Singh, 2018). Various of the inter- and intraspecies genetic interactions, that have allowed leishmania parasites to adapt to such life cycle, are summarized elsewhere (das Chagas et al., 2022). Particularly, *Leishmania* species known to be etiological agents of human leishmaniasis

are a highly genetically diverse and complex group of parasitic protozoans (Herwaldt, 1999). Even though a study by Herwaldt pointed to this diversity more than 20 years ago, the appearance of genomic technologies has allowed us to better understand what drives the genetic diversity in parasitic protozoans (Gibson, 2021). Some examples have been reported in various species of *Leishmania* with high genetic diversity of samples from similar ecological niches or the same infected patient. To mention some examples, studies have been reported in *L. (L.) tropica* (Glans et al., 2021; Lypaczewski & Matlashewski, 2021), *L. (V.) brazilienzis* (Patino et al., 2020; Van den Broeck et al., 2020) *L. (V.) peruviana* (Van den Broeck et al., 2020), *L. (L.) major* (Ghouila et al., 2017), *L. (L.) infantum* (Antoniou et al., 2004; Gouzelou et al., 2013) and *L. (V.) panamensis* (Llanes et al., 2022). The fact that variable genetic species can be found in a single endemic location and even in a sample from a patient, can often lead to problems in diagnosing, vectorial control and applying a successful treatment.

Apart from the genetic diversity that allows the parasite to adapt to the environment and host, its transmission and disease establishment require a vector known as phlebotomine sandfly. These insects belong to the genera *Phlebotomus* and *Lutzomyia,* and the parasite has evolved mechanisms to adapt its life cycle to vectors in these genera. The adaptations are quite complex, and the detailed life cycle can be reviewed elsewhere (Bates, 2007; Serafim et al., 2021; Telleria et al., 2018). In brief, parasites are taken from an infected host by a blood meal, these parasites are found in their intracellular amastigote form. Upon reaching the gut, environmental signals such as pH and availability of free sugar can trigger the transformation of *Leishmania* parasites into their free-living form known as promastigotes. From this point, parasites' development takes place from a procyclic to a metacyclic stage; this change can only take place once the parasite has established itself in the vector's gut, and it continues its way to the sandfly stomodeal valve, here, it generates a blockage with a promastigote metacyclic gel (PMG) which is then released when the sandfly bites another person. Apart from the gel, there

is increasing evidence that endogenous elements from the vector play an important role to achieve successful infection. One such element that seems to actively be involved in contributing the parasite fitness is the sandfly gut microbiome (Dey et al., 2018; Kelly et al., 2017; Sant'Anna et al., 2014). The entire cycle and interactions with other microorganisms take place in such a harsh environment involving both, the host immune systems and the immunity and gut of the sandfly. Despite this, *Leishmania* parasites have found ways to overcome the obstacles and thrive in this cycle.

The last group of factors influencing the epidemiology and transmission of the disease comes from human activity. In this sense, various studies have pointed out that climate change is having a great influence on the spread of leishmaniasis thanks to changes in the ecological niche of sandflies. Based on these studies, models have been developed to predict potential distributions in a future scenario with a warmer climate; the expansion of sandflies may extend higher up north in the OW and NW leading to the spread of leishmaniasis (Chalghaf et al., 2018; Moo-Llanes et al., 2013; Rodríguez-Rojas et al., 2017). Another study investigated the most common environmental factors that can affect the distribution of these phlebotomies and that climate change might alter and it found that precipitation and vegetation patterns would positively affect the spread of these vectors beyond endemic regions (de Santana Martins Rodgers et al., 2019). Recording the dynamics of the actual disease is more crucial than modelling the flow of vectors. In this case, various reports of non-exported leishmaniasis in previously recognized *Leishmania*-free countries in Europe (Gianchecchi & Montomoli, 2020), in Africa (Boudrissa et al., 2012) as well as the United States (Curtin & Aronson, 2021) have been reported. Besides climate change caused by human activity, globalization is another factor to consider, especially the accessibility to travel to virtually any part of the world, which has caused the appearance of imported leishmaniasis cases among travellers (Boggild et al., 2019; Pavli & Maltezou, 2010; Schwartz et al., 2006). Altogether, this information shows how

important it has been to establish control of leishmaniasis in travelers as well as to mitigate the effects of climate change, both of which can greatly influence the spread of the disease.

To sum up the importance of this vector-borne disease, different aspects of its transmission make leishmaniasis very difficult to control. The genetic plasticity of the *Leishmania* parasites improves their fitness, and it can give rise to adaptations to newly found vectors. Equally important is the fact that phlebotomies sand flies have nearly adapted to tropic and subtropical environments, with the risk of spreading their ecological niche due to climate change. It becomes imperative to study the disease dynamics, as it can be a great tool to aid in the astonishing task of controlling leishmaniasis spread.

Leishmania species, host, and vector immunity in the disease outcome

In humans, infection with the *Leishmania* parasite can result in three distinctive pathologies, mainly attributed to the *Leishmania* species responsible for the infection as well as to the host immune response. As mentioned earlier, these three pathologies are CL, MCL, and VL. As reviewed by van der Auwera and Dujardina (2015), all species of pathogenic *Leishmania* parasites can cause to a certain degree a dermal affection (e.g., CL and MCL); however, not all can cause the VL form of the disease. Interestingly, among the species causing dermal lesions, some species only cause CL, some certainly cause MCL, and others cause lesions that can be allocated in a certain spectrum from CL to MCL (van der Auwera & Dujardina, 2015). Since the 70s', the appearance of species responsible for different types of intermediate pathogenic CL or MCL has resulted in the study of the immunological role of the host to either control, modulate, or remain silent against a *Leishmania* infection. A seminal book chapter and various reviews were written on the immunology of leishmaniasis as early as 1971 (Heyneman, 1971; Liew & O'Donnell, 1993; Locksley & Louis, 1992). Nowadays, host immunity is a key aspect when developing potential treatments against the various forms of leishmaniasis (Akbari et al., 2021; Dayakar et al., 2019; dos Santos Meira & Gedamu, 2021;

Ikeogu et al., 2020; Taslimi et al., 2018). In this part of the review, the clinical outcome will be presented in terms of the *Leishmania* species and host immunity, with special attention on leishmaniasis occurring in the NW.

Visceral leishmaniasis (VL) is distinguished by hepato-splenomegaly, pancytopenia, irregular febrile episodes, anemia, and anorexia. If the condition is left untreated, it is fatal in nearly 95% of the cases (World Health Organization, 2023). In the OW, it mostly affects countries in East Africa and India, where the etiological agents are *L. (L.) infantum* and *L.(L.) donovani*. Meanwhile, *L. (L.) chagasi,* later reclassified as *L. (L.) infantum*, is responsible for the majority of VL cases in the NW (McCall et al., 2013; Wamai et al., 2020). On some rare occasions, VL might be caused by *L. (L.) amazonensis* and *L. (L.) tropica*, which are classically known to cause CL in the NW and the OW, respectively (McCall et al., 2013; Wilson et al., 2005). For VL, the appearance of asymptomatic individuals has been an extensive research area (Ibarra-Meneses et al., 2022). The specific determinants that allow the disease to progress to a clinically symptomatic VL in any of the species remain unclear (Singh et al., 2014); however, some reports have mentioned that genetic factors, AIDS, malnutrition, and age, which are tightly related to the immune competency of individuals, are the primary contributors to disease progression [reviewed in (R. Kumar & Nylén, 2012; McCall et al., 2013; Singh et al., 2014)]. Some studies have reported on the molecular and cellular components of the immune response mediated by the host cytokines (Dayakar et al., 2019) and a variety of T-cell subpopulations (Jawed et al., 2019) to potentially influence disease progression. Others have looked up molecular markers that can be used to monitor this progression (Chakravarty et al., 2018; Das et al., 2020). Whether environmental determinants or immunological modulation, or both, are responsible for the progression of asymptomatic individuals to clinical VL is yet to be determined.

