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RESEARCH ARTICLE

Effectiveness of *Aspergillus* sp. extract in denture adhesive on surface roughness of acrylic resin on *Candida albicans* biofilm formation

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ABSTRACT

The denture adhesive increases retention on the denture base and affects oral microorganisms. Adding antifungals to denture adhesives can inhibit the *Candida albicans* biofilms formation and prevent denture stomatitis. The combination of denture adhesives and herbal medicines is an alternative to antifungals, which have few side effects because it is a plant. Moreover, one of them is the endophytic *Aspergillus* sp. extract containing chemical compounds that can inhibit the *Candida albicans* biofilms formation. This study aims to analyze the effectiveness of the endophytic *Aspergillus* sp. extract in denture adhesive materials for *Candida albicans* biofilm formation on acrylic resin surfaces. The research method is to extract the *Aspergillus* sp. extract antibiofilm test. Denture adhesive formulation was adjusted to the standard, and added *Aspergillus* sp. with concentrations of 3.125%, 6.25%, 12%, and 25%. The research sample used hot polymerized acrylic resin. The control group used X denture adhesive and added nystatin, each group suspended by *Candida albicans* for 24, 48, and 72 hours. Examination of biofilm formation activity on the surface of acrylic resin used SEM. The analysis used Two Way Anova. *Aspergillus* sp. extract in denture adhesive effectively prevents *Candida albicans* biofilm formation within 24 hour incubation time. In conclusion, extract of the endophytic *Aspergillus* sp. in denture adhesive can inhibit the formation of Candida albicans biofilm on the surface roughness of acrylic resin.

Keywords: acrylic resins; *Aspergillus* sp.; biofilms; *Candida albicans*; denture adhesives

INTRODUCTION

Central statistics data for 2018 shows that the elderly percentage in Indonesia has doubled, reaching 9.27 percent or around 24.49 million people. It was dominated by the young aged (60-69 years age group or 63.39 percent).[1] Dentures commonly used by the elderly are complete dentures with a base (base plate) as the central part, acting as a substitute for the supporting tissue around the tooth. Removable dentures on the acrylic resin are still the choice for denture manufacture. The use of acrylic resin as a denture base reaches 98%.[2]

Changes in the supporting tissue in the elderly occur physiologically and pathologically to reduce the retention and stabilization of removable dentures, such as hormonal changes, neuromuscular disorders (Parkinson's disease, Alzheimer's disease), xerostomia caused by drugs/radiotherapy, and systemic diseases.[3,4,5] Alternative treatment can be provided by using denture adhesive (PGT).[5] Continuous use of PGT is accompanied by physical limitations for the maintenance and care of dentures after using adhesive, causing uncontrolled use of adhesive and adhesive residue on dentures and supporting tissues, increasing *C.albicans* colonies as a cause of denture stomatitis.[6,7]

Denture adhesives on the market have three packaging: powder, cream, and strips.[8] Each PGT packaging has an elemental composition consisting of an adhesive (methylcellulose, hydroxymethyl cellulose, carboxymethyl cellulose) and synthetic polymers (polyethylene oxide,

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acrylamides, polyvinyl acetate), a preservative and antimicrobial (sodium borate, sodium tetraborate, hexachlorophene or propyl hydroxybenzoate and ethanol), and additives (petrolatum, mineral oil, polyethylene oxide in gel to minimize clumping, peppermint, coloring).[9,10]

Research by Maia et al. on several adhesives showed that PGT materials, which did not have an antifungal component, tended to increase the number of *C. albicans*. Meanwhile, adhesives with an antifungal component decreased the number of *C. albicans*. Research by Rajanam, Manoj, and Nunes states that the effect of PGT is different because it uses different antimicrobials. Many researchers conducted innovations to replace antimicrobials with different antifungals to develop PGT. The researchers used synthetic antifungal agents, which generally inhibited and suppressed the growth of *C. albicans*.[11,12] Other researchers used natural ingredients to inhibit the development of *C. albicans* biofilms, significantly to minimize colonization.[13]

