

EVALUATION OF TOXICITY AND ANTIBACTERIAL ACTIVITIES OF *Andrographis paniculata* HERBAL MOUTHWASH AGAINST ORAL PATHOGENS

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ABSTRACT

Andrographis paniculata (AP) is an ancient herb known for its medicinal and therapeutic values. In this study, we aimed to evaluate the antibacterial activity and cytotoxicity of AP herbal mouthwash. Aqueous extract of AP was used to prepare the herbal mouthwash. The product was tested against selected oral pathogens namely *Actinomyces viscosus*, *Staphylococcus aureus*, *Streptococcus mutans*, *Streptococcus sobrinus*, and *Porphyromonas gingivalis* for its antibacterial activity using the agar well diffusion method. Toxicity analysis was carried out and subjected to cytotoxicity screening using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, *in vivo* study using brine shrimp lethality bioassay, and detection of heavy metals using atomic absorption spectroscopy (AAS). Five AP herbal mouthwash concentrations (62.5, 125, 250, 500, 1000 mg/mL) were developed. AP herbal mouthwash exhibited the strongest antibacterial activity against *A. viscosus*. AP herbal mouthwash (125 to 1000 mg/mL) showed inhibition against *P. gingivalis*, *S. mutans* and *S. sobrinus*, except for *S. aureus* which did not reflect any sign of antibacterial activity. In brine shrimp lethality bioassay, AP herbal mouthwash has LC₅₀ 3255.064 µg/mL. *In vitro* cytotoxic evaluation was done on L929 mouse fibroblast cell lines using MTT assay with IC₅₀ 43.55 mg/mL denotes the mouthwash is non-toxic. Heavy metals content in AP herbal mouthwash falls within the permissible range of plumbum (2.50 ppm) followed by arsenic (1.875 ppm), mercury (0.15 ppm), and cadmium (0.06 ppm). Thus, verifying AP herbal mouthwash is effective in common oral pathogens and has a non-toxic effect.

Key words: *Andrographis paniculata*, antibacterial, brine shrimp, heavy metal, mouthwash, oral pathogens

INTRODUCTION

The ecological imbalance of dental biofilm is usually associated with the attachment of bacteria to the oral surface. Without proper oral hygiene, these bacteria can cause dental caries, periodontitis, halitosis, and inflammation of dental tissue (Yang *et al.*, 2020). Therefore, the inhibition activity of the oral bacteria is important in the prevention and treatment of oral diseases. Prior studies confirmed mouthwash is a versatile cleansing tool because it can fill in the areas that are difficult to reach. In clinical treatment, chlorhexidine is often prescribed to the patient. However, prolonged use is not suggested linked

to tooth discoloration, taste alteration, and other reactions (Van Swaaij *et al.*, 2020). Moreover, many commercial types of mouthwash contain alcohol that is non-biocompatible when in direct contact with mucosal tissue (Su *et al.*, 2019). Considering these, research on finding a safe and effective mouthwash to replace such chemicals is needed.

Medicinal plants have the reputation of being a good pharmaceutical value in herbal medicine. AP is an ancient herb known for its medicinal properties such as antibacterial (Bahari *et al.*, 2021), antifungal (Hossain *et al.*, 2021), and antiviral (Lim *et al.*, 2021) of its ethanol and methanol extracts. The chemical properties of AP have been widely researched and its use in herbal medicine has been widely reported. Additionally, natural product is now gaining a spotlight

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with the belief that it incurs fewer side effects (Zhou *et al.*, 2019). We aimed to evaluate the antibacterial activity of the formulated AP herbal mouthwash at various concentrations onto common oral pathogens such as *S. mutans*, and *S. sobrinus* which are the early colonizer in dental caries, and also periodontal pathogens; *A. viscosus* and *P. gingivalis*. This study also included the clinical strain of *S. aureus* which recently reported high carriage rates in the oral cavity (Eric & Kotey, 2020).

Although many herbal plants contain a lot of medicinal properties, some research has found that certain toxic compounds present in certain plants are harmful to health. For safety evaluation, toxicity investigation of the plant extracts or the formulated herbal medicine is considered the most important step before the product is commercialized for consumer applications.

