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Molecular evaluation of the efficacy of fungal treatment combined with conventional therapies in treating canine leishmaniasis

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ABSTRACT:

— ***Objective:*** Fungal extracts added to conventional treatment for *Leishmania infantum* determine the disappearance of *Leishmania* DNA in fungi-treated dogs. The aim of the present study was to monitor the *Leishmania* load in blood tissue from naturally infected dogs before and after anti-leishmanial therapy associated with fungal extracts.

— ***Materials and Methods:*** A cohort of dogs naturally infected with *Leishmania infantum* were divided into two groups: the control group treated with meglumine stibiate and allopurinol, and the study group who were additionally treated with a mix of fungal extracts (Shiitake 125 mg, Reishi 62.5 mg, Cordyceps 62.5 mg, at the dosage of 34 mg of the extract mixture per kilogram of body weight). We then monitored the parasite presence and blood parameters at different time points.

— ***Results:*** Polymerase Chain Reaction (PCR) analysis revealed that *Leishmania infantum* DNA present at the beginning of the study was no longer detectable for all the fungi-treated dogs, and cyto-histological analyses confirmed the absence of *Leishmania* amastigotes in fungi-treated dogs.

— ***Conclusions:*** The combination of allopurinol, meglumine stibiate, and fungal extracts demonstrates a negative bone marrow PCR and clear bone marrow smears, a result that persists for over a year. These promising results motivate further exploration of fungal extracts as cost-effective and low-toxicity adjuncts in anti-*Leishmania* treatment strategies.

— ***Keywords:*** Fungal treatment, Dogs, *Leishmania infantum,* DNA, PCR, Tropical disease, Parasites, Leishmaniasis.

— ***List of Abbreviations:*** Canine leishmaniasis (CanL), PCR (Polymerase Chain Reaction), canine visceral leishmaniasis (CVL), visceral leishmaniasis (VL), Dehydrated Extract (DE), kinetoplast DNA (kDNA), Acute Phase Proteins (APPs), natural IgM antibodies (IgMn), nitric oxide (NO), Reactive Oxygen Species (ROS), 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR).

INTRODUCTION

Leishmaniasis is a vector-borne disease which can infect both humans and other mammals. It is a heterogeneous disease because, in dependence on the species and host characteristics, clinical manifestations go from asymptomatic infections to cutaneous lesions (cutaneous leishmaniasis), mucosal lesions (mucocu-

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taneous leishmaniasis) or visceral lesions (visceral leishmaniasis)[1].

Canine leishmaniasis (CanL) is a severe infection caused by the protozoan parasite *Leishmania infantum* (syn. *L. chagasi*). Dogs acquire the parasite through the bite of infected phlebotomine sandflies, which are considered the main tanks of infection in the domestic transmission cycle. The promastigote stage of the parasite infects macrophages and monocytes; these then transform into amastigotes, which multiply within the cells and withstand the body's immune response, leading to the disease[2]. The parasite *Leishmania infantum* has been recognized as the highest etiologic agent of canine visceral leishmaniasis, (CVL). Several organs can be affected during the progression of the disease, usually the skin (dermatitis, alopecia, onychogryphosis), lymphoid organs (splenomegaly, adenomegaly), and kidneys (renal disease)[3]. Zoonotic visceral leishmaniasis (VL), represents a serious public health problem in many regions where it is endemic (the Mediterranean basin, Latin America, and parts of central and eastern Asia) for its significant mortality and morbidity rate in the reservoir host. Particularly in Italy, CanL is endemic in northern regions, with a seroprevalence of 21.6%, 29.6% in central regions, and 28.2% in southern regions and islands[4]. However, a portion of the canine population in *Leishmania*-endemic areas does not show symptoms. Diagnosis is based on the set of clinical signs presented by the dog, but since many asymptomatic dogs have not pathognomonic clinical signs, laboratory diagnosis confirmation is needed. Diagnosis of CanL can be executed by direct identification of the parasite through the molecular detection of its DNA, or detection of *Leishmania*-specific antibodies[5].

The standard treatment protocol for dogs includes the combinatorial administration of meglumine and allopurinol[6]; however, long-term treatment is needed, often causing toxicity and side effects that lead to interruption[7]. Moreover, the treatment can only decrease the parasite load without proper elimination, resulting in a re-increase post-therapy and thus possibly contributing to the spreading of Canine Leishmaniasis[8].

