

Different Extracts of *Zingiber officinale* Decrease *Enterococcus faecalis* Infection in *Galleria mellonella*

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Dried, fresh and glycolic extracts of *Zingiber officinale* were obtained to evaluate the action against *G. mellonella* survival assay against *Enterococcus faecalis* infection. Eighty larvae were divided into: 1) *E. faecalis* suspension (control); 2) *E. faecalis* + fresh extract of *Z. officinale* (FEO); 3) *E. faecalis* + dried extract of *Z. officinale* (DEO); 4) *E. faecalis* + glycolic extract of *Z. officinale* (GEO); 5) Phosphate buffered saline (PBS). For control group, a 5 µL inoculum of standardized suspension (10⁷ cells/mL) of *E. faecalis* (ATCC 29212) was injected into the last left *proleg* of each larva. For the treatment groups, after *E. faecalis* inoculation, the extracts were also injected, but into the last right *proleg*. The larvae were stored at 37 °C and the number of dead larvae was recorded daily for 168 h (7 days) to analyze the survival curve. The larvae were considered dead when they did not show any movement after touching. *E. faecalis* infection led to the death of 85% of the larvae after 168 h. Notwithstanding, in treatment groups with association of extracts, there was an increase in the survival rates of 50% (GEO), 61% (FEO) and 66% (DEO) of the larvae. In all treatment groups, the larvae exhibited a survival increase with statistically significant difference in relation to control group (p=0.0029). There were no statistically significant differences among treatment groups with different extracts (p=0.3859). It may be concluded that the tested extracts showed antimicrobial activity against *E. faecalis* infection by increasing the survival of *Galleria mellonella* larvae.

Key Words: *Enterococcus faecalis*, *Zingiber officinale*, host-pathogen interactions, *Galleria mellonella*.

Introduction

One of the endodontic treatment goals is to eliminate the infection within root canals and prevent post-endodontic treatment reinfection. However, Bystrom and Sundqvist (1) showed that microorganisms can survive even after biomechanical preparation and *Enterococcus faecalis* (Gram-positive, facultative anaerobic coccus) has been frequently isolated in root canals exhibiting persistent post-endodontic treatment infections (2,3).

In cases of pulp necrosis with radiographic sign of apical periodontitis and lack of periodontal disease, *E. faecalis* was isolated in 55% (4) and 64% of the cases (2). Microbiological identification and analytic studies of teeth presenting endodontic treatment and periapical lesion verified that this microorganism is the most prevalent species, present in 45% (3), 77% (5) and up to 77.8% (6) of the cases.

Different substances have been used to reduce microorganism counts within root canal system, such as sodium hypochlorite, chlorhexidine, citric acid, saline and alternative therapies using natural medicinal plants that seem to be most promising solutions (7,8).

Zingiber officinale is a plant native to India and China, which has been largely employed as flavoring, in alcoholic beverages and in popular medicine. It has been extensively

used in traditional Chinese medicine to treat headaches, nausea, fever, arthritis, rheumatism and muscle pains. The active constituents of ginger comprise gingerols and shogaols, and the first is the main biological active constituent accounting for the anti-inflammatory and analgesic properties of ginger (9). Analyzing the active constituents of ginger, Lantz et al. (10) verified that both gingerol and shogaol were capable of significantly inhibit the production of prostaglandins E₂ induced by LPS from *Escherichia coli*. The extracts containing mainly gingerol had the capacity of inhibiting COX-2 production, while those containing shogaol did not show effect on COX-2.

Previous studies *in vitro* showed the antimicrobial action of *Z. officinale*, as Kim and Park (11), who evaluated the glycolic extract of *Z. officinale* on *Pseudomonas aeruginosa* biofilm. The authors demonstrated that biofilm development was reduced in up to 56% when the phytotherapeutic drug was added to the bacterial culture. Maekawa et al. (7) also observed antimicrobial action of *Z. officinale* and verified that 20% glycolic extract of ginger (Apis Flora) as intracanal medication for 14 days was effective in eliminating *Candida albicans*, *E. faecalis* and *E. coli*.

