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EPIDEMIOLOGICAL, DIAGNOSTIC AND DRUG DISCOVERY STUDIES OF AN EMERGING INFECTION: HUMAN LEISHMANIASIS

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Abstract

Human leishmaniasis is a vector-borne diseases, caused by the Leishmania parasite and endemic in 100 countries, including southern Europe. The clinical spectrum of Leishmania infection ranges from asymptomatic to visceral leishmaniasis (VL), the latter being fatal if not treated. The real prevalence of asymptomatic infections is unknown, the diagnosis of VL is challenging and lacks standardized methods, and also antileishmanial treatment has critical limitations.

In this scenario, this study aimed (1) to determine the prevalence of asymptomatic infections in blood donors of the Bologna province, where increase of VL cases has been recently reported, (2) to compare the performance of nine different serological tests in the diagnosis of VL in northern Italy, (3) to assess the antileishmanial activity of a library of newly synthesized natural like compounds, ie chalcones.

(1) The screening on samples of blood donors living in the Bologna province, shows a total prevalence of asymptomatic Leishmania infection of 11.5%, suggesting a high circulation of the parasite in this area.

(2) Our findings on serodiagnosis indicate that rK39 immunochromatographic tests are insufficiently sensitive as screening tests for VL, ELISA exhibited suboptimal results in terms of sensitivity, IFAT and WB exhibited excellent sensitivity, but their cost and complexity in execution would not allow their employment as screening tests for VL. These results confirm the complexity of VL serodiagnosis and reveal the variability of diagnostic performance of serological tests.

(3) Screening of 33 newly synthesized chalcones on proliferation of Leishmania promastigote and amastigote revealed that 2 compounds showed a remarkable antileishmanial potency against the parasite, a low citotoxicity against mammalian cells and they also were able to efficiently bind trypanothione reductase - a specific parasite enzyme - suggesting that these compounds should be further evaluated as antileishmanial agents.

Keywords: leishmaniasis, neglected disease, asymptomatic infection, epidemiological study, serodiagnosis, diagnostic test, natural compound, drug discovery, challenging.

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Epidemiological, diagnostic and drug discovery studies of an emerging infection: human leishmaniasis

1. Introduction

Leishmaniasis is one of the most important neglected tropical diseases, which is endemic in around 100 countries including Southern Europe, Asia, Africa, and Latin America, with more than 350 million people living at risk of developing one of the many forms of the disease (1). The presence of leishmaniasis is linked to poverty, but social, environmental and climatologic factors also influence the disease's epidemiology. In the last decades leishmaniasis has spread in industrialized countries and, as a consequence of global warming, in countries previously regarded unfitting for the spread of the microorganism (2-4).

Leishmaniasis is caused by digenetic protozoa parasite from over 20 *Leishmania* species and is transmitted to humans by the bite of infected female phlebotomine sandflies (5). The clinical spectrum ranges from asymptomatic infections to three different manifestations: cutaneous leishmaniasis (CL), mucocutaneous or mucosal leishmaniasis (ML) and visceral leishmaniasis (VL), the latter being the most severe form, which affects internal organs and in the absence of specific therapy is fatal (6).

1.1 The Parasite

The genus *Leishmania* (*L.*) is composed of protozoa belonging to the *Trypanosamatidae* family, order *Kinetoplastida.* There are two subgenera of medical importance that were created on the basis of the parasite developmental patterns within the sandfly gut: *Leishmania* (all development anterior to the pylorus) and *Viannia* (developing in the hindgut and pylorus) (7).

Initially, species classification was based on various extrinsic criteria such as clinical, geographical and biological characteristics. Since the 1970s, intrinsic criteria such as immunological, biochemical and genetic data have been used to define species of *Leishmania*. Today, on the basis

of isoenzyme analysis of the parasite (multilocus enzyme electrophoresis - MLEE), leishmanial species are grouped into complexes (FIG.1) which are distributed in countries based on epidemiology and clinical characteristics (8, 9).

Leishmania subgenus includes the following complexes

- *L. donovani complex,* causing visceral and cutaneous forms in the Old World (species: *L. chagasi; L. infantum; L. donovani*)
- *L. major complex,* causing cutaneous forms in the Old World (species: *L. major*)
- *L. tropica complex,* causing disseminated cutaneous forms in the Old World (species: *L. aethiopica; L. tropica*)
- *L. mexicana complex,* causing cutaneous and mucosal forms in the New World (species: L. mexicana; *L. amazonensis; L. ganhami*)

Viannia subgenus includes the following complexes:

- *L. braziliensis complex,* causing mucocutaneous forms in the New World (species: *L. braziliensis; L. peruviana*)
- *L. guyanensis complex,* causing disseminated cutaneous forms in the New World (species: *L. guyanensis; L. panamensis*).

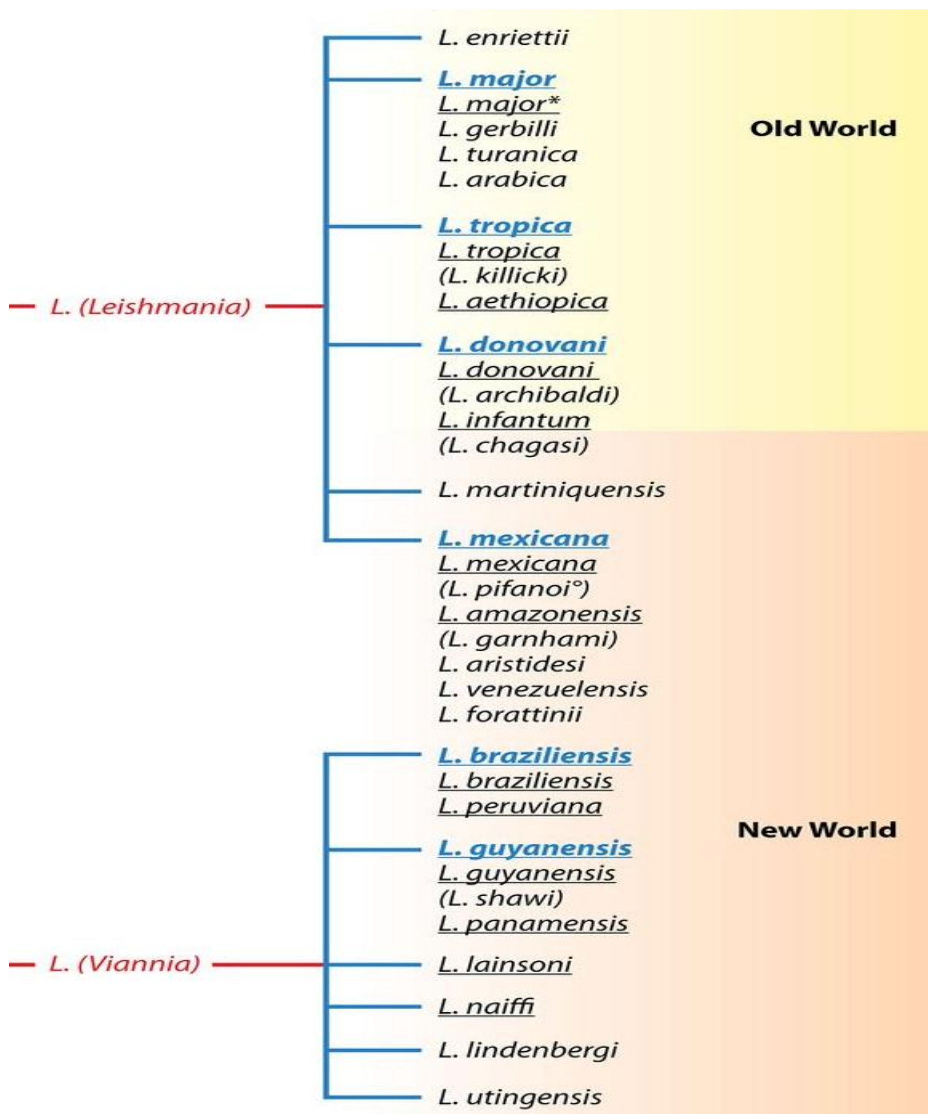


Fig. 1 Taxonomy of the *Leishmania* genus based on MLEE analysis. Modified from Van der Auwera G. et al., 2015

1.1.2 Morphology and life cycle

These protozoa have a complex digenetic life cycle, maintaining their life cycle through transmission between an insect (phlebotomine sandfly) and a mammalian host. Depending on the species, transmission can be zoonotic or anthroponotic. In the anthroponotic cycle, the sole reservoirs are humans with symptomatic or asymptomatic infections, while the zoonotic cycle involves a vector and wild and/or domestic animals as reservoirs, and humans are considered accidental hosts (10) (FIG 2).

The extracellular flagellated, 15-20 μm long motile forms of *Leishmania* spp. are called promastigotes (FIG 3a). They are found within the midgut of the sandfly and progress through various morphologically distinct stages of differentiation to ultimately become the non-dividing, infectious 'metacyclic' promastigotes located at the stomodeal valve (an invagination of the foregut into the midgut). During blood feeding, the sandfly regurgitates metacyclic promastigotes, together with immunomodulatory parasite-derived proteophosphoglycans and various salivary components. Metacyclic promastigotes are phagocytosed by tissue-resident macrophages and dermal dendritic cells (DCs) of the host. Inside the monocyte-macrophages, promastigotes transform into the amastigote life form. Amastigotes do not have an exteriorized flagellum, are 3-5 μm long (FIG. 3b) and live as obligate intracellular parasites in mammalian cells: they develop and replicate within the phagolysosomal niche of the phagocyte until they are released by cell lysis in order to infect other macrophages (11). Amastigotes re-transform into flagellate forms in sandfly midgut after a blood meal, completing the transmission cycle (3, 7, 12). A typical characteristic of the parasite is the kinetoplast, a structure consisting of mitochondrial DNA that is separated from the nucleus (7).

Depending on the species tropism, parasites remain localized at the inoculation site, resulting in the development of CL, they can spread to mucosae (MCL) or to the organs of the mononuclear phagocytic system, giving rise to VL.

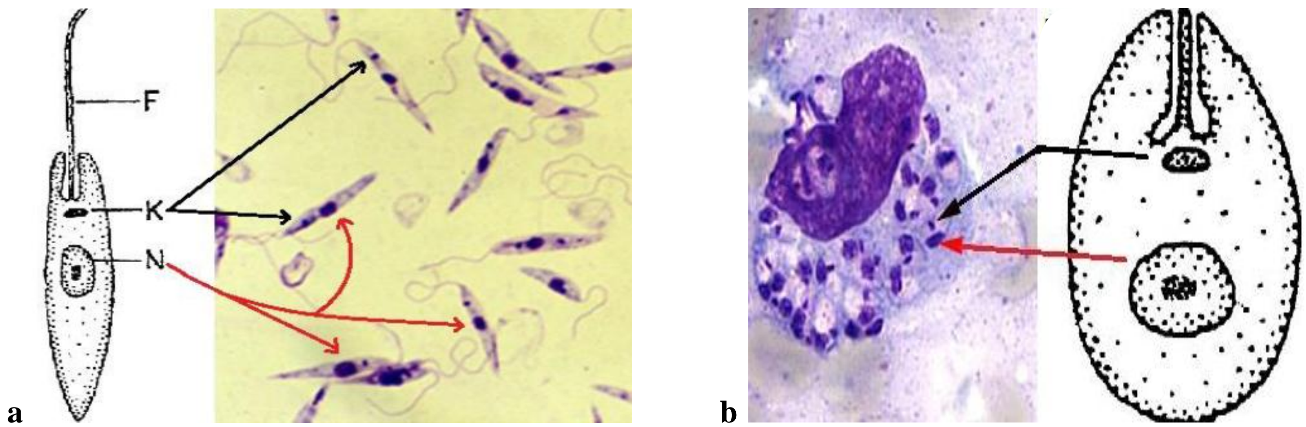


Fig. 3 The two forms of the *Leishmania* parasite. Schematic diagram of a promastigote (a) and amastigotes (b) within a macrophage. Modified from University of Wisconsin (hhttp://www.uwlax.edu/Biology/Zoo-lab/Lab-3—Protozoans) and CDC (https://www.cdc.gov/dpdx/leishmaniasis/index.html)