Similar to VL, mucocutaneous leishmaniasis (MCL) can result in a fatal outcome if appropriate treatment is not given. It is characterized by severe lesions mainly in the nasal mucosa, but reports can also be found on MCL affecting the oral mucosa (Goto & Lauletta Lindoso, 2012). Various symptoms can be encountered in an MCL patient, from clinical manifestations similar to those present in CL sufferers, along with nasal inflammation, ulceration, and perforation of the septum, to affections in the soft palate, larynx, and pharynx (Goto & Lauletta Lindoso, 2012). In the NW, species of the sub-genera *Vianna* are associated with the presence of this form of the disease, in particular *L. (V.) braziliensis, L. (V.) panamensis,* and *L. (V.) guyanensis.* Fewer cases have been reported by species of the sub-genera *Leishmania* with the involvement of the *L. (L.) amazonensis* (Abadías-Granado et al., 2021; Goto & Lauletta Lindoso, 2012; van der Auwera & Dujardina, 2015). In the OW, *L. (L.) major and L. (L.) infantum* can be commonly found in MCL lesions (Abadías-Granado et al., 2021). As it also occurs with VL, the determinants for the damage of mucosal body parts of these species have been largely debated, as it is not clear what drives the mucosal tropism. Some clues suggest a connection to the immune state of the patients in the appearance of the MCL (Reithinger et al., 2007). For instance, a strong correlation has been reported in co-infections with HIV as well as in individuals under the action of an immunosuppressor. Besides the individual characteristics of the immunological state, exogenous factors such as *Leishmania* viruses have also been reported from *L. (V.) panamensis* infections (Mukherjee et al., 2023). So far, much needs to be done to understand the triggers of the mucosotropism observed in these *Leishmania* species responsible for MCL.

The milder form of all the leishmaniasis pathologies is CL, which can be caused by nearly all the *Leishmania* species, and it is characterized by the presence of erythematous papules around the sandfly bite (Ghosh et al., 2023). These papules are often self-resolved by the host immune system; however, on some occasions, other forms of CL, such as diffuse CL

(DCL) or MCL, might develop. The patients' immune states and responses seem to be the main two causative agents for the appearance of these pathologies (Ghosh et al., 2023). As mentioned, all the *Leishmania* parasites affecting humans can cause CL, and the factors that decide the fate of the infections vary from intrinsic to extrinsic ones. Some studies have pointed out the relationship between inoculation size, sandfly components, unbalanced pro- and anti-inflammatory responses, the ability to induce apoptotic mimicry at the bite site, and the progression of CL to other forms such as DCL, MCL, and VL, respectively to each *Leishmania* species (Ghosh et al., 2023). A comprehensive review has been published by Ghosh and colleagues where they outlined the causes for the visceralization or spread of the *Leishmania* site beyond the initial papule in the case of cutaneous infection (Ghosh et al., 2023).

There has been an increasing amount of evidence that other factors beyond the *Leishmania* species, such as the immunological state of the patients, symbiotic relationships of the *Leishmania* parasites with other microorganisms, and bite-dependent factors, can result in a wider spectrum of the disease. This makes leishmaniasis a very complex disease to treat in humans.

Leishmaniasis in Ecuador

Since 2019, a steady number of leishmaniasis cases (~ 1000 cases) have been reported by the Ministry of Public Health of Ecuador in their vector diseases gazettes (Dirección Nacional de Vigilancia Epidemiológica, 2023). In the last report in 2023, up to week 36 of the current year, there were 561 leishmaniasis cases, of which 97.7% (548) corresponded to CL and the remaining percentage corresponded to MCL cases (13). According to data published by the Pan-American Health Organization, visceral leishmaniasis has not been reported in Ecuador ever since there has been a registration of leishmaniasis by this organization (Pan-American Health Organization, 2022). CL and MCL cases have been diagnosed in 22 of the 24 Ecuadorian provinces in 2023; Pichincha, Morona Santiago, and Esmeraldas are the

provinces with the highest reported cases (Dirección Nacional de Vigilancia Epidemiológica, 2023). These three provinces are considered endemic regions of leishmaniasis in Ecuador. The only two places in the country without any form of leishmaniasis are the Galapagos Islands and Carchi Province. According to this epidemiological data, more than half of the cases are found in the population between 20 and 49 years old. Males were the most affected by the *Leishmania* infection, making up to two-thirds of all cases reported this year (Dirección Nacional de Vigilancia Epidemiológica, 2023). This data represents cases that are diagnosed at health facilities such as hospitals, ambulatory clinics, and health subcenters. Unfortunately, there is no active epidemiologic screening in Ecuador, which might lead to an underrepresentation of total cases of leishmaniasis in this country (Hashiguchi et al., 2017).

Challenges in drug screening for leishmaniasis.

Various challenges become relevant when performing the research work needed to test and improve the compounds that were observed to have leishmanicidal activity. Three main strategies that have been used to search for potential drugs are: 1) Phenotypic screening, which relies on discovering drug candidates that affect *Leishmania* viability *in vitro*; 2) Target-based drug discovery, which is mainly driven by developing in-silico drugs that target essential proteins validated by biochemical or biophysical assays; and 3) Drug repurposing, which brings attention due to the lower cost that repurposing can have compared to developing a drug from scratch (Jones et al., 2018). Any strategy can be useful for the development of potential drug candidates to treat leishmaniasis; however, the first two are the most used (Jones et al., 2018; Santos et al., 2008). Despite the method of choice, considerations, going from the genotypic diversity of these parasites to selecting an appropriate *in vitro* and *in vivo* model, should be made to satisfactorily obtain a potential drug hit (Cohen & Azas, 2021). These challenges will be put into context in the next part of the review.

Whether a phenotypic-based or target-based approach is being considered to develop potential drug candidates for leishmaniasis, either will need to be tested in the laboratory. These tests would need to be performed preferably in conditions replicating those observed in the natural environment of the parasites. An excellent *in vitro* model should consider the parasitic cycle of *Leishmania* species (Cos et al., 2006; Pink et al., 2005; Sereno et al., 2007). As mentioned earlier, in the human host, these parasites can be found in their intracellular amastigote form, which greatly differs from the promastigote form found in the sandfly vector (Cohen & Azas, 2021; De Rycker et al., 2013). Differences have been found not only phenotypically but also metabolically for different species of *Leishmania* parasites (Jara et al., 2017; Rosenzweig et al., 2008; Yao & Wilson, 2016). Various cellular processes have also been reported to be different in both parasite stages based on gene expression and regulation (Cortazzo da Silva et al., 2022). These differences allow the parasite to withstand the environment of the sandfly gut and the host immune system (Santarém et al., 2014). It becomes imperative that drug screening protocols take into consideration the choice of an *in vitro* model.

The *in vitro* culturing of *Leishmania* parasites has been the predominant technique to screen for leishmanicidal agents (Cohen & Azas, 2021); however, parasites should be kept in an infective cycle within a mammal model to maintain their phenotypic and genotypic characteristics. Parasites can be kept *in vitro*; however, the passage number is recommended to be below low to preserve the best infective characteristics of the parasites. Some evidence of this effect was reported in literature with *L. (V.) infantum,* where the passage number affected the *in vitro* infection capability due to the inability to differentiate to this amastigote form; however, it is important to mention that significant effects were observed at their 21st passage (Moreira et al., 2012). Similar observations were described for *L. (V.) donovani,* where the authors concluded that changes in genome plasticity and lowered expression of virulence genes were responsible for the observation at passage 10th (Sinha et al., 2018). A similar reduction in

gene expression and infectivity was observed for *L. (L.) mexicana* (Ali et al., 2013). This might not be the case for all *Leishmania* species, as a comparison of infectivity in *L. (V.) major* between cultured and sand-fly derived parasites did not find significant differences in gene expression profiles related to infectivity (Haravani et al., 2023). This result should be considered cautiously as the authors did not perform infectivity tests. Even though *in vitro* culturing is the most used technique to screen for leishmanicidal activity, special attention should be directed to the loss of virulence and changes in genotypic and phenotypic expression when designing experimental activities for drug screening.

Diverse strategies have been published that demonstrate the reduction of genetic and phenotypic changes, enhancement of cell viability, and preservation of infectivity in *in vitro Leishmania* parasites. One of such strategy relies on the proper selection of media and culturing conditions of the parasites, which are not only able to maintain viability but also infectivity; this can be achieved without relying on continuous isolation of *Leishmania* parasites in their amastigote form from either mammal or patient ulcers in the case of cutaneous leishmaniasis, even though it is highly recommended whenever possible to do so (Evans et al., 1989). In this regard of appropriate media usage, keeping parasites in biphasic conditions such as USHMARU (sheep blood agar) and Tobey's have been reported to keep viability (Evans et al., 1989). These media have in common their composition that contains some mammal-blood derived products in the form of solid or liquid phase, and these have consistently been reported as a useful strategy to also maintain parasite infectivity in various species of *Leishmania* (De Almeida Rodrigues et al., 2010; Grekov et al., 2011; Ladopoulos et al., 2015; Merlen et al., 1999). Interestingly, a similar exposure to readily available blood components was reported to enhance cell division and infectivity capability in sandflies (Serafim et al., 2018). Studies on how the selection of appropriate media affects parasites at the expression and genomic level are being published more often; some discrepancies can be found as the interpretation of results

remains complex. Despite this limitation, phenotypically maintaining infectivity and viability by mimicking *in vitro* the parasitic environment remains a valid strategy for purposes such as testing for potential leishmanicidal drugs.