MATERIALS AND METHODS

Preparation of AP herbal mouthwash

AP powder was purchased from Best Farm. Co., Malaysia. One hundred gram plant powder was extracted with one liter of distilled water for 72 h using a Soxhlet extractor (Bahari *et al.*, 2021). Subsequently, the filtered aqueous extract of AP was evaporated with the Buchi R-200 Rotavapor. The dried extract was diluted for the preparation of AP herbal mouthwash into 62.5, 125, 250, 500, and 1000 mg/mL, with the addition of peppermint oil as a flavoring and Tween 20 as solubilizing agents adapted from (Zulkepli, 2012).

Bacterial stock culture

Bacterial stock cultures were obtained from American type culture collection (ATCC) *S. aureus* (ATCC®25923™), *S. sobrinus* (ATCC®33478™), *S. mutans* (ATCC®35668™), *A. viscosus* (ATCC®15987™), *P. gingivalis* (ATCC®33277™). The growth of each bacterial strain from the blood agar was suspended in Mueller Hinton broth (MHB) to acquire turbidity of 0.5 McFarland.

Agar well diffusion

The antibacterial activity of the AP herbal mouthwash was evaluated by the agar well diffusion method (Ahmad *et al.*, 2021). Precisely, 6 mm of wells were punched on Mueller Hinton agar (MHA) and Mueller Hinton blood agar (MHBA). A sterile swab was used to perform bacterial lawn on the plate. 20 µL of AP herbal mouthwash were pipetted into the triplicate wells and additionally incubated aerobically for *S. aureus* and anaerobically for *S. mutans*, *S. sobrinus*, and *A. viscosus*, *P. gingivalis* at 37 °C for 24 h. The diameter of the inhibition zone was measured. Colgate plax® was used as positive control while sterile distilled water as a negative control.

Brine shrimp lethality bioassay

Brine shrimp lethality assay was performed using the nauplii of a simple zoological organism, *Artemia salina* according to the study (Aboalola *et al.*, 2020). This method estimates the cytotoxicity activity by measuring the lethality of the test organism. A stock of AP herbal mouthwash was prepared (10,000 µg/mL) and serially diluted to prepare the concentrations (1000, 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 µg/mL). Artificial seawater was prepared by dissolving 38.2 g of non-iodized sea salt into one liter of distilled water. *Artemia salina* eggs were hatched in the prepared seawater for 24 h. Ten hatched nauplii were transferred into a petri dish containing 4.5 L seawater and 500 µL of AP herbal mouthwash. The test was taken place in an illuminated room for 24 h. Survivors were counted to calculate the lethality percentage by using the formula; Percentage of mortality (%) = No. of dead nauplii / Total no. of nauplii × 100

MTT assay

The cytotoxic effect of AP herbal mouthwash on L929 cells was evaluated by MTT assay as described by Yang *et al.* (2020). L929 mouse fibroblast cells were cultured in Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, USA) and antibiotics (Life Technologies). The culture flask was incubated at 37 °C, 5% CO₂ and 95% humidity. Medium from confluent cell cultures was discarded and rinsed with phosphate buffer saline (PBS). 500 µL of trypsin-EDTA was added and incubated at 37 °C for 15 min. 100 µL of 5 × 10³ viable cells /well were incubated in flat-bottomed 96-well microplates for 24 h at 37 °C in 5% CO₂ until it reached 70% confluence. Then, the old medium was discarded and 200 µL of the new medium was added.

In this study, the cells were treated with 2 µL AP herbal mouthwash at concentration (0.12, 0.24, 0.48, 0.97, 1.95, 3.90, 7.81, 15.62, 31.25, 62.50 mg/mL). Well-containing cell suspension only was used as a control for cell viability. Well containing only complete medium was used as blank control. Each mouthwash dilution was assessed in triplicate. 96-well microplates were incubated for 72 h. The culture medium was discarded, and cell layers were washed with PBS. Next, 30 µL of MTT (Sigma-Aldrich, USA) was added to the wells and incubated for 3 hr. Following incubation, the culture medium was removed and 100 µL of dimethyl sulfoxide (Sigma-Aldrich, USA) was added to the wells to dissolve the formazan crystal. The optical densities (OD) were read at 570 nm using a spectrophotometer. The percentage of the viable cell is calculated as follows; (Cunha *et al.*, 2020); (Mean OD of treated cells / Mean OD of control cells) × 100 %

