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## Activity of gallic acid and its ester derivatives in *Caenorhabditis elegans* and zebrafish (*Danio rerio*) models

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**Aim:** Gallic acid and its ester derivatives have shown antifungal activity *in vitro*. This study was performed to investigate their activity against *Candida albicans* and their toxicity in the animal models *Caenorhabditis elegans* and zebrafish embryos. **Results:** The compounds protected worms from *C. albicans* infection. The dodecyl gallate was the most effective. In zebrafish embryo, gallic acid and dodecyl gallate were the least toxic. **Conclusion:** Gallic acid and its ester derivatives have potential for *in vivo* use against *C. albicans* infection. The antifungal effects and toxicity of gallate esters in these alternative animal models were dependent on carbon chain length.

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**Keywords:** alternative animal models • antifungal development • gallates • gallic acid • infection

*Candida albicans* is still the main causative agent of invasive fungal diseases and is associated with high mortality rates (35–50%), especially in immunocompromised patients and patients undergoing high-risk surgeries. *Candida* infections can be superficial, such as oral or vaginal candidiasis, or systemic [1]. Unfortunately, the current repertoire of antifungal agents is limited and discovery of new antifungal drugs is slow. Furthermore, some antifungals, such as amphotericin B, may be toxic to the host, and fungal resistance to azoles has been described [2]. Thus, interest in discovery of new antifungal agents is increasing.

Gallic acid is a compound derived from the secondary metabolism of various plants. Previous studies have shown that this compound and its alkyl ester derivatives (gallates) possess antioxidant and antimicrobial activities [3,4]. Kubo *et al.*, for example, tested the activity of three gallates against 16 microorganisms and found that octyl gallate showed antifungal activity against *Saccharomyces cerevisiae*, *Zygosaccharomyces bailii*, *Candida albicans* and *Aspergillus niger* [5]. The *in vitro* activity of alkyl gallates against human fungal pathogens, including *Candida* spp., *Cryptococcus gattii*, *Histoplasma capsulatum* and *Paracoccidioides* spp., has also been recently evaluated [6]. In addition, various gallates have shown activity against plant pathogenic fungi [7]. Although the *in vitro* antifungal activity of gallates has been investigated, little is known about the antifungal efficacy and host toxicity of these compounds *in vivo*.

Because of ethical reasons, high cost, new requirements for animal protection and consideration of the three Rs (Refinement, Reduction and Replacement), the use of mammals for *in vivo* tests has become restrictive, which creates the need for standardization of alternative models [8]. *Caenorhabditis elegans* has been used as an alternative to mammals to study host–pathogen interactions and novel antifungals. Furthermore, *C. elegans* and zebrafish models are useful for evaluating the toxicity of new drugs.

*Caenorhabditis elegans* is a free-living nematode found in soil, which presents a number of advantages in experimental assays. Its small size (1 mm), rapid generation and easy laboratory maintenance allow cheap and rapid production [9]. Another advantage is that its genome has been fully sequenced [10]. High genetic homology and conservation of biological functions such as the innate immune response are observed between this nematode and

mammals [11]. The use of *C. elegans* as a host is possible, since *C. elegans* can consume fungi as a food source, and is susceptible to fungal infection. Studies have shown that human pathogenic fungi such as *C. albicans* [12], *Candida krusei* [13], *Cryptococcus neoformans* [14], *H. capsulatum* [15] and *Penicillium marneffeii* [16] can lethally infect *C. elegans*. Additionally, *C. elegans* has been used to screen for potential antifungal compounds against *C. albicans*. One previous study examined 1,266 compounds with known pharmaceutical activities and found 15 that prolonged survival of nematodes infected with *C. albicans*. Two of these compounds, caffeic acid phenethyl ester and fluoroquinolone enoxacin were subsequently tested in a murine model of candidiasis and showed antifungal activity in this model [17]. Another screen was performed using a *C. albicans* infected *C. elegans* model that identified 12 saponin derivatives with antifungal activity [18]. Recently, two natural products, magnolol and honokiol have been shown to prolong survival of *C. elegans* infected with *C. albicans* [19].

Another alternative animal model is the zebrafish (*Danio rerio*). Among many applications, this model has been useful for assessing potential toxicities of new compounds toward developing embryos. The use of zebrafish embryos presents advantages including small size and effective compound absorption by embryos and larvae. Other advantages are that embryos are transparent, allowing visualization of their development, and one couple can generate up to 200 embryos [20]. The genome of this vertebrate has been fully sequenced, and 71.4% of zebrafish genes have human orthologs [21]. Thus, the zebrafish has been increasingly accepted as a model for predicting teratogenic effects of new drugs, nanoparticles and agrochemical agents [22].

This study was performed to investigate the activity against *C. albicans* and the host toxicity of gallic acid and its ester derivatives (hexyl gallate, octyl gallate and dodecyl gallate) in *C. elegans*. We also investigated the embryonic toxicity/teratogenicity of these compounds in zebrafish.