Fungi have been used in Asian traditional medicine for a thousand years to address a variety of health problems, from enhancing immunity to promoting longevity[7]. In modern science, fungi are confirmed to be precious allies for human and animal health, having proved to be able to perform antiviral and antimicrobial activities[9], exhibiting hypocholesterolemic and hypoglycemic properties[7], and even being evaluated as potential anticancer drugs[10]. Furthermore, there's a growing interest in their use as immunomodulators for nutraceutical purposes in both humans and pets. For example, a nutritional supplement, which included *Ophiocordyceps sinensis*, was used in dogs naturally infected with CanL, with no side effects observed and reported disease markers improvement[11,12]. *Ganoderma lucidum*, also known as Reishi, has shown significant *in vitro* and *in vivo* pharmacological activity. It was safely used to address a variety of conditions like inflammation, depression, anxiety, bone disorders, cancer, epilepsy, diabetes, cardiovascular issues, and immune disorders. Its use in food, medicine, and cosmetics is increasing, as shown by a rise in clinical trials, patents, and its inclusion in numerous supplements and cosmetic products products[13]. Shiitake mushrooms (*Lentinus edodes*) are among the richest in beta-glucans, such as deta-1,3-D-glucans and beta-1,6-D-glucans. These are biologically active polysaccharides that have been observed to enhance the immune response by increasing Th1 lymphocytes and are currently an object of study for possible anticarcinogenic activities[14]. Moreover, an *in-vitro* study by Pineda-Alegria et al[15] evidenced *L. edodes* anthelmintic activity against both larvae and eggs of the helminth *Haemonchus contortus*.

Given these premises, the aim of the present study was to monitor the *Leishmania* load in blood tissue from naturally infected dogs before and after anti-leishmanial therapy associated with fungal extracts, with the goal of finding an economically sustainable remedy, having high activity and possibly low toxicity.

MATERIALS AND METHODS

Enrolment Criteria

The study included 40 sick dogs of different ages and breeds living in apartments selected according to specific criteria: *Leishmania spp* DNA in the hematopoietic marrow identified through the qPCR method and blood alterations of ferritin and gamma globulins. Ferritin had to be above 250 ng/ml, and gamma globulins had to be above 1 g/dl. Exclusionary features were severe renal impairment with creatinine greater than 2 mg/dl and severe hepatic impairment with alanine transaminase (ALT) greater than 100 IU/L[16].

Study Design

In this exploratory study, dogs were allocated to either the control group or the fungal-treatment group based on owner consent and according to the availability of clinical cases rather than through a strict randomization protocol, leading to different group sizes. Although it was not feasible to blind the field staff responsible for administering treatments and collecting samples, the laboratory personnel who conducted the PCR analyses and histopathological evaluations were not informed of each dog's treatment status. Due to scheduling constraints and owner availability, dogs in the control group were followed for a shorter period (1-3 months), whereas the treatment group had a follow-up extending to six months or more.

Treatment

With this study, we evaluated the different effectiveness of two therapeutic protocols applied to

two groups of sick dogs. While treatments were primarily administered by owners, regular follow-ups were conducted through phone calls or clinic visits. Additionally, owners received detailed instructions, and medication logs were utilized to track adherence as effectively as possible. The first group, composed of 9 dogs (control group), received the standard treatment as per the guidelines[6]. The treatment is based on meglumine stibiate (Glucantime®, Sanofi Italia, Milan, Italy) subcutaneously for 28 days, reaching a maximum administration of meglumine stibiate equal to 2,800 mg per kilo of body weight in the 28 days of administration. Allopurinol was associated with meglumine stibiate at a dosage of 20 mg per kilo of body weight divided into two doses per day for a total of 6 months. The second group of 31 dogs (study group) was administered 1,690 mg of meglumine stibiate over 28 days of therapy, associated with fungal extracts, thus composed of Shiitake Dehydrated Extract (DE) 125 mg [*Lentinula edodes* (Berk.) Pegler, fungal body], Reishi 62.5 mg D.E. tit. 50% polysaccharides [*Ganoderma lucidum* (Curtis) P., fungal fruiting body] with alpha-glucans, Cordyceps 62.5 mg D.E. tit. 7% cordyceptic acid [*Ophiocordyceps sinensis* (Berk.) Sacc., sporophore, powder], at a dosage of 34 mg total of the extract mixture per kilo of body weight, once a day in the evening, 3 hours after the last meal. Patients in both groups were monitored through general physical examination and complete biochemical tests at time 0 (before the start of therapy) and at the end of 28 days. In both groups, there were no reports of discomfort that would exclude them from treatment. At the end of the 28 days of therapy, both groups underwent a complete examination and blood tests.

DNA Extraction

Genomic DNA was extracted from the defrosted samples of hematopoietic marrow from -80°C using a commercial Speedtools DNA extraction kit (Biotools B&M Labs S.A, Madrid, Spain). DNA extracted was eluted in elution buffer (100 µL) and stored at -20°C. qPCR methods were performed for molecular evaluation, amplifying specific sequences of the minicircle kinetoplast DNA (kDNA). The Sybr Green (Merck KGaA, Darmstadt, Germany) based qPCR assay (Sybr-qPCR) was designed to be amplified by the fluorochrome known as Sybr Green and was performed according to previously described methods[16,17].

