ORIGINAL PAPER



# *IN VITRO* EFFICACY OF GARLIC EXTRACT AGAINST *CANDIDA ALBICANS* BIOFILMS FROM CHILDREN WITH EARLY CHILDHOOD CARIES

**Vidya Carolyn Tjokrosetio[1], Sarworini Bagio Budiardjo[2], Ike Siti Indiarti[2], Eva Fauziah[2], Margaretha Suharsini[2], Heriandi Sutadi[2], Mochamad Fahlevi Rizal[2]**

[1]Postgraduate Student of Pediatric Dentistry Department, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia
[2]Lecturer in the Pediatric Dentistry Department, Faculty of Dentistry, University of Indonesia, Jakarta, Indonesia

## ABSTRACT

**Introduction:** Garlic (*Allium sativum*) is known to be a natural antifungal agent with the ability to penetrate cellular and organelle membranes, resulting in cell death of *Candida albicans*.

**Objectives:** The aim of the present study was to measure the efficacy of garlic extract against *C. albicans* biofilms frequently detected in early childhood caries (ECC).

**Material and methods:** Samples of dental biofilms were obtained from children with ECC, cultured in CHROMagar *Candida* medium, and confirmed using a polymerase chain reaction (PCR) technique. Furthermore, biofilms were established in microplates, incubated for 48 h, and exposed to 10%, 25%, 50%, and 100% garlic extract using 0.2% chlorhexidine (CHX) as a positive control. Cell viability was subsequently measured by the methylthiazolyl-tetrazolium (MTT) assay.

**Results:** There was a statistically significant decrease in the viability of *C. albicans* biofilms at all concentrations of garlic extract ($p \leq 0.000$).

**Conclusion:** Garlic extract is efficacious in reducing the viability of *C. albicans* biofilms from children with ECC.

**Key words:** *Candida albicans*, ECC, garlic extract, biofilm, MTT assay.

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## INTRODUCTION

Early childhood caries (ECC) is defined as the presence of ≥ 1 decayed cavitated or non-cavitated carious lesions, missing teeth due to caries, or any primary tooth with dental restoration afflicting children aged ≤ 72 months. ECC is a biofilm-dependent disease with rapid and aggressive onset and progression of carious lesions, resulting in rampant destruction of the teeth. It is a virulent form of dental caries affecting many children in developing countries. *Streptococcus mutans* has often been cited as the primary microbial pathogen in ECC through its heavy presence in the dental biofilms formed as dental plaque; however, recent studies have also shown that *Candida albicans* is frequently detected along with *S. mutans* in oral biofilms collected from ECC-affected children [1, 2]. *C. albicans* was found in the saliva, plaque, and buccal mucosa of 77%, 83%, and 44% of ECC-affected children, respectively, whereas only 12%, 6%, and 6% of caries-free children had *C. albicans* present in their saliva, plaque, and

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Address for correspondence: Prof. Dr. Sarworini B. Budiardjo, drg., Sp. KGA (K), Lecturer, Department of Pediatric Dentistry, Faculty of Dentistry, University of Indonesia, Building A Level 2, Jalan Salemba Raya No. 4, Jakarta Pusat, Jakarta 10430, Indonesia, phone: +62817704575, +628161875360, fax: +62.21.31931412, e-mail: sarworinibagio@yahoo.com

buccal mucosa, respectively. These results show the potential role of *C. albicans* in the pathogenesis and progression of ECC [1].

Interactions between *C. albicans* and *S. mutans* within biofilms intensify the development of co-species biofilms, causing the onset of rampant caries [1, 2]. The *S. mutans*-derived exoenzyme glucosyltransferase (Gtf) is an exopolysaccharide (EPS) producer that plays a significant role in this cross-kingdom association. Gtf can adhere to the surface of *C. albicans* cells in a hyphal form, converting them to glucan producers and thus promoting the assembly of an EPS-rich matrix scaffold. Further, Gtf enables *C. albicans* to form colonies on the surface of EPS and enhances fungal-bacterial coadherence [1, 3]. *C. albicans* is recognized as a highly acidogenic and aciduric form of fungus that enhances acid production in the co-species biofilms, which further promotes the growth of both species [1].

*C. albicans* biofilms are known to have a unique form and evolution involving the attachment of *C. albicans* cells in yeast form to a surface followed by initial adhesion. The formation continues with the early development of basal layers of yeast cells, hyphae, and extracellular matrix. Mature biofilms contain a substantial number of yeast cells, hyphae, pseudohyphae, extracellular matrix, and water channels that facilitate the movement of nutrients and cell distribution [4]. Mature forms of *C. albicans* biofilms have proven resistant to a variety of conventional antifungal drugs, leading to ineffective treatment [5].

