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Original article

In vitro and *in vivo* antileishmanial effects of aloe-emodin on *Leishmania major*

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ABSTRACT

Cutaneous leishmaniasis is a common parasitic disease that is endemic in some parts of Iran. The drugs of choice used for leishmaniasis therapy are associated with a risk of recurrence and serious adverse effects. Therefore, finding a safe and effective treatment is of great importance. In the present study, the effect of aloe-emodin on the growth of *Leishmania major* amastigotes was evaluated under *in vitro* conditions. In addition, the efficacy of a topical of aloe-emodin ointment was investigated in BALB/c mice with cutaneous leishmanial ulcers. Different concentrations (40 μg/mL, 80 μg/mL, 120 μg/mL, and 160 μg/mL) of aloe-emodin were tested on *Leishmania* amastigotes twice: 24 hours and 48 hours. The induced apoptosis and necrotic effects of two concentrations (40 μg/mL and 120 μg/mL) of aloe-emodin on promastigotes were investigated by flow cytometry. Under the *in vivo* condition, aloe-emodin ointment efficacy was evaluated at two concentrations (i.e., 0.1% and 1%). Serum indicator factors of the test and control groups were tested to evaluate the toxic effects of this compound on the liver and kidney. Results showed that aloe-emodin inhibited the growth of *Leishmania* amastigotes and induced apoptosis in promastigotes. Topical application of aloe-emodin ointment likewise reduced the ulcer size. No significant differences in biochemical analysis were observed between the control and treated groups. In conclusion, aloe-emodin showed antileishmanial effects under *in vitro* and *in vivo* conditions and may be used in clinical trials.

1. Introduction

Leishmaniasis is a vector-born disease caused by protozoan parasites of the *Leishmania* genus and transmitted by the phlebotomine sandfly.[1] The disease is normally localized to the skin and infects dermal macrophages, although metastasis to mucosal tissue and bone marrow can occur.[2] Three forms of the disease are cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (MCL), and visceral leishmaniasis. In CL, manifestations develop from a small nodule to ulcerative wounds.[3] Cutaneous leishmaniasis is endemic in 98 countries and five continents.[4] The five most affected countries are Afghanistan, Algeria, Brazil, Iran, and Syria.[5] Throughout the world, 1–1.5 million new cases of the disease are reported annually.[3] Different drugs such as miltefosin, liposomal amphotericin B, paromomycin, and allopurinol have been used to treat this disease,[6] but the first-line drug treatment for CL is antimony compounds. Two popular types are sodium stibogluconate or pentostam and meglumine antimoniate; however, these compounds have adverse effects, and drug resistance and relapse after treatment can occur.[5,7,8] Factors associated with unsuccessful treatment include the presence of more than three cutaneous lesions, previous treatment, body weight above 68 kg, and an incomplete treatment schedule.[9] Aloe-emodin (1,8-dihydroxy-3-hydroxymethyl-anthraquinone; Fig. 1), an exudate from the aloe plant,[10] is an anthraquinone in aloe vera and other species of the Asphodelaceae and Polygonaceae families. Some studies have indicated that aloe-emodin has antibacterial, antifungal, antiviral, diuretic, immunosuppressive, hepatoprotective, laxative, anti-inflammatory, and anticancer specificities.[11–14] Aloe-emodin reportedly inhibits the replication of enveloped viruses such as herpes simplex virus, influenza virus, and human cytomegalovirus.[15] Aloe-emodin induces apoptosis through the release of apoptosis-inducing factors and cytochrome c from the mitochondria in human gastric carcinoma cells.[16] This compound has a

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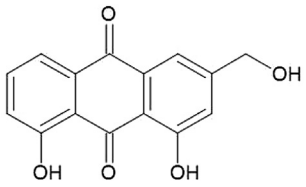

Fig. 1. Chemical structure of aloe-emodin.

considerable antimetastatic capability on a melanoma cell line, which is exerted by inducing cell differentiation.[17] It also has anticancer activity against human promyelocytic leukemia cells[18] and human hepatoma cells.[19] The aim of the present study was to evaluate antileishmanial activity of aloe-emodin under *in vitro* and *in vivo* conditions.

2. Materials and methods

2.1. Preparation of aloe-emodin

Aloe-emodin powder was purchased from the Selleckchem Company (Houston, TX, USA) and dissolved in dimethyl sulfoxide (DMSO) at the concentration of 20 mg/mL. For the *in vitro* study, four concentrations were prepared: 40 μg/mL, 80 μg/mL, 120 μg/mL, and 160 μg/mL. Two concentrations (10 mg/mL and 1 mg/mL) were also prepared for the *in vivo* assay in an ointment base by Eucerin.

