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Leishmaniasis

Knowledge, learning and innovation

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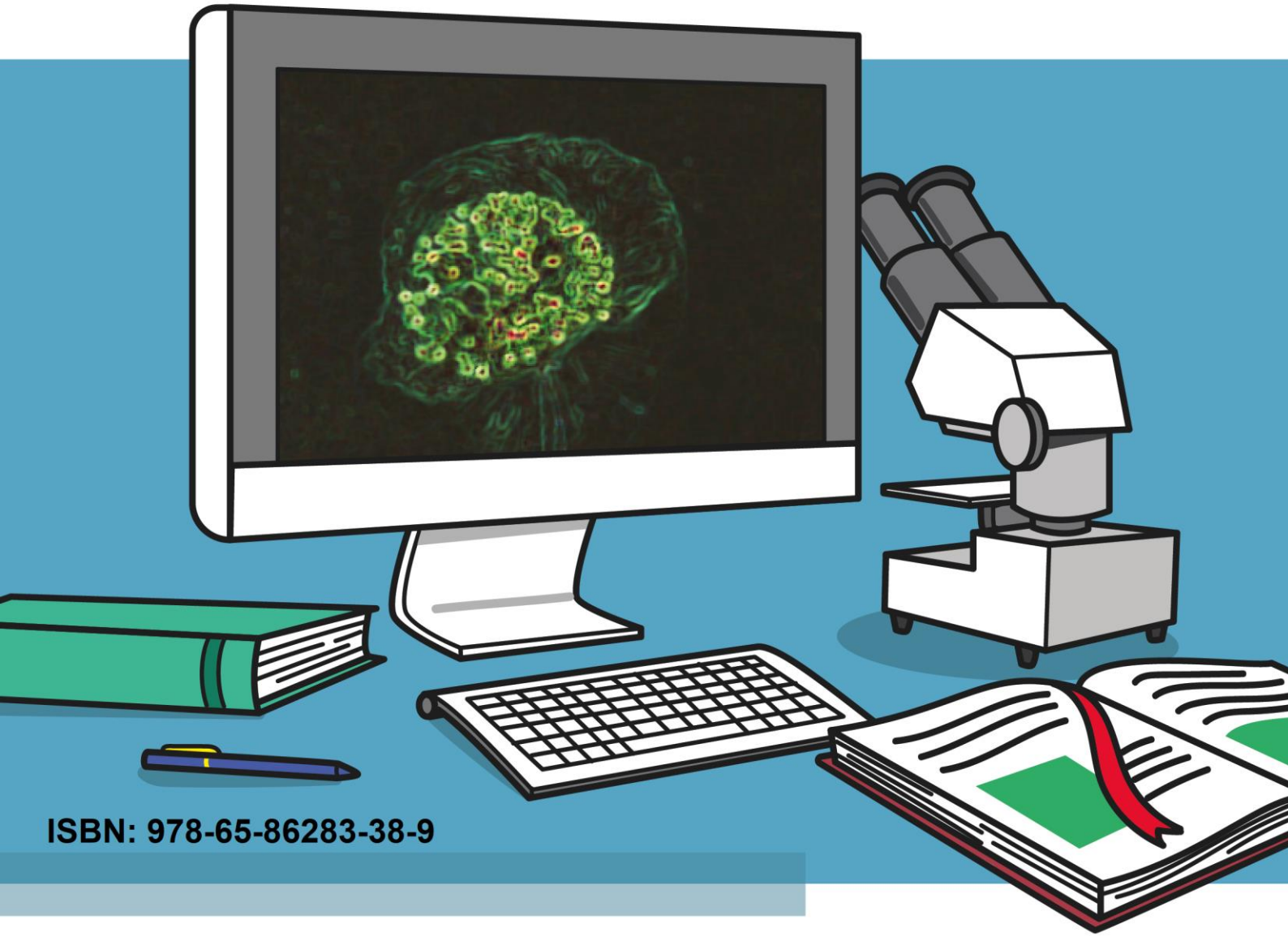
Dilvani Oliveira Santos



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BOOK PRESENTATION

The increase in the number of leishmaniasis cases observed during the last 25 years worldwide is due to some specific factors such as: globalization and climate change. They contribute to the spread of leishmaniasis in non-endemic areas. For example, in the last few decades, the number of cases of leishmaniasis in international travellers (tourists and businessmen) has increased. The literature also reports evidence that global warming will lead to an extension of the distribution of sand flies further north, which in the future, could result in the transmission of leishmaniasis in regions hitherto non-endemic. Other risk factors for the emergence and spread of leishmaniasis are war and other disorders. Nowadays, the outbreak of Cutaneous Leishmaniasis in the Middle East and North Africa represents a huge concern. This Cutaneous Leishmaniasis (CL) epidemic was triggered by the civil war in Syria and the refugee crisis, and now, affects hundreds of thousands of people living in refugee camps or in conflict zones. The most common form of leishmaniasis is Cutaneous Leishmaniasis with 0.7– 1.3 million new cases occurring annually worldwide (World Health Organization- WHO). Leishmaniasis is considered endemic in 88 countries, as more than 12 million people suffer from the disease and a portion of the population of approximately 350 million is at risk of contracting it.

In the book “Leishmaniasis - knowledge, learning and innovation”, Cutaneous Leishmaniasis is approached in different aspects such as: (1) Geographic Challenge; (2) Endemic disease distributed in all Brazilian territories, including the Brazilian border regions with other countries in South America; (3) a complex disease whose treatment remains a challenge and finally, (4) a disease that, in the near future, may have a promising treatment based on less harmful natural products. In short, this book aims to share some knowledge acquired from years of experience in working with this disease. This research also aims to show the formation of an innovative product which may contribute to the pharmacological treatment derived from algae, with leishmanicidal potential and devoid of cytotoxicity to cells human.

We hope that this book can be a pleasant and useful reading,

Dilvani Oliveira Santos

DEDICATION

This book is dedicated to patients affected by Leishmaniasis and health professionals whose work deals this disease.

SUMMARY

“Only Knowledge that makes us better is useful”

(Sócrates)

Stricto Ensu Editora

LEISHMANIASIS – A GEOGRAPHIC CHALLENGE

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ABSTRACT

Leishmaniasis is among the nine major infectious-parasitic diseases worldwide. However, it consists of a social health problem with an increasing number of cases linked to economic development, changes in behaviour and the replacement of forested environments to urbanization. Leishmaniasis is a complex of diseases which occur in tropical and subtropical areas and is found in 98 countries in Europe, Africa, Asia and America. However, over 90% of new cases occur in the following countries: Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Colombia, Ethiopia, India, Iran, Peru, South Sudan, Sudan and Syria. The globalization (circulation of people, products and information has become quick and easy in the world) and the climate changes, surely contributed to the spread of leishmaniasis to non-endemic areas. Besides, over the last decades, the number of cases of leishmaniasis in international travellers (tourists and businesspeople) has increased. And most recently, outbreaks of leishmaniasis have been recorded from refugee camps in many parts of the world. These facts, promoted the increase in the number of leishmaniasis cases observed during the last two decades, putting different professionals and agencies on alert linked to health, including health geographers. Therefore, this chapter aims to address the geography of Leishmaniasis in Brazilian social context, since this Neglected Tropical Disease still remains a major health problem in many endemic countries.

Keywords: Cutaneous Leishmaniasis, Health Geography and Geo-Historical context.

1. INTRODUCTION

Leishmaniasis is a complex of diseases which occur in tropical and subtropical areas and is found in 98 countries in Europe, Africa, Asia and America. However, over 90% of new cases occur in the following countries: Afghanistan, Algeria, Bangladesh, Bolivia, Brazil, Colombia, Ethiopia, India, Iran, Peru, South Sudan, Sudan and Syria. The environment in

which the human population lives affects their quality of life, which in turn depends fundamentally on some essential aspects such as healthy food, basic sanitation and decent housing. Without those essential living conditions, the environment in which you live affects your behaviour and consequently, your physical and mental health. Throughout the nineteenth and early twentieth century, infection diseases were the main argumentation for the advent of urban planning in Europe and the USA (PINTER-WOLLMAN et al., 2018).

At the end of the nineteenth century, sanitation was imposed in Brazil and consisted of urban planning, with the objective of controlling epidemics of infectious diseases (SANTANA; NOSSA, 2005). At that time, there were many poor quality, narrow low-rise and overcrowded apartment buildings in Brazilian cities, and these offered danger of contagion from various pathologies. However, through successful health campaigns, some epidemic outbreaks have been eliminated, such as yellow fever for example (FRANCO, 1969). The disorderly population growth in Brazil associated with immense social inequality are the main causes of many infectious and contagious epidemics in view of the Covid-19 pandemic that so far has claimed more than 155.000 deaths in Brazil (WHO, 2020). At the present time, Brazil represents the ninth largest economy in the world and, incredibly occupies the seventh position in terms of social inequality, according to the WHO (2019). Even today, the worsening of this poor population distribution in large metropolitan urbanities such as São Paulo and Rio de Janeiro, and their favelas and peripheral areas, with virtually no adequate sanitation, end up being the focus of epidemics. The urbanization of diseases such as Leishmaniasis, first occurred due to the vast spread of the urban agglomeration in the cities. With expansion of the cities into the direction of the open landed environments, such as forested areas, a greater contact with the natural vectors of a determined endemic started to be developed, in which these vectors became adapted to the urban environment, representing a danger to the population.

In Brazil, urban peripheral areas, such as suburban places, have suffered, and still suffer with a lack of knowledge about education on public health as well as a quality of infrastructure. This becomes a facilitator for the emergence of certain endemic diseases, such as Leishmaniasis. The urban area, where Leishmaniasis generally occurs or has a potential for further occurrence, is characterized in brazilian urban peripheries, but not only in low-income areas, but also in more central areas, mainly in the Southeast and Midwest regions. Despite the scientific progress in the area of Leishmaniasis, this complex of several forms of pathology continues to be a public health problem in many regions of the world and it is considered as a neglected disease.

Leishmania are protozoa transmitted by insect bites infected sandflies. Among these insects, 98 species of Phlebotomus and Lutzomyia genera have been described as proven or suspected vectors for human leishmaniasis (MAROLI et al., 2013). According reviewed by these authors, only female sandflies bite mammals in search of blood, as blood is important for completing the development of the female insect's egg. Some sandflies have a wide variety of hosts, including canids, rodents, marsupials and hyraxes, while others feed primarily on human blood. Thus, human leishmaniasis may have zoonotic or anthroponotic transmission patterns. According to Steverding (2017), the history of leishmaniasis comprises several aspects such as: the origin of the genus Leishmania in Mesozoic era; its geographical distribution initial evidence of the disease in antiquity; the first reports of the disease in the Middle Ages; and the discovery of Leishmania protozoa as etiological agents of leishmaniasis in modern times. As reviewed by Steverding (2017), regarding the origin and dispersion of Leishmanias, some hypotheses were presented (paleartic, neotropical and super-continental origin, respectively). Ancient documentation and paleoparasitological data indicate that leishmaniasis was already common in antiquity. The identification of parasites of the genus Leishmania, as etiologic agents and, sand fly insects as vectors of transmission of leishmaniasis began in the early 20th century and the discovery of new species of Leishmania and sand fly has intensified in the 21st century.

The increase in the number of leishmaniasis cases observed during the last 25 years throughout the world is due to several factors (STEVERDING, 2017). Globalisation and climate change are two factors that contribute to the spread of leishmaniasis to non-endemic areas (SHAW, 2007). For example, over the last decades, the number of cases of leishmaniasis in international travellers (tourists and businesspeople) has increased (MANSUETO et al., 2014).

However, still according to the review of Steverding (2017), there is also evidence that global warming will lead to an extension of the distribution of sand flies more northwards which could result in the transmission of leishmaniasis in hitherto non-endemic regions in the future (SHAW, 2007; POEPPL et al., 2013).

Other risk factors for the emergence and spread of leishmaniasis are war and unrest (SHAW, 2007). Currently, of great concern is the outbreak of Old World Cutaneous Leishmaniasis in the Middle East and North Africa. This disease epidemic was triggered by the Syrian civil war and refugee crisis and now affects hundreds of thousands of people living in refugee camps or caught in conflict zones (DU et al., 2016; AL-SALEM et al., 2016). Before

the outbreak of the civil war, the annual incidence of OldWorld CL in Syria was estimated to be around 23.000 cases (DU et al., 2016).

2. LITERATURE REVISION

2.1 THEORETICAL REFLECTIONS ON HEALTH GEOGRAPHY

Studies in Health Geography require a good understanding of geographic science, as they involve aspects at times of the physical and biological environment, at times of the human groups that inhabit a space (GUAGLIARDO,

2004). The concern with human health and its relationship with the environment are widely discussed. In this process, health geography plays an important role, as both social and environmental aspects are most often responsible for the problems that afflict the population's health. The association between Geography and Medicine is old and can be identified since Classical Antiquity, which includes the work of Hippocrates (480 BC) on the correlation of the factors: Air, Water and Place, with diseases, being most likely the pioneer of the themes relating Geography to Health. According to Vaz e Remoaldo (2011), Hippocrates' work deals with how the human body would change in an integrated way to the changes that occur in the constitution of nature, that is, it addresses the influence of seasonal changes, climates and winds on the human body and its diseases (SANTOS et al., 2019). Thus, in the Hippocratic period, the environment of cities was already considered a focus of health problems, because for him, diseases were the understanding of the imbalance of different fluids, such as blood, water, etc. To him, health would be the result of the balance of such fluids depending on the environmental conditions of the places. Worldwide, according to Santana e Nossa (2005), the concern with the distribution of diseases and the environment already existed in colonialist Europe, this being the first stage in the formation of social medicine: state medicine, having occurred in Germany, not yet unified, developing in that country, a state medical practice that sought to improve the health conditions of the population. Considering the national literature on health geography in Brazil, we have since the sixteenth century, descriptive treaties, in which some tropical diseases then unknown to Europeans are described as seasonal fevers, dysentery, scabies and, among others, diarrhea. In 1844, SIGAUD published a work on the climate and diseases of Brazil, “Du Climat

et des Maladies du Brésil" Apud Caponi e Opinel (2017), of indigenous communities, African slaves, workers in the gold and diamond mines and healers, among many other aspects of Brazilian life in the nineteenth century.

Research investigations were linked to a health institution with the principle of exterminating the causes of epidemics and endemics. For some time, public health was linked to urban planning and some authors, such as Santana e Nossa (2005), mention urban sanitation as the only remedy to control the transmission processes of infectious and contagious diseases, improving living conditions in cities. However, the organization of the population in the cities is a facilitator for the development of certain diseases (PEREHOUSKEI; BENADUCE, 2007). Mankind created facilities, over the years, for its daily routine, but did not imagine how this could cause so much damage to its health.

In the geographical space, interactions between different segments of human society and nature are usual. The human being is part of nature, not detached from it. Therefore, all its actions have consequences in the living space. If human transformations on space are not harmonious, new diseases may arise or diseases that have already been controlled can re-emerge. In reality, it is observed that the anthropic relationship with nature has become much more complex, because the disorderly growth of cities, representing a greater impact on the environment and, in some cases, can cause loss of quality of life. In this context, some epidemics, such as leishmaniasis, can be observed in several states of Brazil. Most of the concerns surrounding epidemics and pandemics are based on the impossibility of controlling new cases of infection, especially after such diseases are widespread in urban centres. It is important to emphasize, in this context, that the dynamics that involve the so-called global cities (SASSEN, 2005), characterized by the space-time compression, would be the main responsible for the increase in the number of infections in short periods and, also, by the difficulty of control over them. Thus, the continuous displacements that make up contemporary ways of life can organize the knowledge that involves space and spatialities in a prominent level, especially in regard to investments directed to health at a global level (GRAÇA, 2012). Therefore, once these issues are known, it is time to put that knowledge into practice and propose solutions and control measures for the problems encountered. The geography of Leishmaniasis mainly in brazilian social context, reveal aspects related to urbanization.

2.2 LEISHMANIASIS AS A FOCUS OF HEALTH GEOGRAPHY

The urban space is a human construction. It is the result of actions accumulated over time, and engendered by agents that produce and consume space. Thus, it is a constant social production, reflecting the history and present of societies (CORRÊA, 1998). The constant changes in the spatial organization of the city influence the urban environment in several aspects, among which health and climate can be mentioned, as these changes (especially with regard to changes in urban ecosystems), expose the population to the vulnerability of aspects related to diseases (VAZ; REMOALDO, 2011). When exploring the main natural environment, human beings come into contact with a medium not yet adapted to its presence. Therefore, it unawakened wild diseases with the potential to be spread due to urbanization. And, consequently, the urbanization of leishmaniasis can be attributed to the increase in urban population. Before this phenomenon, it was restricted to wild areas and affected men only in regions considered rural, such as farms.

However, from the moment when the human being becomes an urban citizen, it converts into favourable attitudes for the emergence of the phlebotome, a vector of leishmaniasis, which seeks areas rich in organic matter, since it uses it as food for its larval ripening. This process associated with climatic conditions promotes litter decay (leaves and branches, etc.) and makes it easier for the insect to lay its eggs. Leishmaniasis is known as a disease of an area with a dry climate with annual rainfall of less than 800 mm and a physiographic environment composed of valleys and mountains. However, this type of environment has changed with the urbanization of leishmaniasis.

2.3 THE GEO-HISTORICAL CONTEXT OF LEISHMANIASIS

Cutaneous leishmaniasis (CL) has existed for many years in the country and divides three periods in the history of the disease: (1) of uncertain origin and based on vague references, until 1895, the year of clinical observation of the 'Bahia button' and its correspondence with the 'button of the Orient'; (2) extends to 1909, when the etiological agent of “Bauru ulcer” is identified and described; (3) begins in 1910 with the identification of the parasite in mucosal lesions, then incorporated into the clinical picture of the disease (LAINSON; SHAW, 1987; SILVEIRA et al., 1997, VALE, 2005).

Archaeological studies developed in ceramic vases with the reproduction of healthy human figures mutilated by different diseases were able to ensure the occurrence of uta and

espundia - local names for the cutaneous and mucous forms of (CL), respectively - among the Incas during the pre-Columbian era. In the Brazilian territory, a contrasting study of anthropomorphic ceramics produced by our indigenous ancestors, did not allow the same observation, due to their rudimentary character (VALE, 2005). The only sure and, perhaps, oldest indication of the existence of the disease in Brazil is found in a quote in Tello's thesis, "Antiguedad de la syphilis en el Peru", from 1908, concerning the written work, Pastoral Religioso Político Geographico, edited in 1827 (LAINSON; SHAW, 1987).

The second period in the history of CL in Brazil begins with Juliano Moreira, who in 1985 studying the so-called “Bahia button”, related for the first time the “endemic button in hot countries”, when the possible migration of the disease through Syrian forays into the New World in earlier times represents a possibility. In 1903, WRIGHT identified *Helcosoma tropicum* as an agent of the "bud of the Orient", later called *Leishmania furunculosa*, which allowed the association of leishmaniasis with several dermatoses with different names, generally designating the affected geographical regions (LAINSON; SHAW 1987).The great epidemic of ulcer cases accompanied by mucous lesions, in the beginning of the 20th century in the State of São Paulo, with the construction of the Northwest Railway, described as "Bauru ulcer", foreshadows the end of the second period, which culminates with the identification of the agent in 1909, almost simultaneously by Lindenberg and by Carini and Paranhos (LAINSON; SHAW, 1987). The third period was marked by the endemic disease in several parts of the country, such as the Vale do Rio Doce in Minas Gerais, the Amazon region and the south of Bahia (VALE, 2005).

It is worth mentioning that, only after the discovery of leishmania as an etiological agent of the "button of the Orient", Rabello proposed the expression 'cutaneous leishmaniasis" (as before, cutaneous Leishmaniasis was referred as tegumentar Leishmaniasis) since this form of the disease is manifested by cutaneous and mucosal lesions and diverse morphology which allows to distinguish it from the visceral form of leishmaniasis. This author drew attention to the fact that the disease exists and spreads outside virgin forests, referring to several cases observed in the urban area of Rio de Janeiro already at that time. He, then, recognizes that there are many cases of ganglia - mutilating rhinopharyngitis - manifestations of CL. He also comments on the impossibility of distinguishing, at the time, between the leishmanias found in cutaneous leishmaniasis in Brazil and those present in the 'Eastern button'.