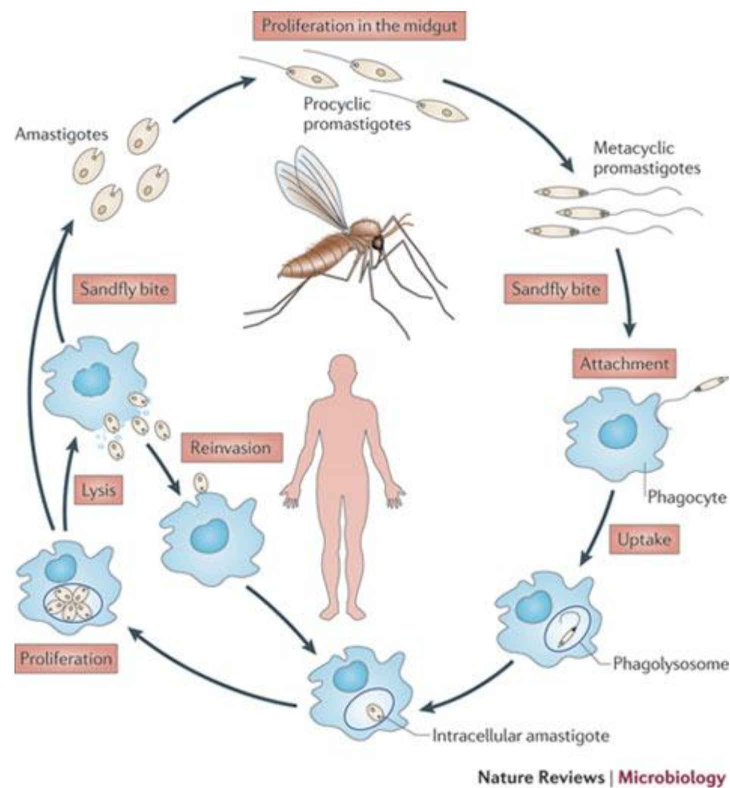


Fig.2 *Leishmania* life cycle. Modified from Kaye P. et al., 2011

1.2. The vector

The blood-feeding phlebotomine sandflies belonging to the genus *Phlebotomus* in the Old World and *Lutzomyia* in the New World, are considered the only natural vectors of leishmanial species. Among the 800 known sandfly species, around ninety three have been positively identified as vectors of leishmaniasis (7, 9). Like mosquitoes, the female needs a blood meal for egg development and only the female is hematophagous. The sandfly is a noiseless 2-3 mm long arthropod, whose colour ranges from black to white and unlike other *Diptera* species characteristically positions its wings at an angle to the abdomen (7, 13). Adults are mainly active in the evening, at night and in the early morning; they are weak flyers with flight speed <1 m/s and flight range of a few hundred meters (14, 15).

Sandflies are widely distributed in Asia, Africa, Australia, Central and South America and Europe. The geographical distribution and density of the phlebotomine sandflies are strongly linked to environmental conditions such as altitude, land cover, vegetation, climatic factors and to the availability of suitable hosts (16). Sandflies are abundant in the Mediterranean basin, the most prevalent phlebotomus species are *P. perniciosus*, *P. tobbi*, *P. ariasi*, *P. papatasi*, *P. perfiliewi*, *P. sergenti* and *P. neglectus* (17).

The sandfly species reported in Italy includes *P. perniciosus*, *P. perfiliewi*, *P. neglectus* and *P.ariasi*. *P. perniciosus* and *P. neglectus* are the most prevalent species observed in central Italy, in pre-Alpine territories and in southern Italy; while *P. perfiliewi* is the most prevalent species in northeastern Italy and *P.ariasi* is found close to the French border.

Transmission routes other than insects have rarely been reported, such as vertical transmission and injection by needles – the latter led to a VL outbreak in the ‘90 among intravenous-drug users in southern Europe (18, 19). Potential transmission of *Leishmania by* organ transplantation, blood transfusion and semen has also been reported (19-24).

1.3 The reservoir hosts

For vector-borne infections, the system comprises one or more vectors and one or more mammalian hosts, which allow the agent to be transferred continuously between them (25). A mammal host responsible for the long-term maintenance of a population of infectious agents is called a reservoir host. The reservoir spectrum that maintains the various species of *Leishmania* is wide; within the zoonotic leishmaniases the reservoir hosts are wild animals, commensals or domestic animals (17, 26, 27). Dogs are the main domestic reservoirs, with widespread dissemination of parasites both in viscera and dermis, but also foxes, jackals and wolves represent sylvatic reservoirs of *L. infantum*, *L. major* and *L. aethiopica*. Rodents, bradypus and foxes are reservoirs of *Leishmania* in the New World. Within anthroponotic leishmaniases, the reservoir host is human (6), who is directly involved as principal reservoir host in VL caused by *L. donovani* and CL caused by *L. tropica*.

1.4 Epidemiology

Leishmaniasis is still one of the world's most neglected diseases. The disease affects the poorest people on earth and is associated with malnutrition, population displacement, poor housing, a weak immune system and lack of financial resources (6, 28). About 350 million people are considered at risk of contracting leishmaniasis and 2 million new cases occur yearly. Control programs are insufficient and morbidity/ mortality from leishmaniasis worldwide show a worrying increasing trend (6).

Leishmaniasis is distributed worldwide with the exception of Australia and Antarctica. Within the Old World it is spread in Asia, the Middle East, Africa and the Mediterranean basin. Within the New World cases of leishmaniasis have been recorded from the southern United States to northern Argentina, with the exception of the Caribbean islands, Chile and Uruguay (6, 7).

It is estimated that about 75% of the total cases of CL are concentrated within 10 countries, 4 of which are in the Americas (Brazil, Colombia, Peru, and Nicaragua). With respect to VL, 90% of

cases are concentrated within Brazil, Ethiopia, India, Bangladesh, Sudan, and South Sudan (29, 30). Almost 90% of MCL cases occur in Bolivia, Brazil and Peru (6).

In southern Europe leishmaniasis is endemic in Spain, Portugal, Greece, France, Italy and the Balkans. Most of the reported cases are due to zoonotic VL, CL is also present (2), but there are probably many more cases than those registered (31). Human leishmaniasis appears not to be limited to the Mediterranean region anymore, it has spread northward (where sandflies were previously thought to be absent or present only in very low densities), as shown by the recent reports in northern Italy and one case in southern Germany (2, 32-34).

1.5 Pathogenesis

The diverse clinical manifestations of *Leishmania*, from cutaneous lesions to systemic VL, are the result of the interplay between the infecting parasitic species and the host's immune response. Parasites such as *L. major* are mainly dermotropic and remain contained at cutaneous levels, while *L. infantum* and *L. donovani* may disseminate into the organs (35, 36).

The sandfly lacerates blood vessels during feeding and parasites are introduced intradermally. Neutrophils are the earliest cells recruited to the site of the sandfly bite and represent the first infected cell population(37, 38). Parasites are taken up essentially by dendritic cells and macrophages that can then leave the injection site. The types of macrophage population infected by the cutaneous and visceral *Leishmania* species differ, possibly depending on the preferential affinity of the parasite to specific macrophage cell surface receptors, however the mechanism is still poorly understood.

Antileishmanial immunity is mediated *via* both innate (macrophages, dendritic cells and neutrophils) and adaptive (T cells) immunity, but the CD4+ T-cell subset is crucial for host defence. In addition, cytokines participate in a network of interactions that induce and control the immune

responses. Conversely, although a strong humoral response is present during the infection, it appears that antibodies play no role in protection (39).

Based on experimental studies in murine models, it is widely accepted that resistance to the disease is correlated with expansion of T-helper-1 (Th1) cells that produce IL-2 and IFN-γ and amplify effector response by activating macrophages to inhibit the growth of the parasite. The development of T-helper-2 (Th2) cells, resulting in IL-4, IL-5 and IL-10 production, or a mixed Th1/Th2 response is associated to disseminated infection (40-42)

Polymorphisms in genes involved in such immune interactions may play a role in the clinical pleiomorphism demonstrated by leishmaniasis (7). Furthermore, acquired immunosuppressive conditions can modulate parasite replication. For example, it was observed that infection with HIV-1 leads to 100–2,230 times increase in the risk of developing VL (43). HIV/Leishmania coinfection include chronic evolution, with a high rate of relapse and poor response to treatment (7, 44, 45).

1.6 Clinical presentation

The clinical spectrum of leishmaniases depends mostly on parasite species and host immune response, ranging from asymptomatic infection (80-95% of affected people) to three main clinical forms: visceral (also known as kala-azar and the most severe form of the disease), cutaneous and mucocutaneous (20, 46). The incubation period between the infecting sandfly bite and symptoms' onset ranges from 1 week – 2 months to a year or more (6).

1.6.1 Visceral leishmaniasis (VL)

VL is caused by parasite belonging to the *Leishmania donovani* complex and is associated with considerable morbidity and mortality. VL is endemic in around 100 countries including Southern Europe, Asia, Africa, and Latin America. More than 90% of new cases reported to WHO occurred in 6 countries: Brazil, Ethiopia, India, Somalia, South Sudan and Sudan.

VL is the most severe form of the disease and is fatal if untreated. It is characterized by irregular fever, weight loss, splenomegaly, hepatomegaly and anaemia (FIG. 4) (6). Parasites proliferate wherever there are cells of the mononuclear phagocyte system, most often in macrophages; Bone marrow cells become infected, and patients develop pancytopenia and immunosuppression, making them susceptible to bacterial superinfections (47).

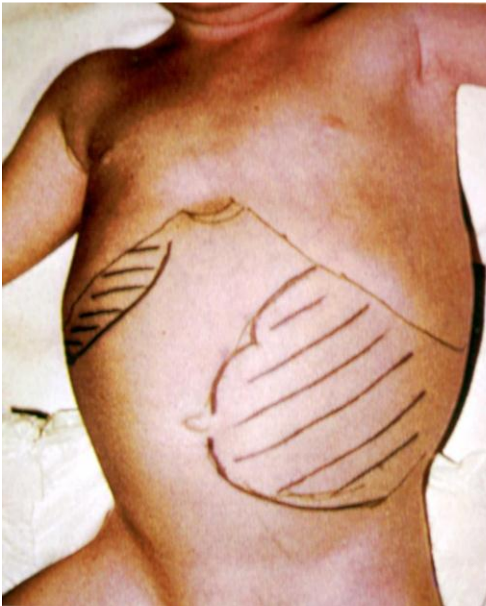


Fig.4 Hepatosplenomegaly in a VL case. Modified fromhttp://www.emedmd.com/content/leishmaniasis

1.6.2 Tegumentary leishmaniasis (CL and MCL)

CL usually occurs in an exposed area accessible to sandflies such as the face, neck and limbs. In the Old World it is caused by parasites belonging to the *Leishmania major* complex, *L. tropica* complex and occasionally by *L. infantum* (*L. donovani* complex). In the Americas, CL is most commonly caused by the species *Leishmania mexicana*, *Leishmania braziliensis*, or *Leishmania panamensi. L. major* and *L. tropica* tend to cause less severe lesions that heal more quickly, and therefore, take on a relatively benign course (46). Conversely, CL in the New World is less likely to resolve without treatment.

Regardless of the infecting parasite species, CL is not a life threatening disease, but healing occurs over several months and may leave a scar and permanent alterations in the skin pigmentation.

Lesions may begin as small red papules and can progress into erythematous nodules, indurated plaques, scaly plaques or ulcers with raised, rolled dusky borders; they may be dry and crusted or be accompanied by exudates (FIG. 5a) (48).

MCL is usually caused by leishmanial species of the New World, but it has occasionally been associated with any species that are endemic in the Old Word (49, 50). In the New World, MCL usually begins with a cutaneous lesion that may be followed by metastasis to the mucosal tissues of the mouth and upper respiratory tract and may result in severe tissue destruction and gross disfigurement (7). Studies have shown that MCL can present from several months to 20 or more years after a cutaneous lesion. Mucosal lesions caused by *L.infantum* are not associated to a previous CL and involve the buccal or laryngeal mucosae in immunocompetent or immunocompromised patients (FIG. 5b)(51).

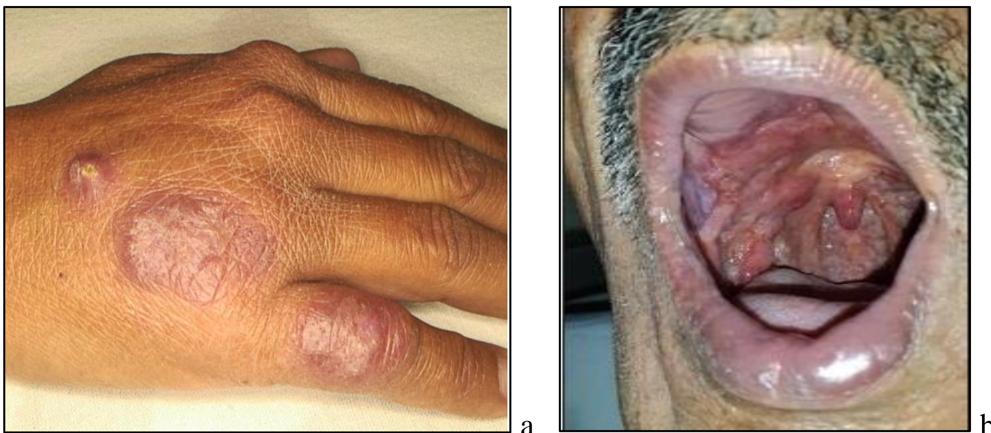


Fig.5 (**a**) nodular lesion of cutaneous leishmaniasis (**b**) ulceration of the oral mucosa in mucosal leishmaniasis. Modified from Nangia A. et al., 2014

1.6.3 Leishmaniasis in immunocompromised patients

Immunosuppression is one of the strongest risk factor responsible for increased vulnerability to a primary *Leishmania* infection and to reactivation of a latent infection. Immunosuppression can also alter disease presentation and treatment response. While historically mainly observed in HIV-infected patients, non-HIV related immunosuppressive conditions are becoming increasingly prevalent globally, mainly because of better medical care of patients with chronic illnesses and the therapeutic use of immunosuppressive drugs as TNF-alpha antagonists (52-54). VL has also been reported after organ transplantation (52, 55). A comparative study of HIV-infected and uninfected Spanish individuals showed that AIDS patients have an incidence of VL that is 418-fold higher than HIV-negative individuals (56).