For thousands of years, garlic (*Allium sativum*) has been widely consumed throughout human history as a food, spice, and traditional medicine. Its efficacy has previously been recognized due to its antibacterial and antifungal activities. Moreover, many studies have demonstrated that garlic serves as a more effective antibacterial and antifungal agent than other existing conventional drugs, possibly due its capacity to fight against a wide spectrum of microbes [6, 7]. However, there is a lack of literature concerning the effects of different concentrations of garlic extract against the viability of *C. albicans* biofilms because previous studies have been more focused on the inhibitory properties of garlic extract.

## OBJECTIVES

The aim of the present study was to assess the efficacy of garlic extract against the viability of *C. albicans* biofilms from children with ECC.

## MATERIAL AND METHODS

### SUBJECTS

The present study was an *in vitro* laboratory experiment testing the viability of *C. albicans* biofilms following administration of garlic extract at various concentrations. Ethical approval of the study was obtained from the Ethics Committee of the Faculty of Dentistry Universitas Indonesia (No: 6/Ethical Approval/FKGUI/III/2018). Written informed consent was obtained from the parents of the subjects prior to study commencement. The subjects included children who visited the Pediatric Dental Clinic of our university and were subsequently diagnosed with ECC. The inclusion criteria included the following: age, 3-6 years old; diagnosis of ECC based on the American Academy of Pediatric Dentistry guidelines [8]; and parental consent. The exclusion criterion was children who had received local or systemic antifungal therapy within 90 days of the sample collection visit.

### SAMPLE COLLECTION

The subjects were required to not brush their teeth 2 h prior to sample collection. A dental plaque sample was collected throughout the whole dentition using a sterile toothpick, which was placed in 1 ml of Sabouraud dextrose broth in a sterilized microfuge tube (Onemed) and transported on ice to the Oral Biology Laboratory of our university. The samples were cultured within 2 h.

### CULTURE IN CHROMAGAR *CANDIDA* MEDIUM

The biofilm samples were centrifuged at 6000 × g for 1 min at room temperature; the supernatant was subsequently discarded, and 100 µl of normal saline was added. Further, the samples were vortex-mixed for 30 s followed by the inoculation of CHROMagar medium with 10 µl of each suspension and 48-h incubation under aerobic conditions at 37°C. Isolates were identified according to the different colors of the colonies on CHROMagar; green colonies represented the existing *C. albicans* species. Hence, these colonies were collected using a sterile loop, with which Sabouraud dextrose broth was inoculated and incubated for 24 h at 37°C under aerobic conditions. The following day, 200 µl of each suspension was obtained for DNA isolation.

### *CANDIDA ALBICANS* IDENTIFICATION BY PCR

*C. albicans* DNA was extracted using TRIzol reagent. The primer pairs used for *C. albicans* identification were based on sequences conferring species specificity of EO3 to *C. albicans* mitochondrial DNA, as described by Miyakawa *et al.*: primer 1 (5'-CACCAACTCGACC AGTAGGC-3') and primer 2 (5'-CGGGTGGTCTATA TTGAGAT-3') [9]. Amplification reactions were performed in a 25 µl reaction mixture containing 12.5 µl Dream Taq, 1.5 µl of each primer, 3 µl of DNA, and 6.5 µl of nuclease-free water. The PCR parameters were as follows: initial denaturation at 95°C for 10 min and

34 cycles comprising 20 s at 95°C, 45 s at 59°C, and 15 s at 72°C, followed by an additional cycle of 5 min at 72°C for chain elongation. All reaction products were analyzed by electrophoresis on agarose gels.

## PREPARATION OF GARLIC EXTRACT

Garlic was purchased from a local market (Pasar Induk Kramat Jati) in Jakarta and stored at 4°C. For extraction, 100 g of garlic cloves were crushed, placed in a vacuum filter, and juice was collected in a centrifuge tube. A volume of 100 ml of 20% ethanol was added, and the tube was shaken 10 times. Following 10-min incubation at room temperature, the solution was centrifuged twice at 5500 rpm for 5 min each time. The obtained garlic extract was diluted in distilled water to obtain solutions of different concentrations: 10%, 25%, 50%, and 100% (undiluted).

