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Original article

# Licochalcone A induces morphological and biochemical alterations in *Schistosoma mansoni* adult worms

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ABSTRACT

This paper is the first report on the *in vitro* effects of licochalcone A, a chalcone isolated from *Glycyrrhiza inflate* Batalin (Leguminosae), on *Schistosoma mansoni* adult worms. *In vitro*, licochalcone A afforded lethal concentrations for 50% of parasites ($LC_{50}$) of 9.12 ± 1.1 and 9.52 ± 0.9 μM against female and male adult worms, respectively, at 24 h. Additionally, the compound reduced the total number of *S. mansoni* eggs and affected the development of eggs produced by *S. mansoni* adult worms. Together, the results achieved after 24 h showed that licochalcone A was 55.7- and 53.3-fold more toxic to *S. mansoni* female and male adult worms than to Chinese hamster ovary fibroblasts cells, respectively. Treatment with licochalcone A elicited drastic changes in the tegument of *S. mansoni* adult worms, as well as mitochondrial alteration and chromatin condensation. Licochalcone A also increased the superoxide anion level and decreased the superoxide dismutase activity in *S. mansoni* adult worms. Overall, our results indicated that licochalcone A displays *in vitro* schistosomicidal activity. This effect may result from increased production of reactive oxygen species (ROS) induced by the action of licochalcone A. The resulting ROS could act on the *S. mansoni* tegument and membranes and help induce the death of *S. mansoni* adult worms.

## 1. Introduction

Schistosomiasis is a neglected tropical disease (NTD) caused by trematode worms of the genus *Schistosoma*. This disease affects more than 200 million people worldwide, and the number of people at risk of infection is estimated to be between 600 and 779 million [1,2]. It causes over 300,000 deaths annually and leads to the loss of 1.53 million active lives per year due to disability of adjusted life (DALYs) [3].

Since the introduction of praziquantel (PZQ) in 1980, patients with schistosomiasis have been treated with this drug [2]. PZQ is successful for many reasons: it is effective against all *Schistosoma* species, it is safe, it is largely available, it treats the disease in a single oral dose, it is inexpensive, and it dismisses the need for direct medical supervision [2]. However, there are also drawbacks to the use of PZQ, such as the emergence of *Schistosoma* strains that are less sensitive to PZQ, the low efficacy of PZQ in juvenile worms (between 7 and 28 days old), and PZQ's failure to prevent reinfection [4,5]. In addition, PZQ tablets are large and taste bitter, and there is no readily available pediatric formulation [6].

Reliance on a single antischistosomal drug is alarming and attracted the attention of the scientific community, who are searching for a lead compound to develop into a novel, inexpensive, safe, and effective drug against schistosomiasis [7,8]. Natural products, such as plants, are a rich and promising source for the discovery of novel biologically active compounds against neglected tropical diseases (NTD), including schistosomiasis [6,9,10].

Among the several classes of secondary metabolites derived from plants, chalcones are a group of plant-derived phenolic compounds belonging to the flavonoids family that possess a wide variety of cytoprotective and modulatory functions. This may have therapeutic potential for multiple diseases [11]. Licochalcone A is a characteristic

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chalcone of licorice; it is isolated from *Glycyrrhiza inflate* Batalin (Leguminosae) roots, a traditional medicinal plant [12]. The compound has been reported to possess anti-inflammatory, antimicrobial, antiparasitic, and antitumor properties [13–20], but effects on *Schistosoma mansoni* have not yet been reported.

This evaluated the effects of licochalcone A on *S. mansoni* adult worm parasites and examined its potential schistosomicidal action. The motility, viability and the alterations on tegument and organelles of *S. mansoni* were evaluated, and the cytotoxic effect on mammalian host cells was determined. In addition, the production of the superoxide anion and the activity of superoxide dismutase (SOD) were evaluated as a parameter of oxidative stress.

## 2. Material and methods

### 2.1. Obtainment of licochalcone A

The dried extract of *G. inflata* was provided by Shanghai Openchem International Co., Ltd. (Shanghai, China). Licochalcone A was obtained according to the methodology described by Fontes et al. [21]. $^{481}$H and $^{13}$C nuclear magnetic resonance (NMR) and spectral data verified the chemical structure of licochalcone A, which agreed with literature data [22,23]. The purity was estimated as above 95% by $^{13}$C NMR and HPLC using different solvent systems.

### 2.2. Parasite maintenance, recovery, and culture

The *S. mansoni* life cycle (LE – Luiz Evangelista strain) is routinely maintained by passage through *Biomphalaria glabrata* snails and BALB/c mice (female, six weeks old, 20–25 g) at the University of Franca. After 50 ± 2 days of infection with 200 ± 10 cercariae, the mice were euthanized, and the *S. mansoni* adult worm pairs were recovered under aseptic conditions by perfusion of their livers and mesenteric veins [24]. The worms were washed in RPMI 1640 medium (Inlab Diagnonóstica, São Paulo, BRA), kept at pH 7.5 with HEPES 20 mM, and supplemented with penicillin (100 UI mL), streptomycin (100 µg mL), and 10% bovine fetal serum (Cultilab, Campinas, BRA). Then, one or twenty adult worms pairs were transferred to a 24-well culture plate or a 25-cm$^2$ culture flask containing 2 mL or 20 mL of the same medium and incubated at 37 °C in a humid atmosphere containing 5% $CO_2$ prior to use.

