ASIAN JOURNAL OF PHARMACEUTICAL AND CLINICAL RESEARCH

INNOVARE ACADEMIC SCIENCES
Knowledge to Innovation

Special Issue (October)

Online - 2455-3891
Print - 0974-2441

Research Article

ANTIBACTERIAL EFFECT OF JAVA TURMERIC ETHANOL EXTRACT AGAINST DUAL-SPECIES *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SANGUINIS* BIOFILM (*IN VITRO*)

AJRINA BUSRI, RIA PUSPITAWATI*, SRI UTAMI

Department of Oral Biology, Faculty of Dentistry, Universitas Indonesia, Jakarta, Indonesia. Email: rpuspitawati2013@gmail.com

Received: 21 April 2017, Revised and Accepted: 13 July 2017

ABSTRACT

Objectives: The minimal bactericidal concentration (MBC) of Java turmeric (*Curcuma xanthorrhiza* Roxb.) ethanol extract is 25% against *Streptococcus mutans* and 15% against *Streptococcus sanguinis* as single species. This study aimed to examine the antibacterial effect of Java turmeric ethanol extract against *S. mutans* and *S. sanguinis* as dual-species. *S. mutans* and *S. sanguinis* compete against each other to obtain nutrients.

Methods: The antibacterial effect of Java turmeric ethanol extract against dual-species *Streptococcus in vitro* was analyzed by measuring the growth of bacteria after exposure to the extract by counting colony formation and quantifying bacterial cell numbers using real-time polymerase chain reaction.

Result: The MBC of Java turmeric ethanol extract against dual-species *Streptococcus* is 10%. *S. sanguinis* is more sensitive to the extract than *S. mutans.*

Conclusions: The antibacterial effect of Java turmeric ethanol extract on *S. mutans* and *S. sanguinis* as single species differs from the effect on the bacteria as dual-species of *Streptococcus*.

Keywords: Dual-species *Streptococcus*, Java turmeric ethanol extract, *Streptococcus mutans*, *Streptococcus sanguinis*.

 DOI: http://dx.doi.org/10.22159/ajpcr.2017.v10s5.23096

INTRODUCTION

Dental caries is a hard tissue disease in teeth caused by the gradual chronic loss of mineral ions from enamel, dentin, and cementum [1]. Dental caries is one of the most frequently seen oral infections in society, regardless of age group and economic status. The United States surgeon general has reported that 45% of children between 5 and 17 years old suffer from dental caries [2]. The prevalence of dental caries in different countries is relatively high, especially in Indonesia. According to the Basic Health Survey 2013, 72.3% Indonesian population was suffering from caries experience [3].

Caries can form as a result of the interaction of a number of factors, including host (tooth structure and saliva), bacteria (cariogenic bacteria, especially *Streptococcus* and *Lactobacilli* sp.), substrate (fermented food), and time [1]. The formation of dental caries begins with the formation of pellicle. Pellicle is a thin layer of the matrix on the tooth surface, which is formed by saliva. The pellicle is colonized by *Streptococcus sanguinis*, which is an early colonizing bacterium [4]. *S. sanguinis* coaggregate with other bacteria, such as *Streptococcus mutans*, to form dental plaque or dental biofilm. Dental biofilm is a soft layer composed of microorganisms cultivating on pellicle and is attached to tooth surfaces [5]. Dental biofilm is normally always present on the tooth surfaces [6]. Biofilm can be composed of many different interacting species [7]. The accumulated different species of bacteria interact in a mechanism called quorum sensing, causing changes in the composition of microbes, which then leads to the formation of caries [6].

Previous studies by Caufield *et al.* (2002) stated that *S. sanguinis* interacts by opposing *S. mutans* to colonize the tooth surface. Both bacteria need tooth surface for colonization. The interaction between *S. sanguinis* and *S. mutans* is affected by environmental factors, such as the density of the colony, the availability of nutrition, and pH [8]. Studies by Kreth *et al.* showed that the interaction between *S. mutans* and *S. sanguinis* was influenced by conditions in the environment. *S. mutans* and *S. sanguinis* produce substances that are able to suppress the growth of other species. *S. mutans* produces mutacin, which has the ability to suppress the growth of *S. sanguinis*. On the contrary, *S. sanguinis* produces hydrogen peroxide, which can suppress the growth of *S. mutans* [9].