Heavy metal analysis by AAS

Four heavy metal elements were analyzed using (Perkin Elmer PinAAcle 900z atomic absorption (AA) spectrometer) such as arsenic (As), cadmium (Cd), plumbum (Pb), and mercury (Hg). Briefly, 25 mL of AP herbal mouthwash (125 mg/mL) was diluted with concentrated nitric acid (HNO₃) at 90°C for 45 min. The process was continued until complete digestion was achieved and the temperature was increased up to 100°C. The solution was boiled for 6-7 hr until the extract become colorless. The solution was filtered and diluted with 100 mL of distilled water. The stock standard solution was prepared by dissolving in appropriate quantities and dried in distilled water. The limit of detection and quantification is 0.06-1.875 ppm (Swargiary & Daimari, 2021).

Statistical analysis

Data analysis was performed using Statistical Package for Social Science (SPSS) software, version 27.0. For the antibacterial study, the Kruskal-Wallis test was used. All results were expressed as median (Interquartile Range) and it is considered significant if ($p < 0.05$). For the cytotoxicity study, the comparison of the cell viability means to the control group was analyzed using a one-way ANOVA test. LC₅₀ and IC₅₀ values were determined by Regression analysis, followed by Probit analysis. Graphical presentations were prepared in Microsoft Excel.

RESULTS

Antibacterial activity of AP herbal mouthwash

In the present study, we compared the antibacterial

activity of five different concentrations of AP herbal mouthwash (62.5, 125, 250, 500, 1000 µg/mL) with the control group (Colgate plax®) mouthwash against five ($n=5$) bacteria isolates (*S. mutans*, *S. sobrinus*, *A. viscosus*, *P. gingivalis*, *S. aureus*). The result in Figure 1 indicated four types of mouthwash (125, 250, 500, and 1000 mg/mL) exerted antibacterial activity against all tested bacteria, except *S. aureus* which did not reflect any sign of antibacterial activity. The Control group has shown antibacterial activity against all bacteria. *A. viscosus* is the most sensitive to the treatment with the highest zone of inhibition (23.63 mm) at 1000 mg/mL AP concentration compared to other bacteria. All concentrations tested displayed a zone of inhibition against *A. viscosus*.

P. gingivalis, *S. mutans*, and *S. sobrinus* showed the highest inhibition zone at the highest AP concentration (1000 mg/mL) with zone inhibition of 14.80 mm, 15.13 mm, and 14.10 mm respectively. At the lowest AP concentration (125 mg/mL), there were the lowest zone inhibitions recorded for *P. gingivalis* (8.95 mm), *S. mutans* (10.75 mm), and *S. sobrinus* (9.33 mm). The results showed the effect of AP concentrations on inhibition zones in concentration-dependent trends (Figure 2).

Since our data did not follow a normal distribution, a non-parametric Kruskal-Wallis test was conducted and presented in Table 1. The P -value is 0.004 indicating there is a significant difference between the antibacterial activity of the AP herbal mouthwash and the control group. Selected oral pathogens receiving 1000 mg/mL AP herbal mouthwash have significantly higher zones of inhibition (median 15.00 IQR 3.40) compared to bacteria treated with the control group

