第 49 卷 第 1 期
2022 年 1 月

湖南大学学报（自然科学版）
Journal of Hunan University (Natural Sciences)

Vol. 49 No. 1
January 2022

Open Access Article https://doi.org/10.55463/issn.1674-2974.49.1.25

# *Candida Albicans* and *Streptococcus Mutans* Biofilms Suppression by Bioactive Compounds Isolated from *Ruta Angustifolia*

**Shafa Noer[1,3], Abinawanto[1]*, Boy M. Bachtiar[2], Anom Bowolaksono[1], Sofa Fajriah[4]**

[1] Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok, Indonesia

[2] Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta Pusat, Indonesia

[3] Department of Biological Education, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indraprasta PGRI, Jakarta Timur, Indonesia

[4] Research Center for Chemistry, National Research and Innovation Agency, Banten, Indonesia

**Abstract:** *Ruta angustifolia* is one of the species of *Ruta* genus that is widely used in Asia as traditional natural medicine, but scientific research related to this plant is still limited. Several studies have stated that this plant has an antimicrobial effect, but no studies have discussed the antibiofilm effect of bioactive compounds isolated from *R. angustifolia*. This study is the first to address this issue. This study aims to see whether the bioactive compounds isolated from *R. angustifolia* can inhibit the formation of biofilms on oral microbes that play the most important role in the construction of dental caries, namely *Candida albicans* and *Streptococcus mutans*. Isolation and identification of bioactive compounds is achieved through Thin Layer Chromatography, Liquid Chromatography with tandem mass spectrometry, and proton nuclear magnetic resonance, respectively. Crystal Violet (CV) method is used to see the total biomass of biofilm, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method is used for the viability test. The morphological visualization of *C. albicans* has also been carried out using a light microscope and a scanning electron microscope. Three compounds that have been isolated are kokusaginine, chalepin, and lindelofine. The CV and MTT data were compared with the control data using an analysis of variance test followed by the Tukey honestly significant difference test, while the morphological data is qualitatively analyzed. From the results of the biofilm test, it was concluded that the three compounds could significantly inhibit the formation of biofilms produced by *C. albicans* and *S. mutans* in mixed species cultures.

**Keywords:** *Ruta angustifolia*, biofilm, *Candida albicans*, *Streptococcus mutans*, bioactive compounds.

# 从芸香中分离的生物活性化合物对白色念珠菌和变形链球菌生物膜的抑制作用

**摘要**：芸香是芸香属的一种，在亚洲被广泛用作传统的天然药物，但与这种植物相关的科学研究仍然有限。几项研究表明这种植物具有抗菌作用，但没有研究讨论从 R. 小叶中分离出的生物活性化合物的抗生物膜作用。这项研究是第一个解决这个问题的。本研究旨在了解从 R. 小叶中分离出的生物活性化合物是否可以抑制口腔微生物生物膜的形成，这些微生物在龋齿的形成中发挥着最重要的作用，即白色念珠菌和变形链球菌。生物活性化合物的分离和鉴定分别通过薄层色谱、串联质谱液相色谱和质子核磁共振来实现。晶体紫（简历）法用于观察生物膜的总生物量，3-（4,5-二甲基噻唑-2-基）-2,5-二苯基溴化四唑（MTT）法用于生

Received: October 21, 2021 / Reviewed: November 15, 2021 / Accepted: December 13, 2021 / Published: January 28, 2022
Fund Project: Universitas Indonesia (Research Grant Contract Number: NKB-618/UN2.RST/HKP.05.00/2020)
About the authors: Shafa Noer, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok, Indonesia; Department of Biological Education, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indraprasta PGRI, Jakarta Timur, Indonesia; Abinawanto, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok, Indonesia; Boy M. Bachtiar, Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta Pusat, Indonesia; Anom Bowolaksono, Department of Biology, Faculty of Mathematics and Natural Sciences (FMIPA), Universitas Indonesia, Depok, Indonesia; Sofa Fajriah, Research Center for Chemistry, National Research and Innovation Agency, Banten, Indonesia
Corresponding author Abinawanto, abinawanto.ms@sci.ui.ac.id

存力测试。还使用光学显微镜和扫描电子显微镜对白色念珠菌的形态进行了可视化。已分离出的三种化合物是到国兔、chalepin 和林德洛芬。将简历和 MTT 数据与对照数据进行比较，使用方差分析检验和图基诚实显着性差异检验，同时对形态学数据进行定性分析。从生物膜测试的结果可以得出结论，这三种化合物可以显着抑制混合物种培养中白色念珠菌和变形链球菌产生的生物膜的形成。

**关键词：** 芸香、生物膜、白色念珠菌、变形链球菌、生物活性化合物。

# 1. Introduction

According to the World Health Organization data, oral diseases pose a huge health challenge to several countries and affect human lives, causing pain, discomfort, disability, and even death [1]. Meanwhile, one of the most common oral problems is dental caries from plaque caused by biofilm that forms on the tooth surface. Biofilm is a complex arrangement produced by microbial interactions and causes resistance to antimicrobial substances [2].

Furthermore, biofilms are formed from colonized microorganisms' interactions in the oral environment. One of the microbes that most commonly causes dental caries is *Streptococcus mutans* [3], while the presence of yeast *Candida albicans* is reported to increase its cariogenicity level [2]. *C. albicans* is a species that causes several infectious diseases [4]. In the oral environment, *C. albicans* is found as a normal flora, but under certain conditions, it causes various health problems [5].

The interaction between bacteria and fungi is either antagonistic or cooperative. Cooperative interactions occur when microorganisms provide substrates or metabolites needed by another microorganism. For example, *C. albicans* is inefficient at metabolizing sucrose and is satisfied by the degradation products of sucrose by *S. mutans* (glucose and fructose). Meanwhile, the presence of *C. albicans* in the biofilm increases the number of exopolysaccharides, thereby contributing to the formation of *S. mutans* microcolonies [6].

Abnormalities in oral conditions that lead to serious disorders usually involve more than one species of microbe [2]. Several studies have shown that the biofilms formed by *C. albicans* and *S. mutans* have a higher cariogenic potential compared to single species biofilms [3]. Furthermore, the biofilms formed from these two species are more tolerant to environmental stresses, including antimicrobial exposure. Therefore, there is a need to develop appropriate therapeutic agents to overcome the biofilms caused by these two species [6]. One of the largest sources of antibiofilm agents believed to have good potency is derived from plants [7].

