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Antimicrobial Activity of Fingerroot [*Boesenbergia rotunda* (L.) Mansf. A.] Extract against *Streptococcus mutans* and *Streptococcus sobrinus*

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The extract of medicinal plants fingerroot [*Boesenbergia rotunda* (L.) Mansf. A.] obtained using 100% methanol was tested for antibacterial activity against two major pathogen of dental carries namely *Streptococcus mutans* KCCM 3309 and *Streptococcus sobrinus* KCCM 3207. The minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC) and time-kill curve on *S. mutans* and *S. sobrinus* were analyzed using Clinical and Laboratory Standard Institutes (CLSI) methods. Preliminary antimicrobial screening showed the mean zones of inhibition for *S. mutans* (9.0 mm) and *S. sobrinus* (8.0 mm). MIC value obtained for *S. sobrinus* and *S. mutans* was 313 µg/ml while the MBC values were 313 µg/ml (*S.mutans*) and 625 µg/ml (*S. sobrinus*). Time- kill curve were obtained at concentrations of 0xMIC, 1/2xMIC, 1xMIC, 2xMIC, 4xMIC and 8xMIC. *S. mutans* was found to be more susceptible to the fingerroot extract than *S. sobrinus*. Time - kill curve showed that the concentration of 8xMIC was able to kill 99.9% of *S. mutans* after 4 hours treatment. These results may be useful for developing fingerroot *B. rotunda* as natural anticariogenic agent in toothpaste or any oral care products such as mouthwash in treatment of dental carries, sore throat and flaming gums.

Keywords: anticariogenic; *Boesenbergia rotunda*; fingerroot; oral bacteria; antibacterial activity.

These days, dental carries is one of the common diseases in human. The tendency to produce dental carries often defined as cariogenic. Cariogenicity of dental carries involves the adherence of bacteria on the tooth followed by the formation of glycocalyx, a sticky glucan that is synthesized by the action of the bacterial enzyme glucosyl transferase on sucrose (Ferrazzano *et al*., 2011). Then, the constant production of glycocalyx by integral bacteria increases the break down of the carbohydrates which then results in the deposits of biofilm (plaque) at low pH level. As this happens, there will be an accumulation of acids and this will lead to the denaturation of enamel (Hamilton-Miller, 2001).

Pathogen gram positive Streptococci sp. is known as common cariogenic bacteria (Ferrazzano *et al*., 2011). The main causative oral disease from Streptococci sp. agents are *Streptococcus mutans* and *Streptococcus sobrinus* (Wang *et al*., 2012; Ruby *et al*., 2002). Oral disease like dental carries is known as a slow progressing disease but the oral bacteria that cause this disease may also lead to other problems such as periodical abscesses on tooth and infections of jaw bones (Chan *et al*., 1989). As a result, controlling and

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reducing oral bacteria are crucial to prevent oral diseases. In the market today, mouthwashes and toothpastes contain fluoride that can prevent formation of dental plaque but some researches reported that the streptococcal strains were able to adapt and survive the presence of high fluoride concentration (Jabbarifar and Tabibian, 2004). This is due to the prolonged fluoride therapy that confers some strains the ability to become fluoride tolerant (King, 1983). Besides that, usage of antibiotic in mouthwashes can elevate the development of multidrug resistant (MDR) strains of bacteria (Fani *et al*., 2007). Due to these problems, there has been a need to find an alternative to the use of fluoride in mouthwash and toothpaste today. In present, more of natural products are preferred rather than conventional medicine such as drugs and pharmaceuticals.

The demand for natural ingredients with medicinal properties has increase rapidly in the recent years as scientists began to unravel the medicinal properties in traditional herbal plants in Asian countries (Bhamarapravati *et al*., 2006). Malaysian plants have a great economic value locally as well as globally as they are utilized to improve the health of consumers in either pure natural form or their derivative forms. The medicinal properties of Malaysian plants come from various parts of plant such as stem, leaves, roots, fruits, and flower (Sharma and Kumar, 2009). *Boesenbergia rotunda* (L.) Mansf. Malaysian common name fingerroot or *temu kunci,* is one of tropical medicinal herbs from the Zingiberaceae family. Fingerroot has been used in traditional medicine as a cough reliever, stomach ache suppresser and post natal care in women (Chan *et al*., 1989). A study had described *B. rotunda* to contain panduratin A, an active compound that is able to reduce the spread of *Streptococcus mutans, Streptococcus sanguis* and *Actinomyces viscosus* in human mouth (Yanti *et al*., 2009). Hwang *et al*. (2010) invented the optimum oral wash formulation of antihalitosis (bad breath) containing panduratin A agent from *B. rotunda* which reported could reduce the effect halitosis by 70-90%.