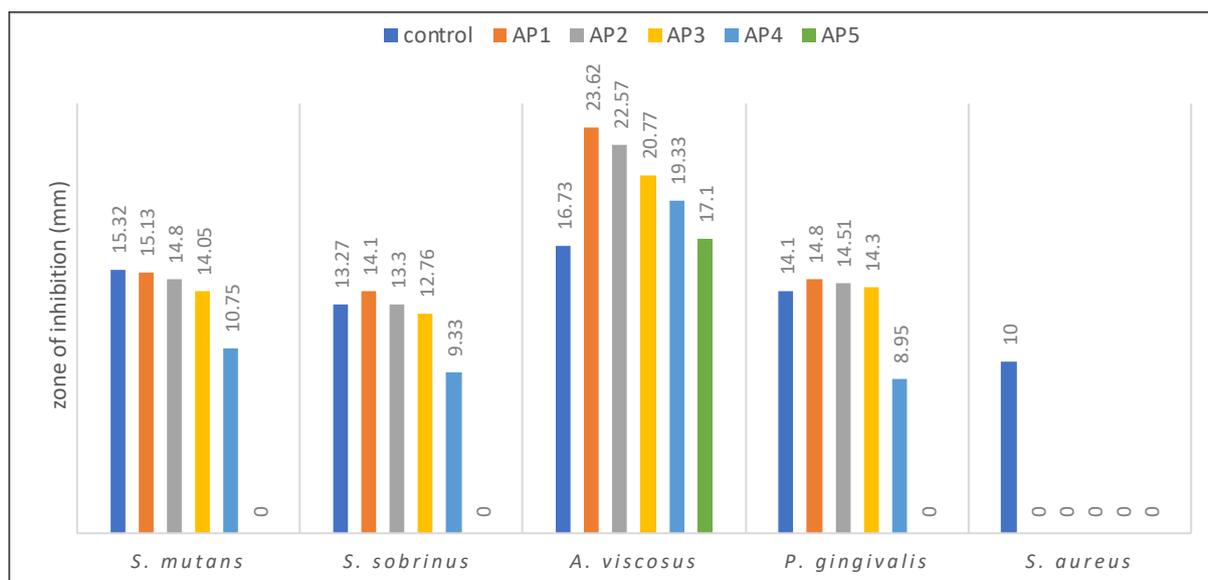


Fig. 1. The bar diagram shows the mean zone of inhibition of the control group (Colgate plax®) and five concentrations of *A. paniculata* herbal mouthwash against selected oral pathogens.

*control (Colgate plax), AP5 (62.5 mg/mL AP mouthwash), AP4 (125 mg/mL AP mouthwash), AP3 (250 mg/mL AP mouthwash), AP2 (500 mg/mL AP mouthwash), AP1 (1000 mg/mL AP mouthwash)

(median 13.50 IQR 3.20). AP herbal mouthwash at a concentration of 125 mg/mL showed minimum inhibitory concentration (MIC) against *S. mutans*, *S. sobrinus*, *A. viscosus*, and *P. gingivalis*.

Brine shrimp lethality bioassay

LC₅₀ value is represented in Figure 2. The results obtained showed a concentration-dependent with the highest mortality (56.67%) recorded at a concentration of 10 000 µg/mL whereas the least mortality (2.22%) was at 50 µg/mL. From the regression probit analysis, the LC₅₀ value of AP herbal mouthwash was 3255.06 µg/mL with a confidence interval of 1358.05 µg/mL to 9134.94 µg/mL.

MTT assay

The result of cytotoxicity is presented in Figure 3. The linear equation indicated that AP herbal mouthwash has IC₅₀ (43.55 mg/mL) with a dose-dependent trend. Cytotoxic activity was only recorded at the highest concentration of AP herbal mouthwash (62.5 mg/mL) with a significant reduction of cell

viability (29.79%). Based on the One-way ANOVA test (Table 2), there is a significant difference in the mean cell viability between the treatment groups ($P < 0.001$). Post-hoc test using Bonferroni showed a significant difference in mean cell viability between cells treated with 62.50 mg/mL AP herbal mouthwash in the control group.

Heavy metals analysis of AP herbal mouthwash

The level of heavy metals is presented in Table 2. The present study revealed that the sample of AP herbal mouthwash contains a negligible amount of heavy metals as per the Poison Act 1952 permissible level. The order of detection rate of heavy metals in AP herbal mouthwash depicted the highest level of Pb (2.50 ppm) followed by As (1.875 ppm), Hg (0.15 ppm), and Cd (0.06 ppm).