## Materials & methods

### Fungi

*Candida albicans* ATCC 90028 was obtained from the collection of the Clinical Mycology Laboratory, School of Pharmaceutical Sciences, UNESP, Araraquara, São Paulo, Brazil, and was maintained in Sabouraud medium at 37°C. For the experiment, *C. albicans* was cultivated in Sabouraud broth at 37°C with shaking. A total of 50 µl of this culture was inoculated onto brain heart infusion agar supplemented with kanamycin (90 µg/ml) and ampicillin (200 µg/ml) and incubated at 37°C for 24 h.

### Drug & compounds

Amphotericin B and gallic acid (3,4,5-trihydroxybenzoic acid) were obtained commercially (Sigma-Aldrich, MO, USA). The ester derivatives (hexyl gallate, octyl gallate and dodecyl gallate) were synthesized according to previously published methods of Morais *et al.* [23]. Stock solutions of amphotericin B or gallic acid and its derivatives were prepared in DMSO and diluted in M9 buffer (KH<sub>2</sub>PO<sub>4</sub>; Na<sub>2</sub>HPO<sub>4</sub>; NaCl; MgSO<sub>4</sub>) for experiments with *C. elegans* and in embryo medium (NaCl; KCl; CaCl<sub>2</sub>.2H<sub>2</sub>O; MgCl<sub>2</sub>.6H<sub>2</sub>O) for experiments with zebrafish to make the concentration of DMSO 1%.

### *C. elegans*–*C. albicans* assay

The *C. elegans* AU37 (*glp-4;sek-1*) strain, obtained from the Caenorhabditis Genetics Center, was grown on nematode growth medium agar plates, fed with *Escherichia coli* OP50 and incubated at 15°C according to standard procedures [24]. For experiments, worms were synchronized by treatment with sodium hypochlorite. Stage L4 worms were added to the center of each plate with an inoculum of *C. albicans* to induce infection. Plates were incubated at 25°C for 3 h. Worms were then washed with M9 buffer, and about 20 worms each were added to wells of 96-well plates containing 60% M9 buffer, 40% brain heart infusion, 10 µg/ml cholesterol in ethanol, 90 µg/ml kanamycin and 200 mg/ml ampicillin. Different concentrations of the control drug amphotericin B (0.1; 0.5; 1 and 5 µg/ml), or gallic acid and hexyl, octyl and dodecyl gallates (0.1; 1; 5; 10; 15; 30; 60 and 120 µg/ml) were added to the wells. The plates were maintained at 25°C, and worm survival was assessed daily for 4 days (at ~24 h intervals), based on their mobility and their shape (rod shaped worms were considered dead and sinusoidal worms were considered alive). On the fourth day postinfection, worms were stained with SYTOX<sup>®</sup> Green (Invitrogen, CA, USA) at a final concentration of 1 µM and were incubated for 15 min at room temperature in a horizontal shaker at 120 r.p.m. Images were captured in the automated microscope IN Cell Analyzer 2000 (GE Healthcare, Buckinghamshire, UK). Three independent experiments were performed with a total of about 60 worms per tested concentration.

### Zebrafish embryo assay

Wild-type zebrafish (*Danio rerio*) were maintained in a temperature-controlled aquarium ( $28 \pm 0.5^\circ\text{C}$ ) with a 14 h light/10 h dark cycle. The housing and breeding of the fish used in this study received appropriate national and institutional approvals (Conselho Nacional de Controle de Experimentação Animal – CONCEA and Faculdade de Ciências Farmacêuticas, permit number 01.0082.2014) and were in accordance with the EU Directive 2010/63/EU. Adult zebrafish (male/female ratio of 1:1 or 1:2 or 2:1) were set up for each mating and embryos were collected from the bottom of the tank. Embryos were washed with embryo medium plus 0.00003% methylene blue and placed into Petri dishes. Those with normal fertilization were selected for testing. Embryos were transferred to 96-well plates (2 embryos per well) in 100  $\mu\text{l}$  embryo medium per well. Gallic acid and hexyl, octyl and dodecyl gallates (100  $\mu\text{l}$ ) were added to achieve concentrations of 0.1; 1; 5; 10; 15; 30; 60 and 120  $\mu\text{g}/\text{ml}$ . Compound exposure was initiated at 2–3 h post-fertilization (hpf). Plates were maintained at  $28^\circ\text{C}$  and were observed for phenotypes of malformation at 5, 24 and 48 hpf. Embryos did not reach free-feeding stage during this time. The effect of compounds on the development of embryos was evaluated using an inverted microscope, and images were captured in the automated microscope IN Cell Analyzer 2000 (GE Healthcare). Values for the 50% lethal concentration ( $\text{LC}_{50}$ ) were calculated. Three independent experiments were performed, with a total of 24 embryos per tested concentration.