Endophytic microbes have great potential in searching for new drug sources because microbes are easy to breed, have a short life cycle, and can produce large amounts of bioactive compounds quickly.[14] The activity they produce is sometimes greater than that of their host. However, there is still little use for endophytic fungi from the sea.[15] Endophytic fungi are found in plant tissue systems, and it does not cause disease symptoms in the host plant. Endophytic fungi can produce antibacterial compounds as potential control agents. To overcome this problem, searching for antifungal compounds from natural resources isolated from extracts of the endophytic *Aspergillus* sp. *RmAk3* is necessary. These compounds must still consider the requirements of biocompatibility, mechanical, and physical properties of acrylic resin.[2]

MATERIALS AND METHODS

The ethical permission of the research was approved by the health research ethics commission, the Medical Faculty of Universitas Sumatera Utara/H. Adam Malik General Hospital board No 587/TGL/KEPK FK USU-RSUP HAM/2018.

Filtration of the fungal mycelium, then the ethyl acetate extract was evaporated *in vacuo* using a rotary evaporator.[16] Then, assessing the forming of *C. albicans* biofilms used the crystal violet technique. Previously *C. albicans* was cultured in trypticase soy broth (TSB), and the quantity was calculated based on the OD value. Quantity calculation used a microplate reader; 24 wells were washed with TSB culture medium coated with saliva, then inoculated with *C. albicans* (10^6 cells) and incubated for 90 minutes at 30 °C without shaking. Each well was added with Sabouraud medium (10, 30, or 50%) and then incubated for 2-3 days at 30 °C.

Candida cells that did not form biofilm on the bottom of the plate were removed and washed

Table 1. Material Composition for Denture Adhesive Formulations

Material	Wt %
Gantrez / maleic acid	37.5
Carboxylmethyl cellulose (CMC)	15.5
Liquid parafin	10.4
White petrolatum	28.5
Versagel MN	5
Colloidal silica	3
Nipazol	0.05
Aspergillus sp	3.125, 6.25, 12.5, 25

Source[11]

twice with 500 µl phosphate buffered saline (PBS), then 50 µl 0.1% crystal violet dye was added for 15 minutes at room temperature. Then, it was washed with PBS to remove crystal violet, which is not absorbed by bacteria. After washing, 200 µl of 98% ethanol was added. In the next step, 200 µl/well of 96% ethanol was added to every 24 wells of the tissue culture plate, then transferred to 96 wells of the microtiter plate. The formed biofilm was measured based on its absorbance value at a wavelength of 490 nm using an Elisa reader.[17]

The test plates were made from hot polymerized acrylic resin (Acron, GC Japan) with 48 plates. It used a primary model of 10 x 10 x 1 mm metal.[18] The plate production follows the manufacturer's instructions. The plate surface was polished using sandpaper of 200, 300, and 400.

Table 1 presents all formulation compositions stirred with a vacuum mixer for 20 minutes. The test used a 20 g denture adhesive formula after adding 3.125%, 6.25%, 12.5%, and 25% *Aspergillus* sp. concentrations.

The prepared denture adhesive was homogenized with 100 mg PBS: 10 ml solution and vibrated. Furthermore, the formed acrylic resin is adapted to a physiological NaCl solution to obtain a uniform absorption pressure, and the acrylic is placed vertically. Afterward, it was incubated in 10 ml of critical saliva in PMSF (10:1) pH 6.5 for 30 minutes. 300 µl of *C. albicans* solution $1.5x10^8$ CFU/ml was added to each acrylic sample.[19] After 15 minutes, the test material (endophytic fungi) was added based on the concentration (3.125%, 6.25%, 12.5%, 25%), 2 mg diluted polident solution, and nystatin in 10 ml PBS pH 7.

