

Differential Expression of Gene of *Streptococcus Mutans* in Response to Treatment with Piper *Betle* Aqueous Extract – A Research Framework

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Abstract—This paper will provide the overview of the research framework to investigate gene expression in *Streptococcus mutans*, the major causative agents of dental caries in response to *Piper betle* aqueous extract which had reported to show anticaries properties. Bacteria culture will be treated with different concentrations of *Piper betle* extract (2, 4 and 10 mg mL⁻¹). Culture treatment with chlorhexidine (0.12%) will be used as comparison and positive control. Real Time PCR will be used to assess the expression of *spaP*, *gbpB* and *gtfB* after the treatment. The findings from this study may provide gene expression profile of *Streptococcus mutans* after treatment with different concentration of *Piper betle* extract.

Keywords—*Streptococcus mutans*, *Piper betle*, gene expression

I. INTRODUCTION

Dental caries is a disease where dissolution of the calcified tissue of the tooth by acid produce by bacteria . It usually begin with the formation on dental plaque that create a hospitable environment for the acid producing bacteria. The consequences of the caries for example pain and loss of teeth do create great impact of on the individual and society. It is also one the common childhood disease in many country and local government had to spent a lot for the treatment and prevention .

Natural products are in great demand due to its extensive biological properties and providing source for the discovery of many types of effective bioactive compounds. Due to controversy that keep on growing discussing the side effects of chemicals used in dental products, numerous research have been carried to obtain antimicrobial properties from plants that suitable for oral hygiene care. There are several reports of the component of plants showing antibacterial activities against oral bacteria. Among the plants are garlic, ginger, cinnamon [15], *Piper betle* [14], green tea leaves, grape seed [19] and magnolia bark extract [9].

This paper will provide an overview of the research framework to investigate the differential expression analysis of virulence genes in *Streptococcus mutans* (*S. mutans*) in response to treatment with *Piper betle* extract. Hence, the objective of this in vitro study is to investigate expression of *S. mutans* genes (*spaP*, *gbpB* and *gtfB*) in response to *Piper betle* aqueous extract.

II. LITERATURE REVIEW

A. Caries

Caries is tooth decay, caused by acids produced by bacteria in dental plaque. It is also reported as infectious transmissible diseases and continue to be a global health concern. The aetiology of caries involved interaction of four major factors which include host susceptibility, microorganisms, diet and time. Caries had been reported to be one of the possible causes for systemic diseases. However few studies also revealed that systemic diseases such as asthma and epilepsy are also associated with higher caries experience (1). Asthmatics are in the high risk of getting caries due to frequent inhalation of dry powder inhalers that contain sugar and decrease of salivary flow rates (20).

B. *Streptococcus mutans*

Streptococcus mutans is a gram positive facultative anaerobes bacterium that commonly isolated from the oral cavity. However it was also reported to be the possible causative agents of cardiovascular disease such as atherosclerotic coronary heart disease [13].

S. mutans generally known to be the most cariogenic oral bacteria due to its ability to produce extracellular polysaccharides (EPS) from carbohydrate. The insoluble EPS will play a role in adhesion of bacteria to teeth which then contribute to the formation of dental biofilm (dental plaque). Facultative anaerobes bacteria that are well protected in the dental plaque and frequently exposed to dietary carbohydrates especially sucrose will produce acid, causing demineralization of the teeth and subsequently lead to caries formation [10].

Among the known virulence genes of *S. mutans* are *spaP* that produce SpaP that act as adhesin by mediating adherence of *S. mutans* to salivary agglutinin-coated hydroxyapatite in the absence of sucrose [4]. Next is *gtfB* that produce glucosyltransferases (GTFs) which synthesizes glucan polymers from sucrose and starch carbohydrate and *gbpB* that produce glucan binding protein (GBPs). Glucans synthesized by GTFs provide binding sites for bacterial adhesion on the tooth surface and also for aggregation with other bacteria. The specific role of GBPs remain unclear but it was found to be associated with *S. mutans* due to protection against dental caries in animal models when given as immunization [18].

Many research studies have been conducted to investigate the pharmacological activities of plant extract against *S. mutans*. Most of the previous results revealed the antimicrobial effect, inhibitory activity and ultra-structural changes to bacterial morphology after the treatment with the selected plants. Among the plants are *Piper betle* that showed anti-virulence property [14] and cranberry juice that had inhibitory effects due to reduction of the hydrophobicity [21]. Limited research was conducted to assess the expression of virulence genes in *S. mutans* after the treatment with any plant extract that showed anti-caries effect.

C. *Piper betle*.

Piper betle or locally known as sireh is belonging to the *Piperaceae* family. The plant is perennial creeper and easily found in the South East Asia region. The leaves are often used for traditional treatment in a form of external application or decoctions for oral consumption [8]. In maintaining good oral health, *Piper betle* extract was reported to reduce the production of methylmercaptan and hydrogen sulfide that cause oral malodor or halitosis. It also showed anticaries properties by reducing the production of acid by *S. mutans* up to 93.5%. This effect could be caused by fatty acids and hydroxyl fatty acids ester components that exist in these plants [14].

The following Table I provides a summary of the literature in the pharmacological activities of *Piper betle* from respective countries.

TABLE I. RESEARCH ON PHARMACOLOGICAL ACTIVITIES OF PIPER BETLE FROM DIFFERENT COUNTRIES.

