



**HAL**  
open science

## New method for screening anti-Leishmania compounds in plants extracts by HPTLC-bioautography

Valentin Hilaire, Gregory Michel, Alissa Majoor, Francis Hadji-Minaglou,  
Anne Landreau, Xavier Fernandez

### ► To cite this version:

Valentin Hilaire, Gregory Michel, Alissa Majoor, Francis Hadji-Minaglou, Anne Landreau, et al..  
New method for screening anti-Leishmania compounds in plants extracts by HPTLC-bioautography.  
Journal of Chromatography B - Analytical Technologies in the Biomedical and Life Sciences, 2022,  
1188, pp.123061. 10.1016/j.jchromb.2021.123061 . hal-03476640

**HAL Id: hal-03476640**

**<https://univ-angers.hal.science/hal-03476640>**

Submitted on 5 Jan 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

New method for screening anti-*Leishmania* compounds in plants extracts by HPTLC-bioautography.

Valentin Hilaire<sup>1,2</sup>, Gregory Michel<sup>3</sup>, Alissa Majoor<sup>3</sup>, Francis Hadji-Minaglou<sup>1</sup>, Anne Landreau<sup>2,4</sup>, Xavier Fernandez<sup>2</sup>

1 : BotaniCert, 4 traverse Dupont, 06130 Grasse, France

2 : Université Côte d'Azur, CNRS, Institut de Chimie de Nice, UMR 7272, Nice, France

3 : Université Côte d'Azur, Inserm, U1065, C3M, Nice, France

4 : Univ Angers, Univ Brest, GEIHP, SFR ICAT, F-49000 Angers, France.

## Abstract

*Leishmania* genus is responsible for leishmaniasis, a group of diseases affecting 12 million people in the tropical and subtropical zone. Currently, the few drugs that are available to treat this disease are expensive and cause many side effects. Searching for new therapeutics from plant species seems to be a promising path. This work proposes an original HPTLC test against parasites, in particular on *Leishmania infantum*, to screen new molecules from plant extracts. The technique uses protozoa transformed to express the luciferase gene to observe the bioautogram in bioluminescence. We have developed two different test protocols based on the two dimorphic stages of the parasite. The free promastigote stage, and an intracellular stage parasitizing macrophage cells called the amastigote stage. These two stages only survive under extremely different conditions which required the development of two very different test protocols. For the promastigote free stage of the protozoa, the direct bioautography technique was chosen while for the intracellular amastigote stage, bioautography by immersion (agar overlay) was required. Amphotericine B was chosen as the reference compound for this assay. The development of each of these two tests made it possible to clearly detect areas of activity on the bioautogram, allowing a rapid and inexpensive screening of the antiparasitic properties of molecules in natural extracts.

## Keywords

HPTLC, *Leishmania infantum*, Direct Bioautography, Agar overlay, plant extracts screening, Anti-*Leishmania*.

## 1 Introduction

Leishmaniasis are vector parasites of reticuloendothelioses. The causative agent is a flagellated protozoan belonging to the genus *Leishmania*. Leishmaniasis is considered by the WHO (World Health Organization) to be an emerging, uncontrolled, and neglected disease [1]. They represent the second highest cause of mortality and the fourth cause of morbidity from tropical diseases. The number of cases worldwide is very high (estimated at 12 million). There are more than twenty species of *Leishmania* around the world, leading either to an asymptomatic carriage, or to various clinical manifestations such as a simple skin papule, a spreading of the mucous membranes of the face or even a visceral stage which is lethal without treatment. The number of therapeutic molecules currently available is limited and they have significant undesirable side-effects [2]. The number of molecules authorized for the treatment of leishmaniasis is very low. Most of these molecules are from synthetic origin and are shared with other diseases. For example, amphotericin B was originally used as an antifungal [3]. However, this molecule is very expensive and has many side effects. In addition, resistance to molecules has appeared in parasites and cases of relapse have been described [4,5,6,7]. Furthermore, plants have always been a common source of drugs, either in traditional medical practices or as sources for purified active pharmaceuticals. The WHO considers more than 60% of the world's population predominately relied on plant-derived treatments in front line treatments [8]. Therefore, research of new antileishmanial drugs focusing on the use of plants seems a promising pathway.

Actual screening *in vitro* methods for anti-leishmanial compounds are 96 well plates assays. Detection methods can be microscopy [9,10] or coloring agents such as resazurin [11], MTT [12], or Alamar Blue® [13]. Some have modified *Leishmania* parasites transfecting  $\beta$ -lactamase gene in order to follow the apparition of a colorimetric compound [14,15]. These microwell screening techniques are well suited to screening libraries of pure molecules. However, these techniques are not efficient for studying plant extracts which are complex mixtures of molecules. Usually, the identification of new active plants compounds requires the preparation of various extracts of plants before assessing the activity of each molecule in the extract. Numerous bio-guided fractionation steps are ultimately necessary which are extremely time-consuming and costly in materials, reagents, and labor [16].

The aim of this work was to present a faster and more effective screening strategy for anti-*Leishmania* bioassays of plant extracts. We have succeeded in developing an HPTLC-bioautography method, including a new biological activity screening test [17,18]. This method has never been adapted to protozoa. Each molecule of the extract can therefore be

evaluated separately from each other and the tedious steps of conventional bioguided fractionation are eliminated. The method has been described and adapted to each of the parasite lifecycle, promastigote and amastigote.