Preparation of Cyto-Histological Samples

Bone marrow smears were prepared using the May-Grünwald Giemsa Kit from Liofilchem S.r.l. (Roseto degli Abruzzi, Italy). The kit contains the May-Grünwald methanol solution of 0.25% (w/v) eosin methylene blue and the Giemsa buffered methanol solution of 0.4% (w/v) azure eosin methylene blue. The staining process was performed following the manufacturer's instructions.

Statistical Analysis

A unified database from the dogs' blood test results was prepared using Python v. 3.11.0 (https://www.python.org/) and its library Pandas[18]. Statistic tests and data visualization were performed using Python's libraries SciPy[19], numpy[20], and Seaborn[21]. The normality of each blood parameter was assessed using the Shapiro-Wilk test, setting the threshold for normality as $\alpha = 0.05$. Having an alpha level below 0.05 level for all the blood indicators, the non-parametric Mann-Whitney U Test was used to assess the statistically significant difference between before and after the cure and between the dogs who received the standard therapy and those who were administered the fungal extracts.

RESULTS

Assessing the Efficacy of Fungal Treatment in Eradicating Leishmania in the Bone Marrow

We started our investigation with a PCR analysis of bone marrow samples from the cohort of dogs aimed at assessing the presence of *Leishmania* parasites. This analysis was conducted before the beginning of the fungi treatment and at several time points post-treatment for the fungi-treated group and after at least one month for the control group. Every dog in the fungal-treated group was tested at least twice. As displayed in Figure 1, the data revealed that, regardless of the number of copy numbers of *Leishmania infantum* DNA present at the beginning of the study, the DNA was no longer detectable for all the fungi-treated dogs, and when tested multiple times, this result was persistent over time. On the contrary, in the second PCR of the dogs that were not treated with fungal extracts, the analysis still displayed the presence of *Leishmania* DNA. However, it is worth noticing that it was performed only one to three months after the treatment.

Optical Microscopy Analysis of Bone Marrow Smears

Cyto-histological analyses were performed simultaneously with the PCR to further confirm the presence or absence of *Leishmania* infection upon enrolment, after fungal treatment, and in the control group. The staining results shown in Figure 2 validate the presence of Leishmania amastigotes. Chronic antigen stimulation signatures were evident at the beginning of the study and in the control group, while the absence of *Leishmania* amastigotes became apparent following fungal treatment.

Comparative Assessment of Blood Parameters

In addition to PCR and cyto-histological analyses, we conducted a statistical evaluation of various blood parameters across four groups: before fugal treatment, after fungal treatment, and in the control group