## VIABILITY TEST USING A 3(4,5-DIMETHYLTHIAZOL-2-YL)-2,5-DIPHENYLTETRAZOLIUM BROMIDE (MTT) ASSAY

Samples of *C. albicans* in Sabouraud dextrose broth were homogenized using a vortex mixer, and 100 µl of each suspension was transferred to a 96-well microplate and incubated at 37°C under aerobic conditions for 48 h. Following incubation, the plates were carefully rinsed with phosphate-buffered saline (PBS) solution, and the *C. albicans* biofilms were exposed to 100 µl of the different garlic extract concentration solutions and to 0.2% CHX as a positive control. Negative controls without testing material were also prepared. The microplate was subsequently incubated at 37°C under aerobic conditions for 24 h. The following day, the microplate was carefully washed with PBS solution, then 50 µl of 5 mg/ml MTT solution was added and incubated at 37°C for 3 h. Acidified isopropanol was added to the microplate, which was placed on an orbital shaker for 1 h. The absorbance was measured at 600 nm using an enzyme-linked immunosorbent assay reader. The viability percentage score was calculated using the optical density (OD) of the treatment and control samples by the following formula:

$$\frac{OD\ treatment}{OD\ negative\ control} \times 100\%$$

# RESULTS

*C. albicans* was identified by the green colonies surfacing on CHROMagar and verified by PCR, the products of which were approximately 125 bp (Table 1 and Figure 1) [9].

The present study aimed to identify the efficacy of garlic extract against *C. albicans* biofilms. The Shapiro-Wilk test for data normality showed a normal distribution; thus, the data were further subjected to a one-way ANOVA followed by a post-hoc analysis to identify any discrepancies in viability scores among the different concentrations of garlic extract.

**TABLE 1.** Primers used for verification of *Candida albicans* by PCR

| Primer name | Sequence (5′-3′) | Expected PCR product size (bp) |
|---|---|---|
| Identification PCR | | |
| Primer 1 | CACCAACTCGACCAGTAGGC | 125 |
| Primer 2 | CGGGTGGTCTATATTGAGAT | |

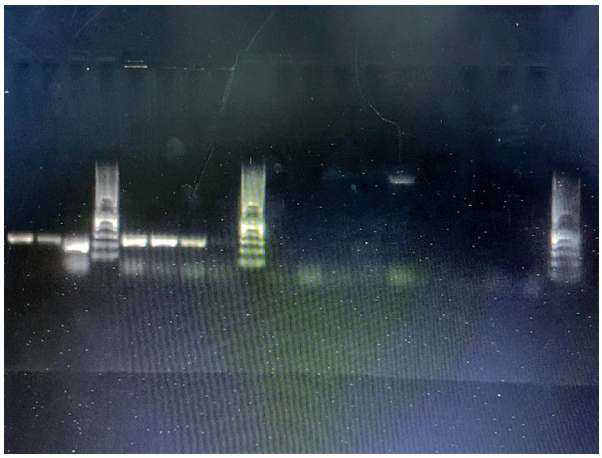

**FIGURE 1.** Agarose gel electrophoresis of polymerase chain reaction products with an expected size of 125 bp

The differences in the *C. albicans* viability scores following administration of various concentrations of garlic extract are summarized in Table 2. The one-way ANOVA revealed significant differences ($p = 0.000$), and post-hoc analysis was subsequently conducted to determine intergroup differences in terms of mean viability scores.

The post-hoc analysis results of the efficacy of different concentrations of garlic extract against the viability of *C. albicans* biofilms are shown in Table 3. A statistically significant difference in terms of the viability of *C. albicans* was noted between the negative control and all treatment groups.

# DISCUSSION

The present study was conducted to identify the efficacy of garlic extract against *C. albicans* biofilms from children with ECC. The results of the fungal culture on CHROMagar *Candida* medium and PCR verification are supported by the literature, in which *C. albicans* has been shown to be frequently detected along with *S. mutans* in high numbers within biofilms formed as dental plaque on the surfaces of teeth in children with

**TABLE 2.** Differences in viability of *Candida albicans* biofilms following administration of various concentrations of garlic extract

| Treatment group | *n* | Viability of *Candida albicans* biofilms Mean ± SD (%) | *p*-value |
|---|---|---|---|
| 10% garlic extract | 3 | 45.65 ± 7.41 | 0.000 |
| 25% garlic extract | 3 | 47.18 ± 16.95 | |
| 50% garlic extract | 3 | 22.35 ± 3.17 | |
| 100% garlic extract | 3 | 23.35 ± 1.49 | |
| Positive control (0.2% CHX) | 3 | 19.31 ± 5.83 | |
| Negative control | 3 | 100.00 ± 34.87 | |

*One-way ANOVA*
**Significant value based on p < 0.05*
*SD – standard deviation*

**TABLE 3.** *Post-hoc* analysis of differences in viability of *Candida albicans* biofilms between treatment groups and negative control

| Treatment group | *p*-value |
|---|---|
| Negative control vs. 10% garlic extract | 0.023 |
| Negative control vs. 25% garlic extract | 0.029 |
| Negative control vs. 50% garlic extract | 0.001 |
| Negative control vs. 100% garlic extract | 0.001 |
| Negative control vs. positive control (0.2% CHX) | 0.001 |

*Bonferroni's post-hoc analysis*
**Significant value based on p < 0.05*

ECC. The interaction of *C. albicans* with bacteria such as *S. mutans* enhances the accumulation and virulence of biofilms, leading to aggressive forms of tooth decay characterized by rampant carious lesions [10].

