Malaysian Journal of Microbiology, Vol 19(2) 2023, pp. 200-210
DOI: http://dx.doi.org/10.21161/mjm.220052

Malaysian Journal of Microbiology
Published by Malaysian Society for Microbiology
(In SCOPUS since 2011)

Comparative antibacterial activity and stability of *Andrographis paniculata* herbal mouthwash and commercial mouthwashes

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Received 25 August 2022; Received in revised form 3 February 2023; Accepted 19 February 2023

ABSTRACT

Aims: Chemical mouthwash has been used for ages as one of the oral hygiene practices, but due to its side effects, mouthwash formulated from plants has become a better alternative. *Andrographis paniculata* (*AP*) is an herb plant known for its antibacterial effects. Thus, this study was aimed to compare the antibacterial activity of *AP* with commercial mouthwash and to observe the stability of mouthwash formulated from *AP*.
Methodology and results: Aqueous extract of *AP* was used to prepare herbal mouthwash. The antibacterial activity of *AP* mouthwash and three commercial types of mouthwash, namely Colgate Plax, Oral B and Listerine, were determined by minimum inhibitory concentration (MIC) through broth dilution method and minimum bactericidal concentration (MBC) against selected oral pathogens; *Staphylococcus aureus*, *Streptococcus mutans* and *Streptococcus sobrinus*. The *AP* mouthwash was stored for six months and a year at three different storages to assess its stability and microbial contamination by the pour and drop plate method. The results indicated that Colgate Plax and *AP* mouthwash have the best antibacterial activity compared to two other commercial types of mouthwash with an inhibition percentage of 95.96% at 15.63 mg/mL for *S. aureus* (Colgate Plax) and 168.45% at 62.5 mg/mL for *S. aureus*, 93.75% at 7.81 mg/mL for *S. mutans* and 98.51% at 7.81 mg/mL for *S. sobrinus* (*AP* mouthwash). The parameters measured remained unchanged during storage except at room temperature, while the pH level ranged from 6.72 to 7.45. The *AP* mouthwash showed stable sterility throughout the study.
Conclusion, significance and impact of study: The *AP* mouthwash shows good antibacterial activity against oral pathogens and is almost similar to other commercial mouthwashes and stable to be used for up to a year. In addition, it has excellent potential as an alternative herbal mouthwash in treating oral pathogens effectively.

Keywords: Andrographis paniculata, antibacterial activity, stability test, mouthwash, oral pathogen

INTRODUCTION

There are about 750 known bacteria species that inhabited the human oral cavity (Szymanska *et al.*, 2020). The surface of the teeth, which is hard and non-shedding, has been a suitable surface for accumulating oral pathogenic bacteria. These bacteria start colonising the teeth by depositing in the oral cavity, followed by the formation of biofilm, which eventually develops as dental plaque. Over time, dental plaque can cause more severe oral diseases, such as dental caries (Dani *et al.*, 2016).

Dental caries is a chronic bacterial disease that results from the demineralisation and destruction of hard tissues by bacteria. Gram-positive bacteria such as *Streptococcus mutans*, *Streptococcus sobrinus* and *Staphylococcus aureus* are examples of the common oral pathogenic bacteria known to cause dental caries (Karpiński and Szkaradkiewicz, 2013; McCormack *et al.*, 2015). The initiation of dental caries can be carried by *S. mutans* as it is able to produce mutacins, which are an important factor in the formation of biofilm on the tooth surface. Besides, in the presence of extracellular sucrose,

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(2) 2023, pp. 200-210
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S. mutans act by metabolising it and synthesising insoluble extracellular polysaccharides which help them to adhere better on the tooth surface. Meanwhile, another species of mutans *Streptococci*, *S. sobrinus* is responsible for the development of dental caries (Merritt and Qi, 2012; Karpiński and Szkaradkiewicz, 2013).

Mouthwash has been used for decades as a solution for dental disease such as gingivitis, gum disease and caries. It is also used as another alternative aside from toothpaste in cleaning the mouth and helps in eliminating smelly breath. Chemical mouthwash usually contains chlorhexidine (CHX) as its main component (Brookes *et al*., 2020). CHX is an ideal broad-spectrum antimicrobial agent against bacteria and fungi effectively and it has been proven to show a positive result in treating dental plaque, gingival inflammation and bleeding. However, the use of CHX has a significant impact on the oral microbiome. Its shifts to a more acidic condition and lower nitrite availability in the mouth but it is unknown whether it promotes a healthy oral microbiome, or it may cause a shift to a microbiome associated with disease (Brookes *et al*., 2020). Besides, CHX has also been reported to have side effects such as staining on the teeth and the ability to cause cross-resistance to antibiotics which eventually contributes to the increase of antibiotic resistance bacteria (Jananya and Promphakkon, 2021).

As these situations have now become challenging and risky to the consumers' health, there is a promising study utilising alternative products which having similar benefits to existing commercial mouthwash but lesser side effects (Chatzopoulos *et al.*, 2022). The discovery of many phytochemicals from medicinal plants has now reached a light lime in producing a new product originating from plants.