### Statistical analysis

Statistical analyses were carried out using GraphPad Prism 5 (GraphPad Software Inc., CA, USA). Survival curves for *C. elegans* worms and zebrafish embryos were plotted using the Kaplan–Meier method and survival differences were analyzed by log-rank (Mantel–Cox). A p-value of  $<0.05$  was considered statistically significant.

## Results

### Efficacy & toxicity of compounds in the *C. elegans*–*C. albicans* model

Worms of *C. elegans* strain AU37 were placed in contact with *C. albicans* for 3 h and subsequently treated with various concentrations of amphotericin B (control), gallic acid and hexyl, octyl and dodecyl gallates. Survival curves indicated that most of the worms infected with *C. albicans* died on the second day. The solvent DMSO at 1% showed no toxicity to the worms. All concentrations of amphotericin B significantly increased the survival rate of worms in a concentration-dependent manner ( $p < 0.0001$  in all cases). The percent survival of *C. albicans* infected worms on the fourth day was 20, 54, 66 and 84% for groups treated with amphotericin B at 0.1, 0.5, 1 and 5  $\mu\text{g}/\text{ml}$ , respectively (Figure 1).

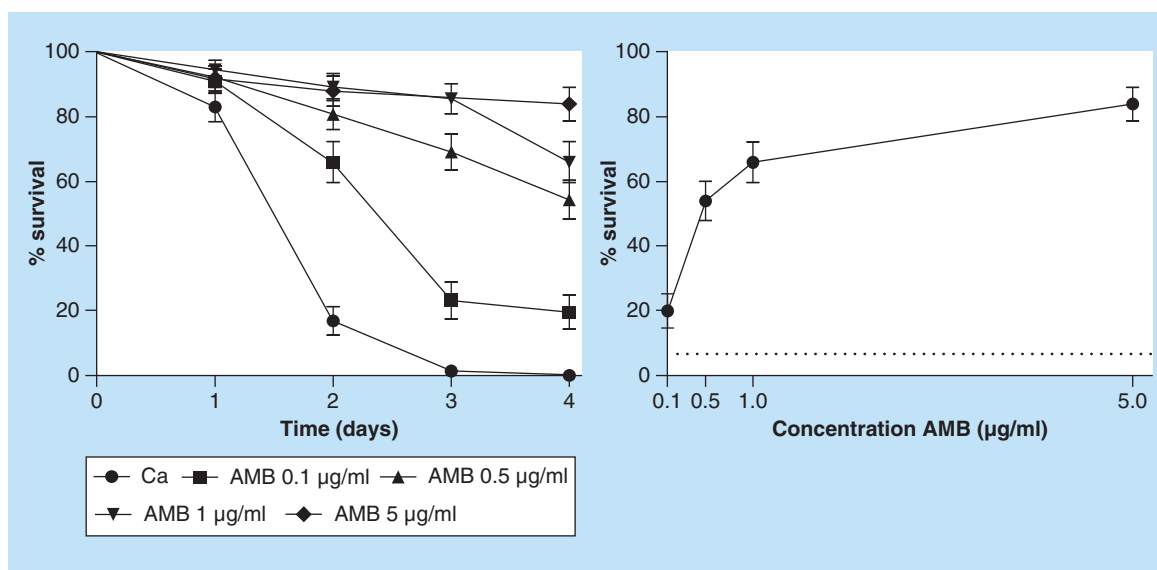
Gallic acid and its ester derivatives were selected for their *in vitro* antifungal activity [6]. The chemical structures of these compounds are shown in Figure 2. Treatment with the lowest concentration (0.01  $\mu\text{g}/\text{ml}$ ) of all compounds was not protective against *C. elegans* infected with *C. albicans* (data not shown). However, at higher concentrations (1, 5, 10, 15, 30 and 60  $\mu\text{g}/\text{ml}$ ) gallic acid and its ester derivatives significantly increased survival rates of *C. elegans* infected with *C. albicans* ( $p < 0.05$  in all cases). At the end of 4 days, gallic acid, hexyl gallate, octyl gallate and dodecyl gallate increased the survival of worms by 13–33, 18–33, 12–31 and 14–46% at tested concentrations, respectively. Dodecyl gallate was determined to be the most effective compound to protect the worms from fungal infection (Figure 3).

Another point to be considered when analyzing concentration response curves is the toxicity of the compounds. A reduction of larval survival rates at higher concentrations of a tested compound may mean that it is toxic at these concentrations. In the present work, nematode survival was diminished at higher concentrations (60 and 120  $\mu\text{g}/\text{ml}$ ) for all compounds, but octyl gallate was the most toxic to the worms (Figure 3). Viable and dead worms are shown in Figure 4, and staining with SYTOX Green (Invitrogen) helped distinguish worms killed by infection or treated with drug and compounds.