DISCUSSION

The aqueous extract was chosen in this study as we wanted to formulate Alcohol-free mouthwash

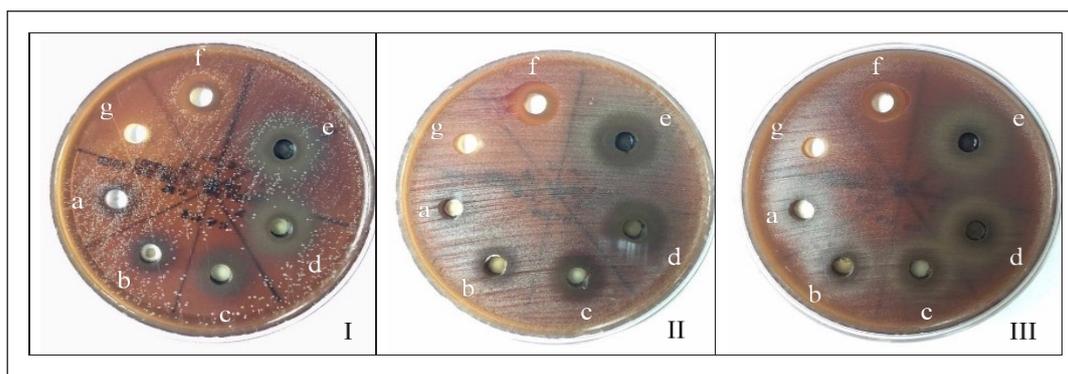


Fig. 2. Agar plates showing zones of inhibition generated by *P. gingivalis* (I), *S. mutans* (II), *S. sobrinus* (III) after being treated with *A. paniculata* herbal mouthwash at concentrations; 62.5 mg/mL (a), 125 mg/mL (b), 250 mg/mL (c), 500 mg/mL (d), 1000 mg/mL (e), and Colgate plax® as positive control (f), sterile distilled water as negative control (g).

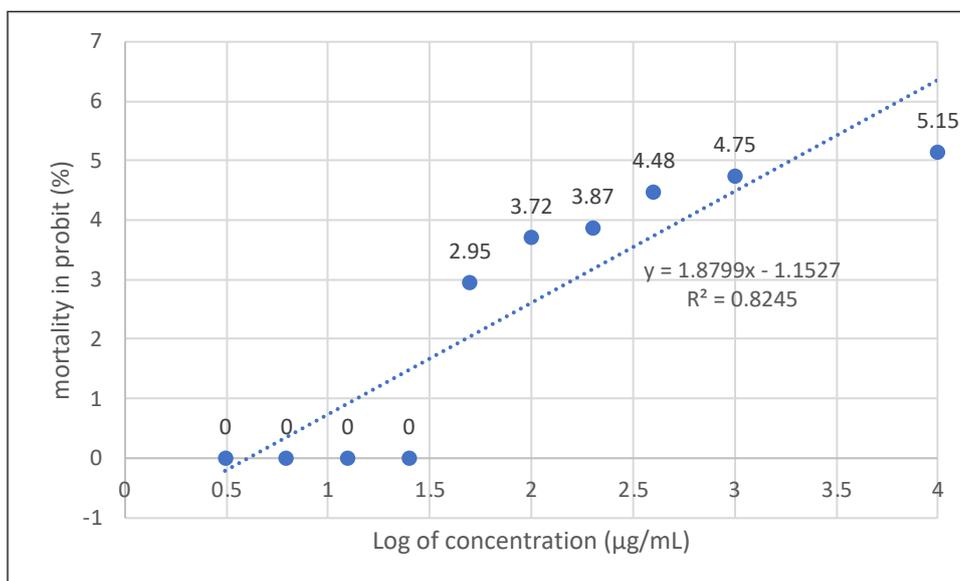


Fig. 3. Determination of LC₅₀ value for *A. paniculata* herbal mouthwash using brine shrimp lethality bioassay.