Many researchers have acknowledged the chemical properties and benefits of herbal plants. *Andrographis paniculata* is one of the herb plants that has been used in ancient oriental and ayurvedic medicine. It is reported that *AP* has antibacterial, antifungal, antiviral, choleretic, hypoglycaemic, hypocholesterolaemia and adaptogenic effects. Its main metabolites, called andrographolide and deoxyandrographolide have been confirmed to reduce plaque accumulation and increase oral hygiene (Panossian *et al*., 2000; Akbar, 2011; Jananya and Promphakkon, 2021). A previous study by Wasman *et al*. (2011) also showed that the ethanolic extract of *AP* provided significant protection against gastric ulcers.

Although the antimicrobial activity of *AP* has been recognised, the study of its antibacterial activity against oral pathogens is still lacking. Moreover, there is no research that has been carried out comparing the antibacterial activity of *AP* with those of commercial mouthwashes. Therefore, research about herbs formulated in mouthwash, especially *AP*, should be conducted to test its antibacterial activity and stability for shelf life. The objectives of this study were to compare antibacterial activity of *AP*-formulated mouthwash with commercial mouthwash, namely Colgate Plax Peppermint Fresh, Oral B 3D White and Listerine Original and observe the stability of *AP* mouthwash.

The development of herbal products is currently gaining attention, with the hope that they will serve as an alternative oral product. As less research was conducted utilising *AP* as an oral product and the natural oral care product now has similar potential to the commercial product (Chatzopoulos *et al*., 2022), this study was aimed to evaluate the antibacterial activity of *AP*-formulated mouthwash and selected herbal mouthwashes against common oral pathogens. Besides, the stability of *AP* mouthwash was also evaluated. To the best of our knowledge, this is the first study where *AP*-formulated mouthwash was investigated in terms of its stability and efficacy compared to commercial mouthwash.

MATERIALS AND METHODS

Preparation of *Andrographis paniculata* herbal mouthwash

AP powder was extracted and formulated using standard ingredients for the preparation of *AP* herbal mouthwash. *AP* powder was purchased from Best Farm Co., Selangor, Malaysia. Extraction was conducted with modification according to Bahari *et al*. (2021). A total of 100 g of *AP* powder was soaked in one litre of distilled water at 100 °C for 72 h by using a Soxhlet extractor. The extract was then filtered through Whatman No. 1 filter paper and evaporated using Rotavapor (Bocchi R-200) and subjected to a freeze-drying method for three days. The concentrated extract in powder form was then diluted for the preparation of 125 mg/mL *AP* herbal mouthwash. An amount of 6.25 g *AP* extract was mixed with certain amount of flavouring agent (400 µL of peppermint oil and 12 g of sorbitol), solubilising agent (706 µL of Tween 20) and preservative (0.05 g of sodium benzoate) and 50 mL of sterile deionised water (adapted from Zulkepeli, 2012).

Test microorganisms

Bacteria used in this study included *S. aureus* (ATCC®25923™), *S. sobrinus* (ATCC®33478™) and *S. mutans* (ATCC®35668™) obtained from American Type Culture Collection (ATCC). The bacteria were subcultured onto blood agar (BA) and incubated in anaerobic condition at 37 °C for 48 h except for *S. aureus*, which was incubated in aerobic condition at 37 °C for 24 h. Preparation of microbial inoculum was conducted by suspending the freshly grown isolates into Mueller-Hinton broth (MHB) (Oxoid), and the densities were standardised to 0.5 MacFarland turbidity standard for further study using the broth dilution method.

Broth dilution method

The broth dilution method was used to determine the lowest concentration of *AP* mouthwash that inhibits the visible growth of the tested bacteria. The method was adapted from Tuan Kub *et al*. (2021) with slight modifications. The minimal inhibitory concentration (MIC) test was performed in a microplate. The *AP* and

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

Malays. J. Microbiol. Vol 19(2) 2023, pp. 200-210
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commercial mouthwash were subjected to two-fold serial dilutions to a final volume of 100 µL at final concentrations of 0.12, 0.24, 0.49, 0.98, 1.95, 3.90, 7.81, 15.62, 31.25 and 62.50 mg/mL. Next, 100 µL of stock solutions of *S. mutans*, *S. sobrinus* and *S. aureus* (0.5 MacFarland) was added to each well to obtain a final volume of 200 µL. Wells containing MHB with *AP* mouthwash and MHB with each commercial mouthwash were used as negative control, while wells containing MHB and bacteria inoculum were used as viability control. Wells containing MHB alone acted as sterility control. All tests were performed in triplicates and incubated at 37 °C for 24 h in anaerobic conditions except for *S. aureus* (aerobic condition). After incubation, the optical density (OD) of the suspension was measured using a spectrophotometer at 620 nm. The percentage of microbial inhibition was calculated by using the below formula. The higher percentage of microbial inhibition indicates the higher inhibition, the lower percentage indicates lower inhibition, while the negative value indicates the growth promotion of microorganisms.

Percentage of microbial inhibition (%)
$= 1 - (OD_t - OD_{Nc}/OD_{Vc} - OD_{Bo}) \times 100$

Where, t: test well (containing MHB, *AP* mouthwash/commercial mouthwash and microorganisms' inoculum); Nc: negative control well (containing MHB and *AP* mouthwash/commercial mouthwash only); Vc: viability control wells (containing MHB and microorganisms' inoculum only); Bo: broth only well (containing MHB only).

Minimal bactericidal concentration (MBC)

Wells with no visible bacterial growth and the nearer wells with bacterial growth (turbid) were seeded on BA and incubated at 37 °C for 24 h in anaerobic conditions except for *S. aureus* (aerobic condition). No growth seen on BA indicated as an MBC endpoint (Azizan *et al*., 2020).