In the past, *Leishmania braziliensis*, a name given by Gaspar Viana, was admitted as the only agent of American cutaneous leishmaniasis (LTA) in the country (SILVEIRA et al.,

1997). Until the beginning of the 1960s, the classifications of parasites were based exclusively on clinical evolutionary behavior, configuring clinical forms of the disease in different geographical regions, since the morphology of parasites under optical microscopy did not allow their distinction (FURTADO, 1994).

In 1961, Pessoa proposed the subdivision of *L. braziliensis* into the varieties *braziliensis, guyanensis, peruviana, mexicana and pifanoi* that would be related to the different clinical forms of the disease in different regions (PESSOA, 1961). Thereafter, the classification of leishmanias was oriented towards the distinction of the *L. mexicana* and *L. braziliensis* complexes, based on more consistent criteria, such as the characteristics of the parasite's behavior in culture media, experimental animals and vectors (LAINSON, 1972). Since then, the advances represented by electron microscopy, molecular biology, biochemistry and immunology have opened new perspectives in the taxonomy of leishmanias (SHAW, 1985).

The new methods that have come to be used in the characterization of leishmanias include mainly the study of the development of promastigotes in the intestine of the phlebotomine vector (SHAW, 1982), the morphometric study of amastigotes and promastigotes in electron microscopy (SHAW, 1976; ALEXANDER, 1978), the electrophoretic mobility of isoenzymes (MILES et al., 1981; MADEIRA et al., 2009), the determination of the fluctuating density of the nucleus and kinetoplast DNA, the analysis of DNA degradation products by restriction enzymes, the radiospirometry, the characterization of specific external membrane antigens by monoclonal antibodies, the DNA / RNA hybridization techniques and the analysis of the kinetoplast DNA by means of the amplification technique by the polymerase chain reaction (DECKER-JACKSON, 1980; McMAHON-PRATT, 1982; BARKER, 1983; WORTH, 1983; JACKSON et al., 1984; ; LOPEZ et al., 1988; GRIMALDI, 1993; LOPEZ et al. , 1993). The most used classifications today for leishmanias follow the taxonomic model proposed by Lainson e Shaw (1987), which divide them into the subgenera Viannia and Leishmania.

The most common form of leishmaniasis is cutaneous leishmaniasis (CL) with 0.7– 1.3 million new cases occurring annually worldwide (WHO, 2016). CL occurs in three different forms, localised cutaneous leishmaniasis (LCL), diffuse cutaneous leishmaniasis (DCL) and mucocutaneous leishmaniasis (MCL). LCL is characterised by skin lesions and ulcers on exposed parts of the body, leaving permanent scars. DCL is a less common and distinguished from LCL by the development of multiple, slowly progressing nodules without ulceration involving the entire body. MCL is restricted to Latin America.

In Brazil, at least, seven Leishmania species responsible for human disease are recognized: the cutaneous form being caused mainly by *L. (V.) braziliensis*, *L. (V.) guyanensis* and *L. (L.) amazonensis* and, more rarely, by *L. (V.) lainsoni*, *L. (V.) naiffi* and *L. (V.) shawi*, while *L. (L.) chagasi* is responsible for visceral disease (GRIMALDI, 1993). Each species has peculiarities in relation to: clinical manifestations, vectors, reservoirs and epidemiological patterns, geographical distribution and therapeutic response. Since Rabello's brilliant historical review in 1925, considerable advances have been made in the knowledge of leishmaniasis, especially in relation to the biology of leishmaniasis and the immunology of the disease. This has allowed the development of new methods for both diagnostic and therapeutic purposes.

However, nowadays it is verified that Leishmaniasis continues to be an important public health problem in several regions of the world, mainly in global south countries, being mentioned as a neglected disease. *Leishmania sp*, life cycle and epidemiology Leishmaniasis is a zoonosis caused by the protozoan of the genus Leishmania, transmitted by the diptera vector of the genus *Phlebotomus* in global north and by the genus *Lutzomyia* and *Psychodopigus*, in the global south (DEANEI; GRIMALDI, 1985; MARSDAN, 1985; GRIMALDI, 1991; SANTOS et al., 2008). There are about 20 species of Leishmania that can cause infections in humans (BANULS, 2007). Among these, the main members of this genus are *L. major, L. mexicana, L. tropica L. amazonensis, L. mexicana, L. braziliensis, L. infantum (in Brazil, L. chagasi) and L. donovani* (LIESE, 2008).

Until the 1950s, cutaneous leishmaniasis (CL) spread practically throughout the national territory, coinciding with the deforestation caused by the construction of roads and the installation of population centres, with a greater incidence in the states of São Paulo, Paraná, Minas Gerais, Ceará and Pernambuco (LAINSON; SHAW, 1987; VALE, 2005). From then, until the 1960s, the disease appears to have declined, with deforestation already completed in the most urbanized regions of the country, in addition to the relative stability of rural populations. However, worryingly, in the last 20 years there has been a marked increase in the endemic, both in magnitude and in geographical expansion, with epidemic outbreaks in the South, Southeast, Midwest, Northeast and, more recently, in the North Region (VALE, 2005). The Amazonian theory was proposed by Marzochi e Marzochi in 1994, based on epidemiological and geographic distribution studies of *Leishmania (Viannia) braziliensis* in different ecosystems, involving different vectors and reservoirs. The authors suggest that human disease arose in the western Amazon region, mainly south of the Maranon-Solimoes-Amazonas river, where *L. (V.) braziliensis* predominates.

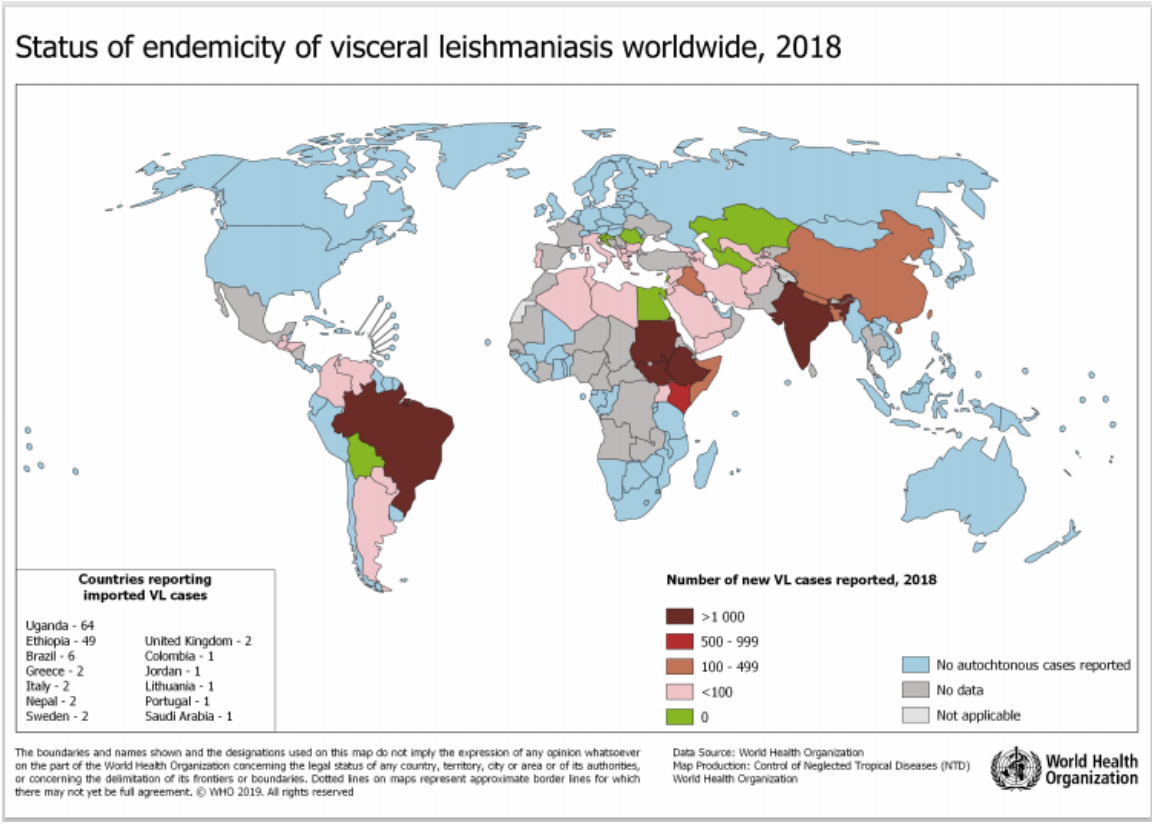
Leishmaniasis is among the nine main infectious and parasitic diseases worldwide. The status of endemicity of Visceral Leishmaniasis (VL) and the Cutaneous Leishmaniasis (CL) in the world can be seen in figure 1. Brazil presents both forms of Leishmaniasis (visceral (Figure 1A) and cutaneous (Figure 1B), while Indian, for example, only present Visceral Leishmaniasis.

However, as it is a neglected tropical disease, it consists of a medical and social problem with an increasing number of cases linked to economic development, changes in behaviour and the environment (CASTELLANO, 2005; VALE, 2005; SANTOS et al., 2008). According to Steverding (2017) leishmaniasis is considered endemic in 88 countries, as more than 12 million people suffer from the disease and a portion of the population of approximately 350 million is at risk of contracting it. In Brazil, the disease has been increasingly expanding in the North, Northeast, Midwest and Southeast regions. Especially in Rio de Janeiro, the presence of vector insects, small mammals as reservoirs of the parasite, in particular the growing number of stray dogs and even domestic ones infected by Leishmania, favor the spread of this disease and the contagion of man, favoring the cycle of protozoan life (RANGEL et al., 1986; SOUZA et al., 2002; MADEIRA et al., 2004; MADEIRA et al., 2006; VARGAS-INCHAUSTEGUI, 2008; DE PAULA et al., 2009; BRITO et al., 2012).

The epidemiological complexity of Cutaneous Leishmaniasis (CL) is characterized by the diversity of Leishmania species, reservoirs, and vectors involved in the transmission cycle. In this context, the literature reported Geographic information systems (GIS) – spatial analysis tools - that allow the conection of host, vector, parasite, and environmental data, to understand the transmission patterns and epidemiology of leishmaniasis, Chagas disease, malaria, dengue, and other infectious diseases, as well as their spatiotemporal distribution (ROGERS; RANDOLPH 2003; RUSHTON, 2003; NUCKOLS, 2004; VIEIRA, 2014).

The identification and monitoring of territorial units of epidemiological significance, as well as the knowledge of the spatial distribution of leishmaniasis cases and the species involved, may help to guide control measures, as reviewed by Miranda et al. (2019).

A



B

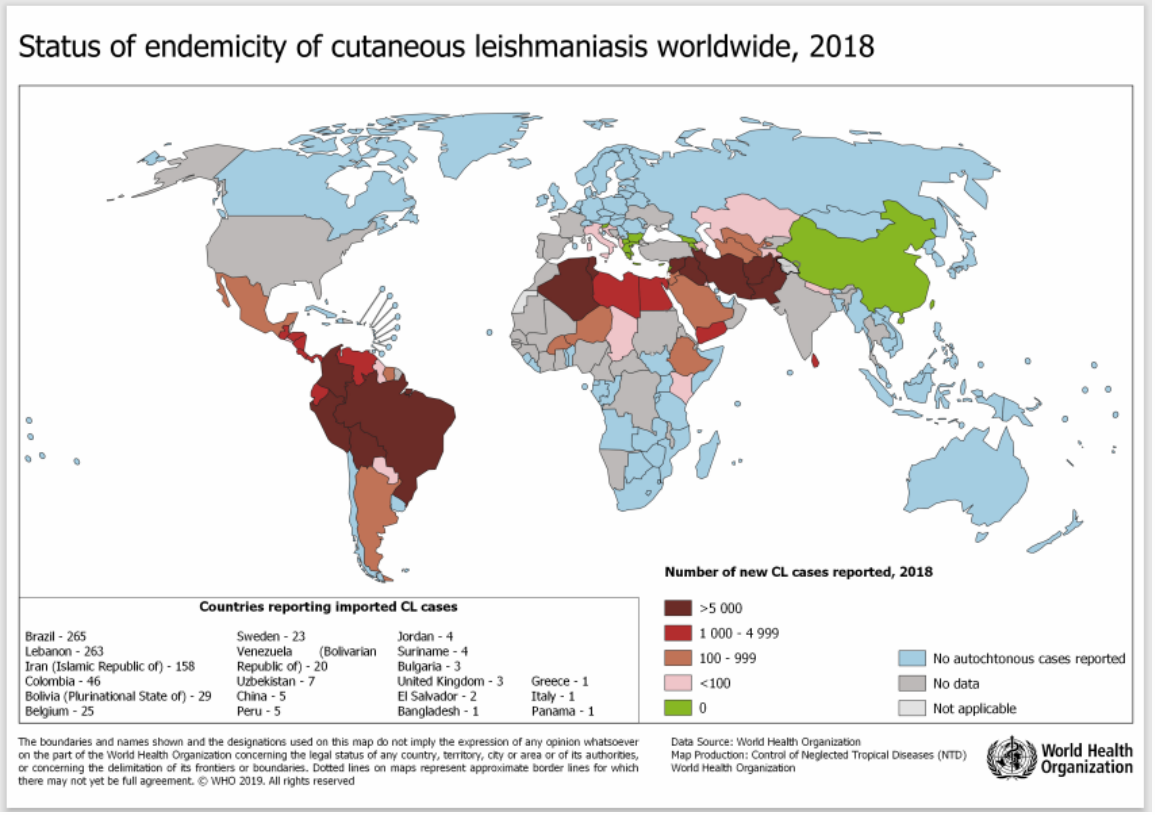


Figure 1. Status of endemicity of Visceral Leishmaniasis and Cutaneous Leishmaniasis in the World.

Source: Leishmaniasis - Epidemiological situation (https://www.who.int/leishmaniasis/burden/en/)

2.4 LEISHMANIASIS - A BRIEF REVIEW OF THE DISEASE, TREATMENT AND DIAGNOSIS

Briefly, leishmaniasis is a disease that can assume three characteristics: visceral, cutaneous and mucocutaneous, in addition to asymptomatic or subclinical forms in resistant individuals. Within this classification, cutaneous and mucocutaneous correspond to the clinical manifestations of cutaneous leishmaniasis.

Visceral leishmaniasis (VL) also known as Calazar, is a more severe pathology than the Cutaneous Leishmaniasis, caused mainly by the species *L. donovani* and *L. infantum / L. chagasi* (SANTOS et al., 2008; MARZOCHI et al., 2009). This disease is characterized by substantial weight loss, high fevers and anemia. The parasite has tropism for the organs, liver and spleen, causing an increase in size and loss of function in addition to other changes. Without proper treatment, the evolution of the disease can progress to death at a rate of 100% within 2 years (WHO, 2018). In Brazil, LV initially endemic to rural regions, suffered an intense expansion to peri-urban and urban areas.

The main reservoirs in proximity to man are domestic dogs (domestic kennels), and the great importance is that those animals can develop the asymptomatic form of the disease and have a high parasitic load on the skin and organs (MARZOCHI et al., 2009; SANTIS et al., 2011). In the case of Cutaneous Leishmaniasis (CL), the main causative species are: *Leishmania (viannia) braziliensis*, *L. (V) guyanensis, Leishmania (Leishmania) amazonensis, L. (V.) lainsoni, L. (V) naiffi, L. (V) shawi* and *L. (V) lindenbergui* (MARZOCHI, 1994; SILVEIRA et al., 2004; COSTA, 2005; OLIVEIRA; BRODSKYN, 2012). Mucocutaneous leishmaniasis (CML) affects mucosal regions - oropharyngeal-, mainly nose and mouth, and can become disfiguring, while it can culminate in multiple ulcerations that destroy the mucosa and reach nearby tissues (WHO, 2012). Cutaneous Leishmaniasis (CL) is characterized by a transient lesion on the skin, in the form of a papule or ulcer “on the edge of a volcano”. It usually occurs in exposed areas, such as the face, arms and legs, and resolves within a few months, although a scar remains at the injury site (WHO, 2012). The species *L. braziliensis* corresponds to the major causative agent of CL in Brazil and mucocutaneous leishmaniasis (MCL) in Latin America (VARGAS-INCHAUSTEGUI, 2008). It represents one of the seven dermotropic species found in Brazil, causing localized, multiple or disseminated lesions on the skin and side effects on the mucosa. Some researchers believe that CML is a variation of LC, as an evolution of the disease (BEDOYA-PACHECO, 2011). Several species of

Leishmania can be transmitted to humans through the bite of the *Lutzomyia intermedia* diptera.

The literature have shown a great concern with European countries, such as France, Italy, Spain and Portugal with the performance of *Leishmania sp* as opportunistic agents. The incidence of such parasites has increased the number of cases of VL, which was considered rare in the region (COURA, 1987; SOONG, 1996; DESJEUX, 2003; ANDROULA et al., 2010; NEGHINA; NEGHINA 2010; ERGEN et al., 2015;). According to Soong et al. (1996), *L. braziliensis* may also be associated with visceral infections, as well as seen in multiple or disseminated lesions, when dealing with HIV positive patients. These patients have a high parasitic burden (SILVA et al., 2002). Diffuse cutaneous leishmaniasis (LCD) produces chronic and disseminated lesions. It consists of one of the most difficult ways to treat LC. The appearance of individuals with LCD is very similar to patients with lepromatous leprosy (WHO, 2012).

This complex of disease has an even worse clinical derivation which is diffuse anergic cutaneous leishmaniasis (DACL). What divides it into two antagonistic poles in response to *L. braziliensis*: mucocutaneous leishmaniasis (CML) and DACL, although, the main causative agent of DACL in Brazil is *L. amazonensis* (SILVEIRA et al., 2009). The response to the treatment of the disease depends on a set of factors, such as the type of infective parasite, its resistance to the drug and the host's cellular immune response to the parasite. Among the species that cause LC, *L. braziliensis* is the most difficult to obtain a therapeutic response (SCHUBACH et al., 1998). According to the treatments proposed by the World Health Organization (WHO) and the Ministry of Health, the use of pentavalent antimonials is initially satisfactory. These drugs have been used since the 1940s in the primary treatment of the disease, mainly N-methylglucamine and sodium stibogluconate.

The dose of 20mg / Sb + 5 / kg / day, intravenously, lasts for 30 days. However, this medication is made with strict control of kidney, liver and pancreatic functions, due to its adverse effects and cytotoxicity (SANTOS et al., 2008; WHO, 2012). In Brazil, Glucantime® is distributed free of charge by the Ministry of health through the public health network, adopting the therapeutic scheme recommended by the WHO. As a second option in cases of resistance, other drugs are used such as: classic amphotericin B (Fungizona®), applied intravenously in the hospital; pentamidine (isothionate and mesylate) applied intramuscularly, less effective and quite toxic; and paromycin, which is highly cytotoxic (SANTOS et al., 2008; WHO, 2012). However, one of the essential problems in the treatment of the disease are the

adverse and toxic effects for the patient. Often, the patient needs to interrupt the treatment to take care of the side effects produced (TIUMAN et al., 2011).

Laboratory diagnosis can be performed using three methods: parasitological, histopathological and immunological. For parasitological analysis, visualization of the parasite is essential for the certainty of the diagnosis, carried out by researching the amastigote forms (intracellular forms of Leishmania) through biopsy of the lesion for anatomopathological examination, culture in artificial media and inoculation in a hamster. While immunological techniques (PCR, serology - ELISA, indirect immunofluorescence, Montenegro's intra-dermo-reaction (MRI), provide greater reliability in diagnosis, although still quite precarious in Brazil due to its high cost (MACHADO et al., 2011; BRITO et al., 2012). The number of animals infected with Leishmania has increased a lot in large urban centers, serving as reservoirs of parasites (MARZOCHI; MARZOCHI, 1994; MADEIRA et al., 2004) and prophylactic measures require planning and development of programs in conjunction with society. It involves education, access to information, treatment and medical assistance, among others.

3. FINAL CONSIDERATIONS

In many endemic regions, leishmaniasis is an epidemiologically unstable disease which has a tendency for unpredictable fluctuations in the number of cases. There are several reasons for this. However, cultural, environmental and socioeconomic factors seem to be the most relevant. Thus, from a geographical point of view, a simple contribution to ameliorate on a short term, the current panorama of leishmaniasis (CL and VL) in Brazil, could come with the following measures:

1. Address the problem of locating people and activities in different Brazilian geographic regions, which are essential focuses of the geography of leishmaniasis;

2. Approach the planning of health care provision that should privilege levels of accessibility, focusing on proximity to the population from the identification of the area with the highest incidence of the disease;

3. It would be very important to create a base map with the following layers of information: Administrative divisions; Demographic data (censuses); Location of health posts; Location of the area with the highest numbers of infected with Leishmania - This would help a lot as a measure of control of Leishmaniasis, which

would have multifunctions such as: diagnosis, notification of cases, treatment and cure of the disease.