## 2 Material and methods

### 2.1 High-performance thin-layer chromatography

All solvents used for plate preparation were of analytical grade from Carlo Erba Reagents (Cornaredo MI, Italy). Amphotericin B (fungizone<sup>®</sup> from Bristol-Myers Squibb (New York, USA)) solution (100µg/ml) was applied with CAMAG ATS4 (Muttenez, BL, Switzerland) on pre-washed (MeOH/H<sub>2</sub>O (8/4 ; v/v)) HPTLC silica gel plates 60 F254 Merck (Darmstadt, Hesse, Germany) on four spots with different concentrations (2 µl, 4 µl, 6 µl and 8 µl applications). Plates were developed with MeOH/H<sub>2</sub>O (90/10 ; v/v) mobile phase on CAMAG ADC2. Photos of the developed plate were taken with CAMAG TLC Visualizer 2 at 366nm.

For the separation of the plant extracts, a raw methanolic extract (1/10 ; v/v) of a plant and an non-polar fraction of this extract were applied on a prewashed HPTLC plate in two separate spots (2 µl). The plate was developed with toluene/acetone (4/1 ; v/v) mobile phase. Photos of the derivatized (Natural Products reagent) plate were taken with CAMAG TLC Visualizer 2 under visible light.

### 2.2 *In vitro* inhibition test on promastigote stage

*Leishmania infantum* MON-1 parasites (MHOM / FR / 94 / LPN101) were isolated from a patient with visceral Leishmaniasis in the Nice region. and 1.6 kb of the firefly luciferase coding region has been added to its genome [19].

Preparation of Culture Medium: The promastigote stage was cultured in Schneider's Insect Medium<sup>®</sup> from Sigma Aldrich (Saint-Louis, MI, USA). In To this medium the following were added: 0.4 g/L of NaHCO<sub>3</sub> from Acros Organics (Antwerp, Belgium), 0.6 g/L of CaCl<sub>2</sub> from Fluka chemie (Buchs, Switzerland), 10% of fetal calf serum, 10 ml of a human urine pool, 0.1% of phenol red from Sigma Aldrich (Saint-Louis, MI, USA), 1 % 1M HEPES pH 7.3, 1% of respectively penicillin and streptomycin, 1% L-glutamine all three from GIBCO Thermo Fisher (Waltham, USA).

Verification of bioluminescence: 1 ml of parasite suspension at  $1.10^7$  parasites/ml (p/ml) was collected and centrifuged. The pellet was resuspended in 100 µl of complete schneider medium added to 100 µl of luciferin from Perkin (Waltham, USA) 8 mg/ml. The suspension

was placed in a well of a 96-well microplate stirred for 5 s and read by luminescence (Gen5 (BioTek, Winooski, USA)).

The developed HPTLC plate was immersed in the parasite suspension at  $1.107 \text{ p/ml}$  using a narrow development and soaking tank  $100 \times 100 \text{ mm}$  from Bionis (Houdan, Yvelines, France), and placed in a wet atmosphere box in the incubator at  $26^\circ\text{C}$  for 6h. After these 6 hours, the plate was immersed in a solution of luciferin at  $8\text{mg/ml}$  and read directly in the Fuji LAS4000® from Fujifilm Life Science (Valhalla, NY, United States) in luminescence mode with a time of exposure of 50s.

### 2.3 *In vitro* inhibition test on amastigote stage

THP-1 cells (ATCC® TIB-202™) are derived from a pre-monocytic human cell line, derived from a child with leukemia. They were cultivated in RPMI Medium 1640® Invitrogen GIBCO® medium from GIBCO Thermo Fisher (Waltham, USA) to which 10% fetal calf serum, 1% glutamine, 1% sodium pyruvate, and 1% penicillin and streptomycin all four from GIBCO Thermo Fisher (Waltham, USA) have been added.

To avoid adhesion to the walls of the culture flasks, cells have been activated with vitamin D<sub>3</sub>. THP-1 cells were activated with  $100\mu\text{l}$  of vitamin D<sub>3</sub> at  $250\text{ng/ml}$ . The activation time was three days long. Activated THP-1 cells were infected by parasites in the promastigote stage. by introducing 20 promastigote parasites into the cell culture for one THP-1 cell (MOI 20). The infection time was 24 hours.

A total of 20 ml of Ficoll® from Cytiva (Marlborough, USA) was placed in respectively two 50 ml centrifuge tubes and the suspension of infected THP-1 cells was split in two ( $2 \times 20 \text{ ml}$ ) and each half was deposited delicately on the surface of Ficoll® using a pipette. Each of the tubes were centrifuged at 2000 rpm for 30 min (acceleration 1 and braking 0). Cells were collected at the Ficoll® / medium interface and washed twice with sterile PBS. The pellet was collected and suspended in 10 ml of THP-1 medium. The cells were counted using a Glasstick® slide from Kova international (Garden Grove, USA).

The developed plate was placed in a  $100 \text{ mm} \times 100 \text{ mm}$  wet box. The suspension was diluted or concentrated to obtain a final concentration of  $1.10^8$  cells in 5 ml of THP-1 medium. This mix was added to 5 ml of liquefied 1.2% agar and homogenized to be finally poured onto the plate. The plate was incubated at  $37^\circ\text{C}$  for 24 h. At the end of these 24 h, the agar was covered with luciferin solution at  $8 \text{ mg/ml}$  and 20 min later the dish was photographed in luminescence mode during 50 s placed in the Fuji LAS4000®.

