Original Article

Antileishmanial Activity of Auranofin Against *Leishmania Major in vitro*

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Abstract

Aim: Despite the various studies done in the field of cutaneous leishmaniasis treatment, there is still no perfect and safe drug for definite treatment of this disease. Therefore, the quest to find an appropriate drug continues. Previous studies have shown that auranofin has anti-leishmanial activity; therefore, in this study; the effect of auranofin on *Leishmania major* was studied. **Materials and Methods:** Effectiveness of four concentrations (1, 2, 4, and 8 µg/ml) of auranofin on *L. major* was studied. After culturing amastigotes and promastigotes of the parasite, IC_{50} was calculated. The ability of auranofin to induce apoptosis in promastigotes was evaluated, and the degree of fragmentation of promastigotes DNA after treatment with auranofin was studied. Subsequently, the ultrastructural changes induced by treatment with auranofin in promastigotes were studied. Using the obtained results, IC_{50} of auranofin against amastigotes and promastigotes was calculated as 1.007 and 2.38 µg/ml, respectively. **Findings:** Showed that auranofin induce apoptosis in *L. major*. The highest rate of apoptosis (%80.1) occurred at the concentration of 8 µg/ml and also auranofin-induced fragmentation of DNA. Considerable changes occurred in the shape of body and free flagellum of the *L. major* promastigotes after treatment with auranofin. **Conclusion:** Based on the results, it can be concluded that auranofin has a considerable anti-leishmanial activity and additional studies in this field will be based on the results.

Keywords: Apoptosis, auranofin, fragmentation, leishmania major, ultrastructural

Introduction

Leishmania is a genus of the flagellate protozoa and belongs to the *Trypanosomatidae* family which is a causative agent of leishmaniasis.[1] The vectors of these protozoa are the *Phlebotomus* sand flies in the ancient world and the *Lutzomyia* in the new world.[2] Cutaneous leishmaniasis is one of the clinical forms of leishmaniasis. This disease has been present in the most parts of the world and is characterized by cutaneous ulcers.[3] According to the WHO reports, 700,000 to one million new cases of cutaneous leishmaniasis occur annually.[4] Various chemical agents such as miltefosine, Paromomycin, Amphotericin B, liposomal amphotericin B, Allopurinol, and Mepacrine are used in the treatment of this disease, but due to the imperfect treatment, relapse of the disease, the toxicity of the usual drugs and remaining wound scar, researchers are still working hard to find the appropriate drug.[5-7] Auranofin is chemically combined with the formula [2, 3,4,6-tetra-o-acetyl-L-thio-b-D-glycopyranp-sato-S-(triethyl-phosphine) gold]. It can be used in human diseases and does not have toxic properties.[8-10] A combination of auranofin consists of gold, phosphine plus ligands of thiol.[11] Auranofin inhibits oxidative/reversible enzymes, such as thioredoxin reductase,[12,13] oxidative/reductase enzymes, including important enzymes in the control of reactive oxygen species and prevents damage to DNA of various cells, such as cancer cells, T-cells, and parasites.[12-14] Oxidative/reductases are enzymes that strongly protect parasitic protozoa.[15,16] In 1985, auranofin was first used to treat rheumatoid arthritis.[17]

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This drug reduces inflammatory reactions in the body and is used to treat rheumatoid arthritis to reduce pain and swelling.[18,19] Furthermore, auranofin inhibits the signaling pathways of IKK/nuclear factor κB and reduces the regulation of the expression of the pro-inflammatory cytokines such as interleukin-1β and tumor necrosis factor-α.[20,21] Studies have shown that auranofin is effective against giardia, especially metronidazole-resistant giardiasis.[16] In a similar study, the effect of auranofin on the inhibition of the glutathione reductase receptor in *Schistosoma mansoni* was evaluated.[22] Recent studies have shown that auranofin has an activity against *Trypanosoma cruzi*[23] *Plasmodium falciparum,*[8] *Leishmania infantum,*[24] *S. mansoni*[25] and *Entamoeba histolytica.*[26] The results of the research indicate that auranofin is active against Gram-positive organisms *in vitro* and in the human body, including the clinical strains of *Streptococcus pneumoniae* and *Staphylococcus aureus*.[27] Antifungal activity of auranofin has been detected against *Candida albicans* biofilms and *Cryptococcus* and *Candida* species.[28,29] Considering the prevalence and complications of cutaneous leishmaniasis and the necessity of its treatment, the aim of this study was to survey the anti-leishmanial activity of auranofin against *L. major* in *in vitro* condition.