Plants belonging to the genus *Ruta* are shrubs often used as traditional medicines from ancient times. These plants have broad pharmacological effects, especially with antimicrobial activity: antibacterial, antifungal, antiviral, and antiparasitic [8]. One of the medicinal plants from this genus often used as traditional medicine is *Ruta angustifolia*.

*R. angustifolia* originates from the Mediterranean region and is commonly found in Asia as a medicinal plant. The height of this plant might reach 1.5 m, and the stem is woody. It grows in mountainous areas with an altitude of 1000 m above sea level. The leaves are light-green compounds with a width of 2-6 mm and a length of 8-20 mm, while the fruit is small, and the seeds are black and have a kidney shape [9].

Over the past few years, various research groups have developed plant-sourced biofilm inhibitory agents [10]. Plants' metabolic compounds function as good therapeutic agents for various oral diseases such as caries and candidiasis [2]. *R. angustifolia* contains several bioactive compounds potentially used to treat various diseases. Some of the bioactive compounds that have been isolated from this plant include graveoline, rutamarin, psoralen, neophytadiene chalepensin, arborinine, kokusaginine, moskachan (A, B, C, and D), bergapten, and chalepin [9].

However, the antibiofilm activity of bioactive compounds isolated from *R. angustifolia* against *C. albicans* and *S. mutans* has not been investigated. Meanwhile, several studies have shown that this plant contains bioactive components with great potential as antibiofilm. Therefore, this study aims to determine the effectiveness of bioactive compounds isolated from *R.angustifolia* leaves as an antibiofilm for *C. albicans* and *S. mutans*.

# 2. Methods

### 2.1. *R. Angustifolia* Extract Preparation

*R. angustifolia* was obtained from the Manoko Experimental Garden, Research Institute for Spices and Medicinal Plants, Lembang, Bandung, West Java, Indonesia. The leaves were separated from the stems and dried in the sun for about seven days. The

extraction used the maceration method with 96% methanol as solvent.

### 2.2. Isolation and Identification of Bioactive Compounds from *R. Angustifolia* Leaf Extract

Thin Layer Chromatography (TLC) separates a mixture of compounds from methanol extracts of *R. angustifolia* leaves. The spot results on TLC are then purified. For obtaining and confirming the structure of bioactive compounds obtained, Liquid Chromatography with tandem mass spectrometry (LC-MS/MS) and Proton Nuclear Magnetic Resonance (H-NMR) are used. Pure compounds were obtained in powder and then dissolved with DMSO up to a 1 mg/mL concentration for further use in this study.

### 2.3. Microbial Preparation and Growth Conditions

The microbes used in this research were *C. albicans* ATCC 10231 and *S. mutans* ATCC 25175. Microbes from stock cultures were grown in solid medium Sabouraud Dextrose Agar (*C. albicans*) and Brain Heart Infusion Agar (*S. mutans*) for 48 hours at 37$^{o}$C (for *S. mutans* under anaerobic conditions). Furthermore, about 2-3 colonies of microbes were then transferred to a liquid medium and incubated for 24 hours. The number of microbes was calculated initially before the study commenced using a hemacytometer ($10^6$ for *C. albicans* and $10^8$ for *S. mutans*).

### 2.4. Saliva Coating

Donor saliva was collected in a sterile tube (15 mL) and then centrifuged at 8000 rpm, 4$^{o}$C for 15 minutes. The supernatant was taken and then sterilized using filter paper with a diameter of 0.22 µm. Each 100 µL was placed into a 96 well plate and then incubated for 1 hour in an incubator at 37$^{o}$C. The salivary fluid was then discarded, and the saliva-coated plate was ready for further testing.

### 2.5. Biofilm Test with Crystal Violet (CV) Method

Microbes with counted cells were put into 96 well plates coated with saliva and extracts with the following conditions; each plate contained 30 µL of bioactive compounds and 70 µL of microbes (for 35 µL of mixed species each). The negative control contained 100 µL of microbe culture without adding extract. This mixture was then mixed using an orbital shaker for about 10 minutes and incubated in an incubator at 37$^{o}$C for 48 hours. After this stage, pH measurements are performed using the pH indicator to determine whether the pH is above or below 6 (the relevant critical pH criteria associated with the dissolution of dental hard tissue is below 5.5) [11]. Furthermore, the supernatant was removed, the plate was washed with 200 L of PBS, and the plate was then left/fixation until the biofilm was dry. 200 µL CV with a concentration of 0.5% was placed into the well and incubated at 37$^{o}$C for 15 minutes. The supernatant containing the remaining CV solution was then discarded, the plate was washed with 200 µL of PBS, and then, 200 µL of 95% ethanol was added. The plate was then inserted into a microplate reader to read the OD value using a 600 nm wavelength.

### 2.6. Viability Test 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) Method

Microbes (calculated cell number) are put into 96 well plates with bioactive compounds with the following composition: each plate contains 30 µL bioactive compound and 70 µL (for mixed species of 35 µL each) microbes. Negative control contains 100 µL of microbes culture (without the addition of bioactive compounds). The mixture was then mixed using an orbital shaker for about 10 minutes and incubated at 37$^{o}$C for 24 hours. The supernatant is then discarded, and MTT reagent is inserted with a concentration of 5 mg/mL in 0.9% NaCl and then incubated at 37$^{o}$C for 3 hours. An acidified isopropanol solution (100 µL) is inserted and incubated at room temperature for 1 hour. Ninety-six well plates were then inserted into the microplate reader to read the OD value using 490 nm wavelength.

### 2.7. Morphological Visualization of *C. Albicans* Using Light and Scanning Electron Microscope (SEM)

The morphology of *C. albicans* in biofilm conditions (single and mixed cultures) is seen using a light microscope (Olympus, Tokyo, Japan) with 40x magnification. For visualization using SEM, biofilms are prepared by culturing *C. albicans* (single and mixed species) on acrylic discs placed in a 24-well plate beginning with 90 min incubation time and continuing for 48 h. After treatment for biofilm formation, specimens are fixated using 1 mL 2.5% glutaraldehyde for 1 hour. Specimens were then dehydrated using ethanol series (10, 25, 50, 75, and 90%) for 20 minutes each, followed by immersion in 100% alcohol for 1 hour [12]. The sample was then dried by overnight incubation at 37°C. Samples that have been dried are sent to PT Cipta Mikro Material (PUSPIPTEK, Serpong, West Java, Indonesia) for visualization using SEM with 1000, 3000, and 5000 x magnification.