CH as potential drug candidates for parasitic diseases.

Different studies and reviews highlight the potential biological activity of CH derivatives [For a comprehensive overview of studies related to CH biological activity please refer to (Gaonkar & Vignesh, 2017; Rammohan et al., 2020; Salehi et al., 2021)]. CHs consist of two aromatic rings that are joined by an α, β-unsaturated carbonyl system. They are synthesized in nature by the shikimate pathway, as they serve as precursors for the synthesis of flavonoids (Raut et al., 2016). In addition, a variety of biologically active heterocycles can be obtained using s as their key precursor (Raut et al., 2016).

The leishmanicidal activity of the CH derivative has also been widely reported. Briefly reviewing the PubMed database, searching for the combined term [(Leishmania) AND (CHs)] yielded 121 studies up to the date this thesis was written. From the first report found in this search, a compound named lipoCH A had high activity against *L. (V.) major* and *L. (V.) donovani* promastigotes and intracellular amastigotes in human monocyte-derived macrophages. The authors reported that the compound might have had leishmanicidal activity by affecting the mitochondria (Chen et al., 1993). In this study, we screened nineteen CH derived compounds for their biological activity against *Leishmania (L.) mexicana* parasites and RAW 264.7 macrophages. The SI was determined, and an infection model was also reported for further screening of improved compounds.

METHODOLOGY AND EXPERIMENTAL DESIGN

CH-derivatives synthesis and characterization

CHs were synthesized and characterized at UTE University. They were kindly provided by Prof. Jorge Heredia (CENBIO, Universidad UTE). Most of the compounds were dissolved in DMSO at 20.0 mM for storage. Solubility was a problem for three compounds, CH 01, stored at 5 mM, as well as CH 02 and CH 14, stored at 10 mM. The chemical structure and denomination of the compounds for the experiments can be found in Figura 1.

Materials and reagents

Dulbecco's modified eagle's medium (DMEM, Corning), Roswell Park Memorial Institute medium (RPMI-1640, Lonza Bio-Whittaker), and Schneider drosophila medium (SDM, Gibco) were purchased from Thermo Fisher Scientific (United States). F~~o~~etal bovine serum (FBS, Eurobio) was purchased from Eurobio Scientific (France). Antibiotic containing penicillin (10.000 IU) and streptomycin (10.000 µg/ml) (Gibco) was purchased from Thermo Fisher Scientific (United States). Ushmaru solid medium with sheep blood agar in flute beak shape was purchased from Agrobiopac (Ecuador). PBS (10X) was purchased from Bio-Basic (United States). Dimethyl sulfoxide (DMSO for molecular biology, Sigma-Aldrich) was purchased from Merck. DAPI (4′,6-diamidino-2-phenylindole, molecular probe) and HCS CellMask red staining (Molecular probes) were purchased from Thermo Fisher Scientific (United States).

L. (L.) mexicana culture – Maintenance of promastigote form

L. (L.) mexicana strain parasites were kindly provided by the Universidad Cayetano Heredia, and its species identification was performed by amplifying and sequencing the cytochrome B gene (Barreiro-Costa et al., 2021). Promastigote forms of the parasite were originally obtained in supplemented SDM (sSDM) with 10% FBS and 1% antibiotics. To help

in maintaining infection fitness without an animal model, parasites were cultured in two phases: a liquid phase in sSDM for 3 days at 25 ^{o}C and a biphasic medium consisting of sSDM in Ushmaru (sheep blood agar in slant) tubes for 2 days at 25 ^{o}C. This method has been reported to be effective in cultivating *Leishmania* parasites for research purposes (Nasiri, 2013, 2017). In brief, promastigote forms of the parasites in sSDM (3 days of incubation) were washed twice by transferring them to a conical tube, centrifuging for 10 minutes (2000 RPM), discarding the supernatant, and resuspending the pellet in PBS (1X). After the second wash, the pellet was redissolved in sSDM and passed through an insulin syringe needle (gauge: 5.00 μm) to break up aggregates that tend to be formed at this growth stage. Afterwards, parasites were counted alive in a Neubauer chamber at the appropriate dilution (1:100). 50.00×10^6 parasites in sSDM (4 mL) were transferred to an Ushmaru tube. After 48 hours, 20.00×10^6 parasites from the biphasic medium were transferred to monophasic sSDM medium in a sealed T25 cell culture flask (no filter) and kept at 26 ^{o}C for 3 days. At this stage, parasites reach confluency, and growth is halted.

***L. mexicana* culture – Obtention of metacyclic infective promastigote form.**

Parasites in sSDM were suspended in supplemented RPMI-1640 medium (sRPMI), which contained 10% FBS and 1% antibiotics; this was done to better obtain metacyclic forms (infective stage) and maintain parasite fitness. In brief, this was performed by replacing the sSDM medium with sRPMI in 20% increments. After rinsing the parasites with PBS 1X, the first change in the medium was performed (80% sSDM: 20% sRPMI). Parasites were left to grow in this medium for both stages, the biphasic medium (2 days) and the monophasic medium (3 days). The motility and constant growth of the parasites were assessed under an inverted microscope to ensure they responded well to the medium change. Parasites were left to grow completely in 100% sRPMI. A growth curve was obtained from the parasites in sRPMI.

Incubation time was determined to be 4 days to successfully observe mostly metacyclic parasites, which were used for further experiments.

Murine macrophages RAW 264.7 culture

Murine macrophages RAW 264.7 (ATCC number) were kindly donated by Dr. Ilya Raskin (Rutgers, The State University of New Jersey, United States). Upon thawing, cryogenically preserved RAW 264.7 (1 mL) were cultivated in a T25 cell culture flask with DMEM supplemented with 10% FBS and 1% antibiotics (sDMEM) and incubated at 37 ºC, 5% CO_2, and 95% relative humidity. Every 48 hours, cells were rinsed with PBS (1X), and the medium was renewed. Cells were only allowed to reach 80% confluency before being used for further experiments, and they were used up to the 25 cell-passage.

Dose-response assays for leishmanicidal activity against *L. Mexicana*

Metacyclic parasites were obtained after 4-5 days of incubation in sRPMI. Parasites were washed twice with PBS 1X and resuspended in 10 mL of fresh sRPMI. Parasites were counted alive using a Neubauer chamber at an appropriate dilution (1:100). 1×10^6 parasites (100 μL, 10×10^6 parasites/mL) were seeded on a 96-well plate with conic bottom (SARSTEDT). Treatments (100 μL) were applied immediately after seeding in triplicate with final concentrations of 0.01–100 μM, of the CH-derived compounds. sRPMI was used as the negative control, 2 μM amphotericin B was used as the positive control, and sRPMI solutions at 2%, 1%, and 0.5% DMSO were used as growth controls. Plates were incubated at 25 ºC for 48 hours.

Dose-response assays for cytotoxic evaluation on RAW 264.7

RAW 264.7 was used at 80% confluency from a T25 cell culture flask. Cells were washed twice with PBS 1X and scrapped out in 5 mL of sDMEM. Resuspended cells were

homogenized and counted by the trypan blue exclusion method in a Neubauer chamber. 6.0×10^3 cells/well (100 μL, 60.0×10^3 cells/mL) were seeded on a 96-well plate (SARSTEDT) and stirred on a plate shaker (10 minutes). Cells were allowed to grow overnight (37 ºC, 5% CO_2, and a humid atmosphere). Treatments (100 μL) were applied in triplicate with final concentrations of 0.01–100 μM. Only compounds with the highest leishmanicidal (IC_{50} <100.0 μM) activity were tested against RAW 264.7. sDMEM was used as a negative control, a 4 mg/mL saponin solution in DMEM was used as a positive control, and sDMEM solutions at 2%, 1%, and 0.5% DMSO were used as growth controls. Plates were incubated at 37 ºC for 48 hours in a 5% CO_2 and humid atmosphere.

