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**ORIGINAL ARTICLE**

## **Antibacterial Activities of *Glycyrrhiza glabra* Linn. (Licorice) Root Extract against *Porphyromonas gingivalis* and Its Inhibitory Effects on Cysteine Proteases and Biofilms**

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### **ABSTRACT**

Little is known about the antibacterial activity of licorice root extract. **Objective:** To investigate the antimicrobial and anti-proteolytic activities of root extract on *Porphyromonas gingivalis* in both planktonics and biofilm cells. **Methods:** *Glycyrrhiza glabra* (*G. glabra*) roots were extracted by 95% ethanol freeze dried and kept at -20°C prior experiments. *P.gingivalis* (ATCC 33277) were cultured and used for experiments. Determination of antibacterial activities of *G.glabra* extracts (lico rice) against *P.gingivalis* planktonic the MIC and MBC were evaluated by agar well diffusion, broth microdilution, and time-killing methods. The crystal violet assay was used to assess the biofilm growth inhibition and the disruption of established biofilm. The Arg - specific proteolytic activities were analyzed using the chromogenic substrates assays using N-benzoyl-DL-arginine-4-nitroanilide hydrochloride and N-(p-tosyl)-Gly-Pro-Lys 4-nitroanilide acetate salt to assess the enzymatic inhibition effects of the extracts compared with the controls. **Results:** The licorice root extract had antimicrobial activities on *P.gingivalis* with MIC and MBC of 62.5µg/ml and 25 µg/ml respectively. The assay showed that Licorice root extractonidazole. Licorice root extract also had effect on *P.gingivalis* biofilms. Quantification by crystal violet staining showed the reduction of biofilm mass in the presence of Licorice root extract. The Arg-and Kgp- proteases activities were also inhibited by the extract in dose dependent manner. **Conclusion:** The results suggested that licorice root extract may has potential therapeutics values as a candidate for periodontal disease.

**Key words:** antimicrobial, biofilm, licorice root extract, *Porphyromonas gingivalis*, proteases activities

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### **INTRODUCTION**

Chronic periodontitis is a common form of periodontal disease that can cause damage of supporting tissue of teeth and lastly tooth loss in adult. This disease has an impact on the major public health in many countries. The proportion of disease is around 30-40% of chronic diseases in adult population. The severe form of chronic periodontitis affecting on patients quality of life is around 5-10%.<sup>1</sup> Not only

being cause of tooth loss in adult but also increasing the risk of the cardiovascular disease. Although chronic periodontitis is a multi-factorial disease which many factors are involved, the causing factor is bacteria.<sup>2</sup> Some certain subgingival plaque microbiota such as *Porphyromonas gingivalis* (*P.gingivalis*), *Aggregatibacter actinomycetemcomitans*, and *Tannerella forsythia* are keys periodontal pathogens. One of the periodontal pathogen, *Porphyromonas gingivalis* is the most studied bacteria and is known

that contains several virulence factors that attenuate the periodontal disease.<sup>3</sup> *P.gingivalis* is a black pigmented Gram-negative facultative anaerobic cocci bacilli. The bacteria is reported as a major opportunistic pathogen in destructive periodontitis. *P.gingivalis* can invade may host cell types such as oral epithelial cells, human gingival fibroblast, osteoblast and a number of host immune cells i.e. monocyte, macrophage etc.<sup>4</sup> In addition *P.gingivalis* endothelial invasion is reported as well as its accumulating in atherosclerosis plaque may rise opportunities associated into many systemic diseases.<sup>5</sup> *P.gingivalis* is rarely found in healthy or mild gingivitis individuals but in chronic periodontitis the bacteria are frequent detected. *P.gingivalis* normally lives below the gingival margin where the environment is favorable. They are in biofilm form by tenacious adhered on the root surface. Biofilm is a bacterial community that possesses vast array of bacteria living together associated with a hard surface and surrounded by polymicrobial extracellular slime layer.<sup>4</sup> The biofilm mixed microbiota is hard to remove or get rid of due to the glue-like structural layer is hinder of antibiotic treatment or antimicrobial approach. Chronic periodontitis therefore is a disease that quite difficult to manage. *P.gingivalis* outer membrane consist of large amount of virulence determinants i.e. fimbriae, hemagglutinin, hydrolytic enzymes i.e. proteases, lipopolysaccharide (LPS), toxic metabolites i.e. butyric acid and toxins etc. These factors assist *P.gingivalis* to invade host cells and impair host response.<sup>5</sup> Major proteases locate on outer membrane of *P.gingivalis* are Arg- and Lys-specific cysteine proteinase or known in terms of RgpAB and Kgp.<sup>6</sup> With the rising resistance to antimicrobial agents including which had been used in dental filed, the development of natural products into alternative antimicrobial treatment is of interest.