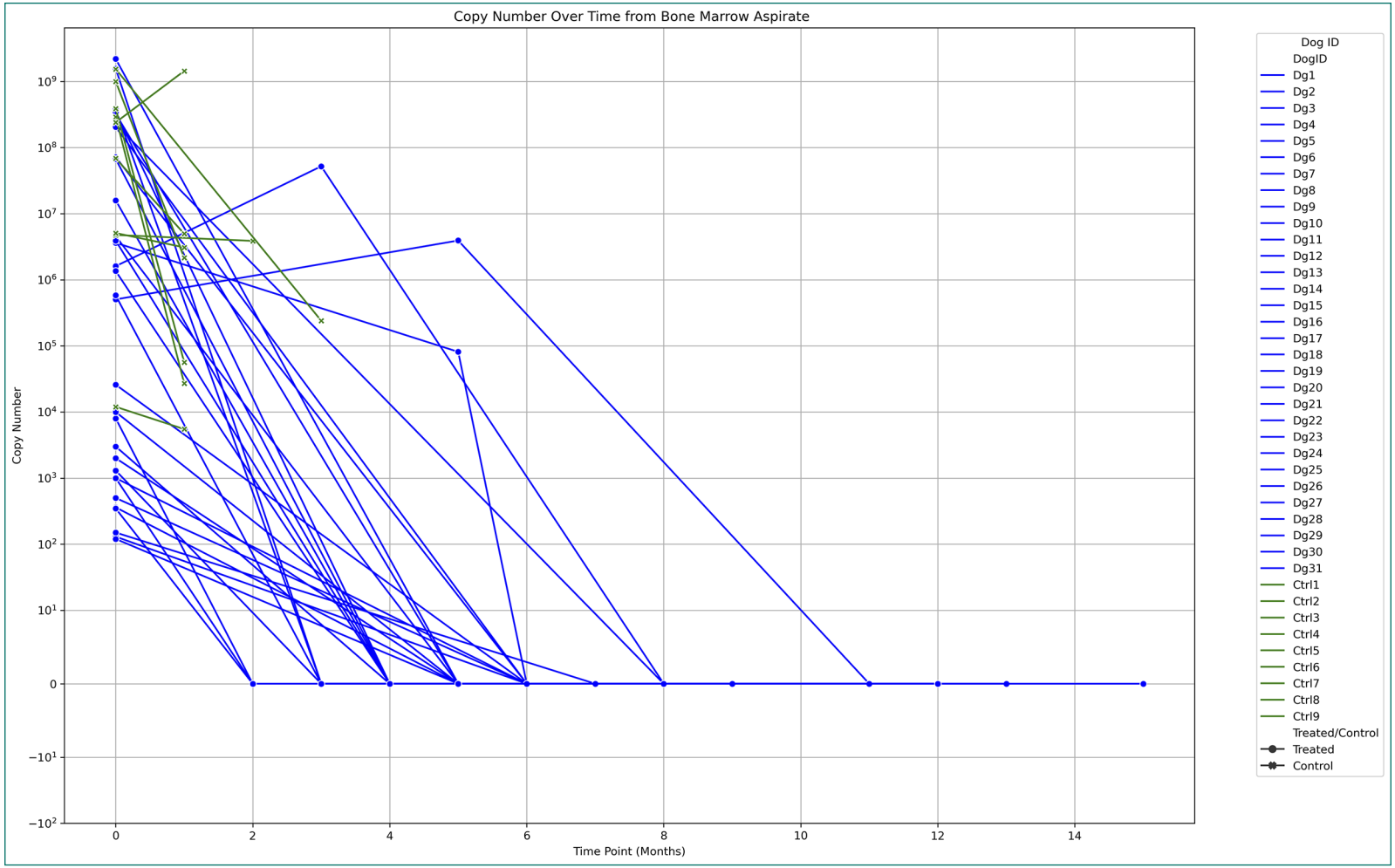

Figure 1. Timeline of *Leishmania infantum* DNA detection from bone marrow aspirate. For each dog in the study group, samples were analyzed at at least two and up to three distinct time points. After 11 months, all dogs that received fungal treatment tested negative for *Leishmania infantum* DNA in the bone marrow. Notably, once testing negative, no dog in the fungi-treated group exhibited a recurrence of *Leishmania* DNA presence.

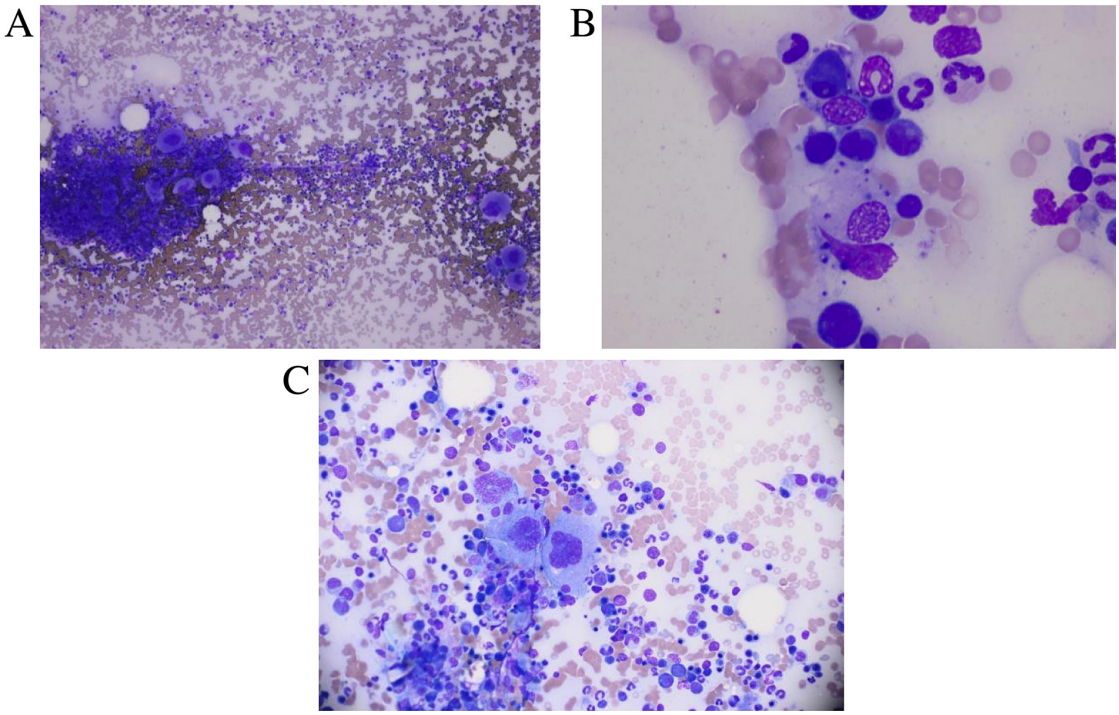

Figure 2. Bone marrow smears using May-Grünwald and Giemsa stain. White blood cell nuclei are stained blue, while their cytoplasm appears in a lighter blue shade. Basophils are identifiable by their dark blue-black granules within the cytoplasm. Eosinophils are distinguished by their bright orange granules in the cytoplasm. Red blood cells are stained red. Images were acquired at approximately 20x magnification for Figure 2A, 100x for Figure 2B and 40x for Figure 2C. **A-B**, show *Leishmania*-positive samples displaying a chronic antigen stimulation. **C**, illustrates a sample taken after fungal treatment in which normal bone marrow was resumed.

