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Original Article

A *Morinda royoc* Root Extract and Fractions Exhibit Antigiardial Activity without Affecting Cell Viability

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Abstract
Background: The gastrointestinal parasite *Giardia lamblia* causes giardiasis. Its treatment with standard drugs produces side effects and improper treatment can generate resistant strains. New antigiardial compounds are needed. An analysis was done to identify the antigiardial activity of *Morinda royoc*, a plant used in traditional Mayan medicine to treat stomach and bowel pain. We aimed to assess the efficacy of *M. royoc* roots against *G. lamblia* and their effect on cells viability.
Methods: A methanol extract was done of the root and then fractionated. The extract and fractions were tested in vitro on *G. lamblia* trophozoites and their effect on cell viability was quantified by flow cytometry. The active extract and fractions were analyzed by gas chromatography–mass spectrometry and high-performance liquid chromatography.
Results: The hexane fraction exhibited potent activity against *G. lamblia* (IC_{50} = 0.08 μg/mL). Its principal component was an anthraquinone-type compound. None of the fractions were toxic to human promyelocytic leukemia, chronic myelogenous leukemia and human mononuclear cells.
Conclusion: The medicinal plant *M. royoc* contains promising bioactive agents with antigiardial activity and deserves further research.

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Introduction

*G*iardia lamblia (syn. *Giardia duodenalis*, *Giardia intestinalis*) is an intestinal parasite that infects humans, domestic animals and wildlife worldwide. It is the causal agent of the disease known as giardiasis, a major cause of diarrhea contracted by ingestion of cysts via contaminated water and/or food. *G. lamblia* spread easily in human populations under conditions of poor hygiene, it can cause asymptomatic, acute or chronic disease. Its symptoms include diarrhea, abdominal discomfort, weight loss and malabsorption (1). The protozoa *G. lamblia* affects adults and children in underdeveloped nations; furthermore, approximately 33% of the population of these nations have presented giardiasis. According to Centers for Disease Control and Prevention (CDC), 15,223 cases were described in 2012 in United States. (2). The main effects of giardiasis in children are: malnutrition, growth decline, little cognitive function and death (3). Due to giardiasis affects development and socioeconomic improvements in developing countries, the WHO included this disease in its Neglected Diseases Initiative since 2004 (4).

Administration of imidazole and nitrofurans is the main treatment for giardiasis. However, treatment with these drugs is not always effective (3). These drugs also cause side effects and their indiscriminate use has generated strains resistant to these drugs (5-7). New, more effective and selective drugs are clearly necessary. Natural products of plant origin are a primary source of new drugs. Sometimes an effective molecule is used in its natural form while others are modified to improve their activity. About 50% of new drugs approved by the United States Food and Drug Administration (FDA) are products of natural origin or their derivatives (8). Mexico has an immensely diverse flora including approximately 26,000 plant species. Many of these are important elements in traditional medicine prac-

tices. The Yucatan Peninsula's flora has received extensive ethnobotanical study and many of its plant species have formed part of the botanical medicinal arsenal of the indigenous Maya culture for thousands of years (9).

Morindeae (Rubiaceae family) is a pantropical group of 160 species assigned to six genera. The most species-rich genus is *Morinda* (10). The medicinal plant *M. royoc* is a shrubby plant distributed throughout southern Mexico, Venezuela and the Antilles. On the Yucatan Peninsula it has various applications in the traditional medicinal practice of the Maya culture. Its fruit is roasted and the juice applied to remove warts. When roasted the roots are used to make a poultice for removal of varicose veins, and when boiled they are used to treat stomach pain and cancer. Other uses in traditional medicine include as a diuretic, an astringent, and as a treatment for snake bites, and kidney and liver problems (11-13).

As part of the search at Mexican Institute of Social Security for new antiprotozoal drugs, the present study objective was to evaluate the effects of an extract and fraction from *M. royoc* L. root on *G. lamblia*.

Materials and Methods

Plant material

Morinda royoc L. was collected in Caucel, Yucatán, México (20°59'52.7"N, 89°42'43.8"W) in June 2018. The plant material was identified by comparison with a voucher specimen from the Unit of Natural Resources of the Scientific Research Center of Yucatán (E. Ucán 1502, CICY).