Licorice root (*Glycyrrhiza glabra* Linn. *G. glabra*) is an ancient herb had been used as traditional medicine and sweetener in various parts of the world for over 4000 years.<sup>7</sup> Historically, the dried rhizomes and roots of licorice were utilized as an expectorant and carminative medicine by the Egyptian, Chinese, Greek, Indian, and Roman civilizations. Licorice was also recorded in medical textbooks in China as a life-enhancing medical agent and included as a part of Chinese traditional medical prescriptions which has been used in several diseases therapy. A water soluble complex isolated from licorice roots consists of triterpene saponins, flavonoids, polysaccharides, pectins, simple sugars, amino acids, mineral salts, and various other substances which accounts for 40-50 percent of total dry material weight.

Licorice plant is from the pea family (*Leguminosae*). The genus *Glycyrrhiza* (*Leguminosae*) is composed of about 30 species including *G. glabra*, *G. uralensis*, *G. inflata*, *G. aspera*, *G. korshinskyi* and *G. eurycarpa*, *G. glabra* including three varieties: Persian and Turkish licorices which are ascribed as *G. glabra* var. *violacea*,

Russian licorice is *G. glabra* var. *gladulifera*, and Spanish and Italian licorices are *G. glabra* var. *typical*. It is also known in names of liquorice (licorice), kanzoh, gancao, sweet root and yasti-madhu. Of 30 species the *Glycyrrhiza* genus consists of about 15 species have been extensively studied so far.<sup>8</sup> Several previous experimental and clinical trial studies revealed varieties of pharmacological activities of licorice root extracts including antimicrobial antifungal, antiviral, anticancer anti-inflammatory antioxidative anticoagulant, immunomodulatory hepatoprotective cardioprotective effects anti-allergic, and anti-radical activity and a protective effect against the peroxidation of liposomal membranes.<sup>9</sup> Amongst those activities mentioned the antimicrobial and antiviral had been mostly studied and reported.<sup>9</sup>

Licorice contains more than 20 triterpenoids and nearly 300 flavonoids. Among them, glycyrrhizin (GL), 18 $\beta$ -glycyrrhetic acid (GA), liquiritigenin (LTG), licochalcone A (LCA), licochalcone E (LCE) and glabridin (GLD) are the main active components which possess antiviral and antimicrobial activities.<sup>5</sup> The antimicrobial activities of licorice and its bioactive compounds had been studied extensively. Previous study revealed that flavonoids isolated were the effective constituents of licorice against methicillin-resistant *Staphylococcus aureus* (MRSA).<sup>10</sup> Glabridin, glabrene and licochalcone A exhibited antimicrobial activity against *Helicobacter pylori* in vitro.<sup>10</sup> The extracts of *G. glabra* were found to have effective antibacterial activity against all the five bacteria, *E. coli*, *B. subtilis*, *E. aerogenes*, *K. pneumoniae* and *S. aureus*.<sup>11</sup> An in vitro study showed the susceptibility of *Salmonella typhi*, *S. paratyphi B*, *Shigella sonnei*, *S. flexneri* and enterotoxigenic *E. coli* to *G. glabra* extract with concentrations lower than 7.5% but at the higher concentration all strains were susceptible.<sup>12</sup> Glycyrrhizol A and 6, 8-diisoprenyl-5, 7, 4'-trihydroxyisoflavone from the root of *G. uralensis* exhibited potent antibacterial activity against *S.mutans* with minimum inhibitory concentrations of 1 and 2  $\mu$ g/mL, respectively.<sup>13</sup>

Recent studies showed promising values of MIC and MBC of licorice extracts on oral bacteria especially *S. mutans*, *A. viscosus*, and *E.faecalis*.<sup>14</sup> Several studies revealed that Licorice has antimicrobial activities on certain pathogens responded to dental caries, periodontitis and oral candidiasis. These studies investigated and revealed the effect of licorice on bacteria and fungi including studies of their roles in immunomodulatory response of host cells.<sup>15,16</sup> A study showed effects of licorice bioactive compounds, 18 $\alpha$ -glycyrrhetic acid (18 $\alpha$ -GA) on alteration of host response to *P. gingivalis* LPS induction after treatment with licorice extracts.<sup>17</sup> These alterations included inhibitory effects on leukocyte adhesiveness, vascular permeability and inflammatory cytokines i.e. IL-1 $\beta$ , IL-6, IL-8, TNF- $\alpha$ , MMP-7, -8, and -9.<sup>17</sup> However, the

effect of licorice on biofilm formation or disruption of preformed *P. gingivalis* biofilm cell has not been clearly studied. In addition, the major protease activity of *P. gingivalis* is investigated to determine whether licorice has ability to inhibit the enzymes or not.

