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In vitro antimicrobial activity of *Antidesma bunius* extracts on oral pathogenic bacteria

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ABSTRACT

Introduction: *Antidesma bunius* is commonly found in Thailand and contains high amounts of antioxidants. The pharmaceutical properties such as antibacterial, anti-inflammatory and anticancer. The aim of this study is to evaluating antibacterial activity against oral pathogens (i.e., *Streptococcus mutans, Straphylococcu saureus* and *Streptococcus pyogenes*) with respect to four different types of *A. bunius* extraction by water including green fruits, red fruits, black fruits, and leaves. **Method:** The extracts of *A. bunius* were examined percent yield, total phenolic compounds, thin layer chromatography and antimicrobial susceptibility testing. **Result and Conclusion**: The percentage of extract yield was found to be the highest in the black fruits (5.20%), followed by red fruits (3.53%) and the leaves (0.71%). The lowest percentage was observed in the green fruits (0.47%). The thin-layer chromatography analysis showed a dark spot of gallic acid, the green and red fruit extract was found to have the same R_f value at 0.92.Total phenolic content was highest in the leaves (59 mg GAE g^{-1}) followed by the green fruits (32 mg GAE g^{-1}) and red fruits (26 mg GAE g^{-1}), respectively, whereas the lowest was found in the black fruits (24 mg GAE g^{-1}). Antibacterial activity of the green fruit extract against *S. aureus* (1.73 cm) was higher than tetracycline (1.05 cm). In contrast, the anti-bacterial activity against *S. pyogenes* was not different (2.46 cm for the green fruits was similar to tetracycline), whereas against *S. mutans,* it was lower (1.96 for the green fruits, 3.86 for tetracycline). Anti-bacterial activity was not observed in the black fruit and leaf extracts. The MIC of the green fruit extract were 0.0125 mg ml^{-1} for *S. pyogenes* and 0.025 mg ml^{-1} for both *S. mutans* and *S. aureus*. Furthermore, the same results were identical for MBC. Results of the current study indicated that the green and red fruit extracts of *A. bunius* had had an inhibitory effect on oral pathogenic bacteria.

INTRODUCTION

Oral bacterial infections, which mainly lead to dental caries, have been recognized as being an important public health problem. Dental caries cause the production of an acidic condition from *Streptococcus mutans* [1]. This bacterial species can be resistant to many antibiotic medicines (e.g., penicillin, erythromycin, tetracycline, and cephalosporin) [2]. Oral lesions associated with trauma are relatively common in dental practices and are attributed to resistant *Staphylococcus aureus* [3]. It has been reported that *S. aureus* can be resistant to the activity of many medicines (e.g., macrolides, lincosamides, aminoglycosides, beta-lactam, penicillins, and cephalosporin). Moreover, oral disease infections caused by *Streptococcus pyogenes* (*S. pyogenes*: Group A beta-hemolytic streptococci) can exert syndromes such as pharyngitis, tonsillitis, sore throats, and lymphadenitis [4]. Due to the fact that they are diverse medical biochemical compounds, herbal extracts have been widely employed to remedy oral diseases and have resulted in lower medication resistance relative to their synthetic compound counterparts [5]. Previous studies have reported that plant derived phenolic compounds could inhibit a number of bacterial diseases including *Streptococci* spp. [6]. Phenolic compounds have antibacterial properties, especially *S. mutans,* through the inhibition of bacterial biofilm formation [7].

Antidesma bunius is commonly found in Thailand and contains high amounts of antioxidants such as flavonoids and phenolic compounds (e.g., anthocyanin, tannin, gallic

acid (GA), catechin, epicatechin, rutin, quercetin, and vitamin C. It has many interesting pharmaceutical properties such as antibacterial and anti-inflammatory properties as well as somatic cell deceleration and anticancer properties [8,9]. In previous studies, it has been shown that when the extracts of green, red, and black fruits as well as the leaves and bark of *A. bunius* had been extracted with 95% ethanol, and 98% methanol they had shown the ability to suppress the activities of two Gram-positive bacteria: *S. aureus* and *Propionibacterium acnes* [10]. Moreover, Sotthisawad and Insin [11] reported the incorporation of *Antidesma thwaitesianum* crude extract into standard formula mouthwash to combat the following three strains of *Streptococcus* as: *Streptococcus constellatus, Streptococcus salivarius,* and *Streptococcus mitis*.