Extraction

Powdered and dried roots of *Morinda royoc* L. (150 g) was macerated three times with methanol (3 × 200 mL) at room temperature. After complete extraction, the methanol was evaporated under reduced pressure to give a semi-

solid extract and it was evaluated against *G. lamblia*. According to antigiardial result, the methanol extract (4.1 g) was resuspended in a solution of methanol-water 1:1 (50 mL) and sequentially partitioned in solvents (50 mL) of increasing polarity (hexane, dichloromethane (DCM), ethyl acetate) and the residue that remained after partitioning produced a polar soluble sample. These fractions were evaporated completely by using rotary evaporator under reduced pressure to obtain dry fractions; afterwards these were evaluated against *G. lamblia*. All solvents of technical grade employed for chromatographic extraction were distilled prior to use.

Antiprotozoal assay

G. lamblia strain IMSS:0696:1 was cultured in TYI-S-33 modified medium, supplemented with 10% calf serum, 1 mg/mL bovine bile, 0.1 g/L streptomycin and 100 U/mL penicillin (reagents purchased from Sigma–Aldrich), and subcultured twice a week; for the assay, trophozoites were tested in their log phase of growth. Stock solution of extract and fractions (1mg dissolved in 1mL of DMSO) were added to microtubes containing 1.5 mL of medium in order to reach concentrations of 1.5 3.1, 6.2, 12.5, 25, and 50 µg/mL, leading to a final concentration of DMSO that was less than or equal to 1%. The solutions were inoculated with trophozoites of *G. lamblia* 4×10^4 and were incubated for 48 h at 37 °C. After time, trophozoites were measured with a hemocytometer (Neubauer chamber), and the percentage of trophozoite growth inhibition was calculated by comparison with the controls, as the negative control, trophozoites were incubated in culture medium with DMSO 1% (Sigma-Aldrich), this DMSO concentration no affect cell morphology and viability; metronidazole was used as positive control (0.1 to 5 µg/mL, Sigma-Aldrich). The 50% inhibitory concentration (IC_{50}) was calculated by Prisma GraphPad. The experiments were done in duplicate and repeated at least three times (14).

Cell culture

Human promyelocytic leukemia cells (HL60 ATCC CCL-240) and chronic myelogenous leukemia (K562 ATCC CCL-243) were purchased from ATCC and authenticated by STR repeats (Biosynthesis, Lewisville TX). Cell lines were grown in a maintenance medium culture: RPMI medium, supplemented with fetal bovine serum (20% in HL60, HMC and 10% in K562 cells), 1% penicillin-streptomycin, these were incubated at 37 °C in a humidified 5% CO_2 atmosphere. The Human Mononuclear Cells (HMC) were obtained from hematologically normal patients undergoing orthopedic surgery. The procedures were performed at the Hematology Department, Medical Specialties Hospital, La Raza Medical Center IMSS, Mexico City, and the Hip Surgery Department, Villa Coapa Hospital, IMSS, Mexico City, respectively. The HMC were isolated using Ficoll Paque Plus (Pharmacia Biotech, Uppsala, Sweden). Cells were then resuspended in RPMI advanced medium and total numbers of nucleated and viable cells were determined with a hemocytometer, using Turck solution and trypan blue stain, respectively. HMC were collected according to institutional guidelines, including written informed consent from each donor.

All procedures were approved by the Ethics and Scientific Committee at IMSS with number R-2013-3602-6 (15).

Cell viability assay

Cells (5×10^4) in maintenance medium were inoculated in a 48-well plate and were evaluated with extracts at different concentrations (6.25, 12.5, 25, and 50 µg/mL) for 48 h. After this time, cells were dyed with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) for 15 min. Then, the cells were analyzed immediately using a FACSCalibur Flow Cytometer (BD Bioscience, USA). Cells with media only (untreated) and with added Dymethyl sulfoxide 1% (DMSO) were also measured as nega-

tive controls, while cells treated with Partenolide 10 μM was used as a positive control in K562 cell line, dasatinib 100 nM was used as a positive control in HL60 cell line and hydrogen peroxide 50 μM (H_2O_2) as a damage control in HMC (15). The experiments were performed in triplicate.