## METHODS

### Licorice preparation

Licorice root extracted (extracted by 95% ethanol) was kindly provided from Thai-Chinese Fragrant Industrial Company (Bangkok, Thailand). Briefly, whole roots of the *G. glabra* plant were collected, washed by tap water and air- at room temperature before grinding to powder with a mechanical grinder. The powder was extracted by maceration in 95% ethanol. Approximately 50g of the powder were soaked in 200ml of for 72 h at room temperature. The extract was filtered through Whatman No. 1 filter paper and then through a 0.45 m membrane filter (Sigma) respectively. The filtrate was evaporated to dryness at room temperature in the evaporator (30g) used. The dried extract was sterilized by overnight UV-irradiation and sterility checked by plating on nutrient agar. The crude extracts were stored at room temperature until used for testing. Making the stock solutions of extracts by dissolving the dried crude extract in phosphate buffer saline pH 7.0 prepared with concentration of 1 mg/ml and kept at 4°C protected from light before used.

### Bacterial and growth conditions

*P. gingivalis* was used in this study was strain ATCC-33277. The bacteria were grown at 37°C in Brain heart infusion broth (BHI) (Oxoid) supplemented with hemin (10µg/mL) and vitamin K (10µg/mL) and incubated under anaerobic condition (N<sub>2</sub>/H<sub>2</sub>/CO<sub>2</sub>: 80/10/10).

### MIC and MBC determination for planktonic cells

Overnight cultures were diluted in fresh BHI media to obtain an optical density at 660nm (OD<sub>660</sub>) of 0.2 which approximately to 10<sup>6</sup> CFU/mL. Equal volume (100µL) of bacterial solution of and a series of 2-fold serial dilutions of the licorice root extracts were mixed into the wells of sterilized-96-microtitre-plates (Nunc). Control wells with no bacteria or compounds were also prepared. Metronidazole was used as a positive control. After an incubation of 48h at 37°C, bacterial growth was recorded visually. The MIC was determined, as the lowest concentration without visible bacterial grown. The MBC was defined as the lowest concentration of each tested substrate from each well showing no visible growth after spread on BHI agar supplemented with hemin and vitamin K showing no colony formation occurred. The MIC and MBC were determined in three independent experiments.

### Time kill curve

Adjusted bacterial solution (10<sup>6</sup> CFU/ml) were

incubated in BHI broth containing supplement as described previously with licorice at a dilution equivalent to the following licorice concentration of 0.5xMBC, 1xMBC, 2xMBC and 4xMBC. At time intervals (0, 2, 4, 8, 12 and 24 hours) of incubation time, aliquots were taken, serially diluted, plated and incubated for 48 h at 37°C under anaerobic condition before colonies count. The control was also assessed in culture medium. Experiments were carried out in triplicate.

### Biofilm inhibitory assay

Briefly, bacterial solution was adjusted to 0.8x10<sup>7</sup> CFU/ml. Equal volume (180 µL) of bacterial solution and volume (20 µL) of dilutions of the licorice root extracts were mixed into the wells of sterilized-96-microtitre-plates to obtain a given final concentration of 0.5xMBC, 1xMBC, 2xMBC and 4xMBC and 10<sup>6</sup> CFU/ml of inoculums in each well. The plates were incubated under anaerobic condition as described previously. At time intervals (48 hours) of incubation time, the plate was shaken at 100 rpm at 37°C and all media was discarded. Each well was washed twice with deionized water and air dried. Crystal violet was used to stain the biofilm formed cells at the bottom of each well at room temperature for 15 mins. Unbound crystal violet was then removed by washing twice with deionized water. To re-solubilize the dye bounded to biofilms, 100 µl of ethanol containing 20% (vol/vol) acetone was added to each well and the plates were submitted to agitation for 15 min. The crystal violet solutions obtained were transferred to a new sterile flat bottom 96-well plate and the absorbance at 600 nm was measured using a microtiter plate UV spectrophotometer (Biorad®, USA). Experiments were carried out in triplicate.

