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Hypotheses

Natural Substances as New Potential Strategies for the Treatment of Leishmaniosisin Dogs

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Abstract: Leishmaniasis is a disease caused by the protozoan parasites *Leishmania*, infecting numerous mammal species. Canine leishmaniasis is potentially zoonotic and causes severe fatal disease in dogs. The discovery of new natural products extracted from medicinal plants or compounds derived from them, such as quercetin, hesperidin, vitamin c, horse chestnut extract and selenium could represent a valuable source of new medicinal agents for treating leishmaniasis in dogs.

Keywords: Leishmaniasis, Dog, Quercetin, Hesperidin, Vitamin C, Horse Chestnut Extract, Selenium

Introduction

Leishmaniosisis a disease caused by more than 20 protozoan parasites of the genus *Leishmania.* These parasites are transmitted by the bite of phlebotomine sand flies and can infect numerous mammalian species, including humans. Leishmaniasis has a distribution ranging through America and Africa and extending into temperate regions of Latin America, Europe and Asia. The World Health Organization has identified human leishmaniasis as one of the most neglected tropical diseases. Visceral leishmaniasis is widespread in more than 80 countries and it is the most serious form, fatal in more than 95% of cases within 2 years after the onset of the disease. Some 90% of all new cases are reported from six countries: Brazil, Ethiopia, India, Somalia, South Sudan and Sudan (www.who.int/leishmaniasis/en/).

In contrast, pets are infected with different *Leishmania* species. *L. infantum* is the most widespread in domestic animals and dogs represent the main domestic zoonotic reservoir for visceral leishmaniasis. In addition, canine leishmaniasis (CanL) causes severe and often fatal disease in this animal species. Clinical diagnosis in cats of Feline Leishmaniasis syndrome (FeL) in endemic areas is not common. This may be due to their grooming habits that seem to minimize the risk for arthropod infection compared to dogs or the subclinical infection that occurs in most infected cats, or finally, because veterinary practitioners do not usually consider this disease in the list of differential diagnosis of their feline patients (Otranto *et al*., 2017). Horses and domestic equines suffer occasionally from single or multiple cutaneous lesions but probably they represent an incidental host of the disease (Gramiccia, 2011).

Clinical Signs in Canine Leishmaniasis

CanL is a severe disease induced by panosomatid species of the genus *Leishmania*. It is estimated that almost thirty species from the genus *Leishmania* can be responsible for this disease. *L. infantum* has been identified as a major, potentially fatal, zoonotic infection in regions of Europe, Africa, Asia and America (Gramiccia and Gradoni, 2005), although other species such as *L. chagasi, L. mexicana, L. braziliensis, L. donovani* or *L. amazonensis* are also potential etiologic agents (Lainson and Shaw, 2005; Alvar *et al*., 2004). Due to their close association with people, dogs are considered the main reservoir of human infection and phlebotomine sand flies are the biological vectors of this protozoal disease (Reguera *et al*., 2016; Baneth *et al*., 2008).

CanL results in several serious clinical signs in infected dogs. The parasite target cell is the macrophage, which becomes the site of parasite replication inhibiting its antimicrobial activities (Gramiccia, 2011). The majority of clinical dogs show poor body condition, generalized muscular atrophy, lymphadenomegaly, excessive skin scaling, decreased appetite and lethargy (Ciaramella *et al*., 1997; Koutinas *et al*., 1999; 2001; Baneth *et al*., 2008). Progressive muscle atrophy is associated with chronic polymyositis characterized by

