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BIOTECHNOLOGY

Larvicidal, cytotoxic and genotoxic effects of aqueous leaf extract of *Jatropha mollissima* (Pohl) Baill

José Rafael da Silva Araujo[1], Maria Wlly da Silva Costa[1], Wallace Baldez Oliveira[1], Reginaldo Roris Cavalcante[2], Pedro Marcos de Almeida[3] and Francielle Alline Martins[1*]

*[1]Centro de Ciências da Natureza, Universidade Estadual do Piauí, Rua João Cabral, 2231, 64002-150, Teresina, Piauí, Brazil. [2]Universidade Federal do Piauí, Teresina, Piauí, Brazil. [3]Centro de Ciências da Saúde, Universidade Estadual do Piauí, Teresina, Piauí, Brazil. *Author for correspondence. E-mail: franufv@yahoo.com.br*

ABSTRACT. The present study aimed to evaluate the larvicidal effect of aqueous leaf extract from *Jatropha mollissima* on the larvae of *Aedes aegypti* and analyze its cytotoxic and genotoxic activity in the *Allium cepa* test. Larvae of the mosquito were exposed to the negative and positive controls (distilled water and diflubenzuron, 0.003 mg mL^{-1}, respectively) and to leaf extract concentrations of 0.001, 0.005, 0.01, 0.02, 0.04, 0.06, 0.08 and 0.1 mg mL^{-1}. The mortality rate was evaluated every 24 hours over five days. For the cytotoxic and genotoxic analyses, roots of *A. cepa* were exposed to the negative (distilled water) and positive control (trifluralin, 0.84 ppm) and to different leaf extract concentrations (0.01, 0.1, 1 and 10 mg mL^{-1}) for 24 hours. The statistical analyses were performed by Kruskal-Wallis test ($p < 0.05$). The leaf extract presented promising larvicidal activity at the concentrations of 0.08 and 0.1 mg mL^{-1}, and none of the concentrations evaluated in *A. cepa* exhibited cytotoxic or genotoxic effect. Since the larvicidal action of *J. mollissima* and the absence of cellular toxicity have been demonstrated, further studies are recommended to determine the mechanism of action of the extract as a possible natural larvicide.

Keywords: *Aedes aegypti*; *Allium cepa* test; chromosome alterations; medicinal plant.

Efeitos larvicida, citotóxico e genotóxico do extrato aquoso das folhas de *Jatropha mollissima* (Pohl) Baill

RESUMO. O presente estudo teve como objetivo avaliar o efeito larvicida do extrato aquoso das folhas de *Jatropha mollissima* sobre as larvas de *Aedes aegypti* e analisar sua atividade citotóxica e genotóxica no teste *Allium cepa*. As larvas do mosquito foram expostas aos controles negativo e positivo (água destilada e diflubenzuron, 0,003 mg mL^{-1}, respectivamente) e ao extrato foliar nas concentrações de 0,001; 0,005; 0,01; 0,02; 0,04; 0,06; 0,08 e 0,1 mg mL^{-1}. A taxa de mortalidade foi avaliada a cada 24horas durante cinco dias. Para as análises citotóxica e genotóxica, as raízes de *A. cepa* foram expostas ao controle negativo (água destilada) e positivo (trifluralina, 0,84 ppm) e nas concentrações (0,01; 0,1; 1 e 10 mg mL^{-1}) do extrato foliar por 24 horas. Análises estatísticas foram realizadas pelo teste de Kruskal-Wallis ($p < 0,05$). O extrato foliar apresentou atividade larvicida promissora nas concentrações de 0,08 e 0,1 mg mL^{-1}, e nenhuma das concentrações avaliadas em *A. cepa* exibiu efeito citotóxico ou genotóxico. Uma vez demonstrada a ação larvicida de *J. mollissima* e a ausência de toxicidade celular, mais estudos são recomendados para determinar o mecanismo de ação do extrato como um possível larvicida natural.

Palavras-chave: *Aedes aegypti*; teste *Allium cepa;* alterações cromossômicas; planta medicinal.