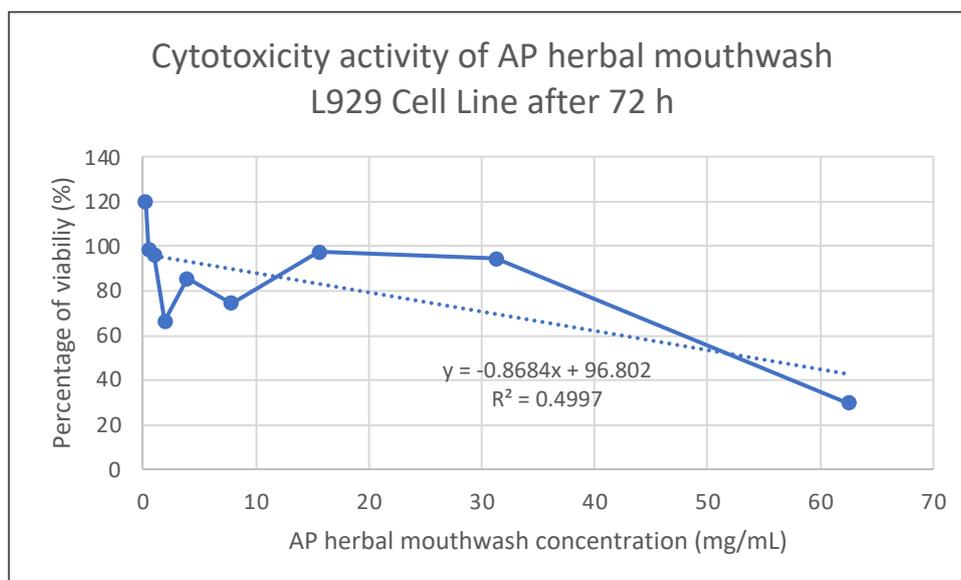


Fig. 4. Determination of IC_{50} value for *A. paniculata* herbal mouthwash using MTT assay.

Table 1. Comparison of antimicrobial activities on different concentrations of *A. paniculata* herbal mouthwash among selected oral pathogens ($n=5$)

Variables	Median (IQR)						Chi-square	P-value*
	Concentration (mg/mL)							
	Control	1000	500	250	125	62.5		
Zone of inhibition (mm)	13.50 (3.20)	15.00 (3.40)	14.40 (3.35)	13.70 (3.20)	9.50 (3.80)	0.00 (0.00)	17.517(2)	0.004

*Kruskal-Wallis test

Post hoc test with Bonferroni corrections

62.5 mg/mL vs control, P -value = 0.043

62.5 mg/mL vs 500 mg/mL, P -value = 0.040

62.5 mg/mL vs 1000 mg/mL, P -value = 0.007

Table 2. Comparison of mean cells viability between treatment groups

Treatment groups	n	Mean (SD) cells viability	F-statistic (df)	P-value*
control	3	2.27 (0.82)		
0.24 mg/mL	3	2.73 (0.87)		
0.48 mg/mL	3	2.24 (0.98)		
0.97 mg/mL	3	2.18 (0.88)		
1.95 mg/mL	3	1.51 (0.58)	3.052	<0.001
3.90 mg/mL	3	1.94 (0.64)		
7.81 mg/mL	3	1.69 (0.28)		
15.62 mg/mL	3	2.22 (0.41)		
31.25 mg/mL	3	2.14 (0.61)		
62.50 mg/mL	3	0.22 (0.26)		

* One-way ANOVA test

Table 3. Result of heavy metals analysis by graphite furnace absorption spectrometry (GFAS) and flow injection mercury system (FIMS) in *A. paniculata* herbal mouthwash

No	Test parameter	Test method	Poison Act 1952 (ppm)	LOD (ppm)	Results (ppm)
1	Arsenic	GFAAS	5.00	1.875	ND
2	Cadmium	GFAAS	0.30	0.15	ND
3	Plumbum	GFAAS	10.00	2.50	ND
4	Mercury	FIMS	0.50	0.06	ND

*ND is not detected

having fewer side effects for the consumer (Sirilun *et al.*, 2016). One study confirmed that alcohol can induce irritation and increase the development of oral cancer (Su *et al.*, 2019). Alcohol causes damage to the oral mucosa and includes epithelial atrophy and a decrease in basal cell size atrophy with associated hyper-regeneration (De *et al.*, 2009). In the previous report by Muhamad Alojidi *et al.*, (2021), they found out that AP aqueous crude extract recorded most of the inhibition zones on *S. mutans*, *S. sobrinus*, and *A. viscosus* ranged from 2.93 ± 5.08 to 12.82 ± 0.85 . Similarly, aqueous extract from the whole plant of AP showed inhibition against *Micrococcus luteus*, *Streptococcus pyogenes*, and *Enterococcus faecalis* with the most effective inhibitory concentration at 1000 $\mu\text{g}/\text{disc}$, providing evidence that AP aqueous extract has a significant antibacterial activity (Sule *et al.*, 2010). The antibacterial activity possessed by this herbal plant is further linked to the presence of andrographolides and arabinogalactan protein ((Hossain *et al.*, 2021).