Thus, the objective of this study is to determine the antimicrobial activity of 1% fingerroot extract against two most common species oral bacteria which are *Streptococcus mutans* and *Streptococcus sobrinus*. The susceptibility of both *S. mutans* and *S. sobrinus* in term of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) towards fingerroot extract as well as the time-kill curve will be determined using Clinical and Laboratory Standards Institute methods (CLSI, 2003). Time–kill curve will also be determined to assess the correlation between MIC and bactericidal activity of *B. rotunda* extract at different concentrations, ranging from 0 × MIC to 8 × MIC.

MATERIALS AND METHODS

Plant materials

Rhizome of fingerroot was collected from herbal market in Pasar Baru Bandung, Indonesia. Samples were deposited in the Laboratory of Natural Products, Institute of Bioscience, Universiti Putra Malaysia.

Plants extract preparation

The air-dried, grinded plant (100 g) was extracted twice with 400 ml of 100% (v/v) methanol for 48 hours at room temperature. The extracts were filtered with Whitman filter paper No.2 using a rotary vacuum evaporator (Heidolph VV2011, Schwabach, Germany) at 50 °C and 150 rpm. The supernatant was collected and then dissolved in 1 ml dimethylsufoxide (DMSO) to obtain stock solution 10%. Maximum concentration of 10% DMSO was found to not kill the two microorganisms tested in this study. This is supported by one which investigated 10% of DMSO or less does not kill or effect growth of bacteria (Torrungruang *et al*., 2007). The final amount of seed extract used was 0.1 gram which diluted with DMSO became 1% of extract. Then, the extract was stored at 4 °C in an air tight Schott bottle.

Tested microorganisms and inoculum preparation

Streptococcus mutans KCCM 3309 and *Streptococcus sobrinus* KCCM 3207 were obtained from the Korean Culture Center of Microorganisms (Seoul, South Korea). Mueller- Hinton broth (MHB) was used for growing the bacteria at 37 °C and for further diluting the bacteria.

Antibacterial assay

The agar diffusion method was used to determine the antibacterial activities of the *B. rotunda* extract. Five millimeters of paper disc were punched into inoculated MH agar. Then, each of

the paper discs were infused with 10 µl concentration of extract. For positive control, 0.05% chlorhexidine was used while 10% dimethyl sulfoxide (DMSO) was used for negative control. Chlorhexidine is the positive control because it has been used for standard antiplaque agent (Eley, 1999). The positive control and negative control were done for both bacteria. The plates were left for 1 hour at room temperature and thereafter incubated at 37 °C for 24 hours. The results collected were based on diameters of zone of inhibition and recorded in millimeters (mm).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Minimum inhibitory concentrations (MIC) of *B. rotunda* extract were determined by microdilution technique from the highest concentration using 0.5 ml volume and sterile 96-wells microplates (Greiner, Germany). For initial dilution of bacteria 1 µg/ml was added to sterile 10 ml Mueller-Hinton broth media. The microdilution was performed at concentration ranging from 0 µg/ml – 5,000 µg/ml. The bacteria were tested in duplicates. For negative control, bacterial suspensions were used while the extract of broths was used as positive control. The plates were incubated for 24 hours at 37 °C and the endpoints were visualized after incubation. The MIC values were recorded by the lowest concentration of the extract that produced no visible growth on wells.

The minimum bactericidal concentration (MBC) values were determined by spreading 10 µl from each wells of MIC into Muller Hinton agar. The plates were then incubated for 24 hours at 37 °C. MBC values were recorded as lowest concentration of antimicrobial agent at which showing no growth on agar plate.

Time-Kill Curve

Time-kill curve were made through calculation of bacteria growth at serial dilution agar on predetermined time (Rukayadi *et al*., 2008). In brief, final concentrations used were 0xMIC, 1/2xMIC, 1xMIC, 2xMIC, 4xMIC and 8xMIC for each streptococcal species. At time of 0 min, 15 min, 30 min, 1 hour, 2 hour and 4 hour, aliquots were incubated in rotary shaker at 37 °C and 10 µl of aliquots part of MIC were transferred to Eppendorf tube containing 990 µl of phosphate broth. Then, 10 µl from aliquots were transferred to other eppendorf tube for dilution concentration until 10^{-6} of concentration. Thereafter, 10 µl from each dilution concentration were spread on nutrient agar to determine the number of colony count in CFU/ml.