Introduction

For thousands of years, several plants have been employed for medicinal purposes, from the simplest types of treatment up to the industrial manufacturing of drugs (Saraiva et al., 2015). Medicinal plants represent the largest sources of phytochemicals, showing various biological activities, such as antimicrobial (Dhale & Birari, 2010), antifungal (Silva et al., 2014), antimalarial (Jansen et al., 2010) and bioinsecticidal effects (Bosire, Deyou, Kabaru, Kimata, & Yenesew, 2014).

The interest as well as the utilization of natural products as potential larvicides have been increasing, as these products are less harmful to non-target organisms (Ali, Ravikumar, & Beula, 2013), besides regulating the growth of insects (Bezerra-Silva et al., 2015). Furthermore, they stand out as alternatives to conventional chemical pesticides, for instance organochlorides and organophosphates, which, upon indiscriminate use, lead to the selection of more resistant vectors, such as *Aedes aegypti* L. (Belinato & Valle, 2015). *A. aegypti* is a domestic,

anthropophilic mosquito with diurnal hematophagous activity, depending on clear water reservoirs for oviposition. It is a cosmopolitan species, occurring in tropical and subtropical regions. Apart from being the vector of dengue, yellow fever and chikungunya, it has recently been described as the main transmitter of the zika virus (ZIKV) in Brazil (Mustafa, Rasotgi, Jain, & Gupta, 2015).

Plant species with insecticidal activity against *A. aegypti* have been studied; however, aiming to reach the less favored layers of the population, studies in species of wide distribution and easy access should be prioritized. In this sense, *Jatropha* L. is one of the most representative genera in various parts of the world, comprising species with larvicidal potential, such as *J. curcas* L., *J. podagrica* Hook and *J. multifida* L. (Rampadarath, Puchooa, & Ranghoo-Sanmukhiya, 2014).

The larvicidal potential of *J. mollissima* (Pohl) Baill leaves has not yet been determined. The species is endemic to the semiarid region of Brazilian's northeast, where it is commonly known as 'pinhão bravo', being widely used in folk therapy. Its latex *in natura* exhibits antimicrobial activity (Rocha & Dantas, 2010), and can be used to treat snakebites and heal wounds (Oliveira, Lins Neto, Araújo, & Albuquerque, 2007). The plant's seeds are used as tranquilizer and antidepressant (Saraiva et al., 2015). Its leaves are antioxidant (Melo et al., 2010), appetite-enhancing, and applied in renal infections (Almeida, Silva, Amorim, Maia, & Albuquerque, 2005), while the stalk presents anthelmintic activity (Ribeiro et al., 2014).

Although *J. mollissima* exhibits various therapeutic advantages, different chemical constituents found in the species can be potentially toxic, mutagenic, carcinogenic and/or teratogenic. Overall, the genus *Jatropha* presents toxic phytochemicals, such as diterpenes and ricin, a toxalbumin that causes vomit, diarrhea, dehydration, and renal and hepatic damage (Sabandar, Ahmat, Jaafar, & Sahidin, 2013). This way, as for any plant extract with larvicidal and/or medicinal potential, the toxic effects of *Jatropha* should be assessed regarding their risks to the environment, and consequently the human health, in the short and long term (Ping, Darah, Yusuf, Yeng, & Sasidharan, 2012).

Among the methods available to evaluate toxicity, mutagenicity and genotoxicity, the bioassay using *Allium cepa* L. can be highlighted, owing to its low cost and reliability. Moreover, it presents good correlation with other test systems, such as the mammalian system using mice (Fedel-Miyasato et al., 2014), cell culture systems (Malini et al., 2010), and the *Oreochromis niloticus* erythrocyte assay (Hemachandra & Pathiratne, 2016), with the advantage of excluding the need for animal use and sacrifice (Leme & Marin-Morales, 2009). Additionally, the *A. cepa* test system has been validated by the World Health Organization, the United Nations Environmental Program, and the United States Environmental Protection Agency (Mauro et al., 2014). According to Leme and Marin-Morales (2009), this test system allows the simultaneous assessment of cytotoxic, genotoxic and mutagenic effects of a given compound, environmental samples or natural products, without the need of performing different assays.

