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Leishmanicidal activity of ishpingo and moringa plants

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DEDICATORIA

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RESUMEN

La leishmaniasis es una enfermedad tropical desatendida que posee una amplia variedad de manifestaciones clínicas, las mismas que incluyen síndromes cutáneos, mucocutáneos asociados a una infección parasitaria y una variante visceral. El uso de medicamentos de primera y segunda línea para el tratamiento de leishmaniasis cutánea presenta graves efectos adversos que deterioran la calidad de vida del paciente, así como un daño orgánico importante que requiere un seguimiento minucioso por el médico, generando poca afinidad del paciente al tratamiento y abandono del tratamiento, el incremento de la resistencia a fármacos en el tratamiento de especies de Leishmania y la falta de vacunas efectivas han convertido a esta enfermedad desatendida una de las principales causas de morbilidad y mortalidad de las enfermedades tropicales . Por lo tanto, ante la falta de herramientas efectivas para tratar y erradicar la enfermedad es necesaria la investigación de nuevos compuestos con potencial leishmanicida en el Ecuador.

Palabras clave: Leishmaniasis, efectos adversos, resistencia, enfermedad desatendida, enfermedad tropical.

ABSTRACT

Leishmaniasis is a tropical neglected disease which causes a range of clinical manifestations including cutaneous and muco-cutaneous syndromes associated with the parasite infection and the visceral variant. The use of first and second-line drugs for the treatment of cutaneous leishmaniasis causes serious adverse effects that affect patient's quality of life, as well as important organic damage that requires close monitoring by a physician, generating a very low patient affinity and abandonment of the treatment, *Leishmania´*s growing resistance to pentavalent antimonials and unsuccessful vaccines convert *Leishmaniasis* one of the most dangerous tropical parasitic diseases that causes of death and disability. Thus, due to the therapeutic tools are not adequate to eradicate the infection it is a necessity the assessment of new potential anti-leishmanial plant compounds of plants traditionally used in Ecuador.

Key words: Leishmaniasis, adverse effects, resistance, neglected disease, tropical disease.

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PART I: GENERAL INTRODUCTION

History of Medicinal plants

Humankind use plants for food and relieve diseases. Magic, religion and experimentation with plants have played a role in medical practices (Kelly, 2009) and plants have been used for medical purposes for thousands of years. Thus, some of the pharmacological knowledge came from this experimentation with plants. This relationship between humankind and the search for active compounds in nature dates from millennials. There is evidence of this connection in written documents, monuments and original plants medicines (Petrovska, 2012).

The oldest written evidence that described the usage of plants for medical uses has been found on a 5000 years old Sumerian clay slab, in which twelve documents for drug preparation described 250 plants some of them containing alkaloids such as henbane, poppy and mandrake (Kelly, 2009). The Chinese Medicinal Book from the Pen T´Sao was written by the Emperor Shen Nung in 2300 BC, who described 365 isolated plant extracts from dried parts of medicinal plants and their prescriptions. In India the holy book of Vedas referred to the use of plants as treatment for diseases. The Ebers Papyrus written in 1550 BC evidences a collection of 700 prescriptions of plants used for therapy such as onion, garlic, willow, etc. According with the Bible and the Jewish book some treatments employing aromatic plants such as myrtle and incense were described. In the Iliad and the Odysseys written by Homer in 800 BC, 63 plants species from Egyptian, Minoan and Mycenaean cultures pharmacotherapy were described (Petrovska, 2012). Dioscorides, the most important writer on plants uses, is consider “the father of the pharmacognosy”, was the pharmacognosist of Nero´s Army and studied the utility of medicinal plants with the Roman Army. In his book “De Materia Medica” he describes 944 drugs with medical properties (Petrovska, 2012).

Galen in 131 AD wrote the first list of drugs “De succedanus”. He mentioned several new drugs that Dioscorides had no described previously. With the discovery of America “Materia Medica” was enriched with new medical plants such as*:* cacao, ratambia, vanilla, mate and tobacco, among others. In XVII century, *Cinchona succirubra* (family *Rubiaceae*) native three of South America is introduced to Europe because its anti-fever, and anti-malarial properties. Some alkaloids were isolated *from C. succirubra* (family *Rubiaceae*) such as: quinine, quinidide, cinchonine, cinchonidine. Quinine was the first anti-malarial treatment and still today is used as prevention and treatment for paludism (Lives et al., 2004). In XIX century was the turning point in the understanding and application of medicinal plants. The isolation of alkaloids from poppy in 1806, ipecac in 1817, cinchone in 1820 and other plants, then the isolation of glycosides represents the beginning of scientific ethnopharmacology. In the 20th Century some authors proposed many methods in order to standardized and stabilized labile medicinal components isolated from fresh medicinal plants. (Petrovska, 2012).

At the present time, biologist described and accepted a number of 374,000 plants species on Earth (Christenhusz & Byng, 2016). It is estimated that 35,000 to 70,000 of those species have used in some cultures for medicinal purposes at one time or another in the history (WHO, 1998). According to World Health Organization reports, about 80% of the population around the world still uses botanical drugs and several of them have their origin to medicinal plants. The estimated of 374,000 plants species described, less than 20% have been investigated for medicinal applications(Sen & Samanta, 2014).

Malaria is a fatal disease caused by a parasite transmitted to the bite of a female Anopheles mosquito. According to the WHO in 2017 there were 219 million cases of malaria in 87 countries and 435 000 deaths were reported in the same year (Hallyburton et al., 2015). Since the isolation of quinine in 1820, the first compound with effective antimalarial activity several natural compounds have been developed. In 1940 chloroquine was used to treat malaria,

in 1970 mefloquine and halofantrine (Tse, Korsik, & Todd, 2019). In 1967 was discovered the antimalarial properties of the leaves of Artemisia annua (family Compositae), traditional medicine used to reduce fever (White & Hien, 2015). The chemical, physico-chemical and antimalarial properties were evaluated in animal models and then in human malaria. In fact, it produced rapid parasite clearance than others antimalarial drugs. Therefore, artemisin are currently the pillar of malaria treatment (Talman, 2019). In 2015 C. Campbell and Satoshi Omura won the Nobel Prize for Medicine or Physiology for their discovery of avermectins, and Tu You You for her contribution to the discovery of artemisinin (White & Hien, 2015). Nowadays there is an emergence of artemisin and artemisin derivates resistance that was demonstrated in 2007 in a clinical trial measuring the rate of parasites expose to the drug (Talman, 2019). New anti-malaria drugs are currently in clinical trials, rosiglitazone, imatinib and sevuparin are in Phase II trials (Tse et al., 2019).

Medicinal plants as future drugs

The most important natural substances isolated from plants which served as therapeutic drugs are mentioned in the following paragraphs. Some of them are associated for their cardiovascular and nervous system effect, and their anti-inflammatory, anticancer, and antimicrobial properties. Plant extracts and phytochemicals are associated to have positive effects on human brain function. *Papaver somniferum* (family *Papaveraceae*) with active compounds morphine and heroin, *Cannabis sativa* (family *Cannabaceae*) with active compound marijuana and *Coffea arabica* (family *Rubiaceae*) with active compound caffeine used for decreasing the acute and chronic pain are widely used and abused around the world (Berman, Symonds, & Birch, 2004; Hill, Palastro, Johnson, & Ditre, 2017).

Plants with promising anti-inflammatory activity: Various natural products suppress the inflammatory response through the inhibition of the signaling cascades. For instance, *Salix*

alba (family *Salicaceae*) was described for Hippocrates for the treatment of pain, inflammation and fever (Kelly, 2009). Alkaloids such as quinoline, isoquinoline, and indole have been used for their anti-inflammatory properties. Alkaloids isolated from *Sophora prostrata* (family *Leguminosae)* have COX inhibitory properties (Changwei, 2009), *Phyllanthus amarus* (family *Phyllanthaceae*) inhibit IL1- b production in inflamed tissues (Candida, 2005) and the phenylpropanoids isolated from *Illicium* species (family Schisandraceae) were found to inhibit the histamine release on rats with leukemia cells (Yakushjin, 1982).