The adaptation process of *C. albicans* biofilm formation on the surface of acrylic resin needed 24, 48, and 72 hour incubation times. Moreover, the incubation was at 37 °C. Acrylic resin coated with biofilm and endophytic fungi was prepared to observe the effect of endophytic fungi on the formation of *C. albicans* biofilm. In the first stage, the acrylic resin was immersed in 0.9% NaCl for 15 minutes and shaken at 500 rpm. Then the part of the acrylic resin that formed the biofilm was immersed in 10 ml of 1% crystal violet for 30 minutes. Then, it was immersed in 0.9% NaCl for 5 minutes above the shaker at 500 rpm. In the next step, 10 ml of 1% safranin was added for 15 minutes, then washed and stored at 4 °C for 48 hours.

Confirmation of biofilm formation was examined with an electronic microscope at 400-1000 x magnification. Then, examining the biofilm with SEM to identify the area and quantity of the biofilm. The area of biofilm formation was then measured with ImageJ. The growth quantity of C. albicans on biofilm formation on acrylic was measured by spectrophotometry based on turbidity at a wavelength of 550 nm. OD 0.08-0.1 (<300 CFU). This indicator is a reference for measuring the number of colonies on the ability to form biofilms after being prepared with endophytic fungi.

RESULTS

Figure 1. 6.25% concentration with 24 and 72 hour incubation time of *Aspergillus* sp. shows better antibiofilm *C. albicans* than other concentrations. Meanwhile, the 48 hour incubation time was relatively stable at all concentrations.

Table 2 two Way Anova analysis shows significant differences ($p < 0.05$) in the activity of *C. albicans* anti-biofilm on the concentration of *Aspergillus* sp. and incubation time. It shows that the concentration of the test material and time influence the ability of the anti-biofilm. Therefore, the increase in the number of active components in each concentration is directly proportional to the increase in the activity of anti-forming *C. albicans* biofilms. However, there was no interaction between concentrations and time on anti-biofilm activity.

Scanning Electron Microscopy (SEM) observations determined the activity of *C. albicans* forming biofilms on *Aspergillus* sp. and the control group. Figure 2 shows that a 6.25% and 25% concentration with 24 hour incubation time suppressed the activity of *C. albicans* to form biofilms, and a small portion of the biofilm matrix was damaged. Meanwhile, nystatin in Figure C shows an excellent antifungal effect; *C. albicans* activity was not found. Biofilm development found