2.2. Leishmania culture

L. major (MRHO/IR/75/ER) promastigotes were cultivated in Roswell Park Memorial Institute (RPMI) 1640 medium, which contained penicillin (100 units/mL), streptomycin (100 μg/mL), and 20% fetal bovine serum, in an incubator 24 ± 2°C. The stationary phase of parasites was obtained by culture promastigotes in Novy–MacNeal–Nicolle medium.

2.3. Antiamastigotes assay

The drug susceptibility of amastigotes in BALB/c mouse macrophages was determined using the modified method by Love et al.[20] In brief, peritoneal macrophages were isolated from the peritoneum of BALB/c mice. They were added onto a glass coverslip in tissue culture on 12-well plates and incubated for 24 hours at 37°C with 5% carbon dioxide. Nonadherent macrophages were removed by washing. Adherent macrophages were adjacent to the stationary growth phase of promastigotes at a parasite/macrophage ratio of 10:1. After 24 hours of incubation under the previous condition, washing was repeated and different concentrations (i.e., 40 μg/mL, 80 μg/mL, 120 μg/mL, and 160 μg/mL) of aloe-emodin were added to the infected macrophages in the wells and incubated separately for 24 hours and 48 hours. The coverslips were stained with Giemsa stain and the number of amastigotes inside the macrophages were counted (100 macrophages per coverslip).

2.4. Flow cytometry analysis

The promastigotes were cultured in 24-well plates (3×10^5 parasites per well) in the absence of aloe-emodin (i.e., negative control group) and in the presence of 40 μg/mL and 120 μg/mL of aloe-emodin. They were incubated at 24°C. The Annexin V FLUOS Staining Kit (Biovision, USA) was used to detect apoptotic and necrotic cells. In accordance with the kit instructions, the promastigotes were collected after a 24-hour incubation and a 48-hour incubation. They were centrifuged at 3000 rpm for 5 minutes. The supernatant was then discharged, and 500 μL binding buffer, 5 μL Annexin V, and 5 μL propidium iodide were added to the residue. The samples were incubated at room temperature and under a dark condition for 5 minutes. They were then obtained by BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and were analyzed by FlowJo Software (BD Biosciences).

2.5. In vivo assay

An ointment was prepared with 10 mg/mL (1%) and 1 mg/mL (0.1%) aloe-emodin in a standard ointment base (Eucerin). Forty female BALB/c mice that were 6–7 weeks old were used in this study. All mice were inoculated subcutaneously in a shaved area above the tail with approximately 2×10^6 stationary stages of Iranian strains of *L. major* promastigotes. Forty mice were divided into four groups with each group containing 10 mice. The groups were classified as follows: Group 1 was treated with the 1% aloe-emodin ointment; Group 2 was treated with the 0.1% aloe-emodin ointment; Group 3 was treated with only the ointment base (i.e., Eucerin); and Group 4 was untreated (i.e., control group). Treatment was initiated when local lesions were obvious. The mice were treated topically twice daily for 30 continuous days. Each week, the lesion size was measured before and after treatment by vernier calipers in two diameters (a, b). The lesion size was calculated by the formula:

$$\text{Lesion size (LS)} = (a + b)/2. \quad (1)$$

2.6. Biochemical analysis

To evaluate the toxic effects of aloe-emodin in the livers and kidneys of the mice, serum samples were collected from a group of 12 mice. Aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, creatinine, urea, sodium and potassium were measured using Pars azmoon kit (Iran) and Hitachi Analyzer (Japan).

2.7. Statistical analysis

Statistical significance between groups was analyzed by one-way analysis of variance (ANOVA) using SPSS version 16 software (SPSS Inc., Chicago, IL, USA) and the obtained $p < 0.05$ were considered significant.

3. Results

3.1. Antiamastigote effect

After adding 160 μg/mL aloe-emodin, the mean number of amastigotes/macrophage after 24 hours and 48 hours was 1.3 and 0.9, respectively, and in the negative control group the mean number was 4.3 and 4.8, respectively. Fig. 2 shows the other concentration results.

3.2. Flow cytometry analysis

The treatment of promastigotes at two concentrations of aloe-emodin (i.e., 40 μg/mL and 120 μg/mL) for 24 hours and 48 hours resulted in necrotic and apoptotic effects in the parasite. The percent of apoptosis in promastigotes induced by 40 μg/mL and 120 μg/mL of aloe-emodin after 24 hours was 13.45% and 68.3%, respectively; after 48 hours, it was 15.43% and 70.2%, respectively (Fig. 3).