Currently, invertebrate animal models, such as the

nematode *Caenorhabditis elegans*, and insets, such as *Drosophila melanogaster* and *Galleria mellonella*, have been used in the study of the microorganism pathogenicity, host-pathogen interaction and as screening for testing new therapies (12). Invertebrates have several advantages over the conventional mammalian models, including lower costs, faster results and fewer ethical issues (13).

G. mellonella is a reliable and proven experimental model for pathogenesis studies and innate immunity, because it has in its hemolymph six types of hemocytes (prohemocytes, coagulocytes, spherulocytes, oenocytoids, plasmocytes and granulocytes), which play an important role in host-pathogen interaction (14,15).

Since studies *in vivo* are crucial for the study of the pathogenesis of micro-organisms and development of alternative treatments, the aim of this study was to evaluate and compare *in vivo* the effectiveness of dried, fresh and glycolic extract of *Zingiber officinale* essential oils against experimental infection by *E. faecalis* in *G. mellonella* model.

Material and Methods

Microorganism and Culture

A standard *Enterococcus faecalis* strain (ATCC 29212) was used. This strain came from the Laboratory of Microbiology and Immunology of the Institute of Science and Technology/UNESP. To perform the *G. mellonella* trials, the microorganism was seeded in Petri plates containing brain heart infusion agar (BHI) (Difco, Detroit, MI, USA) and incubated at 37 °C for 24 h. After this, *E. faecalis* colonies were suspended in sterilized saline solution (0.9% NaCl) to obtain a standardized suspension of 10⁷ cells/mL by spectrophotometry (B 582, Micronal, São Paulo, SP, Brazil).

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Used Phytotherapies

The plant used in this study was *Zingiber officinale* Roscoe. The dried and fresh extracts of *Z. officinale* were purchased at the Collection of Medicinal and Aromatic Plants of the Multidisciplinary Clinical, Biological and Agricultural Research Center of UNICAMP (MCBARS). The MCBARS has the botanical identification of the studied plant, as well as its own store in a herbarium voucher specimen of the University, with registration number 533 corresponding to *Zingiber officinale* Roscoe. The glycolic extracts of *Z. officinale* were purchased from Apis Flora®, Ribeirão Preto, SP, Brazil. The obtained products were used at a 2.5 mg/mL concentration for fresh and glycolic extracts and 5.0 mg/mL for dried extract.

In Vivo Evaluation of the Combination of *E. faecalis* Strain with *Galleria mellonella* Experimental Model

This study employed the methodology described by Mylonakis (14) and Cowen et al. (15) with some modifications. Sixteen randomly chosen *G. mellonella* larvae at final larval stage with body weight of approximately 330±25 mg were used for each group, totalizing 80 animals. The following groups were established: 1) *E. faecalis* suspension (control); 2) *E. faecalis* + fresh extract of *Z. officinale* (FEO); 3) *E. faecalis* + dried extract of *Z. officinale* (DEO); 4) *E. faecalis* + glycolic extract of *Z. officinale* (GEO); 5) Phosphate buffered saline (PBS). In the control group, a 5 µL inoculum of standardized suspension (10⁷ cells/mL) of *E. faecalis* (ATCC 29212) was injected into the last left *proleg* of each larva. For treatment groups, after *E. faecalis* inoculation, the extracts were also injected, but into the last right *proleg* (Table 1). Two control groups were included in the assays as part of this study: one group was inoculated with PBS to enable observation of the demise of the larvae due to physical trauma, and the other received no injection as a control for general viability.

Following inoculation, the larvae were stored in plastic containers at 37 °C and the number of killed *G. mellonella* was recorded daily for 168 h (7 days) to analyze the survival curve. The larvae were considered dead when they did not show any movement after touching. During all assays performed in this study, the larvae did not receive nutrition.