The Ethics Committee for Animal Care of the University of Franca authorized all experiments (Approval number: 028/2012). All animals were handled in agreement with good animal practice guidelines as defined by the University of Franca in accordance with Brazilian law.

### 2.3. Motility evaluation

Motility was examined by visual inspection under an inverted microscope (Carl Zeiss, Göttingen, DEU). One pair of adult worms was transferred to each well of a 24-well culture plate containing the same culture medium described above. Licochalcone A previously dissolved in dimethyl sulfide (DMSO) (Sigma-Aldrich, St Louis, USA) was added to the culture medium to final concentrations of 3.125–200 µM. These concentrations were used because antiparasitic and antitumor activities were previously reported for licochalcone A [15–20]. Motility was evaluated after 6, 12, and 24 h of incubation. Worm motility was classified as normal motility, no motility (no movement after two minutes of observation), decreased motility (decreased motility compared to the negative control groups), and minimal movement (occasional movement of the head and body) [25]. After observation (24 h), the culture medium was removed, fresh culture medium without licochalcone A was added, and motility was re-examined for up to 24 h. As negative control groups, adult worm pairs were incubated in RPMI 1640 medium or in RPMI 1640 medium plus 0.1% DMSO. As a positive control, adult worm pairs were incubated with PZQ (1.56 µM). A total of 16 adult worm pairs were evaluated.

### 2.4. Viability assay

To verify parasite viability, adult worms pairs were incubated for 24 h with licochalcone A at the same concentrations described above, and the viability was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay [26]. Female and male *S. mansoni* worms (separated by action of licochalcone A or separated manually after treatment) were placed individually into wells (96-well plates) containing 100 µL of phosphate saline buffer (PBS) with 5 mg of MTT mL (Sigma-Aldrich) at 37 °C for 2 h. The solution was carefully removed and replaced with 200 µL of DMSO, and the worms were allowed to stand in DMSO at room temperature for 1 h. The absorbance was read at 550 nm using a spectrophotometer (Biochrom Corp, Miami, USA). The experiment was performed in quadruplicate and repeated three times. As negative control groups, adult worm pairs were incubated in RPMI 1640 medium or in RPMI 1640 medium plus 0.1% DMSO. As positive control groups, adult worm pairs were incubated with PZQ (1.56 µM) or heat-killed at 56 °C.

### 2.5. Evaluation of worm pairing, egg production, and egg development

Worm pairing and egg production were also examined by visual inspection under an inverted microscope (Zeiss). One pair of adult worms was transferred to each well of a 24-well culture plate containing the same culture medium described before, and licochalcone A previously dissolved in DMSO was added to the culture medium to final concentrations of 3.125 to 200 µM. After 6, 12, or 24 h, worm pairing and egg production were evaluated. To assess the effect of compound on *S. mansoni* egg development, one adult worm pair was transferred to each well and cultured as described above for 48 h, to allow for egg laying. After 48 h, the worms were removed, and licochalcone A was added at the same concentrations described above for the medium containing eggs. Eggs were maintained at 37 °C in a humid atmosphere containing 5% $CO_2$. After 120 h of culture, egg development was evaluated with an inverted microscope (Zeiss) as described by Michaels and Prata [27]. As negative control groups, adult worm pairs were incubated in RPMI 1640 medium or in RPMI 1640 medium plus 0.1% DMSO. As a positive control group, adult worms pairs were incubated with PZQ (1.56 µM). The experiment was performed in quadruplicate and repeated three times.

### 2.6. Cytotoxicity assay

Cytotoxicity was measured with the aid of an *in vitro* Toxicology Colorimetric Assay Kit (XTT; Roche Diagnostics) according to the manufacturer's instructions. Chinese hamster ovary fibroblasts (CHO-K1 cells) were cultured in HAM-F10 (Sigma–Aldrich) and Dulbecco's modified Eagle's medium (DMEM) (Sigma–Aldrich) (1:1) supplemented with 10% FBS, antibiotics (100 µg/mL streptomycin and 50 U/mL penicillin) and 2.38 mg/mL HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (Sigma–Aldrich) at 37 °C with 5% $CO_2$. For these experiments, cells ($10^4$ cells/well) were plated in 96-well microplates. Each well received 100 µL of DMEM medium (Sigma–Aldrich) containing licochalcone A at concentrations ranging from 1.56 to 800 µM dissolved in 0.2% DMSO for 24 h. Negative (without treatment), solvent (0.2% DMSO), and positive (25% DMSO) controls were included. After incubation at 37 °C for 24 h, the medium was removed, and the cells were washed twice with 100 µL of phosphate-buffered saline (PBS) and exposed to 100 µL of DMEM medium without phenol red. Then, 50 µL of XTT was added to each well. The microplates were covered and incubated at 37 °C for 17 h. The absorbance was read at a test wavelength of 492 nm and a reference wavelength of 690 nm at 690 nm with a spectrophotometer (Biochrom). Three experiments were performed in duplicate.