4. Creating a network information system with other countries in South America, in order to maintain a dialogue about the control of the disease. This would be specially, important to bordering countries who face an increase of cases. It is essential to notify and work in cooperation with neighbouring social contexts.

Altogether, we believe that those apparently simple measures above mentioned will contribute to the control of Leishmaniasis, in a more viable and effective way in Brazil. In the next chapter of this book, Leishmaniasis in the regions bordering Brazil in Latin America and their challenges will be discussed.

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Stricto Sensu Editora

CUTANEOUS LEISHMANIASIS IN LATIN AMERICA - INCLUDING REGIONS BORDERING BRAZIL - A BRIEF REVIEW

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ABSTRACT

In the present chapter, we seek to analyse the epidemiology of Cutaneous Leishmaniasis in Latin America, where 18 out of 20 countries are endemic. Leishmaniasis was also analysed in the borders regions of Brazil with the other countries of Latin America. We review articles related to the topic, accessed through the platforms such as: SCIELO, PubMed, LILACS and BVS. Official data base from the Pan American Health Organization as well as publications of the World Health Organization were also taken into account. Leishmaniasis are diseases with high index of morbidity and mortality, with wide demographic distribution in America and characterized as a severe public health issue. Brazil, Colombia and Peru remain endemic countries with the largest number of case records in Latin America. In 2016, after a some period among the countries with the highest incidence rate, Nicarágua, has joined the countries with the highest number of cases. Leishmaniasis affects mainly the working class and their living and working conditions are linked, both by the environment and by socioeconomic and cultural factors. The analysis and integration of social determinants of health and vigilance actions are extremely important, in order to develop social policies and improvements on diagnostic tests, therapeutic and prophylactic agents for control of this complex of diseases.

Keywords: *Leishmania*, Cutaneous Leishmaniasis, Public Health, Epidemiology, Latin America and Borders areas.

1. INTRODUCTION

Leishmaniasis are zoonosis caused by flagellate protozoan which presents high incidence and wide geographic distribution in the Americas and continue to be a major

challenge for national and regional programs in their pursuit of surveillance, prevention and control of these diseases (OPAS, 2018). According to the World Health Organization (WHO), in 2017, leishmaniasis was endemic in 98 countries and territories on four continents, with an estimated 350 million people living in high-risk areas.

Cutaneous Leishmaniasis (CL) is a disease caused by *Leishmania sp* and it is an autochthonous disease of the American continent (PESSOA; BARRETTO, 1948). The history of the disease mentions pre-Columbian ceramics dating back 400 to 900 years were found in the Americas, produced by Peruvian Indians. The sculptures represented men with lesions on the lips and nose-uta and spúndia, local denomination for CL (BASANO; CAMARGO, 2004). Leishmaniasis can be considered as a complex of diseases, since they present several clinical forms and have long reached the population (MURRAY, 2005; LAINSON, 2010). In the literature, we find several theories about its origin and expansion in the American continent, being Rabello's theory (1925) referenced, describing the history and synonymy of the disease and distinguishing three periods in its history (PESSOA; BARRETTO, 1948; VALE; FURTADO, 2005; FURUSAWA, 2014).

The social-historical transformations are directly linked to infectious and parasitic diseases, and the distribution of Public Health problems. The environment plays a relevant role in the spread of parasitic diseases in man. Therefore it is necessary to maintain the balance of the ecosystem interactions (Figure 1), due to the link between the environment, animal health and human health. In this context, analyzes of the interconnected physical, social, cultural and political environments are extremely important to understand the conditions and determinants of the proliferation of infectious agents and vectors. In the case of a Neglected Tropical Disease, it is worth mentioning that the slow process in the diagnosis and treatment of leishmaniasis, currently due to the pandemic of COVID-19, may cause the intensification of cases of this pathology not only in Brazilian context, but also, in other Latin American countries which shares borders with Brazil. Analyses of the social determinants of health are extremely important, and epidemiology helps us fulfil this great role. In addition, the expansion of leishmaniasis deserves attention and needs to be observed as a result of the adaptation of sandflies (phlebotomines) to altered environments, where humans, domestic and wild animals are available as alternative nutritional resource (XIMENES et al., 2007; ALESSI et al., 2009).

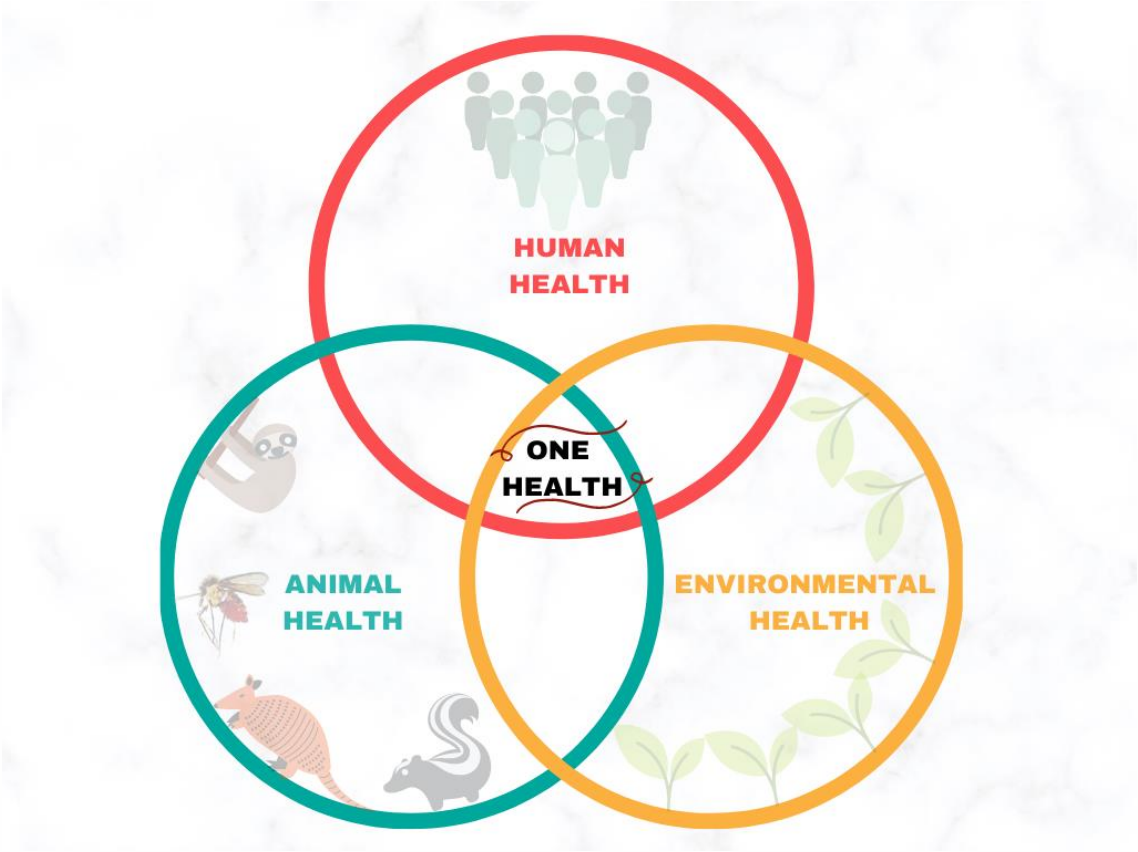

Figure 1. One Health - Term proposed by the World Health Organization (WHO), World Organization for Animal Health (OIE) and United Nations Food and Agriculture Organization (FAO) (personal archive).
(https://www.oie.int/ ; http://www.fao.org/home/en/)

2. METHODS

In this study, there were carefully reviewed articles on Cutaneous Leishmaniasis (CL) in Latin America, published in the last 15 years. The Access was done by platforms such as Pubmed, Scielo, LILACS and BVS. We also analysed the epidemiological reports of the Pan American Health Organization and World Health Organization - PAHO / WHO from 2013 to 2019, articles, publications, reports and manuals of the World Health Organization and the Brazilian Epidemiological Surveillance manuals. We searched for data concerning topics such as the etiological and protozoan agents, reservoir, biological cycle and transmission of Cutaneous Leishmaniasis in Latin America, understanding that they are essential for a review of epidemiology. Besides, we carried out an epidemiological analysis in the region, with further analysis in countries with the greatest number of case register.

3. RESULTS AND DISCUSSION

3.1. ETIOLOGICAL AGENTS OF LEISHMANIASIS

Leishmaniasis are caused by protozoa from genus *Leishmania* which is taxonomically divided into subgenus *Viannia* (BANULS et al., 2007; LAINSON; SHAW, 2010,), which have a wide range of dipteran vectors (LAINSON et al., 2005). In this context, about 900 species of sand flies are described around the world, around 70 are related to the transmission of leishmaniasis (READY, 2013) and in the Americas about 500 species are described. A little more than 30 of those species can be related to the transmission of human leishmaniasis (LAINSON et al., 2005). The dipteran classification and historic-geographical division predominantly employed are in the genus Phlebotomus in Europe, Africa and Asia and the *Lutzomya* genus in the Americas (READY, 2013).

The literature describe approximately 30 species of *Leishmania*, and of these more than 20 are pathogenic, and may cause infections in humans (LYRIO et al., 2017). In Latin America, several species of Leishmania circulate responsible for causing Cutaneous Leishmaniasis (CL). The figure 2 shows the diversity of Leishmania species and vectors in Latin America. As shown in this figure, according to the bulletins of Pan American Health Organization / World Health Organization (2015-2019), *L. braziliensis* is present in all the Latin America countries, except El Salvador and Suriname. On the other hand, *L. amazonensis* is found in all the countries of Latin America, except Costa Rica, El Salvador, Guatemala, Guyana, Honduras, Mexico and Nicaragua.

LATIN AMERICA COUNTRY	*LEISHMANIA SPECIES*	LUTZOMYIA VECTORS
ARGENTINA	*L. amazonensis, L. braziliensis, L. guyanensis.*	*L. whitmani, L. neivai, L. migonei.*
BOLIVIA	*L. amazonensis, L. braziliensis, L. guyanensis, L. lainsoni.*	*L. flaviscutellata, L. nuneztovari, L. carrerai carrerai, L. shawi, L. ayrozai, L. yucumensis, L. llanosmartinsi, L. nuneztovari anglesi*
BRAZIL	*L. amazonensis, L. braziliensis, L. colombiensis, L. lindenbergi, L. naiffi, L. lainsoni, L. shawi.*	*L. flaviscutellata, L. whitmani, L. neivai, L. migonei , L. intermedia, L. complexa, L. wellcomei, L. davisi, L. fischeri, L. pessoai, L. umbratillis, L. ubiquitalis, L. ayrozai, L. paraensis, L. amazonensis, L. salesi, L. shawi, L. reducta, L. olmeca nociva, L. carrerai carrerai, L. intermedia, L. antunesi, L. squamiventris, L. edwardsi, L. anduzei.*
COLOMBIA	*L. amazonenses, L. braziliensis, L. mexicana, L. colombiensis, L. guyanensis, L. panamensis, L. laisoni, L. naiffi, L. venezuelensis.*	*L. flaviscutellata, L. colombiana, L. spinicrassa, L. hartmanni, L. umbratillis, L. longiflocosa, L. trapidoi, L. panamensis, L. gomezi, L. pia, L. towsendi, L. yulli yulli, L. cruciata, L. columbiana.*
COSTA RICA	*L. braziliensis, L. panamensis.*	*L. trapidoi, L. ylephiletor, L. ayrozai, L. umbratillis.*
EL SALVADOR	*L. panamensis, L. infantum.*	*L. cruciata, L. longipalpis, L. evansi.*
ECUADOR	*L. amazonensis, L. mexicana, L. naiffi, L. panamensis, L. braziliensis, L. guyanensis, L. laisoni, L. equatoriensis.*	*L. ayacuchensis, L. carrerai carrerai, L. davisi, L. flaviscutellata, L. gomezi, L. hartmani, L. migonei, L. olmeca bicolor, L. panamensis, L. paraensis, L. salesi, L. sanguinaria, L. shannoni, L. trapidoi, L. ubiquitalis, L. ylephiletor, L. yuilli, L. tortura, L. cruciata.*
GUATEMALA	*L. braziliensis, L. panamensis, L. mexicana.*	*L. olmeca olmeca, L. ovallesi, L. panamensis, L. ylephiletor, L. trinidanensis, L. cruciata, L. shannoni, L. trapidoi.*
GUYANA	*L. braziliensis, L. guyanensis.*	*L. anduzei, L. umbratillis, L. whitmani, L. panamensis.*
HONDURAS	*L. braziliensis, L. panamensis, L. intantum.*	*L. trapidoi, L. panamensis, L. ylephiletor, L. ovallesi, L. longipalpis.*
MEXICO	*L. braziliensis, L. mexicana.*	*L. anthophora, L. cruciata , L. diabolica, L. gomezi, L. hartmani, L. olmeca olmeca, L. ovallesi, L. panamensis, L. shannoni, L. ylephiletor L. deleoni.*
NICARAGUA	*L. panamensis, L. infantum, L. braziliensis.*	*L. cruciata, L. diabolica, L. evansi, L. gomezi, L. longipalpis, L. nuneztovari anglesi, L. ovallesi, L. panamensis, L. sanguinaria, L. shannoni, L. trapidoi, L. ylephiletor.*
PANAMA	*L. panamensis, L. braziliensis, L. guyanensis, L. naiffi, L. colombiensis.*	*L. gomezi, L. olmeca bicolor, L. olmeca olmeca, L. panamensis, L. sanguinaria, L. trapidoi, L. ylephiletor, L. cruciata.*
PARAGUAY	*L. amazonensis, L. lansoni, L. braziliensis.*	*L. migonei, L. neivai, L. pessoai, L. shannoni, L. whitmani, L. intermedia.*
PERU	*L. amazonensis, L. braziliensis, L. colombiensis, L. guyanensis, L. lainsoni, L. panamensis, L. peruviana, L. shawi.*	*L. ayacuchensis, L. diabolica, L. peruensis, L. pescei, L. tejadai, L. verrucarum, L. yuilli, L. flaviscutellata, L. yulli yulli, L. ubiquitalis, L. davisi.*
SURINAME	*L. amazonensis, L. guyanensis, L. lainsoni.*	*L. flaviscutellata, L. umbratilis, L. anduzei.*
VENEZUELA	*L. amazonensis, L. pifanoi, L. venezuelensis, L. braziliensis, L. colombiensis, L. guyanensis, L. infantum, L. mexicana.*	*L. anduzei, L. flaviscutellata, L. gomezi, L. migonei, L. olmeca bicolor, L. olmeca olmeca, L. ovallesi, L. panamensis, L. spinicrassa, L. trinidanensis, L. umbratillis, L. youngi, L. reducta, L. rangeliana, L. longipalpis, L. pseudolongipalpis.*

Figure 2. Layout of Leishmania species and lutzomyia vectors corresponding to endemic Latin American countries (from 2015 to 2019) – source: Pan American Health Organization / World Health Organization.
(www.paho.org/leishmaniasis)

3.2. LEISHMANIASIS RESERVOIRS

The parasite's reservoir is the site / organism used to multiply or differentiate the parasite, without developing the pathology (CERBINO NETO et al., 2009). The main reservoirs of *Leishmania* are wild mammals, whom participate in the primary cycle of transmission, serving as a source of the disease (LYRIO et al., 2017). However, studies suggest that some domestic animals, for example dogs and horses, in certain situations may be responsible for the maintenance of the peridomestic and urban cycle of the CL, stating that species of sand flies are adapted to the home and / or peridomestic environment (BRASIL, 2010; LYRIO et al., 2017). In addition, it is important to emphasize that they are considered to be maintenance reservoirs, mammals capable of infecting and maintaining stable infections by a certain species of parasite, and reservoirs amplifying those that besides maintaining the infections, present a profile that guarantees the transmissibility of this parasite (ROQUE et al., 2010).

3.3. TRANSMISSION AND BIOLOGICAL CYCLE OF LEISHMANIASIS

The transmission of the disease occurs through hematophagous insects called phlebotomines (sandflies), through the bite of infected females, which previously fed blood from an infected mammal (MAROLI et al., 2013).

Phlebotomines (sand flies) measure approximately 1 to 3 mm, have a yellowish body covered by light bristles, wings always kept upright - even when at rest, and a bouncing flight. Their habits are nocturnal or evening, and in periods of rainfall, or in conditions of deforestation, or construction of dams and urbanization, occurs an increase in population density and consequent transmissibility of the disease (BRASIL, 2010). Other factors such as the socioeconomic imbalance of a significant portion of the population also contributes to the aggravation of the situation, due to migratory flows, occupation of land by mining area and, mainly, the continuous degradation of the environment, corresponding to the sandfly past, which initially inhabited wild areas (AMARO, 2012).

In addition, the increase in human travel allowed the spread of infectious agents, introducing them in areas that were previously absent (COLWELL et al., 2011). Other forms of transmission were also described through injecting drugs and blood transfusion (LAINSON; RANGEL, 2005; MAIA-ELKHOURY et al., 2008; BELO et al., 2013).

The development of the Leishmania biological cycle in the hosts begins during the feeding of the blood in the infected vertebrate, the female vector ingests cells from the injured tissue at the site of the bite, mainly tissue macrophages containing amastigote forms in its interior, which are transported to the anterior region of the tract digestive of the insect, where the amastigote will be protected within the peritrophic matrix and after differentiation will become procyclic promastigotes (extracellular form of Leishmania). Subsequently, the peritrophic matrix ruptures and the forms go to the epithelium of the digestive tract and suffer consecutive binary divisions, in the anterior region of the intestine they undergo metacyclogenesis and become highly infective, being called metacyclic promastigote. The transmission of Leishmania occurs during a new blood supply in a host, human or other mammal, regurgitating together the salivary content metacyclic promastigotes forms, being these phagocytosed by the cells of the Mononuclear Phagocytic System and later differentiated in amastigotes (intracelular forms of Leishmania), which will multiply and break these cells and, thus, are released into the bloodstream of the vertebrate host (DESJEUX, 2004; MURRAY et al., 2005; BATES, 2007; REY, 2008; DOSTÁLOVÁ; VOLF, 2012).

Moreover, it is important to note that only metacyclic promastigotes that scaped the lytic action of complement and neutrophils and eosinophils in vertebrate hosts will be phagocytosed by macrophages (SILVEIRA et al., 2008). The forms that adhered to the macrophage and were subsequently phagocytosed will become amastigote form, as previously stated, within the parasitoid vacuole. In addition, after the infection by the parasite, the incubation period of the disease in humans is quite variable, being in average, from two to three months, and can vary from two weeks to two years (BRASIL, 2007). The evolution of the infection will depend on the immunogenic profile of the vertebrate host, being strongly associated with the cellular immune response, and virulence of the infecting *Leishmania* species, resulting in different clinical forms of CL (MOSSER; EDELSON, 1984; SILVEIRA et al., 2008).

3.4. PATHOGENESIS OF CUTANEOUS LEISHMANIASIS

Initially, the lesion appears as unique, however, if the host is bitten several times by the infected vector insect, due to the intense multiplication of protozoa generating irritation or certain mechanical obstruction, it can generate other lesions in the form of erythematous papule that can evolve to a lymph node accompanied by regional adenopathy with or without lymphangitis (RODRIGUES et al., 2006; REY, 2008).

The clinical manifestation will depend on the species of the Leishmania and the patient's immune response, being restricted to the place where the parasite was inoculated, or reaching new sites on the skin and mucosa of the nose, larynx and oropharynx (FERREIRA et al, 2012). *Leishmania* (*Viannia*), *Leishmania* (*Viannia*) *guyanensis*, and *Leishmania* (*Viannia*) *panamensis* (*Leishmania* (*Viannia*) *amazonensis*) are the most important species of *Leishmania* (*Viannia*). CL lesions can range from unapparent forms to disseminated lesions, reaching the skin and mucous membranes (FAGUNDES et al., 2010). The literature reports the occurence lymphadenopathy, which consists of enlarged lymph nodes, close to the site of inoculation of the parasite. These may occur preceding days or weeks to the cutaneous injury and are related to the species *L.* (*Viannia*) *braziliensis* (LESSA et al., 2007). Classically, American Cutaneous Leishmaniasis (ACL) occurs in the following forms: cutaneous leishmaniasis and muco-cutaneous leishmaniasis.