### 2.8. Data Analysis

Each data from each treatment was compared with the control using a one-way analysis of variance (ANOVA) test followed by the TUKEY HSD test. A p-value $< 0.05$ was considered statistically significant.

# 3. Results and Discussion

### 3.1. Isolation and Identification of Bioactive Compounds

This study successfully identified a number of candidate bioactive compounds. From among these candidates, we selected three (kokusaginine, chalepin, and lindelofine) based on their purity levels to further isolate and test their biofilm activity.

Of the three compounds isolated in this study, two were separated from the same plant in previous studies, namely kokusaginine [9] and chalepin [9], [13], [14], [15]. However, to the best of our knowledge, lindelofine is a bioactive compound that has not previously been isolated from *R. angustifolia*. As such, the present study is the first to reveal this. Prior studies have described bioactive compounds isolated from plants that have been tested in terms of their antibiofilm activity, although the three compounds used in this study have not previously been investigated in this regard [7]. Thus, this study is also the first to determine the antibiofilm activity of kokusaginine, chalepin, and lindelofine.

Chalepin is a bioactive compound that belongs to the coumarin group, which comprises compounds that have an aromatic group from the polyphenol group. Previous studies have shown that polyphenols are very well able to inhibit *C. albicans* biofilm [16]. Chalepin has also been investigated as a potential anticancer agent for the treatment of breast [13] and lung [17] cancer due to its ability to induce cell apoptosis. Moreover, chalepin can change the morphology of the cell nucleus, which results in a drastic reduction in the number of cells, the condensation of cell chromatin, and cell death due to fragmented DNA [13]. It can inhibit the replication of the hepatitis C virus [10], and chalepin isolated from *R. angustifolia* has also been studied in silico as a potential COVID-19 treatment targeting the SARS-CoV-2 papain-like protease [15].

Kokusaginine is a quinoline alkaloid that inhibits the action of the topoisomerase type II enzyme, which results in the inhibition of DNA replication [18]. It has also been shown to significantly inhibit the growth of *Mycobacterium smegmatis* [19].

Lindelofine is a compound belonging to the pyrrolizidine alkaloids group. Research concerning this compound remains scarce, especially in terms of its antimicrobial activity. The group is known as a plant toxin because it has the potential to interfere with metabolic activity as well as to form DNA adducts. Herbal products that contain compounds from this group must be consumed appropriately to prevent chronic effects [20].

### 3.2. Inhibition of Biofilm Biomass and Biofilm Cell Viability

In general, the antimicrobial activity of a plant is related to the composition of its secondary metabolites, which include phenolic compounds, anthraquinones, terpenoids, flavonoids, and alkaloids [7]. A previous study found *R. angustifolia* extract to contain steroids, flavonoids, tannins, and quinones, which indicated its significant potential as an antibiofilm [21].

Several studies have reported the antimicrobial activity of *R. angustifolia* extracts such as *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* [22]. However, no prior study has investigated the microbial antibiofilm obtained from this plant. Thus, the present study is the first to explore the antibiofilm potential of bioactive compounds isolated from *R. angustifolia* against both *C. albicans* and *S. mutans*.

In this study, the mixed biofilm formed by *C. albicans* and *S. mutans* cells was found to exhibit a significant increase in total biomass when compared with the control groups formed from only *C. albicans* or *S. mutans*. Moreover, when compared with the controls, the mixed cultures showed significantly lower biofilm formation for all the utilized compounds (kokusaginine, chalepin, and lindelofine) ($P < 0.05$), although the difference was not significant in the single cultures ($p > 0.05$). This indicates that kokusaginine, chalepin, and lindelofine generally inhibit the formation of biofilms produced by *C. albicans* and *S. mutans* in mixed cultures (Fig. 1).

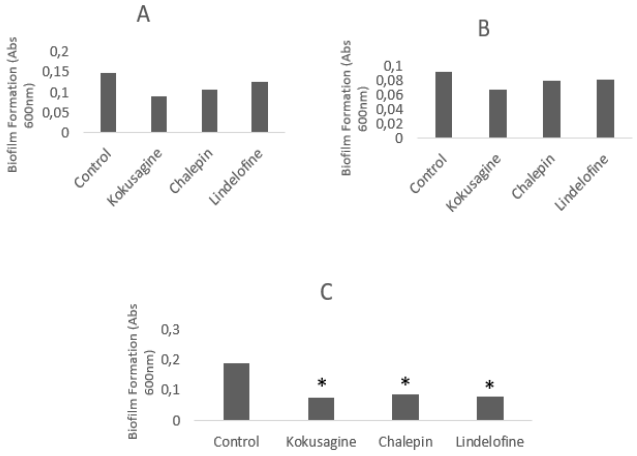


Fig. 1 Effects of bioactive compounds isolated from *R. agustifolia* on *C. albicans* ATCC (A), *S. mutans* ATCC (B), and mixed species *C. albicans* ATCC + *S. mutans* ATCC (C) biomass biofilms with crystal violet (CV) method
* Significantly different from control

A viability test was performed to determine whether the test microbes still survived when treated with the bioactive compounds. When compared with the controls, all the bioactive compounds (kokusaginine, chalepin, and lindelofine) significantly reduced the

viability of the microbes ($p < 0.05$) in both the single and mixed cultures (Fig. 2).

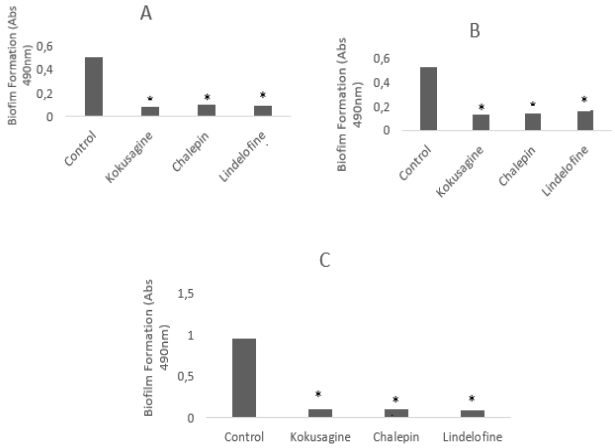


Fig. 2 Effects of bioactive compounds isolated from *R. agustifolia* on *C. albicans* ATCC (A), *S. mutans* ATCC (B), and mixed species *C. albicans* ATCC + *S.mutans* ATCC (C) biofilm cell viability with MTT method
* Significantly different from control

An analysis of the interaction between *S. mutans* and *C. albicans* in an in vitro model of biofilm formation revealed the higher cell viability of *C. albicans* in the mixed biofilms formed by *C. albicans* and *S. mutans* when compared with the single biofilms formed by *C. albicans* or *S. mutans* (Fig. 2). These data indicate that the amount of *C. albicans* in the biofilm was stimulated by the *S. mutans*. Several sources have stated that *S. mutans* increases fungal growth in mixed biofilms [12]. Furthermore, the supernatant obtained from the *S. mutans* culture decreased the viability of the *C. albicans* cells but not the total biomass of *C. albicans.* In the mixed culture, *S. mutans* produced a metabolic product that inhibited the formation of biofilms in *C. albicans* [12].

