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Inhibition Of *Candida albicans* and *Streptococcus mutans* (Single and Mix Species) Biofilms by Crude Extract of *Ruta angustifolia*

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Abstract

Plants from the genus Ruta are known to have many phytochemicals that have potential as drugs and have been widely used as traditional medicines. One of the plants that has the potential to be used as traditional medicine is *Ruta angustifolia*. This plant has been widely used to cure several diseases traditionally, but scientific research on this plant is still rarely done. Several studies have stated that this plant has an antimicrobial effect, but there are no studies that specifically discuss the antibiofilm effect of this plant. This study is the first study to discuss the effect of *Ruta angustifolia* extract on physiological biofilm formation. The purpose of this study was to see whether the extract of *Ruta angustifolia* could inhibit the formation ofphysiological biofilms on the oral microbes that play the most role in the formation of dental caries, namely *Candida albicans* and *Streptococcus mutans*, both in single and mixed species. The method used in this research is Cristal Violet (CV) method for biofilm testing and Total Plate Count (TPC) for viability testing as well as morphological visualization using Scanning Electron Microscope (SEM). The results showed that *Ruta angustifolia* extract in general could inhibit the formation of biofilms produced by *C.albicans* and *S.mutans* in both single and mixed species cultures. The viability test showed that the higher the concentration of the extract, the fewer microbes that could survive.

Key words : *Ruta angustifolia*, Biofilm, *Candida albicans*, *Streptococcus mutans*, Viability

INTRODUCTION

Medicinal plants are widely used for traditional medicine and have a diverse composition of chemical compounds. Natural products derived from plants are one of the potential sources in finding new bioactive compound agents or drugs (Coimbra et al., 2020). Medicinal plants can be used as therapeutic resources for the prevention and treatment of diseases in various forms. For example, in traditional home medicine, plants are used directly such as herbal teas, while for broader phytopharmaceutical uses, plants are made into extracts or fractions of their constituent bioactive components.

Plants belonging to Ruta genus can be a potential source of natural products that have potentially medicinal biological activity. Ruta extracts, essential oils and compounds isolated from this plant have shown various potentials for use in the treatment of various diseases and for pest control (Coimbra, et al., 2020).

Ruta angustifolia is one of the plants of the Ruta genus that has long been trusted and used by the public, especially in Asia, as a medicine for various diseases. However, adequate information about *R.angustifolia* and scientific explanations about the effectiveness of this plant is still very rare, especially research related to the potential of this plant as an antibiofilm. The potential of *R.angustifolia* as a medicinal plant has attracted the attention of phytochemists to reveal the content of natural ingredients in this plant. Previous studies explored the benefits of chemical compounds isolated from *R.angustifolia* leaves and found that these compounds have great potential to be used as antibiofilm candidates (Noer et al., 2018).

Biofilm is a complex three-dimensional form of a community of microorganisms united in an extracellular matrix layer and has unique phenotypic properties when compared to free cells (planktonic cells) (Ganguly and Mitchell, 2011). When microorganisms form biofilms, their control and destruction becomes much more difficult. About 80% of microbial infections in humans are associated with biofilms (Raut and Karuppayil, 2016). It is also estimated that biofilms are associated with 65% of nosocomial infections (Licking, 1999). As great as the impact of biofilms on the world of health is, it is not surprising that research on the search for medicinal ingredients as antibiofilms continues and develops until now.

Candida albicans is a fungal pathogen in humans that can cause candidiasis and other diseases involving polymicrobials because of its ability to form multispecies biofilms (Barbosa et al, 2016) and this relationship increases its infectivity. One of the most common forms of *C.albicans* symbiosis in the oral environment is with *Streptococcus mutans* bacteria and plays a role in the formation of dental plaque (Metwalli et al, 2013).

Candida biofilms and the infectious diseases they cause are a serious threat to health world. The

treatment of Candida infections associated with biofilms has been limited to date because most of the antifungal drugs available fail to eradicate or inhibit the growth of biofilms (Raut and Karuppayil, 2016). The purpose of this study was to see whether *R.angustifolia* extract could inhibit the formation of physiological biofilms on oral microbes that have the most role in the formation of dental caries, namely *C.albicans* and *S.mutans*, both in single and mixed species.