GC-MS analysis

We analyzed methanol extract and hexane fraction of *M. royoc* L roots using gas chromatography-mass spectrometry (GC-MS). Gas chromatograms and mass spectra were obtained on an Agilent Technologies GC-MS instrument (models 6890N and 5975B) using the following chromatographic conditions: split injection; 1 mL sample at 1% concentration; Ultra 1 column (25 m × 0.2 mm i.d.); flow rate 1.0 mL/min (helium as carrier gas); oven temperature program: T1 = 180 °C (3 min), T2 = 280 °C (15 min), gradient 10 °C/min; injector and detector (FID) temperature at 280 °C. We analyze the main compounds presents in the bioactive extracts and there were identified by comparison with NIST 2005 base data, in addition to comparison with literature reports.

HPLC analysis

One milligram of the methanolic extract and fractions was dissolved in 1 mL of methanol. Furthermore, they were diluted with methanol at a final concentration of 50 mg/L. High-performance liquid chromatography (HPLC) was performed on an Agilent 1260 liquid chromatography system fitted with a quaternary pump, auto-sampler, and a diode array detector (DAD) set at 290 nm. It was employed a Zorbax Eclipse plus C-18 (100 x 4.6 mm, 5 µm) column (Agilent) coupled to an analytical guard column (Zorbax Extend C18, Agilent, 4.6 x 12.6 mm, 5 µm), operated with column temperature set at 40 °C. The initial mobile phase consisted in methanol (A)-water (B) (65:35, v/v) at a flow rate of 1.2 mL/min for a linear gradient elution as follows: a hold of 65%A for 15 min; increase to 80% A in 5 min with a hold to for 5 min; finally return to the original conditions in 5 min. The injection volume was 2 µL.

Results

Extraction

Methanol extraction of 150 g dried and powdered *M. royoc* L. root produced 6.4 g crude extract. A 4.1 g aliquot of the methanol crude extract was extracted using the organic solvents n-hexane, dichloromethane and ethyl acetate, as well as a residual methanol-water fraction; the hexane produced 0.82 g, the dichloromethane 0.49 g, the ethyl acetate 0.97 g and the aqueous residual fraction 1.1 g (Table 1).

Table 1: Yield and antigiardial activity of methanol extract of *M. royoc* roots and its fractions

Variable	Yield (%)	IC_{50} µg/mL
Methanol extract	4.26	1.13 ± 0.51
Hexane fraction	0.87	0.08 ± 0.03
Dichloromethane fraction	0.52	1.10 ± 0.23
Ethyl acetate fraction	1.03	1.09 ± 0.34
Methanol-water residual	1.17	5.21 ± 1.28
Metronidazole		0.21 ± 0.01

Antiprotozoal assay

The extract and fractions were screened for their ability to inhibit *G. lamblia* trophozoites.

The methanol extract and polarity ascendant fractions displayed significant inhibitory activity (0.08 - 5.21 μg/mL). However, the hexane

fraction exhibited an inhibitory effect more potent than the initial extract and the other fractions; the least potent was the methanol-water residual fraction (Table 1).

Cell viability assay

In order to evaluate the cytoxicity of methanolic extract and hexanic fraction from *M. royoc*, we carried out the evaluation against leukemia cells and Human Mononuclear cells through cell viability assay. Cell viability in the methanol extract and hexane fraction were assessed with flow cytometry (Fig. 1). Both