### 3.3. pH Estimation

The present study also estimated the pH for each treatment. The control showed that *C. albicans* exhibited a very low pH (3.5) in the single-species biofilm condition. By contrast, the pH of the single-species *S. mutans* was determined to be 8.5, which indicated an alkaline condition. Interestingly, even though the single-species *S. mutans* showed an alkaline pH, it could still be tolerated under mixed-species conditions that became acidic [23]. In the mixed biofilm conditions (*C. albicans* with *S. mutans*), the pH increased slightly to 4.5, although it remained below the critical value. Following contact with all the test compounds (kokusaginine, chalepin, and lindelofine), the pH of the *C. albicans* increased, although it still remained below the critical value (< 5.5). However, under mixed biofilm conditions, the pH increased to 7.5 in all the treatments, which indicated that it had exceeded the critical value (Table 1).

Table 1 pH estimated in biofilm conditions

| Microorganisms | Control | Kokusaginine | Chalepin | Lindelofine |
|---|---|---|---|---|
| *C. albicans* ATCC | 3,5 | 5 | 4,5 | 4,5 |
| *S. mutans* ATCC | 8,5 | 8,5 | 8,5 | 8,5 |
| *C. albicans* ATCC + *S. mutans* ATCC | 4,5 | 7,5 | 7,5 | 7,5 |

These findings suggest that kokusaginine, chalepin, and lindelofine can increase the pH of the mixed-species biofilm (*C. albicans* with *S. mutans*) and, therefore, play an important role in preventing oral problems such as caries and tooth demineralization. Dental caries represent a significant dental problem that commonly occurs in humans. The organic acids produced by the microorganisms in dental plaque play an important role in the development of carious lesions. The microorganisms produce various organic acids through the fermentation of dietary carbohydrates and so lower the pH at the dental biofilm interface to a critical value below 5.5, thereby leading to tooth demineralization [24].

### 3.4. Morphological Visualization

This study also performed a morphological analysis of *C. albicans* using a light microscope and SEM. From the image obtained using a light microscope, it can be seen that in the control condition, the single-species *C. albicans* showed a hyphae morphology (Fig. 3A). In contrast, in mixed-species conditions, *C. albicans* morphology was yeast or pseudohypha (Fig. 4A and 5A). The presence of *S. mutans* in mixed cultures can inhibit the formation of filamentous structures and hyphae because it produces an inhibitory peptide in the early stages of biofilm development [24].

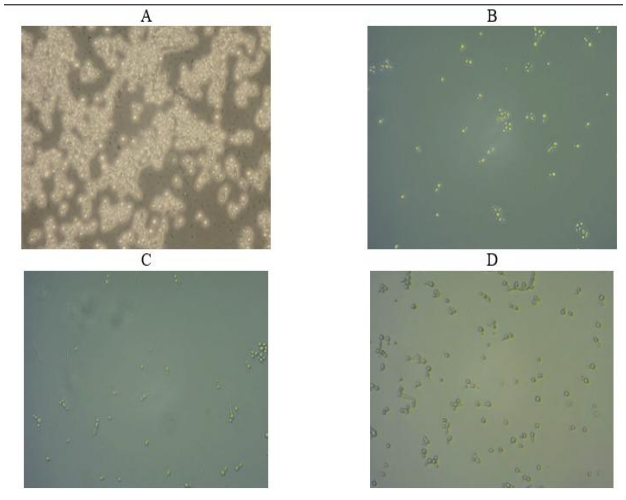


Fig. 3 Illustration of *C. albicans* in a single species biofilm by light microscopy: (A) Control; (B) After treatment with kokusaginine; (C) After treatment with chalepin; (D) After treatment with lindelofine. The image has been edited to adjust brightness and contrast

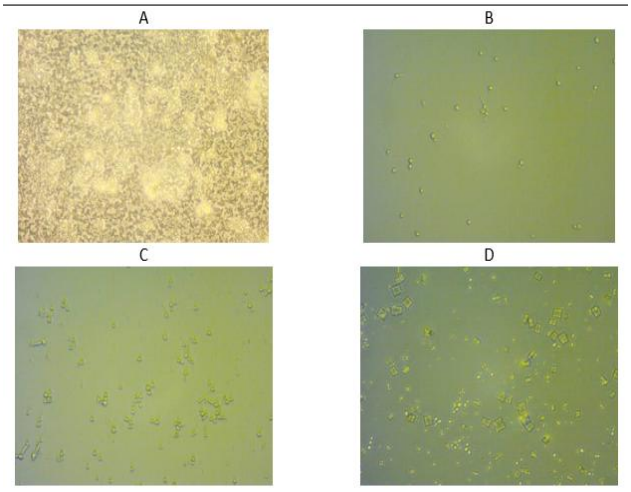


Fig. 4 Illustration of *C. albicans* in mixed-species biofilm with *S. mutans* using light microscopy: (A) Control; (B) After treatment with kokusaginine; (C) After treatment with chalepin; (D) After treatment with lindelofine. The image has been edited to adjust brightness and contrast

Environmental conditions can also affect the morphology of *C.albicans*. At low pH (<6), Candida dominantly grows in the form of yeast, while at pH> 7, hyphal form begins to be induced [24]. This is in line with the pH results (Table 1) that the pH produced by single-species *C. albicans* is lower than in mixed species. The pH in the mixed-species control showed 4.5, and the morphological appearance with SEM showed the yeast form with germ tube (Fig. 5A). Meanwhile, when contacted with the three bioactive compounds (kokusaginine, chalepin, and lindelofine), the pH increased to 7.5, and the morphological appearance with SEM showed a pseudohypha form (Fig. 5B-D).