Pharmacological activities	Country	Author
Antimicrobial	Malaysia	[14]
Anti-oxidants	India	[3,5]
Anti-cancer	Malaysia	[8]
Anti-inflammatory	India	[17]
Fungistatic	Malaysia	[11]
Anti-plaque	Malaysia	[7]

III. RESEARCH FRAMEWORK

The research framework of this study comprises of:

- A. Plant material
- B. Preparation of the aqueous extract
- C. Treatment of bacteria with plant aqueous extract
- D. Isolation of Total RNA
- E. Real Time Reverse Transcriptase-PCR (RT-PCR)
- F. Gel electrophoresis

A. Plant material

Fresh *Piper betle* leaves will be obtained from one source in Selangor.

B. Preparation of the aqueous extract of *Piper betle*

P. betle leaves will be cleaned and put to boil in deionized distilled water. Aqueous extract will be obtained by concentrating decoctions of the fresh leaves of the plant using speed vacuum concentrator. The dried extracts will be refrigerated at -80°C prior to use. The weighed pellet will be

dissolved and diluted to the required concentrations with deionized distilled water.

C. Treatment of bacteria with the plant aqueous extract:

Streptococcus mutans ATCC 25175 primary cultures will be grown in brain heart infusion (BHI). The concentration of cell will be standardized to about 10^8 cells/ml using methods previously described [6]. Bacterial cultures will then be divided into three groups

Group 1 : Preincubate with sucrose 2% for 1 hour. The bacteria culture then will be treated with 2,4 and 10 mg mL⁻¹ of crude aqueous *P. betle* leaves extract. The mixture will be left to incubate for 2 hours.

Group 2 : Preincubate without sucrose for 1 hour. The bacteria culture then will be treated with 2,4 and 10 mg mL⁻¹ of crude aqueous *P. betle* leaves extract. The mixture will be left to incubate for 2 hours.

Group 3 : Chlorhexidine gluconate (0.12%) will be used in the place of the extract. The mixture will be left to incubate for 2 hours.

After the 2 hours incubation, the treated bacterial cells will be precipitated and processed for RNA extraction.

D. Isolation of Total RNA

Total RNA from bacterial precipitation will be obtained using RNeasy Mini Kit (Qiagen, Stanford, CA). The sample will be processed following the recommendation by the supplier. To remove all DNA, the purified RNAs will be treated with DNase I (Ambion, Inc., TX)

E. Real Time Reverse Transcriptase-PCR (RT-PCR)

The expression levels of all the genes will be measured by real-time RT-PCR. The RT-PCR reaction mixture will contain 1x SYBR Green PCR Master Mix (Applied Biosystem), cDNA and appropriate forward and reverse PCR primers. Each assay will perform with at least two independent RNA samples in duplicate and the x-fold change of the transcription level will be calculated by using a software (ABI Prism 7000 SDS Software v1.1 with RQ Study 1.0, Applied Biosystems). RealTime PCR will be conducted using StepOnePlus™ Real-Time PCR. Table II presents the primers that will be selected.

F. Gel electrophoresis

For validation, PCR products will be determined using Agilent Bioanalyzer system. The PCR products will be visualized as a single compact band with expected size.

TABLE II. PRIMERS TO BE USED FOR REALTIME-PCR

Primer	DNA sequence (5'→3')	Gene
gtfB-Fw	AGCAATGCAGCCATCTACAAAT	<i>gtfB</i>
gtfB-Rv	ACGAACCTTGCCGTTATTGTCA	
gpbB-Fw	CGTGTTCGCTATTCGTGAAG	<i>gpbB</i>
gpbB-Rv	TGCTGCTTGATTTCTTGTTC	
spaP-Fw	TCCGCTTATACAGGTCAAGTTG	<i>spaP</i>
spaP-Rv	GAGAAGCTACTGATAGAAGGGC	

IV. EXPECTED RESULTS

The following subsection provide insights on the expected results from the experiments

A. Preparation of the aqueous extract

Aqueous extract will be used instead of ethanol extract due to common form of extract used to detect pharmacological effect of *Piper betle* in previous studies [5, 6, 14] .

B. Treatment with plant extract, chlorhexidine and molecular studies

The expression of *spaP*, *gtfB* and *gpbB* are expected to down regulate after treatments by both *Piper betle* extract and chlorhexidine. This is because microorganisms usually respond to environmental changes by altering the expression of genes and gene products critical to their continued survival [16]. Differential expression of selected genes after the treatment can be confirm using Real Time PCR . RT PCR had been used to obtain differential expression of genes of *Streptococcus pneumoniae* in response to a subinhibitory concentration of penicillin [16].

V. CONCLUSIONS

This paper will provides insights of the research framework to investigate the differential in gene expression of *S. mutans* in response to *Piper betel* extract. In this study, *Piper betle* is chosen due to non-toxic properties of the leaf extract [2,3]. It is also safe for children as it was reported to be prescribed as remedy for cough and cold which was given to children and infant during winter months [12]. This is important as dental caries prevalence and risk factors are the highest among children.

The findings of the expected results may indicate down regulation of genes that involved in attachment and plaque formation by *S. mutans*. The rational strategy to prevent caries that cause by dental plaque bacteria is to inhibit colonization of *S.mutans* Thus, *Piper betle* extract may provide cariostatic effects without disrupting the resident flora.

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