## 3 Results and discussion

### 3.1 Choice of the HPTLC-bioautography method

Among other screening methods, HPTLC-bioautography has been chosen because in this technique the constituents of complex mixtures are first separated by high performance thin layer chromatography (HPTLC), which is already widely used in phytochemistry for quality control, followed directly by the contact with microorganisms. After revelation, spots appear on the bioautogram corresponding to molecules exhibiting an anti-parasitic activity. In addition, the chromatography step will allow to separate molecules that could have antagonistic effects inside the plant extract and whose activity had not been yet revealed. Likewise, this process will also separate the molecules whose activity could have resulted from a synergy with other various compounds. This method was therefore particularly suitable for the discovery of new, active, pure compounds to promote as future drug candidates. Three types of bioautography currently exist [20]. Two of them have been used in this assay development.:

The first method is direct bioautography, in which the microorganism of interest is applied directly onto the developed HPTLC plate surface.

The second bioautography method used is agar overlay: a layer of agar seeded with a given concentration of microorganisms was poured and gelled directly on the plate where the plant extracts had been separated. The active molecules included in the extracts then diffused off the plate and through the agar to interact with microorganisms.

### 3.2 Methods adaptation to parasite stages

As the parasite exists in two different stages, the search for active molecules therefore took place in two different ways. The survival conditions of promastigotes being less demanding than those of amastigotes, thus direct bioautography was chosen for the promastigotes (Fig.1).

Conversely, the agar overlay technique proved to be the best option for attest inhibition of amastigotes. This method fixes the cells and nourishes them properly during this time (Fig.2).

For this development, a strain of *Leishmania* transfected with the gene encoding the enzyme luciferase [19] was used. Thus, the viability of the parasites was directly proportional to the number of photons picked up by the detector of the Synergy II plate reader when the *Leishmania* parasites are brought into contact with the luciferase substrate, luciferin. This molecule is oxidized by luciferase resulting in the production of photons. When the parasites are dying, the photon emission is no longer possible and the decrease in luminescence is directly proportional to the antileishmanial activity of the molecule on the parasites [19].

### 3.3 Test with a standard

The test was developed with an amphotericin B standard as a positive control, which is the gold standard in the treatment of leishmaniasis. The amphotericin B stock solution was chosen at a concentration of 100 µg / ml. Deposits of increasing volumes were made on the plate: 2µl, 4µl, 6µl and 8µl. The plate was then developed using the mobile phase: MeOH / H<sub>2</sub>O (90/10; v / v) allowing the migration of amphotericin B.

### 3.4 Development of *In vitro* inhibition test on promastigote stage

Since HPTLC-bioautography has never been developed for protozoa, the first step in development was to ascertain that promastigotes could survive on the Silica gel plate by doing a test plate with just one spot of amphotericin B and luminescence revelation. Once this step was successful, the adequate concentration to have a sufficiently bright background was searched and set at 10<sup>7</sup> parasites/ml. At this concentration, the active areas were clearly visible. The luciferin concentration was set at 8 mg/ml. Several contact time assays were carried out to find the optimal contact time between the eluted plate and the parasite. It has been found that 6 h was a good compromise between having a fast test and suitable results. Migrated amphotericin B treated under these conditions showed black areas on a luminescent background. Evaluation of the different concentrations of this latter has permitted to determine its proper limit of detection for this test. In fact, the lowest concentration deposited (2 µl in a final solution of 100 µg/ml, i.e. 200 ng deposited on the plate) did not produce visible black spot on the plate while the 2 times higher concentration allowed it (400 ng deposited on the plate) (Fig.3).

### 3.5 Development *In vitro* inhibition test on amastigote stage.

In contrast to most anti-*Leishmania* screening methods, this method uses live, in-host, amastigotes. Usually, to screen the anti-leishmanial activities on the amastigote stage, many scientists use axenic *Leishmania* parasites, which are free forms of the parasite simply approaching the amastigote stage. This use is now criticized [23] because these forms do not exactly mimic the metabolism of the real amastigote stage which is intracellular. For several years now, the C3M research laboratory is working on real amastigote stages that mimic reality more faithfully. However, these stages are extremely delicate to produce and maintain. As the amastigote stage of the parasite can only survive in a host cell, the test conditions have had to be adapted. Longer time was needed for the penetration of the molecule towards and inside the cell to make contact with the parasite, but the THP-1 cells did not survive this time on the silica surface (maybe the nutrient was not enough), therefore agar overlay bioautography was chosen. This method has allowed the cell to remain in a viable environment during the entire contact time. As before, the test was developed with a

plate containing several deposits of an amphotericin B solution (100 µg/ml) of increasing volumes. After all these steps, an 8 mg/ml luciferin solution was poured over the agar before revelation. Kinetic curve has been done to register the delay caused by the luciferin to diffuse through agar and reach the amastigote stage. The plateau of the curve was reached after 20 minutes. Finally, the plates were then photographed 20 minutes after the luciferine pouring. Dark spots corresponded to inhibition zones of amastigote parasites (Fig.4).

This stage has required a much more advanced test development methodology than the promastigote stage given that the development time was much longer. Indeed, many difficulties were overcome during the development of this test, in particular, first the challenge to obtain living and infected cells in the agar gel and then specifically to find the right concentration of cells allowing to obtain an exploitable signal.

### 3.6 Tests on plant extracts

To achieve the development of the test, these methods were carried out also on plant extracts to ascertain the correct progress and the appearance of active molecules in complex matrices. The test was carried out on a total methanolic plant extract as well as on a fraction containing only the non-polar molecules. This plant contains antibacterial compounds and has been screened for anti-leishmanian activity. One molecule in particular was targeted by this test. It is a terpenoid-type compound (revealed in violet with anisaldehyde) and eluting at  $R_f = 0.5$  with the toluene / acetone (4/1; v / v) mobile phase.

