In Vivo Efficacy of Ellagic Acid against *Candida albicans* in a *Drosophila melanogaster* Infection Model

Aline da Graça Sampaio,^{a,b} Aline Vidal Lacerda Gontijo,^a Helena Marcolli Araujo,^b Cristiane Yumi Koga-Ito^a

^aDepartment of Environmental Engineering and Oral Biopathology Graduate Program, São José dos Campos Institute of Science and Technology, Universidade Estadual Paulista (UNESP), São José dos Campos, Brazil

^bInstitute of Biomedical Sciences, Federal University of Rio de Janeiro (UFRJ), National Institute of Science and Technology in Molecular Entomology (INCT-EM), Rio de Janeiro, Brazil

ABSTRACT The aim of this study was to evaluate the antifungal activity and the toxicity of ellagic acid (EA) using a *Drosophila melanogaster* model. *Candida albicans* bacteria were inoculated into Toll heterozygous flies. Survival curves were obtained for the evaluation of the antimicrobial effect and toxicity of EA. A protective effect of EA against fungal infection in *Drosophila melanogaster* was observed at nontoxic concentrations. This study showed that EA is a promising tool for the treatment of candidiasis.

KEYWORDS *Candida albicans*, *Drosophila melanogaster*, candidiasis, ellagic acid

Invasive candidiasis is a major health problem, with a mortality rate of 40% of infected patients (1). Nowadays, there are few available antifungal drugs, and a significant increase in the occurrence of resistance has been observed (2). Therefore, there is a real need of new molecules for the treatment of this disease. Ellagic acid (EA) is a natural bioactive extracted from plants and has presented promising *in vitro* activity on the planktonic form and biofilm of *Candida albicans* (3, 4). Nonetheless, *in vivo* studies to evaluate the antimicrobial activity against systemic infection by *C. albicans* still need to be performed.

The *Drosophila melanogaster* model has been scientifically useful as an initial whole-animal model for identifying new antimicrobial drugs (5–7), since this model overcomes the ethical concerns linked to animal models and enables the ability to work with a larger sample (7, 8). The present study aimed to evaluate the antifungal activity and toxicity of EA in a *Drosophila melanogaster* model infected by *C. albicans*. *Drosophila melanogaster* insects carrying a loss-of-function Toll allele were used in this study, since previous work (7) showed that flies with reduced Toll signaling are more susceptible to fungi and have been successfully used to study the pathogenesis of infection and antifungal activity (7, 8).

EA was purchased from Cayman Chemical (Ann Arbor, MI, USA). *C. albicans* SC 5314 was cultured onto Sabouraud dextrose (SD) agar 24 h prior to the experiments. Heterozygous loss-of-function Toll *Drosophila melanogaster* (Tl[r3]/+) flies (Bloomington Stock Center at Indiana University) were used in order to sensitize the Toll pathway. Flies were cultured in standard cornmeal media and maintained at 19°C during the experimental period. For the experimental procedures, only female flies aged 4 to 10 days were included in the study. In order to validate the systemic candidal infection model, 16 flies subdivided into 3 independent experiments were tested. Flies were anesthetized according to Artiss and Hughes (9). *Candida albicans* SC 5314 was cultured for 24 h at 37°C. A standardized portion (1 mm) of an ultrafine tungsten manipulator probe was then dipped in the center of an isolated colony. The probe was then injected in the lateral region of the fly. The count of CFU per fly (CFU/fly) was performed according to Miles et al. (10).

Received 17 August 2018 Returned for modification 6 September 2018 Accepted 7 September 2018

Accepted manuscript posted online 24 September 2018

Citation Sampaio ADG, Gontijo AVL, Araujo HM, Koga-Ito CY. 2018. In vivo efficacy of ellagic acid against *Candida albicans* in a *Drosophila melanogaster* infection model. Antimicrob Agents Chemother 62:e01716-18. <https://doi.org/10.1128/AAC.01716-18>.

Copyright © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Aline Vidal Lacerda Gontijo, aline.gontijo@gmail.com.

