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In-vitro and *In-vivo* Activities of Phenolic Compounds Against Cutaneous Leishmaniasis

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Abstract: Phenolic compounds (PCs) are well-known phytochemicals found in plants that have been studied for their pharmacological properties. In particular, the potentialities of PCs as anti-leishmanial agents have been reported. In the present study, we evaluated 10 PCs for in-vitro anti-leishmanial activity and two PCs, p-coumaric acid (CA) and gentisic acid (GA) against experimental cutaneous leishmaniasis in BALB/c mice infected with *L. amazonensis*. Five doses of each pure compound were administrated every 4 days at 30 mg/kg by intralesional route and disease progression was compared with animals treated with glucantime (GTM). All tested compounds inhibited intracellular amastigotes growing, with IC50 values between 4.4 and 25.5 μM. Treated animals with GA showed a significant reduction ($p < 0.05$) in the size of lesion and parasite burden compared with animals treated with CA, GTM and untreated mice. The treatment with CA caused reduction of lesion size ($p < 0.05$) compared with GTM and control mice, but had the same ($p > 0.05$) parasite burden as control and GTM treated animals. The present findings established that CA and GA have significant anti-leishmanial effects. Further experiments on formulation design, mechanism of action and probably anti-inflammatory / immune-modulator activity of GA could be encouraged.

Keywords: *Leishmania amazonensis*; cutaneous leishmaniasis; *p*-coumaric acid; gentisic acid; phenolic derivatives.

1. Introduction

Medicinal plants have been used as potential resources of new therapeutic agents. They are diverse, largely productive, biologically active and chemically unique with promising biological therapeutic effects. Among their constituents, phenolic compounds (PCs) constitute one of the main determinant factors of pharmacological potentials [1]. PCs are well-known phytochemicals found in plants that consist of simple phenols, benzoic acid derivatives, cinnamic acid derivatives, coumarins, tannins, lignins, lignans and flavonoids, among others [2]. For many years, PCs have been intensely studied for their pharmacological properties and their usages have considerably increased [3]. Antitumor, pro-apoptotic, anti-angiogenic, anti-oxidant, immune-stimulant and antiparasitic effects have been demonstrated [1,3].

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In recent years, the potentialities of PCs as anti-leishmanial agents have been reported [1,4-6]. Leishmaniasis is an undesirable parasitic disease that affects approximately two million people per year. It is caused by protozoans of the *Leishmania* genus and presents a wide spectrum of clinical manifestations from cutaneous lesions to visceral distress [7-9]. Development of guidelines for the treatment of leishmaniasis remains difficult and available commercial drugs suffer from several limitations such as necessity for long-term treatment, limited effectiveness, significant side effects, and toxicity [9,10]. Consequently, an urgent need exists to discover new drugs that are effective against leishmaniasis.

This study shows the *in-vitro* assessment of ten phenolic compounds (Figure 1), including: cinnamic acid derivatives, flavonols and hydroxybenzoic acid derivatives, against *Leishmania amazonensis*. Potential *in-vitro* chemotherapeutics were also evaluated against experimental cutaneous leishmaniasis in BALB/c mice infected with *L. amazonensis.*