Considering the importance of plants with larvicidal effect as alternatives to conventional chemical pesticides, and the need to perform toxicological studies, the present work aimed to evaluate the larvicidal potential of the leaf extract from *J. mollissima* on the larvae of *A. aegypti*, as well as to analyze its toxic, cytotoxic, genotoxic and mutagenic effects on meristematic cells in the *A. cepa* assay.

Material and methods

Biological material

Leaves of *J. mollissima* were collected from an adult plant in Luís Correia – PI (Northeast of Brazil, geographical coordinates 2° 56' 01.1" S 41° 32' 39.2" W) in January 2015. Herbarium specimens containing leaves, flowers and fruits were stored at the Herbarium Afrânio Fernandes at the *Universidade Estadual do Piauí* (Uespi, Teresina, state Piauí, Brazil; voucher specimen number HAF 03111). The larvae of *A. aegypti* were collected at the foci of the mosquito in the city of Teresina (state Piauí, Brazil) and taken to the Entomology Center at the *Universidade Federal do Piauí*. The seeds of *A. cepa* cv. Vale Ouro IPA-11 used in the bioassays were kindly provided by the Agronomic Institute of Pernambuco (IPA, Recife, state Pernambuco, Brazil).

Leaf extract preparation

Young leaves of *J. mollissima* were dried in incubator at 45°C for 5 days at the Genetics Laboratory of Uespi, and subsequently ground to a fine powder using a blender. The aqueous leaf extract (ALE) was then obtained by diluting 2 g of the leaf powder in 200 mL of distilled water. The solution was agitated for 5 min and stored at 5°C for 48 hours. Next, the solution was percolated through filter paper, and dilutions were made with distilled water to yield concentrations of 0.1, 0.08, 0.06, 0.04,

0.02, 0.01, 0.005 and 0.001 mg mL^{-1} for the larvicidal assays, as well as 10, 1, 0.1 and 0.01 mg mL^{-1} for the *A. cepa* assays.

Colony of the mosquito *A. aegypti*

A. aegypti larvae were collected at the foci of the mosquito in the city of Teresina (PI, Brazil) and taken to the Entomology Center at the *Universidade Federal do Piauí* for screening and establishment of the mosquito population. All collected larvae were identified under a stereomicroscope (Olympus), according to the key proposed by Consoli and Oliveira (1994) and Forattini (2002). Eggs were collected from the colony and placed in mineral water (28 ± 5ºC) for hatching, with 12 hours photoperiod, and fed crushed cat feed until reaching the third stage of development. The male adults were fed 10% sucrose solution, while the females were fed chicken blood.

Larvicidal assay

The bioassay was performed according to the norms for laboratory larvicidal tests (World Health Organization [WHO], 2005), with some modifications. For each Petri dish, 20 mL of the above mentioned concentrations were used. Distilled water was applied as negative control (NC) and diflubenzuron (DFZ) as positive control, at the concentration of 0.003 mg mL^{-1}, as recommended by the municipal health surveillance of the city of Teresina – PI. For each concentration, 20 larvae at the third stage were tested in triplicate. The larvae and the controls were maintained under the same temperature and light conditions. The number of dead larvae was determined every 24 hours from the beginning of exposure, over five days. The test was conducted until death of all the larvae or verification of only pupae in the treatments.

Allium cepa assay

One hundred seeds of *A. cepa* were germinated on Petri dishes containing filter paper moistened with distilled water, at room temperature, at the Genetics Laboratory of the Uespi. After germination, the seeds were transferred to the negative control (distilled water), positive control (trifluralin, 0.84 ppm) for establishment of aneugenic and clastogenic effects (Fernandes, Mazzeo, & Marin-Morales, 2009), or to each aforementioned concentration of the leaf extract (one dish per concentration) for 24 hours. Subsequently, the root tips were fixed in ethanol: acetic acid solution (3:1) and stored at -20°C until slide preparation.

For slide preparation, the root tips were washed three times in distilled water, for 5 min each time, and hydrolyzed at 60°C for 10 min in 1N HCl. After hydrolysis, the root tips were again washed in distilled water and transferred to amber glass bottles containing Schiff's reagent, in which they were kept for 2 hours in the dark. Next, the root tips were washed until complete removal of the reagent, transferred onto slides, squashed with one drop of 2% acetic carmine, and mounted with Entellan® (Almeida et al., 2016).