the presence of mononuclear infiltrates with *Leishmania* amastigotes, neutrophilic vasculitis and IgG immune complexes in muscle tissues in conjunction with serum anti-myofiber antibodies (Vamvakidis *et al.,* 2000). The dermal symptoms include exfoliative, ulcerative, nodular and pustular dermatitis, alopecia and onychogryphosis; in addition, dogs may present with epistaxis, diarrhea and splenomegaly (Noli and Auxilia, 2014; Baneth *et al*., 2008). Other symptoms are the marked enlargement of lymph nodes caused by the increased number and size of lymphoid follicles and the marked hypertrophy and hyperplasia of medullary macrophages in the cords and sinuses (Lima *et al*., 2004). The splenomegaly is associated with increased monocyte and macrophage cellularity and changes in the microvasculature structure with abundant pulp venules and veins and increased reticular fibers (Alexandre-Pires *et al*., 2006). Renal disease can be the result of tubulointerstitial nephritis, glomerulonephritis and amyloidosis with a progression from asymptomatic proteinuria to nephrotic syndrome or chronic renal disease with azotemia (Koutinas *et al*., 1999). Joint and bone lesions are frequently present with periosteal and intra-medullary proliferative lesions and cortical and medullary osteolysis (Nieto *et al*., 1992; Agut, *et al*., 2003; Blavier *et al*., 2001). Ocular lesions are present in 16 to 80.5% of dogs and consist of anterior uveitis, conjunctivitis, dry keratoconjunctivitis and blepharitis (Ciaramella *et al*., 1997; Koutinas *et al*., 1999; 2001; Naranjo *et al*., 2005; Peña *et al*., 2000). Epistaxis, hematuria and hemorrhagic diarrhoea are associated with tissue ulceration and alterations in primary and secondary hemostasis. Hemostatic disorders include platelet aggregation abnormalities leading to platelet dysfunction, thrombocytopenia and decreased coagulation factor activities (Ciaramella *et al*., 2005). Profuse epistaxis can be the only presenting sign of disease and can cause death because of uncontrollable blood loss secondary to pyogranulomatous or lymphoplasmacytic rhinitis, thrombocytopathy and hyperglobulinemia (Juttner *et al*., 2001). Anemia is present in the majority of symptomatic dogs because of chronic renal disease or decreased erythropoiesis caused by chronic disease and can be aggravated by blood loss or immune mediated destruction of red blood cells (Ciaramella *et al*., 1997; Koutinas *et al*., 1999; 2001).

Current Therapies for the Treatment of CanL

Several drugs used for the treatment of CanL are able to improve clinical signs temporarily or cure dogs clinically, but none of these treatments reliably eliminates the infection (Miró *et al*., 2008). Nowadays, chemotherapy is the most efficient treatment for sick dogs and shows benefits in terms of reduction of prevalence and incidence of the disease, as well as in the control of pathogen's life cycle (Miró *et al*., 2011). Current chemotherapy is based on monotherapy or combination therapy of leishmanicidal and leishmaniostatic drugs. Disadvantages of these first-choice drugs are that they require parenteral delivery by veterinarian practitioners and the treatment is long and unpleasant for dogs and owners. Undesirable toxic side effects, particularly initiation or exacerbation of renal disease are not uncommon (Miró *et al*., 2008; Valladares *et al*., 1998). Furthermore, relapses are extremely frequent (up to 74%) in the first year post-treatment (Slappendel and Teske, 1997). Finally, development of drug-resistant strains of the parasite can be a consequence of drug misuse or can be due to host factors (modifications of pharmacokinetic parameters or an alteration in the immunological response to the parasite) and parasite factors (structural modifications of target proteins or mechanisms to evade the host immunological system) (Rhalem *et al*., 1999).

Prevention (or Preventive Measures)

Vaccines are an alternative to chemotherapy drugs. These stimulate the immune response with the aim of preventing infection and disease progression and blocking the parasite's life cycle. Many studies in animal models have shown that after inoculation of infective metacyclic promastigotes by the sand fly, skin dendritic cells are responsible for phagocytosis and antigen presentation to T cells. Afterwards, they migrate to the nearest lymph node to present the antigens to immature T cells and cause proliferation of CD4+ and CD8+ T cells. At this moment, a classical dichotomous cell-mediated response is triggered, this can be protective (Th1) or not protective (Th2), thus resulting in resistance or susceptibility to *Leishmania* infection. Vaccines can adequately boost the immunological system against a potential infection, thus avoiding the toxicity of chemotherapy and the emergence of resistant strains. However, prevention of CanL also involves reducing exposure to vectors using formulations with repellent/insecticide activity such us a large number of insecticide containing deltamethrin or combination of permethrin and neonicotinoid insecticide (Reguera *et al*., 2016).