Maintaining good oral hygiene contributes to preventing the formation of dental caries. There are many different methods to maintain good oral hygiene; among them are mechanical cleansing and the use of chemical agents. The mechanical method involves the use of a toothbrush to eliminate dental biofilm, whereas the chemical method involves using mouthwash as an antimicrobial agent [10]. Mouthwash has the ability to kill bacteria [11]. However, prolonged use of mouthwash may cause tooth discoloration. Besides that, mouthwash that contains alcohol may cause xerostomia and irritation of the mucosa. Thus, the development of alternative agents of caries prevention that are relatively cheap and safe is needed [11].

Currently, traditional medicine is used as an alternative in the prevention and as a cure for diseases, it is relatively cheap and safe. Traditional medicine is a mixture of substances, derived from plants, animals, minerals, or a mixture of these, that have been passed down from generation to generation and have been used as medicine based on experience. Indonesian traditional medicines are divided into herbal medicine (in Indonesia known as jamu), standardized herbal medicine, and fitofarmaka, a form of herbal medicine that has been clinically tested. Jamu is the traditional medicine that has not been subjected to any kind of scientific testing but has been passed down through generations. Standardized herbal medicine has been scientifically tested for its safety and benefits. Fitofarmaka is the natural herbal medicine that has been scientifically proven for its safety and benefits, has undergone preclinical and clinical testing, and has standardized raw materials and products [12].

Indonesia is known as a country with biodiversity and has an abundant agricultural production. There has been an increase in agricultural production, especially in the development of herbal medicine. According to the World Health Organization, there are 20,000 types

of plants in the world that can be used for medicine, and more than 2,200 types of plants are present in Indonesia [13]. Badan Pengawas Obat dan Makanan (the Board of Drugs and Food Supervision) of the Health Department in Indonesia has determined nine leading herbal medicines in Indonesia, including *Curcuma xanthorrhiza* Roxb. or Java turmeric (known in Indonesia as temulawak) [14].

Java turmeric is a member of the Zingiberaceae family and is native to Indonesia. Its establishment as a leading herbal medicine is based on it benefits [13]. It contains a yellow substance (curcumin), starch, protein, fat (fixed oil), cellulose, minerals, and essential oil. Curcumin is used as a food supplement to increase appetite and has anti-inflammatory and antioxidant potentials. The contents of the starch, protein, fat (fixed oil), cellulose, and minerals in Java turmeric can act as a substitute for food. The contents of the essential oil in Java turmeric include active substances such as xanthorrhizol.

Hwang *et al.* (1999) conducted a study using xanthorrhizol isolated from Java turmeric. The research proved that xanthorrhizol had anticaries activities for fighting oral pathogens, especially *S. mutans*. Xanthorrhizol can kill *S. mutans* and shows activities as an antibacterial agent that can prevent the formation of *S. mutans* biofilm.

Based on the research at the Oral Biology Laboratory, Faculty of Dentistry, Universitas Indonesia, it is known that the minimum inhibitory concentration (MIC) of Java turmeric ethanol extract against *S. mutans* is 0.5% (5 mg/mL), and the minimum bactericidal concentration (MBC) is 25% (250 mg/mL). However, Java turmeric ethanol extract has an MIC of 0.5% (5 mg/mL) and an MBC of 15% (150 mg/mL) against *S. sanguins*. Based on previous studies, the effective concentration of ethanol extract varies in different species of bacteria.

METHODS

This study was a laboratory experimental study using the bacteria *S. mutans* ATCC 25175 and *S. sanguinis* ATCC 10556 obtained from the Oral Biology Laboratory of the Faculty of Dentistry, Universitas Indonesia. Before exposure to the extract, the concentrations of the bacteria with dilutions and inoculations on agar were determined. A concentration of 10^5 ml was chosen for both *S. mutans* and *S. sanguinis*. The control group was divided into two main group: Negative control, which are bacteria without any treatment; and a positive control, which are bacteria exposed to chlorhexidine. The dual-species bacteria were obtained by mixing *S. mutans* and *S. sanguinis* in equal concentrations (1:1).

Java turmeric ethanol extract was obtained by a maceration process in Research Center for Spice and Drugs Plant (BALLITRO) and was diluted into different concentrations using dimethyl sulfoxide 10%. The resulting ethanol extract was used in concentrations of 0.2%, 1%, 5%, 10%, and 15%.

The bacterial suspension was mixed with Java turmeric ethanol extract and inoculated on an agar medium. Bacteria that had been exposed to the extract were put in an anaerobic jar filled with N_2 gas balance, 10% CO_2, and 10% H_2, and incubated in an incubator at 37°C for 48 hrs.