A dark spot is visible at  $R_f = 0.5$  after incubation with both promastigote and amastigote stage test methods. These two methods have thus successfully revealed a single compound which shows anti-leishmanian activity for further study (Fig.5).

### 3.7 Advantages and limits

On both obtained bioautograms, a dark spot (at  $R_f = 0.5$ ) has indicated a zone where a molecule with anti-leishmanian activity has migrated. This molecule has showed an activity on both stages of the parasite because of the dark spot appearing on both bioautograms. From these results, the plant extracts evaluation has attested that the assays' methods were suitable for use in the research of natural anti-leishmanian compounds.

One issue is that activity zones appeared slightly diffuse for the amastigote assay. One solution could be to further optimize mobile phase conditions inducing a greater selectivity to obtain an improved separation of the compounds from each plant extract. Otherwise, an alternative could be to use a direct bioautographic method with small amount of agarose (e.g. 0.05%) in the cell suspension in order to fix more medium on the silica plate.

#### 4 Conclusion

This work has enabled the development of an innovative HPTLC-Bbioautography assay to identify anti-leishmanial compounds in crude plant extracts. These types of microorganisms have never been used before in this type of biochemical test. It constitutes a new tool in the search for new natural molecules in the fight against both stages of leishmaniasis: searching for systemic medicines to treat the human disease using the amastigote stage test and for topical medicines to prevent the infection of the parasite using the promastigote stage test. As this test works properly, even at the amastigote stage, these methods could be applied to other intracellular pathogenic microorganisms.

#### 5 Acknowledgement

Authors greatly acknowledge the French Association Nationale de la Recherche et de la Technologie (ANRT) for supporting the CIFRE partnership Botanicert - Université Côte d'Azur.

The authors declare no conflict of interest.

Author names and affiliations.

Valentin Hilaire Université Côte d'azur / BotaniCert

Gregory Michel Université Côte d'azur / INSERM

Alissa Majoor Université Côte d'azur / INSERM

Anne Landreau Université d'Angers/GEIHP

Francis Hadji-Minaglou BotaniCert

Xavier Fernandez Université Côte d'Azur

Corresponding author.