Statistical Analysis

Percent survival and killing curves of *G. mellonella* were plotted and statistical analysis was performed by the Log-rank (Mantel-Cox) test using Graph Pad Prism statistical software. Significance level was set at p<0.05.

Results

The treatment with different extracts of *Z. officinale* was effective in reducing *in vivo* infection in *G. mellonella*.

Table 1. Experimental groups

Group	n	Description
<i>E. faecalis</i> + FEO (2.5 mg/mL)	16	5 µL <i>E. faecalis</i> - left <i>proleg</i> 5 µL FEO - right <i>proleg</i>
<i>E. faecalis</i> + DEO (5 mg/mL)	16	5 µL <i>E. faecalis</i> - left <i>proleg</i> 5 µL DEO - right <i>proleg</i>
<i>E. faecalis</i> + GEO (2.5 mg/mL)	16	5 µL <i>E. faecalis</i> - left <i>proleg</i> 5 µL GEO - right <i>proleg</i>
<i>E. faecalis</i> (Control)	16	5 µL <i>E. faecalis</i> - left <i>proleg</i> 5 µL PBS - right <i>proleg</i>
PBS	16	5 µL PBS - left <i>proleg</i> 5 µL PBS - right <i>proleg</i>

FEO: fresh extract; DEO: dried extract; GEO: glycolic extract; PBS: phosphate buffered saline

Z. officinale at a 2.5 mg/mL concentration of fresh and glycolic extracts, and 5.0 mg/mL of dried extract showed antimicrobial action against *E. faecalis* by protective action against experimental infection in *G. mellonella*.

The infection by *E. faecalis* without any treatment (control group) led to the death of 85% of the larvae after 168 h. However, in the groups treated with essential oils, was verified a 50% increase in the larva survival rates for glycolic extract (Fig. 1A), 61% for fresh extract (Fig. 1B) and 66% for dried extract (Fig. 1C).

In all groups treated with essential oils, the larvae exhibited a survival increase with statistically significant difference in relation to control group, for glycolic extract ($p=0.0444$), fresh extract ($p=0.0013$) and dry extract ($p=0.0022$). There was no statistically significant difference among different extracts of *Z. officinale* showing similarity and effectiveness of different treatments ($p=0.3859$) (Fig. 2). This suggests that the different tested extracts showed antimicrobial activity against *E. faecalis* infection increasing

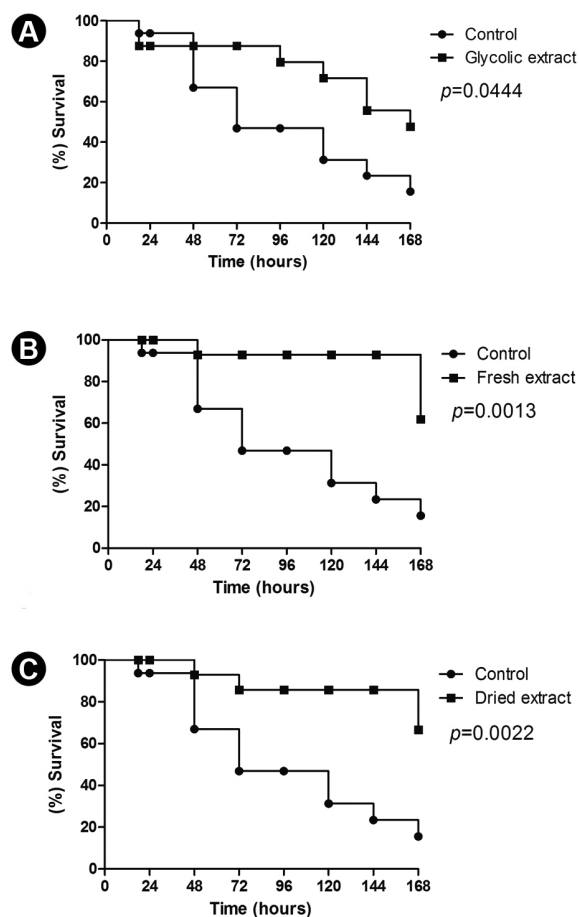


Figure 1. Survival curve of *G. mellonella* infected with *E. faecalis*. A: *E. faecalis* infection and treatment with glycolic extract. B: Infection with *E. faecalis* and treatment with fresh extract. C: Infection with *E. faecalis* and treatment with dried extract.

the survival of larvae of *G. mellonella* when compared with control group.