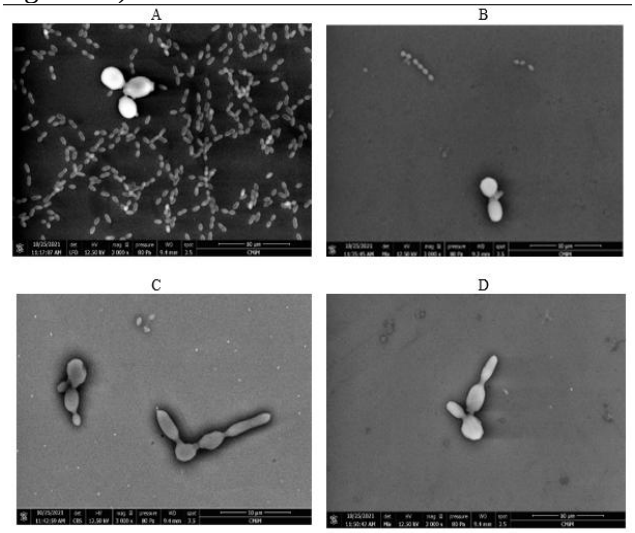


Fig. 5 Illustration of *C. albicans* in mixed-species biofilm with *S. mutans* using Scanning Electron Microscopy (SEM): (A) Control; (B) After treatment with kokusaginine; (C) After treatment with chalepin; (D) After treatment with lindelofine. 3000x magnification

In mixed-species conditions, observations of *S. mutans* using a light microscope and SEM showed that only *S. mutans* mixed species in control were seen forming colonies and interacting with *C. albicans* (Fig. 4A and 5A). Meanwhile, in samples contacting the three bioactive compounds, *C. albicans* and *S. mutans* decreased. Even in observations using SEM, the presence of *S. mutans* was significantly eliminated (Fig. 4B-D, 5B-D). This indicated that kokusaginine, chalepin, and lindelofine effectively inhibited the growth of *C. albicans* and *S. mutans* in single and mixed cultures.

In previous studies, *R. angustifolia* crude extract was shown to potentially reduce biofilm formation because it inhibited hyphae formation in *C. albicans* [25]. The same thing was seen in this study when *C.albicans* was grown in a single culture. In control (Fig. 3A), *C. albicans* can still be seen forming hyphae, but when contacting kokusaginine, chalepin, and lindelofine, *C. albicans* only formed pseudohypha. However, in this study, no drastic changes in the morphology of *C. albicans* yeast cells were seen when grown together with *S. mutans*. Similarly, in mixed culture, *S. mutans* tend to produce signaling molecules that inhibit hyphae formation in *C. albicans* [12].

At least two things explain why the hyphal form has a greater virulence factor than the yeast form. The first is because the hyphae are larger and more complex than the yeast form, so it will be more difficult to be phagocytosed by the body's immune macrophage cells. The second is multiple blastoconidia points on the filament, resulting in increased infectious factors [5]. Farnesol is a natural quorum sensing molecule produced by planktonic cultures of *C. albicans* and acts as an inhibitor of biofilm formation by preventing yeast cell germination into mycelia [24].

Using SEM, this study observed a synergistic relationship between *C. albicans* and *S. mutans* in mixed cultures. From the picture, it can be seen that there was contact or attachment between *S. mutans* cells and *C. albicans* only in control (Fig. 5A). When contacted with the three test compounds, *C. albicans* and *S. mutans* cells did not appear to overlap each other (Fig. 5B-D). It has been widely studied previously that *C. albicans* can increase the production of an exopolysaccharide synthesized by *S. mutans* GTF genes [26]. With the significant elimination of *S. mutans* in this study, the exopolysaccharide matrix formation was hampered due to the test material. This is in line with the appearance of the extracellular polysaccharide matrix (EPS) in the image. Although it is not visible, we indicate that the darker the microbial background color, the higher the EPS concentration. SEM results showed that the control's EPS was thicker than EPS when the sample was contacted with the three bioactive compounds. The extracellular matrix is very important for biofilm formation and determines its virulence level because it blocks the diffusion of substances from the inside to the outside of the biofilm and vice versa, so that acids, which are responsible for

enamel dissolution, accumulated, and saliva cannot perform its neutralization function optimally [6]. The formation of EPS will increase the tolerance of antimicrobial drugs in dual-species biofilms [27].

## 4. Conclusion

Based on the results, it was concluded that kokusaginine, chalepin, and lindelofine isolated from *R. angustifolia* leaves impacted the inhibition of *C. albicans* and *S. mutans* biofilms in terms of total biomass, cell viability, pH conditions, microcolony formation, hyphae formation, and the extracellular polysaccharide matrix construction.

Previous research has succeeded in isolating kokusaginine and chalepin of extracts *R. angustifolia* [9], [13], [14], [15], but this study is the first to isolate lindelofine of this plant. This study is also the first to explore the antibiofilm effect of bioactive compounds isolated from *R. angustifolia* against *C. albicans* and *S. mutans* in mixed culture biofilm. Previous studies have indicated that crude extract of *R. angustifolia* has potential as a *C.albicans* antibiofilm in single culture [25]. Other studies exploring the antibiofilm properties of these two species in mixed cultures include studying the effects of eucalyptus gum [28] and *spent media of Aggregatibacter actinomycetemcomitans* [24].

In general, it can be said that kokusaginine, chalepin, and lindelofine isolated from *R. angustifolia* leaves can be used as an antibiofilm in overcoming oral health problems such as dental caries caused by *C. albicans* and *S. mutans*. However, further studies are needed to identify the detailed mechanism of action. Future research is planned to test the potential of this plant as another microbial antibiofilm agent. So that in the future, *R. angustifolia* can be a natural source solution that can be used as a medicine to overcome many health problems.

## Acknowledgment

The authors are grateful to the Directorate of Research and Development of the University of Indonesia (through a Doctoral PUTI grant) for funding this study with the contract number: NKB-618/UN2.RST/HKP.05.00/2020.