FIG 1 Kaplan-Meier survival curves of Toll heterozygous flies infected by *C. albicans* and treated with ellagic acid (EA) concentrations of 3.2 µg/ml (dark blue line), 6.4 µg/ml (green), and 32 µg/ml (orange line). Control (infected), flies fed through regular culture medium (without EA) and inoculated with *C. albicans* (black line); Control 2 (not infected), flies fed with regular culture medium, not inoculated with *C. albicans* and not subjected to the puncture (light blue line); Control 3 (not infected, but punctured), flies fed with regular culture medium and subjected only to the puncture, but without inoculation of *C. albicans* (red line). $n = 60$ flies/group.

For the evaluation of the protective effect, survival curves were obtained until day 8 ($n = 60$ for each concentration, performed in three independent experiments). Flies were inoculated according to the previously described methodology. Concentrations of 3.2, 6.4, and 32 µg/ml of EA were tested. The concentrations of EA used in the present study were chosen considering a previous *in vitro* study performed by our group, which obtained an MIC of 3.2 µg/ml for *C. albicans* (4). From this value, concentrations 2 and 10 times greater than the MIC were used. Additionally, three control groups were used. Control 1 was used (wild-type flies fed with regular culture medium without EA and inoculated with *C. albicans*) to compare with treatment groups, control 2 (wild-type flies fed with regular culture medium, not inoculated with *C. albicans* and not subjected to the puncture) was used to ensure that all the experimental conditions (e.g., temperature and food) were satisfactory, and control 3 (wild-type flies fed with regular culture medium and subjected only to the puncture, without inoculation of *C. albicans*) was used to certify that the injection procedure did not cause damage to the flies. The toxicity of EA was evaluated at 3.2, 6.4, 32, and 200 µg/ml ($n = 40$ to 60 for each concentration, performed in two or three independent experiments). Prior to the experiments, flies were housed in empty vials for 6 h to starve and then were transferred into vials with EA-containing food, as described previously (7). For an assessment of the EA protective effect, the flies were injected with *C. albicans* suspension as described above and randomly divided into four experimental groups. The flies that died 3 h after inoculation were discarded from the study.

Statistical analyses were performed using GraphPad Prism (version 5.01), and the log-rank (Mantel-Cox) test was applied in order to compare the survival curves plotted in a Kaplan-Meier test. The level of significance was set at 5%.

Systemic infection was induced successfully by *C. albicans* in heterozygous Toll[−] flies using the methodology described above, with fungal counts of $6.7 \times 10^4 \pm 1.9 \times 10^4$ CFU/fly (mean $\pm$ standard error of the mean [SEM]; $n = 16$). This value was greater than those reported in previous studies, which were $\sim 8 \times 10^2$ (7) and $\sim 2 \times 10^3$ CFU/fly (8). However, none of the studies mentioned the values of variations of the mean. In this study, a high SEM was observed ($\sim 2 \times 10^4$); a high number of subjects ($n = 60$ for each concentration) was used, which allows an increase of the statistical significance in the survival curves.

Survival rates of 45%, 33%, and 34% were observed for the groups that received 3.2, 6.4, and 32 µg/ml of EA, respectively (Fig. 1). These survival curves were statistically higher than that in the control group, which presented a survival of 21% ($P < 0.05$). Unexpectedly, the highest survival curve (45%) was observed for the lowest concentration of EA (3.2 µg/ml). This may be explained by the solubility problems of EA at high concentrations (4). Additionally, the flies received EA by food, and, thus, the amount of ingested drug cannot be precisely controlled. Interestingly, the survival median in the 3.2-µg/ml group was 6 days, which is higher than the value of the control group (2 days).

FIG 2 Kaplan-Meier survival curves of healthy, Toll heterozygous flies in ellagic acid (EA) concentrations of 3.2 (blue line), 6.4 (green line), 32 (orange line), and 200 µg/ml (red line) and in the control group (flies fed through regular culture medium without EA; black line). $n = 40$ to 60 flies/group.

The control groups 2 and 3 presented with 86% and 85% survival, respectively. These survival curves are significantly different from all the other curves ($P < 0.001$). As the survival curves were higher than 85% after 8 days, one may conclude that the experimental conditions and the injection procedure were adequate.

The concentrations of EA used in the present study were chosen considering a previous *in vitro* study performed by our group, which obtained an MIC of 3.2 µg/ml for *C. albicans* (4). From this value, concentrations 2 and 10 times greater than the MIC were used.