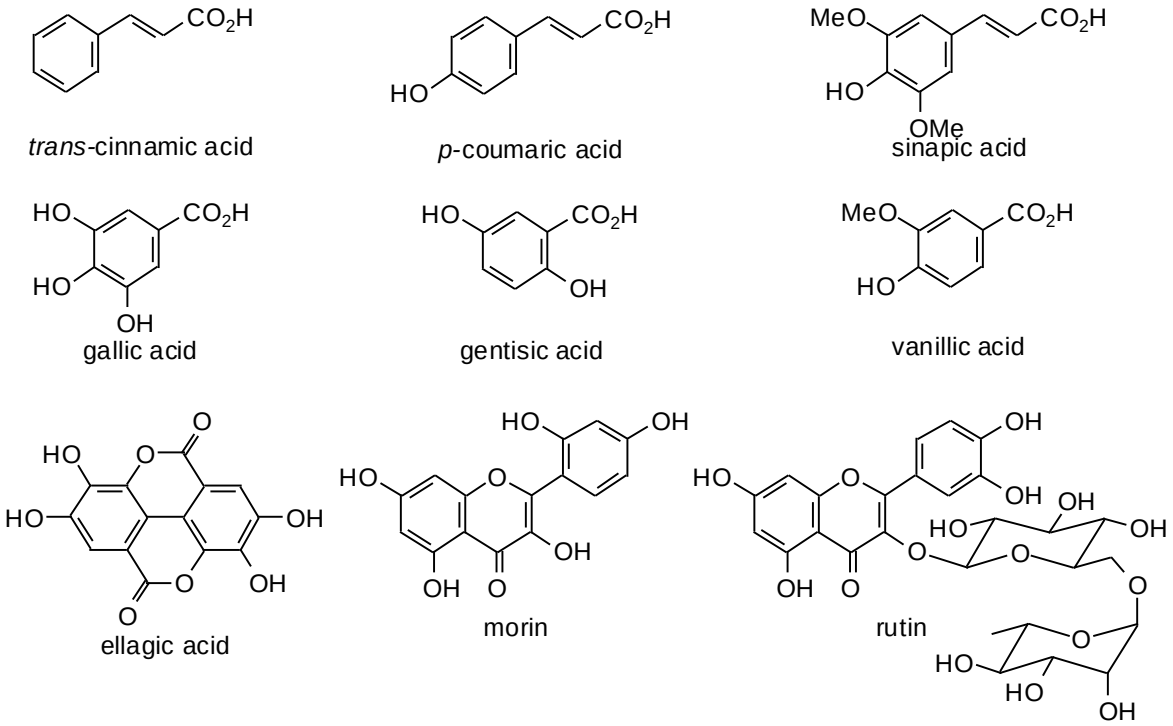

Figure 1. Structures of phenolic compounds examined in this work

2. Materials and Methods

2.1. Parasite

The MHOM/77BR/LTB0016 strain of *L. amazonensis* was kindly provided by the Department of Immunology, Oswaldo Cruz Foundation (FIOCRUZ), Brazil. Parasites were routinely isolated from the mouse lesions and maintained as promastigotes at 28ºC in Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% heat-inactivated fetal bovine serum (HFBS) (Sigma-Aldrich), 100 μg of streptomycin/mL and 100 U of penicillin/mL.

2.2. Compounds

Cinnamic acid, coumaric acid isomers, gallic acid, sinapic acid, gentisic acid and morin (Sigma), rutin Extrasynthese (France), and ellagic acid, vanillic acid were supplied by Aldrich, all of which had > 99% purity. All compounds were dissolved in dimethylsulfoxide (DMSO) at

2 mg/mL. As a reference drug, glucantime (GTM) (Rhône-Poulenc Rorer, Mexico) was used at a concentration of 30 mg/mL in sterile distilled water as stock solution.

2.3. Animals

Female BALB/c mice, with a body weight of approximately 20 to 22 g, were obtained from The National Center of Laboratory Animals Production (CENPALAB, Cuba) and maintained according to "Guideline on the Care and Use of Laboratory Animals". Protocol for animal use was approved by the Ethics Committee from the Institute of Tropical Medicine Pedro Kouri (CEI-IPK 13-10), Havana, Cuba.

2.4. In-vitro Antileishmanial Assay

The activity against intracellular amastigotes was evaluated following the method previously described by Caio *et al.* [11]. The peritoneal macrophages from normal BALB/c mice were harvested and plated in 24-Well Lab-Tek (Costar®, USA) and incubated at 37ºC under an atmosphere of 5% CO_2 for 2 hours. Non-adherent cells were removed and stationary-phase *L. amazonensis* promastigotes were added at a 4:1 parasite/macrophage ratio. The cultures were incubated for further 4 hours and free parasites were removed. Then, 995 μL of RPMI medium (SIGMA, St. Louis, MO, USA) containing 10% HFBS, 100 μg of streptomycin/mL and 100 U of penicillin/mL and 5 μL of the different products dissolved in DMSO were added in duplicate and incubated for an additional 48 hours. The cultures were then fixed in absolute methanol, stained with Giemsa, and examined under a light microscope. The number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages per sample and percentage of infected macrophages. The results were expressed as percent of reduction of the infection rate in comparison to that of the controls, where the infection rates were obtained by multiplying the percentage of infected macrophages by the number of amastigotes per infected macrophage. The median inhibitory concentration (IC_{50}) value was determined from the linear regression of concentration-response curves.