caused a concentration-dependent decrease in the K562 cell line (Fig. 1), with an increasing number of DAPI-stained cells (dead cells). Only a small decrease in cell viability (<12%) was observed after treatment with 1% DMSO, while the positive control (parthenolide) exhibited a significant cytotoxic effect. The assays using the HL60 and HMC cells produced similar results (Fig. 1). The CC_{50} concentration was ≥100 μg/mL in all evaluated cell lines. The dichloromethane, ethyl acetate and methanol-water fractions exhibited no activity (data not shown).
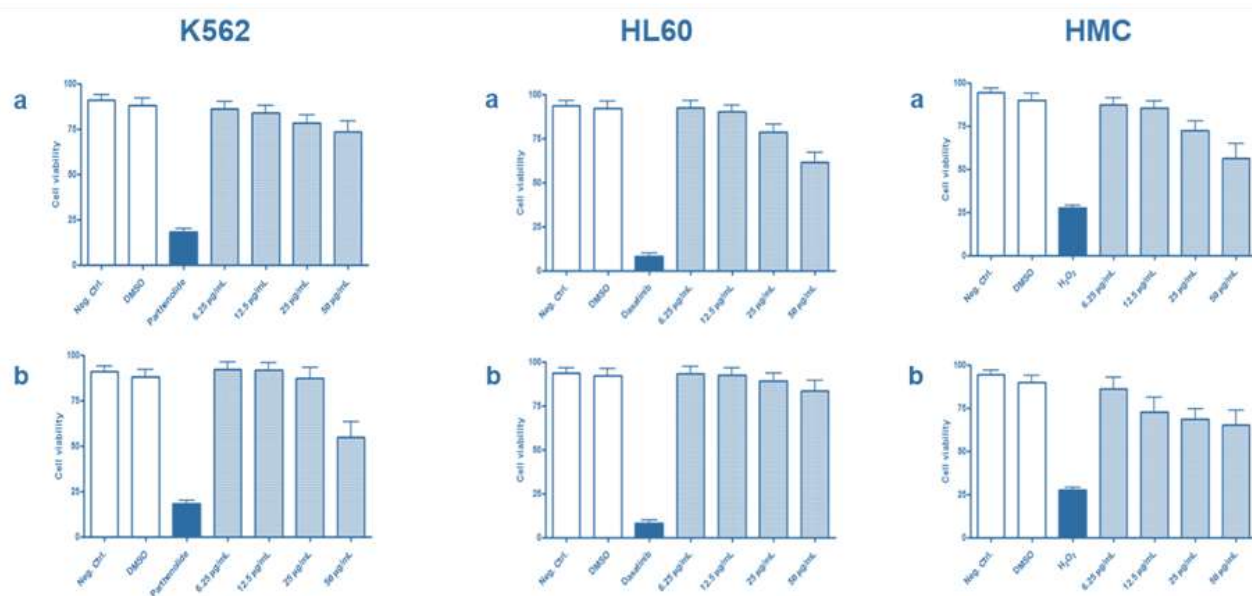

Fig. 1: Effect of *M. royoc* methanol extract (a), and hexane fraction (b) on K562, HL60, and HMC cell viability. DAPI-stained cells were analyzed by flow cytometry

GC-MS analysis

Chromatographic analysis of the *M. royoc* methanol crude extract and hexane fraction identified their principal chemical compounds (Fig. 2). In the methanol crude extract the principal compounds were soranjidiol (RT = 16.6 min, 15.04%, MS (70 eV) m/z rel. int. = 254 [M]$^+$ (100)) and morindone (RT = 14.9

min,13.23%, MS (70 eV) m/z rel. int. = 270 [M]$^+$ (100)). These were confirmed by comparison with previous reports on *Morinda* species and phytochemical analysis of *M. royoc* roots. The major chemical compound identified in the hexane fraction was morindone (RT = 14.9 min, 44.83%, MS (70 eV) m/z rel. int. = 270 [M]$^+$ (100)).
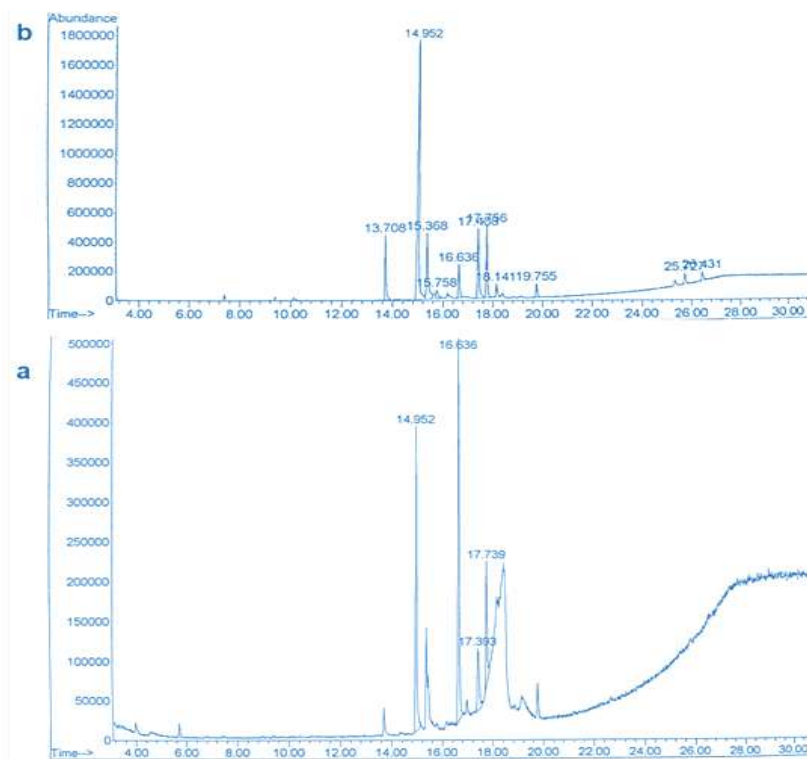
Fig. 2: A typical GC-MS chromatogram of the chemical constituents of methanol extract (a) and hexane fraction (b)