Materials and Methods

R.angustifolia Plant Extract Preparation

The part of the plant that is used as an extract in this plant is the leaf. Samples of *R.angustifolia* were taken from the Manoko Experimental Garden, Research Institute for Spices and Medicinal Plants, Lembang, Bandung, West Java, Indonesia. The leaves are separated from the stems and then dried inthe sun for about 7 days. Extraction using maceration method with 96% methanol as solvent. The extract to be tested was then diluted using sterile distilled water to obtain concentrations of 20%, 50% and 100% (not diluted).

Microbial Preparation and Growth Conditions

The microbes used in this study were *C.albicans* ATCC 10231, *C.albicans* clinical isolate (high caries) 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). About 2-3 colonies of microbes that grow on agar medium are then transferred to liquid medium and incubated for 24 hours. Before being used for research, the number of microbes was calculated using a hemacytometer (the number of microbes used in this study was 10^6 for *C.albicans* and 10^8 for *S.mutans*).

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 put 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 to be used for further testing.

Crystal Violet (CV) Biofilm Test

Microbes whose cells were counted were put into 96 well plates (which had been coated with saliva) along with extracts with the following conditions: each plate contained 30 µL of extract and 70 µL of microbes (for mix species 35 µL each). The negative control contained 100 µL of microbes (without the

addition of extract). This mixture was then mixed using an orbital shaker for about 10 minutes and then incubated in an incubator at 37°C for 24 hours. After incubation, the supernatant was removed and the plate was washed with 200 µL of PBS. The plate is then allowed to fixation until the biofilm is dry. A total of 200 µL CV with a concentration of 0.5% was then put into the well and incubated at 37°C for 15 minutes. The supernatant containing the remaining CV solution was then discarded and the plate was washed with 200 µL PBS and then 200 µL of 95% ethanol was added. The plate is then inserted into a microplate reader to read the Optical Density value using 600 nm wavelength.

Viability Test of Total Plate Count (TPC) Method

The bottom of the well (biofilm) was given 500 µL of PBS solution. Then the bottom of the well is scraped slowly using the tip of the pipette. This solution was then transferred to a microcentrifuge tube and serial dilution was made. The culture was then grown using the spread plate method for 24 hours in an incubator at 37°C with 3 replications. Sabouraud dextrose agar (SDA) media was used to grow *C.albicans*, while brain heart infusion (BHI) media was used for *S.mutans*. The number of colonies that grew was then counted manually.

Morphological Visualization

The morphology of *C.albicans* in biofilm conditions (mixed cultures) is seen using ~~using~~ Scanning Electron Microscope (SEM), biofilms are prepared by culturing mixed species (*C.albicans* and *S.mutans*) on discs placed in a 24-well plate and then incubated for 90 min and continuing for 48h. After treatment to form biofilm (treatment with 100% extract), samples than fixated using 1 mL of 2.5% glutaraldehyde for 1 hour. Samples then dehydrated with ethanol series (10, 25, 50, 75 and 90%) for 20 minutes each, followed by immersion in 100% alcohol for 1 hour (Barbosa et al., 2016). Sample then dried at 37°C, overnight. Samples are sent to PT Cipta Mikro Material (PUSPIPTEK, Serpong, West Java, Indonesia) for visualization using SEM with 1000, 3000 and 5000 x magnification.

Data analysis

Each microbe tested was compared between the control group with various extract concentrations. Data analysis used is one way ANOVA, followed by TUKEY HSD. The data were significant when $p<0.05$.

Results and Discussion

In this study, *C.albicans* and *S.mutans* were used to see whether *R.angustifolia* extract could inhibit these two microbes, either singly or in a mixture. From the biofilm test, it was seen that there were

differences between the control group of microbes (without extract treatment) and the microbes that were contacted with various concentrations of *R.angustifolia* extract (20%, 50% and 100%). The results obtained can be seen in Figure 1.