It has been shown that HIV and *Leishmania* infection reinforce each other, HIV patients are more likely to develop VL, due to reactivation of a dormant infection or clinical manifestation after primary infection (6). At the same time, VL promotes the clinical progression of HIV disease and the development of AIDS-defining conditions and negatively affects the response to antiretroviral treatment (57). Patients characteristically have high disseminated parasite loads and the infection is difficult to cure (58, 59).

1.6.4 Asymptomatic infections

Asymptomatic forms characterize individuals infected by the parasite in apparently healthy condition. Actually, the majority of infected people do not progress to patent leishmaniasis; clinical syndromes caused by *Leishmania* are only the tip of the iceberg. Several prospective studies have documented the ratio of asymptomatic infections with *L. donovani* or *L. infantum* infection to incident clinical cases as 4:1 in Kenya, 4:1 in Bangladesh, 6:1 in Ethiopia, 9:1 in India and Nepal

18:1 in Brazil and 50:1 in Spain, suggesting that many people infected with *Leishmania* develop an effective immune response and do not manifest clinical disease (60-63).

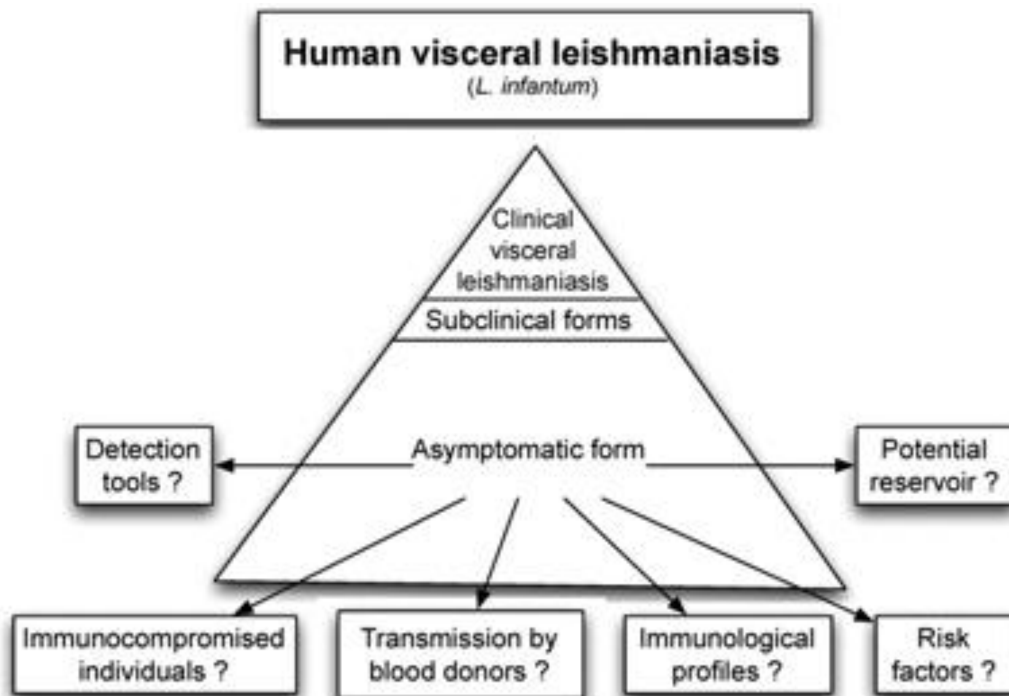


Fig. 4 Human leishmaniasis pyramid and possible involvement of asymptomatic carriers in transmission of leishmaniasis. Modified from Michael G. et al., 2011

1.7 Diagnosis

Microbiological diagnosis of leishmaniasis is fundamental because symptoms are not pathognomonic. For identification of leishmaniasis, clinical suspects must be linked to a positive parasitological and/or serological test (6).

1.7.1 Diagnosis of visceral leishmaniasis

The diagnosis of VL remains challenging, due to the limited sensitivity of microscopy, the poor performance of serological methods in immunocompromised patients and the lack of standardization of molecular tests (64).

The diagnosis of VL is traditionally based on serologic testing and the direct demonstration of *Leishmania* by microscopic examination of bone marrow aspirates, but limits have to be considered, including low sensitivity of serologic testing in immunosuppressed patients, the necessity to use invasive procedures to obtain bone marrow samples, the low sensitivity of microscopy examination (between 53% and 85%) and the need of expert microscopists (65). Over the past 10 years, molecular tests have been developed, and polymerase chain reaction (PCR) represents a highly sensitive technique for the diagnosis of VL (64, 66).

1.7.1.1 Serological testing for VL

Serologic diagnosis is based on the presence of a specific humoral response. Current serological tests available for routine use are employing different techniques, including indirect immunofluorescence test (IFAT), enzyme-linked immunosorbent assay (ELISA), western blot (WB) and direct agglutination test (DAT). All antibody detection tests share the same drawbacks: antibodies remain positive for months after the patient has been cured and do not differentiate between current and past infection (67). Further, serological tests may give false negative results in immunocompromised patients.

IFAT, ELISA and WB exhibit excellent diagnostic accuracy, but their cost and complexity in execution would not allow their employment in developing countries. Therefore, DAT and immunochromatographic tests (ICT) have been produced.

IFAT is based on detecting antibodies, which are demonstrable in the early stages of infection,. It has shown acceptable estimates for sensitivity (87–100%) and specificity (77–100%) (68). IFAT is based on immunological detection of anti-*Leishmania* antibodies in human serum: using a slide with promastigotes fixed on it as antigens and fluorescein-labeled anti-human immunoglobulins.

ELISA is one of the preferred laboratory test for serodiagnosis of VL (68). ELISA is a test for the qualitative determination of specific IgG against *Leishmania* in human serum: on the surface of the microtiter wells, inactivated soluble antigens of *Leishmania* are bound, the human serum anti-*Leishmania* antibodies possibly present in the sample bind with the antigens. A peroxidase conjugated antibody (anti-IgG) with a substrate and a chromogen are added, the bound converts the colorless substrate/chromogen to colored one and the absorption is measured. The sensitivity and specificity of ELISA depends on the antigen used; one of the most commonly used antigens is a crude soluble antigen (CSA). The sensitivity and specificity of ELISA using CSA range from 80 to 100% and from 84 to 95%, respectively.

ICT rK39 rapid test is a non-invasive and cost effective method that has shown high sensitivity and specificity in the Indian subcontinent and southern Europe (68), while in East Africa the estimated sensitivity seems to be lower (69, 70). It is a qualitative test for the detection of antibody to *Leishmania* in serum. The membrane is pre-coated with recombinant parasitic rk39 antigen. During testing, the serum sample reacts with the dye conjugate which has been pre-coated in the test device. The mixture then migrates upward on the membrane chromatographically by capillary action to react with recombinant *Leishmania* antigen on the membrane and generate a line (71).

DAT is an inexpensive and simple-to-perform test, making it ideal for both field and laboratory use. DAT is based on direct agglutination of *Leishmania* promastigotes that react specifically with anti-*Leishmania* antibodies in the serum specimen, resulting in agglutination of the promastigotes. Whole, trypsinized, Coomassie-stained promastigotes can be used either as a suspension or in freeze-dried form that can be stored at room temperature for at least two years (67). DAT is considered to be a highly sensitive and specific method: according to a meta-analysis that included 30 studies, the sensitivity and specificity of DAT were estimated at 94.8 and 97.1%, respectively (68).

For the WB, promastigotes are cultured to log phase, lysed, and the proteins are separated on SDS-PAGE. The separated proteins are electrotransferred onto a nitrocellulose membrane and probed with serum from the patient. This technique provides detailed antibody responses to various leishmanial antigens, and has been found to be more sensitive than IFAT and ELISA, especially in co-infected HIV patients with VL, but the drawbacks of the technique (equipment and time requirements, cumbersomeness, and cost) limit its use to research laboratories or reference laboratories (67, 72).

1.7.1.2 Direct testing for VL

Parasitological diagnosis can be performed by demonstrating the amastigote form of the parasite in tissues, isolating the parasite in cultures, or by molecular assays.

Amastigotes can be observed within macrophages on microscopic examination of Giemsa-stained bone marrow aspirate specimens. The specificity of the method is high, while the sensitivity varies according to the examined aspirates. Clinical samples (bone marrow aspirate or buffy coat from peripheral blood from HIV positive patients) may also be cultured into monophasic (Schneider's insect medium, M199, or Grace's medium) or diphasic (Novy-McNeal-Nicolle medium and Evans modified Tobie's medium) culture media. Cultures should be examined weekly for four weeks and

promastigotes are observed in case of positive culture. Culture from splenic biopsy and from bone marrow aspirates possesses high specificity, nevertheless this technique is tedious, time consuming, requires expertise, expensive equipment and is, therefore, restricted to referral laboratories and hospitals (68).

Molecular techniques can detect nucleic acids unique to the parasite; a variety of nucleic acid detection methods targeting both DNA and RNA have been developed. Amongst these, PCR has proved to be a highly sensitive and specific technique, especially in immunosuppressed patients. This method is minimally invasive as peripheral blood specimens are required, is capable of identifying relapses/reinfections in VL-treated patients, and can provide species identification (73). Different target sequences are used, which include ribosomal RNA genes, kinetoplast DNA (kDNA, mitochondrion genome), miniexon-derived RNA (medRNA), gp63, G6PD and the β-tubulin gene region. Sensitivity is excellent (near 100%) in spleen or bone marrow and can be lower but still good by employing peripheral blood (varying from 70% to 100%) (67, 74). However, PCR techniques remain complex and expensive to perform, and in most VL-endemic countries this technique is not affordable in the diagnostic routine.

1.7.2 Tegumentary leishmaniasis

Clinical manifestations of tegumentary leishmaniasis are not specific and may mimic other skin conditions, such as staphylococcal or streptococcal infection, leprosy, mycobacterial ulcer, cancer, fungal infection, sarcoidosis and tropical ulcer (67, 75). As the clinical presentation of tegumentary leishmaniasis lacks specificity and treatment is costly and toxic, diagnostic confirmation is required. Diagnosis is obtained microscopically by direct identification of amastigotes in Giemsa-stained smears of scraping, fine-needle aspiration or biopsy of lesions. The material obtained by any of these methods can be used for microscopic examination, culture and molecular diagnostic techniques. A combination of microscopy and culture increases diagnostic sensitivity to more than

85% (67). DNA detection by PCR is based on kDNA or rDNA, mini-exon, β-tubulin, gp63, G6PD, cytochrome B gene; it offers the best sensitivity and specificity rates (98.8% and 100%, respectively) (74), but is not currently practical in disease-endemic areas where technical resources are poor (75).

Serological diagnosis has low sensitivity and variable specificity, it does not distinguish between past and present infections; moreover, anti-leishmanial antibodies can be detected by serological tests but they tend to be undetectable or present at low titers due to poor humoral response, therefore serological assays are not the usual diagnostic methods, because they are of little value in the diagnosis of CL; conversely, concerning ML diagnosis, antibody research is useful and shows a good sensitivity (6, 67, 75).

1.8 Treatment

Different chemical compounds have been found to be effective against leishmanisis; however, most are not safe and are difficult to use (76). One of the main challenges is the presence of different *Leishmania* species and various clinical manifestation, complicating the therapeutic approach. There are many available anti-leishmanial agents, but the optimal drug is still out of reach, since almost all available compounds share several limitations; many of them are expensive with severe side and toxic effects or have a marked reduction of therapeutic effectiveness(77, 78).

1.8.1.1 Pentavalent antimonials

Pentavalent antimonials have been used used all over the world since the mid of 1930s for all forms of leishmaniasis. Two antimonials are available: meglumine antimoniate (Glucantime®) and sodium stibogluconate (Pentostam®). They are chemically similar, and their toxicity and efficacy are related to their antimonial content (6).