### Toxicity of compounds in the zebrafish model

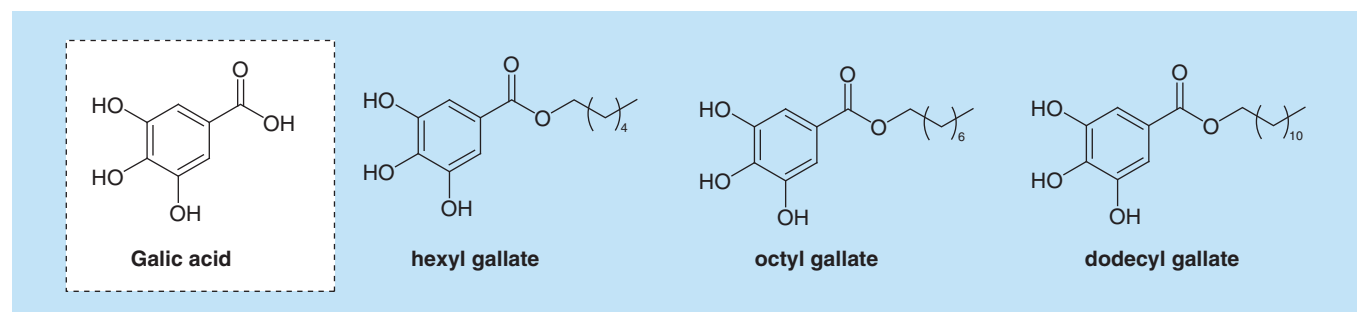
Gallic acid and its derivatives were tested at the same concentrations used in the *C. elegans*–*C. albicans* model (0.1, 1, 5, 10, 15, 30, 60 and 120  $\mu\text{g}/\text{ml}$ ). Based on the percentage of embryos affected at each concentration of each compound, survival curves were plotted (Figure 5). Moreover,  $\text{LC}_{50}$  values for embryotoxic effects or lethality were calculated from concentration–response curves plotted at 48 hpf.

The solvent DMSO at 1% showed no toxic effect in this model. Gallic acid had no toxic effect on zebrafish embryos at up to 120  $\mu\text{g}/\text{ml}$ . Due to the lack of toxicity for gallic acid in the range tested,  $\text{LC}_{50}$  value can be



**Figure 1.** Survival curve and concentration response of amphotericin B from the *Caenorhabditis elegans*–*Candida albicans* model. The graph represents combined data expressed as mean and standard error mean from three independent experiments. Survival curves were plotted using the Kaplan–Meier method, and survival curves were compared by log-rank test. All concentrations of amphotericin B significantly increased the survival rate of worms ( $p < 0.0001$ ).

AMB: Amphotericin B.