RESULTS AND DISCUSSIONS

Dental carries are infectious diseases caused by oral cariogenic species, and these species are moderately resistant to antibiotics (Venditi *et al*., 1989). *Streptococcus mutans* is

Table 1. Inhibition zone diameter of *B. rotunda* extract against oral pathogens

Bacterial species	Inhibition zone diameter (mm)
Streptococcus mutans KCCM 3309	9.0
Positive control (0.05 % Chlorhexidine)	11.0
Negative control (10% DMSO)	-
Streptococcus sobrinus KCCM 3207	8.0
Positive control (0.05% Chlorhexidine)	11.0
Negative control (10% DMSO)	-

-: No inhibition zone

Table 2. MIC and MBC values of *B. rotunda* extract against oral pathogens

Tested bacteria	MIC concentration (µg/ml)	MBC concentration (µg/ml)
Streptococcus mutans	313	313
Streptococcus sobrinus	313	625

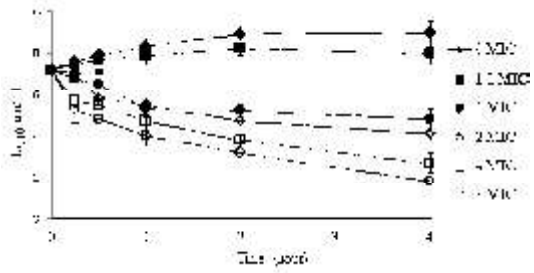


Fig. 1. Time-kill plots for *Streptococcus sobrinus* KCCM 3207 following exposure to *Boesenbergia rotunda* (L.) Mansf. extract.*MIC= 313 µg/ml

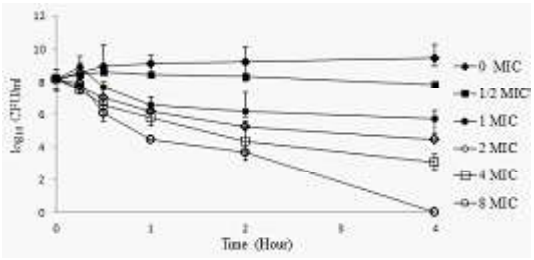


Fig. 2. Time-kill plots for *Streptococcus mutans* KCCM 3309 following exposure to *Boesenbergia rotunda* (L.) Mansf. extract.*MIC=313 µg/ml

related to the formation of dental caries in fissures as well as smooth enamel surfaces, while *S. sobrinus* is more sequestered from the proximate sites of posterior teeth (Lindquist and Emilson, 1989). Controlling these oral pathogens are the key on prevent these diseases. In this study, the antibacterial activities of methanol extract of *Boesenbergia rotunda* (L.) Mansf. against two streptococcal species *S. mutans* and *S. sobrinus* was determined. The results were tabulated in Table 1. Based on the Table 1, preliminary screening showed the diameter of inhibition zone for *S. mutans* and *S. sobrinus* is 9.0 mm and 8.0 mm, respectively. These values were lower than to positive control 11.0 mm for both of the bacteria.

The MIC values result is shown in Table 2. *B. rotunda* extract possesses inhibitory properties against *S. mutans* KCCM 3309 and *S. sobrinus* KCCM 3207 as the MIC value showed 313 µg/ ml for both bacteria. MBC value for *S.mutans* and *S.sobrinus* is 313 µg/ml and 625 µg/ ml, respectively. Numerous studies have demonstrate MICs value againts *S. mutans* with some other natural anticariogenic compound tested such as mangosteen (625 µg/ml) (Torrungruang *et al*., 2007), Sopharae radix (1250 µg/ml) and Coptidis rhizmoma (313 µg/ml) (Yim *et al*., 2013). For *S. sobrinus*, Severino *et al*. (2009) have reported *Hortia oreadica* have MIC value (300 µg/ml), *Kompassia malaccensis* (225 µg/ml) and Perilla seed extract (400 µg/ml) (Yamanoto and Ogawa, 2002). Hwang *et al*. (2004) reported isolated free compound of panduratin A from *B. rotunda* having an MIC value 4 µg/ml against *S. mutans* and *S. sobrinus*. This suggests there are differences on MICs value between free compound and bound compound of the extract. Besides, a paper investigated that *B. rotunda* contains derivatives components alpinitin, pinocembrin and pinostrobin which are active against multi bacteria (Vorathikunchai *et al*., 2005). Therefore, the possibility of other derivatives contained in the extract may have contributed to the antimicrobial activity as well. Therefore, it is difficult to compare directly with previous studies because the factors of different serotypes of streptococci species, sources of *B. rotunda*, different analytical methods and source media. Thus, Aliagnis *et al*. (2001) have classified groups for plant materials based on MIC result; strong inhibitors (less than 500 µg/ml), moderate inhibitors (600-1500 µg/ml), weak inhibitors (more than 1600 µg/ml). Besides that, Duarte *et al*. (2005) had set up a concentration of 2000 µg/ml as highest concentration that can be acceptable for vegetable extract to be antimicrobial.