The objective of this study was to estimate the antibacterial activity of *A. bunius* on three different oral pathogenic bacterial strains including *S. mutans*, *S. aureus,* and *S. pyogenes*.

MATERIALS AND METHODS

Microorganisms

Standard bacterial strains, used to evaluated antimicrobial activity, included the following: *S. mutans* (ATTC 25175), *S. aureus* (ATTC25923), and *S. pyogenes* (ATCC 19615). Moreover, they were obtained from the Department of Medical Science of the Ministry of Public Health.

Plant Material Collection and Extraction

Fresh leaves of *A. bunius* and fruits possessing different colors (i.e., green, red, and black) were collected from Sakon Nakhon Province in Thailand. To obtain an aqueous extract, the fresh leaves were mixed with distilled water. The fruit parts were homogenized using an electric blender and were filtered with a Whatman No.1. The solvent was then evaporated under pressure in a rotary evaporator at 50°C and the extract was stored at −20°C until used.

Determination of Total Phenolic Content

The content of total phenolic compounds in the leaf and fruit extracts of *A. bunius* was measured using the spectrophotometric method [5]. In brief, a 1 mg of sample was mixed with 1 ml of Folin-Ciocalteu's phenol reagent for 5 min. After that, 10 ml of 7% Na_2CO_3 solution was added to the mixture and then of 13 ml of deionized sterilized water was added and mixed thoroughly. After the mixture had been incubated for 90 min, it was kept in the dark at room temperature. Each of the samples was measured using a spectrophotometer at 760 nm. The total content of the phenolic compounds in the extracts was determined by extrapolating the standard GA calibration curve and was expressed in mg of gallic acid equivalents (GAE) per g of dried sample.

Thin-layer Chromatography (TLC) Analysis

TLC was used to identify the active constituents of phenolic compounds such as GA and tannic acid (standard). The plates were coated with silica gel GF 254 (Merck 10 cm × 10 cm). The solvent used water:ethanol:diethyl-ether (0.5:2.5:1.5; vol = 45 ml) in the mobile phase. The standard and samples were spotted on chromatographic plates with micropipettes and were then air dried. The plates were then dipped in the chamber that had been saturated and removed. Active constituents were identified by UV-VIS detectors at a wavelength of 254 nm.

Antibacterial Activity

Antibacterial activity of the extracts against oral pathogenic bacteria was determined using the disc diffusion method. In short, 1 mg of each of the crude extracts was mixed with 1 ml of distilled water and was then diluted by four series of the two-fold serial dilutions (i.e., 0.1, 0.05, 0.025, and 0.0125 mg/ml). The extracts were screened for their antibacterial activity against the *S. mutans, S. aureus,* and *S. pyogenes*. To determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *S. mutans, S. aureus,* and *S. pyogenes,* the aqueous extractions from the different *A. bunius* fruits and leaves were assayed using a broth microdilution technique. The inhibition zone determination was performed using the disc diffusion method. All bacterial strains were inoculated onto agar plates with paper discs, which had previously been soaked in each of the *A. bunius* extracts. Inhibition zones were investigated after the assays had been incubated for 24 h at 37°C.

Statistical Analysis

The data were expressed as means with standard deviations and were calculated using IBM SPSS statistics version 17 (SPSS Inc., USA.).

RESULTS

The percentages of yield from the different *A. bunius* extracts were found to be the highest in the black fruits (5.20%), followed by the red fruits (3.53%), and the leaves (0.71%), respectively. Meanwhile, the lowest percentage of yield was observed from the green fruits (0.47%). The total phenolic content was highest in the leaves (59.40 mg GAE g^{-1}), followed by the green fruits (32.20 mg GAE g^{-1}), the red fruits (25.70 mg GAE g^{-1}), and the black fruits (24.20 mg GAE/g), respectively (Table 1).

Analysis with thin-layer chromatography showed a dark spot of GA, the green and red fruit extracts exhibited an R_f value of 0.92. In addition, it was found that tannic acid, as well as extracts of leaves, green fruits, and red fruits, had shown the spot with an R_f value of 0.74. However, the spot was unclear because the mobile phase may not have been proper for tannin detection (Table 2 and Figure 1).