## References

[1] WORLD HEALTH ORGANIZATION. *Oral Health*, 2020. https://www.who.int/news-room/fact-sheets/detail/oral-health

[2] SALEHI B., KREGIEL D., MAHADY G., SHARIFI-RAD J., MARTINS N., and RODRIGUES C. F. Management of *Streptococcus mutans-Candida spp*. oral biofilms infections: paving the way for effective clinical interventions. *Journal of Clinical Medicine*, 2020, 9(2): 517. https://doi.org/10.3390/jcm9020517

[3] EIDT G., DE ANDRADE C. G., DE CÁSSIA NEGRINI T., and ARTHUR R. A. Role of *Candida albicans* on enamel demineralization and on acidogenic potential of *Streptococcus mutans* in vitro biofilms. *Journal of Applied Oral Science*, 2019, 27: 1–8. https://doi.org/10.1590/1678-7757-2018-0593

[4] CAVALHEIRO M., & TEIXEIRA M. C. Candida Biofilms: Threats, challenges, and promising strategies. *Frontiers in Medicine*, 2018, 5: 28. https://doi.org/10.3389/fmed.2018.00028

[5] TALAPKO J., JUZBAŠIC M., MATIJEVIC T., PUSTIJANAC E., BEKIĆ S., KOTRIS I., and ŠKRLEC I. *Candida albicans*—The Virulence Factors and Clinical Manifestations of Infection. *Journal of Fungi*, 2021, 7(2): 79. https://doi.org/10.3390/jof7020079

[6] LOBO C. I. V., RINALDI T. B., CHRISTIANO C. M. S., DE SALES LEITE L., BARBUGLI P. A., and KLEIN M. I. Dual-species biofilms of *Streptococcus mutans* and *Candida albicans* exhibit more biomass and are mutually beneficial compared with single-species biofilms. *Journal of Oral Microbiology*, 2019, 11(1): 1581520. https://doi.org/10.1080/20002297.2019.1581520

[7] LU L., HU W., TIAN Z., YUAN D., YI G., ZHOU Y., CHENG Q., ZHU J., and LI M. Developing natural products as potential anti-biofilm agents. *Chinese Medicine*, 2019, 14(1): 11. https://doi.org/10.1186/s13020-019-0232-2

[8] COIMBRA A. T., FERREIRA S., and DUARTE A. P. Genus Ruta: A natural source of high value products with biological and pharmacological properties. *Journal of Ethnopharmacology*, 2020, 260: 1-81. https://doi.org/10.1016/j.jep.2020.113076

[9] RICHARDSON J. S. M., AMINUDIN N., and MALEK S. N. A. Chalepin: a compound from *Ruta angustifolia* L. Pers exhibits cell cycle arrest at S phase, suppresses nuclear factor-kappa B (NF-κB) pathway, signal transducer and activation of transcription 3 (STAT3) phosphorylation and extrinsic apoptotic pathway in non-small cell lung cancer carcinoma (A549). *Pharmacognosy Magazine*, 2017, 13(51): S489–S498. https://doi.org/10.4103/pm.pm_13_17

[10] DUTTA K., KARMAKAR A., JANA D., BALLAV S., SHITYAKOV S., PANDA A. K., and GHOSH C. Benzyl isocyanate isolated from the leaves of *Psidium guajava* inhibits *Staphylococcus aureus* biofilm formation. *Biofouling*, 2020, 36(8): 1000–1017. https://doi.org/10.1080/08927014.2020.1842877

[11] XIAO J., HARA A. T., KIM D., ZERO D. T., KOO H., and HWANG G. Biofilm three-dimensional architecture influences in situ pH distribution pattern on the human enamel surface. *International Journal of Oral Science*, 2017, 9(2): 74–79. https://doi.org/10.1038/ijos.2017.8

[12] BARBOSA J. O., ROSSONI R. D., VILELA S. F. G., DE ALVARENGA J. A., DOS SANTOS VELLOSO M., DE AZEVEDO PRATA M. C., JORGE A. O. C., and JUNQUEIRA J. C. *Streptococcus mutans* can modulate biofilm formation and attenuate the virulence of *Candida albicans*. *PLoS ONE*, 2016, 11(3): e0150457. https://doi.org/10.1371/journal.pone.0150457

[13] FAKAI M. I., ABD MALEK S. N., and KARSANI S. A. Induction of apoptosis by chalepin through phosphatidylserine externalisations and DNA fragmentation in breast cancer cells (MCF7). *Life Sciences*, 2019, 220: 186–193. https://doi.org/10.1016/j.lfs.2019.01.029

[14] NAHAR L., AL-MAJMAIE S., AL-GROSHI A., RASUL A., and SARKER S. D. Chalepin and chalepensin: Occurrence, biosynthesis and therapeutic potential.