In order to get ample bactericidal effect, time-kill test was conducted based on the optimal treatment of concentration and time. The rates of bacteria (*S. mutans* and *S. sobrinus)* killed were determined by the exposure to extract at 313 µg/ml of 0xMIC, 1/2xMIC, 1xMIC, 2xMIC, 4xMIC and 8xMIC for 15 minutes, 30 minutes, 1 hour, 2 hour and 4 hour (Figure 1 and 2). Basically, when the extract concentration increased, antibacterial activity will increase as shown by the shorter time to kill the bacteria. As shown on Figure 1 and 2, both strains bacteria were exposed to concentration 1xMIC, 2xMIC, 4xMIC and 8xMIC were able to reduce more than one log colony form unit (cfu) after 15 minutes. For 0xMIC (control) and 1/2xMIC, the CFU was not reduced instead it significantly increased after 15 minutes of treatment. However,

both strains at the concentration of 1/2xMIC showed a decrease of CFU at time of 4 hours. This showed bactericidal activities for both strains at 1/2xMIC concentration of extracts starts only at 4 hours of exposure. Besides, the difference between 2×MIC and 4×MIC was reduced by almost 2 orders at 2 hours of treatment. Eight MIC were found to kill 99.9% of *S. mutans* after 4 hours treatment. *S. sobrinus* are not easily to be killed because *S. sobrinus* is more acidogenic than *S. mutans* (De Soet *et al*., 1989). This is because the availability of sustained acid product at the level below than pH 6 (Nascimento *et al*., 2004).

From the current results and data, *B. rotunda* is proven active against oral pathogenic bacteria. The antimicrobial activity of *B. rotunda* extracts against *S. mutans* was first discovered by Hwang *et al*. (2004). Some of polyphenols isolated from plants conferred as antimicrobial activity either because of growth inhibition against streptococci or because of inhibition of glucosyltransferases (Hada *et al*., 1989). Plus, concentration of 25, 50 and 100 mg/ml of rhizome extract of *B. rotunda* were reported to inhibit candida adhesion to oral surfaces to colonize the mouth and cause oral disease (Sroisiri and Boonyanit, 2010). Toxicity test on high consumption of *B. rotunda* have not been scientifically established yet. However, there is a recent study that demonstrates *B. rotunda* is safe for consumption. Sarathoing *et al*. (2010) reported there were no significant changes in the body weight of *B. rotunda* fed rat. Besides that, he also described that there were no adverse changes on hematological and histopathological parameters used on rats. Thus, with all recent papers reported this paper widely proved the antimicrobial activity of *B. rotunda* as strong inhibitor against *S. sobrinus* and *S. mutans.*

In short, these findings suggest that fingerroot or *Boesenbergia rotunda* (L.) Mansf. could be a highly potential antibacterial agent and would benefit the oral healthcare industry since it is based on natural ingredient. However, the toxicity test has not been done and it should be a main concern, especially in considering the extract to be an oral care product. Therefore, further studies about chemical constituents of plant are crucial because such information may be valuable in finding new sources for synthesis of complex substances. In addition, studies on other oral pathogens such as *Staphylococcus aureus* and *Candida albicans* on their susceptibility to the extract should be done as they are part of the oral flora.

CONCLUSION

In conclusion, the methanol extract of finger root [*Boesenbergia rotunda* (L.) Mansf. A.] was confirmed to have an anticariogenic activity against *S. sobrinus* and *S. mutans*. It was first validated through the preliminary disc diffusion agar test, showed 9.0 mm and 8.0 mm diameter of inhibition zone for *S. mutans* and *S. sobrinus* respectively. Further MIC data supported the preliminary test by showing that *B. rotunda* extract possesses inhibitory properties against *S. mutans* and *S. sobrinus* as the MIC value showed 313 µg/ml for both bacteria. MBC recorded for *S. mutans* and *S. sobrinus* showed the value of 313 µg/ml and 625 µg/ml, respectively. From time-kill curve, eight MIC were found to kill 99.9% of *S. mutans* after 4 hours treatment. *S. sobrinus* are not easily to be killed because *S. sobrinus* is more acidogenic than *S. mutans*.

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