### Biofilm preformed eradication assay

To investigate the ability of licorice root extract to eradicate the formed biofilms, a modified microdilution assay was employed. Microbial biofilms were performed onto the flat bottom of 96-well in microtitre plate. Briefly, the adjusted bacterial solution of 2.5x10<sup>6</sup> CFU/ml was prepared. A volume of 100 µL of bacterial solution was inoculated in each well of 96-well microtitre plate and grow for 48 h at 37°C under anaerobic condition to allow biofilm formation. After incubation, the media in each well of the plate was discarded and each well was then washed three times with sterile PBS to remove non adhered or loosely adhered cells off. Adhered preformed biofilm cells at the bottom were therefore prepared to test with the licorice root extract and control. Licorice root extracted was prepared to attain concentration of 0.5xMBC, 1xMBC, 2xMBC and 4xMBC. All tested antimicrobial solutions were added into each well of 96-well microtitre plate with equal volume. The positive and negative controls were performed as well in other plates. After 48 hr incubation time time, the plate was taken out. Each well media was removed

**Table 1** Antibacterial activity (MIC and MBC) of licorice root extract and metronidazole against *P.gingivalis* ATCC33277 planktonic cells

Agent	MIC (µg/ml)	MBC (µg/ml)
Licorice root extract	62.5	125
Metronidazole	0.125	0.25

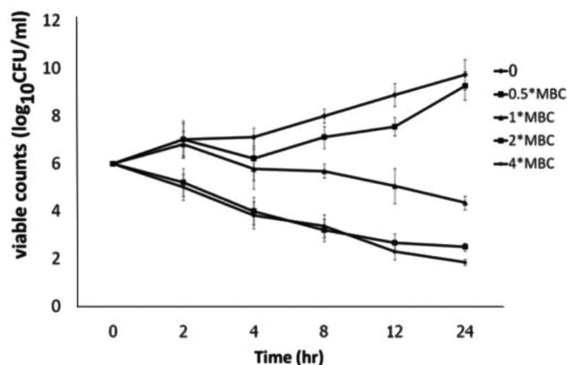
and wash three times with sterile PBS. Aliquots were from taken well showing no visible growth, serially diluted, plated and incubated for 48 h at 37°C under anaerobic condition before colonies count. The positive and negative controls were performed as well in other plates. Experiments were performed in triplicate.

**Arg and Lys-proteases inhibition activities of *P.gingivalis* whole cells**

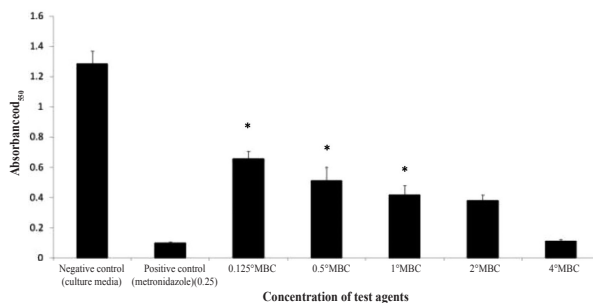
The protease enzymes activities were assess by the colorimetric assay described previously by Pathirana RD et al., 2006. The chhromogenic substrates N-α-benzoyl-Arg-p-nitroanilide (L-BApNA) and N-(p-tosyl—Gly-Pro-Lys 4-nitroanilide acetate salt (GPK-NA) (Sigma) were used for measuring the Arg and Lys-proteases activities respectively. One liters (1000 mL) of *P.gingivalis* overnight grown in BHI broth supplemented with hemin (10µg/mL.) and vitamin K (10µg/mL.) were harvested by centrifugation (at 8000xg, 4°C) for 20 minutes. Cells pellets were then washed twice with 20 ml, and resuspension with 2 ml of ice-cold mixture buffer (comprising 50mM Tris-HCl, 150mM NaCl, 5mM Cacl<sub>2</sub> and 2.8mM cysteine) to obtained cell density approximately of 4.5x10<sup>7</sup>CFU/ml. An aliquot of *P.gingivalis* suspension (2.5µl) was prior treatment with licorice root extract preparation with concentration ranging from 0.5, 1, 2 and 4 folds x MBC for 0 and 90 minutes respectively in mixture buffer in 96-well plates. Then the chromogenic substrate preparations (97.5µl) containing 2mM BApNA or GPK-NA in 30% (vol/vol) isopropanol, 400mM Tris-HCl (pH8), 100mM NaCl and 2mM cysteine was added to give a total volume of 100µl. Sample were removed at time intervals The absorbance at 405 nm was measured, another set of 96-well plates contained only buffer were measured as a negative controls and read blank for subtraction. N-alpha-tosyl-L-lysiny-chloromethylketone (TLCK) was used as the positive control. Experiments were carried out in triplicates.