*C. albicans* biofilms are unique and substantially differ from bacterial biofilms containing hyphae and pseudohyphae. Their properties are remarkably different from those of planktonic or free-living populations, most notably regarding their high-level resistance to most antifungal agents [4, 5]. *C. albicans* biofilms can mature and increase in complexity within 24-48 h, the development of which begins with small microcolonies comprising budding yeast cells that later transform into filaments, forming pseudohyphae and true hyphae. The microcolonies merge into intricate networks to form a monolayer of woven-like structures. At 48 h, the complexity of the biofilms reaches a multilayered biofilm matrix, with all fungal morphologies present in the final biofilm structure [11]. The present study administered garlic extracts to mature *C. albicans* biofilms collected from children with ECC to examine their efficacy.

Garlic has been confirmed to possess antifungal properties against *C. albicans* as it can penetrate cellular and organelle membranes, resulting in damaged organelles and cell death of *C. albicans*. Bioactive compounds found in garlic can affect *C. albicans*' gene expression, metabolic activity, protein processes in the endoplasmic reticulum, meiosis, RNA transport, ribosome biogenesis, RNA degradation, DNA replication, and RNA polymerase activity [5]. The ability of garlic to inhibit growth of *C. albicans* is due to the presence of allicin, a sulfur-containing compound that has antibacterial and antifungal properties [11, 12]. Reportedly, allicin can decrease the metabolism of *C. albicans* cells, causing apoptosis [12, 13].

The antifungal efficacy of garlic extract depends on allicin; however, allicin is an unstable and reactive component known to have the capability to rapidly change within the extract. In the present study, the maceration extraction procedure was applied in a 20% ethanol solution without heating to extract garlic, as described in previous literature. Thus, the extraction of allicin was conducted most efficiently because 20% aqueous ethanol is known to be the most suitable solvent to maintain allicin for up to 2 weeks at room temperature. The hydrophobicity of allicin and the allicin-stabilizing hydroxyl group in the ethanol molecule cause allicin to dissolve more easily in an ethanolic solution [14]. Based on a study by Peters *et al.*, ≥ 30% ethanol at a 4-h exposure time can completely inhibit the metabolism of mature mono- or polymicrobial *C. albicans* biofilms [15]. The 20% ethanolic solution used in the present study to extract the garlic had no significant effect on the viability of *C. albicans* biofilms, suggesting that it did not contribute to any bias. Although we used 20% ethanol to extract the garlic and maintain the allicin within the extract, we diluted the extract with distilled water.

The concentrations of garlic extract used in the present study were 10%, 25%, 50%, and 100%. *In vitro* administration of the garlic extract resulted in a statistically significant decrease in the viability of *C. albicans* biofilms at all concentrations (Tables 2 and 3). A previous study reported that, based on a spectrophotometry technique, the minimum inhibitory concentration (MIC) of pure garlic on the growth of *C. albicans* is 50%, whereas the turbidimetric method showed an MIC for *C. albicans* of 25%. The efficacy of garlic extract increases with increasing concentration [16]. In the present study, the lowest viability value occurred when biofilms were exposed to 50% garlic extract; however, a significant decline in the viability of biofilms was already observed for 10% garlic extract. These results differ from those reported by previous studies because lower concentrations caused a significant decrease in the viability of biofilms, and the efficacy of garlic extract was slightly decreased at 100%. The discordance between the present and previous studies may be attributed to the different types of tests and biofilm development. The MTT assay, a colorimetric assay commonly used in studies on the susceptibility of biofilms to antifungal drugs, was used in the present study [4]. MTT, a yellow tetrazolium salt, is further reduced to insoluble purple formazan

crystals in the presence of metabolic activity, indicating the percentage of viable cells [4, 16, 17].

Garlic extract at 10% was effective against *C. albicans* biofilms, which is in accordance with the results reported by Low *et al*. showing that a fungicidal effect could be observed at a garlic concentration of 100 mg/ml [13]. Garlic extract was suitable for use against *C. albicans*, a result that is supported by a previous study acknowledging the efficacy of garlic extract in improving erythematous lesions caused by denture stomatitis, reporting a patient satisfaction rate of 85% [18]. Furthermore, another study reported that 40% fresh garlic extract remarkably enhanced antifungal activities of fluconazole and itraconazole against *C. albicans* [7].

This was a preliminary study, and the results could serve as the foundation for further studies assessing the antifungal efficacy of garlic extract against *C. albicans* biofilms that increase the severity of ECC.

## CONCLUSION

Based on the *in vitro* results, we can conclude that garlic extract at 10% is effective in decreasing the biofilm viability of *C. albicans*.

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## CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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