The negative result for *S. aureus* is considered acceptable as this bacterium does not have close significant effects on oral disease and it commonly occurs in terms of cross-infection and dissemination from other body sites (McCormack *et al.*, 2015). There was also a lack of evidence that showed that *S. aureus* was a colonizer or infected the oral cavity. For further study, AP herbal mouthwash at a concentration of 125 mg/mL was selected as it exhibited the lowest concentration which exerted antibacterial effects onto almost all the tested bacteria (*A. viscosus*, *S. mutans*, *S. sobrinus*, *P. gingivalis*). Hence, our current finding provided primary data showing mouthwash formulated with aqueous extract of AP could be a potential treatment in eliminating harmful oral bacteria that act as causative agents of oral diseases.

The general conviction of natural product is safer and have fewer side effects than modern drugs demanding needs for further study to assess their toxicology profile as currently available data are still very limited. Hence, this present study focuses on the toxicity evaluation of the AP herbal mouthwash. Brine shrimp lethality test was conducted to represent a preliminary toxicity parameter as it is a convenient, rapid, and simple bioassay. According to Meyer's toxicity index, herbal extract with $LC_{50} < 1000 \mu\text{g}/\text{mL}$ is considered toxic, while $LC_{50} > 1000 \mu\text{g}/\text{mL}$

is considered non-toxic (Daniel & Ibok, 2019). The toxicity of the tested mouthwash by comparison to Meyer's toxicity index showed our formulated AP herbal mouthwash is non-toxic. Supporting our finding, Mamantha (2014) also reported LC_{50} obtained for 10 μg , 100 μg , and 1000 μg aqueous extract of AP showed no toxicity observed, showing values more than 100 $\mu\text{g}/\text{mL}$ and less than 1000 $\mu\text{g}/\text{mL}$.

To gain relevant data on cytotoxicity, we did an *in vitro* study of AP herbal mouthwash onto L929 mouse fibroblast cell lines using an MTT assay. L929 cells were chosen as fibroblast is a type of cell that performs the synthesis of the extracellular matrix and collagen, and is commonly used in cytotoxicity studies associated with dental products (Yang & Kang, 2020). Data demonstrated our formulated mouthwash has an IC_{50} value (43.55 mg/mL). The cytotoxicity activity of AP was categorized according to the National Cancer Institute (NCI) guidelines as follows $IC_{50} \leq 20 \mu\text{g}/\text{mL}$ is highly toxic, and $IC_{50} \geq 500 \mu\text{g}/\text{mL}$ is evaluated as inactive (Goel *et al.*, 2021). Supporting the finding, cytotoxicity analysis of pure compounds (andrographolide, 14-deoxyandrographolide, 14-deoxy-12-hydroxyandrographolide, and neoandrographolide) against monocytic leukemia cells (THP-1) resulted to IC_{50} values exceeding 100 $\mu\text{g}/\text{mL}$, suggesting the absence of cytotoxic activities (Tan *et al.*, 2017). The finding from this study disclosed that AP herbal mouthwash is non-toxic to the L929 mouse fibroblast cells. Thus, the use of this herbal mouthwash did not induce risk in the oral ecosystem.

The level of heavy metals in the AP herbal mouthwash fell within a permissible range established by the National Pharmaceutical Regulatory Agency (NPRA); Pb (10.0 ppm), Cd (0.3 ppm), Hg (0.5 ppm), As (5.0 ppm) (National Pharmaceutical Regulatory Division, 2017). A study reported, the heavy metals content of AP methanol extract; As (0.006 ppm), Hg (0.005 ppm), Cd (0.012 ppm), Pb (3.084 ppm) (Kar *et al.*, 2016). The study of toxicity is a crucial prerequisite in the present-day drug discovery channel to establish safety guidelines for the consumer.

CONCLUSION

In this present study, the AP herbal mouthwash was proved to be effective in inhibiting oral pathogens and

the efficacy was found to be statistically significant. The cytotoxicity results revealed that AP herbal mouthwash is non-toxic regarding its heavy metal and cytotoxicity onto brine shrimp and L929 cell lines. Nevertheless, further, *in vivo* studies and clinical trials need to be conducted to accessing mouthwash safety.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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