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Evaluation of *Terminalia chebula* Retz. Extract Against Caries-associated Bacteria as an Alternative Compound for Oral Care Products

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Abstract

The objective of this study was to investigate the efficiency of *Terminalia chebula* Retz. fruit extract for further use as an active ingredient in oral care products. The quantification of major constituent, the antioxidant activity and antibacterial activity of an extract against *Streptococcus mutans* in both planktonic and biofilm form were measured. The results showed that ellagic acid was the major constituent of an extract and the amount of ellagic acid contained in the extract was 36.671 mg/g. The extract exhibit free radical scavenging activity with IC_{50} value of 0.44±0.01 mg/mL by DPPH assay. For antibacterial activity against *S. mutans, T. chebula* Retz. extract possess high inhibitory effect on *S. mutans* in both planktonic form and biofilm form. The MIC_{50} and MBC value of an extract against planktonic form of *S. mutans* were 0.47±0.28 and 6.25 mg/mL, respectively. For biofilm form of *S. mutans,* the extract has a high capability to preventing biofilm formation and eradicating the existing biofilm. The $MBIC_{50}$ and $MBEC_{50}$ of an extract against biofilm form of *S. mutants* were 4.47±0.32 and 9.64±0.39 mg/mL, respectively.

Introduction

Dental caries is one of the most prevalent infectious diseases which results in destruction of dental hard tissue of both adults and children. It is the most common cause of tooth loss and pain in the oral cavity. Dental caries is a multifactorial disease caused by the interaction of a dental plaque and host factor including teeth, saliva and diet containing sugar (Mathur & Dhillion, 2018; Sim et al., 2016). Among the microorganisms of the oral cavity, *Streptococcus mutans* is considered to be an important pathogen of dental caries. Several studies demonstrated that *S. mutans* is a major cariogenic microorganism especially in regard to

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disease onset. This bacteria is capable of binding to tooth surface by producing an extracellular polysaccharide from sucrose using glucosyltransferase enzyme which promote local accumulation of microbes on the teeth and leads to biofilm formation (Koo et al., 2003). Furthermore, *S. mutans* species are acid resistance and are able to produce organic acid including lactic acid, formic acid and acetic acid during metabolism of fermentable carbohydrate. The acid produced by this bacteria can cause demineralization of tooth enamel and if this process progresses long enough, the end result is a cavity (Colak et al. 2013; Lee, 2013).

Practicing a good oral hygiene routine to remove dental plaque such as brushing, flossing and rinsing is the best way to prevent dental caries and periodontal disease. Chlorhexidine is the most effective anti-plaque agent that has been widely used in antiseptic oral rinse (Brookes et al., 2020). However, several studies demonstrated that chlorhexidine caused a number of adverse effects including taste changes, tooth staining, sore mouth and/or throat, tongue irritation and wheezing/ shortness of breath (Brookes et al., 2020; Sakaue et al., 2018; Van Strydonck et al., 2012). Therefore, natural product such as plant extracts have been attracting much attention as a promising alternative substances for dental caring.

Terminalia chebula Retz. belonging to the family Combretaceae. *T. chebula* Retz. fruit has high contents of phenolic compounds including phenolic, tannin and flavonoid (Nigam et al., 2020). The chief constituents of phenolic are gallic acid, ellagic acid and hydroxy cinnamic acid (Juang et al., 2004; Lee et al., 2017b). The tannins of *T. chebula* Retz. are hydrolysable type and the main compounds among tannin are terflavin A, terchebulin, punicalagin, chebulagic acid, chebulinic acid and corilagin (Juang et al., 2004; Lee et al., 2017b; Lin et al., 1990). Flavonoid found in *T. chebula* Retz. fruit are rutin, quercetin, luteolin, isoquercitin and methylated derivative of quercetin (Kumar et al., 2012; Prakash et al., 2012).