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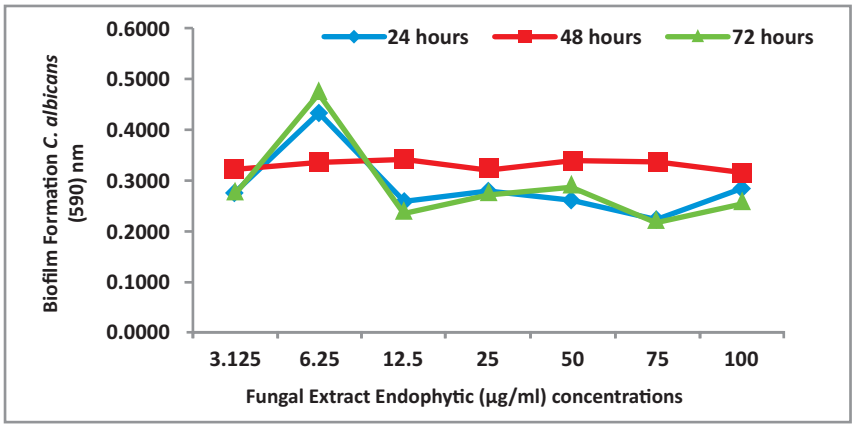
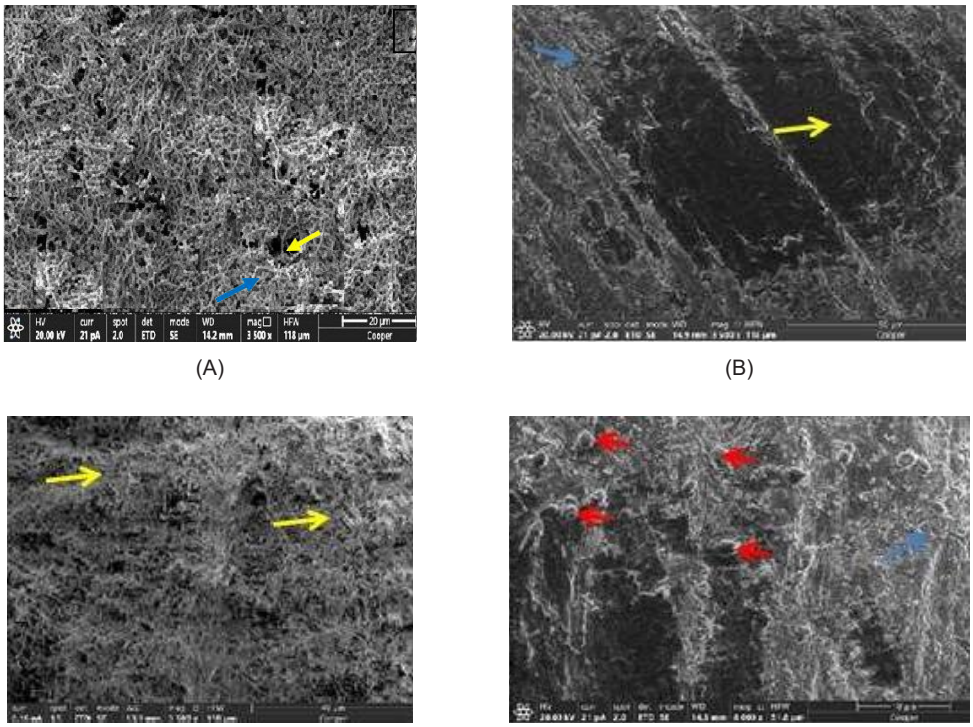


Figure 1. Antibiofilm power diagram of extract fungi *Aspergillus* sp. to *C. albicans*

Table 2. Analysis of anti-forming power biofilm against *C. albican*

Variable Analysis	SS	df	mean	F	p
Concentration	10587.912	5	2117.582	.553	0.003*
Time	14859.693	2	7429.847	1.940	0.000*
Concentration – time	25447.605	7	3635.372	.949	.364

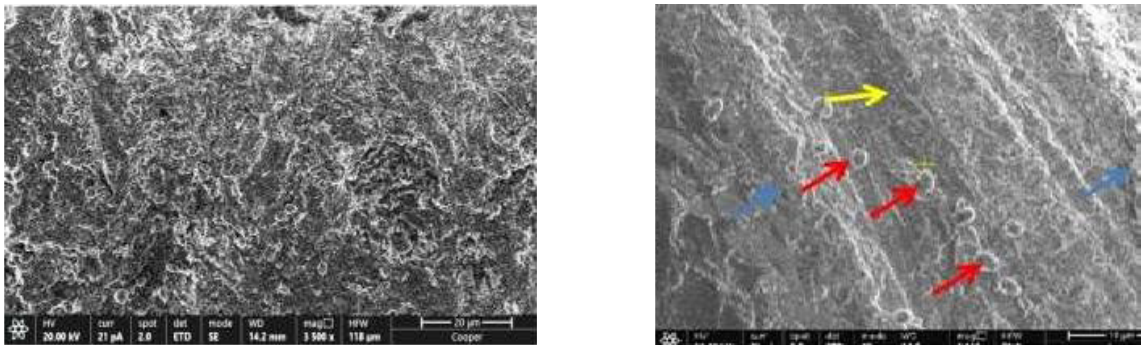
*significant



(A) (B)