Cutaneous Leishmaniasis (LC) is characterized by a typical painless ulcer, rounded or oval in shape, with well-defined and elevated edges, among other signs. Besides, CL may present as localized cutaneous forms, which present lesions or ulcer-like lesions, with a tendency to cure or with a good response to treatment. Another clinical form of Leishmaniasis, the known Disseminated Cutaneous forms (DCL), which is rarely observed in up to 2% of the cases, is characterized by the appearance of multiple papular and acneiform lesions, and the two species known to cause this clinical form, are *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis*. Recurrent forms of the skin presenting lesions with central scarring with infiltrated edges and satellite lesions and Diffuse Cutaneous forms occurring in patients with anergy and specific deficiency in the cellular immune response to *Leishmania* antigen (BRASIL, 2007) are also reported. Recently, a study was published pointing out that the FLI1 gene may be related to the formation of lesions in LC (DA HORA et al., 2020).

Equally important, Muco Cutaneous or Mucosal Leishmaniasis (MCL), are also clinical forms of Leishmaniasis and most cases occur after cutaneous lesions. Some reports show that 3-5% of LC cases can develop mucosal lesions. It is believed that the main cause is the haematogenous dissemination of leishmaniasis inoculated into the skin into the nasal mucosa, oropharynx, palate, lips, tongue, larynx and, exceptionally, trachea and respiratory tree (BRASIL, 2007). Usually these forms are associated with painful symptomatology, difficulty deglutition, sialorrhea, foul smell and bleeding (SANTOS et al., 2013).

3.5. DIAGNOSIS AND TREATMENT OF CUTANEOUS FORMS OF LEISHMANIASIS

For the diagnosis of Leishmanias, different techniques can be used. It is worth mentioning the importance of associating epidemiological data (relating permanence in endemic region), clinical, laboratory, parasite evidence (imprint, histopathology, culture, immunohistochemistry), molecular tests (polymerase chain reaction - PCR), immunological and serology tests (LIMA et al., 2007; COSTA et al., 2014). Despite the existence of several studies proposing the development of new drugs and forms of treatment of leishmaniasis, including the study of the use of larval therapy with Musca domestica in ulcers (REYES PARRADO et al., 2020), we still face several problems with therapies, due to the use of medicines that have been on the market for more than 50 years (SANTOS et al., 2008, LYRIO et al., 2017).

The drugs in question are pentavalent antimonials, N-methyl glucamineantimoniate (Glucantime®) and sodium stibogluconate (Pentostam®), used as the first choice for the treatment of CL (SANTOS et al., 2008; LYRIO et al., 2017). The adverse effects associated with treatment with pentavalent antimonials are: clinical, such as musculoskeletal pain, gastrointestinal disorders and mild to moderate headache; electrocardiographic changes, such as prolongation of QT interval, alteration of ventricular repolarization; ischemic changes; and bigeminated, polymorphic and polyfocalextrasystoles; and laboratory abnormalities, with mild to moderate increases in pancreatic and hepatic enzymes (LYRA et al., 2016). Fatal arrhythmias are rare, with few cases of sudden death, probably related to ventricular arrhythmias (SANTOS et al., 2013). The Brazilian Ministry of Health recommends the use of intravenous or intramuscular Glucantime® dose of 10-20 mg Sb5 + / kg / day for 20 consecutive days for LC and 20 mgSb5 + / kg / day for 30 consecutive days for Mucocutaneous Leishmaniasis (BRASIL, 2010). In cases of non-response, it is necessary to use the drugs of second choice, Amphotericin B and Pentamidine. Amphotericin B can be considered as the first option in the treatment of pregnant women (BRASIL, 2010).

3.6. CUTANEOUS LEISHMANIASIS: EPIDEMIOLOGICAL ANALYSIS IN LATIN AMERICA INCLUDING BORDER REGIONS

Epidemiology plays a very important political and social role in the search for health promotion. As emphasized by Maurício Barreto (1998), as a basic discipline in the field of Collective Health, epidemiology has the responsibility of generating knowledge, information

and technologies that can be used to formulate policies for the promotion, prevention and control of health problems (BARRETO, 1998). According to the Pan American Health Organization PAHO / WHO (2018), Cutaneous Leishmaniasis occur in 20 countries and are endemic in 18 Latin American countries, including Mexico, El Salvador, Costa Rica, Panama, Colombia, Ecuador, Peru, Bolivia, Argentina, Uruguay, Paraguay, Brazil, Suriname, Venezuela, Nicaragua, Honduras and Guatemala and French Guiana (**Figure 3**).

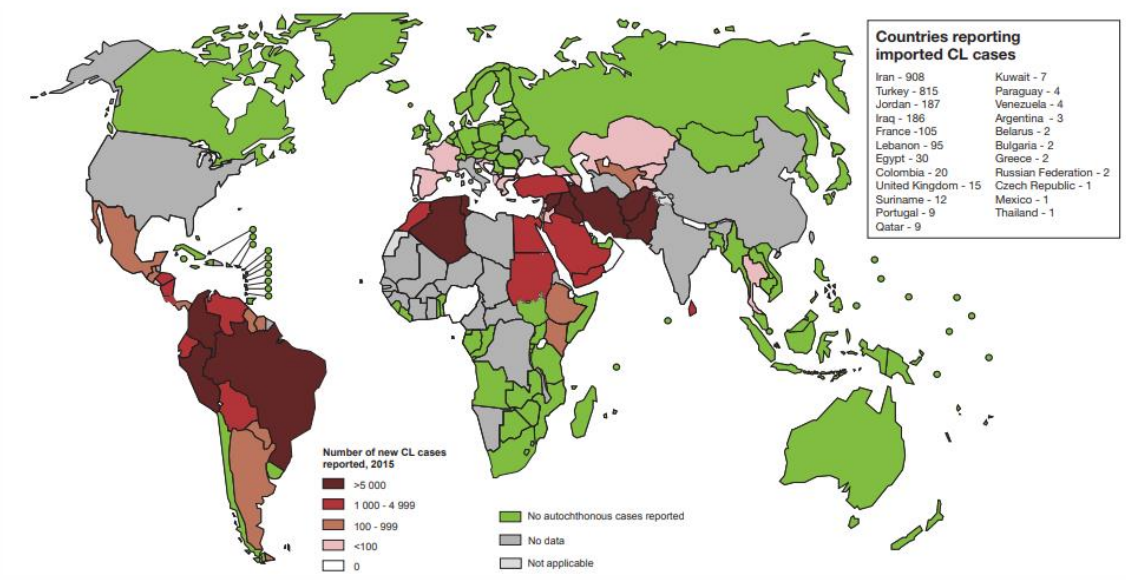

Figure 3. Status of endemicity of cutaneous leishmaniasis worldwide, 2015. Countries reporting imported cases**.**
(World Health Organization, 2018).

In the Americas, CL is a wild, rural and rarely urban disease (NEGRÃO; FERREIRA, 2009), vertebrates acquire the infection when they come in contact with forest areas where there are enzootic species from different *Leishmania* species (PESSOA; BARRETTO, 1948). According to the PAHO / WHO Epidemiological Reports, in the period from 2001 to 2015 (OPAS, 2015), 843,931 new cases of cutaneous leishmaniasis were reported. Furthermore, the historical series shows that from 2009 to 2015 there was a reduction in cases of CL.

In 2013, 16 of the 18 endemic countries reported 47,492 cases of CL and MCL, not counting data from Venezuela and French Guiana (OPAS, 2015). In 2014, 17 of the 18 endemic countries reported 51,098 cases of CL and MCL (OPAS, 2016). In 2015 46,082 cases (OPAS, 2017) were reported and in 2016 48,915 cases were reported (OPAS, 2018)

and, in 2017, 49,959 cases were reported (PAHO, 2019). In the year of 2018, 46,041 cases were reported (PAHO, 2019). Analysing these data, we see a significant increase in the number of cases from 2013 to 2014, and a relative decrease in 2015, which increases again in 2016 (but not reaching nor surpassing the number of cases reported in 2014).

The reduction of cases may be associated to several factors such as the epidemiological surveillance system, which comprises a set of actions that lead to the detection, knowledge, prevention and control of diseases, as well as some local environmental, physical, demographic and social improvements. Consequently, the increase in cases may be linked to failures in surveillance, disasters and environmental degradation, and especially weakening social and public policies, given that poor housing conditions, lack of access to clean water and sanitation, deteriorated environments contribute to the transmission of infection.

The data reported by Regional leishmaniasis Information System-SisLeish/PAHO/WHO (2013) outlines how the surveillance system is organized in the 18 endemic countries (OPAS, 2015). In Mexico, the leishmaniasis Program for the period 2013 to 2018 was elaborated and published with strategies to consolidate surveillance, diagnosis, treatment, promotion and family and collective prevention actions. In El Salvador, maintenance of the surveillance of leishmaniasis in the country was done. However, Costa Rica sought to advance the implementation of the laboratory diagnosis, while Panama measure was to update the norms for surveillance, diagnosis, treatment and control. Colombia strengthened the strategy of integrated management for the prevention and control of leishmaniasis, while Ecuador was in the process of reviewing procedures for the implementation of surveillance and control of leishmaniasis. Peru was in the process of finalizing the technical standards, seeking to progress in the disaggregation of leishmaniasis data to the third subnational administrative level for risk stratification. And, finally, Bolivia organizing to have available information and epidemiological analyses disaggregated to the 3rd subnational administrative level.

Argentina advanced its activities of integrated surveillance and control of the focus of leishmaniasis, while Uruguay advanced towards the elaboration of national guidelines for surveillance and control and, Paraguay invested in vigilance and attention.

In the case of Brazil, an integrated surveillance program seeking to advance the updating of national guidelines was kept. Suriname remained unchanged in the occurrence of cases and advanced in the discussion for the implementation of diagnostic and treatment actions. Guyana invested in training professionals to diagnose and treat leishmaniasis and,

Nicaragua sought to review technical standards for leishmaniasis and implement actions for early diagnosis and appropriate treatment. In the way, Honduras has advanced the structuring of epidemiological surveillance actions and implementation of laboratory diagnosis. Guatemala advanced the update of the National Standards for surveillance and control while in Venezuela, surveillance information was not available in 2013 for SisLeish / PAHO / WHO.

In the analysis of the years 2010 to 2013, Brazil, Colombia, Nicaragua, Panama, Peru, Ecuador, Paraguay and Argentina presented reduced notifications, however, Bolivia, Honduras, Costa Rica, Mexico, Guatemala and El Salvador showed an increase in number (OPAS, 2015). As analysed by PAHO / WHO, the significant reduction in cases could be attributed to several factors, such as the organization of services, the surveillance system, as well as environmental, physical, biological and social aspects (OPAS, 2015).

SisLeish data also shows that males have the highest number of case records Leishmaniasis, being a variable of 99.9% (48,905) and 67.2% (32,886) cases. Children, younger than 10 years old, represented 15, 5% (7,583) of the 99.6% (48,702) records of case available in the system. These data report how important it is to achieve the goals of reducing cutaneous / mucosal leishmaniasis by 90% and reducing the proportion of cutaneous leishmaniasis in children under 10 years old by 50% of the leishmaniasis. The American Action Plan 2017-2022, aims to reduce morbidity and mortality from leishmaniasis in Latin America through strengthening diagnosis, treatment, rehabilitation, prevention, surveillance and control. It is an essential tool for the epidemiological control of the parasitic disease that continues to be a major public health problem, which is directly related to living and working conditions.

Analysing the reports from 2015 to 2019, we can note that in the period of 2010-2015, Nicaragua was among the countries with the highest incidence rate of leishmaniasis. Equaly, Brazil, Colombia and Peru, were also examples of countries with the highest number of cases reported. Data from 2016 shows that Nicaragua is among the countries with the highest number of cases, with an increase in its incidence was 157%. Brazil, despite significantly reducing the number of cases, remains at the top with the largest number of records in Latin America (Figure 4). We also observed an increase in the co-infection of LC / LM and HIV (PAHO, 2019).

YEAR OF NOTIFICATION	LATIN AMERICA COUNTRIES WITH THE HIGHEST NUMBER OF REPORTED CASES
2012	BRAZIL, COLOMBIA, PERU AND NICARAGUA
2013	BRAZIL AND THE ANDEAN SUBREGION
2014	BRAZIL, COLOMBIA AND PERU
2015	BRAZIL, COLOMBIA AND PERU
2016	BRAZIL, COLOMBIA, PERU AND NICARAGUA
2017	BRAZIL, COLOMBIA, PERU AND NICARAGUA
2018	BRAZIL, COLOMBIA, PERU, NICARAGUA, BOLIVIA AND VENEZUELA

Figure 4. Endemic countries in Latin America which presented the highest number of cases registered in the period 2012-2018, according to the statistical data of the Regional Information System for Leishmaniasis - SisLeish / PAHO / WHO.

According to the datas from of the Regional Information System for Leishmaniasis - SisLeish / PAHO / WHO, we observed the permanence of Brazil, Peru and Colombia with the highest number of records for years, and the inclusion of Nicaragua in 2016 and, Bolivia and Venezuela in 2018.

In this perspective, it is extremely important to analyze mainly those countries that have the highest number of case records, due to the need for epidemiological control to reduce the risks not only for the population of such countries, but also of the countries with which they border. It is noteworthy that, although Argentina and Guatemala have lower incidence and numbers of leishmaniasis cases compared to Brazil, Peru, Colombia and Nicaragua, these 2 countries (Argentine and Guatemala) present the largest records of cases at the borders, with a total of more than 40% of the cases of LC in the border areas (PAHO, 2018) (Figure 5).

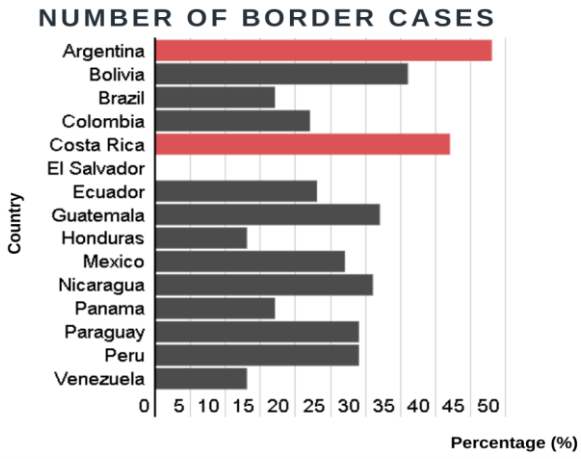

Figure 5. List of Latin American countries and numbers of Leishmaniasis cases registered at the borders according to the statistical data from the Regional Leishmaniasis Information System - SisLeish / PAHO / WHO of 2018. We observed that Argentina and Costa Rica had a higher percentage border cases in 2018.

4. FINAL CONSIDERATIONS

The studies described and accumulated in the last 15 years lead us to draw a common path, which culminates in expansions and increase of indices and numbers of cases of Cutaneous Leishmaniasis in Latin America due to socio-environmental factors (climate changes, deforestation, construction of dams, urbanization), precarious housing conditions, poor nutrition, lack of investment in public policies, as well as the factor of immunological deficiency. They are also related to the working environment and its conditions, lack of knowledge on Health Education, agricultural and food production, and the inherent difficulties of social health services, such as access to health services. Leishmaniasis remains as a great public health problem. However, epidemiological analysis and surveillance are extremely important in order to achieve control of this disease. Scientific research undoubtedly contributes to strengthening and developing prophylactic, immunizing, diagnostic and therapeutic agents which may have a leishmanicide activity and present low toxicity to patients affected by Leishmaniasis, being equaly important that the therapeutic agent is of low cost, having effectiveness, security and efficient distribution. However, scientific research alone will not solve, in short term, the great challenge of controlling Leishmaniasis. In parallel with scientific research, investment on health education and public and social policies is of fundamental importance in order to break the transmission cycle to guarantee the surveillance, prevention and control of this neglected tropical disease.

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Stricto Sensu Editora

TREATMENT FOR LEISHMANIASIS – A CURRENT PERSPECTIVE FOR AN OLD CHALLENGE

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ABSTRACT

Leishmaniasis is a complex of infectious diseases caused by several species of the protozoan Leishmania and transmitted by different species of female sand flies. Its treatment is the same used for more than 70 years, and despite being responsible for reducing the number of active cases, it is still unsatisfactory because of its high toxicity, which can be harmful to the patient. In this context, it is important to investigate new forms of treatment, such as the use of chemical derivatives, bioproducts derived from plants or algae, as well as their secondary metabolites. These derivatives present satisfactory data in the literature on anti-Leishmania, antiprotozoal, antiviral and anti-inflammatory activity. This pioneering review mentions data from citations in the literature on potential alternative drugs, Leishmania - species-specific, for the treatment of Leishmaniasis. The search for new candidates with therapeutic potential (referred to throughout the text as "Alternative treatments") for the different clinical forms of leishmaniasis and with low cytotoxic effect, can contribute to the control of this Neglected Tropical Disease, as well as reducing the appearance of disabilities, sometimes resulting from worsening of the cutaneous and mucocutaneous forms of cutaneous Leishmaniasis. This chapter also discusses the importance of the correct diagnosis for defining the appropriate therapeutic approach for the successful cure of this disease.

Keywords: Leishmaniasis, Relevance of diagnoses, Traditional treatments and Alternative treatments Leishmania species-specific

1. INTRODUCTION

Leishmaniasis are infectious zoonoses that have historical records dated since the colonial period in the Americas. Although its etiological agents are only described in the nineteenth century in India, it is known that Inca and Peruvian ceramic vessels of the pre-

colonial period made representations of individuals with cutaneous and mucosal lesions caused by this complex of diseases (SILVEIRA et al., 2004). Leishmaniasis, therefore, is present even before contact with the European people (ALTAMIRANO-ENCISO et al., 2003, VALE et al., 2005). The dispersion of the disease in Brazil was related to the cycles of coffee in 1840 and rubber in 1912, although later cycles such as gold Mining (1970) and the exploitation of timber in 1980 may also have had participation of this expansion, since they counted on the arrives of people from all parts of the world, allowing not only the entry of man in the forest as the greatest contact of man with the wild reservoirs (PIMENTA et al., 2003; VALE et al., 2005).

In 1909 Gaspar Vianna made the relationship between these parasites with the lesions found in the patients. In that same year, Rabello suggested the denomination of Cutaneous Leishmaniasis (CL), which should represent a different form of that observed in the visceral form. Finally, in 1910, the pathogen correlation was performed with the clinical conditions of the disease (RATH et al., 2003). With the advances of microscopy and molecular biology the species observed were separated in complexes or subgenera called *Leishmania* and *Viannia*, differentiated according to the site of the digestive tract of the insect that presents more favourable conditions for the development and reproduction of parasites. New taxonomies are described as the techniques allow the classification of the existing species, making the current knowledge about the existing coevolution between the parasites and vectors, hosts and reservoirs.

However during the last years, leishmaniasis have become a complex of pathologies that are in great expansion due to the mobility of tourists and immigrants around the world (STRATIGOS et al., 1980; MAAZOUN, 1981; KREUTZER et al., 1993; LAWN et al., 2004; PRATLONG et al., 2004; CARDOH, 2006; AMEEN, 2007; SCHLEUCHER et al., 2008; BERENS-RIHA et al., 2009; ANDROULA et al., 2010; NEGHINA et al., 2010; ERGEN et al., 2015). This fact is very worrying, since it presents two different contexts: (1) they are diseases little known in endemic areas due to the lacking dissemination, and (2) on the other hand, when introduced in developed countries, they are facing to lack the experience of health professionals for diagnosis and treatment, as they are unusual in these territorial spaces. The clinical picture of cutaneous leishmaniasis, for example, is very diverse and ulcerative, nodular and papular lesions may occur. However, these clinical signs may be present in other skin pathological conditions. Thus, differential diagnosis is of utmost importance for immediate treatment for these diseases. Leishmaniasis skin lesions can be confused with frequent skin lesions in the following diseases: leprosy, *Staphylococcus* infection,

sporotrichosis, skin tuberculosis, sarcoidosis as well as neoplasms. Therefore, the health professional's experience in the accurate diagnosis is decisive for the control of this emerging disease. Thus, it takes skill to deal with these two extremes (mentioned above in (1) and (2), which makes this complex pathology increasingly challenging. Despite the great efforts in discovering new forms of treatment, the problem seems far from solved.