T. chebula Retz. fruit has been used as a medicine in many Asian countries from ancient times. The use of *T. chebula* Retz. fruit for treatment includes chronic diarrhea, gastroenteritis, constipation, asthma, dyspepsia, ulcer, cough, skin disease and antiparasitic (Nigam et al., 2020). Several studies reported that *T. chebula* Retz. fruit has various pharmacological activities such as antibacteria (Bag et al., 2009; Bag et al., 2012; Kannan et al., 2009), antifungal (Barazani et al., 2003), antiinflammation (Nair, et al., 2010), antioxidant (Lee et al., 2007a), antidiabetic (Sabu & Kuttan, 2002), anticaries (Rekha et al., 2014), antiproliferative (Saleem et al., 2002) and hepatoprotective activity (Tasduq et al., 2006). A recent study reported that ethanolic *T. chebula* Retz. extract has ability to inhibit the growth of *S. mutans* and can be considered as a promising antibacterial and anti-oral inflammatory agent capable of preventing the development of gingivitis and periodontitis. However, information of the antibiofilm activity of *T. chebula* Retz. extract against *S. mutans* is still limited.

In the present study, we examined the chemical composition and biological activities of ethanolic *T. chebula* Retz. extract including free radical scavenging activity, antibacterial activity against both planktonic form and biofilm form of *S. mutans*.

Materials and methods

1. Preparation of *T. chebula* Retz. extract

The mature *T. chebula* Retz. fruit were collected in November 2018 from Loei Province, Thailand. The plant species was confirmed by the National Park, Wildlife Conservation Department, Ministry of Natural Resources and Environment, Thailand. The fruits were washed thoroughly in tap water and the seeds were separated. The pericarp of the fruit were dried in a hot airoven at 50ºC and were grounded to fine powder using a grinder. Extract of dried *T. chebula* Retz. fruit was prepared by maceration in 70% of ethanol. Briefly, 25 g of *T. chebula* Retz. fruit powder were macerated in 150 ml of 70% ethanol with occasional shaking at room temperature and kept for 24 hours and then was filtered through a Whatman No.1 filter paper. The process was repeated twice using the remaining residues. The pooled filtrate was concentrated using a vacuum rotary evaporator. The extract was then freeze dry and stored in a tight container protected from light in a refrigerator at 4ºC until further use. The yield of extract was calculated using the following equation:

Yield (%) = (Weight of *T. chebula* Retz. fruit extract x 100) / Weight of *T. chebula* Retz. fruit powder)

2. HPLC analysis

According to the previous report, phenolic compound was identified from the fruit of ethanolic *T. chebula* Retz. extract (Prompamorn et al., 2022). In the present study, the chemical constituent presented in the extract

was determined using a high performance liquid chromatography (HPLC). The separation was done in a Verticep^{TM} GES C8 column (4.6 x 250 mm, 5µ particle size), mobile phases consisted of (A) 0.05% trifluoroacetic acid in water, and (B) 0.05% trifluoroacetic acid in acetonitrile using a gradient elution and the flow rate was set at 1.0 mL/min with the controlled temperature at 40º C. Photo diode array 190-400 nm detector was set at the wavelength of 259 nm and injection volume was 10 µL for sample and reference standard (gallic acid and ellagic acid).

3. free radical scavenging activity of *T. chebula* Retz. extract

The free radical scavenging activity of an extract was performed by DPPH assay as described by Yarnpakdee et al. (2015) with slight modification. Briefly, 0.1 mM DPPH (100 µL) in 95 % ethanol was mixed with the sample solution (100 µL) at various concentrations. The mixture was incubated at room temperature for 30 minutes in the dark and the absorbance was measured at 517 nm using the microplate reader. L-ascorbic acid was used as a positive control. The percent DPPH• scavenging activity was calculated using the equation:

$$\text{Inhibition (\%)} = [(\text{Acont} - \text{Atest})/\text{Acont}] \times 100$$

Where Acont and Atest are the absorbance of control reaction and test samples, respectively. Free radical scavenging activity was expressed as IC_{50}, defined as the concentration of the sample required to inhibit 50% of the initial DPPH concentration. The test was carried out in triplicate.

4. Bacterial strain and growth conditions

S. mutans ATCC 25175T was grown on brain-heart infusion agar (BHA) supplemented with 2 % glucose at 37°C. For the preparation of planktonic cultures, colonies were picked and resuspended in brainheart infusion broth (BHB) supplemented with 2 % glucose and the culture was then incubated at 37°C in a shaker incubator for 3–6 hr. until the culture attained a turbidity of 0.5 McFarland Unit.