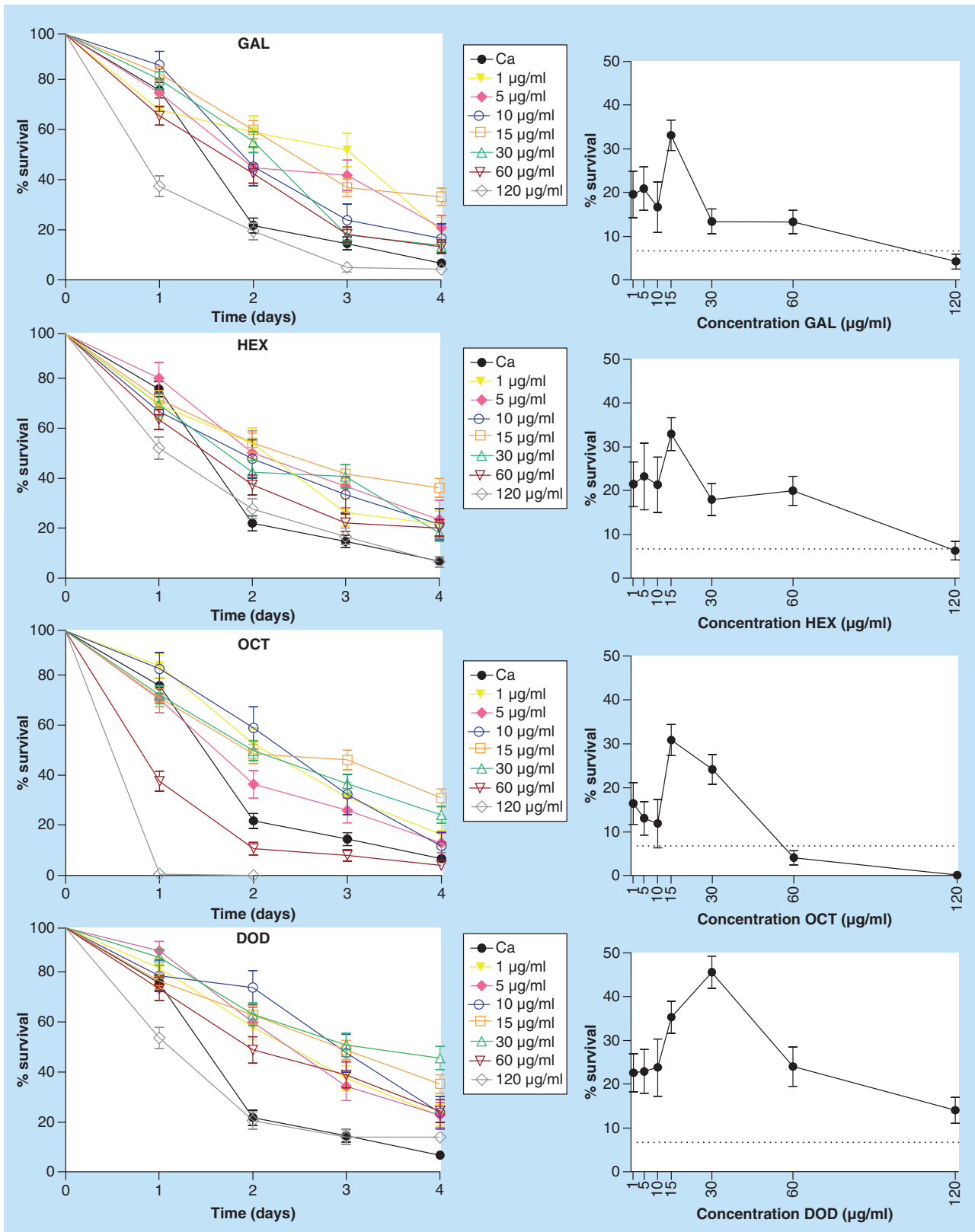


**Figure 2.** Chemical structures of gallic acid and its ester derivatives.

considered to be  $>120 \mu\text{g/ml}$ . For other compounds tested, the number of embryos affected was concentration-dependent. Hexyl gallate and octyl gallate had no toxic effect on zebrafish embryos at up to  $1 \mu\text{g/ml}$ , and presented  $\text{LC}_{50}$  values of 10 and  $4.5 \mu\text{g/ml}$ , respectively. Dodecyl gallate had no toxic effect on zebrafish embryos at up to  $10 \mu\text{g/ml}$ , and presented an  $\text{LC}_{50}$  of  $40 \mu\text{g/ml}$ . Thus, gallic acid and dodecyl gallate had less embryotoxicity than hexyl and octyl gallates. The effect of the compounds was evaluated at 5 hpf in the gastrula stage and at 24 and 48 hpf. At these times, the parameters recommended by the Organization for Economic Cooperation and Development [25] were quantitated: coagulation of fertilized eggs, lack of somite formation, lack of tail detachment and lack of heart beats. In these assays, the only indicator of toxicity observed was the coagulation of fertilized eggs phenomenon (presented as a black nontransparent ovum), an indicator of lethal toxicity when in contact with toxic concentrations of gallates (Figure 6). The stage of embryonic development that was most sensitive to exposure to the compounds was that at 24 hpf, probably because it is the phase where there is extensive cellular proliferation and organogenesis.

## Discussion

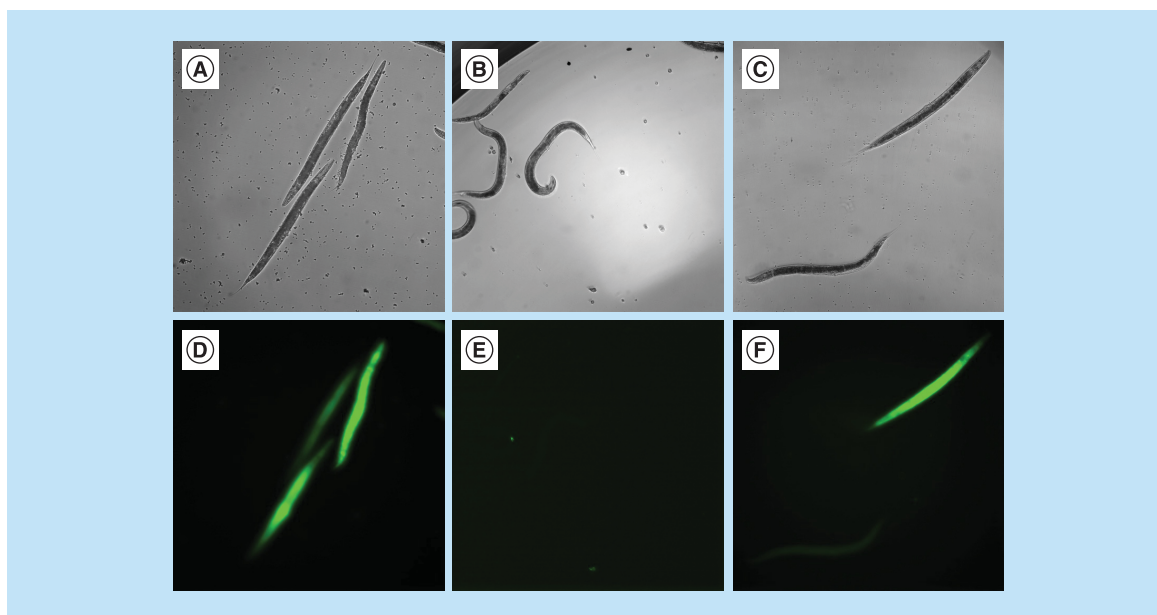
*Candida albicans* is the most common fungal pathogen, and development of new antifungal agents is crucial. In this study, we assess the potential of gallic acid and its ester derivatives with different carbon chain lengths (hexyl