2. LITERATURE REVIEW

2.1 THE DIVERSITY OF SPECIES OF *LEISHMANIA* AND VECTORS

In relation to Cutaneous Leishmaniasis (LC), the *Leishmania braziliensis* and *Leishmania Mexicana* complex are more reported as the etiological agent of Leishmaniasis in the American continent. While *Leishmania tropica* is more related, causing CL on the following continents: Asia, Africa and Europe. The exception to this case is *L. infantum*, which can cause CL and, also Visceral Leishmaniasis. These species are distributed in two subgenera: *Leishmania* and *Viannia*. The first one presents the *Leishmania major, Leishmania tropica, Leishmania mexicana and Leishmania donovani* complexes, while the latter one display *Leishmania guyanensis* and *Leishmania braziliensis* complexes (Figures 1 and 2) (GALATI,2003).

Visceral Leishmaniasis (VL) is caused by the species *Leishmania donovani*, *Leishmania infantum* and *Leishmania chagasi* (Figure 2). The species *L. infantum* predominates in Asia, Africa and Europe while *L. chagasi* is found in America. Some genetic studies through the analysis by molecular techniques of polymerase chain reaction (PCR) have classified *L. infantum* and *L. chagasi* as a single species, or *L. chagasi* as a subspecies of *L. infantum*. Therefore, for LV, the parasites are divided into the complexes *Leishmania donovani, Leishmania mexicana, Leishmania tropica* and *Leishmania major* (GALATI, 2003).

This variety of species responsible for Leishmaniasis demonstrates the importance of studying vector control of phlebotomine through entomological surveillance as one of the forms of prophylaxis. Through the entomological is possible to have greater knowledge of the epidemiological cycle of the disease, since it allows the understanding of dispersion and plasticity, mainly for the vectors that can transmit more than one species of *Leishmania*. It is known that 476 species of phlebotomines are found in American continent and about 40 of

these species are responsible for causing Leishmaniasis (PIRAJÁ et al., 2013). Within this study, *Lutzomyia longipalpis* is considered the main vector for VL (SHERLOCK et al., 2003). It is possible to make a co-evolutionary study that demonstrates the direct relationship between the Leishmania and their corresponding vector, suggesting that Lutzomyia are more susceptible to Leishmania infections than Phlebotomus (PIMENTA et al., 2003).

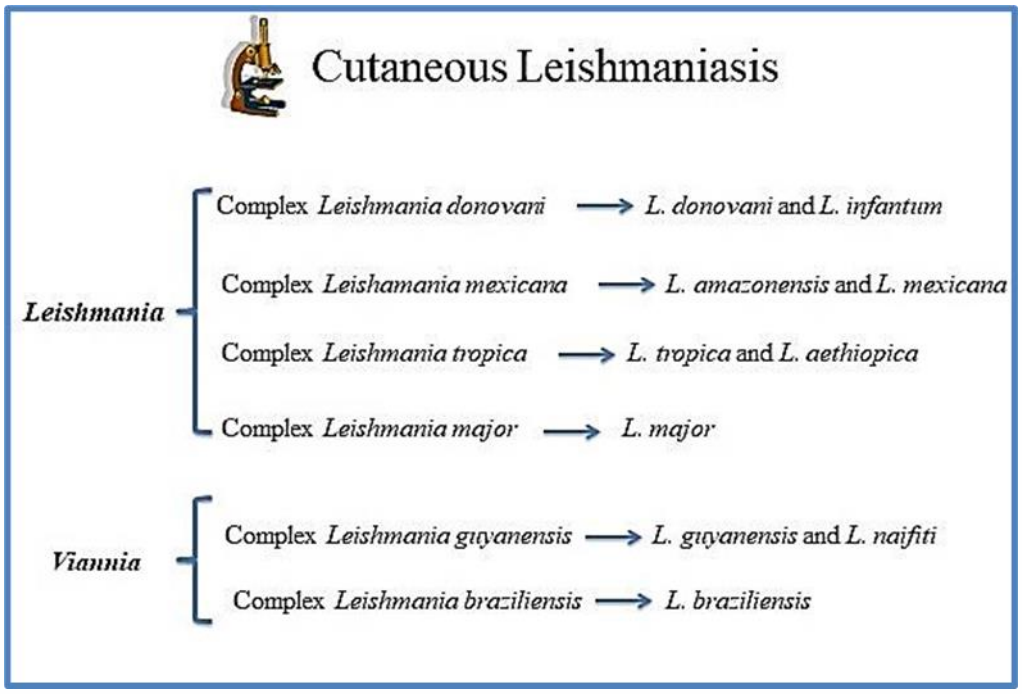

Figure 1. Flowchart of the Leishmania species complex causing Cutaneous Leishmaniasis and its respective parasites in each group.

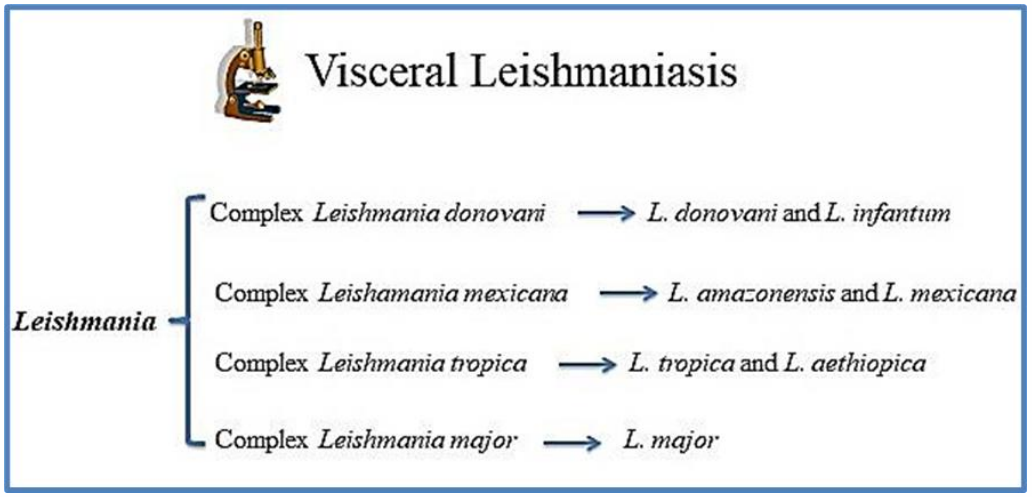

Figure 2. Flowchart of the Leishmania species complex causing Visceral Leishmaniasis and its respective parasites in each group.

2.2 LEISHMANIASIS: A BRIEF REVIEW OF THE EPIDEMIOLOGICAL SITUATION

Recently, major cutaneous leishmaniasis (CL) epidemics have affected different parts of Afghanistan and the Syrian Arab Republic. The World Health Organization (WHO) reported that 2014 was the year in which more than 90% of new cases occurred in Brazil, Ethiopia, India, Somalia, South Sudan and Sudan. In addition, in the following countries leishmaniasis is a concern constant: Afghanistan, Algeria, Brazil, Colombia, Islamic Republic of Iran, Pakistan, Peru, Saudi Arabia and the Syrian Arab Republic.

In several regions of the world, LC is predominantly urban and peri-urban (environments close to homes), the disease is generally characterized by large outbreaks in densely populated cities, especially in areas of war and conflict, and in places where large population migration occurs.

In American continent, the epidemiology of CL is complex due to several factors such as intra and interspecific variations in transmission cycles, reservoir, vertebrate hosts, invertebrate host, many clinical manifestations and different responses to therapy, and the diversity of circulating Leishmania species in the same geographical area. Almost 90% of the cases of mucocutaneous leishmaniasis occur in the Plurinational State of Bolivia, Brazil and Peru.

2.3 LEISHMANIASIS: A CORRECT DIAGNOSIS IS ESSENTIAL FOR THE ASSERTIVENESS IN THE THERAPEUTIC APPROACH

Leishmaniasis presents different forms of clinical manifestations, so each pole of the disease should be diagnosed by one or more methods. The proper and early diagnosis of the disease facilitates not only the correct choice of treatment used as it allows epidemiological control. In some cases, a culture of Leishmania promastigotes forms can be made to identify the species, together with techniques of genetic sequencing.

A major difficulty in the diagnosis of Leishmania infections is due to the fact that the health professional has no experience in recognizing Leishmania within the tissue cells, which hampers the cytopathological analysis of a biopsy, for example. In Brazil this difficulty occurs, but in European countries this difficulty is much greater because health professionals are not familiar with these tropical diseases. For this reason, we illustrate this chapter, with some photomicrographs of the "in vitro" infection of human cells (macrophages and dendritic cells, derived from monocytes obtained from human peripheral blood), experiments routinely

performed in our Laboratory at Federal Fluminense University, RJ, Brazil , with the objective of showing amastigote images (intracellular forms of leishmania), which, certainly, will contribute to diagnostic purposes of LC, in histological sections obtained by biopsies of skin lesions of patients affected by LC (Figure 4).

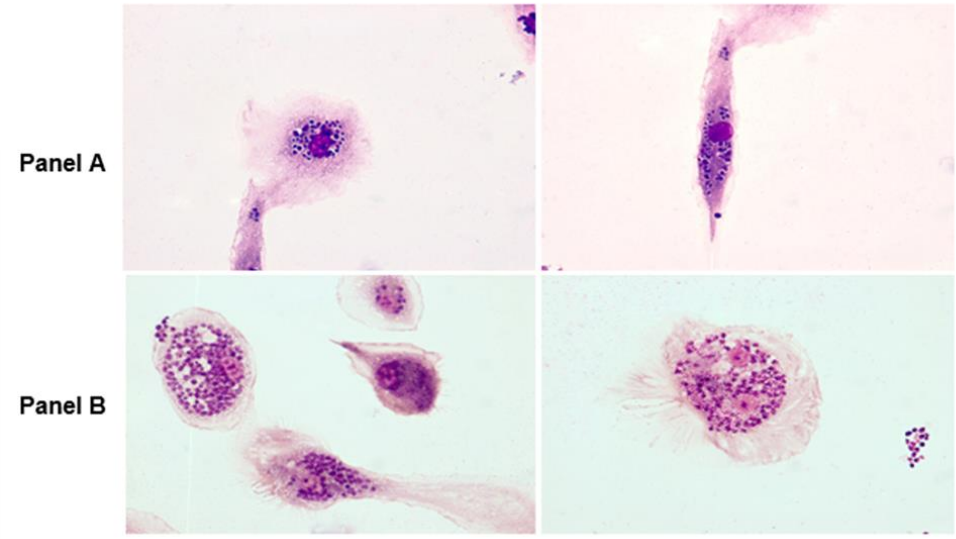

Figure 4 - Optical microscopy analyses of the interaction of *L. braziliensis* with human dendritic cells *in vitro* (panel A) and macrophages (panel B) derived from monocytes isolated from peripheral blood after 16 hours of incubation in RPMI medium 37º C in labtecks with 5% CO_2. Afterwards, the cells were fixed in methanol and stained in Giemsa - (magnification 1000x). Optical Microscope Olympus B x 41 (personal archive).
Note - In Panel A*:* *L. braziliensis* (amastigotes – intracellular form) are inside vacuoles, clearly shown in the citosol of dendritic cells. In Panel B: amastigotes forms are inside the macrophages and situated around the cellular nucleous; specially, in the image of right side in Panel B, a macrophage with two nucleous and many amastigotes, in the cytosol, circulating both nucleous can be observed.

For cases with classical CL, with non-ulcerative lesions, the diagnosis is made through the aspect of the lesion, detection of antibodies by the Elisa method, or by Montenegro intradermal reaction. The latter consists in the observation of a possible late hypersensitivity reaction measured by the presence of inflammation or rash at the site in which the solution of metholylated saline containing inactivated antigens was injected. This form of the disease does not generate systemic impairment and can lead to spontaneous cure, so the patient does not usually seek the diagnosis, making it difficult to treat them. Patients have a characteristic immunity by high IFN-γ and high TNF-α. Their lesions are limited and present parasitic scarcity, making the parasitological diagnosis difficult. In addition, multiple lesions may compromise the final diagnosis since it is not known for certain whether multiple lesions

form a clinical picture through the patient's weakened immune system or as a result of several insect bites (REITHINGER, 2007, PIMENTEL et al., 2014).

For CL, direct parasitological examination is used, through smear of cutaneous biopsies or histological sections. The parasitological exams are the most used, being sensitive only when found a number considered parasites, making it difficult to diagnose in cases where many parasites are not found. In addition to the difficulties above mentioned, the diagnosis is often made late, not only by the lack of preparation of health units but, also by the similarity of the clinical picture of some forms of Leishmaniasis with other diseases (REITHINGER, 2007).

The treatment for CL is usually due to the use of Meglumine antimoniate for 20 days or liposomal amphotericin B for pregnant women and older than 50 years as first-choice drugs and amphotericin B deoxycholate or Pentamidine isetionate (except in pregnant and second choice (GALVÃO et al., 1993, GUIMARÃES et al., 2005, SANTOS et al., 2008, PIMENTEL et al., 2014).

With regard to VL, the affected individual may be asymptomatic with positive serology, but without clinical manifestation, or may present the classical form, with well-characteristic hepatosplenomegaly, severe anemia or leukopenia. When the patient has a high humoral immuneresponse the diagnosis is made through the serum, by gel immunodiffusion, direct agglutination or also by Elisa (REITHINGER, 2007). Besides, there is the acute form of VL, characterized by high and continuous fever, hepatosplenomegaly, weight loss, without leukopenia with prolongation of the disease (RATH et al., 2003). According to MADEIRA et al. (2003), the first choice for treatment is the Meglumine antimoniate for 20 days in uncomplicated cases and as second choice is Liposomal amphotericin B (Figure 5).

As mentioned before, leishmaniasis is considered a complex of diseases due to its range of clinical manifestations, influenced not only by the host's immune system but also by the parasite load and parasite species. In the endemic areas the diagnosis is made mainly by analysis of the biopsy of the lesion (if any) by means of microscopy. (PIMENTEL et al., 2014).

The late diagnosis concomitantly with a non-specific treatment can further aggravate the clinical conditions of the disease and the cases of parasitic resistance to treatment. VL may be mistaken for some lymphoproliferative disorders while CL is mistaken for sporotrichosis, mycoses, dermatitis or allergies. Thus, the treatment of affected individuals in most cases is delayed due to the time spent to arrive at the final diagnosis (MADEIRA et al., 2003, RATH et al., 2003, SANTOS et al., 2008, CONCEIÇÃO-SILVA et al., 2009, PIMENTEL et al., 2014).

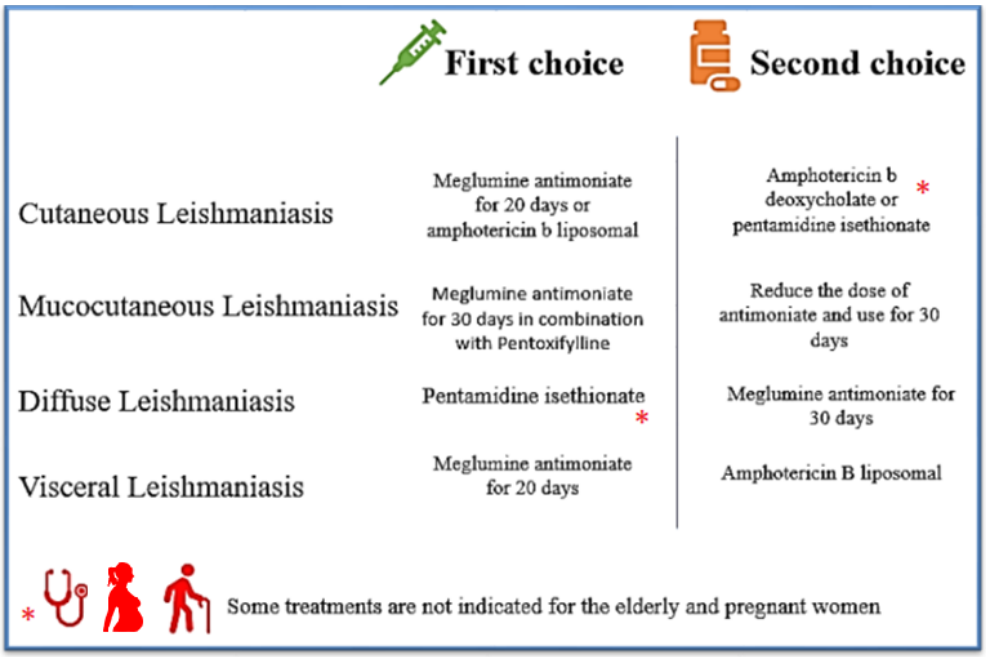

Figure 5. Traditional treatments of Leishmaniasis indicated for each clinical form of the disease.

2.4 WHAT DOES THE SCIENTIFIC LITERATURE REPORT ABOUT RESEARCH WITH NEW DRUGS FOR THE TREATMENT OF LEISHMANIASIS?

Traditional treatments for Leishmaniasis may decrease the number of active cases, but in an unsatisfactory way. Cases of parasite resistance to drugs of choice, non-species-specific treatments, drug toxicity or high cost are only a few negative factors that contribute to reports of treatment abandonment by the patient. With the recurrence of this abandonment, patients become sources of infection for the population, because they prefer to live with the consequences of disease progression than with the side effects caused by their drug's choice (SANTOS et al., 2008; CONCEIÇÃO-SILVA et al., 2009; PIMENTEL et al., 2014). In this context, the importance of the continuous search for alternative or adjuvant treatments that seek to reduce both side effects, its cost and facilitate the drug access by the population is considered essential in the present research. Table 1, below, summarizes all the references used in the discussion that begins throughout the text from the next paragraph.

Firstly, in table 1, we started mentioning some promising reports which are found in the field of botany, through studies with plant extracts and their majority components capable of altering the intracellular metabolism of the parasites tested or destabilizing the action potential of mitochondria of *Leishmania* (BAKKALII et al., 2008; MACHADO et al., 2012; MAKWALI et al., 2012; BEKELE et al., 2013; VILA-NOVA et al., 2013; RODRIGUES et al.,

2013; DASGUPTA et al., 2014; QUEIROZ et al., 2014). The use of natural bioproducts is of great value for the evolution of disease treatments because it presents a lower chance of cytotoxicity to human cells and can be explored and evaluated according to the serial dilution of its fractions, causing the patient be subjected to minimal doses of the bioproduct and have a relevant effect on the regression of Leishmaniasis. Flavonoids are the main chemical components found in vegetables, especially those with high concentrations of chlorophyll, therefore with elevated photosynthesis rates. They are part of a large group of secondary metabolites of the polyphenols class and present low molecular weight. They have great socioeconomic importance because they are present in the manufacture of breads and wines and in the animal eukaryotic cells it is important for the formation of coenzyme A, responsible for the entry of the pyruvate molecule in the mitochondria for cell respiration. Thus, it is probable that by its intrinsic interaction with mitochondria, its excess may cause alteration in the action potential of the mitochondria of the parasite (CROFT et al., 2006).

Alkaloids are substances of basic character derived mainly from plants, but can be found in fungi, bacteria and animal cells. Its chemical formula is composed of nitrogen, oxygen, hydrogen and carbon and its division is in the group of true alkaloids, which possess heterocyclic ring with a nitrogen atom, protoalcaloids, where the nitrogen atom does not belong to the ring heterocyclic and pseudoalkaloids for those that are derived from terpene or steroid and not amino acids. These also present potential antileishmanial activity because they can prevent the transition between the infective forms of the parasite during its evolution cycle, allowing a decrease in infection rates (MAUEL et al., 1993; DELMAS et al., 2004; SANTOS et al., 2008).

Then, nanotherapy deals with the use of nanotechnology to investigate diverse and alternative forms of treatment for diseases. Nanotechnology is the term used in genetic engineering to refer to the study of matter manipulation on the molecular and molecular scale of nanometers. It has been studied to treat neurodegenerative diseases such as Parkinson and Alzheimer, malignant tumors such as melanomas, neurogliomas or diseases such as type 1 diabetes. (NADMAN, 2014; MOL et al., 2015; FIROUZMAND et al., 2015). Equally important are some experimental treatments forms prototypes reported as the main indicators of reducing high parasitic load and symptoms (RANGEL et al., 2011; CHOWDHURY, 2015). The use of nitroimidazoles was discovered in 1959 for the treatment of vaginal trichomoniasis because it is a potent bactericide with excellent activity against anaerobic and protozoan bacteria such as amoebiasis, trichomoniasis and giardiasis. Although its use is more generic,

it has been enhanced with the use of Leishmania amastigotes, as well as the use of Acridines, Coumarins and Benzodiazepines (ARANGO et al., 2010).