Plants for Cardiovascular Disease: According to the WHO, cardiovascular diseases (CVDs) are the first cause of death globally. Many plants have been used for the treatment of CVDs and have a direct effect on the hearth and blood vessels and may cause severe adverse reactions. Glycosides are a cardiac compound isolated from foxglove *Digitalis* spp. which were described from the foxglove plant, *Digitalis purpurea* (family *Plantaginaceae*), for heart failure treatment in 1785. More than 200 years later, glycosides are prescribed for patients with heart failure and atrial fibrillation (Levine, 2019). Other active compounds are used for the treatment of CVDs such as *Tabernaemontana dichotoma* (family *Apocynaceae*) cathafoline that inhibit receptor-operated calcium channels, *Amomum subulatum* (family *Zingiberaceae*) cardamonin that block voltage calcium channel, and plant extract such as ethanolic extract of *Ocimum basilicum* (family *Lamiaceae*) which recovered the arterial pressure and *Allium sativum* oil (family *Amaryllidaceae*) which reduce ventricular tachycardia and fibrillation (Sen & Samanta, 2014)

Plants for the treatment of infectious diseases: The increased of incidence of antimicrobial drug resistance, treatment failure and the shortage of drugs for the treatment of neglected infectious diseases have allowed the study of plant-derived compounds with potential antimicrobial activity. The diversity of plants generates newer potential antibacterial, antiparasitic and antifungal agents. Simple phenols and polyphenols, quinones, flavones,

flavonoids, flavanols, tannins, coumarins, terpenoids, essential oils, alkaloids, polypeptides and other compounds have been demonstrated be effective against viruses, bacteria, parasites and fungi (Gachet et al., 2010). For example, anthraquinones posse antibacterial activity including against *Mycobacterium* species, tannins inhibit the growth of uropathogenic *E. coli,* and alkaloids isolated from plants of the Ranunculaceae family show anti protozoa activity (Sen & Samanta, 2014). The assessment of in vitro anti-protozoal potential of traditionally plants-extracts is a helpful tool to discover new therapeutic alternatives for drug development. Leishmaniasis, African trypanosomiasis, Chagas disease and malaria are life-threatening diseases that represents a great risk to the world population. One of the most relevant of them is leishmaniasis (Gachet et al., 2010). In a study performed in Ecuador a total of 146 plant-extracts were screened against *Plasmodium falciparum, Leishmania donovani, Trypanosoma cruzi* and *Trypanosoma brucei.* The most important plant families described are Asteraceae, Solanaceae, Araceae, Rubiaceae, Fabaceae, Piperaceae and Lamiaceae. Seven plants showed leishmanicidal activity against *L. donovani* amastigotes: *Brugmansia* sp., *Gouania lupuloides* (family *Rhamnaceae*), *Piper* sp. (family *Piperaceae*), *Bocconia integrifolia* (family *Papaveraceae*), *M. guianensis* (family *Primulaceae*) and *Elephantopus mollis* (family *Asteraceae*) (Gachet et al., 2010).

Leishmaniasis

Leishmaniasis is a tropical neglected disease caused by a protozoon of the genus *Leishmania* and is transmitted by the bite of infected female phlebotomine of the genus *Lutzomyia* (Western hemisphere). Leishmaniasis causes a spectrum of diseases. The range of clinical manifestations include cutaneous syndromes associated with the parasite infection and the visceral variant. The cutaneous syndromes include mucosal leishmaniasis (ML), leishmaniasis recidivans (LR), localized cutaneous leishmaniasis (LCL), which is the most

common presentation and the diffuse cutaneous leishmaniasis (Steverding, 2017). Cutaneous lesions may occur on exposed areas of the skin where the sand fly mouth parts can penetrate. Localized cutaneous leishmaniasis (LCL) begins as a red papule and develop into plaque-like lesion, leading to ulceration with an indurated border, multiple lesion may be present (Naomi Aronson, 2019). According to the World Health Organization (WHO) the disease affects the poorest people and is associated with a weak immune system, lack of financial resources, and it is linked to environmental changes such as deforestation and urbanization. Around of 1 million new cases and some 26000 to 65000 deaths occur annually (Naomi Aronson, 2019). According to the Pan American Health Organization, in Ecuador there is an incidence of 22,6 per 100,000 habitants, 1632 cases per year (2015-2017), from which 98,3% are cutaneous leishmaniasis cases (CL) and 1,7% mucosa leishmaniasis (ML) (WHO-PAHO, 2017). It is interesting to notice that in 2016 according to PAHO Leishmaniasis Americas Epidemiologic Report, the percentage of healing was around of 30% whereas in 2019 percentage of healing is around of 99,9%. Despite this data, nowadays there are cases without diagnosis and adequate treatment in rural zones (WHO-PAHO, 2017).

Cutaneous leishmaniasis treatment: Pentavalent antimonials (pentavalent Sb or Sb^{V}), like meglumine antimoniate and sodium stibogluconate are the first-line drugs for the treatment of cutaneous leishmaniasis (Copeland & Aronson, 2015); meanwhile amphotericin B, paramomycin, pentamidine isethionate are considered the second- line drugs (Monzote, 2009; Musa et al., 2012). All of them have been used for decades and exist evidence that exhibited serious adverse effects in the treatment of cutaneous and visceral leishmaniasis (Monzote, 2009).

Adverse effects: A systematic review identified the main associated adverse effects and estimate the frequency of these effects. This systematic review includes 65 studies with a total of 4359 patients from 12 countries from Latin American and the Caribbean, infected with 8

different *Leishmania* species (Oliveira et al., 2011) . The most frequent clinical reported adverse effects of pentavalent antimonials at doses of 10 – 20 mg/kg/day, for the treatment of cutaneous leishmaniasis are local pain 64,3%; myalgia/arthralgia 48,6%; taste alterations 25,3%; headache 23,6%; anorexia 19,4%; asthenia/fatigue 18,9; gastrointestinal disturbances 17,4%; fever 16,7%; cutaneous reactions 5,8%; pancreatitis 3,6% - 4,8%; and thrombophlebitis 3,6%. The most frequently laboratory and electrocardiographic adverse effects of pentavalent antimonials are elevation of lipase/amylase 59,97%; AST/ALT 43,3%; creatine phosphokinase 7,1%; alkaline phosphatase 3,6% - 4,8%; QTc prolongation 16%; ischemic alterations 3,6% and arrythmias 3,3%. For pentamidine isethionate at doses of 2 – 4 mg/kg/day the most frequent clinical adverse effects are anorexia 46,7%; myalgia/arthralgia 24,9%; gastrointestinal disturbances 21,5%; asthenia/fatigue 21,1% and headache 15,2% (Oliveira et al., 2011). Amphotericin B deoxycholate and liposomal Amphotericin B is an alternative for the treatment of cutaneous leishmaniasis. However, a systematic review with 29 patients observed a rate cure of 93,1% and reported that 17,2% of nephrotoxicity, being the most important side effect (Cunha, Leão, De Cassia Soler, & Lindoso, 2015). Other adverse effects have been reported such as cardiotoxicity which occur at doses over 5 mg/kg/day specially in patients with previous cardiovascular events (Autry, Harrison, White, & Miller, 2018), fever 35%, nausea 35%, phlebitis 35%, dorsal pain 25%, vomiting and headache 15% have also been reported (MacHado et al., 2015). And paramomycin is also used as a second line of treatment for cutaneous and visceral leishmaniasis alone or in combination, some adverse effects have reported such as cardiotoxicity, acute pancreatitis, peritoneal hemorrhage and abnormal hepatic function(Musa, et al., 2012).