The effect of compounds in the viability of mouse peritoneal macrophages (host cells for the amastigote form of the parasite) was determined in parallel [12]. Macrophages were collected and incubated as described previously [11]. The medium was removed and 50 μL of RPMI medium (SIGMA, St. Louis, MO, USA) containing 10% HFBS, 100 μg of streptomycin/mL and 100 U of penicillin/mL were added, with additional 48 μL in the wells of the second column, completed with 2 μL of each compound. Subsequently, ten serial dilutions of 1:2 were carried out, taking 50 μL. Then, 50 μL of medium were added in each well to evaluate final concentrations of compounds ranging from 0.04 to 20 μg/mL, which were incubated at 37ºC for 48 hours. Macrophages treated with 1 μL DMSO were included as controls. Then, 15 μL of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (SIGMA, St. Louis, MO, USA) solutions at 5 mg/mL in phosphate-buffered saline (PBS) previously prepared and filtered were added in each well and the plate was incubated under the same conditions. After 3 hours, formazan crystals were dissolved with 100 μL DMSO and optical density was measured as previously described [12]. The Cytotoxic Median Concentration (CC_{50}) was determined by dose-response curves. Selectivity indices (SI) were calculated by dividing the CC_{50} for peritoneal macrophage of BALB/c mice by the IC_{50} for *L. amazonensis* amastigotes.

2.5. In-vivo Antileishmanial Assay

On day 0, normal BALB/c mice received subcutaneous injections in the left hind footpad of 5×10^6 stationary-phase *L. amazonensis* promastigotes. Four weeks post-infection (p.i.) the animals were randomly divided into four groups of eight mice each and the treatment was

initiated with 25 µL of gentisic acid, *p*-coumaric acid or glucantime at a dose of 30 mg/kg dissolved in a mixture 3:7 (v:v) of DMSO : saline solution. Treatments were administered by intralesional route every four days to complete five doses. In parallel, an infected and untreated animal group was included as a control.

Disease progression was monitored weekly by measuring footpad swelling of the lesion diameter between 30 and 60 days p.i., using a digital calliper. Average lesion size was calculated as the differences obtained between infected and uninfected footpads. On day 45 and 60 p.i., three animals of each group were killed by cervical dislocation and parasite burden determined, using the culture microtitration method [13]. Briefly, a sample of the lesion was excised, weighed and homogenized in 4 mL of Schneider's medium. Under sterile conditions a serial four-fold dilution was prepared in 96-well plates. After 7 days of incubation at 28ºC, the plates were examined with an inverted microscope. The final titer was defined as the last dilution for which the well contained at least one parasite. The parasite burden was calculated as follows: parasite burden = (geometric mean of reciprocal titers from each duplicate/weight of homogenized cross section) × 400.

2.6. Statistical Analysis

Statistical differences, classified as $p < 0.05$, between IC_{50} of products against intracellular amastigotes were performance using the Mann-Whitney test. Data on lesion progression and parasite burden determined by the microtitration method were analyzed for statistical significance by the analysis of variance test, followed by a Post Hoc Test (LDS test or planned comparison). Analyses were performed using the StatisticA for Windows Program (Release 4.5, StatSoft, Inc. 1993).

3. Results

3.1. In-vitro Antileishmanial Activity

Activities of ten pure phenolic compounds against intracellular amastigote forms of *L. amazonensis*, as well as their cytotoxicity on mouse peritoneal macrophage are summarized in Table 1. All pure compounds inhibited the growth of *Leishmania* parasites, although the most active compounds were found to be *p*-coumaric acid and gentisic acid. These compounds showed higher activity ($p < 0.05$) than GTM..