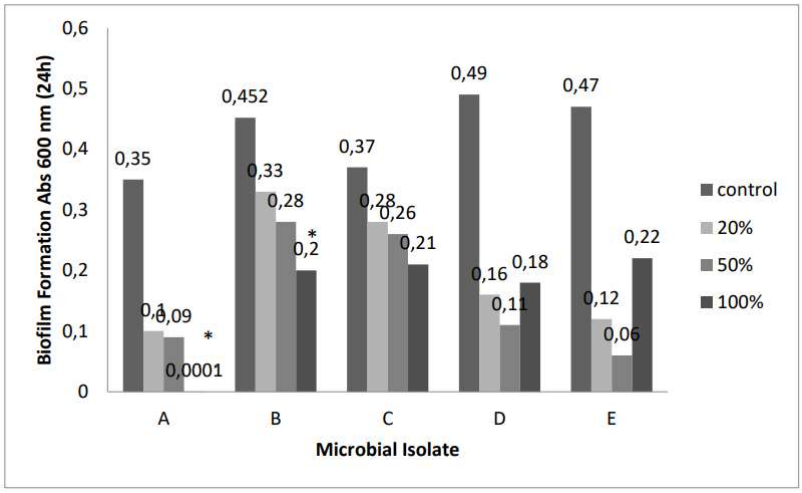

Figure 1. Effect of *R.angustifolia* extract on the formation of *C.albicans* and *S.mutans* biofilms (single and mix species) using the Cristal Violet method. (A) *C.albicans* ATCC, (B) *C.albicans* clinical isolate, (C) *S.mutans* ATCC, (D) *C.albicans* ATCC + *S.mutans* ATCC, (E) *C.albicans* clinical isolate + *S.mutans* ATCC. Data that are significantly different from the control are marked with *.

When compared with the control, each test microbe, both single and mixed cultures, showed lower biofilm formation results at all concentrations of extracts used. This indicates that *R.angustifolia* extract in general can inhibit the formation of biofilms produced by *C.albicans* and *S.mutans* in both single and mixed species cultures. Statistical analysis showed that at 100% extract concentration, biofilms *C.albicans* single species (ATCC and clinical isolate), showed a significant decrease when compared to the control.

Several previous studies stated that there was antimicrobial activity from *R.angustifolia* extracts such as *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli, Bacillus cereus, Pseudomonas aeruginosa* and hepatitis C virus (Kamal et al., 2018; Shuib et al., 2015; Sri et al., 2014). In general, the antimicrobial activity of a plant is related to the secondary metabolites contained by the plant. The secondary metabolites in question are phenolic compounds, anthraquinones, terpenoids, flavonoids,

and alkaloids (Lu et al., 2019). From previous studies, *R.angustifolia* extract was proven to contain qualitative steroids, flavonoids, tannins and quinones, so that it has the potential to be used as an antibiofilm (Noer and Pratiwi, 2016). The compounds contained in the extract of *R.angustifolia* are thought to have inhibitory activity on the *C.albicans* biofilm, especially in the single species state, which showed a significant reduction in this study.

TPC Viability Test

Viability test was carried out to determine whether the tested microbes could still survive when contacted with *R.angustifolia* extract. The results of the study can be seen in figure 2.

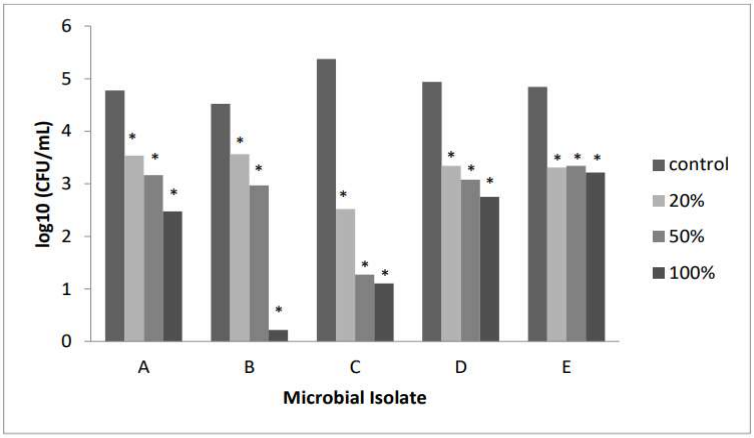

Figure 2. Effect of *R.angustifolia* extract on cell viability of *C.albicans* and *S.mutans* (single and mix species) using the Total Plate Count (TPC) method. (A) *C.albicans* ATCC, (B) *C.albicans* clinical isolate, (C) *S.mutans* ATCC, (D) *C.albicans* ATCC + *S.mutans* ATCC, (E) *C.albicans* clinical isolate + *S.mutans* ATCC. Data that are significantly different from the control are marked with *.