5. Antibacterial activity of *T. chebula* Retz. extract

The antibacterial activity of an extract was performed by agar well diffusion assay according to Ahmed & Beg (2001) with slightly modification. In brief, the broth culture of a mid-log phase of *S. mutans* (approximately 10^7 cfu/mL) was prepared using a brain heart infusion broth (BHI) supplemented with 2 % glucose at 37°C with shaking at 200 rpm. Then, the bacteria suspension was inoculated on BHI agar plate using sterile cotton swab. Subsequently, a hole with a diameter of 6 mm was punched aseptically with a sterile cork borer and a 40 µL of a different concentration of *T. chebula* Retz. extract solution was introduced into the well. The agar plate was then incubated at 37°C for 16-18 hr. 0.5% chlorhexidine was used as a positive control while BHI broth was used as a negative control. Antimicrobial activity was evaluated by measuring inhibition zone diameters. The experiment was performed in triplicate. One way ANOVA was used to calculated the significance of the difference between the experiment and the control sample. Difference were considered significant at $P < 0.05$.

6. Minimal inhibitory and minimal bactericidal concentration of *T. chebula* Retz. extract

Minimal inhibitory concentration (MIC) of *T. chebula* Retz. extract against *S. mutans* was tested using the Clinical and Laboratory Standards Institute (CLSI) recommended broth microdilution assay. The test was performed in a flat-bottom 96-well microtiter plates. *T. chebula* Retz. extract solution was two-fold serially diluted with BHI ranging from 0.097-25 mg/mL and then 100 µL of each concentration of extract solution were given in each well containing 90 µL of BHI broth. Subsequently, 10 µL of working inoculum suspension ($5x10^5$ CFU/mL) was added to the wells. The negative control consisted of BHI broth and inoculum, and the blank control consisted only the BHI broth. The plate was then incubated for 24 hr. at 37ºC. To assess the cell growth, the absorbance of each sample was measured at 600 nm using a microtiter plate reader and compared with the absorbance of negative control. Then, MIC_{50} was calculated by standard plate count method. The MBC was performed by placing each sample dilution on BHA and the plate was then incubated for 24-48 hr. at 37ºC. The lowest concentration of extract that showed no visible growth was taken as the MBC. The experiments were performed in triplicate.

7. Inhibition of biofilm formation of *T. chebula* Retz. extract

The effect of a *T. chebula* Retz. extract on biofilm formation of *S. mutans* was determined using the method described by Mai Nguyen et al. (2017). In brief, 100 µL of the two-fold serial diluted of *T. chebula* Retz. extract ranging from 0.195-100 mg/mL was prepared in the flat-bottomed 96-well plates. Then, an equal volume of *S. mutans* ($1x10^6$ CFU/mL) was added into the well and the plate was incubated for 24 hr. at 37ºC. 0.1%

chlorhexidine was used as a positive control, phosphate buffered saline was used as a non-treated control and BHI broth was used as a blank control. After the incubation, supernatants were discarded and washed three times with phosphate buffer saline to remove non-adherent bacteria. The biofilms that formed in the wells were then stained with 0.1% crystal violet for 10 min. Then, the excess stain was removed by washing three times with phosphate buffered saline and the bound crystal violet was then solubilized in 200 µL of 33% acetic acid per well. The absorbance at 590 nm was then measured. The mean of the three replicates was calculated after subtraction of the blank measurement and the results were expressed as a percentage of biofilm in relation to the untreated control. 50% and 90% of minimal biofilm inhibition concentration ($MBIC_{50}$ and $MBIC_{90}$) were then calculated. Each assay was carried out independently three times.