Materials and Methods

Auranofin was purchased from Santa Cruz Biotechnology Company.

Leishmania major Iranian standard strain (MRHO/IR/75/ER) was cultured in RPMI 1640 containing 15% fetal bovine serum (FBS) () and antibiotic at 24 ± 2°C.

To evaluate the toxicity of different concentrations of the drug, their effect on the macrophage was measured 24 h after culture. MTT method was used for this purpose. J774 macrophage cell line was purchased from Iranian Pasture Institute and cultured in RPMI 1640 containing 10% FBS.[6] Macrophages were cultured in 96 well plates (1×10^5 cells/well), after 24 h, plate was washed and different concentrations (1, 10, 20, 30, 40, and 50 µg/ml) of auranofin were added to wells, then plate was incubated at 37°C with 5% CO_2. After this time, 20 µl MTT (5 mg/ml) reagent was added per well, and plate was incubated for 4 h. Then, supernatant was removed and 100 µl DMSO (dimethyl sulfoxide) was added to each well and after 10 min, OD was read at 540 nm. The percentage of viability of the cell is calculated by following the formula:

[AT-AB]/[AC-AB] ×100

Where, AB, AC and AT are OD of blank well, negative control and treated cells, respectively.

Using the results of the MTT test and IC_{50}, the SI index was calculated.

A 100 µl of the medium contained 10^6 *leishmania* promastigotes cultured in 24 well plates, and different concentrations of Auranofin (µg/ml) were separately added to the wells. Forty-eight hours after adding the auranofin, the number of parasites was counted. All of these experiments were repeated three times and the average of all three replicates was calculated. Furthermore, three wells without auranofin were considered as negative control. IC_{50} of auranofin was calculated after 48 h using GraphPad Prism5 software.

Macrophages were cultured on a glass coverslip in tissue culture 12-well plates (1×10^5 cells/well) (18) and incubated at 37°C with 5% CO_2 for 24 h. Supernatants were removed after 24 h and 500 µl RPMI 1640 containing 10^7 promastigotes in the stationary phase were added to each well. In order to place the parasites inside macrophages, 24 h incubation was done. After this time, different concentrations of auranofin were added to each well and after 24 and 48 h, coverslips were separated and stained with Giemsa stain and amastigote number per macrophages was counted.

The Annexin V-FITC Apoptosis Detection Kit (Biovision; USA) was used. Twenty-four hours after treatment, promastigotes were collected and 500 µl of the bonding buffer was added to each sample. Further in the dark conditions, 5 µl of the Annexin-V and PI solution was added. After 5 min incubation at the room temperature and dark (42), the intensity of the color absorbed by the cells was measured by a flow-cytometric apparatus (BD FACSCanto II Flow cytometer). Finally, the results were analyzed by the special software (Flow jo Software).

To evaluate the fragmentation of DNA, Apoptotic DNA Ladder Detection Kit (abcam) was used. The promastigotes encountered with different concentrations of auranofin are collected after 48 h. According to the Kit protocol, DNA of treated *leishmania* was extracted and electrophoresed in 1.2% agarose gel containing 0.5 µg/ml ethidium bromides. Ethidium bromide-stained DNA was 48 hours after treatment to conduct topographic studies and investigate ultrastructural changes in *L. major,* scanning electron microscopy was used and images were taken. For this purpose, the parasites were washed in sodium cacodylate buffer pH 7.2 and preserve in

