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Research Article

Inhibition of *Streptococcus mutants* by Extracted from *Streblus asper* for Oral Care Product

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Abstract

Background and Objective: *Streblus asper* (*S. asper*) has been used as the Thai folk medicine for the treatment of teeth and mouth. This study was aimed to test antimicrobial activity by using bioactive extracted substances from *S. asper* against *Streptococcus mutants* (*S. mutants*). **Materials and Methods:** The *S. asper* were collected from the northern parts of Thailand and extracted by hot water. *Streptococcus mutants* were collected and screened from volunteers' saliva, then cultured on a nutrient medium. *Streptococcus mutants* was growing by Mitis Salivarius (MS) medium supplemented with sucrose 2.5% w/v. The growth phase during 54 hrs of *S. mutants* in the medium was collected and tested for bacterial inhibition. Extracted substances of *S. asper* against *Streptococcus mutants* were evaluated for percentage of inhibition. **Results:** The values of cell survival of *Streptococcus mutants* when incubate with the leaves, barks and branches of *S. asper* extracted were 25.55 ± 1.26 , 40.46 ± 0.65 and 37.30 ± 3.90 %, respectively. **Conclusion:** It can be concluded that the leaves of *S. asper* extracts presented the best inhibition to *S. mutants* and could be developed it's as a composition of tooth care product for preventing tooth decay.

Key words: Antimicrobial, *Streblus asper*, *Streptococcus mutants*, MS medium, tooth powder, physical assessment, stability test

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Streblus asper (*S. asper*) had been noticed as a great deal of attention similar to tea because its polyphenolic compound is a strong antioxidant and previous studies have many biological activities¹ and parameters of biosorption of metal². This plant has been used in Ayurveda and other folk medicines for the treatment of different ailments including filariasis, leprosy, toothache, diarrhoea, dysentery and cancer³. Chemically, this beverage as leaves tea is characterized by the presence of polyphenolic² and flavonoids⁴ that benefit health promotions. *Streblus asper* leaves and branches are the most popular in the past of Thailand for clean teeth cause of their coarse texture. Moreover in the Ayurveda, these parts have an astringent test for the treatment of wound healing¹. Nowadays this plant is not popular anymore cause of the easy use of toothpaste. But the most important beneficial effects of *S. asper* are the anti-oxidant activity and anti-microbial activity, these activities were arisen by polyphenol components^{1,3,4}. Polyphenols are the most important constituents of tea leaves⁵. The microbe that causes tooth decay is *Streptococcus mutants* (*S. mutants*) and it is a gram-positive bacterium⁶. *Streptococcus mutants* synthesize extracellular water-insoluble glucans by using glucosyltransferase (Gtases). The glucans are synthesized from sucrose by cooperative actions of Gtases and are strong adherence to various solid surfaces including tooth surfaces. The adherent leads to the formation of dental plaque and the development of dental caries^{6,7}. *Streblus asper* is easily found in the northern parts of Thailand. Thai plants were found that extracted or its polyphenol had anti-microbial activities⁸. Some studies had been reported the effects of polyphenols on human health^{9,10}.

The study was aim to test the inhibition of *S. mutants* that is the cause of plaque and caries by the extracted substance of leaves, stem and bark from *S. asper*.

MATERIALS AND METHODS

Study period: The study was carried out at the Research and Development Institute, Lampang Rajabhat University, Thailand from October, 2019 to September, 2020.

Plant sample collection and identification: Leaves, branches and barks of *S. asper* in the local area of Lamphun Province, Thailand were collected for study. (GPS Coordinates 18.595633, 99.010491). The verification of the plant samples was done following the list of plants (<http://www.theplantlist.org>). (Specimen Number: Lamphun001-2019-20).

Plant sample preparation: Each part of the plant samples including leaves, branches and barks from *S. asper* (1.00, 2.00, 4.00, 6.00 and 8.00 g) was prepared by the extraction procedure using 100 mL of water at a temperature between 90-95°C for 5 min. The evaporation step was done at 60.0°C overnight for drying the extracted substances (Buchi, Japan).