MTT assay for evaluation of cell and parasite viability

The reduction of the MTT salt by metabolic activity to form formazan crystal can allow the determination of cell viability after treatments were applied. The differences between controls and treatments can be used to determine IC_{50} and CC_{50}. To achieve this, a MTT solution was prepared in PBS 1X (5 mg/mL). 20 μL of this solution was added to each well after 48 hours of incubation to reach a final 0.45 mg/mL MTT salt concentration. Plates were covered in aluminum foil, mixed in a plate shaker for 10 minutes, and incubated for 2 hours with the conditions mentioned above, depending on the cell type. Plates were then centrifuged for 10 minutes (4000 RPM), and the supernatant was removed. The formazan crystals were resuspended with DMSO (100.00 μL) and mixed for 10 minutes in a plate shaker. Absorbances ($A_{570–630}$) were determined in the plate reader BioTeK ELx808.

Standardization of infection models using *L. mexicana* on murine macrophages RAW 264.7

a. Infection and plating:

5.0×10^4 RAW 264.7 cells (500 μL) in sDMEM (1 mL) were seeded on a 24-well plate for *Leishmania* detection with fluorescent microscopy or seeded in coverslips treated for cell culture for visible microscopy. Cells were incubated overnight (37°C, 5% CO_2, and a humid atmosphere). Wells were gently washed with PBS 1X twice to remove unattached cells. sDMEM (1 mL) with *L. mexicana* parasites in their metacyclic stage (after four days of incubation) was added to the cells at a 10:1 multiplicity of infection (MOI). Infection was allowed to take place for 8 hours (32 °C, 5% CO_2, and humid atmosphere), and non-internalized parasites were removed by washing twice with PBS 1X. The infection model was allowed to take place for 48 hours before being screened by GIEMSA and DAPI staining. RAW 264.7 wells were only allowed to grow in DMEM, and they were used as a negative control.

b. Determination of parasite load on murine macrophages by Giemsa-Wright staining:

The incubation media was removed, and the wells were washed twice with PBS 1X. Cells were fixed with cold methanol for 15 seconds, and they were stained with a 10% Giemsa-Wright solution in ultrapure water (Corning) for 20 minutes. This solution should be freshly prepared, as precipitation of the stain might occur. Wells were washed with PBS 1X twice. The coverslip was carefully fixed to a microscope slide, and cells were observed under an optical microscope.

c. Determination of parasite infection on murine macrophages by fluorescence microscopy with DAPI and Cell-mask staining:

The incubation media was removed, and the wells were washed twice with PBS 1X. Cells were fixed with a 4 % formaldehyde solution for 10 minutes. DAPI (1:2000 dilution) from a 1 mg/mL stock solution was mixed with a cell-mask red solution (20 μg/mL), and cells were incubated with this solution for 30 minutes, protected from light. Wells were washed twice with PBS 1X. Cells were directly observed under a

fluorescence microscope (Nikon, Ts2R inverted fluorescence microscope) with a diascopic and epi-fluorescence illumination model.

Data analysis

Data was initially processed in Microsoft Excel (version 16.70) to determine cell and parasite viability based on the absorbances recorded from the plate reader. Statistical analyses were performed using the GraphPad Prism software (version 10.0.0). The Kolmogorov-Smirnov test was performed to verify the normality of the data. Based on this information, a parametric ANOVA test or its non-parametric counterpart (Krusjal-Wallis) was performed. Significance was set at $p < 0.05$.

RESULTS AND DISCUSSION

Obtention of the metacyclic infective form of *L. mexicana.*

Leishmania metacyclogenesis is a crucial process for these parasites as it allows them to transition from a non-infective procyclic promastigote to an infective metacyclic promastigote, which can infect a potential host and transform into an intracellular amastigote. Mounting research evidence suggests that this is a complex process that takes place in the gut of the sandfly vector, and the details of this process can be reviewed elsewhere (Dostálová & Volf, 2012; Serafim et al., 2021). Replicating metacyclogenesis *in vitro* is important for screening potential drugs both in the free and the intracellular forms of the parasites, as it maintains parasite fitness and infection capability (Cohen & Azas, 2021; Zilberstein & Koren, 2019). Problems have been proposed, such as the loss of relative virulence in various *in vitro* species of leishmania (Ali et al., 2013; Moreira et al., 2012; Ringelmann & Heym, 1991; Santarém et al., 2014). Also, the generation of infective metacyclic promastigotes have been reported to be successfully replicated in certain medium.

Because of the characteristics lost in virulence and the need to generate the infective stages of our parasites, *L. (L.) mexicana* parasites were changed from the SDM to RPMI to allow their growth in this new medium. The results showed that parasites grew slower in RPMI compared to SDM. This resulted in a one-day increase in the incubation period to reach the stationary growth phase (Fig. S1). Something to note is that parasites started to die out after the three-days incubation period in SDM. This was not observed in parasites completely growing in RPMI, as they reached the late log phase at four days and were more resilient to death even on the fifth day (Fig. S1). Parasites accommodated to RPMI could differentiate in a larger proportion to the metacyclic stage with slender bodies and longer flagellum, characteristics that were less frequent in the parasites in SDM (Fig. S2). This observation has also been reported for other *Leishmania* species in which cultivation in this media allows for the better obtention

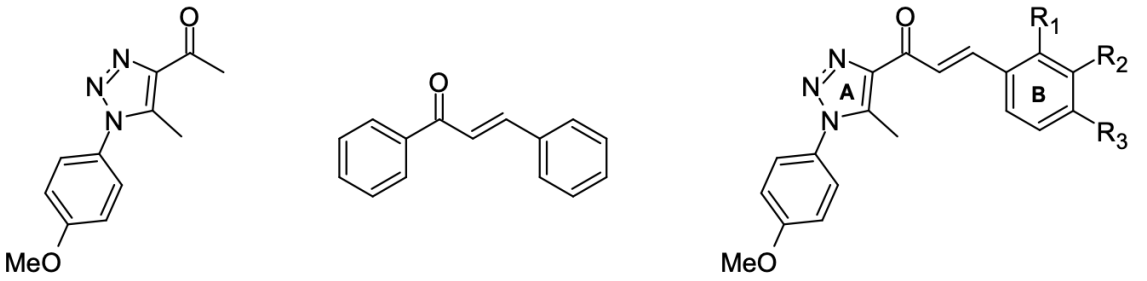
of the metacyclic form, which keeps the parasites' infection capacity as well as its usefulness for research purposes (Castelli et al., 2023; Santarém et al., 2014)

Numerous studies have been reported on methodologies to obtain and select metacyclic forms of the parasites which can be used to perform infection and drug screening assays (Chanmol et al., 2022; Da et al., 1987; Mendes et al., 2019). We were able to obtain a large proportion of metacyclic promastigotes by accommodating parasites in RPMI to perform our drug assay and to proof that infection can take place in the RAW264.7 murine cell line; however, at this stage, we were not interested in isolating and quantifying the conversion rate, our efforts were focused on testing compounds in mostly metacyclic forms. For future studies that might include testing in intracellular amastigote forms, this step must be performed to have a better model of a *Leishmania* infection.

Leishmanicidal and cytotoxic activity of TZL-CH derivatives

First, we investigated the activity of the nineteen TZL-CH derivatives based on the backbone showed in Fig. 1. They were tested on the metacyclic form of *L. (L.) mexicana* parasites. All compounds were tested in 5 different 10-fold dilution concentrations (0.01–100 μM). Figure 1 and Table 2 show the IC_{50} value and the 95% confidence interval of the most active compounds that were calculated from the range of concentrations tested. For the other compounds, it was not possible to determine their IC_{50} as there was no significant reduction in the viability of the parasites even at 100 μM (Table 2, Fig. S4). From this data, two TZL-CHs are the most active: CH02 (IC_{50} = 15.84 μM) and CH09 (IC_{50} = 13.43 μM), with some high to moderate activity against metacyclic promastigotes of *Leishmania* parasites. The other compounds had a moderate activity (IC_{50} > 20 μM), with CH05 (IC_{50} = 66.75 μM) being the least active among these nine compounds (Table 2, Fig. 1). The IC_{50} values obtained for our compounds have a comparable distribution of the biological activity reported for other type of

CH-derivatives against *L. mexicana* (Alkhaldi, 2023; Zheoat et al., 2021) as well as other *Leishmania* species (Bello et al., 2011; Garcia et al., 2021; Gomes et al., 2017).