**Figure 3.** Survival curves and concentration responses of gallic acid, hexyl gallate, octyl gallate and dodecyl gallate from the *Caenorhabditis elegans*-*Candida albicans* model. The graph represents combined data expressed as mean and standard error mean from three independent experiments. Survival curves were plotted using the Kaplan–Meier method. Survival curves were compared with log-rank test. The group of infected but untreated worms is represented by the black line, and the groups treated with different concentrations of the compounds are represented by the colored lines. The gallic acid and its ester derivatives (1–60 µg/ml) significantly increased survival of *Caenorhabditis elegans* infected with *Candida albicans* ( $p < 0.05$  in all cases), but compounds became toxic at 120 µg/ml.

DOD: Dodecyl gallate; GAL: Gallic acid; HEX: Hexyl gallate; OCT: Octyl gallate.





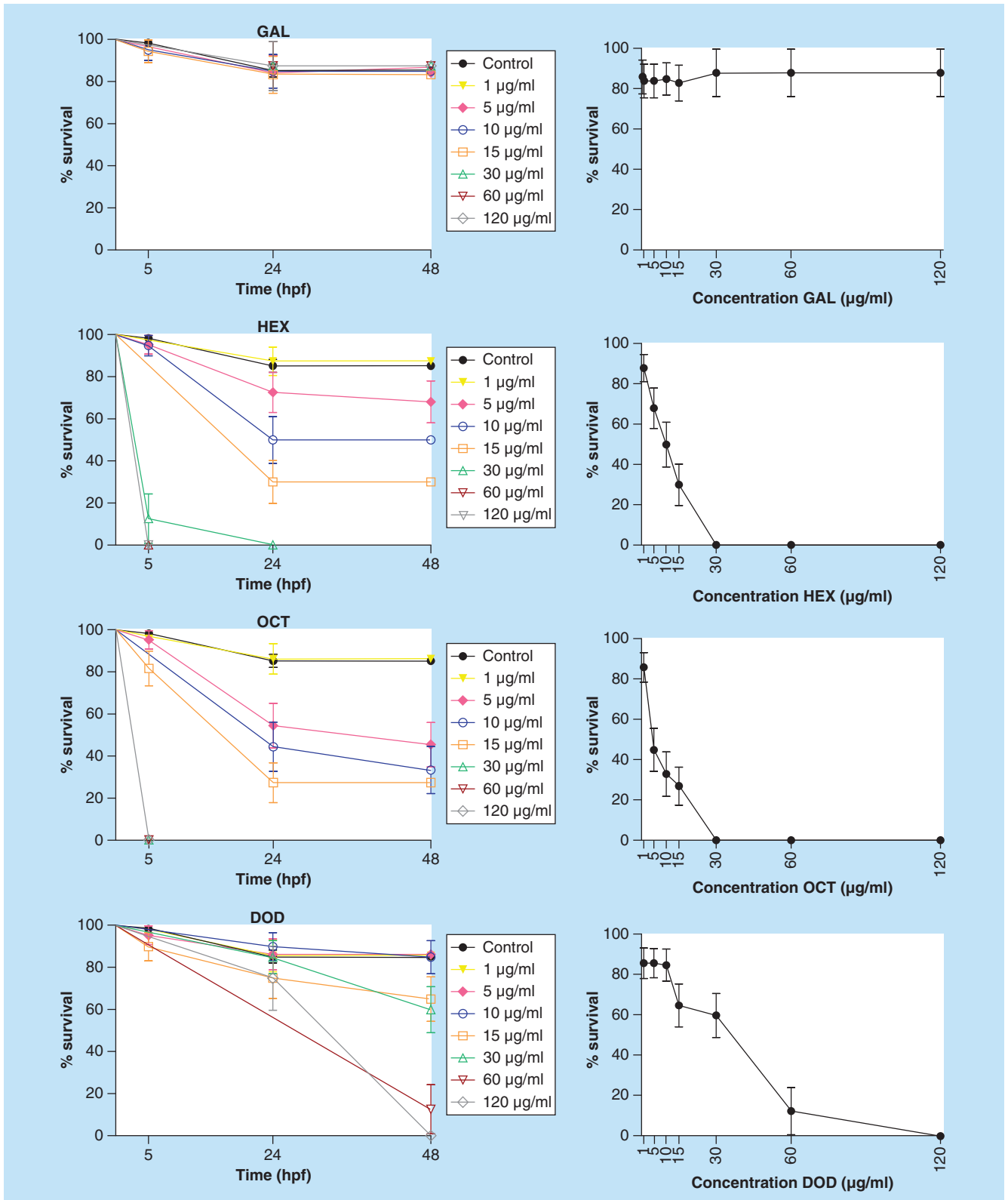
**Figure 4.** Viable and dead *Caenorhabditis elegans* worms in bright field images (above) and dead worms stained with SYTOX<sup>®</sup> Green in fluorescence images (below). Infected and untreated worms (A & D); infected worms treated with amphotericin B at 5  $\mu\text{g/ml}$  (B & E) and infected worms treated with dodecyl gallate at 30  $\mu\text{g/ml}$  (C & F). Images were taken on the fourth day postinfection. Magnification:  $\times 10$ ; Scale bar: 100  $\mu\text{m}$ .