Table 1. Blood parameters tested.

Parameter	Unit	Reference value (min.)	Reference value (max.)
Albumin	%	53.8	64.7
Albumin + globulins[a]	g/dL	2.9	3.7
Alanine transaminase (ALT)	IU/L	0	50
Haptoglobin	mg/dL	10	135
Aspartate transaminase (AST)	IU/L	0	50
Albumin	g/dL	3	3.5
Calcium[a] (Ca)	mg/dL	9.6	11.1
Creatinine	mg/dL	0	1
Eosinophils	units/µL	26	730
Iron (Fe)	µg/dL	84	212
Beta globulins	%	5.9	9.5
Beta + gamma globulins	g/dL	0	3.5
Gamma globulins	g/dL	0	1
Potassium[a] (K)	mEq/L	3.9	5
Lymphocytes	units/µL	942	2,428
DGGR lipase[a] (29-143)	IU/L	29	143
Mean Corpuscular Volume (MCV)	%	64	73
Sodium[a] (Na)	mEq/L	144	150
Phosphorus[a] (P)	mg/dL	2.2	4.4
Total proteins	g/dL	0	7
TIBC-UIBC	µg/dL	100	900
Urea	mg/dL	0	35

[a]Parameter not tested in the control group. Total iron binding capacity (TIBC), unsaturated iron-binding capacity (UIBC), 1,2-o-dilauryl-rac-glycero-3-glutaric acid-(6'-methylresorufin) ester (DGGR).