For the EA toxicity assay, the survival curves for the 3.2-, 6.4-, 32-, 200-µg/ml, and control groups were 78%, 85%, 77.5%, 87%, and 91%, respectively (Fig. 2). No toxicity was observed for any concentration, since survival curves of treatments were comparable to that of the control ($P = 0.0936$). For all concentrations, the tests were performed in three independent experiments, with $n = 60$ for each concentration, except for the 32 µg/ml group ($n = 40$) due to a loss of the fly stock. However, we tested a higher concentration than 32 µg/ml (200 µg/ml), and even for this group, no toxicity was observed.

In conclusion, EA had a protective effect on *Drosophila melanogaster* from infection with *C. albicans*, confirming the antifungal activity reported in a previous *in vitro* study. Additionally, EA showed no toxicity at effective concentrations. This research contributes a new therapeutic strategy for the treatment of candidiasis and encourages the study of EA activity on *C. albicans* in other *in vivo* models.

ACKNOWLEDGMENT

A. D. G. Sampaio was supported by FAPESP (2016/21207-0).

REFERENCES

- Pfaller MA, Diekema DJ. 2007. Epidemiology of invasive candidiasis: a persistent public health problem. *Clin Microbiol Rev* 20:133–163. <https://doi.org/10.1128/CMR.00029-06>.
- Pappas PG, Rex JH, Sobel JD, Filler SG, Dismukes WE, Walsh TJ, Edwards JE. 2004. Guidelines for treatment of candidiasis. *Clin Infect Dis* 38: 161–189. <https://doi.org/10.1086/380796>.
- Teodoro GR, Brighenti FL, Delbem ACB, Delbem ACB, Khouri S, Gontijo AVL, Pascoal ACRF, Salvador MJ, Koga-Ito CY. 2015. Antifungal activity of extracts and isolated compounds from *Buchenavia tomentosa* on *Candida albicans* and non-*albicans*. *Future Microbiol* 10:917–927. <https://doi.org/10.2217/fmb.15.20>.
- Brighenti FL, Salvador MJ, Gontijo AVL, Delbem ACB, Delbem ACB, Soares CP, de Oliveira MAC, Gironi CM, Koga-Ito CY. 2017. Plant extracts: initial screening, identification of bioactive compounds and effect against *Candida albicans* biofilms. *Future Microbiol* 12:15–27. <https://doi.org/10.2217/fmb-2016-0094>.
- Oh C-T, Moon C, Choi TH, Kim BS, Jang J. 2013. *Mycobacterium marinum* infection in *Drosophila melanogaster* for antimycobacterial activity assessment. *J Antimicrob Chemother* 68:601–609. <https://doi.org/10.1093/jac/dks425>.
- Thomsen TT, Mojsoska B, Cruz JCS, Donadio S, Jenssen H, Løbner-Olesen A, Rewitz K. 2016. The lantibiotic NAI-107 efficiently rescues *Drosophila melanogaster* from infection with methicillin-resistant *Staphylococcus aureus* USA300. *Antimicrob Agents Chemother* 60:5427–5436. <https://doi.org/10.1128/AAC.02965-15>.
- Chamilos G, Lionakis MS, Lewis RE, Lopez-Ribot JL, Saville SP, Albert ND, Halder G, Kontoyiannis DP. 2006. *Drosophila melanogaster* as a facile model for large-scale studies of virulence mechanisms and antifungal drug efficacy in *Candida* species. *J Infect Dis* 193:1014–1022. <https://doi.org/10.1086/500950>.
- Glittenberg MT, Silas S, MacCallum DM, Gow NAR, Ligoxygakis P. 2011. Wild-type *Drosophila melanogaster* as an alternative model system for investigating the pathogenicity of *Candida albicans*. *Dis Model Mech* 4:504–514. <https://doi.org/10.1242/dmm.006619>.
- Artiss T, Hughes B. 2007. Taking the headaches out of anesthetizing *Drosophila*. *Am Biol Teach* 69:e77–e80. [https://doi.org/10.1662/0002-7685\(2007\)69\[77:TTHOAA\]2.0.CO;2](https://doi.org/10.1662/0002-7685(2007)69[77:TTHOAA]2.0.CO;2).
- Miles AA, Misra SS, Irwin JO. 1938. The estimation of the bactericidal power of the blood. *J Hyg (Lond)* 38:732–749. <https://doi.org/10.1017/S002217240001158X>.