Leishmanial drugs resistance: It is well known that *Leishmania* species differs in its sensitivity to first and second-line drugs for the treatment of leishmaniasis (WHO, 1999). As we explain above pentavalent antimonials have been used successfully worldwide since the first half

of the XX century. Some aspects that impact the final therapeutic outcome should be considered, an effective immune response is necessary to support anti-leishmania drugs, pharmacokinetics of the drugs, parasite factors can play a role that could explain resistance inherent virulence of the strain, parasite infection with RNA viruses, different *Leishmania* species or strains and factors related to the drug itself and inappropriate dosing by inexpert health workers with subtherapeutic dosage and selection of resistant parasites. However, what does mean the term "drug resistance"? Recent studies indicate that the effectiveness of first-line drugs in some parts of the world (India and Nepal) is decreasing (Ponte-sucre et al., 2017). Antimonial resistance emerges when genetic mutation decreases the parasite´s response to a drug as consequence of antimonial-drug pressure. In fact, in some in vitro studies, resistance selected of arsenic cause cross-resistant to antimony (Ponte-sucre et al., 2017). In addition to this, elevated levels of arsenic in drinking water in north eastern India can offer a plausible explanation of antimony resistance in this region (Perry et al., 2011). Other explanation of this phenomenon is the natural difference of sensitivity between *Leishmania* species to the antileishmanial-drugs. Studies based of amastigote-macrophage in vitro model, showed that *L. brasiliensis* and *L. donovani* were fivefold more sensitive to pentavalent antimonial than *L. mexicana, L. major* and *L. tropica* (S. L. Croft, Sundar, & Fairlamb, 2006). Molecular mechanisms of antimonial drug - resistance have been elucidated, pentavalent antimony (Sb^{V}) is reduced to its trivalent form (Sb^{III}), this reduction occurs in two ways, the first one occurs within the macrophage, the resultant Sb^{III} enters in the cell via AQP1 protein membrane carrier and the second one occurs in the parasite for uncharacterized carrier mechanism within the cell. Antimony pentavalent accumulation inside the parasite is lower in resistant strains when compared with sensitive ones (Perry et al., 2011). A study demonstrated that the overexpression of aquaglyceroporin1 (AQP1), exhibited a sensitivity in wild-type cells whereas gene deletion exhibited resistant effect (Adaui et al., 2011; Asia, America, & Rica, 2017). Reduction of Sb^{v} to Sb^{III} reduce the internalization of the drug and increases the levels of

trypanothione (a rare form of glutathione compound of glutathione and spermidine), which increased thiol redox potential implied in resistance (S. L. Croft et al., 2006) and the expression of three genes coding for the ABC transporter MRPA also confer resistance to pentavalent antimony (Singh, Chatterjee, & Sundar, 2014). Levels of intracellular P-glycoprotein (PgpA), γ -glutamylcysteine synthetase (GCS), and ornithine decarboxylase (ODC) are elevated in resistant strains whereas decreased of Sb reductase is observed in others. The changes described below induce the expression of multi-drug resistance protein 1 (MDR1) efflux in the macrophage, which diminishes the amount of intracellular antimony (S. L. Croft et al., 2006; El Fadili et al., 2005).

Leishmania has a plastic genome, with a potential for aneuploidy, extrachromosomal linear and circular amplification of sets of genes and local copy number variations (CNVs). This plasticity allows an increase in the quantity of transcripts of some genes creation of genetic diversity, and a useful adaptative strategy to elude immunogens such as metalloprotease GP63 (Ponte-sucre et al., 2017).

Therefore, the use of first and second-line drugs for the treatment of cutaneous leishmaniasis cause serious adverse effects that affect patient's quality of life, as well as important organic damage that requires close monitoring by a physician, generating a very low patient affinity, the abandonment of the treatment by the patient and Leishmania´s growing resistance to pentavalent antimonials. Thus, it is a necessity the assessment of new potential anti-leishmanial compounds from plants, traditionally used in Ecuador(Ponte-sucre et al., 2017).

Plants for the treatment of leishmaniasis: Hundreds of studies performed in Latin-America, Europa and Asia reported plant species which have been used in the treatment of Leishmaniasis (Gachet et al., 2010). Berberine, an active compound of *Berberis vulgaris* (Berberidaceae) was evaluated against promastigotes of *L. tropica* and *L. m*ajor, the results exhibited inhibition of growth at doses from 2,1 to 26,6 μg/mL. However, the active principle

berberine exhibited more cytotoxicity in murine macrophage at doses from 27.3 to 362.6 μg/mL. (Mahmoudvand et al., 2014). *Handroanthus serratifolius* (family *Bignoniaceae*) ethanol extract was evaluated against promastigotes of *L. amazoniensis* with no evidence of leishmanicidal effect or citotoxicity (Vanessa et al., 2017). Pentadecane, a floral volatile compound, was evaluated against *L. infantum* parasites showing growth inhibition in promastigotes, amastigotes and resulting in a reduction of macrophage infection (Bruno et al., 2015). Ethanolic extracts of *Astronium fraxinifolium* (family *Anacardiaceae*) and *Plectranthus amboinicus* (family *Lamiaceae*) were evaluated in promastigotes of *L. braziliensis.* For in vivo studies BALB/c mice were infected subcutaneously. The animals showed a significant reduction of the lesions in the 6th week of treatment, however, the results were no comparable with Glucantime (Gomes De Lima et al., 2014). *Eugenia uniflora* (family *Myrtaceae*) essential oil (EuEO) was evaluated against promastigotes of *L. amazoniensis*. According to the study, EuEo was 20 times more toxic to amastigotes than macrophages (Klinger et al., 2013). *Arrabidaea chica* (family *Bignoniaceae*) is a medicinal plant used in Brazil, five fractions obtained from hexanic extract against promastigotes of *L. amazonensis* and *L. infantum* were evaluated. Mitochondrial ultrastructural alterations were observed. Sterols and fatty acids probably are the compounds involved in leishmanicidal activity (Rodrigues et al., 2014). *Ocotea macrophylla* (family *Lauraceae*) and *Zanthoxyllum monophylum* (family *Rutaceae*) plants used in Colombia as traditional medicine in the treatment of infectious diseases, cancers and other diseases were evaluated against *L. panamensis* and *L. major* promastigotes. In that study, two ethanol extracts were evaluated, one from *Ocotea macrophylla* (family *Lauraceae*) and one alkaloid fraction of *Zanthoxyllum monophylum* (family *Rutaceae*). According to that study ethanol extracts and alkaloids fractions are therapeutic options for cutaneous leishmaniasis due to the important activity of the extracts against of promastigotes and

amastigotes. The selective index was more than 10 in the two cases, display promising antileishmanial activity (Chavez, 2014)

Ocotea quixos: It is a plant species of the Lauraceae family (Laurales order). Also known as Ishpink tree (ishpingo) is endemic to the Amazonian regions of Colombia, Ecuador and Peru. *O. quixos* is an extremely aromatic plant which is used in Ecuadorian culinary practices as well as its pharmacological properties in traditional medicine. Nowadays, research analyzes are focused on botanical, biological and chemical properties (Noriega, 2018). *O. quixos* has also been reported to exhibit pharmacological properties, for example it can be used to treat flu, colds, vomiting, gastric and intestinal complaints, diarrhea and as local anesthetic (Mosquera, Parra, & Flor, 2017).

O. quixos tends to grow in the Amazonian region from the southeast of Colombia, Ecuador and to the Hamboyacu Altos area in Peru (Noriega, 2018). The height of the tree ranges between 3 to 6 meters, and presents lauroid leaves, flowers with six sepals and fruits (Noriega, 2018). Chemical studies have focused mainly on the essential oils isolates from chalices and leaves. The main compounds with therapeutic effect isolated from essential oils are humulene, p-cymene, eremofilene, geranial, sabinene, β-caryophyllene, methyl cinnamate, o-methoxycinnamaldehyde (Noriega, 2018) phenolic compounds, cinnamyl alcohol, cinnamyl acetate, cinnamaldehyde (Mosquera et al., 2017).