*Molecules*, 2021, 26(6): 1609. https://doi.org/10.3390/molecules26061609
[15] LASKAR M. A., & CHOUDHURY M. D. Search for therapeutics against *COVID 19 targeting SARS-CoV-2* papain-like protease: an in silico study. *Research Square*, 2020, 1–28. https://doi.org/10.21203/rs.3.rs-33294/v1
[16] RAUT J. S., & KARUPPAYIL S. M. Phytochemicals as Inhibitors of Candida Biofilm. *Current Pharmaceutical Design*, 2016, 22: 1–24. https://doi.org/10.2174/1381612822666160601
[17] RICHARDSON J. S. M., SETHI G., LEE G. S., and MALEK S. N. A. Chalepin: isolated from *Ruta angustifolia* L . Pers induces mitochondrial mediated apoptosis in lung carcinoma cells. *BMC Complementary and Alternative Medicine*, 2016, 16: 389. https://doi.org/10.1186/s12906-016-1368-6
[18] KHAMENEH B., IRANSHAHY M., SOHEILI V., and BAZZAZ B. S. F. Review on plant antimicrobials: a mechanistic viewpoint. *Antimicrobial Resistance and Infection Control*, 2019, 8: 118. https://doi.org/10.1186/s13756-019-0559-6
[19] THAWABTEH A., JUMA S., BADER M., KARAMAN D., SCRANO L., BUFO S. A., and KARAMAN R. The biological activity of natural alkaloids against herbivores, cancerous cells and pathogens. *Toxins*, 2019, 11(11): 656. https://doi.org/10.3390/toxins11110656
[20] EFSA PANEL ON CONTAMINANTS IN THE FOOD CHAIN (CONTAM), KNUTSEN H. K., ALEXANDER J., BARREGÅRD L., BIGNAMI M., BRÜSCHWEILER B., CECCATELLI S., COTTRILL B., DINOVI M., EDLER L., GRASL-KRAUPP B., HOGSTRAND C., HOOGENBOOM L. (R.), NEBBIA C. S., OSWALD I. P., PETERSEN A., ROSE M., ROUDOT A.-C., SCHWERDTLE T., VLEMINCKX C., VOLLMER G., WALLACE H., RUIZ J. A. G., and BINAGLIA M. Risks for human health related to the presence of pyrrolizidine alkaloids in honey, tea, herbal infusions and food supplements. *EFSA Journal*, 2017, 15(7): e04908. https://doi.org/10.2903/j.efsa.2017.4908
[21] NOER S., & PRATIWI R. D. Uji kualitatif fitokimia daun *Ruta angustifolia* (Qualitative test of phytochemicals of *Ruta angustifolia* leaves). *Faktor Exacta*, 2016, 9(3): 200-206. http://dx.doi.org/10.30998/faktorexacta.v9i3.879
[22] KAMAL L. Z. M., HASSAN N. M., TAIB N. M., and SOE M. K. Graveoline from *Ruta angustifolia* (L.) Pers. and its antimicrobial synergistic potential in erythromycin or vancomycin combinations. *Sains Malaysiana*, 2018, 47(10): 2429–2435. https://doi.org/10.17576/jsm-2018-4710-19
[23] BOWEN W. H., BURNE R. A., WU H., and KOO H. Oral Biofilms: Pathogens, Matrix and Polymicrobial Interactions in Microenvironment. *Trends in Microbiology*, 2019, 26(3): 229–242. https://doi.org/10.1016/j.tim.2017.09.008
[24] BACHTIAR E. W., & BACHTIAR B. M. Effect of cell-free spent media prepared from *Aggregatibacter actinomycetemcomitans* on the growth of *Candida albicans* and *Streptococcus mutans* in co-species biofilms. *European Journal of Oral Sciences*, 2020, 128(5): 395–404. https://doi.org/10.1111/eos.12725
[25] NOER S., BACHTIAR B. M., and BOWOLAKSONO A. Inhibition of *Candida albicans* hypha formation in biofilm formation by *Ruta angustifolia* extract. *AIP Conference Proceedings*, 2021, 2331: 050003. https://doi.org/10.1063/5.0041682
[26] WU R., TAO Y., CAO Y., ZHOU Y., and LIN H. *Streptococcus mutans* Membrane Vesicles Harboring Glucosyltransferases Augment *Candida albicans* Biofilm Development. *Frontiers in Microbiology*, 2020, 11: 581184. https://doi.org/10.3389/fmicb.2020.581184
[27] IKONO R., VIBRIANI A., WIBOWO I., SAPUTRO K. E., MULIAWAN W., BACHTIAR B. M., MARDLIYATI E., BACHTIAR E. W., ROCHMAN N. T., KAGAMI H., XIANQI L., NAGAMURA-INOUE T., and TOJO A. Nanochitosan antimicrobial activity against *Streptococcus mutans* and *Candida albicans* dual-species biofilms. *BMC Research Notes*, 2019, 12(1): 383. https://doi.org/10.1186/s13104-019-4422-x
[28] WIJAYA C. H., SEPTIANA S., BACHTIAR B. M., and YULIANA N. D. Cajuputs candy impairs *Candida albicans* and *Streptococcus mutans* mixed biofilm formation in vitro. *F1000Research*, 2020, 8: 1923. https://doi.org/10.12688/f1000research.20700.2

**参考文:**

[1] 世界卫生组织。口腔健康，2020 年。https://www.who.int/news-room/fact-sheets/detail/oral-health
[2] SALEHI B.、KREGIEL D.、MAHADY G.、SHARIFI-RAD J.、马丁斯 N.和 RODRIGUES C.F. 变形链球菌-念珠菌属的管理。口腔生物膜感染：为有效的临床干预铺平道路。临床医学杂志，2020, 9(2): 517. https://doi.org/10.3390/jcm9020517
[3] EIDT G.、DE ANDRADE C.G.、DE CÁSSIA NEGRINI T. 和 ARTHUR R.A. 白色念珠菌对牙釉质脱矿质和变形链球菌体外生物膜的产酸潜力的作用。应用口腔科学杂志，2019，27：1-8。https://doi.org/10.1590/1678-7757-2018-0593
[4] CAVALHEIRO M. 和 TEIXEIRA M.C. 念珠菌生物膜：威胁、挑战和有希望的策略。医学前沿，2018 年，5：28。https://doi.org/10.3389/fmed.2018.00028
[5] TALAPKO J.、JUZBAŠIC M.、MATIJEVIC T.、PUSTIJANAC E.、BEKIĆ S.、KOTRIS I. 和 ŠKRLEC I. 白色念珠菌 — 感染的毒力因素和临床表现。真菌杂志，2021, 7(2): 79. https://doi.org/10.3390/jof7020079
[6] LOBO C. I. V.、RINALDI T. B.、CHRISTIANO C. M. S.、DE SALES LEITE L.、BARBUGLI P. A. 和 KLEIN M. I. 变形链球菌和白色念珠菌的双物种生物膜与单物种生物膜相比，表现出更多的生物量并且互惠互利。口腔微生物学杂志，2019, 11(1): 1581520. https://doi.org/10.1080/20002297.2019.1581520
[7] LU L., HU W., TIAN Z., YUAN D., YI G., ZHOU Y., CHENG Q., ZHU J., and LI M. 开发天然产物作为潜在的抗生物膜剂。中医，2019, 14(1): 11. https://doi.org/10.1186/s13020-019-0232-2
[8] COIMBRA A. T.、FERREIRA S. 和 DUARTE A. P. 芸香属：具有生物学和药理特性的高价值产品的天然来源。民族药理学杂志，2020，260：1-81。https://doi.org/10.1016/j.jep.2020.113076
[9] RICHARDSON J. S. M.、AMINUDIN N. 和 MALEK S. N. A. 查乐平：来自芸香的化合物在 S 期表现出细胞周期停滞，抑制核因子-卡帕乙 (NF-κB) 通路、信号转导和