3.2. In-vivo Antileishmanial Activity

Four weeks after infection of BALB/c mice with *L. amazonensis*, skin lesions at the inoculation site were evident in all animals, at which time treatment commenced. Treated animals with PCs showed a significant reduction ($p < 0.05$) in the lesion size compared with positive and negative controls, from week 7 p.i. until the end of experiment at 10 weeks p.i. (Figure 2A). Cutaneous lesions in treated animals with both compounds were reduced. However, the effect was also statistically higher ($p < 0.05$) in animals treated with gentisic acid than those treated with coumaric acid the last two weeks of experiment.

In addition to lesion size, parasite burden in infected sites was also determined. The superiority of gentisic acid treatment was also evident, due to the fact that the parasite burden was significantly lower ($p < 0.05$) with respect to animals treated with GTM and control mice at 6 and 10 weeks p.i (Figure 2B). However, the coumaric acid treatment only caused a significant decrease ($p < 0.05$) of parasite burden at 10 weeks p.i. compared with untreated animals. Additionally, no difference ($p > 0.05$) was observed in comparison with animals treated with GTM. The potential effect of gentisic acid can be macroscopically visualized in Figure 2C.

Table 1. Antileishmanial and cytotoxic activities of phenolic compounds.

Classification	Compounds	Amastigotes IC_{50}[a] ± SD[b] (μg/mL-μM/L)	Macrophages CC_{50}[c] ± SD (μg/mL-μM/L)	SI[d]
Cinnamic acid derivatives	*o*-Coumaric acid	2.3 ± 0.3 - 13.8 ± 1.5	23.8 ± 2.2 - 145.0 ± 13.3	11
	p-Coumaric acid	1.5 ± 0.5* - 9.0 ± 1.7*	22.5 ± 0.7 - 136.8 ± 0.4	15
	Sinapic acid	4.6 ± 0.7 - 20.7 ± 1.6	>20 - > 90	> 4
	trans-Cinnamic acid	2.3 ± 0.5 - 15.5 ± 1.6	25.7 ± 2.7 - 173.6 ± 1.8	11
Flavonol derivatives	Morin	4.2 ± 0.7 - 14.0 ± 1.8	15.7 ± 1.4 - 51.8 ± 0.5	4
	Rutin	2.7 ± 0.6 - 4.4 ± 0.5	17.3 ± 1.8 - 28.3 ± 0.3	6
Hydroxybenzoic acid derivatives	Gallic acid	2.6 ± 0.1 - 15.4 ± 0.8	9.0 ± 0.5 - 52.7 ± 3.2	3
	Gentisic acid	1.2 ± 0.4* - 7.6 ± 1.3*	42.4 ± 2.6 - 275.0 ± 16.7	35
	Vanillic acid	4.2 ± 0.2 - 25.2 ± 6.6	57.0 ± 2.3 - 338.9 ± 7.6	14
Other	Ellagic acid	7.7 ± 2.5 - 25.5 ± 4.1	15.1 ± 0.6 - 50.1 ± 2.0	2
Glucantime[e]		11.0 ± 3.4 - 21.7 ± 6.7	> 1500 - > 2954	> 130

[a] IC_{50}: Median inhibitory concentration. Concentration that cause 50% of growth inhibition.
[b] SD: Standard deviation.
[c] CC_{50}: Medium cytotoxic concentration. Concentration that cause 50% of mortality.
[d] SI: Index of selectivity. CC_{50} for macrophages / IC_{50} for intracellular amastigotes (μg/mL).
[e] Glucantime: Reference drug.
* Asterisk indicates statistical differences ($p < 0.05$) compared with studied compounds and glucantime.

In addition to pharmacological assays, preliminary toxicity observations were performed. In animals treated with pure compounds, no deaths or weight lost greater than 10% were observed.