Screening test of bacteria: The collection and screening test had performed by selective medium (mitis salivarius medium, MS) (Criterion, U.S.A) from the saliva of 25 boys and girls volunteers (age between 3 and 6 years). *Streptococcus mutants* had been screened out the other Streptococci by 2.5% of sucrose in the presence or absence of bacitracin. The technique of streak plate had been utilized to choose the single colony of *S. mutants* and subsequently cultured in MS broth by variation of time and incubated at a temperature of 37.0°C for 0-54 hrs. The suspension of bacteria was determined growing phase at OD 600 nm of wavelength. Furthermore, the certification of the morphology of the single colony and the cell had been performed under a microscope and gram staining. The optimum condition had been utilized for further experiments.

Counting test of bacteria: The working solution was diluted ten-fold using the preparation procedure by adding 1 mL of microorganisms to the solution of phosphate buffer 9 mL at pH 7.2. The counting procedure was performed for determining the numbers of cells by using the Petroff-Hausser cell counting chamber (Northern chemical, Thailand). The suspension had been stained using crystal iodine (Northern Chemical, Thailand) and counted under microscopy (Northern Chemical, Thailand).

Measurement of bacteria survival and growth: The preparation of each part of plant samples including leaves, branches and barks of *S. asper* at 1.00, 2.00, 4.00, 6.00 and 8.00 (%w/v) had been done using one millilitre of extracted of each part of *S. asper*. Then, 1 mL of *S. mutants* (10⁶ cells) and 8 mL of MS broth were added to the suspension. Distilled water was used as a control. The incubation of suspensions had been performed using a shaker incubator (IKA, KS4000I control, U.S.A). At 18 hrs the inhibition test was investigated by spectrophotometer (Agilent-6890N, Thailand) at the wavelength of 600 nm.

Formulation of tooth powder: The formulation of tooth powder was performed using the tropical usage for clean teeth. The formulation compositions were 2.00 g of white clay,

5.00 g of salt, 1.00 g of Borneo camphor and 1.00 g of camphor. The concentration of the optimum point for inhibition was chosen for tooth powder formulation. Each extract will be used as an ingredient in the powder formulation.

Assessment of formulation

Physical parameters: Each formulated powder has investigated the appearance, colour and homogeneity. The procedure of formulated powder was following:

- **Thermal stability:** The examination of the formulation was performed in 60-70% RH and $37.0 \pm 1.0^\circ\text{C}$ room. The freeze-thaw condition ($0-4^\circ\text{C}$ and room temperature) for 4 cycles had been utilized for the thermal cycle. To achieve the assessment of the cream examination, the result should not be separated from oil or liquidity in the cream
- **pH determination:** The differentiation of pH points had performed mostly in the range from 5-9 for the formulation. The differentiation of powder pH presents around pH 6-9. The dilution of the formulated powder was done to 10% dilution with distilled water. The differentiation of pH ranges of mixtures was conducted using a pH meter
- **Foaming:** The formulation of powder was diluted to the point of 10% dilution using distilled water. The differentiation of the height of foaming was conducted using 100 mL of the cylinder after the top of liquids was placed at the middle of the cylinder

Statistical analysis: All samples were analyzed in triplicate. All values were presented as Mean $\pm$ SD. Significant differences between means were examined by the SPSS software program using one-way ANOVA. A probability value of $p < 0.05$ was adopted as the criteria for significant differences.

RESULTS

Plant sample preparation: The extraction procedure was performed by using each part of plant samples including leaves, branches and barks from *S. asper* in 100 mL of hot water ($90-95^\circ\text{C}$) for five minutes. The process of extraction was belonging to tea preparation and state with the Thai ancient plant extraction. The leaves, branches and barks of *S. asper* extracts were evaporated. Then, the pale greenish powder was derived. The yield of each extract was about two percent of fresh samples. These resulting extracts will be used in further experiments.