(C) (D)

Figure 2. Biofilm profile *C. albicans* on the surface of acrylic resin with concentrations of: A (6.25% 24 hours), B (25% 24hours), C (nystatin 24 hours), D (product X 24 hours). Blue arrow (biofilm), yellow arrow (damaged biofilm matrix), red arrow (cell *C. albicans* which has been damaged). Magnification 3500x



(A) (B)

Figure 3. Biofilm profile *C. albicans* on the surface of acrylic resin with concentrations of: (A) (6.25% 48 hours), (B) (25% 48 hours). Blue arrow (biofilm), yellow arrow (damaged biofilm matrix), red arrow (cell *C. albicans* which has been damaged). Magnification3500x

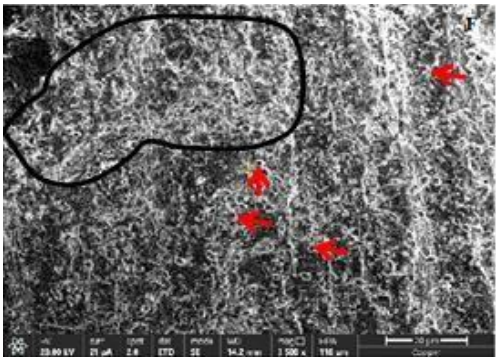


Figure 4. Biofilm profile *C. albicans* on the surface of acrylic resin with product X48 hours, red arrow (cell *C. albicans* that have been damaged), dark circles (biofilm along with cells) *C. albicans* which is still intact). Magnification 3500x

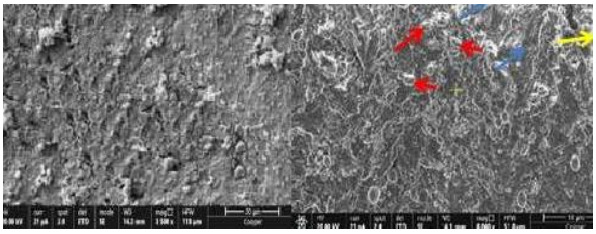


Figure 5. Biofilm profile *C. albicans* on the surface of acrylic resin with concentrations of: A (6.25% 72 hours), B (25% 72 hours). Blue arrow (biofilm), yellow arrow (damaged biofilm matrix), red arrow (cell *C. albicans* which has been damaged). Magnification3500x

only biofilm mass damaged after interacting with nystatin. Product X showed an increased mass of biofilm and *C. albicans* cells. In contrast, product X could not stem the growth of *C. albicans* which was characterized by an increase in biofilm and the development of *C. albicans.*

Figure 3 shows 6.25% and 25% concentrations for 48 hours. Besides suppressing biofilm formation, it also prevents the development of *C. albicans* (the number of *C. albicans* cells is limited and has been separated from other parent cells). Figure 4 shows the mass of biofilm and *C. albicans* cells increased in product X, where product X could not stem the growth of *C. albicans,* which was characterized by an increase in biofilm and the development of *C. albicans*. Figure 5 shows No hyphae and pseudohyphae, meaning that C. albicans failed to thrive. Anti-biofilm activity of *C. albicans* decreased at 72 hours, with a relatively increased number of *C. albicans* cells even though the cells had been damaged.

DISCUSSION

Figure 1 shows that the *Aspergillus* sp. extract has anti-biofilm activity against *C. albicans*, especially at 24 and 72 hour incubation times; the extract has better anti-biofilm activity against *C. albicans* than other concentrations. Meanwhile, it was relatively stable at all concentrations at 48-hour incubation time. According to Gulati, anti-biofilm activity aligns with the theory of biofilm formation, where 48 hours is considered the start of the biofilm matrix's maturation phase. In this condition, there is no interaction activity with several active components from drugs or plant

extracts.[19] Moreover, 24 hours is considered the biofilm matrix formation phase by *C. albicans*. In this phase, the adaptive response of the extract to biofilm cells is higher than *C. albicans* activity, so *C. albicans* experiences a shock response to its environment. In addition, the 72 hour incubation time is considered the stage of biofilm dissemination, the excellent ability to inhibit biofilms by *Aspergillus* sp. at 72 hour incubation time.[20]