4. Discussion

In this study, all compounds showed potent anti-leishmanial activity with IC_{50} values below 5 μg/mL and different toxicity levels, with SI between 2 and 35. Among the ten tested compounds, only the anti-leishmanial activity of gallic acid had been evaluated previously, and in this case, an IC_{50} of 4.4 μg/mL against amastigotes of *L. donovani* (a causal agent of visceral leishmaniasis) was reported [14], which are in concordance with our results against *L. amazonensis* (a causal agent of cutaneous leishmaniasis). This compound has been extensively studied due to its ability to induce cytokine production, especially of interferon-gamma (IFN-γ), which could be used as a beneficial indirect effect in various infectious conditions, including leishmaniasis [15].

The most promising compounds were *p*-coumaric acid and gentisic acid, which showed better *in-vitro* results than GTM, the reference drug used in this study. In addition, the activity of other clinical drugs against *L. amazonensis* have been reported, *e.g.*, pentostam that showed an IC_{50} value of 20 μg/mL [16], higher than the PCs studied herein. *p*-Coumaric acid is a hydroxy derivative of cinnamic acid and one of the most abundant phytochemicals that has demonstrated anti-oxidant [17] and anti-inflammatory [18] activities. In parallel, gentisic acid is a dihydroxybenzoic acid derivative that has been studied for its anti-oxidant [19] and anti-helmintic [20] properties. Therefore, these derivatives were selected for chemotherapeutic tests in the murine model of experimental cutaneous leishmaniasis.