Bacterial screening and counting: The technique called streak plate was used in this study. The saliva of 25 boys and girls volunteers (aged between 3 and 6 years) were collected and screened by the selective medium. The screen out of *S. mutants* from the other *Streptococci* was carried out by 2.5% of sucrose and bacitracin.

The *S. mutants* have been collected with the single colony of *S. mutants* and subsequently cultured in MS broth by variation of time and incubated at 37.0°C for 0-54 hrs. The suspension of bacteria was examined the phase of growth as presented in Fig. 1. The initial phase was 0-18 hrs. The log phase of the growth of *S. mutants* was 36 hrs. of incubation and continue the stationary phase to 54 hrs. At the time of incubation, each condition had been observed the same morphology and its colour and confirmed under the microscope. The MS medium and MS medium with 2.5% of sucrose were utilized for the screening and the results showed the different morphology and colour of a single colony.

The results of the MS medium with 2.5% of sucrose plus bacitracin presented the similarity of morphology and colour of a single colony. On the other hand, the concentration of bacitracin ($1, 2 \mu\text{mL}^{-1}$) addition has not displayed the variation of the bacteria number. After screening the other bacteria, the best optimum of *S. mutants* screening was found 36 hrs. of time used for incubation with 2.5% sucrose and $1 \mu\text{mL}^{-1}$ of bacitracin. At the 36 hrs of incubation time exhibited that a single colony had a similar shape and colour and with the screening under a microscope. Each cell had the same morphology. This condition will be utilized for *S. mutants* inhibiting by the extracts of plant samples including leaves, stem and bark from *S. asper*.

Bacteria survival and growth measuring: The *S. mutants* with leaves, branches and barks of *S. asper* were incubated at 0.00, 1.00, 2.00, 4.00, 6.00 and 8.00 (% w/v) and subsequently evaluated by spectrophotometer at the wavelength of 600 nm. The experimental results presented that the leaves extract of *S. asper* exhibited the best inhibition performance to *S. mutants* (Fig. 2). The extracts from the leaves of *S. asper* at the concentration 4.00 (% w/v) presented the best reduction amount of *S. mutants* to $25.55 \pm 1.26\%$ when compared to control (0.00% w/v of *S. asper* extract).

The *S. asper* extracts at the concentration of 6.00 and 8.00% w/v also exhibited the inhibition effect on the growth of *S. mutants*. On the other hand, these results gave no statistical difference to the *S. asper* extracts at the concentration of 4.00% w/v. For each part of *S. asper*, the leaves extract presented the best effect for the inhibition of *S. mutants* because it showed the lowest amount of % cell *S. mutants* survival (Fig. 2.).