The essential oils from *O. quixos* have been reported to exhibit antimicrobial and antifungal properties particularly against *S. aureus, P. aeruginosa*, *S. cereviseae* and *Candida albicans* (Noriega, 2018).

Moringa oleifera: *M. oleifera* is a fast growing, drought- resistant tree, widely distributed and cultivated in hedges and in house yards. It grows well in hot dry lands and humid tropics (Morton, 1991). *M. oleifera* is an indigenous tree of Asia from Pakistan, India, western and sub-Himalayan tracts, it is now distributed in Arabia and Africa; Cambodia, Philippines,

the Caribbean Islands, North America, Central and South America (Farooq Anwar, Sajid Latif, 2009). The height of the tree varies from 5 to 10 meters, it tolerates a wide range of rainfall and a pH of 5.0 – 9.0 (Farooq Anwar, Sajid Latif, 2009). In America *M. oleifera* has acquired numerous names: acacia, árbol de las perlas, árbol de espáragos, benbom, cedro, chinto de borrego, moringa, palo de aceite, palo de Abejas, perlas del oriente, etc. (Farooq Anwar, Sajid Latif, 2009).

Moringaceae-Brasicales order has been reported to be rich in bioactive compound such as, natural antioxidants, antibacterial, antifungical, antihypertensive, diuretic, cholesterol lowering, antispasmodic and antitumor activities (Farooq Anwar, Sajid Latif, 2009). The root, leaves, stem bark, gum, flower and seeds, are used as a nutritive vegetable supplement for its high nutritional value as well as its medicinal properties. *M. oleifera* leaves have been found to contain β-carotene, vitamin C, essential amino acids, calcium, potassium, ascorbic acid, phenolics, flavonoids and carotenoids (Farooq Anwar, Sajid Latif, 2009). In some countries, it is known as "mother best friends" because increase woman´s milk production (Estrella, Bias, Man, David, & Taup, 2018). Whole-gum exudate contain L-arabinose, -galactose, L-rhamnose, -mannose and -xylose while a degrade-gum glucuronic acid and L-mannose which are generally used for dental caries, to relieve headaches, fever, dysentery, and asthma (Farooq Anwar, Sajid Latif, 2009). Nine amino acids have been isolated from flowers such as such us cystine, methionine, lysine and tryptophan as well as D-glucosa, sucrose, alkaloids, potassium and calcium. Flowers have a high medicinal value like as relieve muscle diseases, hysteria, tumors; lower the serum cholesterol, triglyceride, VLDL, LDL and phospholipids (Farooq Anwar, Sajid Latif, 2009). Antibacterial compounds such as ptrigostermine, 4-α-L-rhamnosyloxy benzyl isothiocyanate and aglycone of deoxy-niazimicine isolated from de root possesses antibacterial and fungicidal effects (Farooq Anwar, Sajid Latif, 2009). The juice from the stem bark show

effect against *S. aureus* while the fresh leaf juice inhibits the growth of *P. aeruginosa* and *S. aureus* (Cáceres, 1991).

PART II: SCIENTIFIC ARTICLE

Leishmanicidal activity of ishpingo and moringa plants

Introduction

Leishmaniasis is caused by a protozoa of the genus *Leishmania*. It is a tropical neglected disease and is transmitted by the bite of an infected female phlebotomine of the genus *Lutzomya* in the New World. Leishmaniasis causes a spectrum of diseases. The range of clinical manifestations include cutaneous syndromes associated with the parasite infection and the visceral variant (Aronson, 2019). According to the World Health Organization (WHO) the disease affects the poorest people and is associated with lack of financial resources, a weak immune system, linked to environmental damages such as deforestation and urbanization. Around of 1 million new cases and some 26000 to 65000 deaths occur annually (WHO, 2010).

According to the Epidemiological Report of the Americas of the Pan American Health Organization, only 49,1% of the cases progressed to clinical cure, 0.03% resulted in death and 50.7% of the progression is unknown or unspecified probably due to the serious adverse effects on patient's quality of life, as well as an important organic damage that requires close monitoring by a physician, generating a very low patient affinity, abandonment of the treatment and *Leishmania´*s growing resistance to pentavalent antimonials (Catta-Pretta, 2018). Additionally, the lack of an effective vaccine makes leishmaniasis a catastrophic disease that is difficult to eradicate.

Pentavalent antimonial (Sb^{V}) drugs have been used worldwide for over six decades with little evidence of resistance (Ponte-sucre et al., 2017). The 95% of untreated patients with cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL) respond to pentavalent antimonial, the recommended first-line treatment. However, in the last decade in some parts of

the world evidences significant drug resistance, increasing the treatment dose from 10 mg/kg (600 mg maximum) to 20mg/kg/day (850 mg maximum), for a regimen of 6 to 10 days to a regimen of 30 days according to the recommendations of World Health Organization (WHO) Expert Committee recommended (S. Croft, 2006).

The study of plants is widely practiced for the control of bacterial, fungal and parasitic infections, including leishmaniasis (Chavez, 2014). Several compounds have been isolated from plant extracts as leishmanicidal agents, almost all of them have not been studied further (REF). *Ocotea quixos* (Lam.) Kosterm. (family *Lauraceae*), known as ishpingo, and *Moringa oleifera* Lam (family *Moringaceae*) are best-known edible plants used for its medicinal, antibacterial, antifungal and antiprotozoal activities. With the high percentage of treatment failure and the increase of pentavalent drug resistance (Croft, 2006), the objective of this study is to evaluate the in-vitro potential leishmanicidal effect of these plant-extracts: *Ocotea quixos* ethanolic extract (OQEX), *Moringa oleifera* aqueous (MOAX) and *Moringa oleifera* ethanolic extract (MOEX). All tests were performed in triplicate and IC_{50} were calculated. A significant effect of the MOEX extract on promastigotes of *Leishmania mexicana* was observed in the screening tests, adjusted *p* value (<0,0001). In the dose-response assay, the IC_{50} for *L. mexicana* was 7.874 µg/mL (CI 0.8873 - 44.59) and the IC_{50} for the RAW macrophages was 12.95 µg/mL (CI 7.255 - 23.95). However, the selectivity index (SI) was 1.64. It is interesting to note that some biological activities of natural products can be evaluated against promastigotes of *Leishmania* spp. According to the literature, several compounds with potential leishmanicidad activity have been isolated from *M. oleifera* such as isothiocyanates and polyphenols (Tumer, 2015).

Material and methods

Plant material and extraction

Leaves of *Ocotea quixos* were collected from the Province of Morona Santiago, Canton Macas, Ecuador. The identification was made with vegetal species of *O. quixos* from Universidad Politécnica Salesiana herbarium. The leaves were dried and the 50:50 hydroalcoholic extract was obtained by percolation. The hydroalcoholic extract was lyophilized and 20,0 mg of material was solved in 1 mL of DMSO. The physicochemical characteristics of *Ocotea quixos* ethanolic extract are pH 6,605 ± 0,5429; density of 0,95 ±0,03 g/cm^3 and total solids of 1,9870 ± 0,7661 g. The main metabolites found in the phytochemical screening are polyphenols, tannis, catechins, quinones, alkaloids and coumarins.

Leaves of *Moringa oleifera* were donated from Ecuamoringa Guayas, Ecuador. The identification was made with vegetal species of *M. oleifera* from San Francisco de Quito University herbarium. The leaves were dried and ground. Soxhlet extraction with distilled water and methanol was performed. The aqueous and methanolic extracts were lyophilized and 20,0 mg of material of each extract was solved in 1 mL of DMSO and 100% ethanol respectively (Fig.1). The physicochemical characteristics of *Moringa oleifera* methanolic extract are pH 6,28; density 0,82 and suspended solids 0,000270889. The metabolites characterization; antioxidant activity and total phenols are being performed and will be reported shortly.