The green and red fruit extracts at 0.2 mg/ml had been able to inhibit the growth of all three oral pathogenic bacteria (Table 3 and Figure 2). The green fruit extract had had a greater inhibitory effect on *S. aureus* than the tetracycline which had been used as a positive control. The inhibitory effect of the green fruit extract on *S. pyogenes* had not differed, whereas relative to tetracycline, the green fruit extract was lower for *S. mutans*. The red fruit extract was found to have exhibited an

Table 1: The percentages of yield and total phenolic contents of different *Antidesma bunius* extractions

Extracts	Extract yield	% yield	Total phenolic contents (mg GAE g^{-1})
Leaf	1.34	0.71	59.40±0.42†
Green fruit	0.79	0.47	32.30±0.59
Red fruit	6.87	3.53	25.70±0.51
Black fruit	9.47	5.20	24.20±0.33

†Mean with standard deviation. GAE: Gallic acid equivalents

Table 2: The R_f value of *A. bunius* extracts

Extracts	R_f value
Gallic	0.92
Tannic acid	0.74
Leaf	0.74
Green fruit	0.74, 0.92
Red fruit	0.74, 0.92
Black fruit	-

A. bunius: Antidesma bunius

Table 3: The inhibition zones of different *A. bunius* extractions

Extracts	Inhibition zone size (cm)		
	S. mutans	***S. aureus***	***S. pyogenes***
Tetracycline††	3.86±0.08†	1.05±0.05	2.46±0.04
Green fruit	1.96±0.04	1.73±0.04	2.46±0.17
Red fruit	1.26±0.17	1.00±0.00	1.43±0.08
Black fruit	-	-	-
Leaf	-	-	-
Deionized sterilized water†††	-	-	-

†Mean with standard deviation, ††Tetracycline as a positive control, †††Deionized sterilized water as a negative control. *A. bunius: Antidesma bunius, S. mutans: Streptococcus mutans, S. aureus: Staphylococcus aureus, S. pyogenes: Streptococcus pyogenes*

inhibitory effect on all three bacteria. However, its effect was found to be lower than that of tetracycline. The extracts from the black fruit and the leaves had not shown any inhibitory effect on the tested bacteria.

With respect to all three bacteria, the green fruit extract had shown a lower MIC and MBC than the red fruit extract (Table 4). The lowest MIC and MBC of the green fruit extract were found in *S. pyogenes* (0.0125 mg/ml), while the red fruit extract in both *S. mutans* and *S. pyogenes* had been equal to 0.05 mg/ml. The diameter of the inhibition zone obtained from the MIC of the green fruit extract for *S. mutans* was: 0.90±0.10 cm, for *S. aureus* was 0.80±0.04 cm, and for *S. pyogenes* was 0.70±0.00 cm (Table 5 and Figure 3). Meanwhile, the inhibitory effect of the inhibition zone with the red fruit extract had been 0.80±0.17 cm on *S. mutans*, 0.70±0.00 cm on *S. aureus*, and 0.96±0.04 cm on *S. pyogenes*.

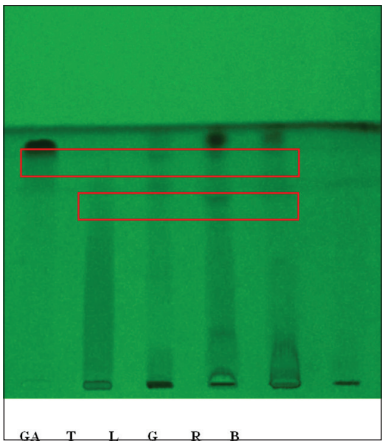

Figure 1: Thin-layer chromatography of *Antidesma bunius* extracts. GA: Gallic acid, T: Tannic acid, L: Leaf extract, G: Green fruit extract, R: Red fruit extract, B: Black fruit extract