Discussion

The treatment with different extracts of *Z. officinale* was effective in reducing *in vivo* infection in *G. mellonella* and probably the biologically active compounds of the extracts were responsible for this reduction. The composition of ginger extracts is very variable, with predominance of biologically active components as gingerols, shogaol, paradols and gingerone. Gingerol and shogaol ratio within the extracts seems to be responsible by the main pharmacologic activities of *Z. officinale*, but dependent on factors as rhizome origin, maturity and preparation methods (9). Moreover, gingerone also present in oils and rhizomes, and is capable of reducing biofilm formation and consequently the *in vivo* infection (16).

Z. officinale at a concentration of 2.5 mg/mL of fresh and glycolic extracts and 5.0 mg/mL of dried extract showed antimicrobial action against *E. faecalis* by protective action against experimental infection in *G. mellonella*. The exact antimicrobial mechanism by which *Z. officinale* acts on microorganisms still needs to be clarified (17). However, it may be anticipated that different action mechanisms of this phytotherapeutic that lead to microbial reduction may be partly due to its hydrophobicity. As a result, there is disruption of the cell membrane lipid bilayer, making it more permeable, causing leakage of the vital cells content (18).

For this study, the GEO group showed increase of 50% of larva survival, while FEO and DEO groups presented 61% and 66% of survival in relation to control group, respectively. This difference found between the groups probably occurred because although GEO had a water-soluble vehicle, it came from a not fresh commercial product, different from the other rhizomes. Both fresh and dried rhizomes were obtained without manufacturing, which may assure purity of the extract and preservation of the product components. To the best of our knowledge,

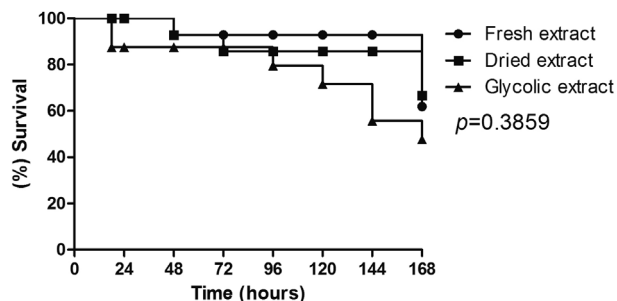


Figure 2. Comparison of the survival curve of *G. mellonella* larvae infected by *E. faecalis* and treated with different extracts.

this is the first study on extracts of plants to treat microbial infections in *G. mellonella* model, consequently there are no studies with similar methodology in order to compare and better discuss the present findings. Even so, these results agree with most published reports regarding the *in vitro* antimicrobial activity of ginger (19,20).

The increased larvae survival in the treated groups suggests that the active compounds of different extracts associated to the immune system of the larvae were able to control the infection for a longer time compared to the control group. With these results, it may be confirmed that *G. mellonella* is a suitable model for the study of alternative therapies to fight microbial infection. In agreement with this, Apolônio et al. (21) evaluated the adaptation response of *Staphylococcus aureus* and *Listeria monocytogenes* to the essential oil of eugenol in *G. mellonella*. The adaptation to eugenol was done by sequential exposure of the pathogens to increasing concentrations of this essential oil. After this, they evaluated the impact of adaptation to eugenol on virulence of these bacteria using *G. mellonella* larvae. As control group, the authors injected these bacteria into the larvae without previous treatment with eugenol. The sequential exposure of *S. aureus* and *L. monocytogenes* to different concentrations of eugenol affected their virulence, increasing the survival of larvae in comparison with the control group and evidencing the great potential of eugenol to fight these human pathogens.