New Alternatives from Natural Products

Extracts from medicinal plants or compounds derived from them are a valuable source of new medicinal agents for the treatment of leishmaniasis. There is a large range of families and species of plants with a potentially active leishmanicidal effect. The leishmanicidal effect may reside in phytochemical components such as flavonoids and, specifically, quercetin, which is a strong candidate in the combination therapy for the treatment of (Marìn *et al*., 2009)

Quercetin

Quercetin is a naturally occurring flavonoid isolated from *Fagopyrum esculentum (Polygonaceae)* (Polonio and Efferth, 2008). This compound shows potent anti-*Leishmania* activity *in vitro* on the amastigote stage of *L. donovani* and *L. amazonensis*. Most of the beneficial health effects of this flavonoid are attributed to its antioxidant and chelating activities, via scavenging of reactive oxygen species/metal chelation and stimulation/inhibition of enzyme activities/signal transduction pathways. Concerning its mechanism of action, quercetin is able to induce topoisomerase II-mediated kinetoplastid DNA minicircle cleavage in *L. donovani* promastigotes and intracellular amastigotes. The treatment of promastigotes with quercetin leads to cell cycle arrest in the G0/G1 phase followed by apoptotic cell death. Quercetin is a specific inhibitor of topoisomerase I, which is an unusual bi-subunit topoisomerase in *Leishmania*. Additionally, this compound can chelate iron, which translates into a decreased availability of the iron dependent ribonucleotide reductase, a rate limiting enzyme for DNA synthesis (Polonio and Efferth, 2008; Sen and Chatterjee, 2011; Sen *et al*., 2005; Fiorania *et al*., 2010; Sen *et al*.,2008; Da Silva *et al*., 2012).

In recent years, quercetin is becoming increasingly popular as a dietary supplement for pets. This compound has been suggested to have various beneficial effects like preventing cancer, suppressing inflammation or decreasing fat absorption and age-related diseases that are associated with increased oxidative stress (Reinboth *et al*., (2010).

In a study by Reinboth *et al*. (2010), a single administration of quercetin in dogs at the dose of 150 mg/subject (about 10 mg kg^{-1}) showed a maximum plasma concentration (C_{max}) of 234 nmol L^{-1} (equivalent to 0.77 µg mL^{-1}) and a very low bioavailability (4%). These results are in agreement with different studies present in literature on other animal species. Indeed, in rats and pigs the absolute bioavailability of quercetin was only 5 and 1%, respectively (Wessmann *et al*., 2000; Chen *et al*., 2005). Due to the low bioavailability of quercetin after oral administration in different animal species, a study performed by Medalpharma sas industry in 2012 (internal data) investigated the pharmacokinetics of quercetin in dogs after a single administration at 150 mg kg^{-1} b.w. No visible side effects were observed immediately after the single administration or over the subsequent seven days. The C_{max} of quercetin after a single dose (150 mg kg^{-1} b.w.) was 11 µg mL^{-1}. Findings of this study suggest that, if compared with a previous study in dogs (Reinboth *et al*., 2010; dose: 10 mg kg^{-1}; C_{max}: 0.77 µg mL^{-1}), quercetin is expected to have linear first-order kinetics in dogs for doses ranging from 10 to 150 mg kg^{-1}. Unfortunately, no pharmacokinetic/pharmacodynamic studies are available concerning a clinical dose of quercetin for the treatment of CanL.

The IC 50 (50% inhibition concentration of cell growth) value against *L. donovani* and *L. amazonensis* intracellular amastigotes is 1.0 µg mL^{-1}. If this value is multiplied for 24 h, a value of $AUC_{0\text{-}24h}$ of 24 µg*h mL^{-1} is calculated. This parameter represents the theoretical value to maintain the plasma concentration of quercetin above the IC 50 for 24 h. Bearing in mind the dose proportional feature of quercetin and tha ta dose of 150 mg kg^{-1} showed a value of $AUC_{0\text{-}24h}$: 129 µg*h mL^{-1} (Medalpharma sas, internal data, 2012), it can be calculated that a dose of 28 mg kg^{-1} produces an $AUC_{0\text{-}24h}$ 24 µg*h mL^{-1} maintaining the plasma concentration of quercetin above the IC 50 for one day.