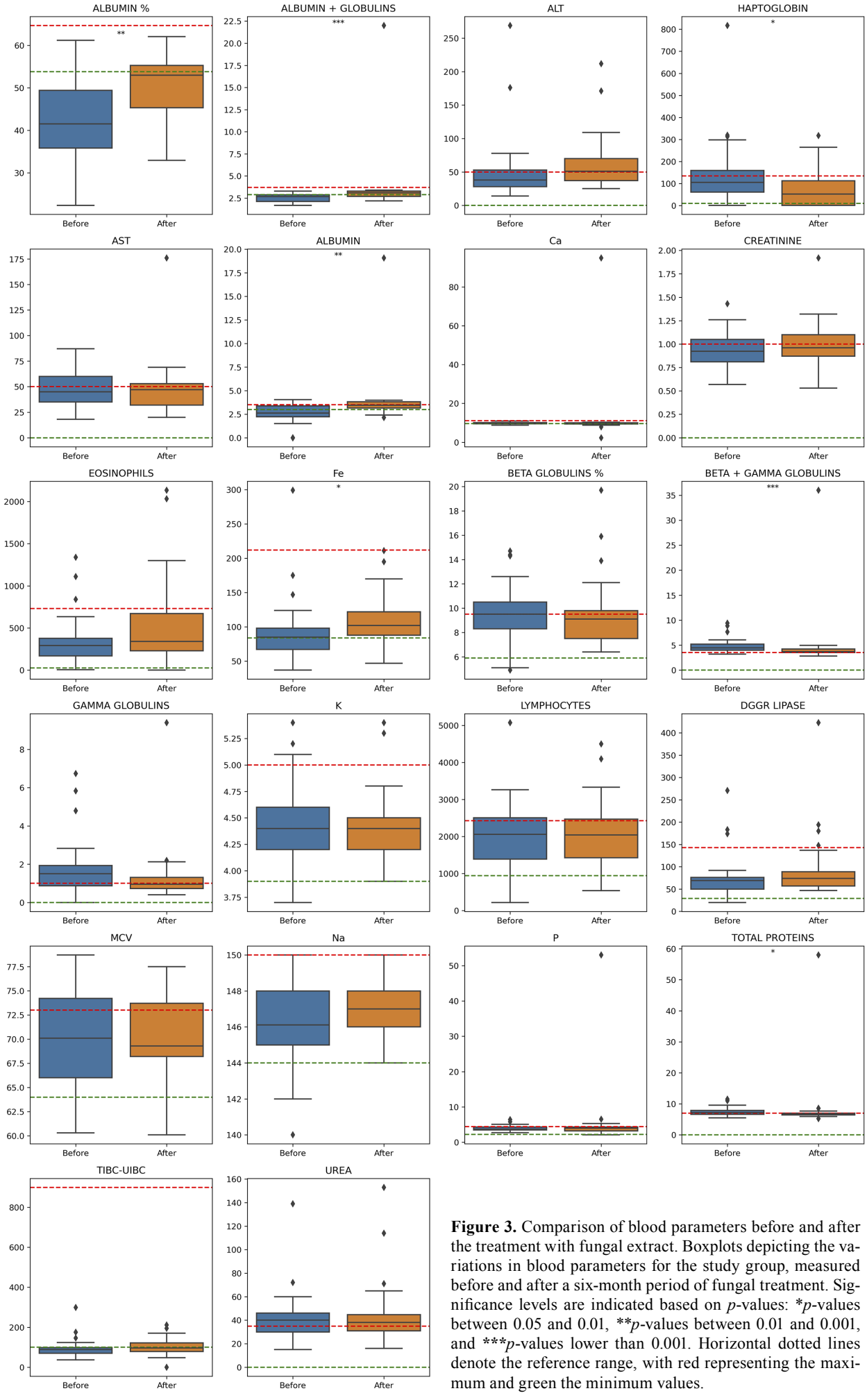

Figure 3. Comparison of blood parameters before and after the treatment with fungal extract. Boxplots depicting the variations in blood parameters for the study group, measured before and after a six-month period of fungal treatment. Significance levels are indicated based on *p*-values: **p*-values between 0.05 and 0.01, ***p*-values between 0.01 and 0.001, and ****p*-values lower than 0.001. Horizontal dotted lines denote the reference range, with red representing the maximum and green the minimum values.

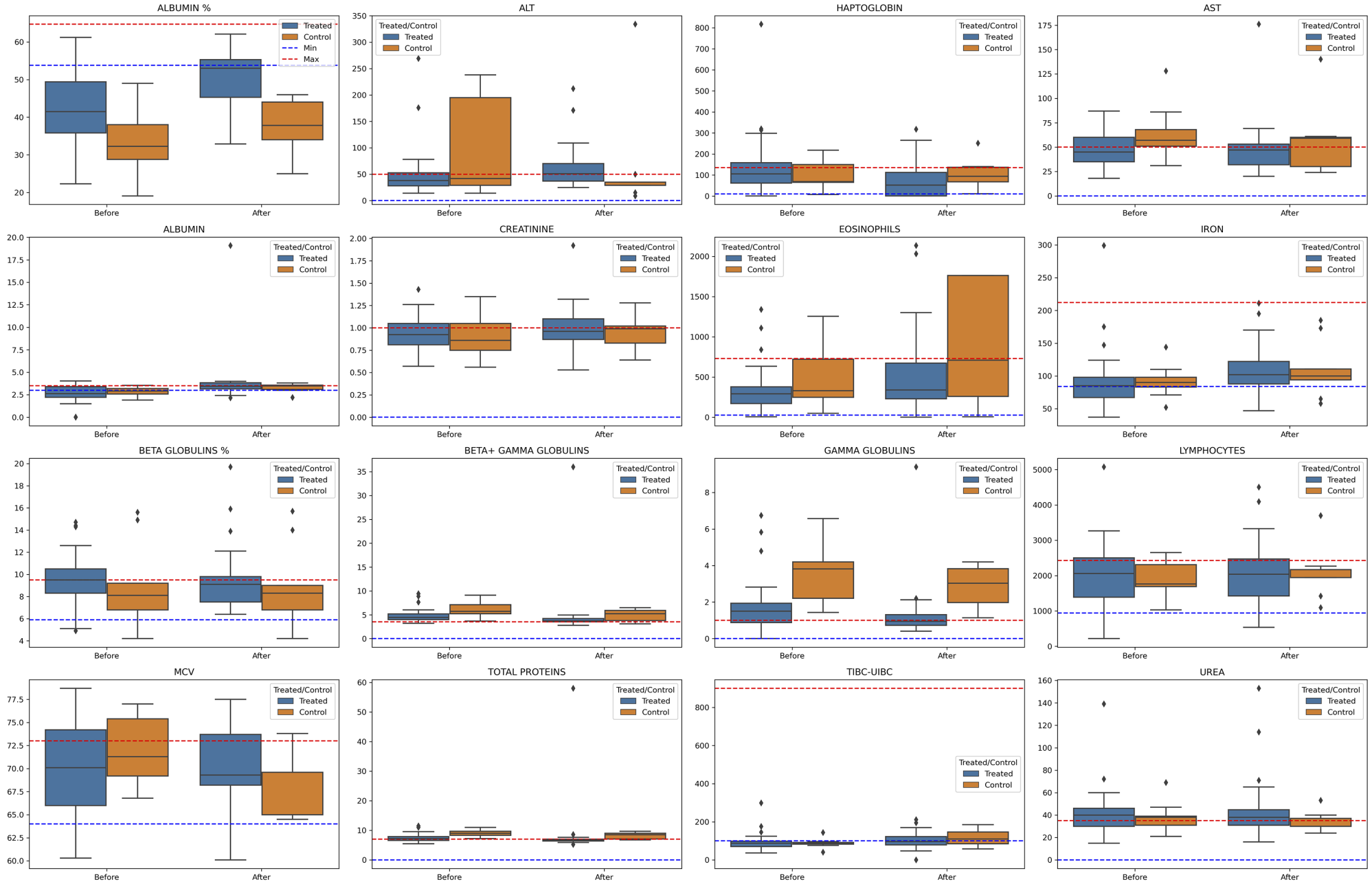

Figure 4. Comparison of blood parameters before and after the treatment with fungal extract and with control group. Boxplots illustrate changes in blood parameters within the study group, evaluated both pre- and post-six months of fungal therapy, alongside comparisons with the group assessed before and after the treatment period. The reference range is marked by horizontal dotted lines, where red indicates the upper limit and blue signifies the lower limit.

after one to three months. Blood parameters assessed are listed in Table 1, along with their respective unit of measurement and reference values.