G. mellonella model was used in this study to evaluate the antimicrobial action of *Z. officinale* on *E. faecalis* infection. The *G. mellonella* experimental model is recent but it is increasingly gaining acceptance in the scientific community. It has presented some advantages as host model, for example: the larvae can be stored at a temperature range from 25 °C to 37 °C, thus making easier the temperature conditions under which the pathogenic microorganisms exist, both in an environmental niche and in mammals (13). Recently, Mesa-Arango et al. (22) evaluated antifungal effectiveness in this infection model and verified that amphotericin B, caspofungin, voriconazole and fluconazole had a protective effect on animals at concentrations similar to those applied in humans, concluding that *G. mellonella* provided a simple and viable model to study drug effectiveness.

This study verified that *E. faecalis* infection in *G. mellonella* was lethal to 85% of animals after 168 h of inoculation. Additionally to Dentistry interest, currently *E. faecalis* has become an important hospital pathogen and most of nosocomial infections have been caused by two species of the genus: *E. faecalis* and *Enterococcus faecium* (23). Because of the great importance of these bacteria nowadays, some studies have evaluated the infection with *Enterococcus* spp. in *G. mellonella* and found a positive

correlation between virulence and host response (24,25).

The results found by the present study corroborate those from *in vitro* studies found in the literature while simultaneously indicating the need of further studies employing invertebrate models and different trials (phagocytosis, counting of microbial load in hemolymph, tissue cultures, hemocyte count and melanization assays) that may allow analyzing both host and microorganism genes and helping to elucidate the unique characteristics of *Enterococcus* virulence, like its capacity of inducing a persistent infection.

Within the limits of this study, it may be concluded that the tested essential oils exhibited antimicrobial activity against *E. faecalis* infection by increasing the survival of *G. mellonella* larvae.

Resumo

Extratos seco, fresco e glicólico de *Zingiber officinale* foram obtidos para avaliar suas ações por meio de ensaio de sobrevivência em *G. mellonella* contra infecção por *Enterococcus faecalis*. Oitenta larvas foram divididas em: 1) Suspensão de *E. faecalis* (controle); 2) *E. faecalis* + extrato fresco de *Z. officinale* (FEO); 3) *E. faecalis* + extrato seco de *Z. officinale* (DEO); 4) *E. faecalis* + extrato glicólico de *Z. officinale* (GEO); 5) Solução tampão fosfato salina (PBS). Para o grupo de controle, 5 µL de inóculo de suspensão padronizada (10⁷ células/mL) de *E. faecalis* (ATCC 29212) foi injetado na última proleg esquerda de cada lagarta. Para os grupos com tratamento, após a injeção de *E. faecalis*, os extratos foram injetados na última proleg direita. Após as injeções, as lagartas foram armazenadas a 37 °C e o número de animais mortos foi registrado diariamente em 168 h (7 dias) para analisar a curva de sobrevivência. As lagartas foram consideradas mortas quando elas não mostraram qualquer movimento após o toque. A infecção por *E. faecalis* levou à morte de 85% das lagartas após 168 h. Não obstante, nos grupos de tratamento com associação dos extratos, houve um aumento nas taxas de sobrevivência de 50% (GEO), 61% (FEO) e 66% (DEO) das lagartas. Em todos os grupos com tratamento, as lagartas apresentaram um aumento na sobrevivência, com diferença estatisticamente significativa em relação ao grupo controle (p=0,0029). Não houve diferença estatisticamente significativa entre os tratamentos com os diferentes extratos (p=0,3859). Pode concluir-se que os extratos testados mostraram atividade antimicrobiana contra a infecção por *E. faecalis*, aumentando a sobrevivência das lagartas de *G. mellonella*.

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