Tyr **CH 0** **CH 01 – CH 21**

CH 01	$R_1= R_2= R_3 = H$	**CH 08**	$R_1= R_2= H, R_3 = CF_3$	**CH 16**	$R_1= R_2= H, R_3 = SCH_3$
CH 02	$R_1= R_3= H, R_2 = F$	**CH 09**	$R_1= R_3 = H, R_2= NO_2$	**CH 17**	$R_1= R_2= H, R_3 = OCF_3$
CH 03	$R_1= R_2= H, R_3 = F$	**CH 10**	$R_1= NO_2, R_2= R_3 = H$	**CH 18**	$R_1= H, R_2=$ O-Bz, $R_3= F$
CH 04	$R_1= CF_3, R_2= R_3 = H$	**CH 11**	$R_1= OH, R_2= R_3 = H$	**CH 19**	$R_1= OCH_3, R_2= R_3 = H$
CH 05	$R_1= F, R_2= R_3 = H$	**CH 12**	$R_1= H, R_2=OCH_3, R_3=F$	**CH 20**	$R_1= R_2= H, R_3= OCH_3$
CH 06	$R_1= R_3 = H, R_2= CF_3$	**CH 13**	$R_1= R_2= H, R_3 = Br$	**CH 21**	$R_1= R_2= H, R_3= N-(CH_3)_2$
		CH 14	$R_1= R_2= H, R_3 = NO_2$		

Fig. 1: CH-derivatives were based on this TZL ring (Tyr). Two substituents remained constant for the TZL ring **A** (*p*-anisyl and CH_3). Modifications were performed in ring **B** of the structure to test for potential leishmanicidal activity. Compounds were synthesized by C-S condensation. CH 0 was used as a reference for classic CH derived compounds.

Table 2: Cytotoxic and leishmanicidal activity of CH-derived compounds determined by MTT colorimetric test. Compounds were tested for 48 hours in *L. mexicana* metacyclic promastigotes (4-5 days old cultures) and RAW 264.7 murine macrophage. CC_{50} and IC_{50} were determined from three independent assays in triplicates using GraphPad Prism 10 from a non-linear regression model.

Compound	**IC_{50} (µM)**	**IC_{50} 95% CI (µM)**	**CC_{50} (µM)**	**CC_{50} 95% CI (µM)**	**SI (CC_{50}/IC_{50})**
CH 01	26.71	17.33 – 41.17	9.09	7.63 – 10.82	0.34
CH 02	15.84	11.56 – 21.70	10.88	9.23 – 12.81	0.69
CH 03	> 100.00	ND	ND	ND	ND
CH 04	> 100.00	ND	ND	ND	ND
CH 05	66.75	52.93 – 76.19	73.96	59.49 – 91.96	1.11
CH 06	33.42	26.19 – 42.64	7.19	6.13 – 8.42	0.22
CH 08	60.85	48.20 – 76.82	20.98	16.75 – 26.27	0.34
CH 09	13.43	9.69 – 18.59	5.67	4.76 – 6.76	0.42
CH 10	> 100.00	ND	ND	ND	ND
CH11	53.22	34.56 – 81.97	4.06	3.53 – 4.67	0.08
CH12	> 100.00	ND	ND	ND	ND
CH 13	> 100.00	ND	ND	ND	ND
CH 14	> 100.00	ND	ND	ND	ND

CH 16	> 100.00	ND	ND	ND	ND
CH 17	> 100.00	ND	ND	ND	ND
CH 18	25.83	15.56 – 42.90	1.95	1.46 – 2.59	0.08
CH 19	29.56	18.83 – 46.43	4.76	3.97 – 5.71	0.16
CH 20	> 100.00	ND	ND	ND	ND
CH 21	> 100.00	ND	ND	ND	ND
CH 0	> 100.00	ND	ND	ND	ND
Tyr	> 100.00	ND	ND	ND	ND

Compounds synthesized for this study have an α, β-unsaturated ketone linking a phenyl and a TZL rings, resembling a CH (Fig. 1). The TZL scaffold by itself did not have any effect on the viability of the promastigotes at the concentration tested (Tyr, IC_{50} > 100 µM, Table 2, Fig. S4). The presence of the two rings linked by the α, β-unsaturated carbonyl has previously been identified as the part of the CH structure responsible for its biological activity (Dhaliwal et al., 2022). Our compounds, due to the synthetic route proposed, inherently had the *p*-anisyl and methyl substituent attached to the TZL ring. These two modifications on this heterocycle ring showed that our base scaffold (CH01) with no modifications to the **B** ring ($R_1 = R_2 = R_3$ = H) can have significant leishmanicidal and cytotoxic activity (IC_{50} < 30.0 µM, CC_{50} < 10.0 µM, Table 2, Fig. 2 - 3) compared to the CH CH 0 (IC_{50} > 100 µM). This makes our scaffold and synthetic route proposed an ideal initial molecule that can be fined tune for both leishmanicidal and cytotoxic activity.

At this stage in our study, we were interested in the effect that changes to the phenyl ring **B** of our TZL-CH may have on its biological activity (Fig. 1). Further modifications to the TZL ring **A** were not explored. Only nine of the TZL-CHs evaluated showed activity against leishmania parasites, and most of them have fluorine in their structures. The two most active compounds CH02 and CH09 (IC_{50} < 20.0 µM, Table 2, Fig. 1–2) have electron withdrawing substituents in *meta* position, fluorine, and nitro group, respectively. It is worth mentioning that the addition of NO_2 groups in either ring of the CH moiety has been one of the major

strategies to modify the biological activity of this class of compounds (Assolini et al., 2020; de Mello et al., 2018; Salehi et al., 2021).

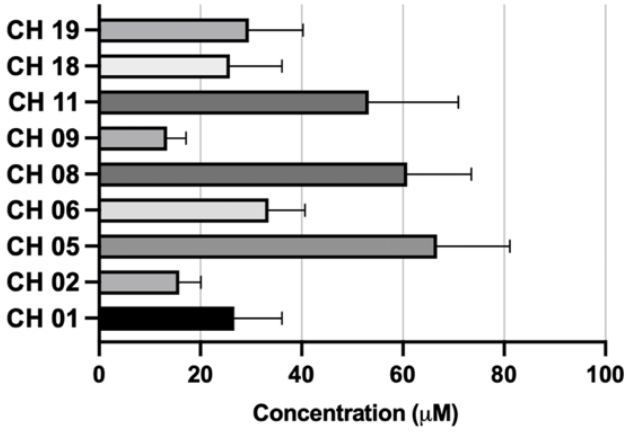


Fig. 2: IC_{50} of the most active CH derivates against promastigote forms of *L. mexicana* parasites maintained in sRPMI. Values were calculated from MTT colorimetric assay using RPMI as the control group to calculate the viability percentage. A non-linear regression model was fitted to the results using the Log (Concentration) and percentage viability as the X and Y axis respectively in PRISM 10.0 (Fig. S3).

The next moderately active compounds are CH01, CH05, CH06, CH08, CH11, CH18, and CH19 (IC_{50} < 100.0 µM, Table 2, Fig. 2). It was observed that the modifications on these compounds (Fig. 1) compared with CH01 had a varied effect on its biological activity against the *L. (L.) mexicana* parasites as their activity was both slightly and significantly reduced depending on the substituents. Regardless of the type of substituent, *meta*-substituted compounds are shown to be more active than *ortho*- and *para*-substituted compounds. For example, comparing the activity of one of our most potent compounds (CH02) with its fluorine atom in the *meta* position to CH05 with the same substituent in the *ortho* position, a significant reduction in activity is observed from 15.84 to 66.75 µM (Table 2, Fig. 2). A similar trend was observed for CH04, CH06, and CH08, which have a -CF_3 substituent in *ortho*, *meta*, and *para* positions, respectively. On the other hand, only compounds CH11 and CH19 with electron-donating substituents OH and OMe in the *ortho* position, respectively, displayed some activity. In the case of the nitrated compounds CH09, CH10, and CH14, only CH09 (*meta*-substituted)

exhibited activity; however, the presence of the NO_2 group in another position completely diminished their biological activity (CH10 and CH14).

The potential mechanisms of action for this type of molecules have not been well stablished, however some evidence suggests that they might interfere with some dehydrogenases and fumarases in the parasitic respiration chain (Ming et al., 2001; Zhai et al., 1999).

As has been observed for our compounds, the position of the substituents tested had a wide range of effects on our initial scaffold. Similar trends were also noted by Tajuddeen and colleagues in their review of the CHs with potential biological activity (Tajuddeen et al., 2018). Further analysis, such as determining their Structure Activity Relationship (SAR) with enzymes and cellular environment, can shed some light on our observations. For example, an improvement in other CH-derived molecules' biological activity has been attributed to the electronegative property of fluorine atoms and its inducible effects, which render the molecule more lipophilic (Bello et al., 2011). Structural and protein-interaction studies should be performed to assess the potential of these molecules as lead compounds. Potential targets for CH derived compounds include the parasite arginase (Garcia et al., 2021) and other enzymes related to the respiration chain in these parasites (Chen et al., 1993; de Santiago-Silva et al., 2023).