Results of comparison between fungi-treated dogs before and after the treatment (Figure 3) show a significant increase in albumin and iron, a significant decrease in haptoglobin and total globulins values, and an overall tendency to return within the reference interval in the samples taken after the fungi extract treatment, with an exception for alanine transaminase and creatinine.

Then, we compared the fungi-treated dogs before and after the treatments with the control group who underwent the standard therapeutic protocol. Interestingly, results shown in Figure 4 evidence a significant decrease of gamma globulins (p-value < 0.0004) after the fungal extract treatment, compared to the control group.

DISCUSSION

In the study group, the reduction in baseline parameters was greater than in the control group. None of the dogs in the control group achieved the disappearance of *Leishmania* DNA at the end of treatment. The study group continued the administration of meglumine stibiate until parasitological negativity was achieved, confirmed by the impossibility of finding the DNA of *Leishmania spp.* in blood samples. Once this condition was reached, the study group continued the administration of the fungal extracts. In none of the dogs in the study group, there was the detection of parasite DNA and recrudescence of symptoms six months after stopping the treatment with meglumine stibiate, while in the control group, the presence of *Leishmania spp.* DNA was detected. Furthermore, bone marrow cyto-histological preparations confirmed the absence of *Leishmania* amastigote and the restoration of physiological bone marrow cytology in the study group upon fungal treatment, while this failed to be observed in the control group, in which the signatures of *Leishmania* infection remained visible. Nevertheless, recurrence prevention has only been observed in the fungal-treated group during the monitored follow-up. Because we lack equivalent long-term data for the control group, we cannot conclusively attribute the prevention of recurrence solely to the fungal extract.

Various blood parameters have been tested at different time points in the study group and the control group. Among these parameters, haptoglobin and albumin are part of the acute phase proteins (APPs), plasma proteins whose concentration is known to change in case of inflammation, infection, or injury[22]. High levels of haptoglobin have been previously identified during acute phase response in canines with Canine Leishmaniasis[23,24]; furthermore, studies[25] reported a decrease in APP concentrations coinciding with the resolution of clinical symptoms. In accordance with this, we observed a statistically significant decrease in haptoglobin after the fungal treatment compared to the control group. Similarly, albumin levels have been observed to increase upon the disappearance of clinical manifestations[26]. Accordingly, our results show a significant increase in the albumin levels after fungal treatment. A third blood parameter that was significantly increased after the fungal treatment was iron. Iron is known to play a dual role in *Leishmania* infections; in fact, it supplies the pathogen with essential nutrients while simultaneously enhancing the host's antimicrobial defences[27]. In humans, *Leishmania* secures iron supplies in phagosomes by increasing iron intake, upregulating *TFR1* expression, and preventing its extracellular export, promoting ferroportin downregulation mediated by hepcidin[28,29], while in a mouse model, the blockage of iron export occurs *via* the downregulation of its exporter *NRAMPI*[30]. Interestingly, in mice models[30,31], iron supplementation was able to decrease parasite burden and contain the infection by inducing the production of reactive oxygen species (ROS) at the site of infection; this may suggest that the observed increase in iron levels in the fungi-treated dogs may not only be a mere consequence of a decrease in parasite activity but may also help in consolidating the parasite elimination. Hypergammaglobulinemia is a common signature of Leishmaniasis observed in humans, dogs, and ferrets[32,33]. An increase in gamma globulins, which includes IgM, may indicate a heightened immune response. However, this immune response may inadvertently facilitate the genetic exchange and diversity of *Leishmania* parasites, potentially impacting the infection dynamics and treatment outcomes; in fact, natural IgM antibodies (IgMn) play a role in facilitating genetic exchange in *Leishmania* parasites. IgMn from *Leishmania*-free animals bind to the parasite, causing significant changes in parasite transcript and protein expression. This binding leads to the formation of parasite clumps, which are crucial for *Leishmania* hybridization[34]. Notably, we observed an overall decrease in globulin levels (both beta and gamma) upon fungal treatment.