Quantification of *S. mutans* and *S. sanguinis* in the dual-species biofilm before and after exposure to Java turmeric ethanol extract was done. Sterile saliva was put into a 24-well plate and incubated at 37°C for 60 minutes to form pellicle and also biofilm by adding bacteria. The biofilm was divided into two groups: Single species (control) and dual species (with and without exposure to Java turmeric ethanol extract). The biofilm sample was incubated at 37°C for 24 hrs. The bacterial DNA in the biofilm was extracted using a thermal shock. The concentration and purity of the DNA sample were then evaluated. Bacterial DNA was then quantified using real-time polymerase chain reaction (PCR).

The data obtained from the antibacterial test of Java turmeric ethanol extract was then analyzed with the Shapiro–Wilk test for normality. The data on the total number of colonies were not normally distributed and were tested with non-parametric tests: Kruskal–Wallis and Mann–Whitney tests, to determine whether there was a significant difference between the two variables. However, the quantification results were relatively normally distributed and were further tested with an unpaired t-test. The statistical test performed had a significance of 0.05 ($p=0.05$) and a reliability of 95% ($\alpha=0.05$).

RESULTS

The results of the experiments on the number of colonies formed after exposure to Java turmeric ethanol extract are presented on Figs. 1-3.

The statistical test used was a Kruskal–Wallis test, and the results showed that there was a significant difference between the number of colonies of bacteria in every treatment group. In the Mann–Whitney test, there was a significant difference between the negative control group and the groups treated with Java turmeric ethanol extract. There was a significant difference between the positive control group and the single-species *Streptococcus* groups treated with Java turmeric ethanol extract at concentrations of 0.2%, 1%, 5%, and 10%, and the dual-species *Streptococcus* group treated with 0.2%, 1%, and 5% concentrations. There was no significant difference between the positive control group and the single-species *Streptococcus* groups treated with Java turmeric ethanol extract at a concentration of 15%, and the dual-species *Streptococcus* group treated with 10% Java turmeric ethanol extract.

The results of the quantification using real-time PCR with a relative count of $2^{-\Delta\Delta Ct}$ is shown in Fig. 4. The relative quantification is done to obtain values for gene expression from the target. Gene expression represents the number of bacteria used in a sample. The relative count

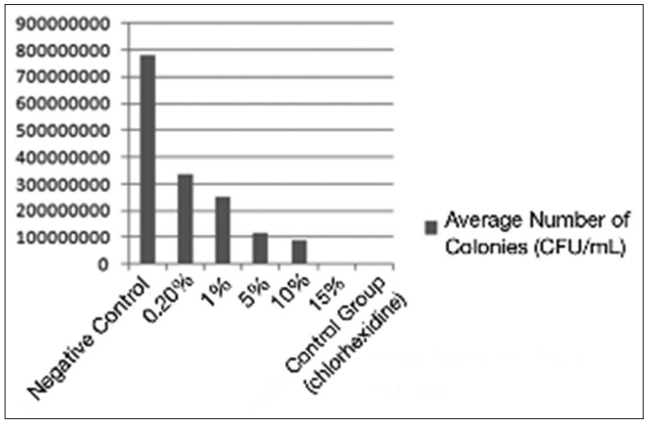

Fig. 1: The average number of colonies of *Streptococcus mutans* (CFU/mL) after exposure to Java turmeric ethanol extract

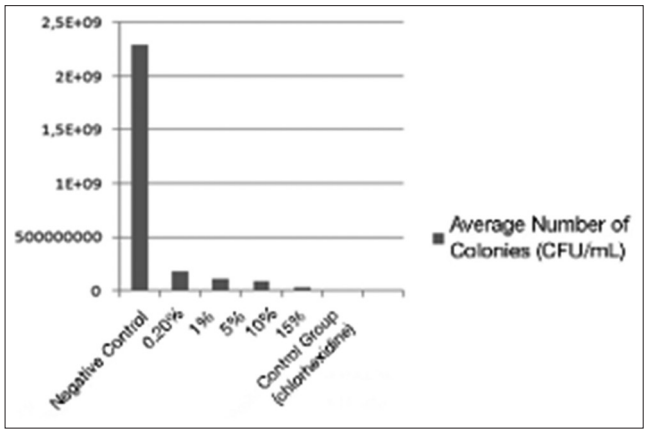

Fig. 2: The average number of colonies of *Streptococcus sanguinis* (CFU/mL) after exposure to Java turmeric ethanol extract

produces a bacterial expression ratio between the groups studied and the control groups. The control groups are single-species *Streptococcus* bacteria. After that a statistical test was conducted using an unpaired t-test to evaluate the significant difference between two unrelated groups. There were different quantities of *S. mutans* and *S. sanguinis* in the biofilm of dual-species *Streptococcus* without exposure to Java turmeric ethanol extract, but the difference was not significant. After exposure to Java turmeric ethanol extract, the difference in the quantities of *S. mutans* and *S. sanguinis* in the biofilm of dual species *Streptococcus* was significant.