8. Eradication of biofilm formation of *T. chebula* Retz. extract

The eradication of biofilm formation of *T. chebula* Retz. extract against *S. mutans* was determined according to Teanpaisan et al. (2014). In brief, 200 µL *S. mutans* ($1x10^6$ CFU/mL) was inoculated into each well of the flat bottom 96 well microtiter plate and incubated for 24 hr. at 37ºC to prepare the biofilm. After biofilm formation, the medium was carefully discarded. The non-adherent cells were then washed three times with sterile phosphate buffered saline. After that 100 µL of the two-fold serial diluted of *T. chebula* Retz. extract ranging from 0.195-100 mg/mL was added to the biofilms and incubated at 37ºC for 24 hr. The adherent bacteria were then washed three times with sterile phosphate buffered saline. The numbers of surviving bacteria were stained with 0.1% crystal violet for 10 min. Then, the excess stain was removed by washing three times with phosphate buffered saline and the bound crystal violet was then solubilized in 200 µL of 33% acetic acid per well. The absorbance at 590 nm was then measured. The mean of the three replicates was calculated after subtraction of the blank measurement and the results were expressed as a percentage of eradication of biofilm formation. 50% and 90% of minimal biofilm eradication concentration ($MBEC_{50}$ and $MBEC_{90}$) were then calculated. 0.1% chlorhexidine was used as a positive control, phosphate buffered saline was used as a non-treated control and BHI broth was used as a blank control. Each assay was carried out independently three times.

9. Statistic analysis

Data were expressed as mean and standard deviation (S.D.) by computational analysis from triplicate independent experiment. Statistic comparisons were made by one way ANOVA. $P < 0.05$ was considered statistically significant.

Results and discussion

1. Preparation of *T. chebula* Retz. extract

T. chebula Retz. fruit extract appeared as dark-brown powder. The crude extract was shown in Fig. 1. The yield of an extract was 55.86%.

Fig. 1 *T. chebula* Retz. fruit extract

2. HPLC analysis

The chromatograms of ellagic acid standard compound, gallic acid standard compound and *T. chebula* Retz. fruit extract ware shown in Fig. 2. The quantification of major constituent of *T. chebula* Retz. fruit extract by HPLC indicated that ellagic acid was the major constituent of an extract. From the chromatogram, the content of ellagic acid and gallic acid in the extract was 36.671 mg/g crude extract and 14.542 mg/g crude extract, respectively. The high amount of ellagic acid and gallic acid found in the extract are in agreement with previous research (Lee et al., 2017b; Saha & Verma, 2018). However, in the present study, the content of ellagic acid was higher than reported previously (Pfundstein et al., 2010). This difference may be due to the difference in the extraction process. Several studies reported that both ellagic acid and gallic acid possess various pharmacological properties such as antioxidant, antiinflammation and antimicrobial (Rios et al., 2018; Sarjit et al., 2015; Yang et al., 2015; Yan & Zhou, 2020).

3. Free radical scavenging activity of *T. chebula* Retz. extract

The free radical scavenging activity of *T. chebula* Retz. fruit extract was performed by DPPH assay which is simple, rapid, inexpensive and widely used method to

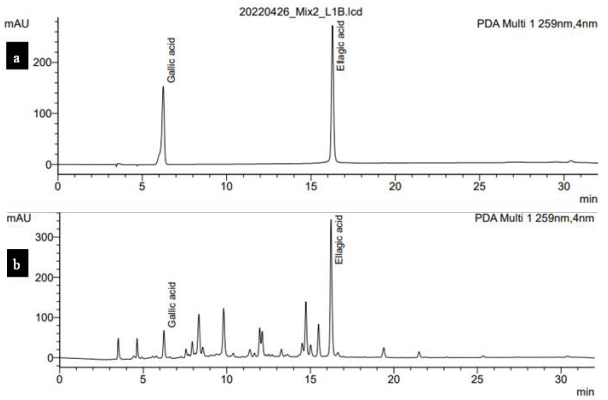

Fig. 2 High-performance liquid chromatography chromatogram of (a) gallic acid and ellagic acid standard and (b) *T. chebula* Retz. fruit extract

estimate an antioxidant activity of natural products (Kedare & Singh, 2011). According to the experiment, the extract exhibit antioxidant activity with IC_{50} value of 0.44±0.01 mg/mL while the IC_{50} value of a positive control, L-ascorbic acid 0.0586±0.002 mg/mL which are in agreement to previous studies. The antioxidant activity of an extract probably exerted by ellagic acid which is considered one of the major antioxidant molecules. The chemical structure of ellagic acid containing two lactones and four hydroxyl groups, enables scavenging a wide variety of reactive oxygen species (Ratnam et al., 2006). Several studies reported that polyphenol from extract of plants can enhance anticaries activity by elimination of oxidative stress which can lead to inflammatory processes and gingivitis (Pytko-Polończyk et al., 2021)