Although antimonials are the most commonly used medication against leishmaniasis, their exact mechanism of action remains unknown. Several *Leishmania*-killing mechanisms have been

attributed to pentavalent antimonials including apoptosis, disturbance of fatty acids β-oxidation, adenosine diphosphate phosphorylation, and redox balance, inhibition of the glycolysis pathway and ability to act on infected macrophages eliciting an oxidative/nitrosative stress against internalized parasites (79, 80).

They are associated with serious adverse side effects, including arthralgias, myalgias, leukopenia, pancreatitis, liver toxicity, cardiotoxicity and cardiac arrhythmia in the patients, and increased parasite resistance (81).

1.8.1.2 Amphotericin B

An alternative drug to the antimonials is the antifungal macrolide, amphotericin B, that has shown optimal results on MCL and VL. Two forms of amphotericin are available: conventional amphotericin B deoxycholate and liposomal amphotericin B (Ambisome®), the lipid formulations of amphotericin B, that have been developed to improve the pharmacokinetic characters of the drug, the tolerability in the patient and to minimize the side effects (82).

Amphotericin B controls *Leishmania* infections through two distinct mechanisms. The first includes, auto-oxidation of amphotericin, leading to the production of free radicals. The second mechanism requires the binding of amphotericin to sterols in the membrane of the parasites, which makes pores that cause an ion imbalance (80).

Amphotericin B exhibits high efficacy (90–100%) even in *Leishmania*/HIV co-infection cases (82). However, it is an expensive drug and it has many adverse effects including fever, thrombophlebitis and occasionally hypokalemia, nephrotoxicity, liver disorder, myocarditis (83).

1.8.1.3 Miltefosine

Miltefosine is an alkyl phosphocholine analogue, originally developed as an oral anticancer drug then implicated as anti-leishmanial agent. It is the only oral medication available against VL and

CL, with a cure rate of 95%. This drug is well tolerated for the treatment of all forms of leishmaniasis (6, 82).

The mechanism of anti-leishmanial action of miltefosine remains unclear, but parasite apoptosis preceded by drug intracellular accumulation has been described. Other possible mechanisms include cytochrome c oxidase inhibition, which leads to mitochondrial dysfunction and immunomodulation (79). Miltefosine commonly induces gastrointestinal side-effects such as anorexia, nausea, vomiting, diarrhea and recent studies have pointed out that miltefosine has a potential teratogenic effect (6, 79).

1.8.1.4 Paromomycin

Paromomycin is an antibacterial aminoglycoside which has been used for the treatment of VL in a parenteral formulation and in both topical and parenteral formulations for the treatment of CL (82, 84). This dryg has a high efficacy (94.6%) and low price (82).

Paromomycin can affect ribosomal activity, inhibiting protein synthesis and mitochondrial membrane potential, which deprives the parasite of energy, other mechanisms of action were also suggested for this drug, such as alteration on membrane fluidity and lipid metabolism (80, 82).

Paromomycin showa a low incidence of side effects, including reversible ototoxicity, vestibular instability and increase of hepatic transaminases. Renal toxicity is rare (6, 82).

1.8.1.5 Azoles medicines

These oral imidazole-derived antifungal agents have variable efficacy in leishmaniasis treatment. They have been reported to be effective in the treatment of VL cases, either alone or in association with other anti-leishmanial agents (82). Azoles exert their effects through inhibition of some key enzymes in the synthesis of ergosterol. Adverse effects include gastrointestinal symptoms and hepatotoxicity (84).

1.8.2 Treatment of leishmaniasis in immunocompromised patients

The criteria used to assess response to the antileishmanial treatment in the immunocompetent patients are not applicable in the immunocompromised host. as immunosuppressed patients tend to have several relapses of VL (85).

Leishmaniasis in HIV-positive patients is particularly severe; HIV and *Leishmania* infection reinforce each other, VL negatively affects the response to antiretroviral treatment and is difficult to cure in co-infected patients. The co-infection is usually associated with low cure rate, high relapse rate and treatment-associated mortality particularly with antimonials (85). The treatment of VL/HIV co-infection cases is similar to that of the immunocompetent VL patients with some precautions in doses due to low CD4 counts, (combined with leukopenia and lymphocitopenia) and prolonged therapeutic regimen., WHO recommends liposomal amphotericin B as a preferred therapy because of its safety profile. Miltefosine had an acceptable safety profile, too and combined therapy is often recommended (6, 82).

1.8.3 Anti-leishmanial drug candidates with focus on natural and natural like products

Given the above mentioned scenario, development of new, less toxic and more cost-effective drugs, which could become available for low-income populations to treat the disease has become a necessity (81). In recent years, there has been growing interest in alternative natural products and plant compounds as promising class of drug candidates against leishmaniasis (79), since these products may be safe, effective and economically feasible for the treatment of leishmaniasis. The secondary metabolites of plants have been demonstrated to be beneficial against diverse groups of pathogens such as viruses, bacteria and fungi. Various herbal formulations and plant secondary metabolites such as isoflavonoids, polysaccharides, tannins, indoles and glucans are known to have different pharmacological profiles. Some important categories of natural products that have shown good or excellent anti-leishmanial properties are the quinones, the alkaloids (i.e. indoles, quinolines,

steroidal alkaloids) (86, 87), the terpenoids (i.e. monoterpenes, sesquiterpenes, saponins) (87-91), the sterols, the phenolics (i.e. flavonoids, chalcones, coumarins, tannins, lignans) (87, 89). In addition, substantial evidence from the literature indicates that essential oils (EO), volatile mixtures of compounds obtained from spices, aromatic herbs, fruits and flowers possess a wide spectrum of biological activities, among which it has been shown that several EOs, or their constituents, have inhibitory activity on *Leishmania* (92-95).

Within alkaloids, quinolines isolated from *Galipea longiflora* have been shown effect against New World cutaneous leishmaniasis in BALB/c mice and in vitro activity against the promastigote form of *Leishmania* (96). Indoles have been reported to exhibit significant antileishmanial activities against the promastigote form of *L. amazoniensis, L. major* and *L. donovani* (97). Steroidal alkaloids inhibits the growth of *L. amazoniensis, L. donovani* and *L. braziliensis* (97).

Within terpenoids, monoterpenes shows antileishmanial activity against promastigotes of twelve *Leishmania* species; an *in vitro* study indicates that the monoterpene linanool also exhibits strong antileishmanial activity against intracellular amastigotes (98). Sesquiterpenes have strong inhibitory effect against promastigotes of *Leishmania* and the sesquiterpene endoperoxide isolated from *Artemisia annua*, artemisinin (an established anti-malarial drug), demonstrated anti-promastigote activity, anti-amastigotes activity and *in vivo* activities in a murine model of infection. In fact, in a BALB/c model of VL, orally administered artemisinin effectively reduced both splenic weight and parasite burden (99, 100). Saponins isolated from *Astragalus oleifolius*, show notable growth inhibitory activity against amastigotes of *L. donovani*; moreover, none of these compounds possesses cytotoxicity on mammalian cells. Saponins obtained from the leaves of *Hedera helix* and *Buddleja madagascariensis* show leishmanicidal activity against *L. infantum* and *L. tropica*. Other saponins show potent antileishmanial activity against *L. major* (101).

Within sterols, compounds isolated from the Mexican plant *Pentalinon andrieuxii* exhibit significant *in vitro* antileishmanial activity against the promastigote and amastigote forms of *L. mexicana* (102).

Within phenolics, flavonoids seem to be promising drugs candidates for the treatment of all forms of leishmaniasis (103). Chalcones have been observed to possess potent antileishmanial activity, with strong inhibition of *L. major, L. tropica, L. braziliensis* , *L. donovani*, *L. amazonensis* and *L. infantum* (104, 105). Coumarins are an important class of polyphenolic compounds that shows significant *in vivo* antileishmanial activity against *L. amazonensis* and *L. major* (106). Lignans show activity against the amastigotes and the promastigotes form of different species (97).

Despite many compounds from plants and natural products-inspired molecules have shown good activity against Leishmania *in vitro* and in murine models, due to lack of investments in the field of neglected diseases such as leishmaniasis none of them has undergone clinical evaluation (83, 97).

2. Aim of the study

Human leishmaniasis is spread worldwide, is endemic in around 100 countries including Southern Europe, Asia, Africa, and Latin America and causes high morbidity and mortality, but still it is included in the neglected tropical diseases, based on the limited resources invested in diagnosis, treatment and control, and on its strong association with poverty (4).

The incidence of human leishmaniasis has displayed a sharp increase since the early 1990s in Mediterranean countries of southern Europe. In recent years, a northward spread of leishmaniasis has been observed in Mediterranean Europe (2).

The real prevalence of this parasitic disease is unknown, but it has been demonstrated that clinical leishmaniasis cases usually represent the tip of an “infection iceberg”; several prospective studies have documented that many individuals that are infected with *Leishmania* do not manifest clinical disease, remaining asymptomatic. Nevertheless, data about the real prevalence of asymptomatic human carriers are scarce.

The diagnosis of VL is challenging and lacks standardized methods. Most of data about the reliability and accuracy of diagnostic methods are from studies performed in India and Brazil, not reflecting the current situation for leishmaniasis in the Mediterranean Europe.

Also chemotherapy has critical limitations, since currently available treatments are toxic, expensive and require long term regimens. Further, drug resistant *Leishmania* parasites are becoming a serious problem in the Indian subcontinent.

In this scenario, this study aimed to:

- determine the prevalence of asymptomatic infections in a pool of blood donors in the municipality of Valsamoggia (Bologna province), where increase of VL cases has been reported in 2013 and 2014 (study I);

- compare the performance of nine different serological tests in the diagnosis of autochthonous VL in northern Italy and to determine a valid screening test for serodiagnosis of VL (study II);

- assess the antileishmanial activity of a library of newly synthesized chalcones, by evaluating the ability to affect parasite growth and the affinity of novel molecules toward the parasitic trypanothione reductase enzyme, in order to identify novel potential antileishmanial drugs (study III).

3. Methods

The research was carried out at the Regional Reference Centre for Microbiological Emergencies (CRREM), at the Sant'Orsola-Malpighi University Hospital of Bologna, Italy.

3.1 Study I

3.1.1 Study design

For the first study there were analyzed samples of blood donors residing in Valsamoggia, an Italian district of 30.071 citizens in the Bologna province, Emilia-Romagna region. The study was designed as evaluation of the prevalence of asymptomatic carriers residing in Valsamoggia district. 260 blood donor's samples were collected at the CRREM laboratory from 1 April 2014 to 31 March 2015, none of the donors have shown previous clinical manifestation of VL. Samples were taken from the Transfusion Unit, Maggiore Hospital of Bologna and sent to the CRREM laboratory within 5 days from the collection of blood sample. The prevalence of asymptomatic infections were evaluated with a serological assay and a molecular assay.

3.1.2 Serological and molecular tests

Samples were centrifuge at 1500 rpm for 10 minutes, 500 µl of serum and 300 µl of buffy coat were collected for each blood sample then stored at – 20 °C.

Serological testing was performed by a commercial western blot kit: Leishmania WESTERN BLOT IgG – LDBio Dianostics® (Lyon, France). The western blot technique provides detailed antibody responses to various leishmanial antigens.

Specific 14-kDa and 16-kDa proteins of *L.infantum* are electrotransferred onto a nitrocellulose membrane and probed with serum from the patient. The presence on the strip of the bands is indicative of the evidence of specific anti-Leishmania IgG in the serum.

Home-made molecular methods were used to detect leishmanial DNA in buffy coats. Nucleic acids were extracted from 300 µL buffy coat with QIAsymphony SP/AS instruments, Qiagen® (Hilden, Germany). DNA was eluted in 200µL of elution buffer. DNA was amplified employing one real-time PCR assay, (18) that amplifying the kinetoplast(k)DNA (19). Primers (15 pmol of RV1 5'-CTTTTCTGGTCCTCCGGGTAGG-3', 15 pmol of RV2 5'-CCACCCGGCCCTATTTTACACCAA-3' for kDNA PCR) were synthesized by PrimmBiotech (Milan, Italy) and 50 pmol of TaqManprobes (FAM-TTTTCGCAGAACGCCCCTACCCGC-TAMRA for kDNA PCR) were synthesized by IDTDNA (Leuven, Belgium). The real-time PCR assays were performed by employing the CFX Real Time PCR detection System (Bio-Rad, California, USA). β2-microglobulin real-time PCR assay was run simultaneously as a control of amplification of the extracted DNA.