DISCUSSION

After exposure to Java turmeric ethanol extract, the bacteria were incubated for 48 hrs, which is the optimal time for *S. mutans* and *S. sanguinis* growth. Antibacterials are more effective during the division of bacteria when cell walls containing phospholipids become thinner, enabling antibacterials to penetrate, and cause lysis [15].

An increase in the concentration of Java turmeric ethanol extract causes a reduction in the number of colonies of *S. mutans* and *S. sanguinis.* The results of this research show that the MIC of Java turmeric ethanol extract to *S. mutans* and *S. sanguinis* as single and dual species was 0.2%. The MBC of Java turmeric ethanol extract to *S. mutans* and *S. sanguinis* as single species was 15% but was 10% for dual-species *Streptococcus*. These results show that the antibacterial effect of Java turmeric ethanol extract on dual-species *Streptococcus* is lower than its effect on single species of *Streptococcus*. This may be due to the competition between *S. mutans* and *S. sanguinis*. The competition between *S. mutans* and *S. sanguinis* is affected by their environment. In an environment with limited nutrition, *S. mutans* and *S. sanguinis* produce substances that can inhibit the growth of other species. *Streptococcus mutans* produce mutacin to inhibit the growth of *S. sanguinis*. Meanwhile, *S. sanguinis* produce hydrogen peroxide, which is able to inhibit the growth of *S. mutans*. In this study, the dual-species *Streptococcus* was in an environment of limited nutrition because sucrose was not added to its medium. Based on the results of the colony count of single species compared with the negative control group, Java turmeric ethanol extract causes a greater reduction in the number of *S. sanguinis* (98%), compared to *S. mutans* (52%). It can be assumed that *S. sanguinis* is more sensitive to Java turmeric ethanol extract, whereas *S. mutans* is more resistant. This may be because *S. mutans* produces a dextran capsule that encapsulates its cell walls, making it stronger and more resistant to antibacterial penetration.

Java turmeric ethanol extract contains starch, curcuminoid, and essential oils. Essential oils contain active substances such as xanthorrhizol. Xanthorrhizol contains phenol and hydrocarbol compounds. Phenol compounds consist of different compounds derived from plants of the same characteristics, having an aromatic ring and containing one or more hydroxyl groups (-OH). This compound interacts with bacterial cells through adsorption involving a hydrogen bond. At high concentrations, xanthorrhizol coagulates with cellular protein and cytoplasmic membranes and undergoes lysis. However, at low concentrations, xanthorrhizol will bond with cellular proteins and form a phenol complex protein with weak bonds and will dissociate. Phenol damages the cytoplasmic membrane and causes leakage of the cell wall, eventually causing cell death.

Fig. 4 indicates an increase in the growth of *S. mutans* and the inhibition of *S. sanguinis* in dual-species *Streptococcus*. This shows that in a dual-species environment, *S. mutans* competes against *S. sanguinis* without antibacterial intervention. *Streptococcus mutans* is more dominant and is able to inhibit the growth of *S. sanguinis*. In dual-species *Streptococcus* exposed to Java turmeric ethanol extract, *S. mutans* can be seen as being more dominant ($p<0.05$). This is due to the sensitivity of *S. sanguinis* to Java turmeric extract and competition against *S. mutans.*

Further studies are recommended to examine the efficacy of Java turmeric ethanol extract toward other cariogenic *Streptococcus* bacteria, and also the effect of Java turmeric extract on the virulence of those cariogenic bacteria.

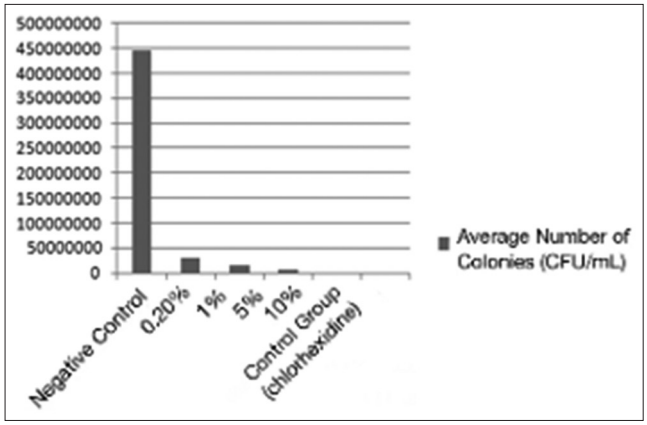

Fig. 3: The average number of colonies of dual-species *Streptococcus* (CFU/mL) after exposure to Java turmeric ethanol extract