Finally, worth mentioning, furans which are chemical compounds widely used in laboratories as solvents, due to the fact that their reaction with hydrogen, catalyzed by palladium, provides tetrahydrofuran. When it is administered in low doses can be very efficient against the proteins of the microorganisms (VENAZZI et al., 2006). This action is similar to quinolines (GUGLIELMO et al., 2009), quinoxalines / quinoxalines and quinones (NEW et al., 1981 ; MEHEUS et al., 2010). Purines are nitrogenous bases that make up the nucleotide.

Adenine and Guanine are purines which, through hydrogen bonds, bind to the pyrimidines Thymine and Cytosine, respectively (SUFFIA et al., 1995; SUNDAR et al., 2009). Generally, these molecules are little soluble in water, of neutral pH and quite abundant in the nature. By manipulating its chemical components, it is possible to modify the morphological and functional structure of the amastigotes and promastigotes of Leishmania. In addition, it is possible to destabilize the production of adenosine triphosphate (ATP), which is essential for the metabolic activity of organisms (CROFT et al., 2003; CROFT et al., 2006; PEREZ-VICTORIA et al., 2003). Although the chemical compounds above can be effective against these protozoa, they cannot alter the production of cellular nitric oxide, which is caused by azoles. Amino acids have the ability to affect the mammalian cytochrome P450 system and are responsible for drug changes that are contrary to various drugs and chemical compounds, including nitric oxide produced by human macrophages or fungal microorganisms (LIU; WELLER, 1996; JHINGRAN et al., 2009). Thus, they can be used for the purpose of destabilizing the plasma membrane of protozoa and fungi because they influence the metabolism of ergosterol.

Table 1. A brief review of the scientific literature reporting some promising results obtained from the experimental treatment for Leishmaniasis.

Author and year of publication	Alternative treatment	Objective
Makwali et al. 2012, Bakkalli et al. 2008, Machado et al. 2012, Bekele et al. 2013, Vila-Nova et al. 2013, Rodrigues et al. 2013, de Queiroz et al. 2014, Dasgupta et al. 2014, Ghorbani et al. 2017	Plants extracts	Reach the action potential of the mitochondria of the parasite – *L. major, L. amazonensis, L. tropica e L. infantum*
Chen et al. 1993, Croft et al. 2006, Taslimi et al. 2018	Flavonoids	Reach the action potential of the mitochondria of the parasite – *L. major, L. amazonensis* e *L. donovani*

Mauel et al. 1993, Delmas et al. 2004, Santos et al. 2008, Reguera et al. 2016	Alcaloids	Inhibit the transformation of promastigote into amastigote – *L. major, L. donovani e L. mexicana*
Pierson et al. 2010, Verma et al. 2012, Okwor et al. 2016,Apostolopoulos et al. 2018	Terpenoids	Increases the production of nitric oxide by macrophages - *L. amazonensis, L. tropica e L. braziliensis*
Chen et al. 1994, Zhai et al. 2001, Shivahare et al. 2014	Cyanobacteria extracts	Decrease infectivity of amastigotes – *L. donovani, L. mexicana e L. majo*r
Nadman, 2014, Firouzmand et al. 2015, Mol et al. 2015, Gonçalves et al. 2019	Nanotherapy	Act on the liposomes of macrophages - *L. major*
Rangel et al. 2011, Chowdhury, 2015	Imunotherapy	Decrease the side effects of traditional treatments – *L. donovani*
Arango et al. 2010	Nitroimidazoles	Increases the destruction capacity of amastigotes within macrophages - *L. donovani*
Nascimento et al. 2014	Vitamin A	Activation of monocytes against *Leishmania – L. infantum*
Mesa-Valle et al. 1996, Ghorbani et al. 2017	Acridines	Decrease the proliferative capacity of the parasite – *L. infantum e L. donovani*
Arango et al. 2010	Cumarines	Affects the amastigote forms – *L. donovani e L. panamensis*
Grant et al. 2004, Ghorbani et al. 2017	Benzodiazepines	Inhibit kinases, important for the proliferation of amastigotes – *L. mexicana*
Venazzi et al. 2006, Apostolopoulos et al. 2018	Furans	Decrease the parasitic *L. donovani e L. amazonensis* no fígado (LV)
Brummit et al. 1996	Piridines	Decrease the proliferative capacity of the parasite – *L. donovani*
Guglielmo et al. 2009, Lackovic et al. 2010	Quinolines	Inhibit GDP-pyrophosphatase - *L. amazonensi*s
New et al. 1981, Bodhe et al. 1999, Meheus et al. 2010, Okwor et al. 2016	Quinozalines	Decrease the proliferative capacity of the parasite – *L. chagasi, L. donovani e L. infantu*m
Sundar et al. 2011	Quinones	Decrease the proliferative capacity of the parasite – *L. amazonensis*
Thakur, 1993, Berman, 1997	Thiophenes	Decrease the proliferative capacity of the promastigote – *L. infantum, L. donovani e L. majo*r
Paris et al. 2004, Taslimi et al. 2018	Triazines	Increases the production of nitric oxide by macrophages - *L. donovan*i

Suffia et al. 1995, Croft et al. 2003, Perez-Victoria et al. 2003, Sundar et al. 2009	Purines and pyrimidines	Act on the promastigotes and amastigotes forms – L. amazonensis, L. tropica, *L. donovani e L. infantu*m
Liu et al. 1996, Jhingran et al. 2009, Okwor et al. 2016	Azoles	Increases the production of nitric oxide by macrophages - *L major, L. donovani e L. amazonensis*

3. FINAL CONSIDERATIONS

Leishmaniasis is a complex of diseases that vary in clinical forms, which can make it difficult to achieve a correct diagnosis. This disease also faces the challenge of resistance and abandonment to the usual treatment. As we are dealing with a group of diseases caused by a great diversity of Leishmania species, the correct form of combat would be better referenced to the parasitological target in question, that is, the species of the parasite. The future of leishmaniasis treatment is in the hands of greater scientific research and pharmacological and governmental investments in new potential drugs. Ideally, these new forms of treatment should be more selective in their targets, being species-specific, reducing the dose of the medication exposed to the patient and, reducing the deleterious effects to solve frequent problems such as: the abandonment of the specific treatment for leishmaniasis, as well as the emergence of drug resistance. We believe that, together, these factors would contribute to the achievement of the control of this neglected tropical disease that affects a huge number of people in Brazil and in the world, which can lead to physical disabilities and, sometimes, death.

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Stricto Sensu Editora

ANTI-LEISHMANIAL ACTIVITY OF MARINE NATURAL PRODUCTS FROM THE GREEN ALGAE *PRASIOLA CRISPA* AGAINST THE EXTRACELLULAR FORM OF *Leishmania (V.) braziliensis*

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ABSTRACT

Introduction: The present study investigates extracts and fractionates isolated from 5 marine algae species for their anti-leishmanial effects as potential non-toxic alternatives for treating this neglected disease. Methods: The effects of algal extracts were tested on *Leishmania braziliensis* (promastigote, or infecting form) and human macrophages. Specifically, the research involved analyzing: (1) the viability of *L. braziliensis* as monitored by Trypan Blue staining, (2) the viabilities of both *L. braziliensis* and human macrophages by cell metabolic activity using the Alamar Blue assay, and (3) the ability of parasites to form promastigote rosettes in vitro. Results: The results of the antileishmanial profile of six crude extracts (obtained from five different algal species) showed that the green alga, *Prasiola crispa*, had the highest activity; thus, this alga was selected for additional testing. Subsequently, nine serially diluted [12.5 µg/ml-100 µg/ml] *P. crispa* fractions were tested for anti-leishmanial effects against the infecting form (promastigote) of *L. braziliensis*. One fraction was selected, due to its enhanced ability to reduce the metabolic activity, to kill parasites, and to inhibit promastigote rosette formation even at the lowest concentration [6.25µg/ml]. Additionally, no cytotoxic effect was observed in human macrophages incubated with any of the fractions. No previous studies have shown the presence of anti-leishmanial effects from *P. crispa*. Conclusions: The results are very promising, since the algal derivates tested here not only act on the infecting form (promastigotes) of *L. braziliensis* but also display low or no toxic effect on human macrophages.

Keywords: *Leishmania braziliensis* killing, Promastigotes, *Prasiola crispa*, Extracts and fractions, Human macrophages e Cytotoxicity assay.

1. INTRODUCTION

Leishmaniasis is a zoonotic disease caused by over twenty protozoan species of the genus Leishmania. The disease pathology results in high rates of morbidity and mortality throughout the world (COURA et al., 1987; KILLICK, 1997; BRANDONISIO et al., 2002; ALMEIDA et al., 2003; BRAVO; SANCHEZ, 2003; GONTIJO; CARVALHO, 2003; GONTIJO; MELO, 2004; REITHINGER et al., 2007; WHO, 2010; MCCONVILLE et al., 2012; ALVAR et al., 2012; MACHADO; PENNA, 2012; BRITO et al., 2012; ARCE et al, 2013; DE VRIE, 2015; WHO, 2016, KHOSRAVANI et al., 2016; THIES et al., 2016; AL-JAWABREH et al., 2017; OZKEKLIKEI et al., 2017; HOLAKOUIE-NAIENI, 2017; KHEZZANI; BOUCHEMAL, 2017). The anthropogenic impact on forests increased contact between humans and the invertebrate vectors, resulting in an enhanced spread of the disease. According to the World Health Organization (WHO, 2016), leishmaniasis is ranked as the ninth most severe infectious disease in the world. Cutaneous leishmaniasis is a neglected pathology that still significantly impacts health and economics in developing countries (REITHINGER et al., 2007; WHO, 2016).

Concern is ongoing even in developed nations, due to the possibility of the disease affecting tourists returning from countries where the parasite is endemic (CARVALHO, 2002; CARVALHO et al., 2015; ORYAN, AKBARI, 2016). Although leishmaniasis-specific therapies have reduced the number of active cases, the rate of new cases per year has not decreased significantly (WHO, 2016; SANTOS et al., 2008). In addition, patients who are "cured" of the infection may relapse, with subsequent tissue damage (WHO, 2016). In the Western hemisphere, leishmaniasis has two types of clinical manifestations: visceral leishmaniasis (VL) and American tegumentary leishmaniasis (ATL) (BERENGUER et al., 1998; AZULAY, DERMATOLOGÍA, 2008).

Brazil has the highest prevalence of ATL cases in the Western hemisphere (VITA et al., 2016). In the last 20 years, ATL has increased in almost all Brazilian states, and epidemic outbreaks have occurred in the Southeast, Midwest, Northeast and regions such as the Amazon (GONTIJO, 2004; MCCONVILLE, 2012). ATL is even found in major urban and peri-urban areas, such as Rio de Janeiro (GONTIJO, 2003; MCCONVILLE, 2012; VITA et al., 2016). The characteristics and severity of human infection by *L. (Viannia) braziliensis* are determined by the initial events in the host-parasite interaction, which are decisive in the development of a protective induced immune response (ALMEIDA et al., 2003).

In humans, clinical and immunological studies of leishmaniasis suggest that a benign evolution of this disease depends on the appropriate enhancement of the cellular immune response (ALMEIDA et al., 2003). Furthermore, leishmaniasis can be caused by different species of Leishmania, including *L. (Viannia) braziliensis*, *L. guyanensis (Viannia)*, *L. (Viannia) naiffi*, *L. (Viannia) shawi*, *L. (Viannia) lainsoni*, *L. (Leishmania) amazonensis, L. (L.) mexicana*, *L. (Viannia) panamensis*, and *L. pifanoi* (SAVOIA, 2015). The species involved depends on the geographical distribution.

The disease may appear via simple or diffuse ulcerations on the skin, especially on the face, causing mutilation and disfigurement (WHO, 2016). Currently, the drugs used to treat leishmaniasis have several issues, including high toxicity and many adverse side-effects, leading to treatment withdrawal by patients and the emergence of resistant parasite strains. In addition, the high and increasing cost of the drugs used may lead to inadequate treatment (RAMOS et al., 1990; OLLIARO; BRYCESON, 1993; RATH et al., 2003; ROSA et al., 2003; BRAY et al., 2003; SINGH; SIVAKUMAR, 2004; CROFT et a., 2006; SANTOS et al., 2011).

Since the 1940s, the primary treatment against Leishmaniasis has included pentavalent antimonial compounds, mainly in the form of sodium stibogluconate and Nmethylglucamine antimoniate (SAVOIA, 2015; KHOSRAVANI, 2016;). Other drugs-such as pentamidine, amphotericin B, and paromomycin - are used as a second option in resistant cases, despite their great toxicity to the host (OLLIARO, 1993; BRAY, 2003, SINGH, 2004). Recently, resistance to pentamidine has also been described in the literature (RAHT et al., 2003), as well as difficulties in treating immunesupressed patients (e.g., those with HIV) for whom conventional drugs are less effective and higher doses of drugs and long-term treatment are commonly needed (RAMOS et al., 1990).

Different clinical responses pentavalent antimonial treatment pose a problem for the healing process. These drugs can accumulate in tissues such as the spleen and liver and cause myalgia, pancreatitis, cardiac arrhythmia, and hepatitis. Furthermore, resistance can be acquired to these compounds (ROSA et al., 2003; CROFT et al., 2006), leading to reduction or withdrawal of treatment (SANTOS et al., 2011). Therefore, new leishmanicide agents that cause less deleterious effects to humans are needed. Recently, scientists have begun to investigate marine organisms as potential sources of unique drugs against various diseases-including cancer (TEIXEIRA, 2004; TEIXEIRA, 2013), AIDS (DESJEUX, 2003; MOLINA et al., 2003; FROTA et al., 2012), and Leishmaniasis (SHAW, 1981; QUINDERÉ et al., 2014), with promising results.

Marine life originated about 3.5 billion years ago and includes the most diversified organisms on the planet. These organisms synthesize many unique substances for communication, defense, reproduction, and metabolism. Currently, several research groups in Brazil are investigating and characterizing substances from marine algae, fungi, and invertebrates and their potential uses against serious diseases (SANTOS et al., 2008; WHO, 2010). In this context, the present study investigated the effects of substances isolated from algae on promastigotes - the infective form of *L. braziliensis* - and human macrophages. The results showed a new bioproduct that is present in algal fractions derived from the green alga *P. crispa* and that shows potential leishmanicidal activity.

2. METHODS

2.1 ALGAE

The algae were collected in several locations on the Brazilian coast. *Bryothamnion triquetum* were collected by scuba diving in the Atol das Rocas Biological Reserve in the Rio Grande do Norte State. *Gracilaria caudata* were collected from a marine culture in the coastal zone of Icapuí, Ceará State. *Osmundaria obtusiloba* were collected from the Rasa Beach in Armação de Búzios, in the north of the State of Rio de Janeiro. *Kappaphycus alvarezii* (a cultivated seaweed) were collected in Paraty, in the south of the State of Rio de Janeiro. *Prasiola crispa* were collected from ice-free areas near the Polish Arctowski Station, Admiralty Bay, King George Island (lat.61º50-62º15'S, long. 57º 30-59º 00W), Antarctica. *P. crispa* was identified by Dr. Yocie Yoneshigue-Valentin (Biology Institute, Federal University of Rio de Janeiro) and transported by Dr. André Chariston Dal Belo (University of Pampa, Rio Grande do Sul State, Brazil). All seaweeds were transported to the Algamar Laboratory at University Federal Fluminense in Niterói, RJ, Brazil (Authorization SISBIO 10594).

2.2 *Leishmania (Viannia) braziliensis*

The protozoan in the infecting form (promastigote) was provided by the Laboratory of Surveillance for Leishmaniasis of the National Institute of Infectology (INI), FIOCRUZ (Laboratório de Vigilância em Leishmanioses do Instituto Nacional de Infectologia (INI),

FIOCRUZ). Samples of *L. braziliensis* were isolated from ulcerative lesions of dogs, diagnosed with leishmaniasis, and then cryopreserved. To ensure infectivity, the parasites were cryopreserved during the first culture passage. When necessary, samples were thawed and cultured in liquid Schneider's medium and kept in the incubator (BOD) at 28°C. The parasites used for interaction with the human macrophages were in the stationary phase, between the 3rd and 5th day of cultivation.

2.3 HUMAN MONONUCLEAR CELLS

The human cells were obtained from the buffy coat (a by-product of the separation of whole blood components) collected from healthy volunteers donated by Dr. Carmen Nogueira from the Hemotherapy Service of the University Hospital Clementino Fraga Filho (HUCFF), Federal University of Rio de Janeiro (UFRJ). This project was approved by the Research Ethics Committee of HUCFF (UFRJ) CAAE - 0157.0.197.000-09.

2.4 CHEMICALS

The primary chemical used was Glucantime® (N-methyl meglumine antimoniate) Sanofi-Aventis Farmacêutica Ltda, at the amount of 300 mg/ml of injectable solution. The other chemicals and reagents were purchased either from Sigma-Aldrich (St. Louis, MO), Gibco (Grand Island, NY), or Vetec Química Fina (Duque de Caxias, RJ, Brazil), except when specified below.

2.5 PROCESS FOR OBTAINING EXTRACTS AND FRACTIONS FROM ALGAE

The air-dried material algae were extracted with organic solvents at room temperature; the solvents were evaporated under reduced pressure. This study investigated the extracts (3mg) and fractions derived from different species of marine algae. The EtOAc extract was obtained from *Osmundaria obtusiloba*; the EtOH extract was obtained from *Kappaphycus alvarezii* and *Gracilaria caudate,* respectively; the EtOAc extract was obtained from *Prasiola crispa*; and the CH2 CL2 extract was obtained from *Bryothamnion triquetrum*.

2.6 PROCESS FOR OBTAINING FRACTIONS DERIVED FROM *Prasiola crispa*

The results of the anti-leishmanial profile of the six crude extracts obtained from five different algae species showed that the green alga, *Prasiola crispa*, had the highest activity; thus, this alga was selected for additional testing. After the accompanying fauna was manually sorted, the algawas dried at room temperature, crushed in a blender, and weighed. It was then subjected to an exhaustive extraction, characterized by the passage of solvents with increasing polarity (dichloromethane, ethyl acetate, acetone and methanol). The EtOAc extract (940 g) was then filtered; after being stored under refrigeration, the mass was weighed to determine the final yield. The EtOAc extract obtained from *P. crispa* was subjected to silica gel column chromatography eluted with pure hexane, n-hexane/CH_2 Cl_2, CH_2 Cl_2, CH_2 Cl_2/ EtOAc, and EtOAc. The fractions used were from the *Prasiola crispa* extract with the solvent ethyl acetate, receiving the respective numbering of F5, F7, F8, F9, F10, F11, and F12. These fractions were eluted with a mixture of CH_2 Cl_2/EtOAc (from 80:20 to 50:50).

2.7 EFFECTS OF ALGAE CRUDE EXTRACTS ON *L. braziliensis* VIABILITY

The extracts derived from algae species as previously described were tested for their effect on *Leishmania braziliensis* promastigote viability. To that end, promastigotes ($1x10^6$ cells/ml) were incubated with Schneider's medium pH 7.2, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FBS) at 28°C, for 24 to 48h in the presence or absence of a specific algae crude extract [50 µg/ml and 100 µg/ml]. Glucantime® [200 µg/ml] was used as a positive control. Controls with and without Dimethyl sulfoxide (DMSO) were also tested. The parasite viability was verified through Trypan Blue staining, by counting the number of parasites in a Neubauer chamber using conventional optical microscopy (Olympus BX41). Experiments were done in duplicate. The analyzed data were computed in the GraphPad Prisma® program, through which graphs were created to compare the parasites' survival / mortality differences in each of the experimental models with the algae different extracts. Subsequent experiments only examined the effect of *P. crispa* extracts on *L. braziliensis* viability, since this alga presented the best leishmanicidal potential.

2.8 EFFECTS OF FRACTIONS DERIVED FROM *P. crispa* ON *L. braziliensis* VIABILITY

The parasite viability with the *P. crispa* fractions was carried out as described above. To investigate the most active potentiality of fractions derived from algae, the determination of the concentration which was able to inhibit 50% of the growth of *Leishmania (V.) braziliensis*, after 24h incubation (EC 50), was calculated.