It shows that this test material can prevent colonization and increase quorum-sensing formation in *C. albicans* to prevent intra- and inter-cell communication of other pathogens involved in biofilm formation. Serrano-Fujarte (2015) strengthens the findings of this study; the incubation time determines the increase in biofilm formation for 24-72 hours.[21] Therefore, this difference shows that *C. albicans*, exposed to the test material, experiences a decrease in static energy as a loop phase to form pseudohyphae and hyphae to prevent biofilm formation activity, attachment transition, colonization, and matrix formation, which are characteristic of biofilm formation at 24 hours or the intermediate phase.[14] Baboni (2010) reported that *C. albicans* is highly sensitive to forming biofilm when the environment changes.[21] In addition, these differences are also influenced by the interaction of *C. albicans* with other oral microorganisms when forming quorum-sensing in biofilm formation.[22]

The *C. albicans* biofilm formation starts from (1) The attachment of the fungal cell forms to the surface. (2) Initiation of cell proliferation and branching formation in the cells' basal layer. (3) Maturation, including hyphal growth, concurrently produces extracellular matrix material. (4) Release of the fungal cell form from the biofilm, forming a new place.[23] Meanwhile, the growth conditions of the biofilm are controlled in four stages: the early stage, where the fungus attaches to the substrate to form a biofilm, then forming coaggregation and colonization.[24]

Then the intermediate stage, where *C. albicans* cells grow and proliferate on the surface of host cells. Then attachment to the surface forms the transition from blastospores to hyphae and pseudohyphae. Therefore, it is possible to extract *Aspergillus* sp. at a 6.25% concentration and incubated for 24 hours, 48 hours, and 72 hours to prevent the formation of biofilms, starting with the coaggregation and colonization stages, and the stages where maturation and dissemination occur.

The analysis results in Table 2 show no significant difference in anti-biofilm formation in endophytic extracts based on incubation time ($p > 0.05$). Meanwhile, based on the concentration of *Aspergillus* sp. extract showed a significant difference in *C. albicans* anti-biofilm activity ($p < 0.05$). It shows that the concentration of *Aspergillus* sp. strongly affects anti-biofilm activity because of the biotolerance of natural ingredients against pathogens. As a natural ingredient, *Aspergillus* sp. extract can be antioxidant and antifungal.[25] Flavonoid activity can inactivate enzymes, transport proteins, prevent adhesion, and harm pathogens' cell membranes. Thus, *C. albicans* fails to ferment carbohydrates to lower the pH in biofilms.[26] *C. albicans* has a high acid tolerance and is capable of producing acid even under low pH conditions.[27]

Therefore, the increase in the number of active components in each concentration is directly proportional to the increase in anti-biofilm formation activity against *C. albicans*. Another critical factor in forming *C. albicans* biofilms is the presence of temperature changes to increase attachment, coaggregation, and protease production.[28] Related to this research, *Aspergillus* sp. can prevent the development of *C. albicans* biofilms because it can form covalent bonds to activate cysteine residues, which then activate UDP-N-acetylglucosamine to form hydrogen bonds. As a result, it inhibits HWP protein synthesis (hypha wall protein), the production site for proteins in biofilm formation.[29]

In addition, it can be assumed that *Aspergillus* sp. can prevent *C. albicans* adhesion by inhibiting phosphoenolpyruvate synthetase. Moreover, the *Aspergillus* sp. extract has

antifungal properties by inhibiting the mechanism of action of the pH-independent effect of binding to host receptor proteins.[30] Thus, the results of this study can be assumed that the *Aspergillus* sp. extract as an antifungal can inhibit quorum-sensing signals in the adaptation phase of biofilm formation to form colonies and aggregation in host cell invasion.[31]