Pr. Xavier Fernandez

Groupe MVBV

Institut de Chimie de Nice

UMR 7272 Université de Nice-Sophia Antipolis / CNRS

Parc Valrose, 06108 Nice Cedex 2

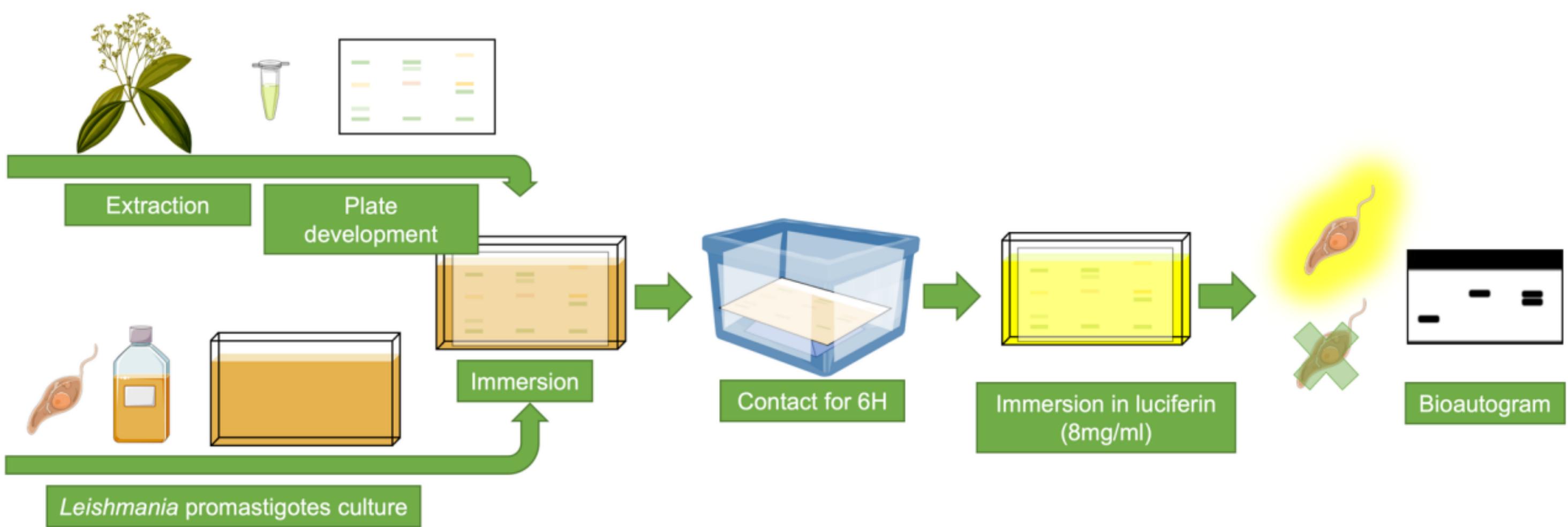
Tel: +33 4 89 15 01 36

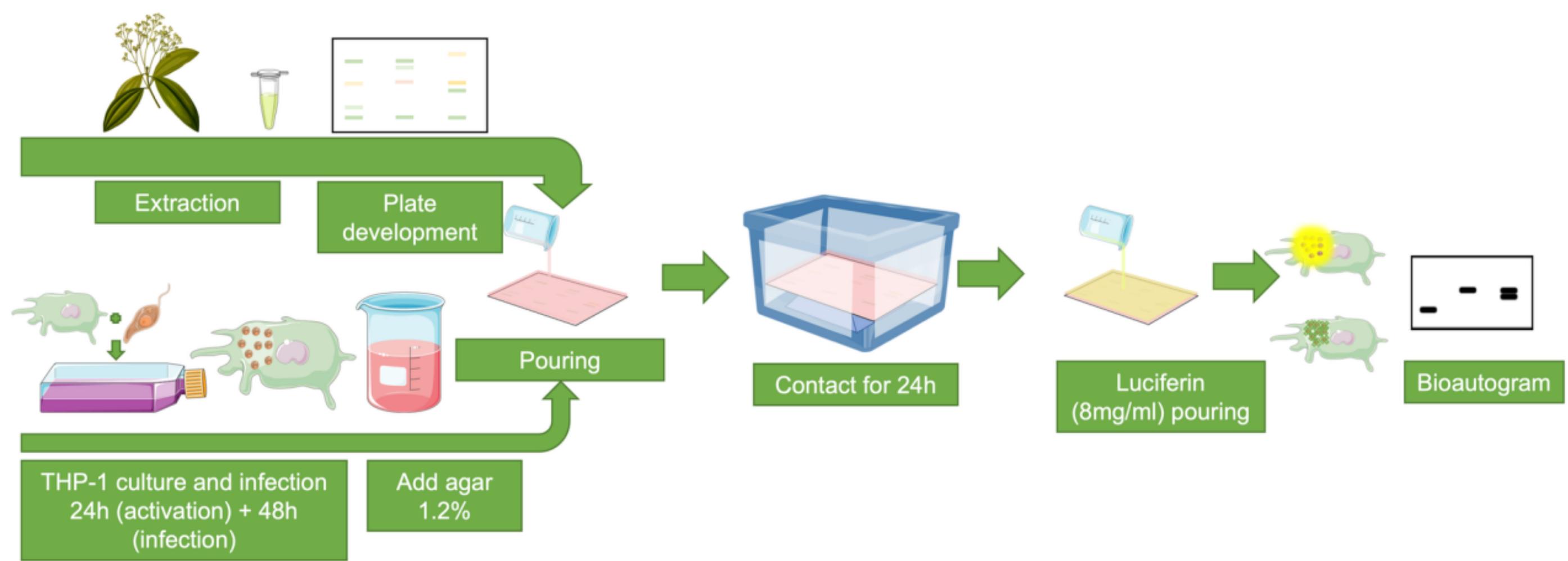
## References

- [1] N. Feasey, M. Wansbrough-Jones, D.C.W. Mabey, A.W. Solomon, Neglected tropical diseases, *Br. Med. Bull.* 93 (2010) 179–200. <https://doi.org/10.1093/bmb/ldp046>.
- [2] S. Burza, S.L. Croft, M. Boelaert, Leishmaniasis, *Lancet.* 392 (2018) 951–970. [https://doi.org/10.1016/S0140-6736\(18\)31204-2](https://doi.org/10.1016/S0140-6736(18)31204-2).
- [3] N.R.H. Stone, T. Bicanic, R. Salim, W. Hope, Liposomal Amphotericin B (AmBisome®): A Review of the Pharmacokinetics, Pharmacodynamics, Clinical Experience and Future Directions, *Drugs.* 76 (2016) 485–500. <https://doi.org/10.1007/s40265-016-0538-7>.
- [4] A.A. Kassardjian, K.M. Yim, S. Rabi, T.Z. Liang, G.H. Kim, M.T. Ochoa, M. V. Sattah, I.Z. Ahronowitz, Diffuse cutaneous leishmaniasis and HIV co-infection: A case report and review of the literature, *J. Cutan. Pathol.* (2021) cup.13993. <https://doi.org/10.1111/cup.13993>.
- [5] T.R. de Moura, M.L.B. Santos, J.M. Braz, L.F.V.C. Santos, M.T. Aragão, F.A. de Oliveira, P.L. Santos, Â.M. da Silva, A.R. de Jesus, R.P. de Almeida, Cross-resistance of *Leishmania infantum* isolates to nitric oxide from patients refractory to antimony treatment, and greater tolerance to antileishmanial responses by macrophages, *Parasitol. Res.* 115 (2016) 713–721. <https://doi.org/10.1007/s00436-015-4793-4>.
- [6] S. Hendrickx, P.J. Guerin, G. Caljon, S.L. Croft, L. Maes, Evaluating drug resistance in visceral leishmaniasis: The challenges, *Parasitology.* 145 (2018) 453–463. <https://doi.org/10.1017/S0031182016002031>.
- [7] A.W. Pountain, S.K. Weidt, C. Regnault, P.A. Bates, A.M. Donachie, N.J. Dickens, M.P. Barrett, Genomic instability at the locus of sterol C24-methyltransferase promotes amphotericin B resistance in *Leishmania* parasites, *PLoS Negl. Trop. Dis.* 13 (2019) 1–26. <https://doi.org/10.1371/journal.pntd.0007052>.
- [8] A.G. Atanasov, B. Waltenberger, E.M. Pferschy-Wenzig, T. Linder, C. Wawrosch, P. Uhrin, V. Temml, L. Wang, S. Schwaiger, E.H. Heiss, J.M. Rollinger, D. Schuster, J.M. Breuss, V. Bochkov, M.D. Mihovilovic, B. Kopp, R. Bauer, V.M. Dirsch, H. Stuppner, Discovery and resupply of pharmacologically active plant-derived natural products: A review, *Biotechnol. Adv.* 33 (2015) 1582–1614. <https://doi.org/10.1016/j.biotechadv.2015.08.001>.