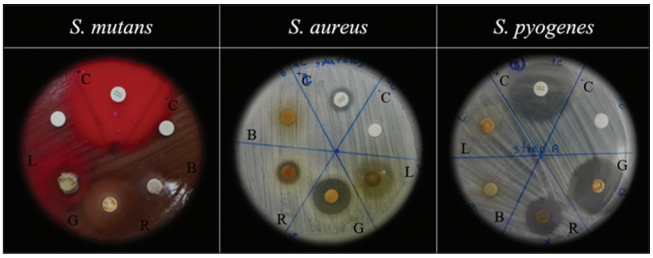

Figure 2: Inhibition zone of *Streptococcus mutans*, *Staphylococcus aureus,* and *Streptococcus pyogenes* as concentration at 0.2 mg/ml of the green and red fruit extracts of *Antidesma bunius*. $^{+}$C: Tetracycline, $^{-}$C: Deionized sterilized water, L: Leave extract, G: Green fruit extract, R: Red fruit extract, B: Black fruit extract

DISCUSSION

The current study has aimed to quantify the extracts' percentages of yield and total phenolic contents as well as to evaluate the inhibitory effects of the green, red, and black fruits of *A. bunius*, and its leaves on oral pathogenic Gram-positive bacteria. The percentages of yield for the black and red fruits were found to be higher than the green fruits and leaves, and this factor has been attributed to the higher sugar content of the two former types of *A. bunius* [10]. Moreover, previous studies have shown that the percentage of yield for the squeezed black fruits had been 8.39%, and when the extracts had been macerated with 95% ethanol, the percentage was found to be 7.39% [8]. For black fruits, the data indicated that when the fruits had been squeezed, the percentage of yield from the extraction had been found to be higher than 95% ethanol had been used in the extraction process.

The concentration of the total phenolic compounds in the leaves and green fruit extracts was higher than in the red and black fruits. This finding was consistent with Sakhunkhu and Chaiyarit [12], using the high-performance liquid chromatography technique in their research, had been able to elucidate the types of antioxidants contained in the leaves and male flowers of twenty *A. bunius* varieties. They noticed that the leaf extract generally possesses higher amounts of GA (up

Table 4: MIC and MBC of the green and red fruit extracts of *A. bunius* to different oral pathogenic bacteria

Extracts	MIC (mg/ml)			MBC (mg/ml)		
	S. mutans	***S. aureus***	***S. pyogenes***	***S. mutans***	***S. aureus***	***S. pyogenes***
Green fruit	0.025	0.025	0.0125	0.025	0.025	0.0125
Red fruit	0.05	0.1	0.05	0.05	0.1	0.05

MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, *A. bunius: Antidesma bunius, S. mutans: Streptococcus mutans, S. aureus: Staphylococcus aureus, S. pyogenes: Streptococcus pyogenes*

Table 5: The diameter of the inhibition zones of different oral pathogenic bacteria affected by different concentrations of the green and red fruit extracts of *Antidesma bunius*

Extract concentrations (mg/ml)	Inhibition zone size (cm)					
	S. mutans		***S. aureus***		***S. pyogenes***	
	Green fruit	**Red fruit**	**Green fruit**	**Red fruit**	**Green fruit**	**Red fruit**
0.1	1.53±0.08‡	1.10±0.03	1.46±0.10	0.70±0.00*	2.66±0.10	1.30±0.06
0.05	1.10±0.10	0.80±0.17*	1.16±0.04	-	1.93±0.04	0.96±0.04*
0.025	0.90±0.10*	-	0.80±0.04*	-	1.23±0.04	-
0.0125	-	-	-	-	0.7±0.00*	-
+C†	4.30±0.20	4.30±0.20	1.13±0.04	1.13±0.04	2.86±0.04	2.86±0.04

*Minimum inhibitory concentration, †Tetracycline, ‡Mean with standard deviation. *A. bunius: Antidesma bunius, S. mutans: Streptococcus mutans, S. aureus: Staphylococcus aureus, S. pyogenes: Streptococcus pyogenes*

to 41 mg GAE g^{-1}) as compared to the flowers (up to 17 mg GAE g^{-1}), whereas, the *A. bunius* fruit was found to contain 32 mg GAE g^{-1} of phenolic compounds [13]. The results of the previously mentioned study are similar to the findings of Jorjong *et al.* [9], who noted that the *A. bunius* fruit had 31 mg GAE g^{-1} of phenolic compounds. In addition, Butkhup and Sammapitto [14] showed that extracts of *A. bunius* fruits had the following total phenolic contents: Green fruits (13.51 mg GAE g^{-1}), red fruits (10.67 mg GAE g^{-1}), and black fruits (8.66 mg GAE g^{-1}). These results suggested that phenolic components of the green fruit of *A. bunius* had exhibited a major effect of antiradical activity.