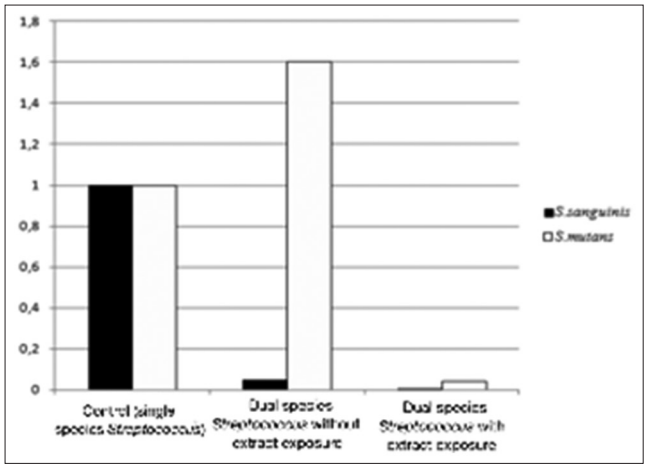

Fig. 4: The relative average of *Streptococcus mutans* and *Streptococcus sanguinis* with and without Java turmeric extract exposure on dual-species *Streptococcus* with real-time polymerase chain reaction

CONCLUSION

Based on this research, it can be concluded that the antibacterial effect of Java turmeric ethanol extract toward *S. mutans* and *S. sanguinis* as single species is different compared to its effect on them as a dual-species *Streptococcus*. Exposure of dual-species *Streptococcus* biofilm to 15% Java turmeric ethanol extract caused a greater decrease in the growth of *S. sanguinis* compared to *S. mutans.* This shows that *S. sanguinis* has greater sensitivity to Java turmeric ethanol extract than *S. mutans.*

REFERENCES

1. Mount GJ, Hume WR. Preservation and Restoration of Tooth Structures. 2nd ed. Queensland: Rob Watts; 2005.
2. Allukian M Jr. The neglected epidemic and the surgeon general's report: A call to action for better oral health. Am J Public Health 2000;90(6):843-5.
3. Indonesian Ministry of Health. Basic Health Survey. Jakarta: Indonesian Ministry of Health; 2013.
4. Shay K. Infectious complications of dental and periodontal diseases in the elderly population. Clin Infect Dis 2002;34(9):1215-3.
5. Pintauli S, Hamada T. Menuju Gigi Dan Mulut Sehat: Pencegahan Dan Pemeliharaan. Medan: USU Press; 2008.

6. Marsh P, Martin MV. Oral Microbiology. 4th ed. Oxford: Wright; 1999.
7. Burmølle M, Webb JS, Rao D, Hansen LH, Sørensen SJ, Kjelleberg S. Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl Environ Microbiol 2006;72:3916-23.
8. Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. Sensors (Basel) 2012;12(6):2519-38.
9. Kreth J, Merritt J, Shi W, Qi F. Competition and coexistence between *Streptococcus mutans* and *Streptococcus sanguinis* in the dental biofilm. J Bacteriol 2005;187(3):7193-203.
10. Lewis DW, Ismail AI. Periodic health examination, 1995 update: 2. Prevention of dental caries. The canadian task force on the periodic health examination. CMAJ 1995;152(21):836-46.
11. Nurdeviyanti N. Iodized table salt inhibit the growth of *Streptococcus mutans in vitro*. Thesis. Denpasar: Universitas Udayana; 2011.
12. Indonesian Food and Drugs Administration. Kriteria dan tatalaksana pendaftaran obat tradisional. Obat Herbal Terstandar Dan Fitofarmaka. Jakarta: Indonesian FDA; 2005.
13. Pribadi ER, Raharjo M. Efisiensi pemupukan NPK pada temulawak. Littri 2008;14(4):162-70.
14. Mangunwardoyo W, Deasywaty D, Usia T. Antimicrobial and identification of active compound *Curcuma xanthorrhiza* roxb. Int J Basic Appl Sci IJBAS-IJENS 2012;12(1):69-78.
15. Jenkins S, Addy M, Wade W. The mechanism of action of chlorhexidine. A study of plaque growth on enamel inserts *in vivo*. J Clin Periodontol 1988;15(7):415-24.