转录激活非小细胞肺癌 (一个 549) 中的 3 (统计数据 3) 磷酸化和外在凋亡途径。生药学杂志，2017，13(51): S489–S498. https://doi.org/10.4103/pm.pm_13_17
[10] DUTTA K.、KARMAKAR A.、JANA D.、BALLAV S.、SHITYAKOV S.、PANDA A.K. 和 GHOSH C. 从番石榴叶中分离出的异氰酸苄酯可抑制金黄色葡萄球菌生物膜的形成。生物污垢，2020，36(8): 1000–1017。https://doi.org/10.1080/08927014.2020.1842877
[11] XIAO J.、HARA A. T.、KIM D.、ZERO D. T.、KOO H. 和 HWANG G. 生物膜三维结构影响人牙釉质表面的原位 pH 分布模式。国际口腔科学杂志，2017，9(2): 74–79. https://doi.org/10.1038/ijos.2017.8
[12] BARBOSA J. O.、ROSSONI R. D.、VILELA S. F. G.、DE ALVARENGA J. A.、DOS SANTOS VELLOSO M.、DE AZEVEDO PRATA M. C.、JORGE A. O. C. 和 JUNQUEIRA J. C. 变形链球菌可以调节生物膜形成并减弱白色念珠菌的毒力。公共科学图书馆一期，2016 年，11(3) : e0150457 。https://doi.org/10.1371/journal.pone.0150457
[13] FAKAI M. I.、ABD MALEK S. N. 和 KARSANI S. A. chalepin 通过乳腺癌细胞中的磷脂酰丝氨酸外化和脱氧核糖核酸片段化诱导细胞凋亡 (MCF7)。生命科学，2019 年，220 : 186–193 。https://doi.org/10.1016/j.lfs.2019.01.029
[14] NAHAR L.、AL-MAJMAIE S.、AL-GROSHI A.、RASUL A. 和 SARKER S.D. Chalepin 和 chalepensin：发生、生物合成和治疗潜力。分子，2021，26(6): 1609. https://doi.org/10.3390/molecules26061609
[15] LASKAR M. A. 和 CHOUDHURY M. D. 寻找针对非典-冠状病毒-2 类木瓜蛋白酶的新冠肺炎的治疗方法：一项计算机研究。研究广场，2020 年，1-28。https://doi.org/10.21203/rs.3.rs-33294/v1
[16] RAUT J. S. 和 KARUPPAYIL S. M. 植物化学物质作为念珠菌生物膜的抑制剂。当前的药物设计，2016，22：1-24。https://doi.org/10.2174/1381612822666160601
[17] RICHARDSON J. S. M.、SETHI G.、LEE G. S. 和 MALEK S. N. A. Chalepin：从芸香中分离。个人在肺癌细胞中诱导线粒体介导的细胞凋亡。BMC 补充和替代医学，2016，16：389。https://doi.org/10.1186/s12906-016-1368-6
[18] KHAMENEH B.、IRANSHAHY M.、SOHEILI V. 和 BAZZAZ B. S. F. 回顾植物抗菌剂：机械观点。抗菌素耐药性和感染控制，2019，8:118 。https://doi.org/10.1186/s13756-019-0559-6
[19] THAWABTEH A.、JUMA S.、BADER M.、KARAMAN D.、SCRANO L.、BUFO S.A. 和 KARAMAN R. 天然生物碱对食草动物、癌细胞和病原体的生物活性。毒素，2019，11(11): 656. https://doi.org/10.3390/toxins11110656
[20] 欧洲食品安全局食品链污染物小组 (康塔姆)，KNUTSEN H. K.，ALEXANDER J.，BARREGÅRD L.，BIGNAMI M.，BRÜSCHWEILER B.，CECCATELLI S.，COTTRILL B.，DINOVI M.，EDLER L.，GRASL-KRAUPP B.、HOGSTRAND C.、HOOGENBOOM L. (R.)、NEBBIA CS、OSWALD IP、PETERSEN A.、ROSE M.、ROUDOT A.-C.、SCHWERDTLE T.、VLEMINCKX C.、福尔默 G.、WALLACE H.、RUIZ J. A. G.，和 BINAGLIA M. 与蜂蜜、茶、草药输液和食品补充剂中存在的吡咯里西啶生物碱有关的人类健康风险。欧洲食品安全局杂志，2017 年，15(7)：e04908。https://doi.org/10.2903/j.efsa.2017.4908
[21] NOER S., & PRATIWI R. D. 芸香叶植物化学定性试验（芸香叶植物化学物质的定性测试）。确切因素，2016，9(3): 200-206 。http://dx.doi.org/10.30998/faktorexacta.v9i3.879
[22] KAMAL L. Z. M.、HASSAN N. M.、TAIB N. M. 和 SOE M. K. 墓碑来自芸香(大号.) 个人。及其在红霉素或万古霉素组合中的抗菌协同潜力。马来西亚圣贤，2018，47(10): 2429–2435 。https://doi.org/10.17576/jsm-2018-4710-19
[23] BOWEN W. H.、BURNE R. A.、WU H. 和 KOO H. 口腔生物膜：微环境中的病原体、基质和多微生物相互作用。微生物学趋势，2019，26(3): 229–242 。https://doi.org/10.1016/j.tim.2017.09.008
[24] BACHTIAR E. W. 和 BACHTIAR B. M. 由伴放线菌聚集杆菌制备的无细胞用过的培养基对共种生物膜中白色念珠菌和变形链球菌生长的影响。欧洲口腔科学杂志，2020，128（5）：395–404 。https://doi.org/10.1111/eos.12725
[25] NOER S.、BACHTIAR B. M. 和 BOWOLAKSONO A. 芸香提取物对生物膜形成过程中白色念珠菌菌丝形成的抑制作用。AIP 会议论文集，2021 年，2331：050003 。https://doi.org/10.1063/5.0041682
[26] WU R., TAO Y., CAO Y., ZHOU Y., 和 LIN H. 变形链球菌膜囊泡含有葡萄糖基转移酶增强白色念珠菌生物膜发育。微生物学前沿，2020，11: 581184. https://doi.org/10.3389/fmicb.2020.581184
[27] IKONO R.、VIBRIANI A.、WIBOWO I.、SAPUTRO KE、MULIAWAN W.、BACHTIAR BM、MARDLIYATI E.、BACHTIAR EW、ROCHMAN NT、KAGAMI H.、XIANQI L.、NAGAMURA-INOUE T. 和 TOJO A. 纳米壳聚糖对变形链球菌和白色念珠菌双物种生物膜的抗菌活性。BMC 研究笔记，2019，12(1): 383. https://doi.org/10.1186/s13104-019-4422-x
[28] WIJAYA C. H.、SEPTIANA S.、BACHTIAR B. M. 和 YULIANA N. D. 卡朱普特糖果损害白色念珠菌和变形链球菌在体外混合生物膜形成。F1000 研究，2020，8：1923。https://doi.org/10.12688/f1000research.20700.2