HPLC analysis

Analysis by HPLC coupled to a DAD detector set at 290 nm with a Zorbax Eclipse plus C-18 column was used to generate constituent complexity profiles of the methanol extract and hexane fraction (Fig. 3). The methanol extract (A) exhibited at least three major resolved peaks (1 to 3 min) and the hexane fraction (B) just one major constituent (RT: 9 to 10 min).
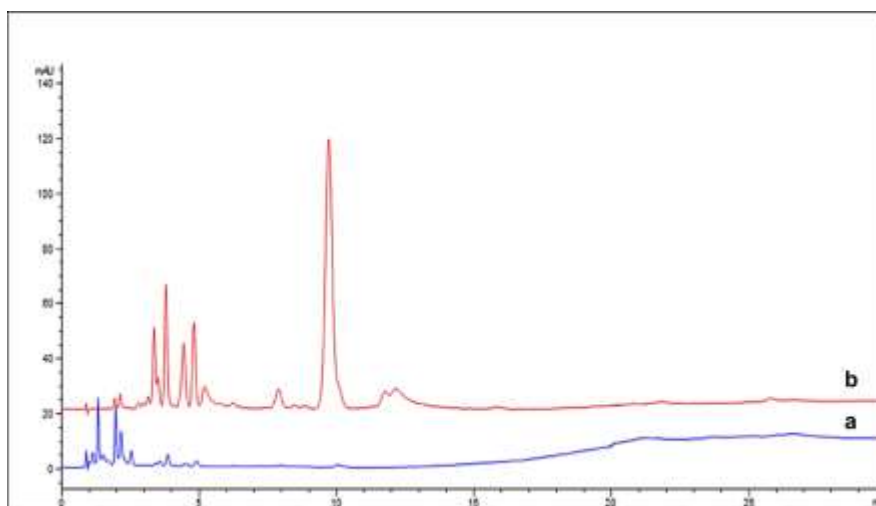

Fig. 3: HPLC chromatograms from *M. royoc* methanolic extract (a) and hexane fraction (b) at 290 nm

Discussion

As part of our research aimed at identifying new antiprotozoal agents in medicinal plants and explaining their use in traditional medicine in Mexico, we evaluated the activity of *M. royoc* root extract and fractions against *G. lamblia*. Both the extract and fractions were active against *G. lamblia* trophozoites (IC_{50} < 5.21 µg/mL); indeed, the hexane fraction was more active (IC_{50} = 0.08 µg/mL) than the positive control (metronidazole). To the best of our knowledge, this is the first report of the antigiardial activity of *M. royoc* L. root extract and its fractions. Giardiasis is one of the main causes of diarrhea among children and causes problems such as malabsorption and loss of weight, leading to delayed growth and development. Metronidazole is still the principal treatment for giardiasis, although it has reported toxicity and resistance. New alternative treatments for giardiasis are therefore necessary, and renewable natural sources such as *M. royoc* are promising.