Stability study

AP mouthwash was tested to determine its stability for freshly prepared, six months and one-year storage. As 62.5 mg/mL *AP* mouthwash showed the optimum antibacterial activities in the previous experiment, stability testing was conducted using this concentration which includes colour, odour, pH, texture, viscosity and microbial contamination (Amiera *et al.*, 2021).

Characteristics study of *AP*

A method from the National Pharmaceutical Regulatory Agency Malaysia (NPRA) (2016) was referred to store the *AP* mouthwash. Three batches of *AP* mouthwash were stored at three proposed storage temperatures which are fridge (5 ± 3 °C), air conditioning (25 ± 2 °C) and room temperature (30 ± 2 °C). The mouthwash was observed for day-1, six months and one year. The colour, pH, odour and texture were recorded to determine the changes in the quality of *AP* mouthwash with regard to the shelf life.

Microbial contamination

Potato dextrose agar (PDA) and Nutrient agar (NA) were used to test microbial contamination in *AP* mouthwash preparation by using drop plate and pour plate methods. Microbial contamination of different temperature of *AP* mouthwash was tested after day-1, six months and a year of storage. *AP* mouthwash was subjected to two-fold serial dilution to obtain final concentrations of 62.5 mg/mL, 31.25 mg/mL, 15.63 mg/mL and 7.81 mg/mL.

Drop plate method

This methodology was adapted from Schweitzer *et al.* (2022). Each agar was divided into five quadrants, each quadrant reserved for one concentration in the series. One drop of each *AP* mouthwash concentration (62.5, 31.25, 15.63 and 7.81 mg/mL) was dropped on the reserved quadrant. The plates were incubated at 37 °C for 24-48 h.

Pour plate method

This methodology was adapted from Bala *et al.* (2017). One mL of each *AP* mouthwash concentration (62.5, 31.25, 15.63 and 7.81 mg/mL) was pipetted onto the centre of the sterile Petri dish. Molten-cooled PDA agar and NA agar were then poured into the Petri dish containing the inoculum and mixed well. The agar was allowed to solidify completely without disturbing it. The plate was incubated in an inverted position at 37 °C for 24-48 h.

RESULTS AND DISCUSSION

Antibacterial activity of *Andrographis paniculata* and commercial mouthwash

The determination of MIC was based on the clear appearance of the MHB indicating that there is no visible growth after being incubated with the tested microorganisms. Based on Table 1, the MIC and percentage of inhibition of Colgate Plax is at 7.81 mg/mL (78.05%) for *S. aureus,* 15.63 mg/mL (83.23%) for *S. mutans* and *S. sobrinus* (65.31%). For Oral B, only two pathogens were being inhibited, *S. mutans* and *S. sobrinus* at 62.5 mg/mL (76.42% and 77.64%). Inhibition of *AP* mouthwash at 15.63 mg/mL for *S. sobrinus* was 40.90% and at 31.25 mg/mL for both *S. aureus* and *S. mutans*, 218.44% and 189.19%, respectively. As for Listerine, none of the microorganisms showed inhibition growth. According to Tuan Kub *et al.* (2021), the concentration of mouthwashes which exceeds 90% of the percentage of microbial inhibition is considered effective MIC. Thus, there were only two mouthwashes showed effective MIC, including Colgate Plax (95.96% at 15.63

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Table 1: Percentage of microbial inhibition of *Andrographis paniculata* and commercial mouthwashes towards *S. aureus*, *S. mutans* and *S. sobrinus*.