4. Antibacterial activity of *T. chebula* Retz. extract

A preliminary efficacy assessment of *T. chebula* Retz. fruit extract against *S. mutans* was performed using the agar well diffusion assay. The antimicrobial effect was detected by the formation of an inhibition zone around the well. Results obtained in this assay showed that *T. chebula* Retz. fruit extract exhibited strong inhibitory activity against *S. mutans* and the degree of inhibition increased with concentration (Fig. 3). The antibacterial activity of an extract are shown in Table 1. The efficiency of *T. chebula* Retz. fruit extract against *S. mutans* in planktonic form was measured by broth microdilution assay. The MIC_{50} value of an extract was 0.47±0.28 mg/mL and the MBC value of an extract was 6.25 mg/mL. The results were in accordance with previous studies which reported that ethanolic extract of *T. chebula* Retz. fruit possess anticaries activity (Aneja & Joshi, 2009; Lee et al., 2017c; Nayak et al., 2014). The exact mechanism of antibacterial of an extract is probably exerted by ellagic acid, the major constituent of *T. chebula* Retz. extract according to the present study. The mechanism of which may be associated with reducing bacterial adhesion, biofilm formation and destroying bacterial cell membrane (Loo et al., 2010; Miklasińska-Majdanik et al., 2018; Yan & Zhou, 2020).

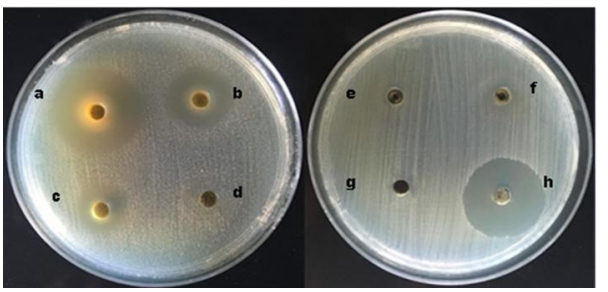

Fig. 3 Zone of inhibition of *S. mutans* tested with *T. chebula* Retz. extract at a concentration of (a) 100 mg/mL, (b) 50 mg/mL, (C) 25 mg/mL, (d) 12.5 mg/mL, (e) 6.25 mg/mL, (f) 3.125 mg/mL, (g) negative control and (h) positive control

Table 1 Antimicrobial properties of *T. chebula* Retz. fruit extract against *S. mutans* using agar well diffusion assay

Concentration of *T. chebula* Retz. fruit extract	Zone of inhibition (mm.± SD)
100	1.60±0.01[a]
50	1.37±0.02[a]
25	0.8±0.04[a]
12.5	-
6.25	-
0.2% chorhexidine	2.93±0.04[b]

Remark: (-) indicates for no inhibitory effect, experiment with 3 replicates. The different superscript letter in the same column represents significant difference when compared with each concentration in variables at $p<0.05$ (one way ANOVA)

5. Antibiofilm activity of *T. chebula* Retz. extract

Strong production of biofilm is an important virulent factor of *S. mutans*. The ability of *T. chebula* Retz. fruit extract to inhibit biofilm formation and eradication of biofilm of *S. mutans* were measured and the results are shown in Table 2. Results reveled that the extract has efficacy to inhibit the biofilm formation and eradication of biofilm of *S. mutans*. The concentration of $MBIC_{50}$ and $MBIC_{90}$ of an extract against *S. mutans* biofilm were 4.47±0.32 mg/mL and 79.32±1.13 mg/mL, respectively (Fig. 4). Whereas the concentration of $MBEC_{50}$ and $MBEC_{90}$ of an extract were 9.64±0.39 mg/mL and 151.70±0.88 mg/mL, respectively (Fig. 5). For positive control, the concentration of chlorhexidine at 0.1% showed 100% of inhibition of biofilm formation and