Toxicity was evaluated by the mean root length variation (in centimeters) of 30 roots per treatment. The experimental unit consisted of one individual root per specimen. Cytotoxicity and genotoxicity were evaluated by scoring 5,000 meristematic cells (experimental unit: slide with 500 cells, with a total of 10 analyzed slides per treatment) under light microscope (Olympus CX 21) at 400x magnification. The assessed aspects were: (1) mitotic index (MI; cytotoxicity); (2) micronuclei (MN; mutagenicity at chromosome level); and (3) chromosome alterations (genotoxicity). The latter included alterations resulting from aneugenic activity (*e.g.* C-metaphases, metaphases with chromosome adherence, lost chromosomes, multipolar anaphases, binucleate cells, polyploid metaphases, among others) or clastogenic effects (*e.g.* chromosome fragments in metaphase or anaphase, chromosome bridges, and others). Micronuclei may arise from both aneugenic or clastogenic effects.

Statistical analysis

Mortality rates and assessment of toxicity, cytotoxicity, genotoxicity and mutagenicity were evaluated by the non-parametric test of Kruskal-Wallis, followed by *a posteriori* test of Student-Newman-Keuls ($p < 0.05$) using the BioEstat 5.3 program (Ayres, Júnior, Ayres, & Santos, 2007).

Results and discussion

In the larvicidal test, the mortality rate (%) of the larvae in the presence of DFZ was significant when compared to the negative control. The mortality rates at the concentrations of 0.001, 0.06, 0.08 and 0.1 mg mL^{-1} of *J. mollissima* ALE were statistically equal to those of DFZ (Figure 1); at 0.001, 0.005, 0.01, 0.02, 0.04 and 0.06 mg mL^{-1}, the rates were statistically similar to those of the NC.

In the toxicity evaluation, the mean lengths of *A. cepa* radicles at the different concentrations of ALE (0.01, 0.1, 1 and 10 mg mL^{-1}) did not present

significant statistical differences when compared to the NC (Table 1). Similarly, no significant statistical difference was observed between the mitotic index (MI) of the treatments and of the NC (Table 1).

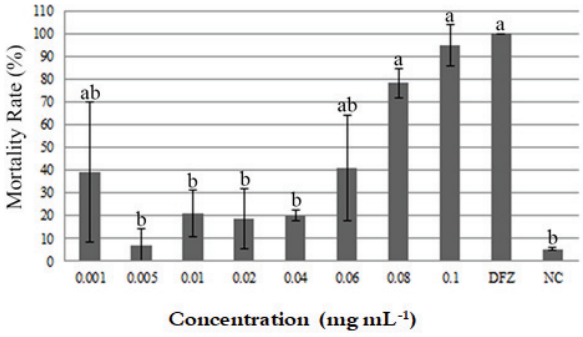

Figure 1. Mortality rate (%) among *Aedes aegypti* larvae evaluated every 24 hours, over five days, from beginning of exposure to different concentrations of aqueous leaf extract of *Jatropha mollissima*. Averages followed by the same letter were not significantly different by the test of Kruskal-Wallis with *a posteriori* test of Student-Newman-Keuls ($p < 0.05$). The results refer to the analysis of 60 third-stage larvae per treatment. NC: Negative control. DFZ: Diflubenzuron, positive control (0.003 mg mL^{-1}).

Table 1. Mean length values for root tips and mitotic indexes in meristematic cells of *Allium cepa* after 24 hours exposure to the aqueous leaf extract of *Jatropha mollissima* at different concentrations.

Treatment mg mL^{-1}	Mean Root Length (cm)	Mitotic Index (Mean ± SD)
Distilled Water	2.98 ± 1.04	124.00 ± 35.60
0.01	2.61 ± 1.09	180.00 ± 41.70
0.1	2.98 ± 1.23	169.00 ± 38.35
1	2.73 ± 1.16	178.00 ± 37.50
10	2.40 ± 0.99	148.00 ± 56.00
Trifluralin (0.84 ppm)	1.39 ± 0.24*	85.70 ± 12.27*

Distilled Water: negative control. Trifluralin: positive control. SD: standard deviation *Significant by Kruskal-Wallis test with *a posteriori* Student-Newman-Keuls test (*$p < 0.05$). The results refer to the mean length value of 30 roots and analysis of 5,000 cells per treatment.