Mouthwash concentration (mg/mL)			0.12	0.24	0.49	0.98	1.95	3.9	7.81	15.63	31.25	62.5
Microbial inhibition (%)	Colgate	*S. aureus*	11.63 ± 7.93	17.24 ± 3.08	14.77 ± 6.62	9.57 ± 4.07	12.13 ± 14.03	26.65 ± 12.73	78.05 ± 32.23	95.96 ± 1.22	95.71 ± 1.61	93.4 ± 1.99
		S. sobrinus	0.83 ± 0.95	7.66 ± 20.95	-5.18 ± 2.18	7.04 ± 10.44	-1.66 ± 2.51	3.52 ± 2.51	56.11 ± 18.01	83.23 ± 3.88	76.4 ± 5.52	60.46 ± 6.25
		S. mutans	33.56 ± 1.71	44.67 ± 23.82	33.79 ± 5.54	39.91 ± 2.58	41.5 ± 4.14	48.53 ± 3.42	70.98 ± 3.42	65.31 ± 2.97	62.59 ± 2.45	60.77 ± 5.20
	Listerine	*S. aureus*	5.69 ± 10.29	8 ± 11.21	7.67 ± 10.43	8.33 ± 14.07	6.27 ± 17.92	11.3 ± 11.93	14.52 ± 16.22	11.47 ± 2.81	9.57 ± 2.49	13.28 ± 5.10
		S. sobrinus	7.66 ± 10.94	11.59 ± 4.04	11.59 ± 3.06	6.42 ± 5.60	7.45 ± 9.40	7.25 ± 5.06	12.01 ± 2.00	8.49 ± 2.94	12.83 ± 0.72	24.43 ± 0.95
		S. mutans	33.33 ± 7.36	31.75 ± 0.79	33.33 ± 3.12	46.26 ± 5.01	32.88 ± 2.83	32.88 ± 1.71	53.97 ± 3.07	30.61 ± 13.40	47.85 ± 1.04	60.77 ± 3.07
	Oral B	*S. aureus*	11.22 ± 5.39	11.72 ± 2.00	7.34 ± 12.04	5.86 ± 4.03	3.47 ± 7.69	0.66 ± 8.38	1.57 ± 4.57	8.66 ± 4.74	24.09 ± 3.13	41.34 ± 3.10
		S. sobrinus	3.31 ± 6.37	0.41 ± 3.74	3.11 ± 9.40	4.76 ± 2.80	-0.62 ± 2.71	4.55 ± 4.66	6.21 ± 6.72	4.76 ± 1.29	15.73 ± 3.64	77.64 ± 0.62
		S. mutans	41.5 ± 1.80	39.46 ± 3.12	42.86 ± 10.02	41.72 ± 2.08	39.23 ± 2.83	43.08 ± 1.71	43.76 ± 1.04	55.1 ± 5.57	58.5 ± 3.12	76.42 ± 11.08
	AP	*S. aureus*	13.31 ± 7.73	22.87 ± 13.42	10.56 ± 44.44	16.35 ± 2.15	24.75 ± 9.44	16.06 ± 9.23	22.58 ± 9.26	40.96 ± 24.15	67.58 ± 29.39	168.45 ± 38.43
		S. sobrinus	86.09 ± 0.23	84.69 ± 0.56	83.59 ± 0.99	82.19 ± 1.26	82.34 ± 1.41	83.91 ± 1.78	93.75 ± 1.93	155.94 ± 4.90	218.44 ± 17.01	261.09 ± 39.14
		S. mutans	89.66 ± 0.72	88.55 ± 0.45	86.01 ± 1.20	82.24 ± 0.78	81.62 ± 1.23	88.51 ± 1.23	98.51 ± 4.91	142.57 ± 4.52	189.19 ± 9.89	200.88 ± 9.29

Note: value ± standard deviation, number of replicates: 3.

mg/mL for *S. aureus*) and *AP* (168.45% at 62.5 mg/mL for *S. aureus*, 93.75% at 7.81 mg/mL for *S. mutans* and 98.51% at 7.81 mg/mL for *S. sobrinus*). Generally, *AP* mouthwash seemed to show increasing trends in the percentage of microbial inhibition. However, contradictory results were also demonstrated for *S. sobrinus*, which showed negative results indicating the bacterial growth promotion at 0.49 mg/mL (-5.18%), 1.95 mg/mL (-1.66%) of Colgate treatments and 1.95 mg/mL (-0.62%) of Oral B treatment. These inconsistent trends might be due to the reason that they contained certain chemicals that were not well homogenised with MHB.

The MBC value indicates the minimum concentration of an antimicrobial agent required to kill most of the viable microorganisms. Colgate Plax showed positive MBC towards all tested microorganisms at concentrations of 7.81 mg/mL for *S. sobrinus* and *S. aureus* and 15.63 mg/mL for *S. mutans* (Figure 1). For Oral B, positive MBC can only be seen for *S. sobrinus* at 62.5 mg/mL (Figure 2), while MBC was not detected at all tested concentrations for Listerine (Figure 3). As for *AP* mouthwash, positive MBCs were recorded for all tested bacteria except for *S. aureus*. The herbal mouthwash showed MBC for *S. mutans* and *S. sobrinus* at concentrations of

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

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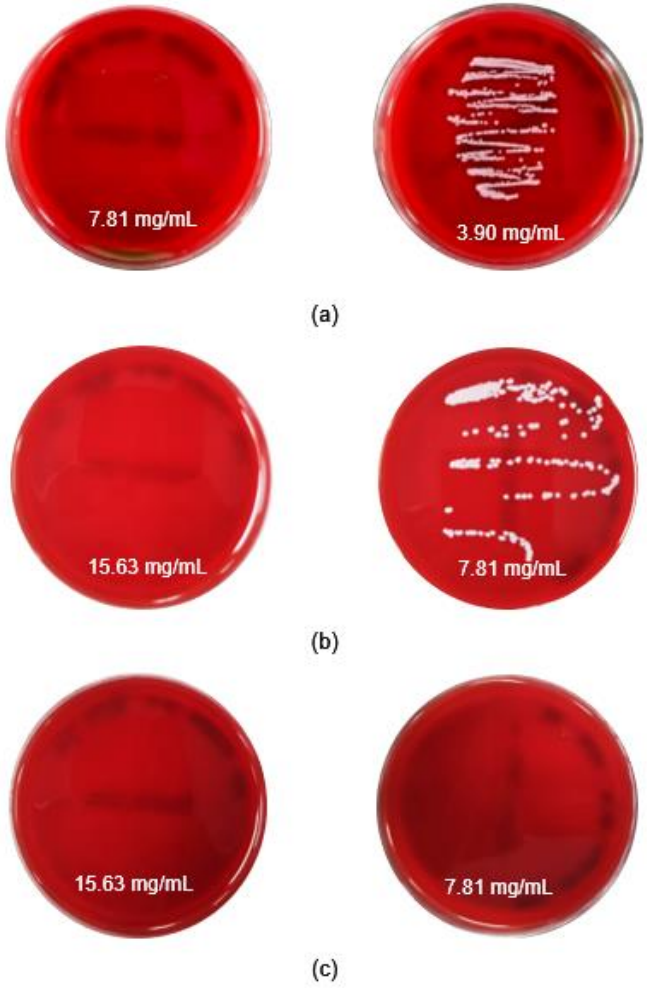

Figure 1: The MBC of Colgate mouthwash towards oral pathogens; (a) *S. aureus*, (b) *S. mutans*, (c) *S. sobrinus.* Note: The values are concentration of the commercial mouthwash used.