gallate, octyl gallate and dodecyl gallate) to increase the survival of *C. elegans* infected with *C. albicans*. In previous studies, these compounds demonstrated excellent *in vitro* antifungal activity, including against *C. albicans* [5,6]. Other studies have demonstrated that gallates had a synergistic effect with imidazole, itraconazole and fluconazole, reducing the MIC of these drugs against *C. albicans* [26,27]. Fujita and Kubo suggested that gallates are capable of damaging the fungal cell membrane, which gives them their fungicidal effect [28]. The mechanism of action of dodecyl gallate has also been related to its inhibition of oxygen consumption and NADH oxidase in the membrane of *Pseudomonas aeruginosa* [29].

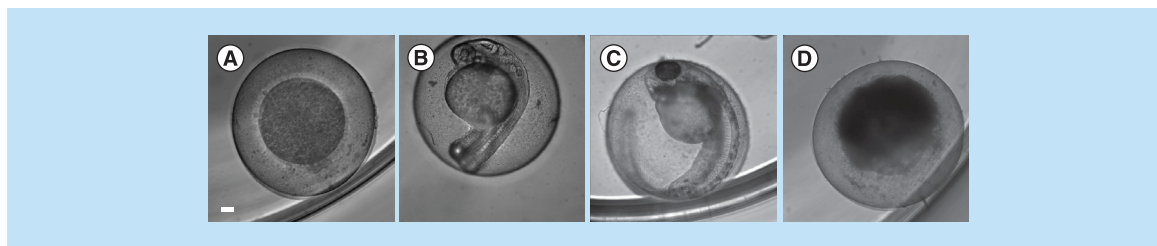
The nematode *C. elegans* has been used to evaluate virulence of various fungi [12–16]. Moreover, previous studies have used *C. elegans* to evaluate conventional and novel antifungal compounds against *Candida* species [13,17–19]. In this regard, correlation was observed between antifungal activity in *C. elegans* and in a murine model of candidiasis [17]. We used the *glp-4/sek-1* double mutant strain AU37 for analysis of *C. elegans* survival curves with *C. albicans*. This strain has been very useful in studies of interaction between *C. elegans* and fungi, because the *glp-4* mutation makes worms unable to reproduce at 25°C, and the *sek-1* mutation increases sensitivity to various pathogens [30].

Using the *C. elegans* infection model, we characterized the antifungal activity of gallic acid and its esters *in vivo*. All compounds significantly protected *C. elegans* from *C. albicans* infection at concentrations above 1  $\mu\text{g/ml}$ . Additionally, dodecyl gallate increased the worms' survival by 46% at 30  $\mu\text{g/ml}$ , and was the most effective compound. Previous studies showed that alkyl esters had more favorable pharmacological properties than those observed for gallic acid [3,31], which have been correlated to the amphipathic character of these derivatives. The hydrophobic moiety seems to contribute to activity, presumably by increasing affinity for cell membranes and cell permeability [32]. Furthermore, an increase in carbon chain length of the gallates resulted in increased *in vitro* antifungal activity. The MIC for *C. albicans* ATCC 90028 of both gallic acid and hexyl gallates was 62.5  $\mu\text{g/ml}$ , whereas the MIC of octyl gallate and dodecyl gallate was 8 and 2  $\mu\text{g/ml}$ , respectively [5,6]. Thus, the *in vivo* anticandidal profiles of gallic acid and its esters in *C. elegans* correlated very well with their observed *in vitro* activity.

Some studies have reported that toxicity can be observed concomitantly with antifungal efficacy in the *C. elegans*–*C. albicans* model [17,30]. At high concentrations (60 and 120  $\mu\text{g/ml}$ ), it appears that gallic acid and gallates are toxic to the nematode. Octyl gallate was the most toxic compound.