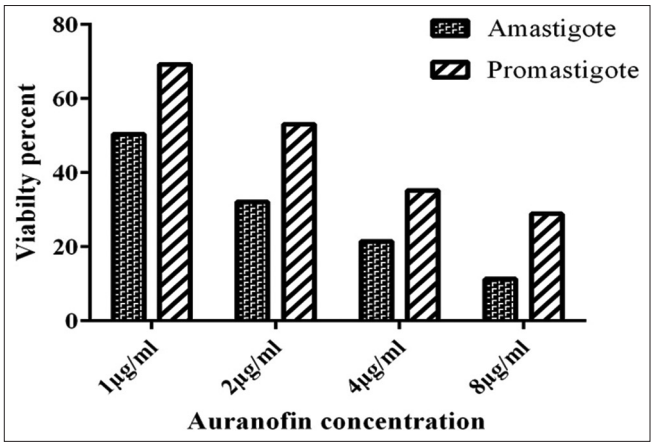

Figure 1: Viability percent of amastigote and promastigote of *L. major*, 24 h after treatment with auranofin

a primary fixative (2.5% glutaraldehyde in 100 mM sodium cacodylate buffer) and then samples were fixed in 2% OsO_4 in cacodylate buffer and then washed with distilled water and then immersed in ethanol 50%–70% for dehydration, then the samples were immersed in hexamethyl-disilazane and finally after coating with gold, images were taken by the scanning electron microscope.

Results

The results showed that auranofin has good antileishmanial effect. After 24 and 48 h, viability percent of promastigotes and amastigotes decreased significantly (differences between groups were significant based on drug concentration and time), and these results were more evident at the concentration of 8 µg/mL [Figures 1 and 2]. According to the obtained results, the IC_{50} of auranofin after 24 h was calculated as 2.38 and 1.007 µg/ml against promastigotes and amastigotes, respectively.

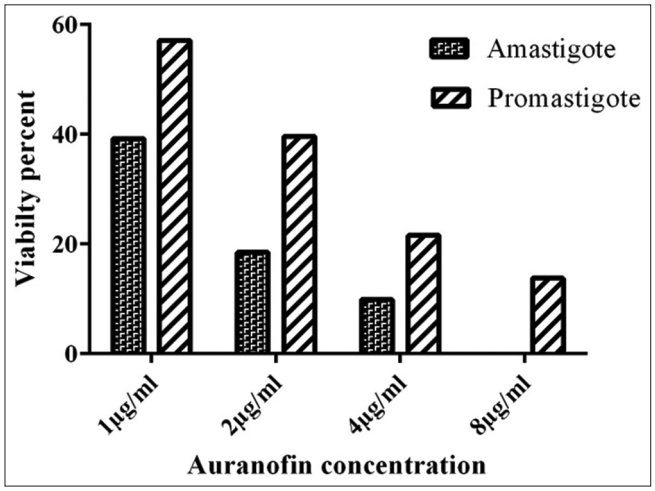

Figure 2: Viability percent of amastigote and promastigote of *L.major*, 48h after treatment with auranofin

CC_{50} (50% cytotoxic concentration) was determined and then SI (selectivity index) was calculated by the following formula: SI = CC_{50} against j774 cell line/IC_{50} against amastigote. SI was 11.7 µg/ml.

Auranofin-induced apoptosis in promastigotes of *L. major* 48 h after treatment. Early and late apoptosis, necrosis, and live cell percent are presented in [Figure 3]. The highest percentage of induction of apoptosis was observed in the 8 µg/ml.

Electrophoresis of the extracted DNA from the treated *L. major* promastigotes showed that auranofin-induced fragmentation in the promastigotes. Fragmentation is quite evident and is lower at 1 µg/ml concentration than the other concentrations of auranofin, while in the untreated (control) group, the fragmentation has not occurred [Figure 4].

Auranofin has caused a change in the shape of the body and free flagellum of the *L. major* promastigotes. In the control group, shape of promastigote was normal, but in treated sample, the changes were quite evident [Figure 5].