2.9 EFFECTS OF FRACTIONS DERIVED FROM *P. crispa* ON ROSETTE FORMATION OF *L. braziliensis*

Promastigotes ($1x10^6$ cells/ml) were incubated with Schneider's medium pH 7.2, supplemented with 10% (v/v) heat-inactivated fetal calf serum (FBS) at 28°C, for 24h in the presence or absence of fractions derived from algae [6.25 µg/ml-100 µg/ml]. Glucantime® [200 µg/ml] was used as a positive control. Controls were also performed with and without DMSO. The induction of rosette formation of promastigotes of *L. braziliensis* was verified by phase contrast optical microscopy (Olympus BX41). Photomicrography was performed to document rosette formation.

2.10 *L. braziliensis* VIABILITY TEST: ALAMAR BLUE ASSAY

Parasite viability with the *P. crispa* fractions was carried out as described above. Untreated controls were performed with and without DMSO. Parasites were harvested and re-suspended in HANK's Balanced Salt Solution (HBSS), and the parasite number was counted in a Neubauer chamber. Then, promastigotes ($5x10^6$ cells/ml) were incubated with Alamar Blue (10% v/v) for 6h at 28°C, according to the protocol described by Raz et al. (1997). The absorbance was measured at 570nm with a spectrophotometer (Spectramax M4 Molecular Devices, Software Softnsx Pro 6.3). *L. braziliensis* lysed by addition of 0.1% Triton X-100 were used as positive control. Experiments were performed in duplicate.

2.11 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC) AND PURIFICATION OF MONOCYTES

PBMC were isolated from the buffy coat through Ficoll-Hypaque Gradient (density 1077), following the LaBiopAC (UFF) protocol described by (SANTOS et al., 2001). The

monocytes were isolated from the lymphocytes through "cold" aggregation assay (SANTOS et al., 2001), then cultured at $2x10^6$ cells/ml in an RPMI medium supplemented with 10% SFB in Labteks (for analysis by optical microscopy) in a 96-well plate (for analysis by spectrophotometry) and kept in an incubator at 37°C with 5% CO_2 for 10 days, for differentiation into macrophages.

2.12 CYTOTOXICITY ASSAY

The cytotoxic effect of fractions derived from the algae was assayed using human macrophages through Alamar Blue assay (RAZ et al., 1997; NOGUCHI, 1926). Human macrophages ($1x10^6$ cells/ml) were treated for 24 hours in an RPMI medium supplemented with 10% FBS at 37°C in the presence or absence of fractions derived from algae [12.5µg/ml to 100µg/ml]. Glucantime® [200µg/ml] was used as a control. Untreated controls were performed with and without DMSO. Human macrophages were harvested and re-suspended in HANK's Balanced Salt Solution (HBSS), and the cell number was counted in a Neubauer chamber. Afterwards, cells ($1x10^6$ cells/ml) were incubated with Alamar Blue (10% v/v) for 6h at 28°C, according to the protocol described above and mentioned in (RAZ et al., 1997; NOGUCHI, 1926). The absorbance was measured at 570 nm with a spectrophotometer (Spectramax M4 Molecular Devices, Software Softnsx Pro 6.3). Cells lysed by an addition of 0.1% Triton X-100 were used as positive control. Experiments were performed in duplicate.

2.13 STATISTICAL ANALYSIS

The results were represented graphically in histograms, for which we used the Student's t-test and the analysis of variance (ANOVA) with the Tukey test to determine significant differences. All statistical tests were performed with GraphPad Instat software version 6.05, GraphPad Software, San Diego, California, USA, 8 www.graphpad.com Copyright 1992-1998 or SPSS Software. The results were considered significant at $p<0.05$.

3. RESULTS

3.1 OPTICAL MICROSCOPY ANALYSIS OF THE ANTI-LEISHMANIAL POTENTIAL OF CRUDE EXTRACTS DERIVED FROM ALGAE AGAINST *L. braziliensis*, MEASURED BY PARASITE VIABILITY THROUGH TRYPAN BLUE STAINING

To determine the algae-derived crude extract with the highest leishmanicidal activity, the 6 algae extracts and fractions were screened into two different concentrations [50 and 100 µg/ml]. Then, promastigotes of *L. braziliensis* (1x10^6 parasites/ml) were incubated for 24 and 48 hours in Schneider's medium at 28°C in the presence or absence of the crude extracts of algae. This first set of experiments was analyzed by optical microscopy. As shown in figure 1, extract 3 - derived from *Prasiola crispa* - presented the best antileishmanial potential against *L. braziliensis*, with consistent results in both concentrations studied and in both time periods of incubation (killing 90-100% of parasites), comparable to the percentage of parasites killed under Glucantime® treatment. The extracts identified as E1, E2, E5, and E6 also presented anti-leishmanial activity in a 24h period of time, but this activity was not intensely maintained over a 48h period of incubation. In contrast, the extract E4 showed no anti-leishmanial activity against *L. braziliensis*.

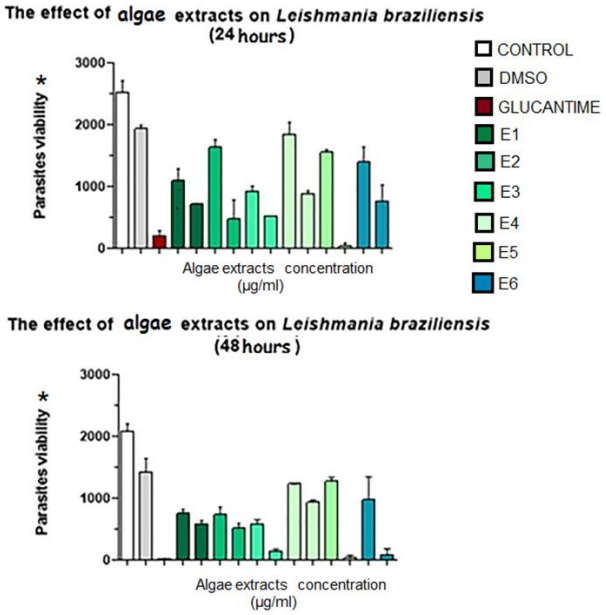

Figure 1: Effect of algae extracts on *Leishmania braziliensis*. Promastigotes of *Leishmania braziliensis* (1x10^6 cells/ml) were incubated in Schneider's medium at 28°C in the presence or absence of the crude extracts of algae (50 and 100 µg/ml respectively) for 24 and 48h of incubation. Glucantime® was used as a control. Controls with and without DMSO were also conducted. Parasite viability was verified through Trypan Blue staining. Parasites were counted in Neubauer chamber by using Conventional optical microscopy (Olympus BX41). Experiments were performed in duplicate. The analyzed data were computed in the GraphPad Prisma program. * (Parasites number x 10^3).

3.2 OPTICAL MICROSCOPY ANALYSIS OF THE ANTI-LEISHMANIAL POTENTIAL OF THE FRACTIONS DERIVED FROM *P. crispa* AGAINST *L. braziliensis*, MEASURED BY PARASITE VIABILITY THROUGH TRYPAN BLUE STAINING

Since *P. crispa* was seen to present comparable results to Glucantime® (the drug used for treating Leishmaniasis), experiments were done to investigate anti-leishmanial activity in fractions derived from *P. crispa*. Experiments were conducted with 9 fractions derived from *P. crispa* (E3), in serial concentrations [12.5µg/ml-100µg/ml] and in the presence of $1x10^6$ *L. braziliensis*/ml. Untreated controls of *L. braziliensis* were performed with and without DMSO (0.8% v/v) (Figure 2), according to the routine protocol used in our Lab. Conventional optical microscopy was used to evaluate the parasite viability, measured by Trypan Blue staining, after incubation of *L. braziliensis* (1 x 10^6 parasites/ml) in Schneider's medium, in the presence or absence of the fraction derived from *P. crispa* for a determined period of time at 28ºC in a 96-well culture plate. The ability to induce rosette formation was analyzed by optical microscopy and photomicrography documentation. Some of these results were expressed via computations in the GraphPad Prisma® program, through which graphs were created to compare the survival/mortality parameters in order to demonstrate the parasite viability in each of the experimental conditions testing treatment by the different fractions of *P. crispa*. Each experiment was performed in duplicate.

Thus, the figure 2 shows the results obtained after treating *L. braziliensis* with 7 different fractions derived from *P. crispa*. Figure 2 clearly demonstrates that, comparable to the results obtained with Glucantime®, the fractions 7, 10, and 11 have presented the best anti-leishmanial activity against *L. braziliensis* in the concentrations tested [6.25 µg/ml-100 µg/ml], either in 24 or 48h of incubation. The parameter of parasite viability monitoring was analyzed through Trypan Blue staining. In this context, the stained cells were the dead cells, which showed permeability to Trypan Blue staining after being treated with either Glucantime® or fractions derived from *P. crispa*.

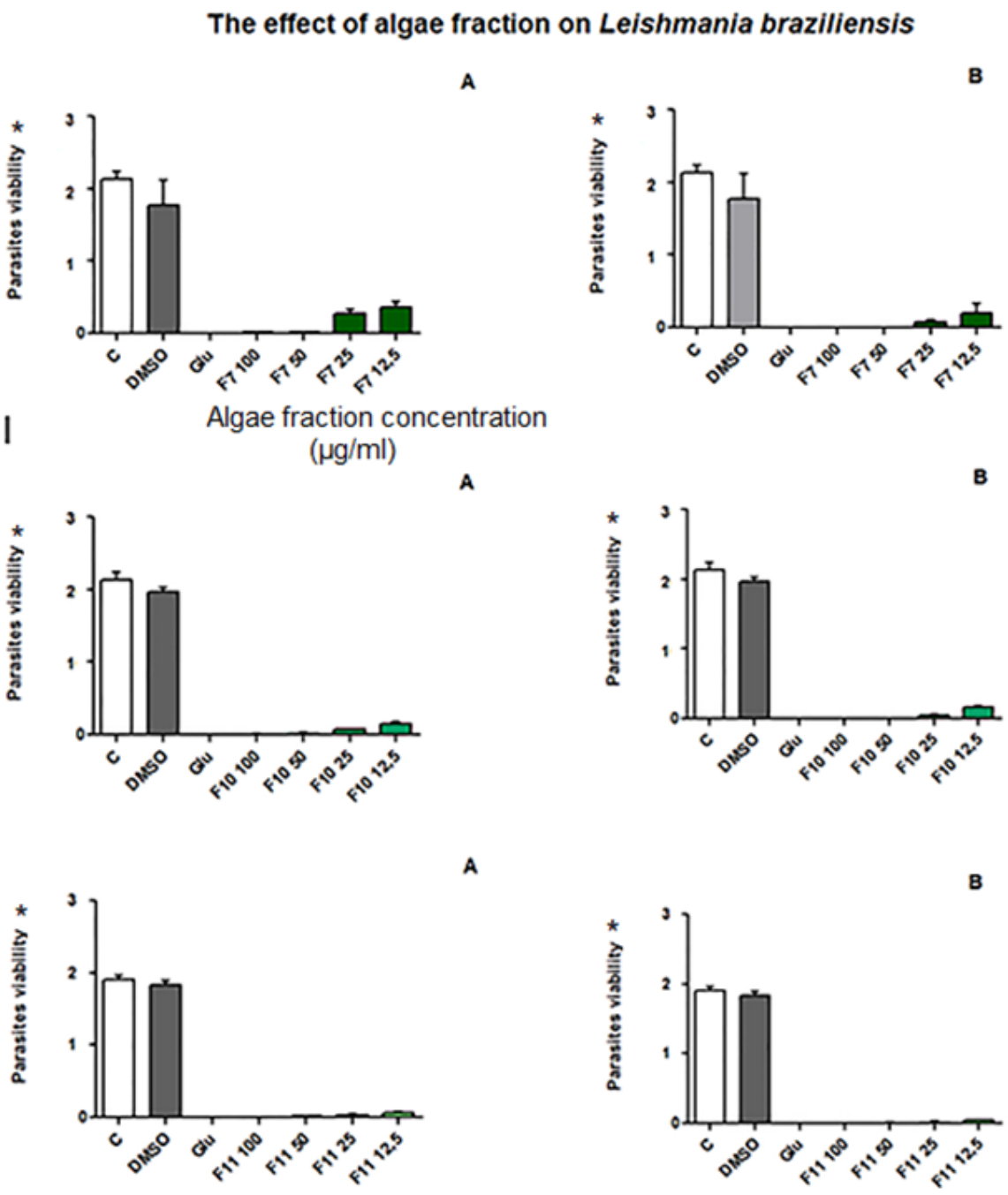

Figure 2. Effect of algae derived fractions on *Leishmania braziliensis*. Promastigotes of *Leishmania braziliensis* (1x10^6 cells/ml) were incubated Schneider's medium at 28°C in the presence or absence of the fractions derived from algae for 24 and 48 h. Glucantime® was used as control. Controls with or without DMSO were also done. Parasite viability was verified through Trypan Blue staining. Parasites were counted in a Neubauer Chamber using conventional optical microscopy (Olympus BX41). Experiments were performed in duplicate. The analyzed data were computed in the GraphPad Prism program. * (Parasites number x 10^3)

3.3 OPTICAL MICROSCOPY ANALYSIS OF THE EFFECT OF THE FRACTIONS DERIVED FROM *P. crispa* ON THE ROSETTE FORMATION OF PROMASTIGOTES OF *L. braziliensis*

The promastigote of Leishmania may present in different morphological types, including procyclic, nectomonad, leptomonad, metacyclic, and haptomonad forms, but also as groups of promastigotes growing in clusters, known as rosettes (RAZ et al., 1997; NOGUCHI, 1926). Although, Leishmania has several morphological forms, rosettes have not been much studied, even though they are usually found in the midgut of the insect vector (NOGUCHI, 1926; FELICIANGELI et al., 1998; LAINSON, 1973). Thus, we investigated the effect of algae derivatives on the formation of *L. braziliensis* rosettes, since it is another, important approach to evaluating the promastigote viability of *L. braziliensis*.

Rosette formation is used as parameter to measure biological functions based on the integrity of specific surface molecular components of *L. braziliensis*. The top of figure 3 shows the spontaneous induction of promastigote rosettes, indicating the parasite's biological activity in Schneider's medium (functional assay). The image (b) shows that DMSO had no influence on the biological activity of the parasite. In the presence of Glucantime® (**c**), no rosettes were seen, indicating the efficacy of this treatment against *L. braziliensis*. After treatment of the promastigotes with fraction 7 derived from *P. crispa*, even in the lowest fraction concentration tested [6.25 µg/ml], the induction of exuberant rosette formation (d) was comparable to that observed in the control situation (without any treatment, *L. braziliensis* spontaneously form rosettes, as seen in (a). Similarly to fraction 7, fraction 11 failed to inhibit rosette formation in the lowest concentration tested [6.25µg/ml], although it was able to inhibit rosette formation in the highest concentration tested [100 µg/ml] (Figure 3, "h" and "i", respectively). Remarkably, fraction 10 inhibited rosette formation (Figure 3, "f" and "g") in both the lowest and highest concentrations used [6.25 µg/ml and 100 µg/ml, respectively]. Observation by the phase contrast optical microscope showed that the rosettes adhered to the culture plate independent of the flow of the culture medium (data not shown). Leishmania appear to be attached to one another when arranged in rosettes.

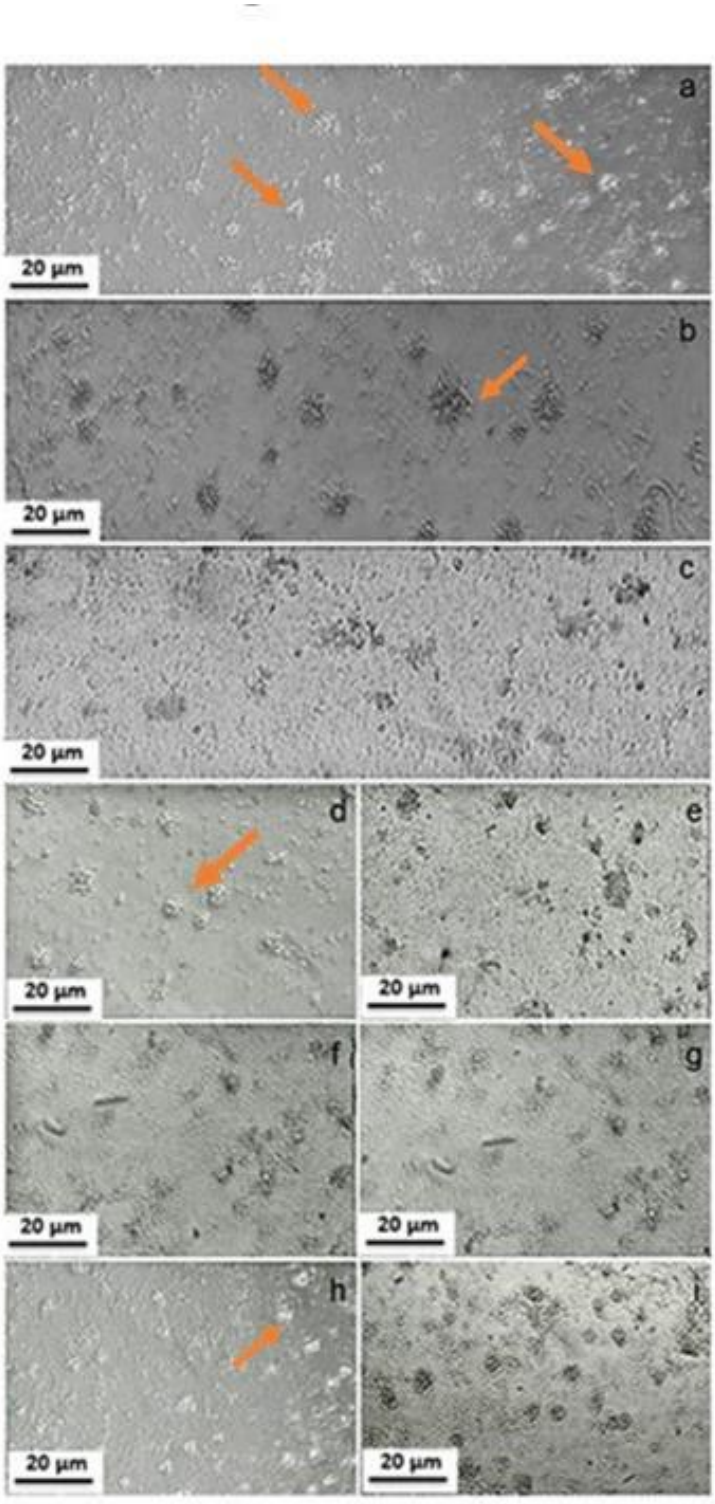

Figure 3. Effect of algae derived fractions on *Leishmania braziliensis* rosette formation. Micrograph taken of a culture of promastigotes of *L. braziliensis* incubated with either Glucantime®, DMSO, or fractions derived from *P. crispa* for 24 h at 28°C. Rosettes are observed by Phase Contrast Optical Microscopy. Scale bar: 20 µg/ml. (a) *L. braziliensis* control; (b) *L. braziliensis* incubated with DMSO; (c) *L. braziliensis* incubated with Glucantime®; (d) and (e) *L. braziliensis* incubated with fraction 7 derived from *P. crispa* in concentrations 6.25 and 100 µg/ml, respectively; (f) and (g) *L. braziliensis* incubated with fraction 10 derived from *P. crispa* in concentrations 6.25 and 100 µg/ml, respectively; (h) and (i) *L. braziliensis* incubated with fraction 11 derived from *P. crispa* in concentrations 6.25 and 100 µg/ml, respectively. Arrows shows rosette formation.

3.4 EVALUATION OF THE ANTI-LEISHMANIAL POTENTIAL OF THE FRACTIONS DERIVED FROM *P. crispa* AGAINST *L. braziliensis*, MEASURED BY ALAMAR BLUE STAINING: ANALYSIS BY SPECTROPHOTOMETRY

Alamar Blue was used to monitor the metabolic activity of *L. braziliensis*, and the results were analyzed by optical densitometry and measured by spectrophotometry. The figure 4 shows the results obtained with the fractions 7, 10, and 11 in different concentrations [12.5 µg/ml-100 µg/ml], reducing the metabolic activity of *L. braziliensis* comparable the results obtained with Glucantime®. All these results were comparable to the results obtained with treatment of *L. braziliensis* by Triton X-100—the positive control used (Figure 4B). The control situations (*L. braziliensis* in Schneider's medium and *L. braziliensis* incubated in the presence of DMSO diluted in Schneider's medium) displayed an enhanced parasite metabolic activity (toward 1.5).