**Statistical analysis**

The results were obtained from three separated experiments and presented as mean + SD. The data were analysed using the one-way ANOVA with post-hoc test and considered statistically significance if p< 0.05 by SPSS software ver.17 (SPSS INC., Chicago, IL, USA).



**Figure 1.** Licorice extracts has antimicrobial effect on *P.gingivalis* viability. The numbers of viable bacterial colonies in the tested-licorice treated group (B) were significantly lower than those in control group (A).



**Figure 2** Effect of licorice root extract on *P.gingivalis* biofilm formation. Inhibition of *P.gingivalis* biofilm formation was determined in the presence of different licorice concentrations exposed for 48 hours. The result is presented as stained crystal violet at OD 570nm. (\* indicates statistical difference between tested groups and controls, p<0.05). Each data point represents the mean and standard deviation of three replicates.

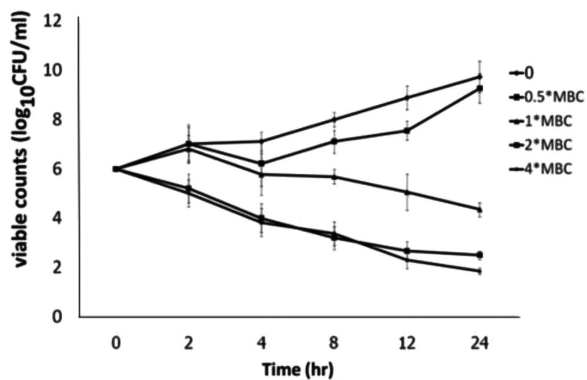
**RESULTS**

**Determination of MIC and MBC of licorice on *P.gingivalis* planktonic cells**

The licorice root extract showed marked antibacterial activities against *P.gingivalis*. The declining of colonies forming units were obviously observed in licorice treated group compared to control ( Figure 1).The MIC and MBC values of 6.25-12.5µg/ml against *P.gingivalis* were obtained. MIC and MBC of licorice root extract on *P.gingivalis* planktonic were 62.5µg/ml and 125µg/ml respectively. While the MIC and MBC of metronidazole on *P.gingivalis* were 0.125mg/ml and 0.25µg/ml respectively (Table 1).

**Time killed assay showed that licorice root extract could inhibit growth of *P.gingivalis***

Time killed curves were performed for *P.gingivalis* in the presence of licorice root extract with the concentration as 0.5 1 2 4 folds its MBC and different exposure interval time were also determined. The



**Figure 3** Time-kill curves for *P.gingivalis* (planktonic cells) in the presence of licorice root extract at different concentration as 0.5 1 2 and 4 folds of MBC. Data are expressed as CFU/ml recovered at each time point.

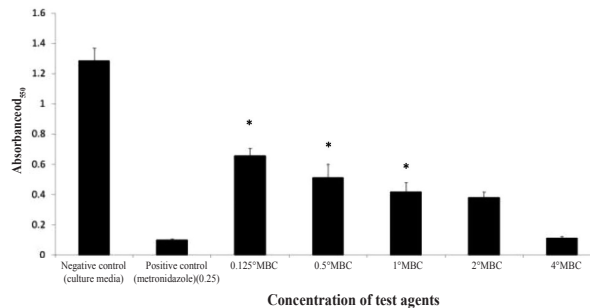
results showed the inhibitory effect of the extract on *P.gingivalis* growth. This is a dose dependent manner with higher concentration the survival of *P.gingivalis* was declined. At the lower concentratin the licorice root extract reduced number of the *P.gingivalis* only 2 log dropped whereas at the higher concentration the ability to kill *P.gingivalis* was obviously shown ( $p < 0.05$ ). The inhibitory effect on *P.gingivalis* growth was seen immediately at the concentration as 2 and 4 folds of MBC. Control cell suspension showed no drop in viability over the same period compared to test groups (Figure 2).