**Figure 5.** Survival curves and concentration responses for gallic acid, hexyl gallate, octyl gallate and dodecyl gallate from the zebrafish model. The graph represents combined data expressed as mean and standard error mean from three independent experiments. Survival curves were plotted using the Kaplan–Meier method, and survival curves were compared by log-rank test. The group of untreated embryos is represented by the black line and the different concentrations of compounds are represented by the colored lines. Gallic acid was not toxic to the embryos at all concentrations tested, and gallates were toxic in a concentration-dependent manner. The compounds significantly reduced the survival rate of embryos at concentrations  $\geq 5 \mu\text{g/ml}$  compared with control ( $p < 0.05$ ) for hexyl and octyl gallates, and  $\geq 15 \mu\text{g/ml}$  compared with control ( $p < 0.05$ ) for dodecyl gallate. DOD: Dodecyl gallate; GAL: Gallic acid; HEX: Hexyl gallate; OCT: Octyl gallate.



**Figure 6. Representative images of zebrafish embryonic development.** The control group with normal development at 5 hpf (A), 24 hpf (B) and 48 hpf (C), respectively. Example of malformation (coagulation) caused by octyl gallate at 15  $\mu\text{g/ml}$  exposure (D). Magnification: 10 $\times$ ; Scale bar: 100  $\mu\text{m}$ . Hpf: Hours post fertilization.

Evaluation of developmental and reproductive toxicity is also important in drug discovery. For this purpose, the zebrafish has been used as a rapid, easy and efficient animal model, which combines the biological complexity of vertebrate systems with the possibility of high-throughput screening, and can detect potential teratogenic effects of new drugs [33,34]. Moreover, studies have demonstrated that zebrafish responses to compound toxicities are predictive of mammalian responses [34–36]. In addition, zebrafish, especially transgenic lines, have emerged as a useful model for evaluating mechanisms of specific diseases such as cancer and infection. In this respect, the transparency of zebrafish embryos and the use of fluorescent reporter techniques allow observation of tumor formation or microorganism colonization and immune system action. In addition to providing detailed understanding of the mechanisms of a disease, zebrafish could be an *in vivo* drug screening model to identify targets and determine efficacy, contributing to development of preclinical agents [37–39].

Taking advantage of the zebrafish model, we evaluated the effects of gallic acid and its esters in embryo assays. Our results demonstrated that gallic acid has no toxic effect on zebrafish embryos at all concentrations tested (up to 120  $\mu\text{g/ml}$ ). However, gallates demonstrated clear concentration-dependent toxicities that were significantly influenced by their carbon chain length. Gallate with eight carbon atoms in the side chain (octyl gallate) was more toxic than gallate with six carbon atoms (hexyl gallate) in zebrafish. However, when the alkyl chain was longer (dodecyl gallate), the ester compound was less toxic, suggesting a cut-off phenomenon for chain length [5,6]. The  $\text{LC}_{50}$  of dodecyl gallate was 40  $\mu\text{g/ml}$ , which is 20 $\times$  higher than its MIC against *C. albicans* (2  $\mu\text{g/ml}$ ), providing a good therapeutic window.

The *C. elegans*–*C. albicans* and zebrafish embryo assays are shown to be suitable as screening tool intermediates between preliminary *in vitro* evaluation and more conclusive analyses in mice and rats. The observed antifungal and toxic effects of gallates in alternative animal models depend on carbon chain length. Dodecyl gallate demonstrated good *C. elegans* protection by inhibiting *C. albicans* growth. Moreover, the toxic concentrations in nematode and zebrafish were much higher than the MIC value. Thus, dodecyl gallate should be a potential anticandidal compound *in vivo*.

### Future perspective

There are good evidences supporting alternative animal models as useful, facile and inexpensive models to evaluate toxicity and efficacy of antifungals. Furthermore, they can reduce the number of mammals needed for *in vivo* experimentation. In *C. elegans* and zebrafish models, dodecyl gallate was potent against *C. albicans* and showed low embryotoxicity. Thus, the compound can be considered a candidate antifungal agent.

### Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.



## Executive summary

**Candidiasis**

- *Candida albicans* is the major cause of invasive fungal disease.

**Antifungal agents**

- The repertoire of antifungal agents is limited; toxic effects on the host and selection of resistant strains are reported. Thus, interest in discovery of new antifungal agents is increasing.
- Antifungal activity of gallic acid and its ester derivatives was confirmed in several *in vitro* studies.

**Alternative animal models**

- *Caenorhabditis elegans* and zebrafish (*Danio rerio*) have been useful, rapid and cheap models to study fungal infection and drug efficacy and toxicity.

***In vivo* effect of gallic acid and its esters derivatives**

- The carbon chain length of gallates is responsible for the *in vivo* antifungal activity and toxicity of the compound, in which dodecyl gallate appears to be the most interesting compound.

**Ethical conduct of research**

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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