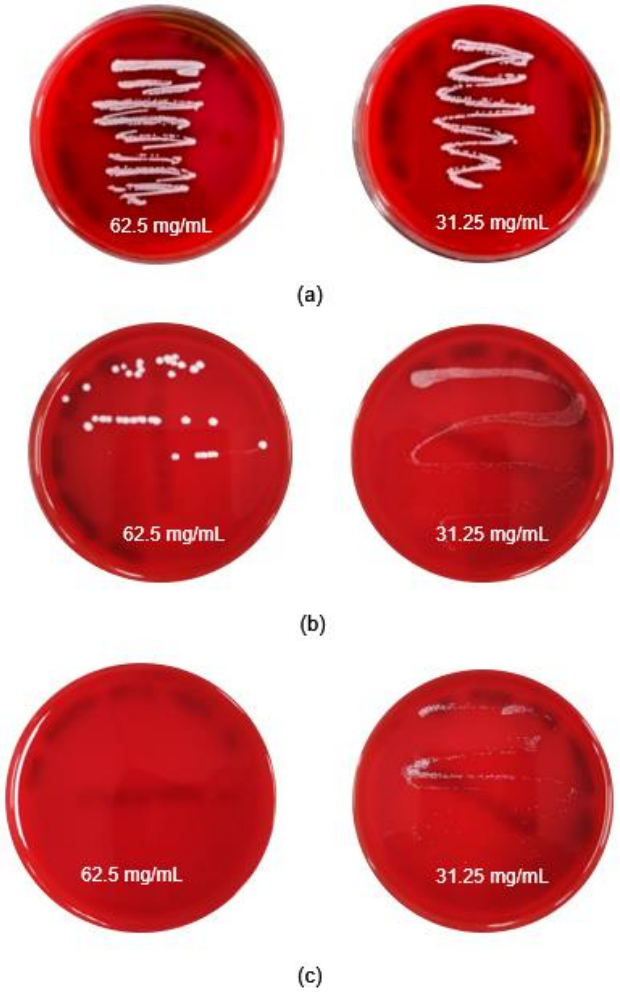

Figure 2: The MBC of Oral B mouthwash towards oral pathogens; (a) *S. aureus*, (b) *S. mutans*, (c) *S. sobrinus*. Note: The values are concentration of the commercial mouthwash used.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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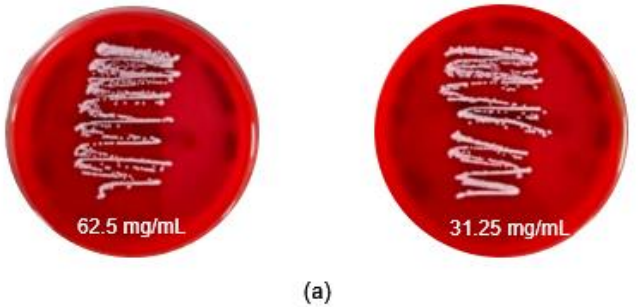
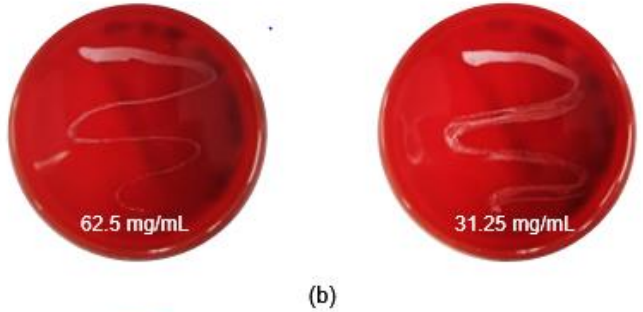




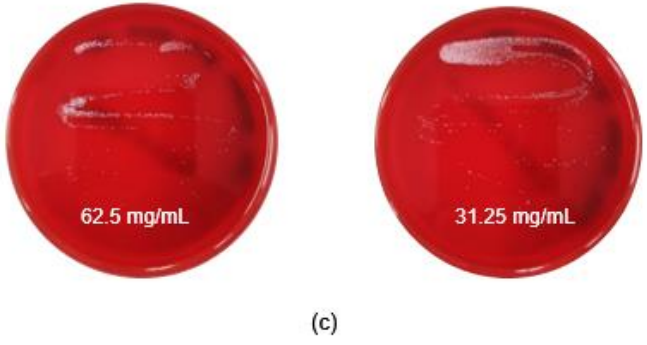
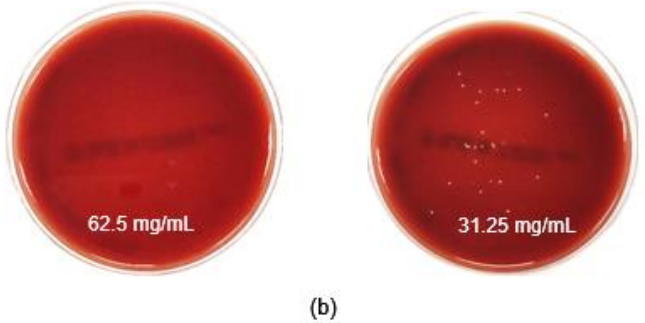


Figure 3: The MBC of Listerine mouthwash towards oral pathogens; (a) *S. aureus*, (b) *S. mutans*, (c) *S. sobrinus.* Note: The values are concentration of the commercial mouthwash used.