**Biofilm assay showed that licorice root extract could inhibit on biofilm formation and eradicate established biofilm**

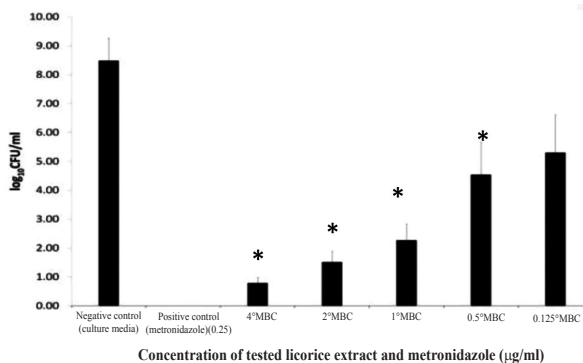
Biofilm inhibition assay was used to determine the ability of licorice root extract for inhibition and/or eradication of *P.gingivalis* biofilm. Measuring absorbance of *P.gingivalis* biofilm stained with crystal violet exhibited that licorice root extract could inhibit biofilm formation compared to that of the negative control (Figure 3). Although, the efficiency of inhibition was not obvious as seen in the positive control (metronidazole) ( $p < 0.05$ ), the pattern of inhibition appeared to be concentration-dependent manner. It is noted that the level of Minimal Bacteriocidal eradication concentrations (MBECs) used to eradicate biofilm cells is nearly 5-10 folds of that found in planktonic condition (Figure 4). Similarly, the degree of eradication of *P.gingivalis* biofilm was a dose-dependent manner for licorice root extract; however, it was not as good as those appeared for metronidazole ( $p < 0.05$ ).

**Effect of licorice root extract on *P.gingivalis* proteases**

The licorice root extract could inhibit both Rgp- and Kgp-proteinase activities of *P.gingivalis* whole cells at the subinhibitory concentration.



**Figure 4** Effect of licorice root extract on *P.gingivalis* biofilm formation. Inhibition of *P.gingivalis* biofilm formation was determined in the presence of different licorice concentrations exposed for 48 hours. The result is presented as stained crystal violet at OD 570 nm. (\* indicates statistical difference between tested groups and controls,  $p < 0.05$ ). Each data point represents the mean and standard deviation of three replicates.



**Figure 5** Eradication of established *P.gingivalis* biofilm by licorice root extract. *P.gingivalis* 48 hour-preformed biofilm was tested with different concentration of licorice compared to positive and negative controls The result is presented as Viable survival counts of established *P.gingivalis* biofilm after exposure 48 hours (\* indicates statistical difference between tested groups and controls,  $p < 0.05$ ). Each data point represents the mean and standard deviation of three replicates.

Licorice root extract reduced significantly Rgp- and Kgp-proteinase activities compared to negative control ( $p < 0.05$ ) and the inhibitory effect on these proteinases activities was dose- dependent (Figure 5). However at the highest tested concentration (8\* MBC) the efficiency still less than N-alpha-tosyl-L-lysiny-chloromethylketone (TLCK) which was positive control ( $p < 0.05$ ).

**DISCUSSION**

Chronic periodontitis is a major public health problem in many countries. *P.gingivalis* and its virulence factors such as Rgp and Kgp-proteinases are associated with pathogenesis. To date, little is known about therapeutics agents that target could specifically to *P.gingivalis*