Discussion

Despite extensive research in the treatment of this disease, researchers have still failed to introduce an effective and

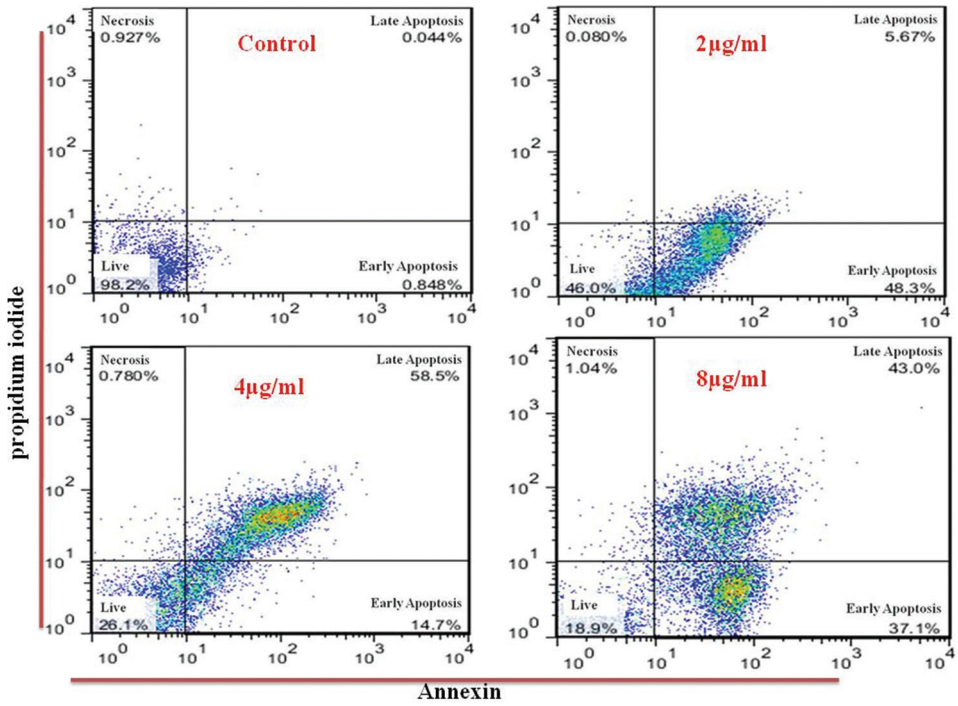

Figure 3: Flow cytometry anlysis. Promastigotes staining with Annexin V and Propidium Iodide after treatment with different concentrations of auranofin after 48 h

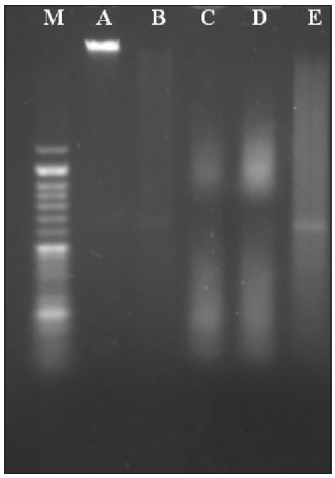

Figure 4: Gel electrophoresis of extracted DNA of *L. major* after treatment with different concentrations of auranofin. M: marker, a: without treatment, b: 1μg/ml, c: 2μg/ml d: 4μg/ml, e: 8μg/ml