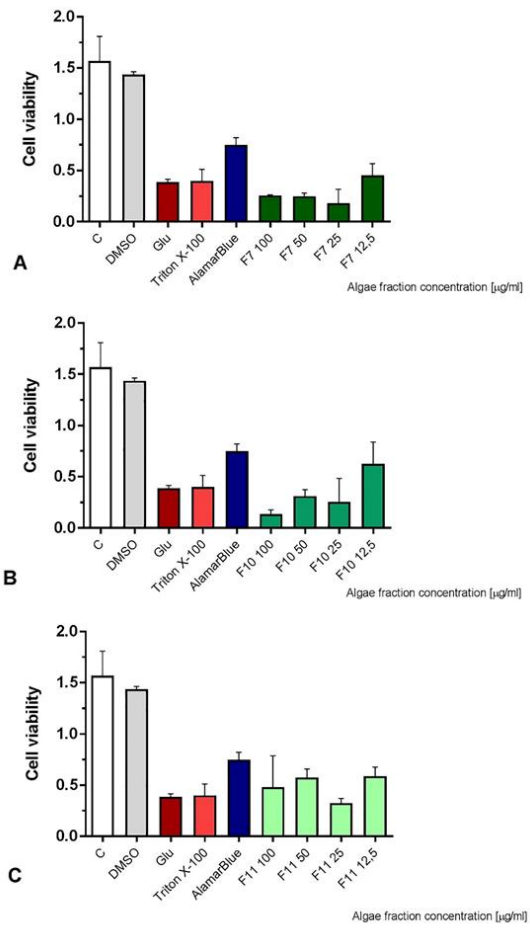

Figure 4: Effect of algae derived fractions on metabolic activity of *Leishmania braziliensis*. Promastigotes of *Leishmania braziliensis* (1x10^6 cells/ml) were incubated in Schneider's medium at 28°C in the presence or absence of fractions 7, 10, and 11 derived from algae for 24 h. Glucantime® was used as a control. Controls with and without DMSO were also performed. Alamar Blue was used to monitor the metabolic activity of *L. braziliensis*, and the results were analyzed by optical densitometry and measured by spectrophotometry. Fraction 7, 10, and 11 [12.5 µg/ml-100 µg/ml] reduced the metabolic activity of *L. braziliensis* to levels

comparable to the results obtained with Glucantime®. Triton X-100 was used as a control. Control situations (*L. braziliensis* in Schneider's medium or *L. braziliensis* incubated in the presence of DMSO diluted in Schneider's medium) displayed an enhanced parasite metabolic activity. Experiments were performed in duplicate. The analyzed data were computed in the GraphPad Prisma program.

3.5 EFFECTS OF FRACTIONS DERIVED FROM *P. CRISPA* ON HUMAN MACROPHAGES VIABILITY, MEASURED BY ALAMAR BLUE STAINING: ANALYSIS BY SPECTROPHOTOMETRY

The cytotoxic potential of fractions derived from *P. crispa* on human macrophages was investigated by measuring the metabolic activity of the cell through Alamar Blue staining (Figure 5). The results show that fractions 7, 10, and 11 have no deleterious effect on the metabolic activity of macrophage, indicating that these fractions were not cytotoxic to this human cell.

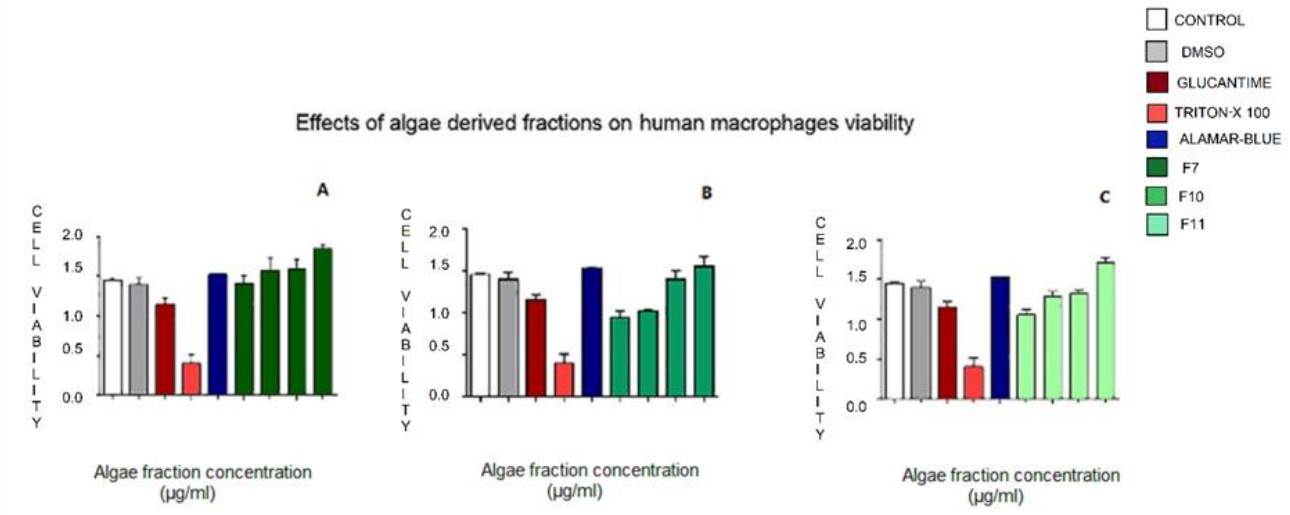

Figure 5. Effect of algae derived fractions on metabolic activity of human macrophages. Fractions 7, 10, and 11 [12.5 µg/ml-100 µg/ml] have no effect on the reduction of metabolic activity of human macrophages, indicating that these fractions were not cytotoxic to this human cell. Experiments were performed in duplicate. The analyzed data were computed in the GraphPad Prisma program.

3.6 LINEAR REGRESSION ANALYSIS OF THE RESULTS OF *L. braziliensis* BEING TREATED BY SOME FRACTIONS DERIVED FROM *P. crispa* AND EC50 CALCULATION

Conventional optical microscopy analysis showed the killing of *L. braziliensis* after treatment with the fractions derived from *P. crispa*, as shown in Figure 2. Thus, these quantitative results underwent linear regression analysis to determine their statistical significance. Figure 6 shows that fraction 10 and fraction 11 derived from *P. crispa* displayed

the greatest anti-leishmanial activity against *L. braziliensis*, as shown by two different profiles of statistical analysis (R1 and R2) with all the points lined in a curve (Figure 6). In addition, the effective concentration that was able to inhibit 50% of *L. braziliensis* viability after 24h of treatment (EC50) was analyzed, which revealed that fractions 10 and 11 (EC50 22 µg/ml and 41 µg/ml, respectively) showed greater anti-leishmanial activity against *L. braziliensis* than Glucantime® (EC50 89 µg/ml) (top-right insert, Figure 6). EC50 and graphs were determined using "Microcal Origin" program. The results were analyzed using Student's t-test, and the level of significance was set at $p<0.05$.

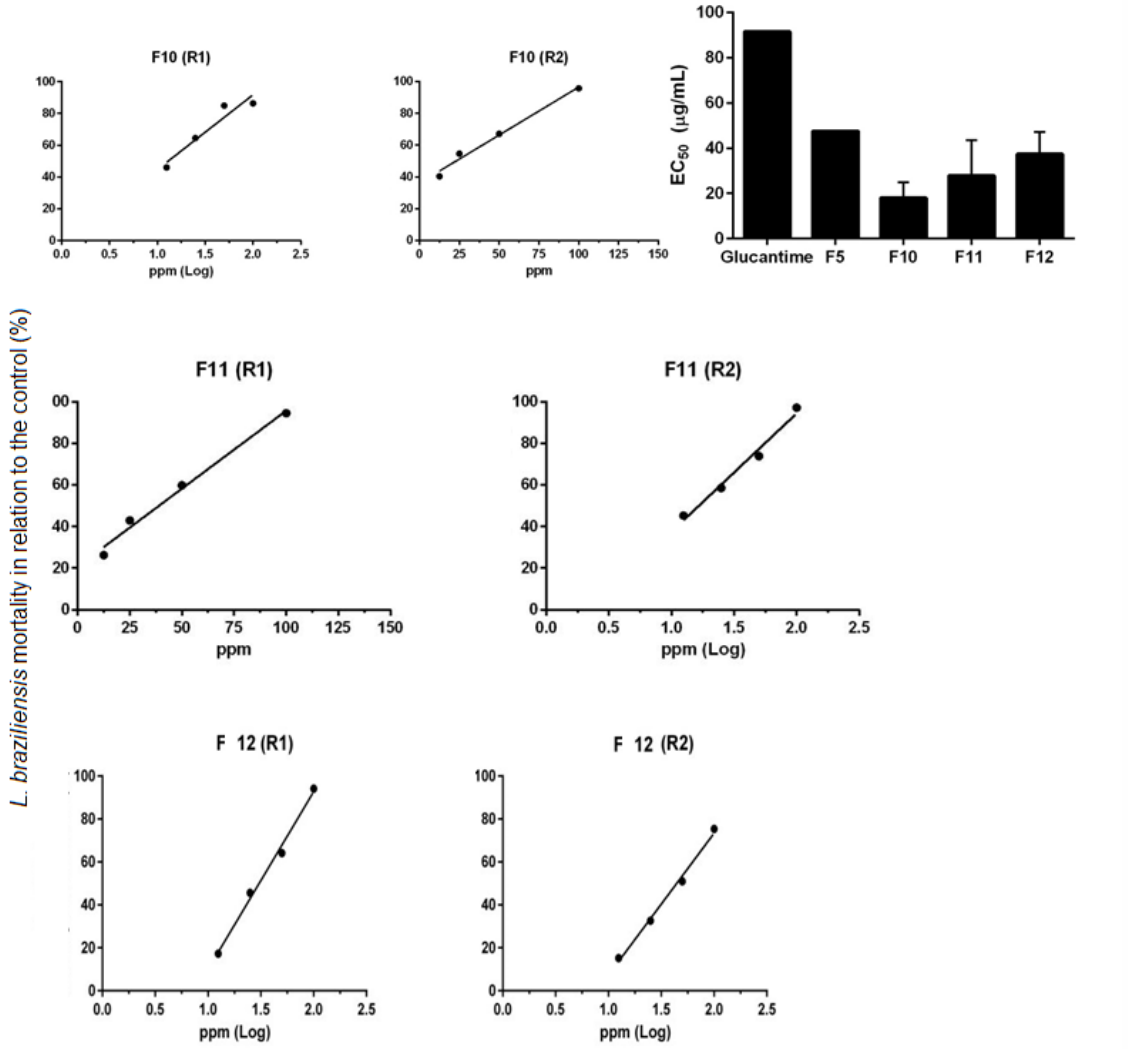

Figure 6. EC50 analysis. Graphical representation of the linear regressions for the fractions with and without transformation of the concentrations for Log10, according to the coefficient of determination (R^2).

The slopes of all regressions were statistically significant ($p<0.05$). All tests were conducted with two replicates (R1 and R2), with the exception of fraction 5; Note Insert in the top right of the figure: Graphic (top right, in black) of the concentration of fractions [5, 10, 11, 12 µg/ml] effective in eliminating 50% (EC50) of *L. braziliensis* parasites. Data analyzed in GraphPad Prism version 6.05. The EC50 values were established by solving the equations of each regression for the coordinate value of 50 (Y=50). Values of $p<0.05$.

4. DISCUSSION

The results presented here refer to the two phases of investigation: (1) the bioproducts, where extracts of various species of algae were tested for their anti-leishmanial potential against the extracellular form of *Leishmania braziliensis* (promastigote); and (2) the extracts, wherein purified fractions derived from algae were tested for the best antileishmanial activity against the extracellular form of *L. braziliensis*. In this context, the extract derived from *Prasiola crispa* was chosen, as its parasite-killing effects are comparable to those of Glucantime®, the drug used to treat Leishmaniasis (FELICIANGELI, 1998; SANTOS et al., 2008). Consequently, we performed several experimental in vitro tests to check the antileishmanial activity in fractions derived from *P. crispa* against *L. braziliensis.*

Briefly, different drugs are available to treat leishmaniasis, including: (a) an antimonial, which is not approved by the USA's FDA (Food and Drugs Administration); (b) Pentostam, permitted by the CDC (Center for Disease Control and Prevention) and used mainly in English-speaking countries; and (c) Glucantime®, which is used in Brazil and other Latin American countries. According to the WHO (WHO, 2016), Glucantime® varies in its therapeutic efficiency depending on the country, and treatment protocols are determined according to the geographical area (WHO, 2016). Glucantime® shows leishmanicidal activity (FELICIANGELI, 1998; RAHT et al., 2003). This medicinal product is contraindicated in cases of hypersensitivity to meglumine antimoniate and to other components of the product, as well as in patients with renal, cardiac, or hepatic deficiency (LAINSON, 1973).

Glucantime® is the first-line treatment for cutaneous Leishmaniasis, despite the many toxic effects related to its systemic administration, including acute renal failure, hepatic toxic effects, fever, arthralgia and myalgia, nausea and vomiting, and skin rash. Despite several reports concerning the anti-leishmanial effect of algae derivates on the treatment of Leishmaniasis - recently reviewed by (LÓPEZ et al., 2016) - to our knowledge, this work is the first to demonstrate the anti-leishmanial effects of fractions derived from *P. crispa* against the *Leishmania* genus.

So far, the literature does not include any report concerning the activity of *P. crispa* against any protozoan, including species of the genus Leishmania. Therefore, the present work breaks new ground in this subject, since it investigated the effects of this marine bioproduct against *L. braziliensis* and consequently discovered a new candidate in the alternative treatment of Leishmaniasis. In the present study, fractions derived from *P. crispa*

were selected, as they presented comparable anti-leishmanial effects to Glucantime®; these effects were determined by using three different methods to evaluate anti-leishmanial potential. The optical microscopy analysis showed that fractions 7, 10, and 11 presented the best antileishmanial activity against *L. braziliensis* when the parameter of cellular viability was analyzed through Trypan Blue staining.

The induction of rosette formation in promastigotes was another parameter investigated in this study; here, fraction 10 was chosen as the best compound that effectively inhibited rosette formation in both the smallest and the highest concentrations used [6.25 and 100µg/ml], as shown by phase contrast optical microscopy analyses. This method is very useful in detecting biological functions of the promastigotes of *L. braziliensis*, which depend on flagellar motility and extracellular matrix integrity on the parasite surface to successfully form rosettes. Rosette formation ability is often used as method forscreening drugs that treat Leishmaniasis, as reported by Jain et al. (2012). Furthermore, Iovannisci et al. (2010) reported that promastigotes of *L. major* strain LV39 (also known as MRHO/SU/59/P) express either poly α 2,8 N-acetyl neuraminic acid (PSA) or its partially de-N-acetylated derivative NeuPSA, which is associated with promastigote cells arranged in rosettes. Interestingly, these authors hypothesize that rosettes initiate mating in Leishmania, during which PSA/NeuPSA expression plays an important role. Since rosettes are a distinct form of the Leishmania life cycle, they can be considered a target for new forms of treating and preventing leishmaniasis. These authors pointed out the probability of the occurrence of in vitro genetic recombination without passage of promastigotes through insect vectors.

Nevertheless, Iovannisci et al. (2010) were the first to present molecular, physiological, and morphological evidence that rosettes represent a genuine stage in the life cycle of Leishmania, and these authors suggest that NeuPSA-based vaccines or drugs can also be useful in preventing or treating leishmaniasis (IOVANNISCI et al., 2010). Furthermore, sialic acids on the surface of developed *Leishmania donovani* are implicated in mediation of the binding, phagocytosis, and modulation of innate immune response and in signaling pathways to establish successful *L. donovani* infection in the host (IOVANNISCI et al., 2010; JAIN et al., 2012). Curiously, Iovannisci et al. (2010) reviewed that *L. major* promastigote rosettes are very similar to early stages of the green alga Chlamydomonas, in which cell-cell contact occurs through a process called agglutination, as described by Wilson (2008) and Adair et al. (1982) This process is mediated by specific adhesion glycoproteins, called agglutinins, seen along the length of the flagella (ADAIR et al., 1982).

The spectrophotometry analysis of the anti-leishmanial potential of the fractions derived from *P. crispa* measured by Alamar Blue staining indicated that fractions 7 and 10 have a strong reduction effect on the metabolism activity of *L. braziliensis*, and this reduction was much more pronounced than the results obtained with Glucantime®. Still, the effects of Glucantime® on the metabolic activity of *L. braziliensis* was comparable to the effects of the compound Triton X-100 on the metabolic activity of *L. braziliensis* (the parasite viability - measured through optical density detection-was ≤ 0.5). Alamar Blue® is an indicator for metabolic cell function and (NOGUCHI, 1926; RAZ et al., 1997). Fraction 11 also presented a reductor effect of the cell metabolic activity in *L. braziliensis* (toward ≤0.5 optical density).

After reductor effects were detected on *L. braziliensis* metabolic activity after treatment by fractions derived from *P. crispa*, our results showed that fractions 7, 10, and 11 had no cytotoxic effects on human macrophages, as shown by Alamar Blue assay. Interestingly, all the tested fractions tended to raise the metabolic activity on human macrophages, and especially fraction 7 presented an exuberant effect of inducing metabolic activity of human macrophages in all the tested concentrations, compared to fractions 10 and 11. Experiments are being conducted in our lab to better understand the beneficial effects of these fractions on human macrophages, since the latter are important cells in immune and inflammatory responses and are also known to be host cells for *Leishmania braziliensis*. According to this assay, Glucantime® did not alter the metabolic activity of human macrophages.

To evaluate significant differences in the quantitative results obtained by optical microscopy, linear regression of the results was calculated. The majority of the points identified in the screening of the different *P. crispa* fractions and the respective concentrations were lined in the curves, as shown in figure 6, indicating that all the investigated fractions were effective in killing *L. braziliensis* ($p<0.05$). However, the analysis of the concentration that was able to inhibit 50% of the *L braziliensis* viability after 24h of treatment (EC50) revealed that fractions 10 and 11 (EC50 22 µg/ml and 41µg/ml, respectively) are the best candidates, with better anti-leishmanial profiles against *L. braziliensis* than Glucantime® (EC50 89 µg/ml) (top-right insert, Figure 6).

With all the evaluated parameters taken together, the fraction 10 (F10) derived from *P. crispa* seems to be more suitable for follow-up investigation concerning the antileishmanial activity seen in compounds derived from *P. crispa*. This fraction was effective in all the investigations performed in this study: F10 induced the death of *L. braziliensis*, inhibited the formation of rosettes even in the smallest concentration used, inhibited cellular metabolism (therefore decreasing the cellular viability) and was able to kill 50% of Leishmania parasites

(EC50 = 22 µg/ml) with an anti-leishmanial potential superior to that with Glucantime® (EC50 = 89 µg/ml); furthermore, it was not cytotoxic to human macrophages.

The current treatment of leishmaniasis has many issues, including costs, varying degrees of toxicity, route of administration, and frequency of resistance. In the case of pathogens like Leishmania that present many stages of life, it is necessary to study all forms of this specific parasite, including the rosettes. In the present work, the first test of antileishmanial activity in fractions derived from *P. crispa* involved the classical extracelular form-promastigote and promastigote rosettes. Tests with rosette formation continue to be performed in our lab, and assays at the intracellular-amastigote stage are still being conducted by our group. Together, these data open new horizons for anti-leishmanial therapy.

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The increase in the number of leishmaniasis cases observed during the last 25 years worldwide is due to some specific factors such as: globalization and climate change. They contribute to the spread of leishmaniasis in non-endemic areas. For example, in the last few decades, the number of cases of leishmaniasis in international travellers (tourists and businessmen) has increased. Other risk factors for the emergence and spread of leishmaniasis are war and deforestation. Leishmaniasis is considered endemic in 88 countries, as more than 12 million people suffer from the disease and a portion of the population of approximately 350 million is at risk of contracting it. In the book **"Leishmaniasis - knowledge, learning and innovation"**, Cutaneous Leishmaniasis is approached in different aspects such as: (1) Geographic Challenge; (2) Endemic disease distributed in all Brazilian territories, including Brazil's border region with other South American countries; (3) a complex disease whose treatment remains a challenge and finally, (4) a disease that, in the near future, may have an innovative product which may contribute to the pharmacological treatment derived from algae, with leishmanicidal potential and devoid of cytotoxicity to cells human.

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