3.2 Study II

3.2.1 Study design

This study was designed as a retrospective comparative study of serological methods performed on77 human serum samples chosen from those arrived to the CRREM laboratory between 2013 and 2015. During 2013-2015, samples of patients with suspected VL residing in the Emilia-Romagna region were sent to the CRREM laboratory, where microbiological diagnosis of VL was performed by molecular and serological methods. Autochthonous VL was confirmed in 27 out of 77 cases and dismissed in the other 50 cases. Specificity, sensitivity, positive predictive value (PPV), negative predictive value (NPV) of nine commercial serological assays were evaluated. Diagnosis of VL was established by the case definition of the World Health Organization, which describes a case of VL as a patient showing characteristic clinical signs with serological and/or parasitological confirmation. Standardized clinical evidence were combined with frontline diagnosis by serology

supported by real-time PCR as second line parasitological test. Subsequently, samples were archived and stored at – 80 °C.

3.2.2 Test methods/serological assays

Index tests included nine serological assays, all of them were commercial products.

IFAT: Leishmania-Spot IF – BioMérieux® (Marcy l'Etoile, France). It detects IgG antibodies against *L.infantum*; promastigote form is the antigen, obtainted from in vitro culture, inactivated and coated on slides. Samples were considered positive when specific IgG titer was equal or above 1/160.

ICT: i) Leishmania Dipstick Rapydtest – APACOR® (Wokingham, England); ii) On Site Leishmania IgG/IgM Combo Rapid Test – CTK Biotech ® (San Diego, USA); iii) Leishmania Strip Quick Test – CYPRESS Diagnotics ® (Langdorp, Belgium). A qualitative membrane-based immunoassay with nitrocellulose strips impregnated with recombinant K39 Leishmania antigen, it detects specific antibodies against rK39. Tests were performed according to the manufacturers'instructions.

ELISA: i) Leishmania ELISA IgG + IgM – VIRCELL Microbiologists® (Granada, Spain); ii) Leishmania Ab ELISA – CYPRESS Diagnotics ® (Langdorp, Belgium); iii) Leishmania infantum IgG – ELISA - NOVATECH® (Bologna, Italy); iv) Leishmania Ab - RIDASCREEN® (Darmstadt, Germany).

Tests were performed according to the manufacturers'instructions. For the detection of antibodies all tests used soluble inactivated antigens of *L.infantum*. OD were determinated at 450 nm, Samples were classified as positive, negative, or indeterminate according to the manufacturers'cut off values.

WB: Leishmania WESTERN BLOT IgG – LDBio Dianostics® (Lyon, France). The western blot technique provides detailed antibody responses to various leishmanial antigens.

Specific 14-kDa and 16-kDa proteins of *L.infantum* are electrotransferred onto a nitrocellulose membrane and probed with serum from the patient. The presence on the strip of the bands is indicative of the evidence of specific anti-Leishmania IgG in the serum.

3.3 Study III

3.3.1 Study design

Thirty-three newly synthesized chalcones have been screened to assess their anti-leishmanial potential. The study was designed as analysis of synthetic natural product-inspired molecules as anti-leishmanial candidates, by evaluating the ability to affect parasite growth and to inhibit the protozoan enzyme trypanothione reductase (TR), an essential enzyme for *Leishmania* metabolism, that is not present in human cells.

3.3.2 Chalcone synthesis

All the chalcone derivatives used in the present study have been readily synthesized through the classic base-catalyzed Claisen-Schmidt procedure. In particular, the selected acetophenone was reacted at room temperature with the appropriate aldehyde in ethyl alcohol and in the presence of a 50% KOH/H2O solution, to give the desired final compounds. The acetophenone intermediates were obtained by reaction of 2,4-dihydroxyacetophenone with the appropriate alkyl bromide to obtain the 2-OH,4-alkoxyacetophenones;reaction with methyl iodide allowed obtaining the 2-methoxy,4-alkoxyacetophenones.

3.3.3 Drug susceptibility tests

Promastigote forms of reference strain of *L. donovani* (MHOM/NP/02/BPK282/0cl4) were cultured at 26°C in a liquid custom made medium. Cell culture THP1 (human leukemia monocytic cell line), used for the anti-amastigote assay, were cultured at 37°C in liquid medium. Cell culture VERO

(kidney of African green monkey cell line), used for the cytotoxicity assay, were cultured at 37°C in liquid medium.

The promastigote growth inhibition assay was carried out incubating promastigotes in 96-well plates with tested compounds (at micromolar concentration, range 40 µM – 1.5 µM), the effect of the chalcones was evaluated by the AlamarBlue® assay (Thermo Fisher Scientific, Massachusetts, USA). To estimate the concentrations at which compounds cause 50% inhibition of growth (IC50) the AlamarBlue method based on monitoring the reducing environment (reduction of resazurin into fluorescent resorufin) of proliferating cells was employed and absorbance was measured at 550 nm and 630 nm.

For the anti-amastigote assay, human acute monocytic leukemia cell line (THP1) infected with promastigotes was used. Cells were seeded in a 96-well plate, after 48 h promastigotes were inoculated, phagocytized and transformed into amastigotes and after 24 test compounds were added. After a 72 h incubation, wells were washed, fixed and Giemsa stained and the infectivity index (% of infected macrophages x average number of amastigotes per macrophage) was determined microscopically.

3.3.4 Cytotoxicity assays

For the citotoxicity test, mammalian kidney fibroblast cells (Vero cell line) were seeded in 96-well plates and incubated with test compounds (at micromolar concentration, range 40 µM – 1.5 µM) at 37°C. The effect of the chalcones was evaluated by the AlamarBlue® assay after 72 h of incubation. Absorbance was measured at 550 nm and 630 nm.

3.3.5 Enzymatic screening

The ability of the compounds to bind the enzyme trypanothione reductase (TR) was assessed by Surface Plasmon Resonance studies whereas the ability to inhibit TR was assessed using

spectrophotometric TR assays by following the NADPH oxidation at 340 nm. This part was performed by the Institute of Molecular Biology and Pathology of CNR in Rome.

4. Results

4.1 Study I

For the first study, 260 samples of blood donors residing in a municipality of the Bologna province (ie Valsamoggia) were collected and sent to the CRREM laboratory from 1 April 2014 to 31 March 2015. The prevalence of asymptomatic infections was evaluated by WB and by real-time PCR.

Antibody anti-*Leishmania* were detected by WB in serum of 27 out of 260 blood donors, with a seroprevalence of 10.4% (Fig.1). Fifteen out of 27 positive recognized the 16-kD antigen (P16 band), 10 sera out of 27 recognized the 14-kD antigen (P14 band) and 2 out 27 recognized both the specific bands (P14 and P16) (Fig. 3). Following the manufacturers' instructions, sera were considered WB positive if one band or both bands were detectable.

Leishmania DNA was detected by real-time PCR in buffy coat of 4 out of 260 blood donors (Fig 2), samples were considered positive when the threshold cycle (CT) value was not higher than 38 cycles (Fig.4).

Among the samples positive for *Leishmania* kDNA, 1 out of 4 corresponded with a positivity to anti-*Leishmania* antibody by WB with the presence on the strip of band P14. In the other three PCR cases, no specific antibodies were detected.

4.2 Study II

Since 2013, blood samples from patients with a clinical suspicion of VL and resident in the Emilia-Romagna region were sent to the C.R.R.E.M. laboratory; microbiological diagnosis of VL was performed by molecular and serological methods. Samples were kept frozen at –80° after tests execution.

The samples for the study were selected in April 2014 and analyses were performed between June 2015 and September 2015. 77 serum specimens were chosen, 27 patients were identified as VL

cases while in the remaining 50 patients VL was excluded by parasitological and serological analysis. The mean age of patients was 44 (range 1 month – 87 years). 51 patients were male (66%), 26 were female (34%). Fifteen patients were children (range 1 month – 13 years), 12 patients (15.6%) were HIV-positive and one patient suffered of a hematological malignancy in terminal stage (Fig.5).

4.2.1 Performance of the ICT

The diagnostic performance of the ICTs was low; as shown in Table 1, the sensitivity of ICT1 (Leishmania Dipstick Rapydtest – APACOR®) was 70%, the sensitivity of ICT2 (Leishmania IgG/IgM Combo Rapid Test – CTK Biotech ®) was 63% while ICT3 (Leishmania Strip Quick Test – CYPRESS Diagnotics ®) showed the lowest sensitivity, 52%. Specificity was uniformly high for all the ICTs (98%). The average of positive predictive value (PPV) and negative predictive value (NPV) were 95% and 83%, respectively. When evaluating sera from the five immunocompromised VL-positive patients, only one out of 5 tested positive with ICT2 and ICT3, while ICT1 tested positive in 2 out of 5 cases.

Among the VL negative cases, one out of 50 showed a positive result with ICT1 and ICT2. As the patient was not included in the study as a VL case and had no previous history of VL, this result was considered as a false positive result .

4.2.2 Performance of the ELISA

ELISA showed better performance than ICTs, by exhibiting an average sensitivity of 70%. As shown in Table 1, sensitivity of ELISA1 (Leishmania ELISA IgG + IgM – VIRCELL Microbiologists®) was 74%, sensitivity of ELISA2 (Leishmania Ab ELISA – CYPRESS Diagnotics) was 81%, sensitivity of ELISA3 (Leishmania infantum IgG – ELISA - NOVATECH®) and ELISA4 (Leishmania Ab - RIDASCREEN®) was 63%. In contrast, no significant difference was found between tests' specificity, with an average value of 97%. ELISA1 showed a PPV of 95%

and a NPV of 88%, ELISA2 showed a PPV of 100% and the best NPV (91%), ELISA3 showed a PPV of 100% and a NPV of 83%, while PPV and NPV values of ELISA4 were significantly lower, 81% and 82%, respectively.

Among the immunocompromised VL-positive patients, 2 out of 5 tested positive with all the ELISA.

4.2.3 Performance of the IFAT

As shown in Table 1, the diagnostic performance of IFAT to identify VL was significantly higher, with a sensitivity of 96%, a specificity of 100%, a PPV of 100% and a NPV of 98%.

Concerning the immunocompromised VL-positive patients, 4 out of 5 tested positive (antibody titer > 1/80) or borderline (antibody titer = 1/80) , the last case exhibited a negative result.

4.2.4 Performance of the western blot test

WB test exhibited en excellent sensitivity with a value of 96%, but modest specificity, with a value of 88%; the PPV was 81% and the NPV was 98% (Table 1).

4.2.5 Comparison of the tests

The sensitivity of ICTs was modest, with an average value of 62% (Table 1). ELISA showed a better performance than ICTs (average sensitivity of 70%), with high fluctuation of sensitivity among the 4 different kits (range 63%-81%), ELISA2 exhibited the best performance (81%). Conversely, IFAT and WB exhibited en excellent sensitivity, both with a value of 96%.

The specificity of the tests ranged from 88% to 100% (Table.1). All tests except for WB exhibited high specificity values (>92%).

The PPV values ranged from 90% to 100% except for WB (81%) and ELISA Ridascreen (81%). Conversely, the NPV was lower (range 79%-86%) except for WB, IFAT and ELISA2, respectively, 98%, 98% and 91% (Table 1).

By restricting results to immunocompetent patients (n=65), the overall sensitivity of serological tests increased significantly; ICTs range was 59%-77%, ELISA range was 68%-91%, IFAT and WB reached a sensitivity of 100% (Table 2). No significantly different specificity-values were recorded by restricting results to immunocompetent patients (Table 2).

The overall sensitivity and specificity of index tests is summarized in Table 1. Sensitivity values ranged from 52% to 96%, whereas specificity values ranged from 88% to 100%.

4.3 Study III

For the third study, 33 chalcones were obtained through the classic base-catalyzed Claisen-Schmidt procedure (as shown in Scheme 1). The A-ring of the chalcone scaffold was properly functionalized at the 2 and 4 positions: the C-4 position was occupied by a suitable alkoxy chain namely 3,3-dimethylallioxy (or prenyloxy) and propargyloxy (O-R), affording series 1 and 2, respectively; the C-2 position was differently functionalized by introduction of hydroxy, methoxy, prenyloxy, and propargyloxy groups (O-R1). In order to perform a Structure Activity Relationship (SAR) study and to explore the chemical space of the target, as B-ring, a pyridine or a substituted aryl function bearing some selected substituents (R2) such as methoxy, bromo, nitro, and fluorine were introduced (Scheme 2). This part was performed in collaboration with Dr. Belluti from the Department of Pharmacy and Biotechnologies (University of Bologna).

4.3.1 In vitro antileishmanial activity of chalcones

The *in vitro* antiparasitic activity of the newly synthetized chalcones against a reference strain of *L.donovani* (MHOM/NP/02/BPK282/0cl4) was determined. Drug susceptibility tests were

implemented on promastigotes at micromolar concentrations (range 40 uM – 1.5 uM) and the effect of the chalcones was evaluated by the AlamarBlue ® assay. The citotoxicity of the chalcones was analyzed on mammalian cell line (Vero cell line) by the same assay.