Analysis with thin-layer chromatography showed dark spots with green and red fruit and had the same R_f value as GA. This is similar to previous study which suggested that *A. bunius* fruit had bioactive constituents (i.e., anthocyanin, tannins, GA, catechin, epicatechin, rutin, and cuercetin) and Vitamin C [8,9].

In this study, the effectiveness of all *A. bunius* extracts was found to inhibit growth and to kill oral pathogenic bacteria (i.e., *S. pyogenes*, *S. mutans*, and *S. aureus*). Moreover, this finding was consistent with the results of a previous study by Thananant and Satnako [15]. They reported that the extracts of green, red, and black fruits of *A. bunius* as well as its leaves and bark which had been extracted with 95% ethanol and 98% methanol had been able to suppress the activity of two Gram-positive bacteria, *S. aureus* and *P. acnes*. In addition, Mwangomo *et al.* [16] noted the antibacterial effects that the stem bark and roots of *Antidesma venosum* had had on Gram-positive bacteria (i.e., *S. aureus*, *Bacillus anthracis*, *Bacillus subtilis*, *Streptococcus faecalis*, and *Bacillus cereus*) when it had been extracted with dichloromethane, petroleum ether, and methanol. It was also reported that the red fruit extract had exhibited a higher inhibitory effect on the activity of *Pseudomonas fluorescens* and *B. subtilis* than extracts from the black fruit.

These results suggest that the phenolic components of *A. bunius* fruit have a major effect on antiradical activity. The phenolic compound could generate antibacterial effects by disrupting the cell walls of bacteria which protect the bacteria from toxic compounds [17]. However, in this study, the leaves of *A. bunius* exhibited the highest phenolic content but showed less antimicrobial activity. This result is similar to a previous study that examined the leaf and seed of *A. bunius* and determined its antibacterial activity against *Campylobacter jejuni* using disc diffusion methods. The data reported that the leaves and seeds had not showed an inhibition zone [18]. Because phenolic compounds are bioactive molecules, the high phenolic levels had not necessarily correlated with the antibacterial activities. With respect to the biological activity related to their molecular structure (hydroxyl group or phenolic ring), phenolic compounds have the capacity to attach proteins and bacterial membranes to form a complex [19]. Moreover, it was shown the phenolic content from the berry extracts had not affected the *Salmonella* strain which was similar to the results of this study [20]. Furthermore, these results supported the previous finding that the extracts of green and red fruits from *A. bunius* had the active constituent of GA as tested by TLC. In addition, regarding the effects of GA on oral bacteria as described by some alkyl esters, it was reported that there was relative antimicrobial activity with lipophilic properties, and it was suggested that the possible site of action was within the cytoplasmic membrane. The mechanisms of action had explained that GA had exhibited the following antimicrobial activities: (1) The inhibition of extracellular microbial enzymes required for microbial growth or (2) the direct action on microbial metabolism through the inhibition of oxidative phosphorylation. In regard to the antimicrobial activity of GA, anti-adhesion might be one active mechanism [21]. Moreover, the acidic property (pH 3-4) of the *A. bunius*