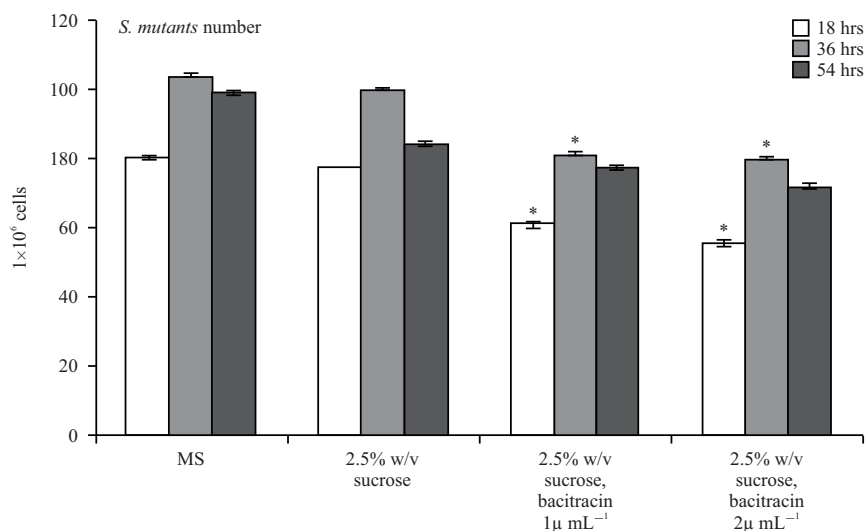


Fig. 1: Variation of media supplement and the number of *S. mutants*

Streptococcus mutants were screened by MS medium with the supplementary with 2.5% w/v of sucrose and 1 μmL^{-1} of bacitracin. The best time of screening *S. mutants* was 36 hrs of incubation time and the *was showed the statistic difference at $p < 0.05$ to MS medium supplemented with/sucrose and bacitracin

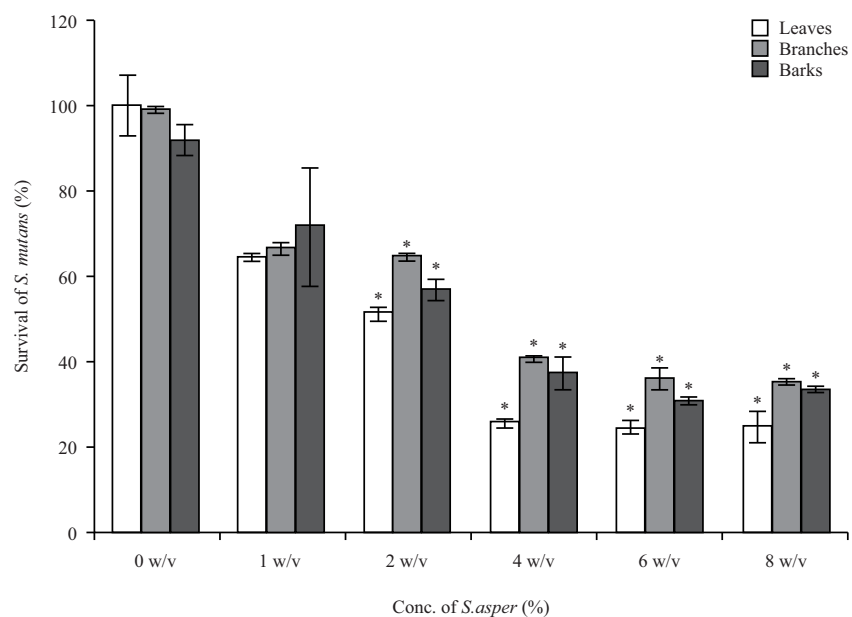


Fig. 2: Survival of *S. mutants* after the process of incubation with the extracts of *S. asper*

Best concentration of *S. asper* extract that presented the lowest amount of cell *S. mutants* survival was the extracts of leaves part of *S. asper* at the concentration of 4.00% w/v and the *was presented the statistic difference at $p < 0.05$ to control (0.00% w/v of *S. asper* extract)

Table 1: Physical assessment of formulated tooth powder

Physical assessments	Percent of leaves of <i>S. asper</i> powder in formulated tooth powder					
	0.000	1.000	2.000	4.000	6.000	8.000
Colour	Pale green	Pale green	Pale green	Pale green	Pale green	Pale green
Homogeneity	Yes	Yes	Yes	Yes	Yes	Yes
Thermal stability	Stability	Stability	Stability	Stability	Stability	Stability
pH	7.0	6.8	6.9	6.9	6.9	6.9
Foaming	-	+++	+++	+++	+++	+++

+++ : Present and - : Absent

Tooth powder formulation: The physical examination including colour, homogeneity, thermal stability, pH and foaming of formulated powder had been performed (Table 1). The difference to the 0.000% of the leaves of *S. asper* was not presented and similar ranges of pH and foaming to leaves of *S. asper* were observed. The pH of a solution of powder was measured to evaluate the possible side effects due to acidic or alkaline pH, which can cause oral irritation. Generally, the subject should have a pH value of 6-9.