Compounds **1-33** were tested for their antiparasitic activity against the promastigotes, intracellular form of the parasite. Among them, for analogues **17-33** no antileishmanial effect was observed on *L. donovani* promastigotes (Table 4). Derivatives **1-16** turned out to effectively inhibit the parasite growth (IC_{50} was calculated: a value that represent the concentration of a compound that causes 50% growth inhibition) with variation of the growth rate, ranging from 21.5 μM of the compound **12** to 3 ± 1.3 μM of the compound **6** (Table 3).

Considering series 1, the analogues with a simple phenyl function as B-ring and different substituents on the 2-position (**7**, **19-21**), were, at the maximum tested dose, inactive with the exception of compound **7** which showed a double-digit inhibitory activity (IC_{50} = 15 μM). In this context, the nature of the substituent at the 2-position of the A-ring markedly affected the inhibitory behavior; the 2-methoxy-4-prenyloxy substitution pattern proved to be the most favorable and it was kept for all the remaining compounds of this series. The presence of a heterocyclic furyl group (compound **18**) led to a loss of activity while a pyridyl moiety showed interesting IC_{50} values, ranging from 5 μM to 10.5 μM, notably the substituent at the 2-position (hydroxyl or methoxyl) did not significantly influenced potencies. The presence of electron-donating moieties such as methoxy groups on the B-ring (sub-set of compounds **8**, **23**, **24**), gave different results, as only the bulky 3,4,5-trimethoxylated analogue **8** proved to inhibit parasite growth with IC_{50} value of 11 μM. The effect of the electron-withdrawing groups namely bromo, nitro, and fluoro (compounds **5**, **6**, and **22**, respectively) in the para position was also investigated; compound **6** with the nitro substituent resulted to be the most active of all the series as it showed IC_{50} value in the single-digit micromolar range (1.5 μM), followed by the bromo-derivatives compound **5** (IC_{50} = 16 μM) which resulted two-times les active than compound **6**.

Regarding series 2, the functionalization B-ring gave different results: regarding to the para position, the bromo moiety allowed obtaining good antileishmanial agents as compounds **12** and **15**, the 2-methoxylated and 2-propargyloxylated analogues, respectively, showed micromolar activities being as active as compound **5**; surprisingly, inactive compounds were obtained with the insertion of a nitro substituent (compounds **25** and **26**). The same detrimental effects were elicited by the 3,4,5-trimethoxy substitution pattern (compounds **27**, **28**). Interestingly, compound **16** turned out to effectively inhibit leishmania growth. The pyridine-based subset (compounds **9-11**, **13**, **14**) gave, analogously to series 1, excellent results, as low micromolar activities were generally detected.

In parallel, the cytotoxicity of the molecules was tested using mammalian kidney epithelial cells (Vero cell line) for the compounds which have shown any inhibition against promastigotes (**1-16**). Data were expressed as 50% citotoxic concentration (CC_{50}). Moreover the selectivity index (SI) was calculated based on CC_{50} / IC_{50} ratio.

The tested compounds generally displayed moderate to very low cytotoxicity. with CC_{50} values superior to 30 μM. In particular, analogues **5**, **6**, **12** , **15**, and **16** were characterized by the absence of toxicity that, combined with an effective inhibition of parasite proliferation, led to a promising selectivity index (SI). On the contrary, the pyridine-based chalcones, due to their moderate cytotoxic effects, showed an unfavorable SI with values ranging from 2 to 3.8.

These assays allowed to identify two lead molecules, compounds **6** and **16**, as the most promising of the series. Indeed, these molecules showed a remarkable antileishmanial potency against the extracellular form of the parasite (IC_{50} = 3 ± 1.3 μM and 12.5 ± 2.25 μM, respectively), coupled with an excellent SI (above 20). These two compounds were further evaluated on intracellular amastigotes of *L.donovani* in THP-1 cells (human acute monocytic leukemia cells line). Evaluation on the inhibition of the growth of the intracellular form of *Leishmania* confirmed the low micromolar trend of inhibition. Notably, compound **16** (IC_{50} = 4.5 ± 2 μM) was more active than

compound **6** (IC_{50} = 14 ± 7.8 μM) in blocking the growth of amastigotes, highlighting divergent abilities in inhibiting promastigote and amastigote growth for the two compounds (Figure 6).

4.3.2 Evaluation of the Inhibition of trypanothione reductase (TR)

This part of the study was performed in collaboration with dr. Andrea Ilari from CNR, Institute of Molecular Biology and Pathology of Rome. Compound **6** and compound **16** also showed a high binding affinity to TR (Kd = 0.6 μM and 2.4 μM, respectively), resulting in an effective enzyme inhibition (IC_{50} = 0.8 μM and 3 μM, respectively). A peculiar feature of this ADPH-dependent disulfide oxidoreductase is the presence of cysteine residues in the catalytic triad, which could explain the affinity of the compounds for this enzyme.

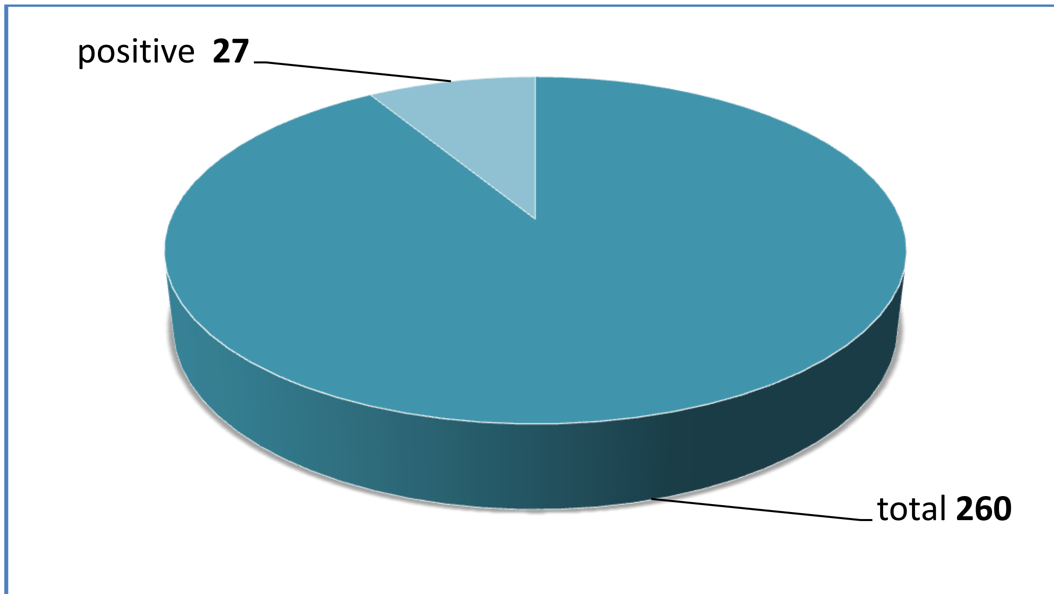


Figure 1 Pie chart representingblood donors that tested positive by western blot for antileishmanial antibodies.

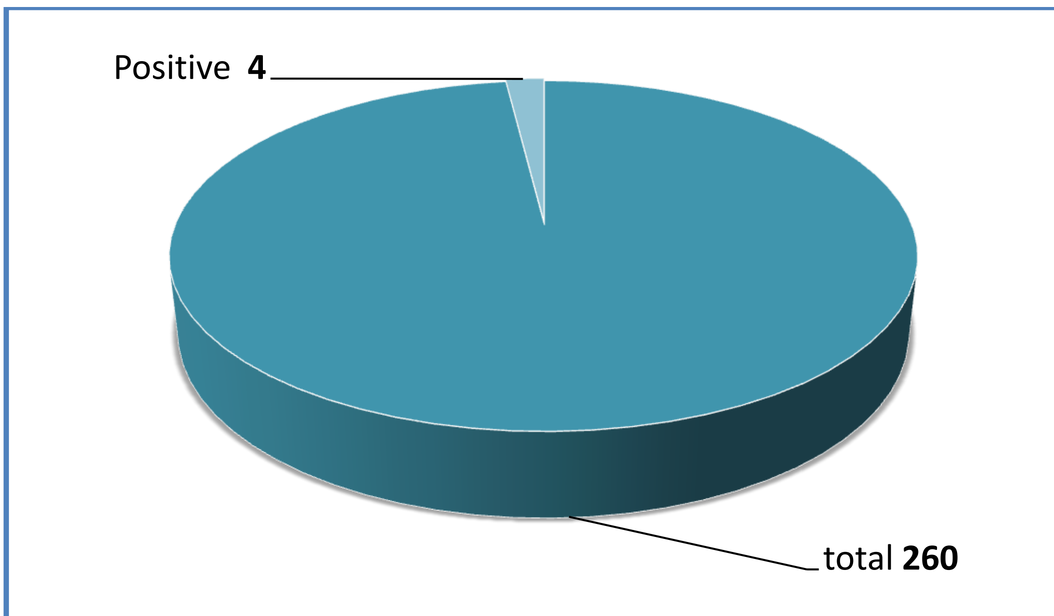


Figure 2 Pie chart representing the blood donors that tested positive by real-time PCR for leishmanial DNA.

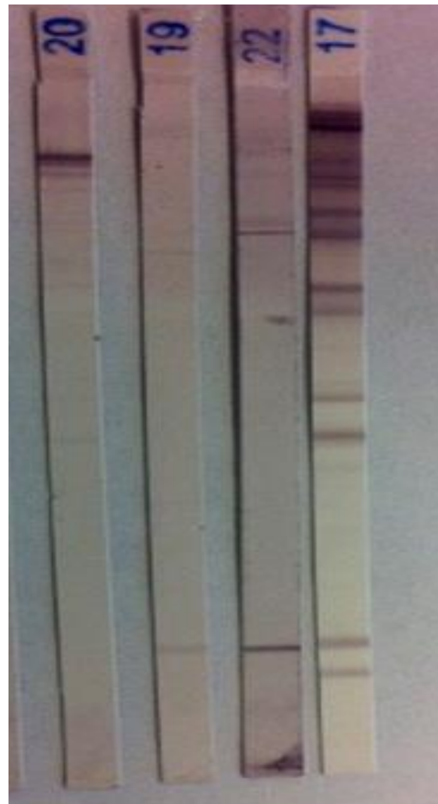


Figure 3 WB strips: nr. **17** a positive case with P14 and P16 band. Nr. **22** a positive sample with P14 band. Nr. **19** low positivity of P14 band. Nr **20** a negative sample.

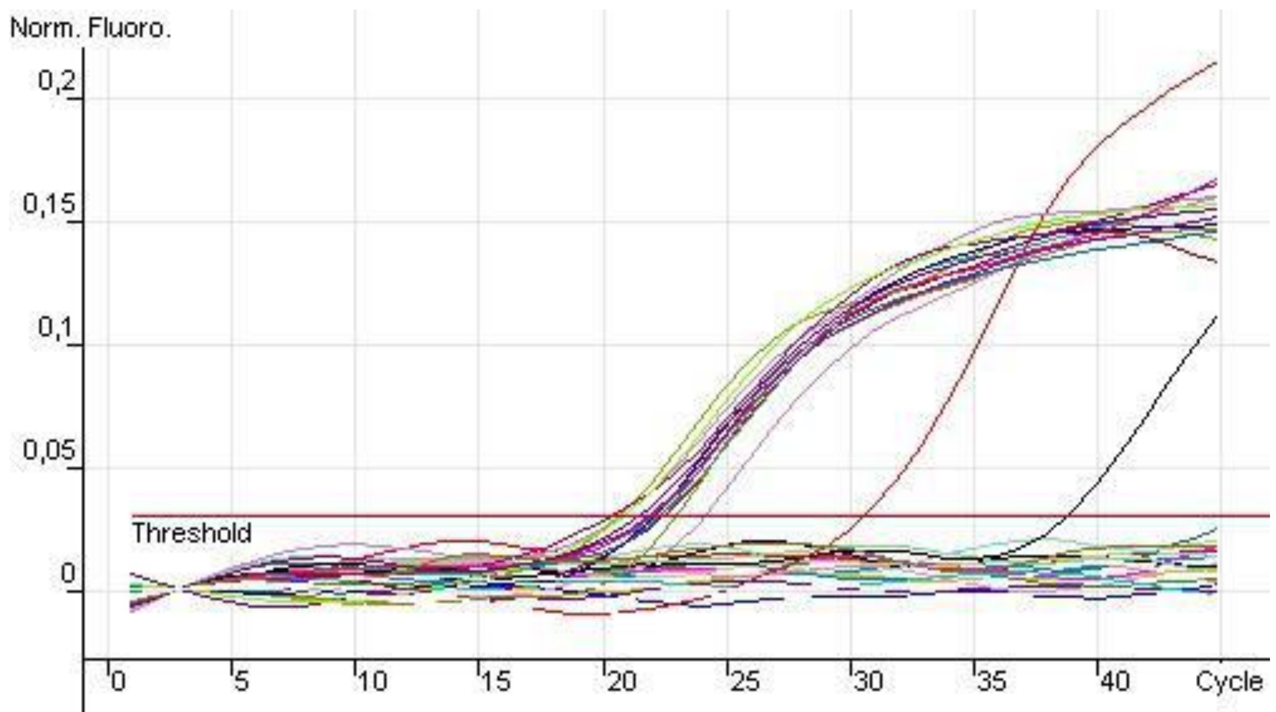


Figure 4 Real-time PCR of (scrivi almeno il gene di Leishmania che vai a testare..). Amplification plot with β2-globulin curves (mixed colours), the positive control curve (purple) and a positive curve (blue) from a blood donor sample.