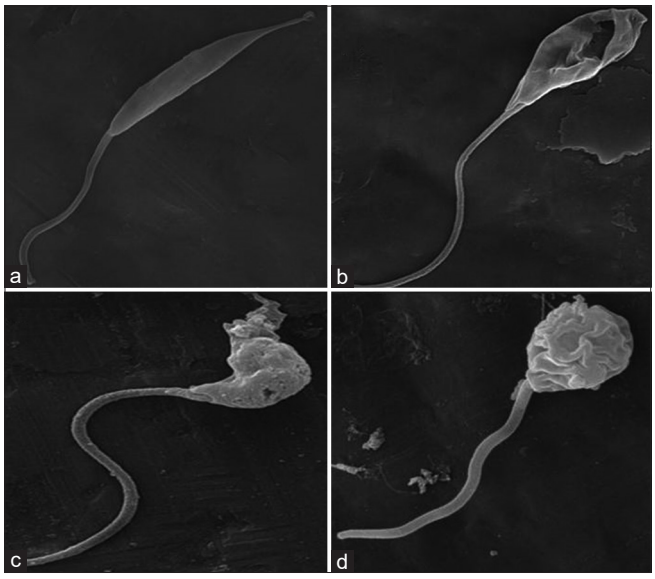

Figure 5: Changes in body shape and flagella of promastigote after adding auranofin in culture media. a: Control group (without auranofin), b: 2μg/ml of auranofin, c: 4μg/ml of auranofin, d: 8μg/ml of auranofin

nontoxic drug that has no adverse effects on the body.[30] Various herbal extracts, alone or in combination, as well as many chemical drugs, have been investigated for the treatment of this disease, but similar studies have continued to achieve this goal.[30,31] In recent years, the use of gold compounds has been used to treat many diseases and against a wide range of microorganisms.[19,32] Auranofin is a compound containing gold drug that was approved by the US Food and Drug Administration in 1985 for the treatment of rheumatoid arthritis.[16,33] The results of this work showed that IC_{50} against promastigote stage was 2.38 μg/ml. In addition, the findings of this study showed that auranofin had an inhibitory effect on the amplification of intramacrophage amastigotes. The IC_{50} against the amastigotes stage was 1.007 μg/ml. Generally, the effect of this drug on both forms was time and dose dependent, so that with increased concentration and duration of effect, the growth rate of the parasite decreased. The SI index calculated in this study was 11.7 which indicated that the drug was a nontoxic drug. Flow cytometry test was performed using two colors of annexin-V and propidium iodide. Flow cytometric results showed that the highest apoptosis (80.1%) was induced by 8 μg/ml of auranofin after 48 h, this result indicates that cell death occurs in the induction of apoptosis and cell death has not occurred through necrosis. The highest incidence of necrosis was <2%, indicating that auranofin is an effective agent for the induction of apoptosis. The qualitative changes that occurred in *L. major* DNA due to the presence of auranofin were investigated in this study. The DNA of the parasites affected by auranofin was fragmented, indicating the destruction of the parasite and the formation of apoptotic fragments. A study conducted by Sharlow *et al.*(2014) showed that auranofin possesses inhibitory effects on the growth of promastigote and intracellular amastigotes and the EC_{50} obtained from the amastigotes of *L. major* and *L. amazonnesis* was calculated to be 0.07 and 0.27 μM, respectively.[34] Kaiser *et al*. in a study on *Trypanosome brucei rhodesience* using auranofin, it was concluded that the antiparasitic activity of auranofin against this protozoa is desirable and significant and this action is performed by inhibiting kappa B kinase and thioredoxin reductase (IC_{50} = 0.01 μm).[35] In another study, Tejman-Yarden *et al*. showed that auranofin has antiparasitic effects against *Giardia lamblia* (especially metronidazole resistant *Giardia*) by inhibiting TrxR.[16] In another study, da Silva *et al*. showed that auranofin has antiparasitic activity against *T. cruzi*.[23] Previous studies have shown that triple cytotoxic compounds of gold can cause cell death through apoptosis. Nevertheless, for most gold compounds, apoptosis is mainly caused by direct mitochondria damage and is not the result of a primary DNA lesion.[36-38] In this framework, the pivotal role of thioredoxin reductase has been considered as a possible target for gold compounds. The prevention of thioredoxin reductase activity may eventually lead to a change in mitochondrial membrane potential, Cytochrome c release, and subsequently an apoptotic event.[36]

Conclusion

Auranofin has an anti-leishmanial activity, which was evident from the results obtained from various tests. Given the suitability of SI for drug use, auranofin can be a good choice for complementary and animal studies in the treatment of cutaneous leishmaniasis.

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Conflicts of interest

There are no conflicts of interest.

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