With regard to genotoxicity, all tested concentrations of ALE presented non-significant total index of chromosome alterations in meristematic cells when compared to NC (Table 2). Moreover, the mean value of each chromosome abnormality was evaluated and no significant occurrence of these alterations was observed at the applied ALE concentrations; therefore, ALE is not considered to have genotoxic effect (Table 2). In addition, also the mean value of MN in the treatments was not significant in comparison to NC, indicating absence of mutagenicity at chromosome level.

Dengue, chikungunya and zika have become a problem around the world, having serious negative effects on the human health and stimulating different governmental entities to search for solutions to the epidemiological increase of these diseases (WHO, 2016). Control of the respective viruses has been based on intervention in the life cycle of the vector mosquito *A. aegypti*, based on chemical larvicides and insecticides. However, development of resistance by the mosquito has been reported as a result of the wide application of these agents, for instance DDT, pyrethroids and DFZ (Ishak, Jaal, Ranson, & Wondji, 2015). In this sense, the search for plants with larvicidal potential has become a viable alternative, owing to their less harmful nature both to humans and to the environment (Ali et al., 2013).

In the present study, the aqueous leaf extract of *J. mollissima* at the highest concentrations (0.08 and 0.1 mg mL^{-1}) showed promising effects against third-stage larvae of *A. aegypti*, with mortality rates higher than 75% (Figure 1). This result is in agreement with the World Health Organization (WHO, 1981). Similar observations have been reported for the aqueous extract (Bassem, Larbi, & Hela, 2014) and the petroleum ether (Rahuman, Gopalakrishnan, Venkatesan, & Geetha, 2008b) of *J. curcas* leaves. These results may be related to the presence of β-sitosterol, which presents high larvicidal activity (Rahuman, Gopalakrishnan, Venkatesan, & Geetha, 2008a). This phytochemical has also been isolated in *J. mollissima* (Santos & Mukherjee, 1992) and might justify the larvicidal action observed in the present study.

Table 2. Mean of chromosome alterations in meristematic cells of *Allium cepa* radicles after 24 hours exposure to the aqueous leaf extract of *Jatropha mollissima*.

Chromosome Alteration	Negative Control	Treatments Aqueous Extract (mg mL^{-1})				Positive Control
	Distilled water	0.01	0.1	1	10	Trifluralin (0.84 ppm)
CA	1.3 ± 1.1	1.6 ± 2.5	3.5 ± 2.1	3.3 ± 1.7	3.5 ± 2.5	5.7 ± 3.6*
C-m	1.6 ± 0.8	0.2 ± 0.3	1.8 ± 1.3	1.6 ± 1.2	1.5 ± 0.6	11.5 ± 7.2*
PC	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	4.1 ± 4.6*
CL	0.0 ± 0.0	0.2 ± 0.2	0.4 ± 0.4	1.0 ± 0.4	0.1 ± 0.1	1.3 ± 0.9*
BC	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.3 ± 0.2	1.3 ± 0.8*
MA	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NB	0.0 ± 0.0	0.2 ± 0.3	0.7 ± 0.6	0.2 ± 0.2	0.2 ± 0.3	4.3 ± 1.9*
CB	0.2 ± 0.1	0.9 ± 1.2	0.3 ± 0.3	0.2 ± 0.2	0.1 ± 0.1	2.1 ± 1.5*
MN	0.1 ± 0.2	0.8 ± 0.6	0.1 ± 0.2	0.4 ± 0.5	0.2 ± 0.3	10.4 ± 3.9*
CF	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.2	0.0 ± 0.0	0.6 ± 0.3*
Total	3.3 ± 2.3	3.9 ± 5.1	7.3 ± 5.2	6.9 ± 4.6	5.9 ± 3.9	41.3 ± 13.7**

CA: Chromosome Adherence. C-m: C-metaphase. PC: Polyploid Cell. CL: Chromosome Loss. BC: Binucleated Cell. MA: Multipolar Anaphase. NB: Nuclear Bud. CB: Chromosome Bridge. MN: Micronuclei. CF: Chromosome Fragment. *Significant by Kruskal-Wallis test with *a posteriori* Student-Newman-Keuls test (**$p < 0.01$; *$p < 0.05$). The results refer to the analysis of 5,000 cells per treatment.