The results showed that when compared with the control, all concentrations of the extract could significantly reduce the viability of the tested microbes. The higher the extract concentration, the lower viability level of *C.albicans* and *S.mutans*. This indicates that *R.angustifolia* extract was very effective in reducing the vibiality of *C.albicans* and *S.mutans* cells, both in single and mixed cultures.

The mixed species of biofilm (*C.albicans* and *S.mutans*) showed a higher number of CFU/biofilm (log) when compared to single species *C.albicans* or *S.mutans* (Lobo et al., 2019). From several sources

it is said that *S.mutans* can increase fungal growth in mixed biofilms (Barbosa et al., 2016; Sztajer et al., 2014). It can be seen in the figure that in mixed cultures, the number of *C.albicans* cells tends to be higher than in single cultures. It has also been reported that the supernatant from *S.mutans* culture can reduce the viability of *C.albicans* cells, but cannot reduce the total biomass of *C.albicans* (Barbosa et al.,2016).

Morphological Visualization Using SEM

In a previous study, *R.angustifolia* extract was shown to have the potential to reduce the potential for biofilm formation because it inhibited hypha formation in *C.albicans* (Noer et al., 2021). However, inthat study, the *C.albicans* used were in a single culture (single species). In this study, observations were made on the morphology of *C.albicans* after contact with crude extract of *R.angustifolia* (100%) in mixed culture with *S.mutans*.

In the picture (figure 3) it is clear that there is a significant difference between the control and the treatment. In the control, both *C.albicans* ATCC (figure 3A) and *C.albicans* clinical isolate (figure 3B) showed close contact between the two microbes in fairly dense colonies. However, after being contacted with *R.angustifolia* extract, the cell density was reduced, it even looked very significant for *S.mutans* cells (3C-D figure). This is in line with the results of the significantly reduced cell viability of *S.mutans* after contact with *R.angustifolia* extract (figure 2).

In contrast to previous studies conducted on a single culture, in this study, the morphological differences of *C.albicans* can be seen. If in previous studies, the control showed that *C.albicans* had formed hyphae, but in this mixed culture, the control showed that *C.albicans* was still in the form of yeast or pseudohypha. This could be because in mixed cultures, *S.mutans* can also produce signaling molecules that inhibit hypha formation in *C.albicans* (Jarosz et al., 2009). In the treatment image with *R.angustifolia* extract (3C-D figure), the hypha morphology began to appear again, this was due to the elimination of *S.mutans* due to the presence of the extract so that there was no inhibitory molecule from this bacterium for the formation of *C.albicans* hypha.

When compared between controls using *C.albicans* ATCC and *C.albicans* clinical isolates, there are also differences between the morphology of *C.albicans* formed in the same incubation time (24 hours). In *C.albicans* ATCC, the morphology is in the form of germ tube yeast, while for *C.albicans* clinical isolate, the morphology is in the form of pseudohyphae. This difference can indicate that clinical

isolates show a higher level of malignancy. This is because hypha formation is one of the key processes in the successful formation of biofilms (Bachtiar et al., 2014).

Treatment for oral biofilm-associated diseases is viewed as complicated due to its multifactorial etiological nature (Ikono et al., 2019). So that this study which showed significant biofilm inhibition results for *C.albicans* clinical isolate was an appropriate solution if used as an antibiofilm material, especially those caused by oral *C.albicans* and *S.mutans*.