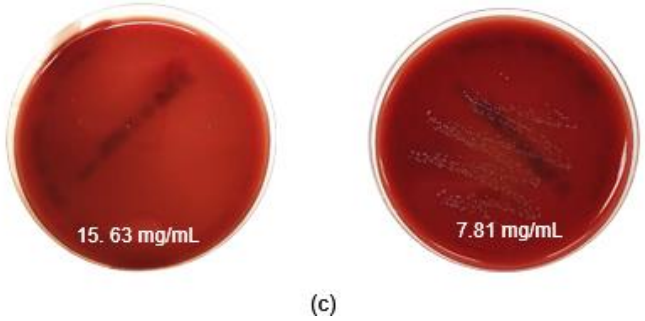


Figure 4: The MBC of *Andrographis paniculata* mouthwash towards oral pathogens; (a) *S. aureus*, (b) *S. mutans*, (c) *S. sobrinus.* Note: The values are concentration of the mouthwash used.

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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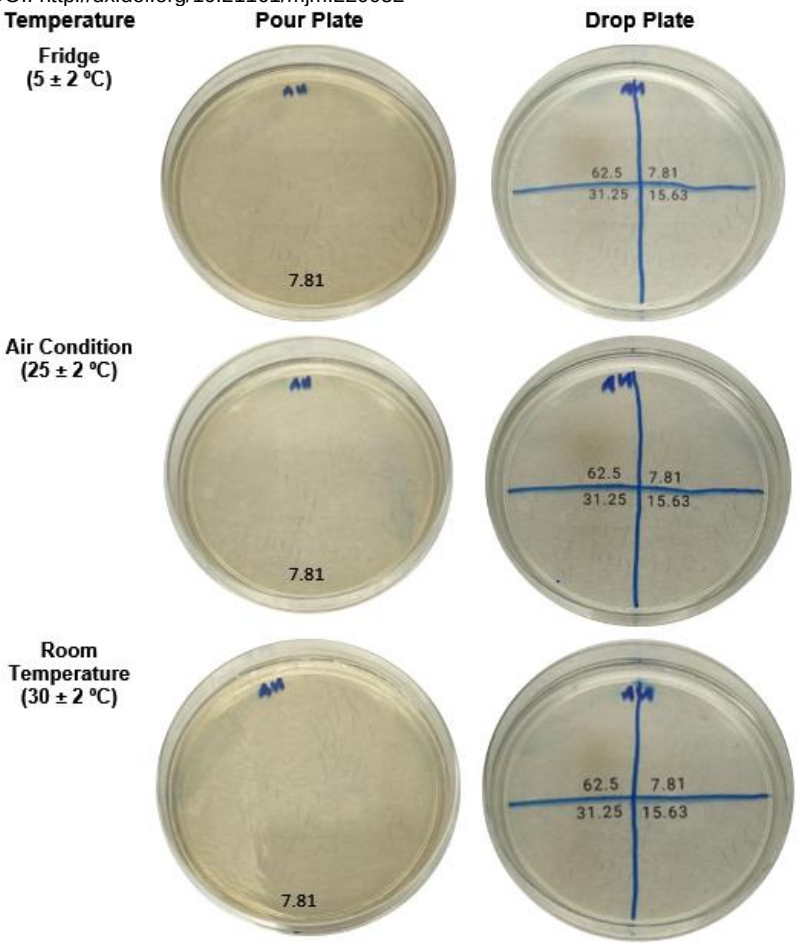

Figure 5: Microbial contamination testing of *Andrographis paniculata* herbal mouthwash after one year onto nutrient agar at different storage conditions. Note: The values are concentration of the mouthwash used (mg/mL).