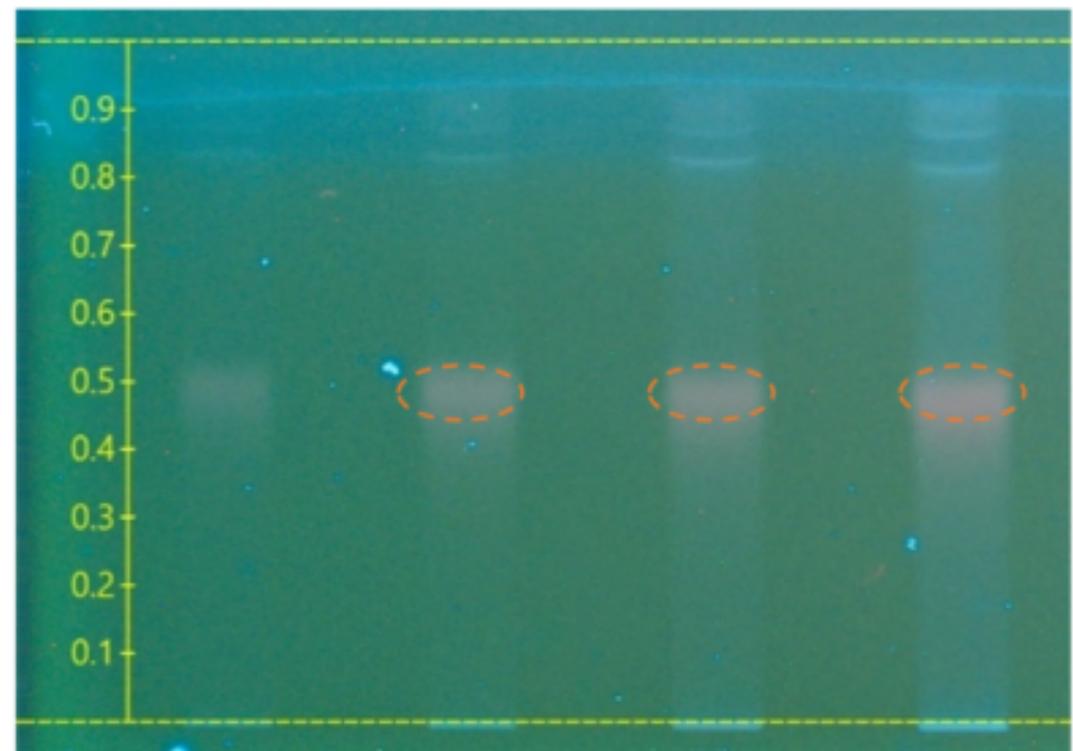
- [9] I. Roquero, J. Cantizani, I. Cotillo, M.P. Manzano, A. Kessler, J.J. Martín, C.W. Mcnamara, Novel chemical starting points for drug discovery in leishmaniasis and Chagas disease, *IJP Drugs Drug Resist.* 10 (2019) 58–68. <https://doi.org/10.1016/j.ijpddr.2019.05.002>.
- [10] J.L. Siqueira-neto, O. Song, H. Oh, J. Sohn, G. Yang, J. Jang, J. Cechetto, C.B. Lee, S. Moon, A. Genovesio, T. Christophe, L.H. Freitas-junior, Antileishmanial High-Throughput Drug Screening Reveals Drug Candidates with New Scaffolds, *PLoS Negl Trop Dis.* 4 (2010) 1–9. <https://doi.org/10.1371/journal.pntd.0000675>.
- [11] D. Paape, A.S. Bell, W.P. Heal, J.A. Hutton, R.J. Leatherbarrow, E.W. Tate, D.F. Smith, Using a Non-Image-Based Medium-Throughput Assay for Screening Compounds Targeting N-myristoylation in Intracellular *Leishmania* Amastigotes, *PLoS Negl Trop Dis.* 8 (2014). <https://doi.org/10.1371/journal.pntd.0003363>.
- [12] S. Khanra, Y.P. Kumar, J. Dash, R. Banerjee, In vitro screening of known drugs identified by scaffold hopping techniques shows promising leishmanicidal activity for suramin and netilmicin, *BMC Res. Notes.* (2018) 1–6. <https://doi.org/10.1186/s13104-018-3446-y>.
- [13] M. Ohashi, J. Agyapong, F. Ayertey, M. Amoa-bosompem, K.D. Kwofie, R. Adegle, M. Mamfe, K. Baffuor, A. Owusu, I. Tuffour, P. Atchoglo, T. Uto, F. Aboagye, A. Ampomah, R.A. Alexander, K.N. William, K. Anyan, I. Ayi, D. Adjei, B. Kwadwo, A. Koram, D. Edoh, S. Yamaoka, Y. Shoyama, *In vitro* antiprotozoan activity and mechanisms of action of selected Ghanaian medicinal plants against *Trypanosoma*, *Leishmania*, and *Plasmodium* parasites, *Phytotherapy.* (2018) 1–14. <https://doi.org/10.1002/ptr.6093>.
- [14] S. Mandal, M. Maharjan, S. Ganguly, M. Chatterjee, S. Singh, F.S. Buckner, R. Madhubala, High-throughput screening of amastigotes of *Leishmania donovani* clinical isolates against drugs using a colorimetric  $\beta$ -lactamase assay, *Indian J Exp Biol.* 47 (2011) 475–479.
- [15] F.S. Buckner, A.J. Wilson, Colorimetric assay for screening compounds against *Leishmania* amastigotes grown in macrophages., *Am. J. Trop. Med. Hyg.* 72 (2005) 600–605.
- [16] A. Urbain, C.A. Simões-Pires, Thin-Layer Chromatography for the Detection and Analysis of Bioactive Natural Products, in: *Encycl. Anal. Chem.*, Wiley, 2020: pp. 1–29. <https://doi.org/10.1002/9780470027318.a9907.pub2>.
- [17] I.M. Choma, E.M. Grzelak, Bioautography detection in thin-layer chromatography, *J. Chromatogr. A.* 1218 (2011) 2684–2691. <https://doi.org/10.1016/j.chroma.2010.12.069>.