Eosinophils are key players in the body's immune system, actively fighting various infections, including parasites, bacteria, viruses, and certain types of cancer[35]. Parasitic infections are the third cause of eosinophilia in dogs[36]. In *Leishmania* infections, there is a significant interplay between eosinophils and dermal tissue-resident macrophages. Eosinophils, by producing interleukin (IL)-4, are instrumental in promoting the proliferation of dermal tissue-resident macrophages (TRMs) and maintaining their M2-like phenotype. This interaction is further enhanced as IL-4 stimulated TRMs to produce C-C motif chemokine ligand 24 (CCL24), which attracts more eosinophils, thereby creating a cycle that supports the immune response against the *Leishmania* infection, but at the same time, is exploited by the parasite to perpetrate the infection[37]. During the early stages of *L. major* infection in mice,

eosinophils, rather than innate lymphoid type-2 (ILC2) cells, are the main IL-4 source[38]. In our study, we observed that control groups infected with Leishmania maintained high levels of eosinophils, while the study cohort exhibited only slight eosinophilia after a few months, suggesting effective intervention. In fact, the treatment might successfully modulate the immune response, reducing the need for eosinophil involvement. This decrease in eosinophilia in treated groups could confirm a reduced inflammatory response and a potential resolution of the infection.

It is hypothesized that the antiparasitic effects of the fungal extract might be due to enhanced nitric oxide (NO) production in macrophages. Macrophages are crucial in fighting pathogens, primarily through generating reactive oxygen radicals and nitric oxide[39]. Research[40] indicates that *Ganoderma Lucidum* polysaccharide extract boosts nitric oxide production, thereby enhancing the antimicrobial activity of macrophages. This connection could explain the extract's effectiveness against parasites. Furthermore, previous studies[41] demonstrated that in human macrophages, beta-glucans, which are the main components of the fungal cell wall, are able to induce epigenetic modifications leading to the upregulation of IL-32 and control of *Leishmania braziliensis* infection in transgenic mice. However, the proposed nitric oxide (NO)-based mechanism is currently speculative and is primarily supported by existing literature[42-44] indicating that fungal polysaccharides may stimulate macrophage activation. We acknowledge the necessity of conducting direct measurements, such as NO levels, cytokine profiles, and macrophage activation markers, in future studies to validate this hypothesis. One important limitation of our study is the absence of a formal randomization process and the unequal sample sizes in the control and treatment groups, posing a risk of selection bias. Therefore, future research should adopt a randomized study design to confirm and extend the preliminary findings reported. Furthermore, in our study, the owners of the control-group dogs were only available for follow-up over a shorter timeframe. Consequently, the duration of observation differed between the control and treatment groups, limiting direct long-term comparisons; for this reason, future studies should aim for uniform follow-up intervals across all groups to strengthen the validity of our outcomes. Another important point is the difference in meglumine stibiate dosage between the control and treatment groups. While the control group received the standard dosage according to established guidelines, the study group was administered a lower total dose alongside fungal extracts. This difference was not intended as a dose-response comparison but rather to assess whether fungal extracts could serve as adjunctive therapy, potentially reducing the reliance on high doses of meglumine stibiate. In fact, we noted that although a lower stibiate was used in the treatment group, the fungal extracts might have played an adjunctive role, thereby compensating for the reduced stibiate. However, we acknowledge that this variation may introduce a confounding factor when interpreting the results. Future studies should standardize meglumine stibiate dosing across groups to better isolate the specific effects of fungal extracts and minimize potential bias.

CONCLUSIONS

In conclusion, our preliminary study reveals that dogs with *L. infantum*, when treated with allopurinol, meglumine stibiate, and fungal extracts, demonstrate a negative bone marrow PCR and clear bone marrow smears after six months, a result that persists for over a year, although further analysis at more extended time points is needed to confirm these findings. Notably, following the fungal treatment, acute phase proteins, globulins, and eosinophil counts tend towards normal levels. Considering the existing literature, we can speculate that the cause of this parasite eradication could be the result of the increased nitric oxide production by macrophages induced by the fungal extracts. Still, further studies involving quantification of NO levels, more extended follow-up for control dogs and standardized meglumine stibiate dosage will be essential to confirm this proposed mechanism. Nonetheless, these promising findings motivate further studies on the use of fungal extracts as low-cost and low-toxicity adjuvants in anti-*Leishmania* therapeutic approaches.

Ethics Approval:
All dogs that took part in this research were naturally exposed to sand flies and were naturally infected with CanL. This research was led in compliance with the national and international guidelines and received approval from the Ethics Committee of the University of Study of Campania "Luigi Vanvitelli" (protocol code 12478/20 – 12/05/2020) prior to the beginning of the study.

Informed Consent:
Dog owners provided written consent upon enrolment. Bone marrow sampling procedures were performed under anesthesia.

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Conflict of Interest:
The authors declare no conflict of interest.

Authors' Contributions:
F.F. processed the experimental data, performed the analysis, drafted the manuscript, and designed the figures; C.M. contributed to the research implementation, results analysis, and the writing of the manuscript; G.B.,

A.D.B. conceived and planned the analysis, enrolled the subjects, and provided the raw data.

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Data Availability:
The datasets present in the current study are available from the corresponding author upon reasonable request.

AI Disclosure:
Artificial Intelligence was not used in the production of the study and its figures.

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