Collection of skin samples

Individuals with clinical and epidemiological suspicion of cutaneous leishmaniasis (CL) were provided by the Instituto Nacional en Salud Pública INSPI. For the diagnosis of CL we took punch- biopsy specimens by aspiration with 0,5 mL sterile Phosphate-buffered saline (PBS) in a sterile needle for parasites culture and dermal scrapings for microscopy diagnosis from (2 -4 mm) at the active border of the lesion. The parasites were isolated according to the CDC Practical Guide for Specimen Collection and Reference Diagnosis of Leishmaniasis. Then, we placed the sterile aspirate into USHMARU bifasic médium (15% rabbit defibrinated blood) supplemented with 18 ul of Gentamicin at a concentration of 40 mg / ml and PBS.

Finally, each 3 or 4 days the isolates were washed with Phosphate-buffered saline (PBS) and were stained with Giemsa for promastigotes microscopy identification.

DNA extraction

DNA was isolated using Cetyl Trimethyl Ammonium Bromide (CTAB) protocol. Cell pellet was incubated of each sample obtained by centrifugation (5 min / 10,000 rpm) with 700 uL of CTAB for 2 hours at 65 ° C. In order to obtain the rupture of the cell membranes, the samples were shaken every 15 minutes, then 700µl of chloroform: isoamyl alcohol (24: 1) were added. The samples were centrifugated for 5 minutes at 12000rpm. 2 phases were obtained, the lower or organic (chloroform / isoamyl), the upper or aqueous with the DNA suspension, and an interface (white) containing the cellular proteins. Then 500µl of the upper phase were transferred to a new reticulated Eppendorf tube, without touching the interface to avoid contamination with cellular proteins in the sample. 1000µl of concentrated ethanol was added and stored at -20 ° overnight. After 24 hours the samples were centrifugated at 13200rpm for 11 minutes. The supernatant was discarded carefully. The DNA pellet was washed by adding 1000µl of 70% ethanol. The sample was centrifuged at 13200rpm for 10 minutes and the supernatant was discarded. The DNA was quantified in NanoDrop equipment. Finally, 2 µL of DNA each sample were used to PCR amplification

PCR amplification

Polymerase chain reaction was performed to generate 120 (pb) HSP70 amplicon using forward primer (5′-CACTCCCCCTTCCTCTCAG-3’) and the reverse primer (5′ TTCCCTTCTGAGCCAATCAC-3′) (Nicolas, et al., 2002). The PCR protocol for amplification was: denaturation at 94 °C for 3 min; followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 30 s, extension at 72 °C for 30 s; and a final extension step of 10 min at 72 °C. Five microliters of DNA obtained from each clinical sample were used for the analysis on a 2% agarose gel to verify the presence and size of amplified product.. Negative

controls were always included, along with a positive one consisting of DNA from *L. panamensis*. The DNA obtained was sent to Macrogen for sequencing the HSP 70 fragment.

Culture and maintenance of the parasite

L. panamensis promastigotes were grown in Schneider's Drosophila Medium supplemented with 10% fetal bovine serum (FBS), 1% streptomicin (100mg/mL) and penicillin (100 U/mL) at 27°C. Promastigotes were washed with Phosphate-buffered saline (PBS) supplemented with 18 ul of Gentamicin at a concentration of 40 mg / mL and passaged each 3 or 4 days at fresh Schneider's Drosophila Medium.

Macrophage RAW 264.7 cell line

RAW 264.7 macrophage cell line were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 35°C in 5% of CO2 atmosphere for 72h. RAW Cell line were washed with DMEM (Dulbecco's Modified Eagle Medium) and passaged each 7 days at fresh DMEM Medium. This cell line was kindly donated by Dr. Ilya Raskin from Rutgers University, NJ, USA.

***Leishmania mexicana* promastigotes culture**

L. mexicana promastigotes culture and experiments were performed in the Biomedical Center of the UTE University. *L. mexicana* promastigotes were grown in biphasic medium (USHMARU) for one week, then was cultured in Schneider's Drosophila Medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 27°C. Promastigotes were washed with Phosphate-buffered saline (PBS) and passaged each 7 days at fresh Schneider's Drosophila Medium. The *L. mexicana* was kindly donated by Universidad Central del Ecuador.

Screening assay

Leishmanicidal activity*: L. mexicana* promastigotes were cultured in 25cm^2 flasks with Schneider's Drosophila Medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 27°C without CO_2. The samples were incubated in 5 to 6 mL of medium for 7 days. Promastigotes viability was assessed after addition of Trypan Blue (1:20) and counted in Neubauer chamber. The assay was performed in 96-well culture plates and each well contained 5 x 10^6 promastigotes with 200 µL of final volume. Triplicate conditions were performed. The assay was made with 1% DMSO and 1% ethanol final concentration as controls, 1 µM amphotericin B as positive control, OQEX, MOAX and MOEX at final concentration of 100 µg/mL and untreated control (Fig.2). After exposure to the extracts for 48 h in culture medium, 20 µL of MTT was added to each well. The plate was incubated at 27°C for 2h in darkness. Finally, 50 µL of DMSO were added in each well to solubilize the formazan and after 15 min the absorbance was measured at 570 nm and 630 nm of wavelength using a microplate reader, a BioTek Synergy HT spectrophotometer.

Cell viability*:* RAW 264.7 cell line were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 35°C in 5% of CO_2 atmosphere for 7 days. RAW 264.7 cell line viability was assessed after addition of Trypan Blue (1:1) and counted in a Neubauer chamber. The assay was performed in 96-well culture plates and each well contained 5 x 10^4 cells with 100 µL of final volume. Triplicate conditions were performed. The assay was made with 0,5% DMSO and 0,5% ethanol (final) as controls, 3% of saponin (final volume) as positive control, at final concentration of 100 µg/mL and untreated control (Fig.2). After exposure to the extracts for 48 h in culture medium, 10 µL of MTT was added to each well. The plate was incubated at 35°C in 5% of CO_2 atmosphere for 2h in darkness. Finally, 100 µL of DMSO were added in each well to solubilize the formazan and after 15 min the absorbance was measured at 570 nm

and 630 nm using a microplate reader a BioTek Synergy HT spectrophotometer. Data of viable parasites and cells were analyzed with the statistical software GraphPad Prism version 8.

Dose response assay

Leishmanicidal activity*: L. mexicana* promastigotes were cultured in 25 cm^2 flasks with Schneider's Drosophila Medium supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 27°C without CO_2. The samples were incubated in 5 to 6 mL of medium for 7 days. Promastigotes viability was assessed after addition of Trypan Blue (1:20) and counted in Neubauer chamber. The assay was performed in 96-well culture plates and each well contained 5 x 10^6 promastigotes with 200 µL of final volume. Triplicate conditions were performed. The assay was made with 1% DMSO and 1% ethanol (final volume) as controls, 1% Amphotericin B (final volume) as positive control, *Moringa oleifera ethanolic extract (MOEX) at* different concentrations: 100µg/mL; 10 µg/mL; 1 µg/mL; 0,1 µg/mL; 0,01 µg/mL and untreated control (Fig. 3). After exposure to the extracts for 48 h in culture medium, 20 µL of MTT was added to each well. The plate was incubated at 27°C for 2h in darkness. Finally, 50µL of DMSO were added in each well to solubilize the formazan and the plate was shaken for 15 min. The absorbance was measured at 570 nm and 630 nm using a microplate reader, a BioTek Synergy HT spectrophotometer.