The cytotoxic activity of the most active leishmanicidal compounds was tested on murine macrophages RAW 264.7 (Table 2 and Figure 3). Compounds showed much higher activity against our murine cell line, which resulted in a low SI (SI < 1.5); this represents a problem when testing the compounds in an intracellular amastigote model. In a separate study, similar findings were observed for the cytotoxic profile of other CHs with similar characteristics on their **B** ring (González et al., 2020). Before testing the compounds in a murine infection model, the SI should be improved. It is well known that an SI > 10.0 is suitable for a

potential leishmanicidal agent. In this study, compounds were only modified at the B ring site of the molecule; in other studies, modifications to the A ring have shown a significant improvement in the tolerability of CH-derived compounds (Tajuddeen et al., 2018) as well as an improvement in their leishmanicidal activity (de Mello et al., 2018). In the review done by Tajuddeen, it was pointed out that adding bulky substituents in specific positions in the A ring can effectively reduce the cytotoxic activity in lymphocytes, while the study by de Mello found that electronegative groups on this same ring can substantially increase leishmanicidal activity; thus, selection of appropriate groups should be done as the A-ring modifications can influence both the CC_{50} against mammalian cells and IC_{50} against *Leishmania* parasites (Boeck et al., 2006; de Mello et al., 2018; Liu et al., 2003). Some of such strategies mentioned in the literature are the addition of more oxygenated groups, long alkyl chains, or even naphthalene-based CHs (de Santiago-Silva et al., 2022; Hernández-Rivera et al., 2023).

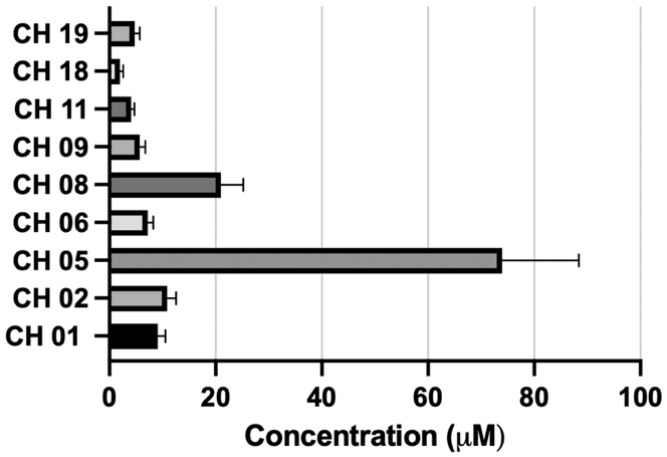


Fig. 3: CC_{50} on RAW 264.7 murine macrophage of the most active CH derivates against promastigote forms of *L. mexicana* parasites. Values were calculated from MTT colorimetric assay using DMEM as the control group to calculate the viability percentage. A non-linear regression model was fitted to the results using the Log (Concentration) and percentage viability as the X and Y axis respectively in PRISM 10.0 (Fig. S4).

Proof-of-concept: Infection model using *L. mexicana* on the murine macrophage (RAW 264.7).

The most common strategy for finding candidates against *Leishmania* parasites is phenotypic screening. In this regard, the next step towards searching for a potential leishmanicidal compound after determining its activity against the promastigote form is to test it on the intracellular form of the parasite. As mentioned earlier, compounds tested for this study did not have a great selectivity index, which could render interpretations from results on intracellular amastigotes as meaningless. Despite this, we aimed to establish an infection model for RAW 264.7 and *Leishmania (L.) mexicana*. From preliminary results, RAW 264.7 cells were able to support an infection from the *L. mexicana* parasite. Distinctive features were found in the DAPI staining screening, such as the nuclear and kinetoplast DNA of the *Leishmania* parasite in the proximity of RAW 264.7 macrophages (Figs. 4C and 4D). On the Giemsa-Wright based screening, the features observed with DAPI were also visible under the light microscope. In addition to this, the cell boundaries can also be observed (Figs. 4A and 4B). This preliminary assay confirms that *L. mexicana* conserved *in vitro* at our laboratory can transform into both metacyclic promastigotes (Fig. S2) and intracellular amastigotes in an active infection in RAW 264.7 (Fig. 4, Fig. 5).

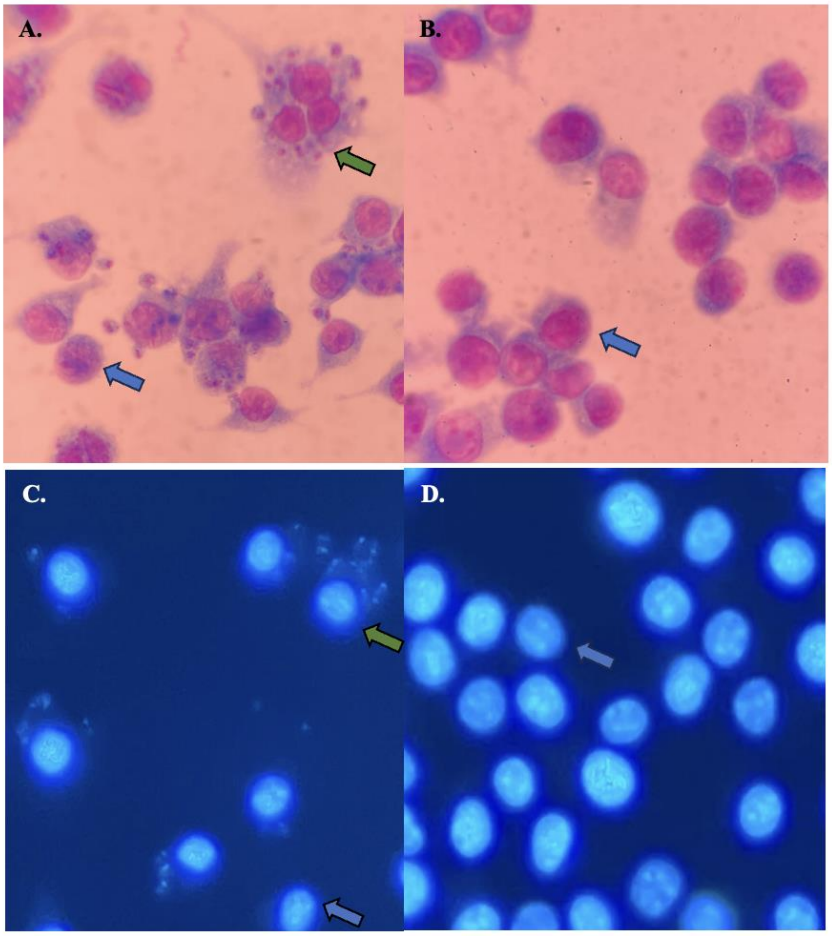


Fig. 4: RAW 264.7 infection model with *L. mexicana* parasites. Infection was performed with parasites at their late log phase (4th day of incubation), where mostly metacyclic promastigotes can be found (Fig. S2). The MOI employed was 10:1, cells were allowed to adhere to the plate overnight, and infection was allowed to take 8 hours at 32 ºC, 5% CO_2, and in a humid atmosphere. Free promastigotes were washed and allowed to continue the infection for 48 hours. Giemsa-Wright staining was performed with a 10% G-W solution (20 minutes) after the cells were fixed for 30 seconds in pure methanol. DAPI staining was done with a 1:2000 dilution from a DAPI solution (1 mg/mL) for 1 minute after cells were fixed in paraformaldehyde (4%) for 15 minutes. Blue arrows represent non-infected cells pointing to the nuclei and the cytoplasm. Green arrow shows an infected murine macrophage. (**A and C**) Model of infection plate. (**B and D**) Negative controls.