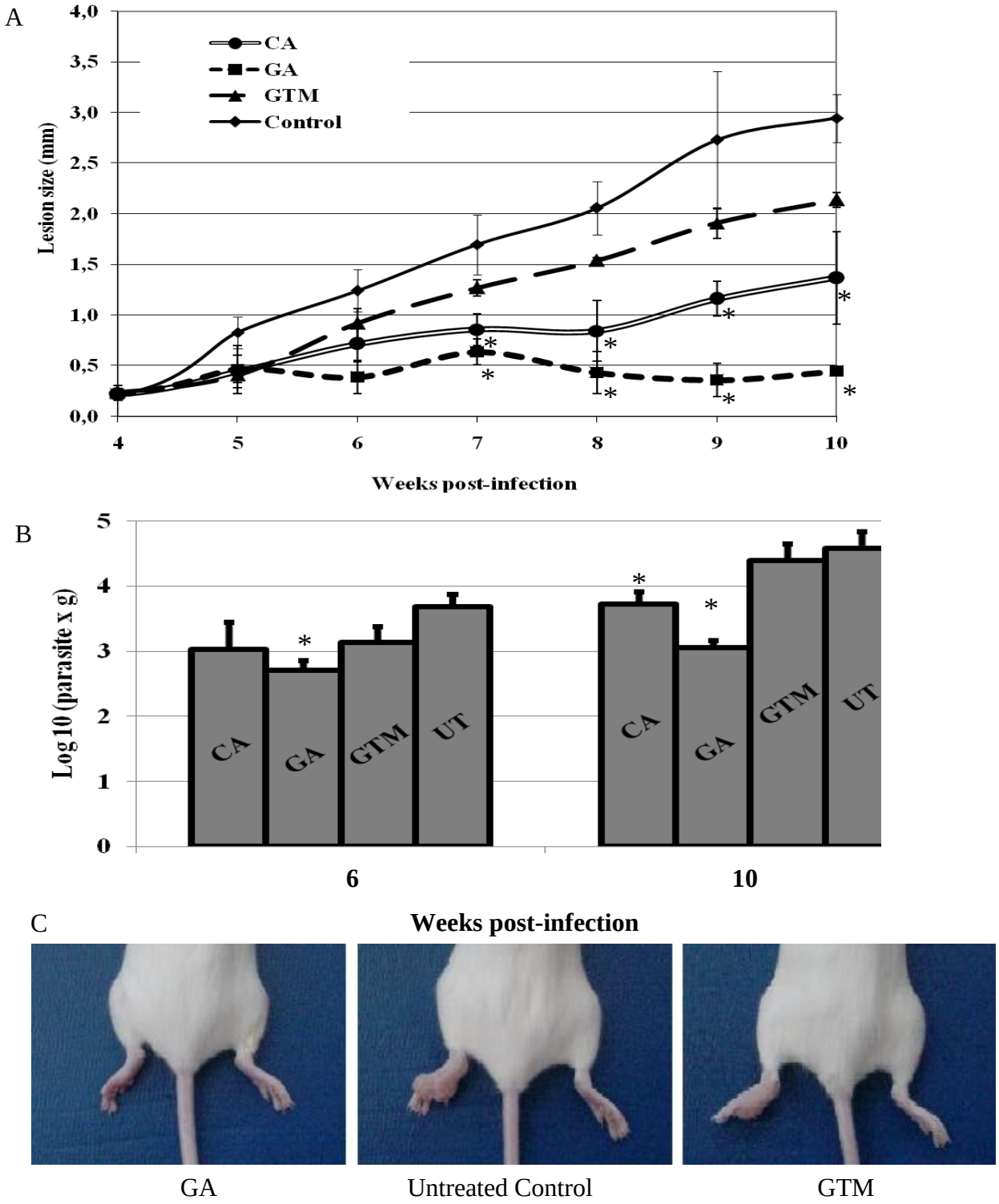

Figure 2. Effect of phenolic compounds on BALB/c mice infected with *L. amazonensis*. The results are express as mean ± standard deviation. A: Lesion size; B: Parasite burden. C: Infected footpads from BALB/c mice (Pictures were taken at 10 weeks p.i). GA: gentisic acid; CA: *p*-coumaric acid; GTM: glucantime; UT: infected and untreated animals used as control. *: Statistical differences ($p < 0.05$) with respect to control animals.

In this study, BALB/c mice infected with *L. amazonensis* and intralesionally treated with PCs controlled the development of cutaneous leishmaniasis, which was demonstrated by reduction of both lesion size and parasite burden. In addition to direct anti-leishmanial action of PCs demonstrated by *in-vitro* and *in-vivo* effects, indirect activity in the cutaneous model could also contribute to the control of infection. For example, an extract of the mushroom *Tremella fuciformis*,where coumaric acid and gentisic acid were identified, demonstrated anti-inflammatory activity through inhibition of nitric oxide production and inducible nitric oxide synthase [21]. In cutaneous leishmaniasis it has been demonstrated that inflammatory molecules play an essential role in diseases progression, thus, anti-inflammatory agents could also contribute to control lesion thickness and infection.

No previous report about the *in-vivo* assessment of coumaric acid and gentisic acid were found in the literature. However, a hydroalcoholic extract of Brazilian green propolis, which contained coumaric acid, displayed a decrease on lesion development in experimental cutaneous leishmaniasis [22]. Additionally, PCs have demonstrated potential anti-leishmanial *in-vivo* effects, *e.g.*, ferulic acid, rosmarinic acid, caffeic acid [5] and quercetin glycosides [23].

Although a superior anti-leishmanial effect of both coumaric acid and gentisic acid were obtained compared with reference drug GTM, a complete cure was not evident since persistent parasites were quantified at the inoculation site. Nevertheless, the dosing scheme of compounds used in this investigation was only five doses every 4 days at 30 mg/kg. It may be possible to increase the dose of studied compounds or number of application with the aim to improve the anti-leishmanial effect. In addition, this approach should be feasible based on the observation that no acute toxic effects in treated animals, through deaths and weight lost, were apparent.

A key goal in pharmaceutical development is a good understating of *in-vitro* and *in-vivo* performance [8]. Based on the *in-vitro* results, gentisic acid was selected as the most promising compound among the ten PCs. Furthermore, the anti-leishmanial potential of gentisic acid against cutaneous leishmaniasis was also demonstrated in the *in-vivo* study compared to GTM. The specific differences between both coumaric acid and gentisic acid suggest that the two compounds have distinct impacts on target structures and functions in the *Leishmania* parasite.

In summary, the present finding establishes that coumaric acid and gentisic acid have significant anti-leishmanial effects. Further experiments on formulation design by parallel desirable administration route and mechanism of action studies should be performed. Additional approaches to elucidate possible anti-inflammatory and immune-modulator activity of both PCs, but in particular of gentisic acid, is encouraged.

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