Cell viability*:* RAW 264.7 cell line were grown in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS), 1% streptomycin (100mg/mL) and penicillin (100 U/mL) at 35°C in 5% of CO2 atmosphere for 7 days. RAW 264.7 cell line viability was assessed after addition of Trypan Blue (1:1) and counted in Neubauer chamber. The assay was performed in 96-well culture plates and each well contained 5 x 10^4 cells with 100 uL of final volume. Triplicate conditions were performed. The assay was made with 0,5% DMSO and 0,5% ethanol (final volume) as controls, 8% of saponin (final volume) as positive control, *Moringa oleifera ethanolic extract (MOEX) at different* concentrations: 100µg/mL; 10

µg/mL; 1 µg/mL; 0,1 µg/mL; 0,01 µg/mL and untreated control (Fig. 3). After exposure to the extracts for 48 h in culture medium, 10 µL of MTT was added to each well. The plate was incubated at 35°C in 5% of CO_2 atmosphere for 2h in darkness. Finally, 100µL of DMSO were added in each well to solubilize the formazan and after 15 min the absorbance was measured at 570 nm and 630 nm using a microplate reader, a BioTek Synergy HT spectrophotometer. IC_{50} of parasites and cells were analyzed with the statistical software GraphPad Prism 8.

Statistical analysis

Screening assay was performed in triplicate using Ordinary one-way ANOVA of multiple comparisons with Dunnett´s multiple comparisons test. The dose response assay was performed in triplicate and IC_{50} were calculated by non-linear regression: log(inhibitor) vs. response (three parameters) using the statistical software GraphPad Prism 8version?.

Results

PCR extraction and DNA sequencing

A fragment of 1,3 kpb was obtained of PCR extraction. Ten µL of DNA was send to DNA sequencing at Macrogen. Forward and reverse DNA was BLAST obtaining *L. panamensis/guyanensis* strain.

Screening assay results

Leishmanicidal activity: Leishmanicidal activity was evaluated by measuring promastigote mitochondrial activity using MTT colorimetric assay as described previously with three potential leishmanial activity plant-extracts. *O. quixos* ethanolic extract (OQEX), *M. oleifera* aqueous extract (MOAX) and *M. oleifera* ethanolic (MOEX), showed 4,2%, 10,2% and 78,6% of growth inhibition respectively at 100 µg/mL (Fig.4). For OQEX and MOAX no significant effect was found against *L. mexicana* promastigotes, however, MOEX exhibited remarkable leishmanicidal activity analyzed with Ordinary one-way ANOVA of multiple comparisons with Dunnett´s multiple comparisons test with adjusted *p* value (<0,0001).

Cytotoxicity: Cytotoxicity activity was evaluated by measuring mitochondrial activity using MTT colorimetric assay as described previously in Raw cell line with three potential leishmanial activity plant-extracts. *O. quixos* ethanolic extract (OQEX), *M. oleifera* aqueous extract (MOAX) and *M. oleifera* ethanolic (MOEX), exhibited 22,52%, 62,11% and 92,78 of growth inhibition respectively at 100 µg/mL (Fig.4.). No significant cytotoxic effect was observed in OQEX, while for MOAX and MOEX exhibited cytotoxic activity analyzed with Ordinary one-way ANOVA of multiple comparisons with Dunnett´s multiple comparisons test with adjusted *p* value (<0,0001).

Dose response assay results

In the dose-response assay the IC_{50} for *L. mexicana* was 7.87 µg/mL (95% CI 0.8873 - 44.59) and the IC_{50} for the RAW cells was 12.95 µg/mL (95% CI 7.255 - 23.95) (Fig.2). The selectivity index (SI) was 0,608 (Table. 1). This result shown no selective effect on leishmania and RAW macrophages cell line. The observed antileishmanial inhibition of these *Moringa oleifera* ethanolic extract (MOEX) is therefore considered as nonselective (Fig.5).

Conclusions and discussion

In this study, we analyzed the leishmanicidal effect of three medicinal plant-extracts: *Ocotea quixos* ethanolic extract (OQEX), *Moringa oleifera* aqueous (MOAX) and ethanolic extract (MOEX) against promastigotes of *Leishmania mexicana.* Finally, we analyzed a of MOEX dose-response leishmanicidal effect in order to elucidate this extract as a promising compound in the treatment of leishmaniasis.

Cutaneous leishmaniasis is a tropical neglected disease whose first and second-line treatment cause serious adverse effects, important organic damage, that requires close and prolonged monitoring by a physician generating low patient affinity and abandonment of the treatment (Ponte-sucre et al., 2017). Additionally, leishmania´s growing resistance to pentavalent antimonials, the absence of an effective vaccine, the rapid growth of urban

population, the adaptation of certain vectors to urban conditions, the movement of rural people and their domesticated animals is affecting the epidemiology of infectious diseases creating a favorable conditions for urban transmission (Neiderud, 2015). For these considerations today there are no effective therapies and we study three plant-extracts of two promising candidates as alternative drugs, but they have not been characterized.

The study was carried out in two stages: In the first screening trial, three extracts, OQEX, MOAX and MOEX were studied. In the second trial, we analyzed a dose-response effect of MOEX against *L. mexicana* promastigotes and Raw cells. Leishmanicidal activity against promastigotes of *L. mexicana* and the cytotoxic activity with Raw cell line were measured using MTT colorimetric assay. Our results exhibited that MOEX has a remarkable leishmanicidal and cytotoxic effect (Fig.4). However, once the dose response test was performed, the Selectivity Index (SI) obtained was 0,608. According to the literature an Selectivity Index (SI) < 10 for active extracts usually indicates that its activity is due to a general toxicity (Gachet et al., 2010). For instance, this result exhibited strong non-selective action against *L. mexicana* promastigotes and RAW cells.

The general toxicity of *M. oleifera* ethanolic extract may be due to the presence of direct and indirect antioxidant activity attributed a combination of polyphenols and Isothiocyanates (ITCs), antioxidants present in moringa leaves which were isolated by fast centrifugal partition chromatography (Tumer, 2015). Isothiocyanates are natural plant products and have been evaluated for their antimicrobial and anti-cancerous properties. ITCs exhibit their antimicrobial effect through hydrolysis products of the glucosinolate sinigrin, show a synergy with antibiotics and are used for food preservation and control of soil-borne disease. Additionally, exhibited effect on prokaryotic membranes, inhibition of enzymic or regulatory (quorum sensing, QS) activities, bacterial respiratory enzymes and induction of heat-shock and oxidative stress responses (Dufour, Stahl, & Baysse, 2015). Polyphenols are important antioxidants that are in

the most plant foods (Pulido, Bravo, & Saura-Calixto, 2000). Some studies attribute antimicrobial properties such as antibacterial and antifungal activity (Mosquera et al., 2017). Growth inhibition was demonstrated against *Staphylococcus epidermidis, Staphylococcus aureus, Escherichia coli, Candida albicans, Streptococcus pyogenes* and *Streptococcus mutans* (Noriega & Cesare, 2008).

The work here presented is an important approach to screening potential plant extracts which have application in the treatment of tropical diseases. The use of plant extracts represents an alternative of medicinal treatments. Natural products such as edible plants offer unlimited opportunities for new medicines. With the emergence of microbial resistance and adverse effects, research in the ethnopharmacology has grown. Standardization of extract methods and antimicrobial activity assay is necessary to assure the quality control for natural plant extracts. More studies will characterize the promising plant extracts

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TABLES AND FIGURES

Table 1. Dose response assay. *Half maximal inhibitory concentration (IC_{50})* and *Selective index of MOEX*

	IC_{50}	Confidence interval	SI
Leishmania mexicana	7,874 µg/mL	0,8873 - 44,59	
Raw cells line	12.95 µg/mL	7.255 - 23.95	
Selective index			0,608

Table. 1. In the dose-response assay the IC_{50} for *L. mexicana* was 7.87 µg/mL (95% CI 0.8873 - 44.59) and the IC_{50} for the RAW cells was 12.95 µg/mL (95% CI 7.255 - 23.95). The selectivity index (SI) was 0,608. This result shown no selective toxicity on leishmania and RAW macrophages cell line.