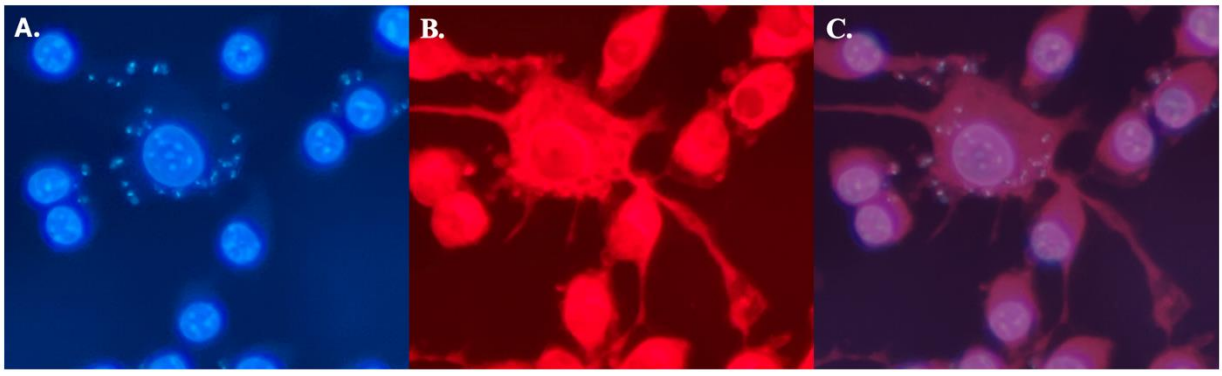


Fig. 5: RAW 264.7 infection model with *L. mexicana* parasites. Infection was performed with parasites at their late log phase (4th day of incubation), where mostly metacyclic promastigotes can be found (Fig. S2). The MOI employed was 10:1, cells were allowed to adhere to the plate overnight, and infection was allowed to take 8 hours at 32 ºC, 5% CO_2 and humid atmosphere. DAPI and Cell-Mask staining was done with a 1:2000 dilution from a DAPI solution (1 mg/mL) and 20 (µg/mL) for 30 minutes after cells were fixed in paraformaldehyde (4%) for 15 minutes. **A.** DAPI only. **B.** Cell mask only and **C.** Merge image.

CONCLUSION

In this study, the evaluation of the leishmanicidal and cytotoxic activity of 19 triazole-CH derived compounds was reported. It was found that nine compounds showed low to moderate activity against the *L. (L.) mexicana* parasite in its promastigote form. From these active compounds against the parasites, the cytotoxic activity was characterized, and all compounds showed higher activity against RAW 264.7 macrophages compared to their leishmanicidal activity, except for one compound (CH 05), which showed relatively the same activity for both parasites and murine macrophages. This resulted in a very low selectivity index (< 1.11) for all the compounds. Their biological activity was explained based on the electronegative properties of the decorating groups in ring B of the CH-derived compounds, with special attention to their position in the phenyl ring. The two most active compounds were CH 02 and CH 09; they have electron-withdrawing substituents in *meta* position, fluorine, and nitro group, respectively, which influenced their activity. Improvements can be considered for these two compounds, as they are potential scaffolds for developing molecules with high leishmanicidal activity and low cytotoxicity. A rational approach would be to create variants with A-ring modifications, as some modifications can cause their cytotoxicity profile to be significantly better. In addition, SAR studies could be performed to test for modifications before being tested *in vitro*.

Finally, confirming that infection could be possible with our *Leishmania (L.) mexicana* and the murine cell line in our laboratory can pave the way to generate more clinically relevant *in vitro* models for further compound testing. This process can be automated with appropriate software tools to improve the screening for leishmanicidal compounds for endemic species circling in Ecuador.

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SUPPLEMENTARY INFORMATION

TABLE OF IMAGES

FIGURES

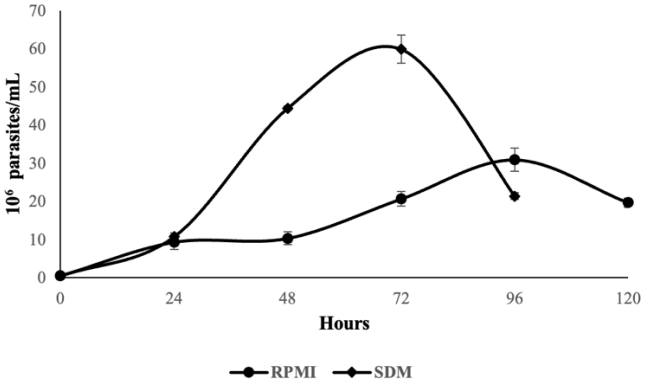

Fig. S1: Growth curve of *Leishmania (L.) mexicana* accommodated to sRPMI. For comparison, growth curve in sSDM is also shown. Cell densities were significantly higher at 48 and 72 hours with much higher growth in sSDM medium. After 72 hours, parasites in this medium quickly died out. Results shown represent three replicates taken at different time points.

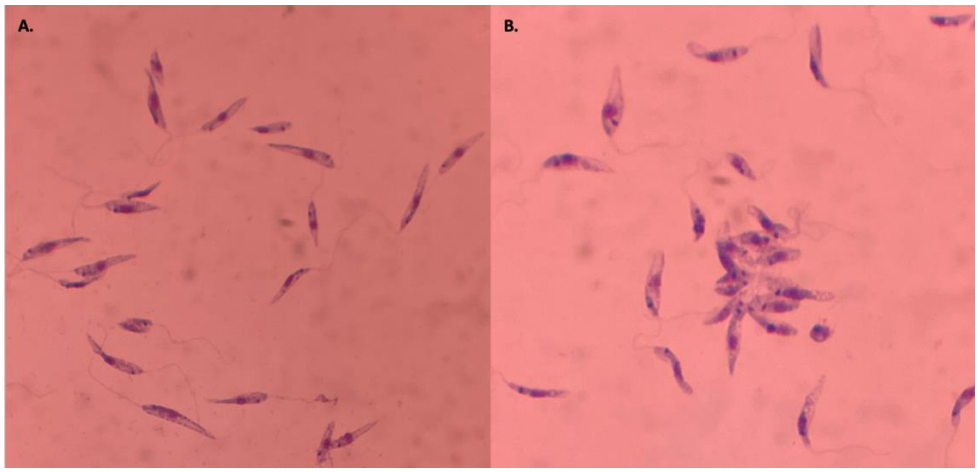

Fig. S2: Giemsa micrograph of leishmania parasites at different development stages. Magnification 10.000 X. **A**. Mostly metacyclic *L. mexicana* forms observed with elongated body shape and kinetoplast DNA closer to the flagellum. Parasites were obtained from RPMI-1640 medium at 4 days of incubation time in monophasic medium, after 2 days incubation time in biphasic medium (Sheep blood agar/sRPMI). **B**. Procyclic *L. mexicana* forms observed with an ovoid shape and kinetoplast DNA closer to the nuclei. Parasites were obtained from SDM medium at 3 days of incubation time in monophasic medium, after 2 days incubation time in biphasic medium (Sheep blood agar/sRPMI)