Hesperidin

Hesperidin is an abundant flavonoid produced by citrus cultivations and it is the major product of oranges and lemons. It is usually administered in association with vitamin C to increase the efficacy of the compound and it has been shown to possess an excellent safety profile (Garg *et al*., 2001).

Due to its broad spectrum of activities, hesperidin is effectively used as a supplemental agent in numerous treatment programmes. One of the main effect of hesperidin is on the vascular system. Hesperidin supplementation has been used in patients suffering with blood vessel disorders including those involving abnormal permeability and fragility of capillary walls (resulting in oedema, bleeding and hypertension) (Garg *et al*., 2001). The role of hesperidin in increasing capillary resistance has been attributed to its inhibitory effect on the action of hyaluronidase enzyme that accentuate capillary permeability and fragility. In a study on human patients, hesperidin inhibited epinephrine and ADP-induced platelet aggregation at a concentration of 0.08 mg mL^{-1}. In another study on horses, hesperidin was found to effectively reduce aggregation of erythrocytes. This decrease in erythrocyte aggregation may explain the beneficial effects of hesperidin on the abnormal permeability and fragility of capillary walls. Hesperidin has been shown to possess significant anti-inflammatory and analgesic effects. This effect could be attributed to the inhibition of the release of histamine from basophils by hesperidin or its metabolic products. This compound showed anti-infective and anti-replicative activities *in vitro* against several plant and animal microbes (Garg *et al*., 2001). Finally, hesperidin has been reported to possess antioxidant and immuno-suppressant properties. Indeed, after intragastric administration of hesperidin at the dose of 50 mg kg^{-1}, this compound suppressed bacterial alpha-amylase antibody production in mice and significantly increased the development of immunological memory in the cellular immune response (Kim and Cho, 1991).

Concerning the pharmacokinetics of hesperidin, in a study by Ameer *et al*. (1996), the authors evaluated the oral absorption of hesperidin in healthy young human volunteers at the dose of 500 mg/subject (about 7 mg kg^{-1}) showing a low bioavailability (about 25%). Furthermore, a study performed by Medalpharma sas industry in 2012 (internal data), reported the pharmacokinetics of hesperidin in dogs after a single administration at the dose of 150 mg kg^{-1} b.w. No visible side effects were observed immediately after the single administration or over the subsequent seven days. The C_{max} of hesperidin after a single dose (150 mg kg^{-1} b.w.) was 7 μg mL^{-1}.

No pharmacokinetic/pharmacodynamic studies are available concerning the clinical dose of hesperidin for the treatment of CanL. From the studies reported above it can be summarized that doses from 7 to 50 mg kg^{-1} might produce suitable plasma concentrations of hesperidin to effectively treat CanL, while higher doses up to 150 mg kg^{-1} were shown not to evoke appreciable adverse effects.

Vitamin C

Leishmaniosis is accompanied by severe anemia and shortened lifespan of erythrocytes. Several lines of evidence suggest an important role of enhanced lipid peroxidation in the pathogenesis of hemolytic anemia. In healthy animals the cells invest much of their metabolic activity in the reductive processes that combat the threat of oxidation. Glutathione (GSH), with glutathione redox cycle in the cell, is involved in several defence processes against oxidative damage. Obviously, a deficiency of these antioxidants increases cells' susceptibility to oxidative damage.

Vitamin C (ascorbic acid) is a powerful antioxidant that reacts rapidly with a variety of oxidants, including the rather poorly reactive superoxide anion radical (Bildik *et al*., 2004). It is transported into erythrocytes as dehydroascorbic acid, which is then reduced to ascorbate via a GSH-dependent reaction.

A study by Field and Rekers, (1949) showed that supplementary treatment with vitamin C in dogs (100 mg orally three times daily for 1 week) might potentiate the anti-hemorrhagic action of the flavanones quercetin. Furthermore, vitamin C is usually administered in association with another flavonoid hesperidin to increase the efficacy of the final compound, this combination has been shown to be extremely safe and without side effects (Garg *et al*., 2001).

In light of the features mentioned above, vitamin C might be a potential new natural compound for the treatment some clinical signs of CanL.