eradication of biofilm of *S. mutans* when compared to the control group. According to the present study, the antimicrobial effect of *T. chebula* Retz. extract on *S. mutans* was found to be less for the biofilm form than the planktonic form at approximately 10% which is in accordance with previous reports. These results are in agreement with previous researchers. The experiments conducted by Davies (2003) and Verderosa et al. (2019) indicated that bacteria in biofilms are inherently more tolerant to antimicrobial treatment when compared directly to planktonic cells of the same strain. In the biofilm form, bacteria has a limited growth rate and has lower metabolism rate than the planktonic form, therefore the bacteria are less susceptible to the antimicrobial agents and are protected from the antimicrobial action (Gilbert et al., 2002; Shemesh et al., 2007; Singh et al., 2017). Furthermore, the biofilm obstructed the penetration of antimicrobial substances from outside (Bowen & Koo, 2011). Shemesh et al. (2007) and Svensäte et al. (2001) have also demonstrated that *S. mutans* cells which form biofilm exhibit a different expression of some proteins in comparison to planktonic culture, for example, an increasing of exopolyphosphatase expression and decreasing of lactate dehydrogenase or pyruvate kinase expression.

Table 2 Inhibitory efficacy of *T. chebula* Retz. fruit extract against planktonic form and biofilm form of *S. mutans* using broth microdilution assay

***S. mutans* form**	**Inhibitory efficacy of *T. chebula* Retz. fruit extract against *S. mutans* (mg/mL)**					
	MIC_{50}	**MBC**	**$MBIC_{50}$**	**$MBIC_{90}$**	**$MBEC_{50}$**	**$MBEC_{90}$**
planktonic	0.47±0.28	6.25	-	-	-	-
Biofilm	-	-	4.47±0.32	79.32±1.13	9.64±0.39	151.70±0.88

Remark: (-) indicates for no test, experiment with 3 replicates

Conclusion

This study suggested that *T. chebula* Retz. fruit has highly effective antibacterial activity against both planktonic form and biofilm form of *S. mutans*, an important pathogen of dental caries. The extract acts as an antibiofilm agent by preventing biofilm formation and eradicating the existing biofilm. The extract also possess free radical scavenging activity. Therefore, the extract has a potential and might be further used as an active ingredient in oral care products for prevention of dental caries in terms of biofilm formation and eradication of biofilm caused by *S. mutans*. However, the toxicity and the mechanisms of action against *S. mutans* of an extract needs further study and evaluation.

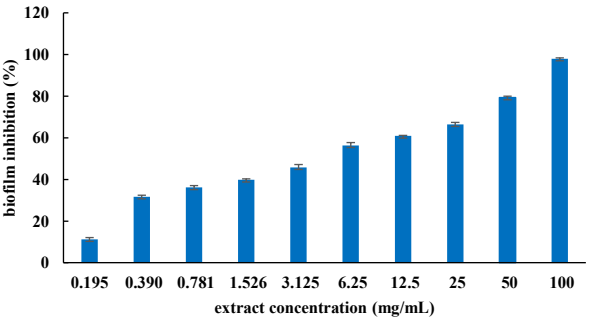

Fig. 4 Inhibition of biofilm formation of *S. mutans* by *T. chebula* Retz. extract at various concentration

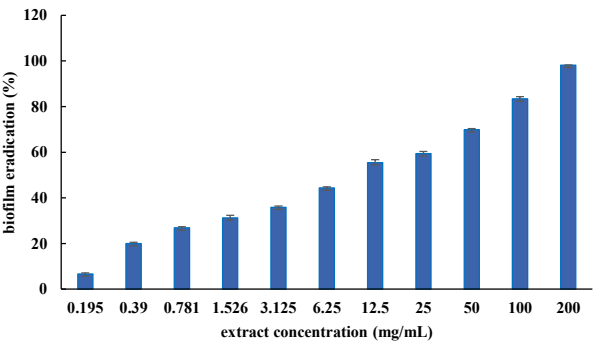

Fig. 5 Eradication of biofilm of *S. mutans* by *T. chebula* Retz. extract at various concentration

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