In addition, the occurrence of other phytochemicals, such as tannins, saponins, coumarins and alkaloids, in the leaves of *J. mollissima* (Araújo, Alencar, Amorim, & Albuquerque, 2008; Melo et al., 2010) may be related to their larvicidal activity. Tannins significantly reduce the growth and survival of larvae, as these molecules inactivate digestive enzymes and give rise to a tannin-protein complex of difficult digestion (Cavalcante, Moreira, & Vasconcelos, 2006). Saponins also reduce the activity of digestive enzymes and the absorption of nutrients by the larvae, and further decrease the mucosa lining of their digestive system, rendering it corrodible (Bagavan, Rahuman, Kamaraj, & Geetha, 2008). Coumarins, as well as alkaloids, may act as inhibitors of acetylcholinesterase (AChеI), an enzyme that promotes the uptake of acetylcholine at the neuronal synapses, thus leading to paralysis and death of the larvae (Khanikor, Parida, Yadav, & Bora, 2013). The activity of AChеI has been confirmed in the leaf extracts of *J. curcas* and *J. gossypiifolia* (Feitosa, Freitas, Luz, Bezerra, & Trevisan, 2011). However, the presence of the mentioned phytochemicals at lower concentrations (0.005, 0.01, 0.02 and 0.04 mg mL^{-1}) might possibly have a protective effect, thus justifying the absence of larvicidal potential. In addition, the inactivity (mortality rate < 25%, Figure 1) of the extract at the cited concentrations is in agreement with the WHO (1981). Moreover, the concentrations of 0.001 and 0.06 mg mL^{-1}, which were statistically equal to both controls, may reflect the action of phytochemicals presenting double effect (toxic and protective), as observed for tannins by Chung, Wei, and Johnson (1998). These concentrations of the extract are weakly promising according to the WHO (1981), as they present mortality rate of 25-50% (Figure 1).

The ALE was evaluated with regard to toxicity, cytotoxicity, genotoxicity and mutagenicity in the *A. cepa* bioassay, which presents agreement with other bioassays, such as those in mammals (Fedel-Miyasato et al., 2014). Thereby neither toxic nor cytotoxic activity was observed at the studied leaf extract concentrations, as also verified by Melo et al. (2010) in Hep-2 and NCI-H292 tumor cells. According to those authors, the results may possibly be related to the tannins with antioxidant action, thus justifying the absence of cytotoxic effects.

In addition, the non-significance of mean values for chromosome alterations (total or individual) at the applied ALE concentrations reinforces a protective action of the cited compounds in the meristematic cells of *A. cepa*, not interfering with the processes of chromatin condensation, polymerization of the spindle fibers and/or cell cytokinesis (Leme & Marin-Morales, 2009). Moreover, the non-significant detection of chromosome losses, breakage and/or nuclear buds in the present study also emphasizes the attainment of non-significant mean numbers of MN, as these structures arise from occurrence of those chromosome alterations (Fenech et al., 2011). Hence, the concentrations evaluated in the *A. cepa* test demonstrated that the leaves of *J. mollissima* are not genotoxic and/or mutagenic.

The present tests showed promising results for *J. mollissima* ALE at the two highest concentrations applied against *A. aegypti* larvae. The facts that no concentration showed cytotoxic, genotoxic and/or mutagenic activity, and that the *A. cepa* test system is recognized as an important approach in ecotoxicology assessments (Leme & Marin-Morales, 2009) reinforce the advantage of using this extract in control programs, and recommend the performance of further studies the study continuity.

Conclusion

The results of this study show that the aqueous leaf extract of *J. mollissima* contains larvicidal compounds, as the assay demonstrated mortality among the larvae of *A. aegypti*. Moreover, its non-toxicity was further validated by the test system using *A. cepa*. This outcome suggests the possible use of *J. mollissima* leaves against the mosquito *A. aegypti*; nevertheless, more studies are recommended, such as for the isolation, identification and analysis of compounds derived from *J. mollissima* that show larvicidal properties.

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