DISCUSSION

The most basic misperception has to do with the various parts of the *S. asper* evergreen plant. The general processing techniques are brewing or using hot water extract. The leaves of *S. asper* are composed of organic substances that may be related to the defence of the invading pathogens, bacteria and fungi^{3,8,10}. The basic organic substances are polyphenol and flavonoids which are generally found by the process of hot water extraction¹¹. Asperoside, Strebloside and Mansonin are the major glycoside of *S. asper* that display the biological activity^{1,3}. Some research presented the cytotoxicity of *S. asper* extract to human cancer cells that exhibited the health promotion to human¹² and anti-diarrhoea in Albino rat¹³. Triterpenoids, polyol, sugar acid, aldehyde, diterpene, terpene, carboxylic compounds, acid and sugar are also found in the extracts of *S. asper* leaves¹². The polyphenol presented effectiveness in the inhibition performance of bacteria^{14,15}. The polyphenol substances in plants showed good properties to the growth of *S. mutants* and also presented the activity against its biofilm formation^{6,16}. *Streptococcus mutants* from volunteers' saliva were enormous by nutrient medium, then, selected and screened by MS medium.

The counting process was utilized to determine the number of bacteria at the 4th of ten-fold dilution. Our result was shown the maximum number of streptococci bacteria when incubated at 36 hrs, it is a log phase of growth. The reduction of bacteria number has been observed when added sucrose/bacitracin. It was shown the other streptococci were screened out by the condition of this study. Recently, previous studies reported the addition of sugar and bacitracin in MS medium base resulted in the inhibition of all oral streptococci except *S. mutants*¹⁷⁻²⁰. The experimental results show the MS medium supplemented with sucrose of 2.5% and 1.0 unit of bacitracin was the best condition for selecting *S. mutants*. This condition presented the statistical difference in the number of

the bacterium to MS medium base or the addition of MS medium with sucrose of 2.5%, but the number of bacteria growth 2.5% sucrose and 1.0 unit of bacitracin MS medium did not exhibit statistical difference to the sucrose of 2.5% and 2.0 unit of bacitracin MS medium (Fig. 1.). The 1.0×10^6 cells of *S. mutants* in the log phase had been incubated with the extracts of *S. asper* at different ranges of concentrations. Our experimental results showed the extracts of the *S. asper* leaves at the concentration of 4.00, 6.00 and 8.00% w/v presented statistical difference in the percentage of inhibition to other concentrations but the extracts of the *S. asper* leaves at the concentration of 4.00% w/v exhibited the best concentration that effect to cell *S. mutants* survival (Fig. 2.). The cell survival values of *S. mutants* when incubated with the extracts of leaves, barks and branches of *S. asper* were 25.55 ± 1.26 , 40.46 ± 0.65 and $37.30 \pm 3.90\%$, respectively. The *S. asper* which has a lot of polyphenol substances present the inhibition ability to microbes. These results were found similar to previously reported that *S. asper* extract showed the potency of inhibition to microorganism^{1,3}. Our result belongs to previous results reported that tea polyphenol could present the inhibition ability to the growth of *S. mutants* by its antioxidant substances^{21,22}. Our data also evidence to Thai medicinal plant that has ability against to oral microorganism or used as oral care²³⁻²⁵. The tooth cleaning powder of *S. asper* extract presented pale greenish with fine powder (data not show). This formula has been utilized for Thai traditional usage for tooth and gum care.

CONCLUSION

The *S. mutants* have been screened and selected by mitis salivarius medium supplemented with the sucrose of 2.5% and 1.0 U of bacitracin. The extracts of the leaves part of *S. asper* at the concentration of 4.00% w/v presented the best concentration for inhibition activity of *S. mutants*. The extracts of the *S. asper* leaves could be the simple substances for providing tooth powder, mount wash and gum care.

SIGNIFICANCE STATEMENT

This study presented that the extracts of *S. asper* have some activities against microbial. The *S. asper* extracts possibly used to develop to be the ingredient in the tooth care products that can protect people from gum or tooth diseases.

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