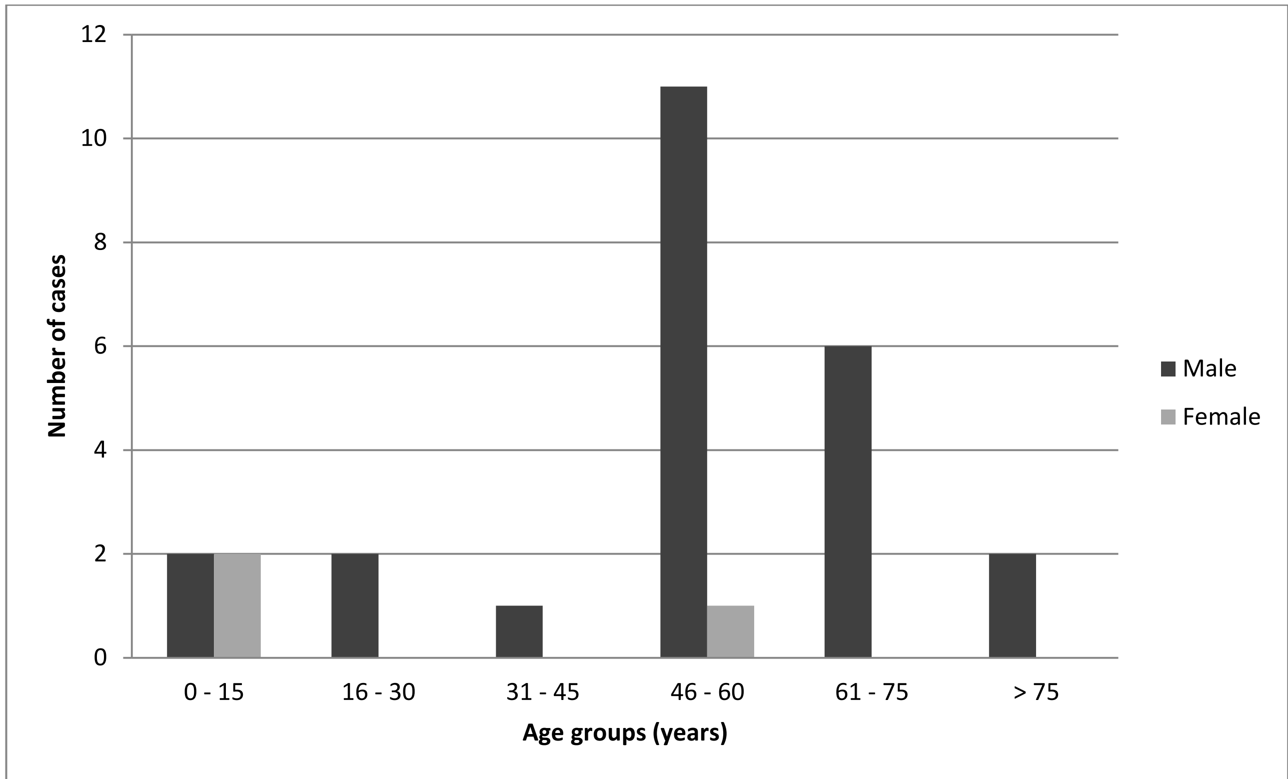


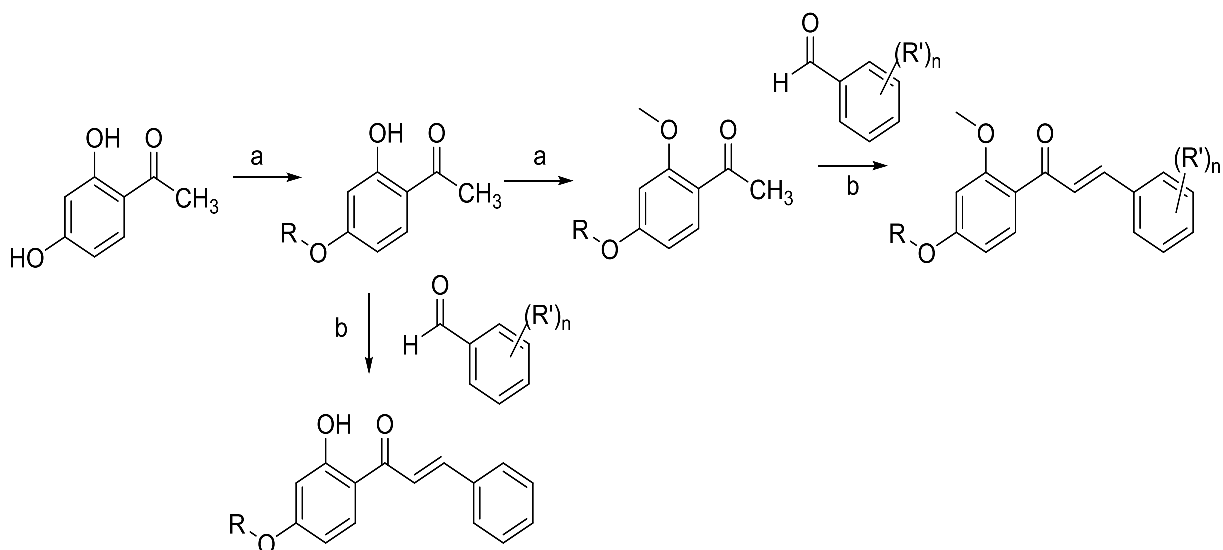
Figure 5 Age and sex distribution of the VL cases included in Study 2 (n=27)

		IC 95%			IC 95%	
TEST	**Sensitivity %**	**LL**	**UL**	**Specificity %**	**LL**	**UL**
ICT 1	70	50	85	96	85	99
ICT 2	63	42	80	98	88	100
ICT 3	52	32	71	100	91	100
ELISA 1	74	53	88	98	88	100
ELISA 2	81	61	93	100	91	100
ELISA 3	63	42	80	100	91	100
ELISA 4	63	42	80	92	80	97
IFAT	96	80	100	100	91	100
WB	96	80	100	88	75	95

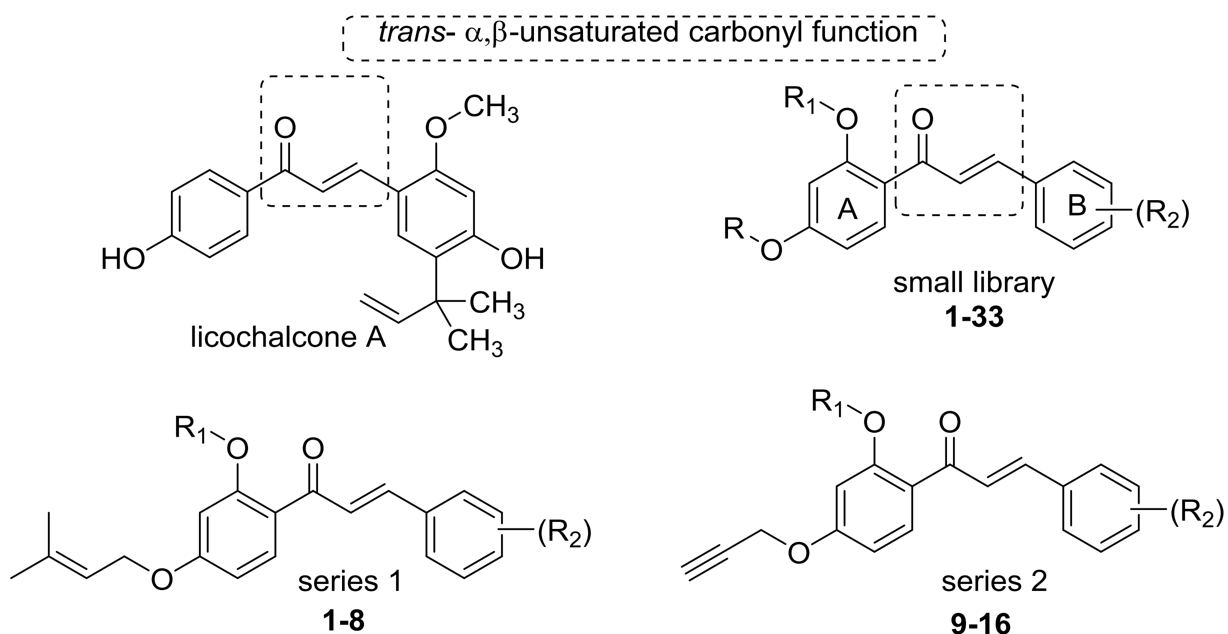
Table 1 Performance of serological tests for VL diagnosis on the study population (n=77)

		IC 95%			IC 95%	
TEST	**Sensitivity %**	**LL**	**UL**	**Specificity %**	**LL**	**UL**
ICT 1	77	54	91	95	83	100
ICT 2	73	50	88	98	86	100
ICT 3	59	37	78	100	90	100
ELISA 1	82	59	94	98	86	100
ELISA 2	91	69	98	100	90	100
ELISA 3	68	45	85	100	90	100
ELISA 4	68	45	85	91	77	97
IFAT	100	81	100	100	90	100
WB	100	81	100	86	71	94

Table 2 Performance of serological tests for VL diagnosis in immunocompetent subjects (n=65)



Scheme 1 Reagents and conditions: a) alkylbromide/ K2CO3, acetone, reflux; b) KOH/H2O, EtOH, rt, 18h



Scheme 2 Structure of licochalchone A and General structure of the synthetized compounds

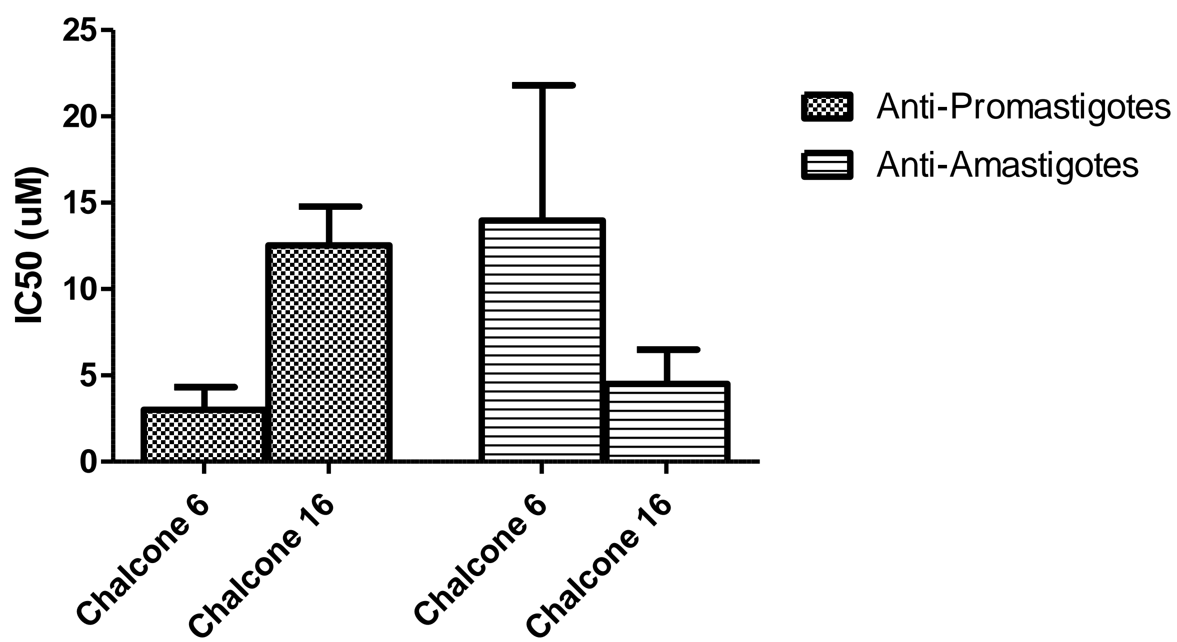


Figure 6 Comparison between antipromastigote and antiamastigote activity of compound **6** and compound **16.**

		In vitro activity		
Compound	**Structure**	**Promastigotes IC_{50} (μM)[1]**	**Vero CC_{50} (μM)[2]**	**SI[3]**
1		5	40	8
2		8.5	32	3.8
3		10.5	32	3
4		10.5	40	3.8
5		16	> 300	> 18.7
6		1.5	> 300	> 200
7		15	100	6.6
8		11	40	3.6
9		17.5	40	2.3
10		4	20	5
11		9.5	20	2.1
12		21.5	> 80	3.7
13		7	20	2.8
14		4	8	2
15		15	> 200	13.3
16		15	> 300	> 20

Table 3 Chemical structure of active compounds, in vitro antileishmanial activity against promastigotes of *L. donovani*, cytotoxicity and selectivity index (SI) of the synthesized compounds

Compound	Structure	Promastigote (IC50 µM)	Vero CC50 (µM)	SI
17		n.i.	n.d.	n.d.
18		n.i.	n.d.	n.d.
19		n.i.	n.d.	n.d.
20		n.i.	n.d.	n.d.
21		n.i.	n.d.	n.d.
22		n.i.	n.d.	n.d.
23		n.i.	n.d.	n.d.
24		n.i.	n.d.	n.d.
25		n.i.	n.d.	n.d.
26		n.i.	n.d.	n.d.
27		n.i.	n.d.	n.d.
28		n.i.	n.d.	n.d.
29		n.i.	n.d.	n.d.
30		n.i.	n.d.	n.d.
31		n.i.	n.d.	n.d.
32		n.i.	n.d.	n.d.
33		n.i.	n.d.	n.d.