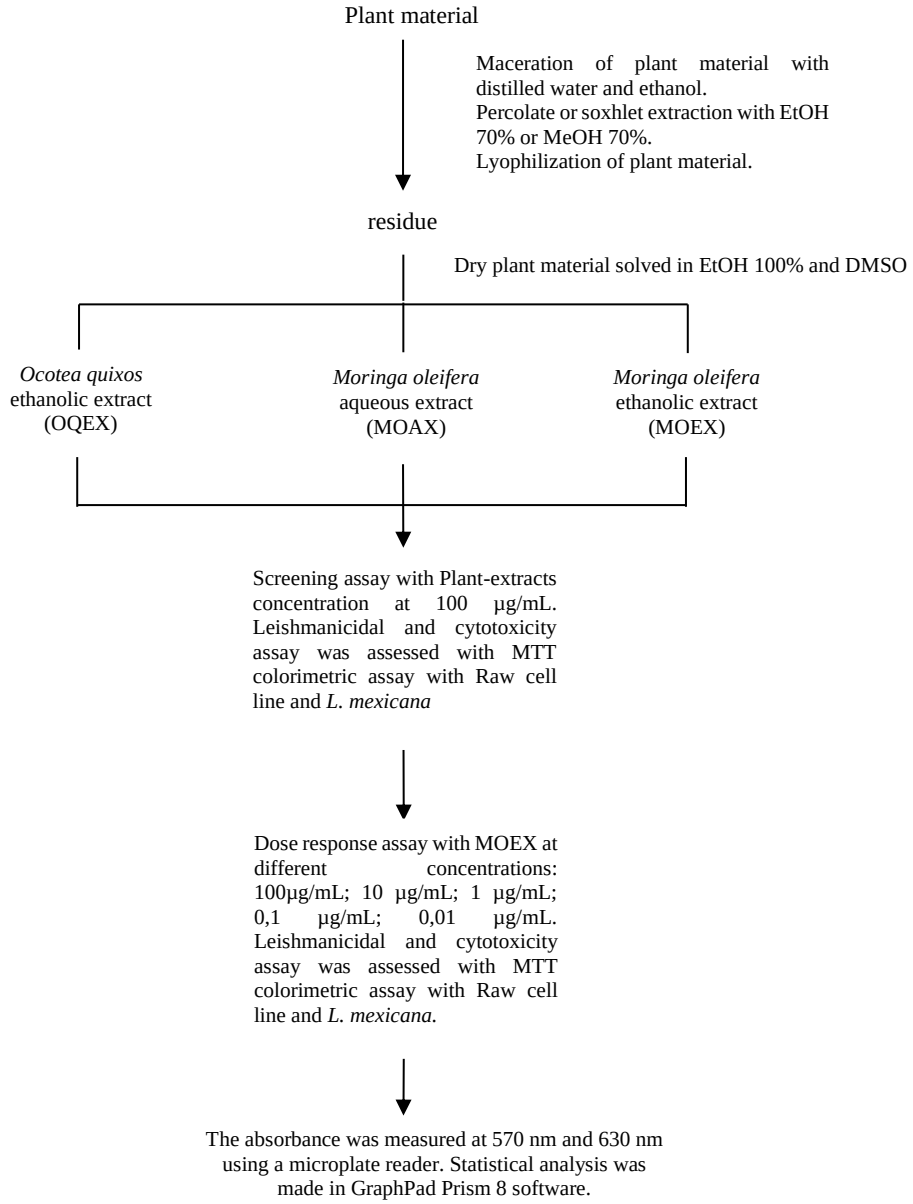
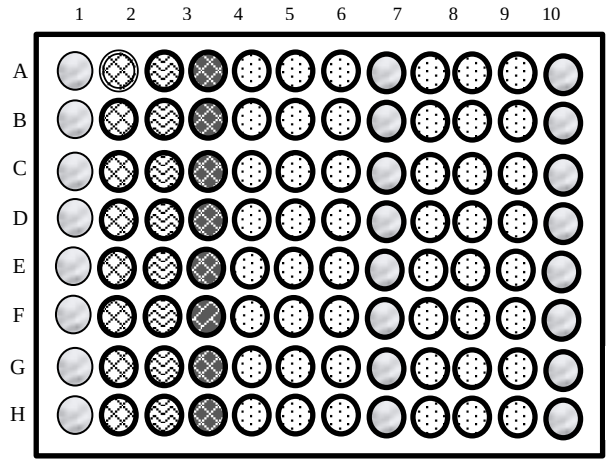
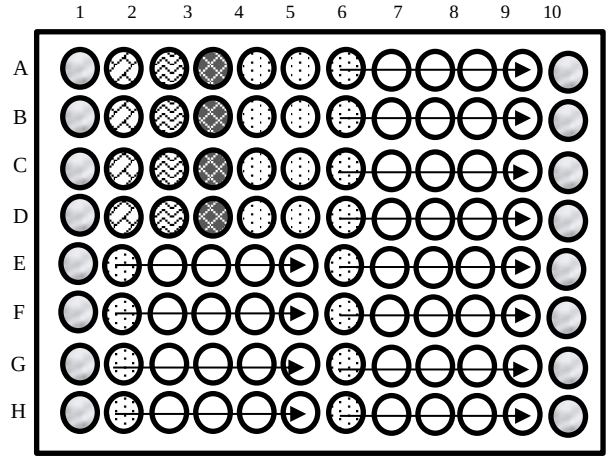


Figure. 1. Scheme of plant material extraction, analysis of screening and dose response assay of 3 plant-extracts: *Ocotea quixos* ethanolic extract (OQEX), *Moringa oleifera* aqueous extract (MOAX) and *Moringa oleifera* ethanolic (MOEX). MTT colorimetric assay was performed to evaluated cytotoxicity and leishmanicidal activity against Raw cell line and *Leishmania mexicana*. Finally, Inhibitory concentration IC_{50} and selective index (SI) was evaluated from *Moringa oleifera* ethanolic (MOEX). The absorbance was measured at 570 nm and 630 nm using a microplate reader. Statistical analysis was made in GraphPad Prism 8 software.



Template of screening assay with 3 plant-extracts OQEX, MOAX and MOEX.



Template of dose response assay of *Moringa oleifera* ethanolic extract (MOEX) with 5 serial dilutions scheme *(Series for dose range 100µg/mL – 0,01 µg/mL).*

Legend

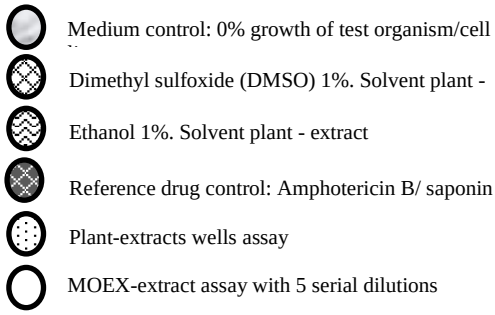
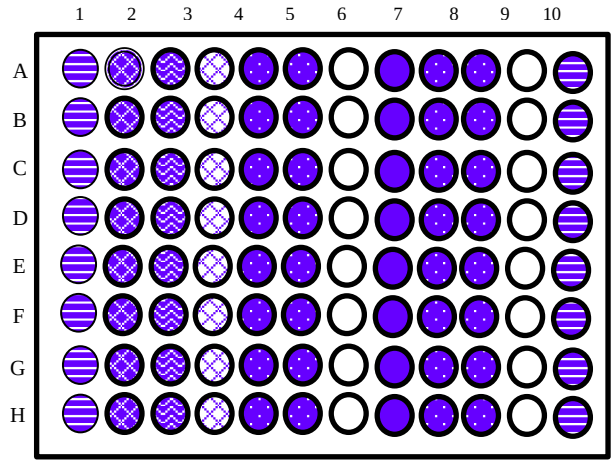
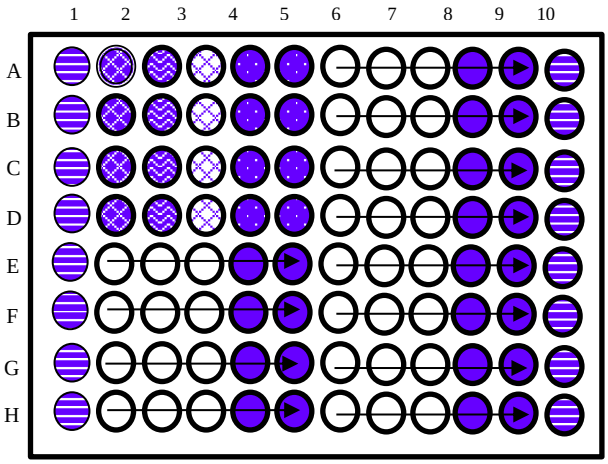