Horse Chestnut Extract

The active compound β-aescin is the main saponin isolated from the seeds of the horse-chestnut *Aesculus hippocastanumi* (Sapindaceae). A study of Vermelho *et al*. (2014) showed a β-aesc in value equal to 1.04±0.23 ug mL^{-1} for *L. infantum* amastigotes and the Horse Chestnut Extract (HCE) contains 70% esc (Guillaume and Padioleau, 1994).

Concerning its mechanism of action, HCE showed antiradical activity in both *in vitro* and *in vivo* studies inhibiting both enzymatic and non-enzymatic *in vitro* lipid peroxidation (in the concentration range 5×10^{-6} to 5×10^{-4} g mL^{-1}). Furthermore, additional studies have shown the veinotonic and lymphagogue properties of HCE at different dosages. Indeed, HCE dose dependently contracts the canine saphenous isolated vein for more than 5 h. In the perfused canine saphenous vein, HCE (25-50 mg in bolus) increases the venous pressure of the normal vein and the pathological vein by increasing the contractile response to noradrenaline. During the perfusion in the inverse direction of the blood flow, a clear contracting effect on the valves is also noted with HCE. In the anaesthetized dog, HCE in situ improves the femoral vein compliance and opposes the venous distension obtained during clamping in a carotido-femoral perfusion with constant flow. HCE significantly increases femoral venous pressure and flow, together with thoracic lymphatic flow, while respecting the arterial parameters (2.5 and 5 mg kg^{-1} intravenously). In regards to the vasculotropic action, HCE dose dependently diminishes the cutaneous capillary hyper permeability induced either by injections of inflammatory agents such as histamine and serotonin in the rat (100 to 400 mg kg^{-1} orally), or by an irritating agent like chloroform applied in the rabbit (50 to 300 mg kg^{-1} orally and 2.5 to 5 mg kg^{-1} intravenously). It significantly increases the vascular resistance in guinea pigs fed a scorbutigenic diet as measured by the petechia method (50 to 400 mg kg^{-1} orally). Regarding anti-edema and anti-inflammatory activities, HCE decreases the formation of edema induced in the rat's hind paw, one of lymphatic origin, the other of inflammatory origin (200 to 400 mg kg^{-1} orally). In an experimental model of pleurisy in rats, HCE suppresses plasmatic extravasation and leucocytes emigration into the pleural cavity (200 to 400 mg kg^{-1} oraly; 1 to 10 mg kg^{-1} intravenously). Finally, HCE decreases connective tissue formation in the subchronic, inflammatory granuloma model in the rat (400 mg kg^{-1} orally and 5-10 mg kg^{-1} subcutaneously) (Guillaume and Padioleau, 1994).

Due to its broad mechanism of action, HCE might be considered a suitable supplementary therapy for the treatment of the main clinical signs of CanL.

Selenium

The final compound considered in the present work is selenium, a micronutrient element with broad functions in biological systems. Selenium ions (SeO_2 is the chemical form of selenium) have antioxidant, cancer preventing and antiviral activities and also appear to

improve the immune response of hosts against various species of bacteria and viral antigens (Beheshti *et al*., 2013; Rayman, 2012; Soflaei *et al*., 2014; Tapiero *et al*., 2003). The IC_{50} of selenium for *L. infantum* is 50 µg mL^{-1} (Soflaei *et al*., 2014).

Considering selenium activities and its IC_{50} value, this compound might be used against leishmaniasis with dose-dependent anti-leishmanial activities.

Conclusion

*Leishmaniosis*is a neglected, potentially lethal infectious disease for human and pets caused by parasites of the genus *Leishmania* that affects many developing countries.

The treatment of leishmaniosis in pets is challenging and lengthy. Therapeutic problems include the toxicity of available drugs and increasing drug resistance. Leishmaniosis has received scarce attention from governments and the pharmaceutical industries until the past decade.

The development of new parasite targets and synthetic drugs along with research on natural products such as quercetin, hesperidin, vitamin C, horse chestnut extract and selenium might represent a possible strategy for the discovery of new therapeutic agents against *Leishmania*. Although further studies are required to determine if these natural products can be used in the same formulation, these preliminary findings pave the road for their use as a supportive treatment for leishmaniosis in dogs.

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Author's Contributions

De Vito Virginia and Giorgi Mario: Contributed to conception of the study, analysis of the literature and wrote the manuscript.

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