- [18] T.T. Häbe, M. Jamshidi-Aidji, J. Macho, G.E. Morlock, Direct bioautography hyphenated to direct analysis in real time mass spectrometry: Chromatographic separation, bioassay and mass spectra, all in the same sample run, *J. Chromatogr. A.* 1568 (2018) 188–196. <https://doi.org/10.1016/j.chroma.2018.07.002>.
- [19] G. Michel, B. Ferrua, T. Lang, M.P. Maddugoda, P. Munro, Luciferase-Expressing *Leishmania infantum* Allows the Monitoring of Amastigote Population Size , *In Vivo* , *Ex Vivo* and *In Vitro*, *PLoS Negl Trop Dis.* 5 (2011) 1–7. <https://doi.org/10.1371/journal.pntd.0001323>.
- [20] K. Ciura, S. Dziomba, J. Nowakowska, M.J. Markuszewski, Thin layer chromatography in drug discovery process, *J. Chromatogr. A.* 1520 (2017) 9–22. <https://doi.org/10.1016/j.chroma.2017.09.015>.



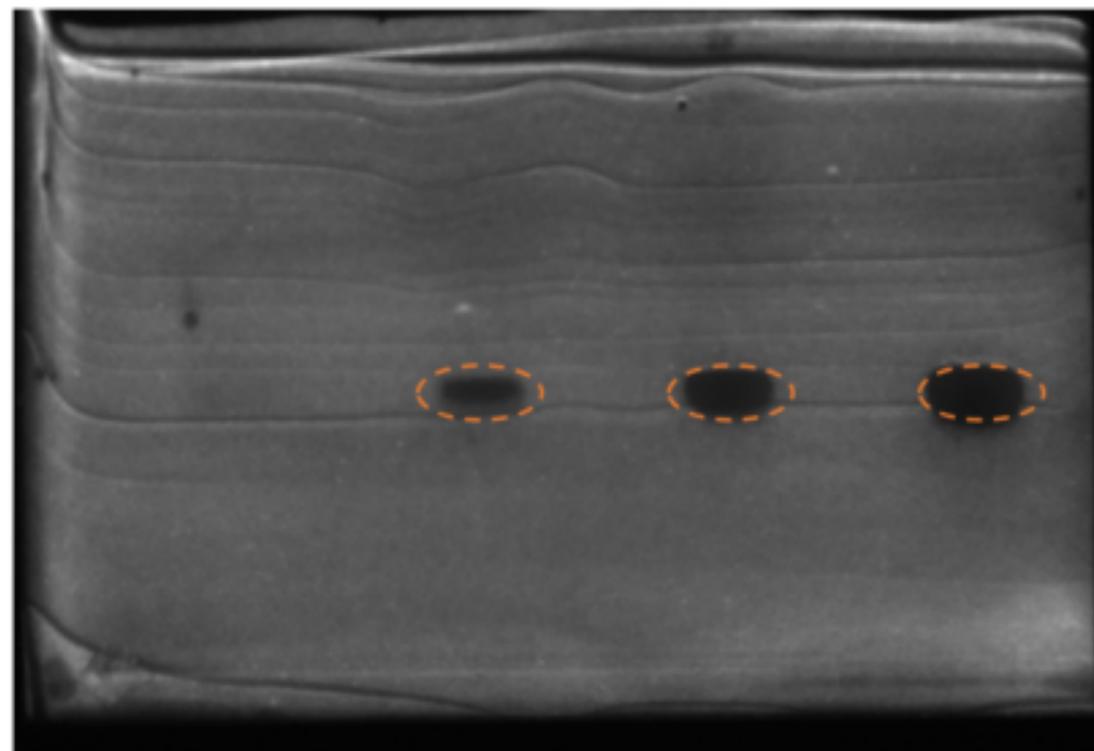


UV 366nm



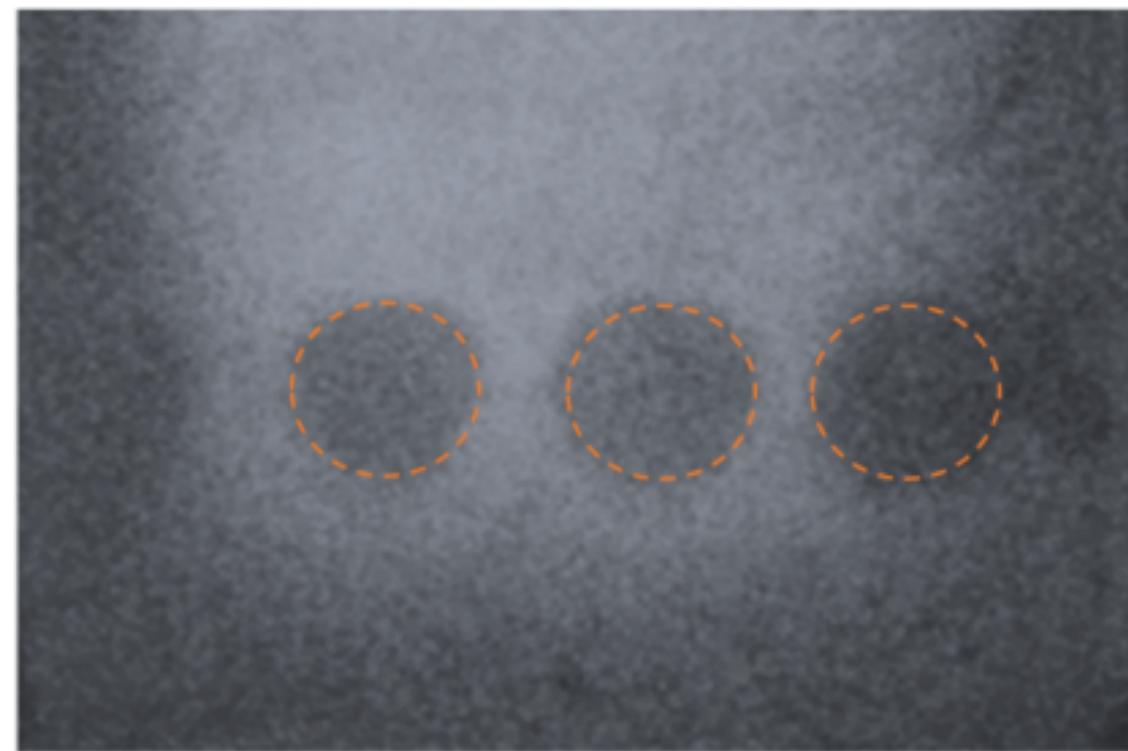
200ng 400ng 600ng 800ng

Promastigote bioautogram



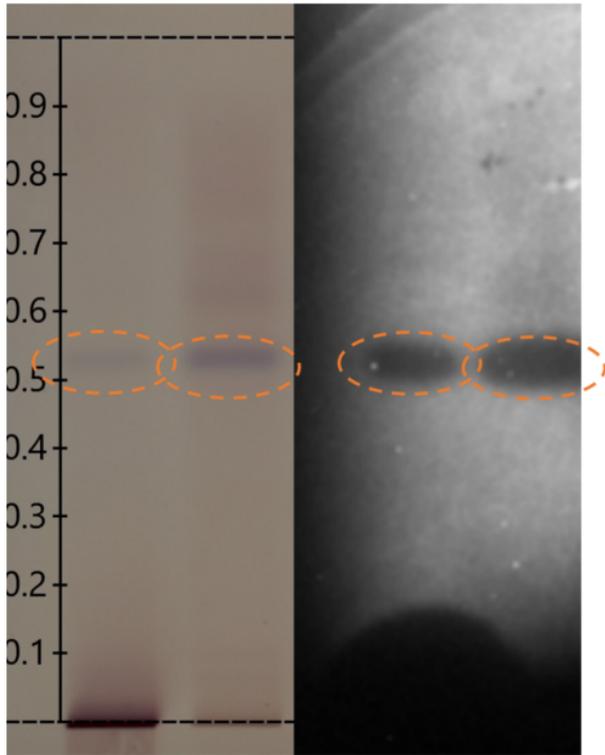
200ng 400ng 600ng 800ng

Amastigote bioautogram



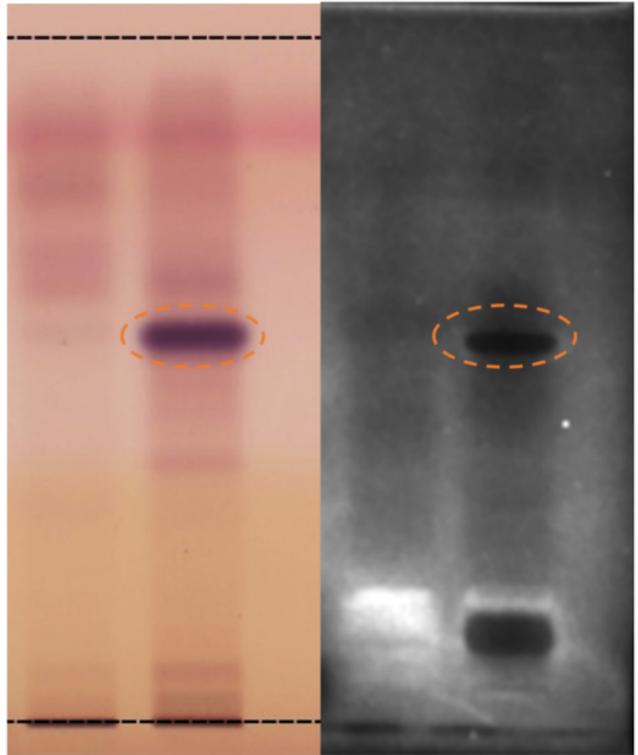
200ng 400ng 600ng 800ng

### Amastigote stage test



1 2  
Anisaldehyde Bioautogram

### Promastigote stage test



1 2  
Anisaldehyde Bioautogram