Figure. 2. Templates of screening and dose response assay of 3 plant-extracts: *Ocotea quixos* ethanolic extract (OQEX), *Moringa oleifera* aqueous extract (MOAX) and *Moringa oleifera* ethanolic extract (MOEX) against Raw cell line (Cytotoxicity assay) and *Leishmania mexicana* assay (leishmanicidal activity) (common 96-well microplate format for all assays, separate plates are used for replicate testing). The absorbance was measured at 570 nm and 630 nm using a microplate reader.



MTT colorimetric assay template of screening assay with 3 plant-extracts OQEX, MOAX and MOEX.



MTT colorimetric assay template of dose response assay of *M. oleifera* ethanolic extract (MOEX) with 5 serial dilutions scheme *(Series for dose range 100µg/mL – 0,01 µg/mL).*

Legend

- Medium control: 0% growth of test organism/cell
- Dimethyl sulfoxide (DMSO) 1%. Solvent plant -
- Ethanol 1%. Solvent plant - extract
- Reference drug control: Amphotericin B/ saponin
- Plant-extracts wells assay
- MOEX-extract wells with growth organism
- MOEX-extract wells with organism growth

Figure. 3. MTT colorimetric assay templates of screening and dose response assay of 3 plant-extracts: *O. quixos* ethanolic extract (OQEX), *M. oleifera* aqueous extract (MOAX) and *M. oleifera* ethanolic extract (MOEX) against Raw cell line (Cytotoxicity assay) and *Leishmania mexicana* assay (Leishmanicidal activity). MOEX dose response assay was made with 5 serial dilutions scheme (100µg/mL; 10 µg/mL; 1 µg/mL;0,1 µg/mL; 0,01 µg/mL). The absorbance was measured at 570 nm and 630 nm using a microplate reader. In purple, wells with organism growth. In white, growth organism inhibition. Inhibitory concentration IC_{50} and selective index (SI) was evaluated from (MOEX).

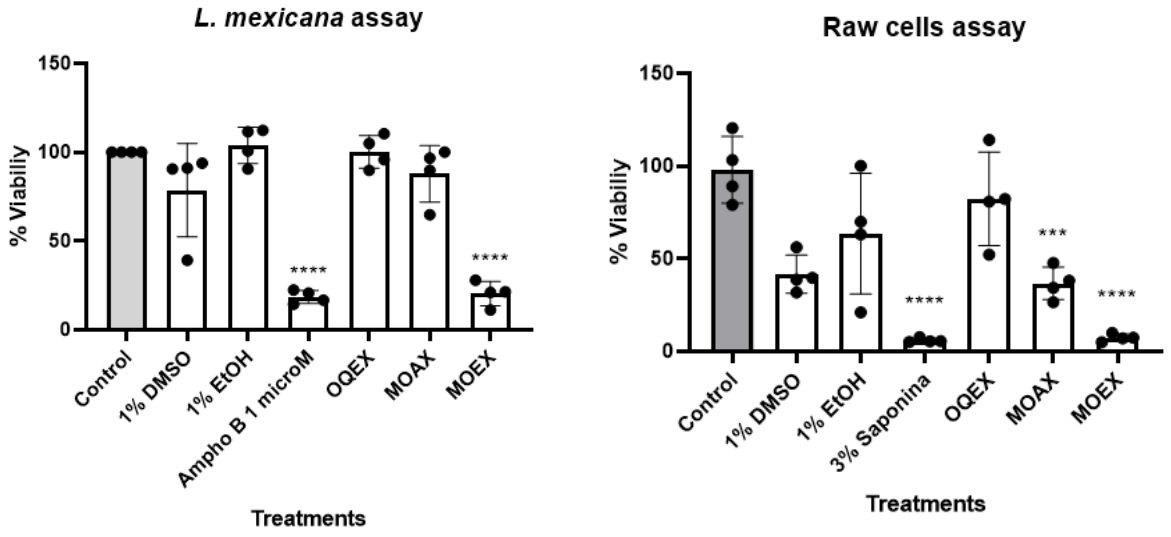


Figure. 4. Screening assay of 3 plant-extracts: *Ocotea quixos* ethanolic extract (OQEX), *Moringa oleifera* aqueous extract (MOAX) and *Moringa oleifera* ethanolic (MOEX) against Raw cell line (cytotoxicity assay) and *Leishmania mexicana* assay (leishmanicidal activity). A significant effect of the MOEX extract on promastigotes of *Leishmania mexicana* was observed in the screening tests, adjusted *p* value (<0,0001). In the dose-response assay the IC_{50} for *L. mexicana* was 7.87 µg/mL (95% CI 0.8873 - 44.59) and the IC50 for the RAW cells was 12.95 µg/mL (95% CI 7.255 - 23.95). The selectivity index (SI) was 1.64. This result shown no selective toxicity on leishmania and RAW macrophages cell line.

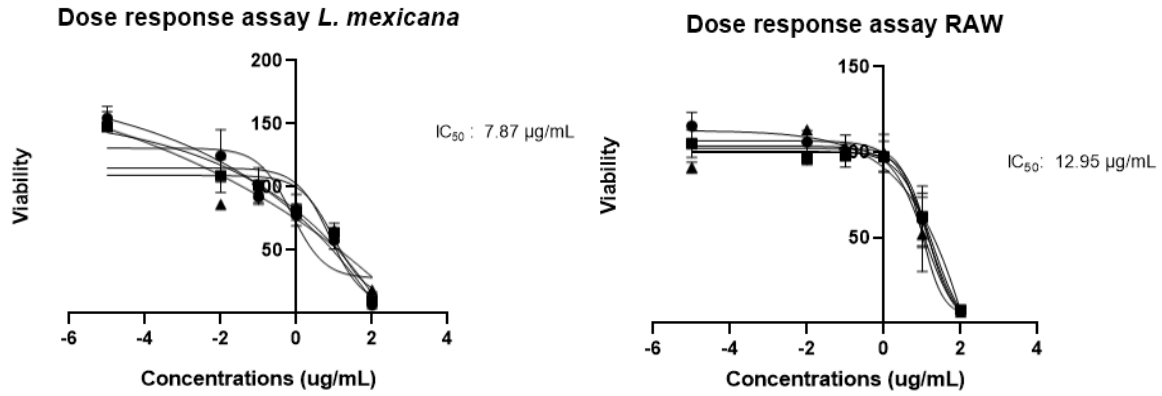


Figure. 5. Dose response assay of MOEX: *Moringa oleifera* ethanolic (MOEX) against Raw cell line (cytotoxicity assay) and *Leishmania mexicana* assay (leishmanicidal activity). IC_{50} for *L. mexicana* was 7.87 µg/mL (95% CI 0.8873 - 44.59) and the IC_{50} for the RAW cells was 12.95 µg/mL (95% CI 7.255 - 23.95). The selectivity index (SI) was 0,608. This result shown no selective toxicity on leishmania and RAW macrophages cell line.