proteinases. Therefore, the alternative agents derived from natural products may have chance to develop such an agent that provide both antimicrobial activities and proteinase inhibitory action on *P.gingivalis*.<sup>17</sup> Our results showed that licorice extract has antimicrobial activities against on *P.gingivalis* both planktonic and biofilm forms. The results were in agreement with previous studies showing antimicrobial activities of other herbal extracts against *P.gingivalis* however the values of MIC and MBC were different. The antibacterial of Pomegranate extract inhibits the biofilm formation and inhibits the growth of periodontal pathogens at lower concentrations. Pomegranate crude extract showed MBC of plantokic at 0.1 mg/ml which is higher than our results.<sup>18</sup> *Dodonaea viscosa* var. *angus tifolia* with MBC of 0.04mg/ml and 0.09mg/ml (extracted by ethanol and methanol) whereas used acetone is 0.04mg/ml all of agents is which is better than licorice root extract.<sup>19</sup> These extracts showed the MICs and MBCs lower than our results. *Coptidis rhizoma* (*Ranunculacea*) aqueous extract also has antimicrobial activities against *P.gingivalis* with (MIC ranging between 31–250µg/ml).<sup>20</sup> *Hamamelis virginiana* (*Hamamelidaceae*) leaves extract showed antimicrobial activity with MIC values generally <512µg/ml against *P.gingivalis*.<sup>21</sup> Bacterial adhesion is essential for biofilm formation. Defective adhesion leads to biofilm failure. The adhesion possibly does not involve the bacterial growth. Impairment of bacterial adhesion and biofilm formation by a pathway that does not influence bacterial growth is a characteristic for antivirulence therapies, one of the recent promising alternatives to combat pathogenic microorganisms, particularly *P.gingivalis*.<sup>22</sup> Licorice root extract inhibit growth of planktonic cells and also inhibit the biofilm formation of *P.gingivalis*. The eradication or disruption established biofilm (24 hours) was achieved as well with the MBEC 62.5mg/ml. Important, the proteinase activity of the organisms by Rgp-proteinase was also reduced by approximately 50% as we known the enzyme is an important virulence factor of *P.gingivalis*. With the actions of the proteinase *P.gingivalis* can invade host cells and impaired the host immune responses.<sup>5</sup> In addition, the enzyme is essential for *P.gingivalis* because it protein and iron provider for *P.gingivalis* for growth and survival including mechanisms of invading host cells.<sup>22</sup> Other natural substrates such as polyphenols and catechins from cranberry and green teas had been studied and also suggested for their antimicrobial and antiproteolytic properties against *P.gingivalis*.<sup>23</sup> Recent study reported the inhibitory effect of the prenylated flavonoids on against *P.gingivalis* gingipain activities and also their biofilm formation and growth.<sup>22</sup> A study on biofilm inhibit of polyphenol such as Theaflavins showed the inhibition of *P.gingivalis* initial biofilm formation at MBIC<sub>50</sub> = 31.25µg/ml; MBIC<sub>90</sub> = 62.5µg/ml respectively however it could not totally reduced the in vitro pre-established *P.gingivalis* biofilm.<sup>24</sup> The proteinase activities such Rgp-proteinase was decreased about 3

folds by licorice extract. These results were in consistent with other studies demonstrating reduction its activities by many natural derived active compounds. Bapna hydrolysis from the studies effect of S-PRG showed inhibition of the enzyme activities in the presence of this substrate. Mechanism was suggested by the metal salts and ion control that regulate bacterial enzyme activities.<sup>23</sup> Rgp-proteinase is required metal ions to achieve maximal enzyme activities. Over time 30 minutes of incubation time the S-PRG decreased enzyme activity of Rgp-proteinase at each time point approximately 2/3 times of controls.<sup>24</sup> In contrast the experiment of effect of lactoferrin on proteinase of *P.gingivalis* showed reduced enzyme activity over 90 minutes at different concentration which is dose-dependent manner and the experiment explained the results by slope of inactivation rate which differently among lactoferrin different concentrations. Our results showed the inhibition of proteinase activities the same as study of lactoferrin did but however calculate in term of inactivation rate. The licorice extract used in the present study is lower than lactoferin.<sup>25</sup> *Dodonaea viscosa* var. *angus tifolia* also reduced Rgp and Kgp-proteinase activities and its action was also dose dependent manner but the affinity with Kgp-proteinase is higher that Rgp-proteinase did (average 30% and 50% reduction respectively) at the 0.02mg/ml of extraction concentration which is higher than our results. In this study, there was no intervention time to compare. The experiment was done over 90 minutes of exposure. Cranberry polyphenol fraction from two studies showed abilities that response to arg- and lys-gingipain reduced activities (0.1mg/ml).<sup>25</sup> Results from this study showed that licorice extract has a potential inhibitory effect on *P.gingivalis*. *P.gingivalis* has been considered as a certain periodontal pathogen in destructive periodontitis. *P.gingivalis* contains protease enzymes which are arg- and lys-specific cysteines. Both proteinases are implicated that play an important role in initiation and progression of chronic periodontitis.<sup>26</sup> The results showed the reduced absorbance after *P.gingivalis* whole cells were incubated with licorice extract. This result referred to the ability of the cell membrane proteins on BAPNA and H-Gly-Pro-pNA was impaired after treat with the licorice root extract. These findings may be explained by the reasons that proteins and peptides were used as substrates in the colorimetric assay had not been cleared by the enzymes which were inhibited by the licorice root extract. Here we have shown that both enzymes were completely inhibited by the licorice extract. The licorice extract used in this study showed ability to assist on *P.gingivalis* planktonic and biofilm with sub-MIC level concentration. Due to the widespread use of antibiotics leads to increasing emergence of bacteria and spread of antibiotics resistance. Therefore searching for alternative agents that derived or extracted from nature is of interest. Licorice shows effectiveness on *P.gingivalis* particularly in its major proteases enzymes. *P.gingivalis* biofilm formation has been