Table 4. Chemical structure of inactive compounds, in vitro antileishmanial activity against promastigotes of *L. donovani*, cytotoxicity and selectivity index (SI) of the synthesized compounds. n.i.; Not inhibiting up to 40 µM. n.d.: Not determined due to the low antileismanial potency.

5. Summary of the studies included in this thesis and general conclusions

Leishmaniasis is considered an emerging pathogen and as a consequence of global warming, may spread in countries previously regarded unfitting for the diffusion of this parasite. In southern Europe, leishmaniasis is endemic in different countries such as Spain, Portugal, Greece, France, Italy and the Balkans. In the last decades, *Leishmania* has spread northward (where sandflies were previously thought to be absent or present at low densities), as shown by recent reports from northern Italy and southern Germany (32-34).

In this regard, in the last few years, a dramatic increase of VL cases has been reported in the Emilia-Romagna region in north-eastern Italy, with a cluster of cases in some municipalities, among which there is the Valsamoggia district.

The real prevalence of this parasitic disease is unknown, but it has been demonstrated that the majority of infected individuals do not progress to patent leishmaniasis, therefore clinical cases usually represent the tip of an "infection iceberg". Nevertheless, even in asymptomatic carriers VL can develop because of reactivation from the previous dormant infection; this phenomenon occurs mainly upon a heterogeneous collection of medical conditions that hamper the immunological control to the parasite. The main cause of *Leishmania* reactivation is HIV co-infection. while the vast majority of non-HIV-related immunosuppressive conditions fall under the realm of transplantation medicine, rheumatology and haematology. VL reactivation has been reported after different types of organ transplantation, or also associated with the use of various immunosuppressive drugs (107).

Considering the number of individuals at risk of Leishmania reactivation, asymptomatic infection is one of the great challenges of the VL disease and several questions remain unresolved regarding these asymptomatic infections, for example whether asymptomatically infected persons are infectious to the sandflies and which factors can predict the development of VL. Because

identification of asymptomatic *Leishmania* infections is difficult, the real prevalence of asymptomatic carriers in endemic areas is largely unknown and epidemiological studies are needed to answer these questions.

In our epidemiological study (Study 1), we screened peripheral blood samples obtained from 260 blood donors living in Valsamoggia, a municipality of the Bologna province where human leishmaniasis is endemic. The *Leishmania*-specific antibodies were revealed by Western blotting in the sera of 27 donors (10.4%), which showed the characteristic 14- and/or 16-kDa bands. In addition, the presence of the parasitic DNA was evident in the blood of 4 healthy donors (1.5%), one of these DNA-positive individuals also showed antileishmanial antibodies. Taking together these results, the total prevalence of asymptomatic Leishmania infection in the Valsamoggia municipality was 11.5%. This finding suggests a high circulation of the parasite in the Bologna area. In line with the literature (108), our study confirm a high proportion of asymptomatic *Leishmania* carriers in endemic areas and how the clinical cases usually represent just the tip of an “infection iceberg”.

False-positive and false-negative results cannot be excluded when one serological test is used to detect *Leishmania* infection, nonetheless Western blot has excellent sensitivity and specificity (109) and false-negative results are rare in our diagnostic experience and according to the literature. However, it is known that in asymptomatic carriers antileishmanial antibody titre in serum is low and antibodies may be absent (60, 110). According to this, we detected the presence of leishmanial DNA in the blood of three healthy donors that tested negative for specific antibodies.

Concerning molecular test, kDNA real-time PCR is known to be a highly sensitive and specific method to detect leishmanial DNA (74, 111). In accordance with the literature, we also demonstrated a an excellent sensitivity of this real-time PCR assay for the detection of VL cases (REF).

The fact that kDNA amplification was negative in 26/27 samples with a positive *Leishmania* serology could be explained by the presence of low parasite density and by the fact that parasitemia is probably episodic, hence, few targets were available for PCR amplification.

The importance of our results lies in the recognition of the presence of cryptic *Leishmania* infection among blood donors. At present, leukocyte depletion is performed in all blood products in Italy, but there are not enough evidence that show this is sufficient to prevent the transmission of *Leishmania* with blood transfusion. *Leishmania* testing is not mandatory in blood banks, but knowledge of positive *Leishmania* serology should impose blood elimination (112). Thus, a broader discussion would be required regarding the need to implement specific screening strategies for *Leishmania* in blood donors and to quantify the risk of *Leishmania* transmission by blood transfusion and by graft transplant.

An additional challenge for VL, due to the limited resources invested, is the lack of standardized diagnostic methods. Human leishmaniasis is spreading in Europe and Italy in areas previously considered to be not-endemic or at low endemicity. For example, the Emilia-Romagna region, an endemic region with a low prevalence of cases, assisted to an increased number of autochthonous VL cases in the last years (34).

In this scenario, the need of appropriate diagnostic methods seems mandatory. A few data are available about Mediterranean VL and information about diagnostic assays are scarce or relating to tropical and subtropical countries with a different socio-economic status compared to our situation.

For this reason, the aim of Study 2 was to compare the effectiveness of nine commercially available serological tests to detect VL cases. These tests were evaluated for diagnostic sensitivity and specificity against two different populations, 27 VL positive cases and 50 VL negative subjects from the Emilia-Romagna region.

In contrast to previous evidences (71, 113), we found that ICTs based on rk39 are less effective than other serological tests, showing low sensitivity and specificity. This low performance, compared to studies in other countries (i.e. India, Nepal) (114), could be connected to genetic differences in human populations that lead to a strongest or lower host immune response against the antigen used in the ICTs. Another explanation for the variable sensitivity of the ICTs in different regions of the worls could be attributed to genetic variability of the parasitic strains circulating in different areas (115).

Concerning ELISA, all but one kit exhibited moderate results in terms of sensitivity; conversely, IFAT and WB exhibited excellent sensitivity, but low specificity was observed for WB. The reason for the lower specificity of WB may be the high efficiency in detecting antileishmanial antibodies, also in asymptomatic carriers probably present within the VL-negative group.

A known obstacle to serodiagnosis of VL is the low antibody response within immunocompromised subjects; by restricting the results of serological tests to immunocompetent patients, the average sensitivity increased for each assay, with optimal sensitivity of ELISA 2, IFAT and WB, 91%, 100%, 100%, respectively.

It is known that a high sensitivity in serological test is crucial in the diagnosis of VL as missing VL diagnosis would lead to patients death. Our study highlights the complexity of VL serodiagnosis; the different tests that we evaluated revealed variable diagnostic performance, none of these tests appeared to be optimal for the use as screening test, even though ELISA 2 outperforms all of the other ELISA tests and ICTs. IFAT and WB showed optimal sensitivity, but their cost and complexity in execution would not allow their employment as screening tests, nonetheless these tests are recommendable as confirmatory test in the diagnosis of Mediterranean VL or as front line tests when the number of samples in the diagnostic routine is limited.

Finally, it is important to highlight that when serological results in immunocompromised patients are negative, molecular tests are needed before exclusion of VL.

As leishmaniasis is a neglected disease, treatment options are limited and therapy is often a challenge. All currently used first-line and second-line drugs for the treatment of leishmaniasis have several drawbacks including cost, toxicity, invasive route of administration and risk of emergence of drug resistance. Despite the urgence for the development of new drugs for treating this parasitic infection, a recent study has revealed that during the last 6 years, only one drug has been approved for the treatment of leishmaniasis (87). Historically, natural products (NP) are a good strategy when searching for new bioactive compounds, as they provide a basis for both design and synthesis of derivative compounds that can optimize biological activity and minimize side effects (116).

Combining the need for new active compounds against leishmaniasis and the known potential of natural products to provide chemical structures, which can be used as prototypes for new drugs, Study 3 aimed to highlight the use of a group of natural products, ie chalcones, as potential source of anti-leishmanial agents.

Chalcones (1,3-diaryl-2-propen-1-ones), members of the flavonoid family, occupy a special role among the biologically active NPs, since the trans-α,β-unsaturated carbonyl function, being a Michael acceptor system, could establish covalent linkages with the cysteine residue of a number of target proteins. Therefore, chalcones could be regarded as a class of protein-reactive compounds and, as expected, they have a large variety of biological activities, including antioxidant, antibacterial, antiprotozoal, antimutagenic, antimitotic, antimetastatic, and anti-inflammatory activity (117).

The antiparasitic properties of natural chalcones have been extensively investigated, in particular, licochalcone A (Scheme 2), a constituent of roots and rhizomes of various species of *Glycyrrhiza L.*

(licorice root), was found to be endowed with antiparasitic effects namely leishmanicidal and antiplasmodial activity (118, 119).

A series of chalcone analogues with various structural features were prepared based on natural product lead and tested for their antileishmanial activity. Among the 33 tested compounds, the derivatives from **1** to **16** turned out to effectively inhibit the promastigotes growth in *in vitro* evaluation against *L.donovani*. In particular, the analogues **6** and **16** were found to be the most promising of the series as they showed a remarkable antileishmanial potency against the extracellular form of the parasite, coupled with an excellent SI (above 20) and a recognizable activity against the amastigote stage of the parasite in *L.donovani* infected macrophages.

For pathogens displaying several life stages like *Leishmania*, there is a need to determine the best parasite stage to target. In the case of *Leishmania* there are two options: first, targeting the extracellular living promastigote stage, second, the intracellular amastigote stage. The first option meet the reproducibility, rapidity and low cost requirements for high-throughput screenings, due to the ease in manipulating promastigotes. The intracellular amastigote stage has been logically designated as the more relevant target for primary screening against *Leishmania*, but methods are labor intensive and would not support automation as the required Giemsa staining of infected macrophages is cumbersome as it needs manual counting. Thus, screening against the intracellular parasite stage were performed only on the two analogues that showed the best activity against the extracellular parasite stage.

Why compound **16** affects intracellular parasite better than compound **6**, in contrast with their inhibition on the extracellular parasite stage is not clear, but we can speculate that compound **16** has a better host cell-dependent mechanism of action than compound **6**.

Several antileishmanial drugs present a non-specific mode of action that accounts for their high toxicity. Thus, the discovery of new chemical entities targeting specific and indispensable

components of parasite metabolism is a priority. The thiol-dependent redox metabolism, required for DNA synthesis and defense against oxidative stress, is one of the unique metabolic features that distinguish trypanosomatids from humans and offer reliable molecular targets for selective drug development (120). In the thiol metabolism, it has already been shown that trypanothione reductase (TR), an enzyme that belong to the protein family of FAD disulfide oxidoreductases, becomes an attractive target for the development of new drugs; our experiments show that TR is strongly inhibited *in vitro* by chalcones, especially by compounds **6** and **16**.

In conclusion, the good inhibition of *L.donovani* replication the lack of toxicity on human cells and the ability to bind and likely inhibit a crucial parasite enzyme such as TR are all factors that highlight the potential for our newly synthetized chalcones to be optimized and developed into more specific agents against leishmaniasis. Therefore, efforts will be put to the modification of these scaffolds in order to synthetize better antileishmanial chalcones, to try to identify new critical targets and additional mechanism of action.

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