The hexane fraction of *M. royoc* root had potent activity against *G. lamblia*, and did not exhibit complexity in the GC-MS and HPLC analyses. The observed antiprotozoal activity correlates with this fraction's anthraquinones content, suggesting its use as a phytomedicine in pharmaceutical applications. This is supported by studies of the antiprotozoal activity of anthraquinones from *Morinda* species. From roots cultured *in vitro* of *M. royoc*, the anthraquinone morindone showed antifungal (MIC = 1.9 µg/mL), and antibacterial activity (MIC = 15 µg/mL) (16). Three anthraquinones isolated from the stems of *M. elliptica* R. (5-hydroxy-2,2-dimethyl-4H-anthra[2,3-d][1,3]dioxine-6,11-dione; lucidin; and rubiadin-1-methyl ether) exhibited activity against both *Entamoeba histolytica* and *G. lamblia* (MIC = 7.8-125mg/mL) (17). The lucidin had the lowest MIC values (MIC = 31.25 vs. *E. histolytica* and 7.80 µg/mL vs. *G. lamblia*) but it was still less active than the metronidazole standard. In another study the anthraquinone lucidin-ω-isopropyl isolated from the roots of *M. panamensis* Seem exhibited activity against *Trichomonas vaginalis* (1.32 µg/mL) at a level slightly higher than the metronidazole control (6 µM = 1.03 µg/mL) (18).

The cell viability assays using human mononuclear cells, and the K562 and HL60 cell lines identified no toxicity at concentrations higher than those with antigiardial activity. In a similar study, an extract of root of the native plant *M. officinalis* and another extract of its hairy roots cultured *in vitro* had no toxicity to splenic lymphocytes in vitro (19). Additionally, the extracts attenuated dextran sodium sulfate-induced chronic ulcerative colitis in mice by regulating inflammation and lymphocyte apoptosis, as well as mitigating the symptoms of colitis, including diarrhea, body weight loss, colon shortening, histological damage and decreased inflammatory cytokine levels. A study evaluating the cytoprotective effects of a *M. officinalis* methanol extract against hydrogen peroxide-induced oxidative stress in Leydig TM3 cells found that treating the cells with 250 µg/mL of extract had a significant protective effect in the cell viability assay (20).

The GC-MS analysis identified the major components of the methanol extract and hexane fraction. Based on the profiles these are anthraquinone type compounds (21). The plant species of Rubiacea family are characterized by the production of anthraquinones-type compounds, these have been isolated from the roots. The *M. royoc* methanol extract had two major constituents, soranjidiol and morindone, while morindone was the only major constituent in the hexane fraction. These findings agree with previous reports of seven anthraquinones from *in vitro* cultured *M. royoc* L. roots, two of which were morindone (9,10-anthracenedione,1,2,5-trihydroxy-6-methyl-, molecular weight 270 g/mol) and soranjidiol (9,10-anthracenedione,1,6-dihydroxy-2-methyl-, molecular weight 254 g/mol) (22). Both have been reported in different *Morinda*

species. Perhaps one of the most studied species of this genus is *M. citrofolia*, commonly known as "noni". Anthraquinone compounds have been isolated mainly from this species, and morindone found to be produced mainly in its roots (23-26). Morindone and soranjidiol have also been isolated from the stems and roots of *M. elliptica* (17, 27). The HPLC analysis supported the GC-MS chromatogram in that it identified a single major compound in the *M. royoc* hexane fraction. This result will be important for standardizing the extract for future *in vivo* assays and possible applications as a phytomedicine.

The antigiardial activity shown in the present results support its traditional medicinal uses. For example, in Cuba *M. royoc* root has multiple uses including as an herbal supplement with purported stimulating, revitalizing and antistress activity, and as an ingredient in drinks claimed to have digestive, cleansing or aphrodisiac properties (22). An alcoholic extract of *M. royoc* root is also used in a nutritional supplement known as PV-2 (28).

Conclusion

A methanol extract and its hexane fractions from *M. royoc* root exhibited clear antigiardial activity in vitro, providing evidence-based support for its ethnomedical use. This is the first report of this kind of activity with this extract. In vivo and toxicological studies are planned to better define the potential therapeutic benefits of this extract. *M. royoc* clearly contains promising bioactive compounds and deserves extensive further research. The present results constitute an initial study in the ongoing search for new antiprotozoal agents and suggest that *M. royoc* root extracts are a potential prototype phytomedicine for development of new drugs against *Giardia lamblia*.

Acknowledgements

Conflicts of interest

We declare that there is no conflict of interest regarding the present study.

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