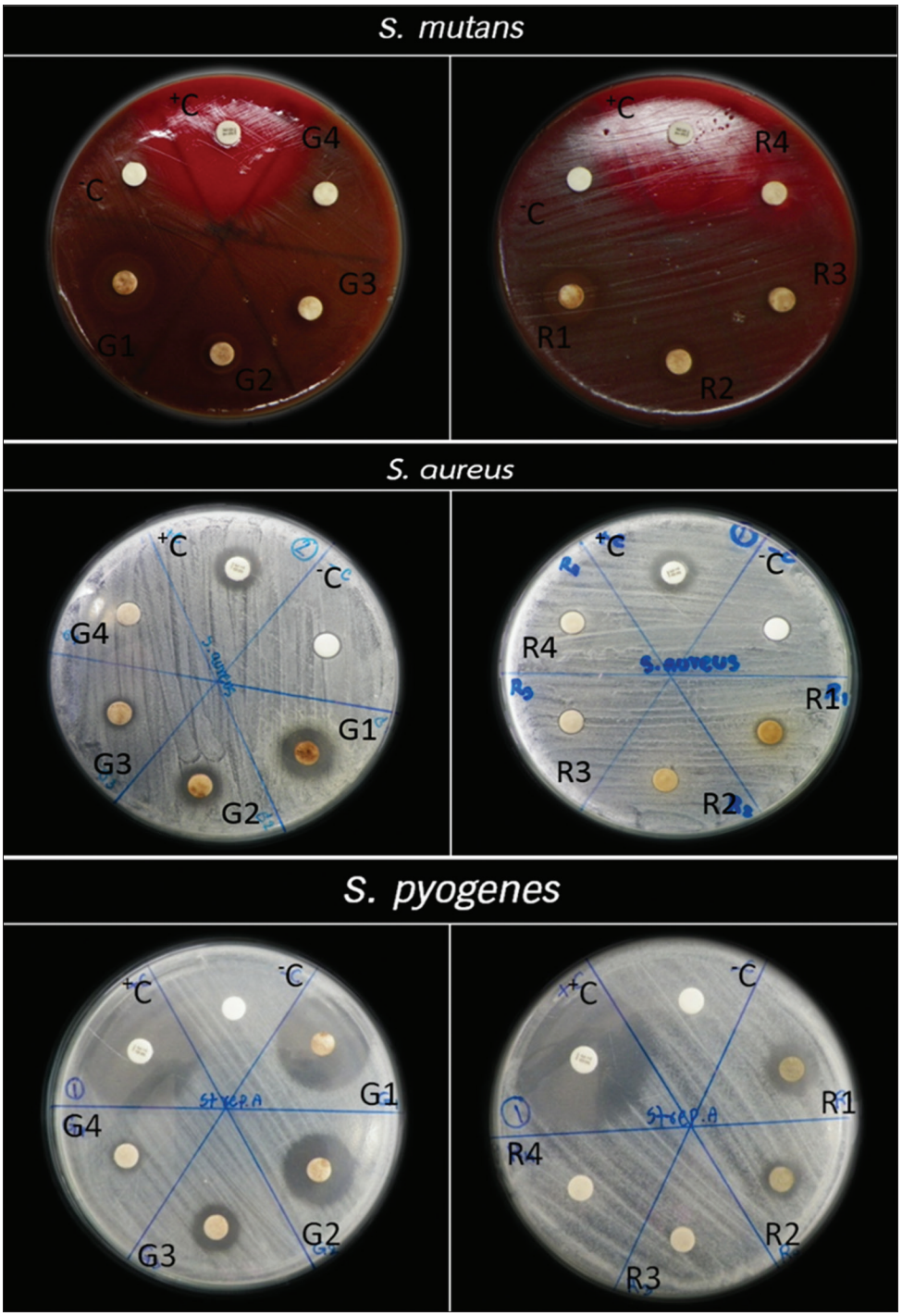

Figure 3: Minimum inhibitory concentrations of the green (G) and red (R) fruit extract of *Antidesma bunius* on *Streptococcus mutans*, *Staphylococcus aureus,* and *Streptococcus pyogenes*. $^{+}$C: Tetracycline, $^{-}$C: Negative control (deionized sterilized water), G1: 0.1 mg/ml, G2: 0.05 mg/ml, G3: 0.025 mg/ml, G4: 0.0125 mg/ml, R1: 0.1 mg/ml, R2: 0.05 mg/ml, R3: 0.025 mg/ml, R4: 0.0125 mg/ml

extracts, which had led to sub-lethal injury and to the breakdown of the bacterial cell membranes, may also have been another mechanism of inhibitory and bactericidal effects [22].

CONCLUSIONS

The present study concluded that the extracts of *A. bunius* green and red fruits possessed pharmaceutical properties of inhibitory and bactericidal effects on oral pathogenic bacteria. Interestingly enough, *A. bunius* extract has the potential to be developed as a phytopharmaceutical product that can relieve dental problems.

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