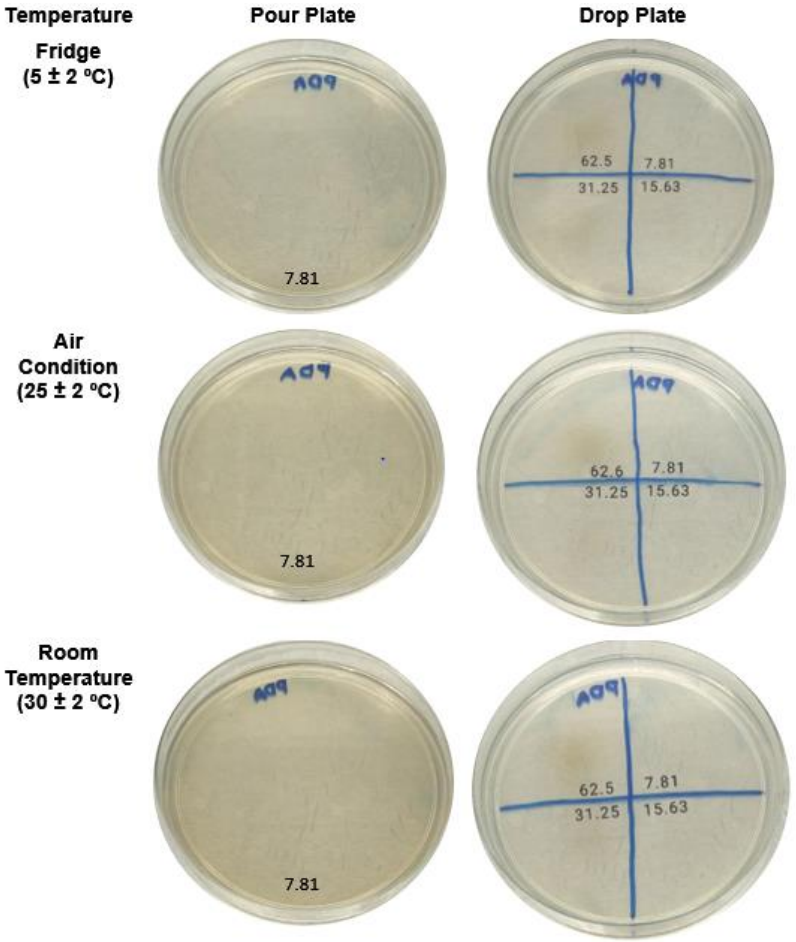

Figure 6: Microbial contamination testing of *Andrographis paniculata* herbal mouthwash after one year onto potato dextrose agar at different storage conditions. Note: The values are concentration of the mouthwash used (mg/mL).

ISSN (print): 1823-8262, ISSN (online): 2231-7538

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Table 2: The characteristics of *Andrographis paniculata* at different durations and storage conditions.

Proposed storage	Parameter	Day-1	Six months	One year
Fridge (5 ± 2°C)	Colour	Brown	Brown	Brown
	Odour	Sweet minty	Sweet minty	Sweet minty
	pH	7.23	6.95	6.76
	Texture	Liquid	Liquid	Liquid
	Viscosity	Low	Low	Low
Air condition (25 ± 2 °C)	Colour	Brown	Brown	Brown
	Odour	Sweet minty	Sweet minty	Sweet minty
	pH	7.45	7.02	6.80
	Texture	Liquid	Liquid	Liquid
	Viscosity	Low	Low	Low
Room temperature (30 ± 2 °C)	Colour	Brown	Brown	Brown
	Odour	Sweet minty	Sweet minty	Minty, brackish smell
	pH	7.38	6.86	6.72
	Texture	Liquid	Liquid	Liquid, formed little precipitate
	Viscosity	Low	Low	Low

62.5 and 15.63 mg/mL, respectively (Figure 4). Most of the MBC values of these mouthwashes were similar to MIC values which indicated that the mouthwashes required the same amounts of concentrations to inhibit and kill the microorganisms. Amongst these three commercial mouthwashes, Colgate Plax showed the best antimicrobial activity, followed by Oral B and Listerine. Our findings are in agreement with the present study by Demirel *et al*. (2015), which reported good microbial activity of Colgate Plax and Oral B onto several oral pathogens.

As this research only used a diluted form of mouthwash, it might be the reason why Listerine mouthwash didn't inhibit any tested pathogens. The concentrated form of Listerine mouthwash used in daily usage by consumers might inhibit oral pathogens. Cetylpyridinium chloride (CPC) in Colgate Plax and Oral B was found to be more active in destroying the integrity of bacterial cell membranes than eucalyptol oil in Listerine (Demirel *et al.*, 2015).

The positive results for *AP* mouthwash were supported by the finding by Zhang *et al*. (2021), which confirmed the remarkable antibacterial activity of the bioactive compound; andrographolide found in the *AP* plant. Besides, another study by Tuan Kub *et al.* (2021), *AP* methanol extract at a concentration 5% (w/w) demonstrated anti-microbial activity against *Staphylococcus epidermidis*, *S. aureus*, *Propionibacterium acnes* and *Candida albicans.* Aqueous *AP* extract was also proven to have anti-bacterial activity against *Shigella* sp. (Parveen *et al.*, 2019). Our study also demonstrated that *AP* mouthwash is more effective in inhibiting tested microorganisms compared to the other three commercial types of mouthwash, but in terms of bactericidal, Colgate Plax showed better results than *AP*. To date, there is a limited report in the scientific papers comparing the antibacterial effects of *AP* and commercial mouthwash. However, research from Muhamad Alojid *et al.* (2021) has shown that *AP* aqueous crude extract is effective in inhibiting *S. aureus*, *S. mutans* and *S. sobrinus* and recorded MIC at 125 mg/mL for almost tested microorganisms which were higher than our findings. The difference between MIC of *AP* mouthwash might be due to the different methods used in performing the MIC (agar diffusion method), which was found to be less reliable compared to the broth dilution method (Tuan Kub *et al.*, 2021).

In another study, *AP* chloroform extract obtained lower MBC (1.5 mg/mL) for *S. aureus* than those reported in our study. *AP* chloroform extract consists of a high total of aromatic compounds comprising of phenols, aromatic carboxylic acid and esters that make it's antimicrobial activities better than other *AP* extracts (Roy *et al.*, 2010). A study conducted by Plianrungsi and Kulthanaamondhita (2021) comparing the anti-plaque activity of *AP* mouthwash and CHX mouthwash has shown that *AP* mouthwash significantly reduced plaque accumulation, comparable with CHX mouthwash. CHX has been proven to have better antimicrobial activity against oral pathogens compared to CPC (Demirel *et al.*, 2015).