Figures 2, 3, and 4 show that the *Aspergillus* sp. extract can suppress the formation of biofilms. The ability of *Aspergillus* sp. extract prevents biofilm formation and colonization on the surface of acrylic resin; this test material can prevent the transition from blastospores to hyphae.[32] Ganguly (2011) clarifies that in vitro experiments show that the *C. albicans* biofilm formation is through a series of sequential stages: adhesion, initiation, maturation, and dissemination.[33] The series of phases have specific effects on the host, the *Aspergillus* sp. extract can prevent a series of *C. albicans* biofilm formation activities based on the findings of this study.[34]

Specifically, PMMA acrylic resin is a primary material for dentures due to its good working properties, such as simple preparation and installation, reasonable accuracy, stability in the oral environment, aesthetic aspects, and affordability. However, acrylic resin is a polar molecule that absorbs PMMA, so it is easily damaged and smells terrible.[33] Loss of water absorption on the acrylic resin surface can cause hydrophobicity, which is beneficial for the development of *C. albicans* which contributes to surface hardening and irregularity in the acrylic resin surface can support increased colonization of fungi that trigger denture stomatitis by *Candida* sp. Denture stomatitis is one of the most common problems in removable dentures, with a 25-65% prevalence. The use of antifungals, such as the azole group and nystatin-based antifungals, cannot work effectively on the inner surface of the denture base because the biofilm is resistant to these antifungals.[35]

According to Andreotti (2018), the surface of acrylic resin is susceptible to fungal colonization because surface roughness is one of the factors that helps the fungus attach. Damage to the surface of the acrylic resin, such as scratches, cracks, and porosity, can increase the attachment of pathogens and allow spread and infection. Therefore, the application of *Aspergillus* sp. is possible on the surface of acrylic resin to prevent the biofilms formation by *C. albicans*. Based on the incubation time, the results of this study showed no difference ($p > 0.05$) in the surface roughness of acrylic resin after interaction with *C. albicans* and exposure to *Aspergillus* sp. Meanwhile, based on the concentration, the analysis results did not show a difference ($p > 0.05$). Therefore, changes in the acrylic resin surface after being adapted to *C. albicans* and the test material were affected by the concentration of *Aspergillus* sp. and incubation time.[36]

The protection mechanism by *Aspergillus* sp. on *C. albicans* activity indirectly prevents a number of *C. albicans* cell surface proteins from increasing adhesion. In addition, it is possible that several active antifungal components possessed by *Aspergillus* sp., such as 7-pentadecyne,-9-methylene, Hexadecanoic acid, 9,12-octadecadienoic acid (linoleic acid (LA) and 4 – Isopropyl - 1,6 - dimethyl -1,2,3,4,4A,7-hexahydro naphthalene can prevent the surface hydrophobicity activity of *C. albicans* cells and the biofilms formation because these chemical compounds can prevent water absorption or can cover the porosity of acrylic resin.

This study did not examine the active compounds of *Aspergillus* sp., which play a direct role in the activity of *C. albicans* in the biofilm formation and the adhesion intensity of acrylic resin surfaces. Future research needs to purify several active compounds from *Aspergillus* sp. involved in the *C. albicans* biofilms formation on the surface of acrylic resin.

CONCLUSION

The endophytic *Aspergillus* sp. extract added in PGT effectively prevents the *C. albicans* biofilms formation. Specifically, a 6.25% concentration showed anti-biofilm activity at all incubation

times. Therefore, the concentration of *Aspergillus* sp. strongly affects anti-biofilm activity. The endophytic *Aspergillus* sp. extract in PGT with 24 hour incubation time can suppress the activity of *C. albicans* to form biofilms.

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