decreased when tested with licorice extracted. The ability to inhibit biofilm formation is a time- and dose-dependent manner. Even though biofilm eradication was not completely accomplished at the MBC obtained from planktonic cells, increasing concentration as 2, 4 or 8 folds showed markedly declined established biofilm cells. *P.gingivalis* biofilm inhibitory activity may be resulted from the effect on arg- and lys-proteinase activities. This possibly explains by the mechanisms employed by the proteinase inhibitor, TLCK which also inhibited *P.gingivalis* biofilm formation. Because the exposure of cryptic receptors to enhance binding of *P.gingivalis* is depended on the proteinase activity, the biofilm formation decreased therefore partly resulted from this reason. This finding was in agreement with the study of garlic extract.<sup>20</sup> The resistance of antibiotics is increasing nowadays is a critical problem as especially in hospital management. The hospital infection or community infection is a serious problem due to the increasing antibiotic resistance. Normally, microorganisms live together in term of biofilm causes serious infectious problem and resists to antibiotics much higher than planktonic.<sup>26</sup> Microbiological tests with the planktonic were widely to test the antibacterial activity of many compounds or crude extract from plants. However the susceptibility may be different when test with biofilm.<sup>27</sup> This fact may explain the treatment failures and enhancing resistance of clinical strains to antimicrobial agents. The licorice extracts maybe involved in the detachment of planktonic cells from the biofilm. Probably licorice extract influenced the membrane integrity in all organisms and helped to eradicate most biofilm cells. The mechanism of action of licorice extract is thought to involve membrane disruption by the lipophilic compounds and thereby inhibit respiration and ion transport processes in the bacterial cells. A study of Terpenes showed that the compound is involved in the fatty acid composition of the cell membrane, hydrophobicity which leads to the eradication of the biofilm.<sup>27</sup> Bacteria living as biofilm are often more difficult to eradicate compared to the planktonic mode of growth.<sup>26</sup> Planktonic cells forms biofilms by adhering to each other strongly via fimbriae. Besides fimbriae, bacteria also use quorum sensing to coordinate the formation of biofilms. Quorum sensing (QS) is a cell to-cell signaling mechanism which often linked to the establishment of complex communities of bacteria. The QS presents between the bacterial inhabitants has led to development of the biofilms. *P. gingivalis* uses quorum sensing to coordinate the formation of biofilms.<sup>23</sup> Recently the *P.gingivalis* is considered as the key stone in chronic periodontitis.<sup>28</sup> *P.gingivalis* gingipains were suggested to play role in multispecies biofilm model by mean regulate the qualitatively and quantitatively composition of polymicrobial biofilms. The recent experiment on natural polyphenol substance such as theaflavins exhibited the antimicrobial effects against both planktonic culture and biofilm of *P. gingivalis*.<sup>29</sup> Theaflavins inhibited the proteinase activities of

gingipains in a dose-dependent manner after 4 hours exposure to 50 and 100 µg/ml of theaflavin.<sup>29</sup>

## CONCLUSION

The licorice root extracts from this present study showed the potential to be a natural potent *P.gingivalis* gingipains inhibitor. Licorice extract has the abilities to inhibit *P.gingivalis* biofilm formation and eradicate the established biofilm. Moreover it exhibited the inhibitory effect especially on *P.gingivalis* cell-surfaced proteinases activities which is necessary for the nutrient acquisition and growth and adhesion on host cells. These accumulative results suggest that the licorice extract has multifaceted functions in attenuation of *P.gingivalis* virulence which is promising characteristics in developing drugs or agents for the future periodontal therapy. Although *P.gingivalis* is a keystone of chronic periodontitis, other species in the subgingival microbiota in the red complex such as *T.denticola* or *T.forsythesia* still needed to study their effect on these bacterial protease activities including the immunomodulatory roles of the licorice. Taken together, based on our results and licorice is derived from natural which is known biocompatible, licorice extracts might be useful in prevention and treatment of periodontal diseases. However, the further research works on the development and clinical trials are required. Therefore, it may help to discover and develop distinctive natural derived entity for new therapeutic agents in the future.

## CONFLICT OF INTEREST

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