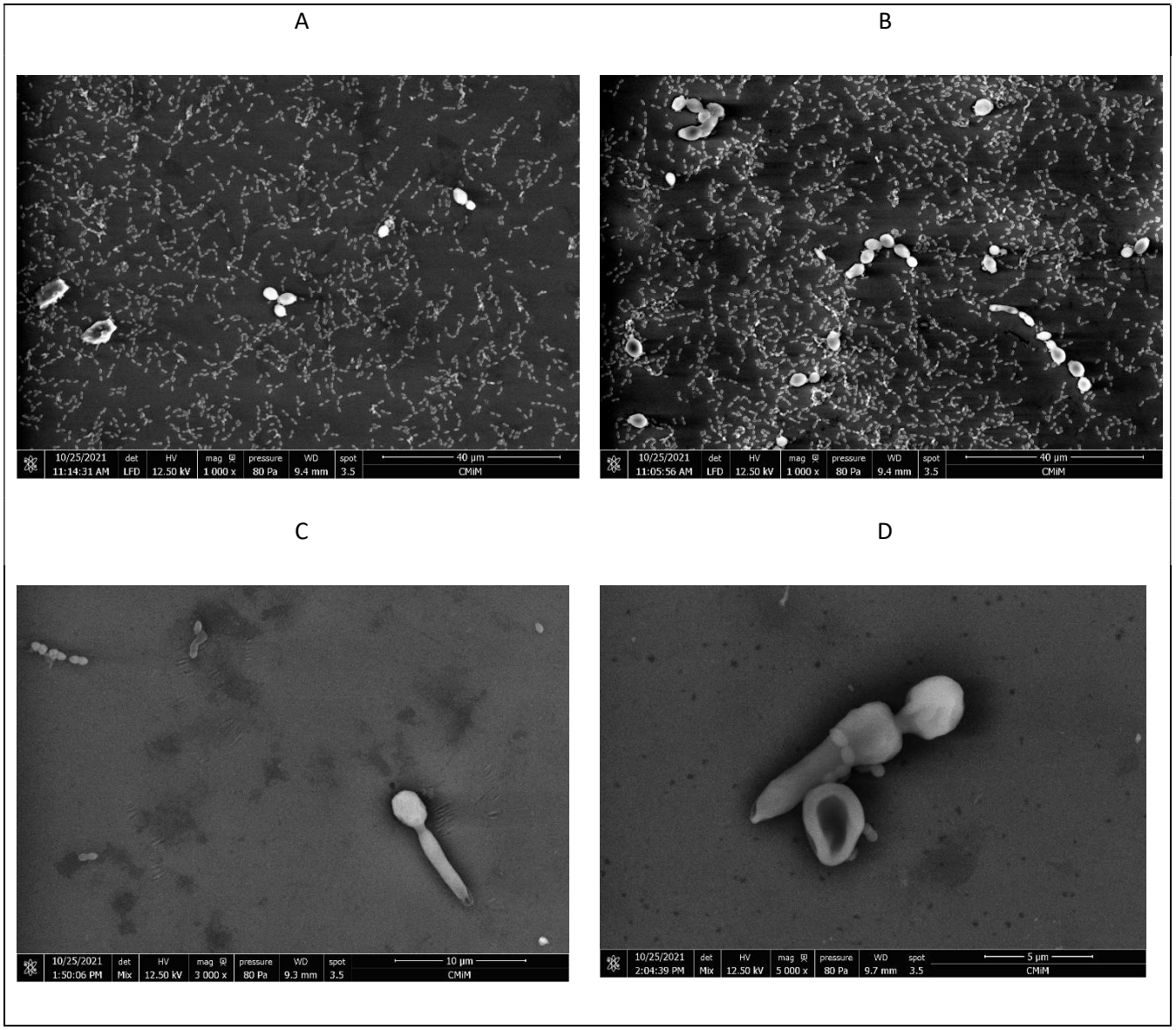

Figure 3. Illustration of *C.albicans* in mixed species biofilm with *S.mutans* using Scanning Electron Microscopy (SEM): (A) *C.albicans* ATCC + *S.mutans* (Control); (B) *C.albicans* clinical isolate + *S.mutans*

(Control); (C) *C.albicans* ATCC + *S.mutans* (100% *R.angustifolia* extract); (D) *C.albicans* clinical isolate + *S.mutans* (100% *R.angustifolia* extract).

Conclusion

The conclusion of this study is that *R.angustifolia* extract can inhibit the formation of biofilms andreduce the viability of *C.albicans* and *S.mutans* in both single and mixed cultures. So it can be said that this plant extract can be used in overcoming oral health problems such as dental caries, especially those caused by *C.albicans* and *S.mutans*. Further research is needed to identify what compounds play a role in this inhibition and the detailed mechanism of action.

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