As the *AP* plant is widely available in Malaysia and the *AP* mouthwash exhibited antibacterial activity towards most of the tested oral pathogens similar to that commercial mouthwash, the potential use of *AP* must be exploited in the pharmaceutical industry. Hence, this study also provides the recommended concentrations beneficial in developing non-alcohol formulation herbal products.

Stability test

Characteristics studies of Andrographis paniculata mouthwash

The physical changes of *AP* mouthwash for day-1, six months and one year remained consistent except for room temperature storage (Table 2). The odour and texture gave a less minty smell or brackish smell and formed precipitated at the bottom of the tube after one year. This might be due to the deterioration of chemical properties or diterpenoids such as andrographolide, andrographic acid and andrographidine in *AP* mouthwash

 ISSN (print): 1823-8262, ISSN (online): 2231-7538

during the storage process. This result has corresponded to the previous study, which showed decrease in the chemical stability of powdered *AP* by around 8% to 25% after three months at room temperature storage, while another study reported the stability of andrographolide are better in lower temperature (Pholphana *et al.*, 2004; Yan *et al*., 2018). Another study by Sirilun *et al.* (2016) also demonstrated that herbal mouthwash formulated from betel, green tea, clove, black galingale, mangosteen and noni plants showed the opaque form after 28 days of accelerated stability test due to the emulsification of peppermint oil. This result was in agreement with our study which showed a little precipitation might be due to the same reason. In contrast, there was also research onto herbal mouthwash formulated from *Euphorbia hirta* L.*,* which recorded the stable preparation properties with no separation using a freeze-thaw stability test (Iskandar *et al.*, 2022).

Referring to our study, the pH level decreased in each storage condition after one year. As referred to the Table 2, the pH level of *AP* in fridge storage ranged from 7.23 to 6.70, in air condition, 7.45 to 6.80 and in room temperature, 7.38 to 6.72. These results tend to agree with the research by Iskandar *et al.* (2022) on *Euphorbia hirta* L. which showed the variation of pH results after the stability study. This is possibly due to the reduction of active ingredient concentrations from evaporation during the storage process or might be due to temperature, humidity or additives used (Iskandar *et al.*, 2022).

The range for salivary pH is 6.2 to 7.6, with the average pH at 6.7 (Kalyani and Leelavathi, 2019). This pH of saliva would change whenever individuals were infected with a disease. Patients with chronic gingivitis had more alkaline saliva (pH above 7.0), whereas those with chronic periodontitis had more acidic saliva (pH below 7.0) (Baliga *et al*., 2013). Since all the pH results in the proposed storage are generally within the pH range of the salivary pH, thus it is still considered stable to be used in a span of one year.

Microbial contamination of *Andrographis paniculata*

For the microbial contamination testing using pour plate and drop plate methods, all the culture plates of *AP* at concentrations 62.5 mg/mL, 31.25 mg/mL, 15.63 mg/mL and 7.81 mg/mL showed no signs of growth after 48 h of incubation for day-1, six months and a year storage onto PDA and NA (Figure 5 and 6). This may indicate there are significant antibacterial and antifungal properties of *AP* mouthwash naturally. Studies have shown that *AP* has antibacterial effects against microorganisms, especially Gram-positive bacteria including *Streptococcus* species and *Candida albicans* (Deepak *et al.*, 2014). The metabolite, andrographolide, is a diterpene lactone that plays an important role in inhibiting microbial growth. Zhang *et al.* (2020) stated that andrographolide could prevent the formation of bacterial biofilms, the production of virulence factors, adhesion between bacteria and the destruction of bacterial integrity. Other than andrographolide, the peppermint oil added to the mouthwash has been known for its phytochemical content, antibacterial effect, antioxidant and preventing microbial growth (Tyagi and Malik, 2011).

Most herbal plants have their own phytochemical, which act as antimicrobe against pathogens. Thus, many herbal products have natural sterility due to the presence of the certain chemical compound. This statement is supported by a study by Ahmad *et al.* (2018), who demonstrated that polyherbal mouthwash formulated with 30% Neem, 30% Clove, 20% Cinnamon and 10% Liquorice aqueous extract maintained sterile condition throughout the accelerated stability study.

CONCLUSION

This study shows that *AP* mouthwash possesses good antibacterial activity against selected oral pathogens and is comparable to Colgate Plax and Oral B mouthwash. *AP* is able to inhibit all tested microorganisms at lower concentrations (31.25 mg/mL), however, higher concentrations are required to kill *S. mutans* and *S. sobrinus* (62.5 mg/mL). Therefore, *AP* mouthwash at 62.5 mg/mL concentration has a good potential against common oral pathogens in terms of alternative product development. As most the existing commercial products seemed to lead the side effects, the advantage of this product must be further explored in the future.

The stability study of *AP* mouthwash also confirmed that this mouthwash is proven to be stable over year of storage with the best-proposed storage at 25 ± 2 ºC. However, since research regarding *AP* is still scarce, further research and clinical trials should be conducted to confirm the medicinal use of *AP*.

ACKNOWLEDGEMENTS

We would like to acknowledge the Ministry of Higher Education Malaysia for the financial support via Fundamental Research Grant Scheme (FRGS) with Project Code: FRGS/1/2022/SKK12/USM/03/5.

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 ISSN (print): 1823-8262, ISSN (online): 2231-7538