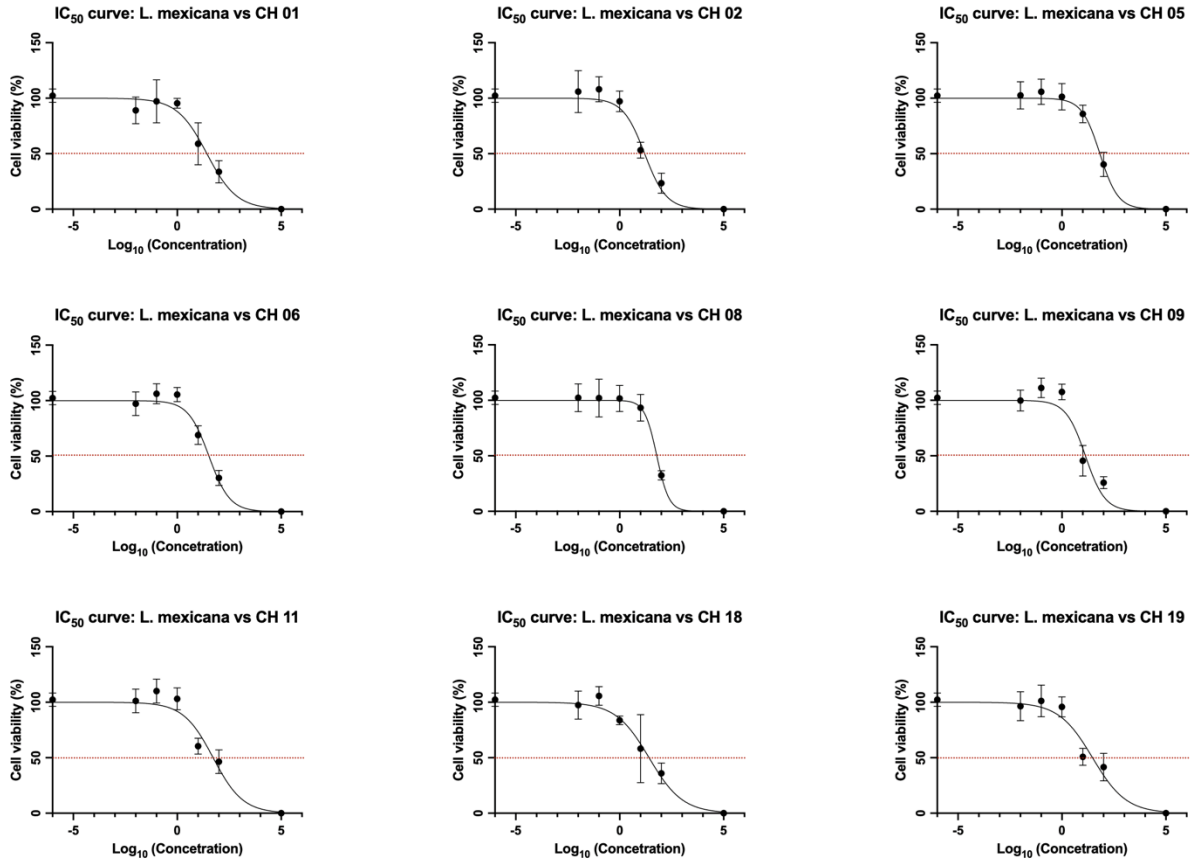

Fig. S3: Determination of IC_{50} from growth of *Leishmania mexicana* in the most active compounds. IC_{50} was determined from fitting a non-linear regression curve in PRISM 10.0. Detailed parameters are shown in the data analysis section.

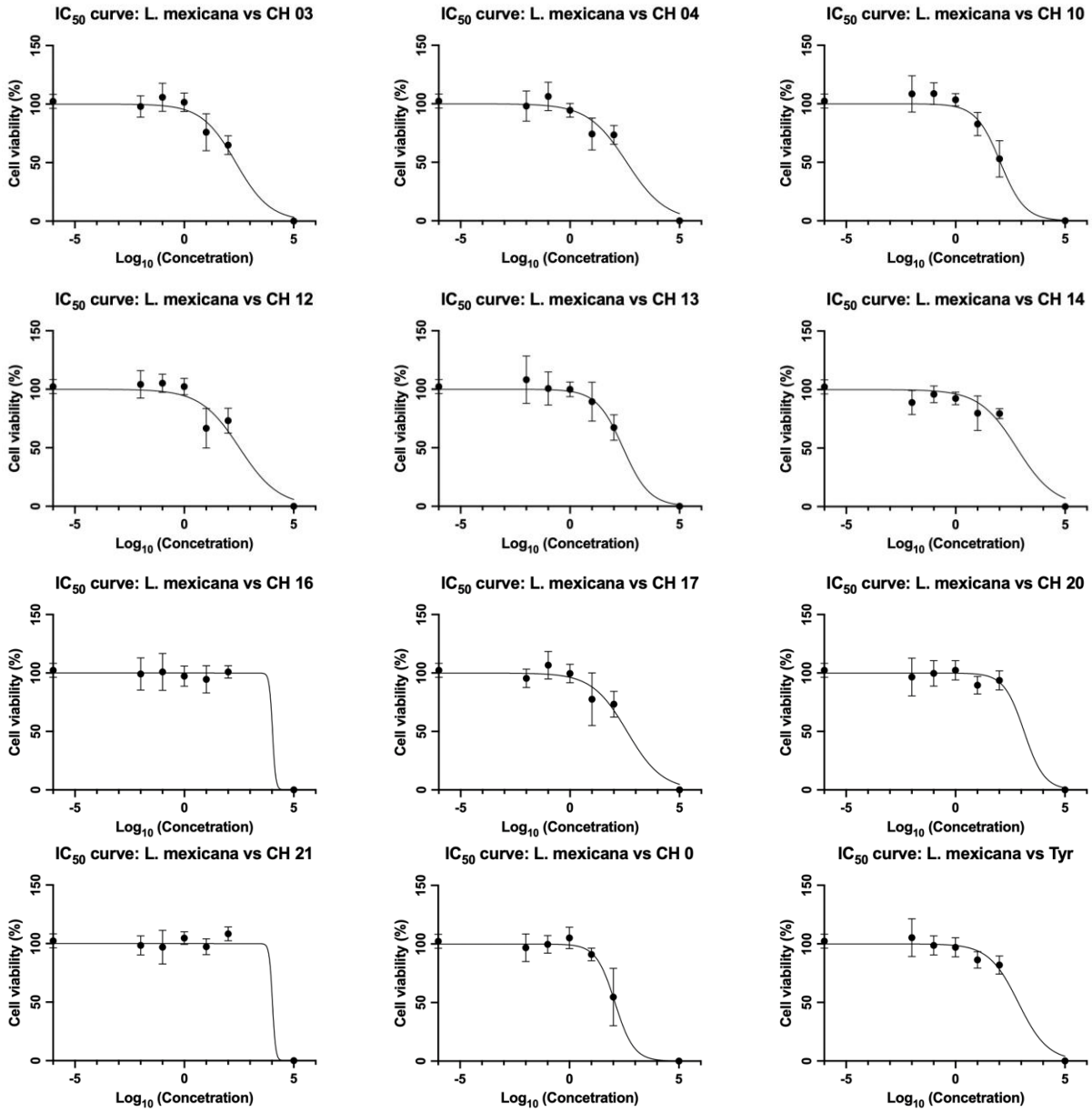

Fig. S4: Determination of IC_{50} from growth of *Leishmania mexicana* in non-active compounds. IC_{50} was determined from fitting a non-linear regression curve in PRISM 10.0. Detailed parameters are shown in the data analysis section.

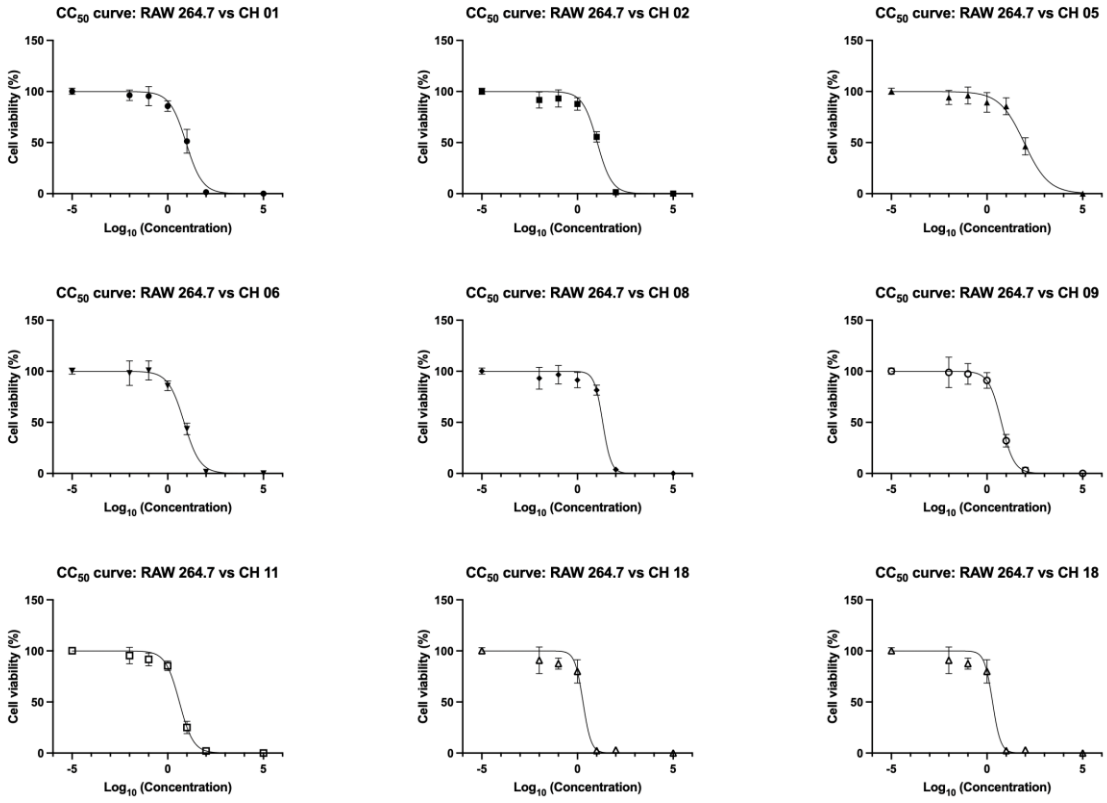

Fig. S5: Determination of CC_{50} from growth percentage of RAW 264.7 of most leishmanicidal compounds. CC_{50} was determined from fitting a non-linear regression curve in PRISM 10.0. Detailed parameters are shown in the data analysis section.