Malays. Appl. Biol. (2021) 50(3): 163–167

ANTIBACTERIAL ACTIVITY OF *Andrographis paniculata* AQUEOUS EXTRACT AGAINST ORAL PATHOGENS

ANIS AMIERA MUHAMAD ALOJID[1], TUAN NOORKORINA TUAN KUB[1,2*], HALIJAH MIRAN[3], SUHARNI MOHAMAD[4], TUAN NADRAH NAIM TUAN ISMAIL @TUAN MANAH[4], MOHAMAD EZANY YUSOFF[4], ROSMANIZA ABDULLAH[1,2], SITI ASMA' HASSAN[1,2], ZETI NORFIDIYATI SALMUNA[1,2] and NUSAADATUN NISAK AHMAD[5]

[1]*Department of Medical Microbiology & Parasitology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan*
[2]*Medical Microbiology & Parasitology, Hospital Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan*
[3]*Department of Pharmacology, School of Medical Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan*
[4]*School of Dental Sciences, Universiti Sains Malaysia, 16150 Kubang Kerian, Kelantan*
[5]*Faculty of Business Management, Universiti Teknologi MARA, Puncak Alam Campus, 42300 Selangor*
[*]*E-mail: tnkorina@usm.my*

Accepted 8 October 2021, Published online 31 December 2021

Oral diseases occur due to poor oral hygiene practices, leading to the accumulation of dental plaque on the teeth surface. Dental plaque is a complex biofilm made up of hundreds of oral pathogens (Benahmed *et al*., 2021). Concerning oral hygiene, mouthwash containing antimicrobial agents is used to improve oral health. However, particular ingredients in mouthwash have been discovered to become toxic when swallowed and inhaled. Mouthwash highly contains alcohol tends to irritate and be destructive to the mucous membrane. In addition, active ingredients, such as chlorhexidine also known to produce brown discoloration of the teeth, while excessive use of antibiotic drugs has led to an alarming increase in antibiotic resistance to oral pathogens although it is considered as the "gold standard" (Van Swaaij *et al*., 2020). Thus, it's necessitating the need for a new novel antimicrobial in dental health. Nowadays, mouthwash formulation from natural ingredients is an improvement of dental products. *Andrographis paniculata* (AP) is one of the potential medicinal herbs with antimicrobial properties. The plant is known as '*Hempedu Bumi*', 'Bile of Earth' or literally 'King of Bitters'. A study conducted by Alexander (2017) revealed that the AP methanol extract is effective against *Staphylococcus aureus, Klebsiella pneumonia,* and *Salmonella typhi.* Due to the potential of AP as an antibacterial agent, this study tested oral pathogens, such as *Porphyromonas gingivalis, Streptococcus mutans, Streptococcus sobrinus, Staphylococcus aureus*, and *Actinomyces viscosus,* using AP aqueous extract. As there is still a lack of standard methods for testing herbal extract, this study evaluates two methodologies: well diffusion and disc diffusion methods. This study also embarks to compare the efficacy of AP extract and commercial mouthwash, namely Colgate Plax®, used as a control.

Firstly, the ground dried AP powder from the whole plant was purchased (Best Farm Co.) and 120 g of AP powder was extracted using the Soxhlet instrument. One liter of distilled water was heated at 100°C, and the extraction process was performed for 48 h. Finally, the extract was evaporated and freeze-dried (Gahlot *et al*., 2018). Bacterial stock cultures were obtained from American Type Culture Collection (ATCC). *Staphylococcus aureus* (ATCC®25923™), *Streptococcus sobrinus* (ATCC®33478™), *Streptococcus mutans* (ATCC®35668™), *Actinomyces viscosus* (ATCC®15987™), and *Porphyromonas gingivalis* (ATCC®33277™). The bacteria were cultured onto blood agar. All bacteria were incubated at 37°C in anaerobic conditions for 24 h except *S. aureus,* incubated in aerobic condition. Growth from freshly sub-cultured isolates was suspended in 5 mL of Mueller Hinton Broth (MHB) to obtain turbidity of 0.5 McFarland standard (Bernardo *et al*., 2021). Mueller Hinton Agar (MHA) plates were inoculated with *S. aureus*. Meanwhile, Mueller Hinton Blood

* To whom correspondence should be addressed.

Agar (MHBA) plates were inoculated with *P. gingivalis, S. mutans, S. sobrinus,* and *A. viscosus.* All plates were made in triplicates (n=3). AP aqueous extract was prepared at concentrations of 0.125 g/mL, 0.25 g/mL, 0.5 g/mL, and 1.0 g/mL were subjected to a serial dilution until they reached 1 (undiluted), 1:2, 1:4, 1:8, and 1:16 dilution. For the disc diffusion method, discs were impregnated with 20 µL of each prepared dilution. For control, an undiluted commercial mouthwash product was used, Colgate Plax® and distilled water were used as negative controls. The plates were incubated at 37°C for 24 h accordingly. The diameter of inhibition zones was observed and measured (Azizah *et al*., 2020). As for the well diffusion method, each well was filled with 60 µL of AP extract dilutions, as prepared earlier. Colgate Plax® was considered a positive control and distilled water as a negative control. The diameter of inhibition zones (mm) was measured after 24 h (Nath, 2018). Data analysis was performed using the Statistical Package for Social Sciences (SPSS) software, version 25.0. A generalized linear model test was run, followed by the analysis of variance one-way ANOVA for each experimental group. It is considered significant if the p-value is less than 0.05 (p<0.05).

Table 1 and Table 2 demonstrate the inhibition zone for the disc diffusion method and well diffusion method, respectively. The study revealed the AP crude extract was effective in inhibiting the growth of *S. aureus, S. mutans, S. sobrinus, A. viscosus,* and *P. gingivalis* (p<0.05) from concentrations of 0.125 g/mL to 1.0 g/mL (Figure 1 & Figure 2). Results showed that using the well diffusion method, the highest inhibition zone for the highest concentration of AP crude extract was ranged at 2.83 ± 4.91 mm – 14.37 ± 0.83 mm (Table 1), while for the disc diffusion method was at 2.93 ± 5.08 mm – 13.37 ± 0.57 mm (Table 2). Furthermore, among these two methods, the well diffusion method showed greater antibacterial activity against the test organisms as compared to the disc diffusion method. In this present work, the antibacterial activity of the AP crude extract showed that most of the inhibition zones recorded on *S. mutans, S. sobrinus,* and *A. viscosus* ranged from 2.93 ± 5.08 to 12.82 ± 0.85 mm.

Commercial mouthwash, Colgate Plax used as a control, also demonstrated high antimicrobial activity against all tested pathogens. It might be due to the reciprocal effect resulting from different chemical compositions in the control mouthwash formulation as compared to the experimental AP crude extract. A study done by Alash and Mohammed (2019) revealed that Colgate Plax mouthwash is the most effective mouthwash when compared to other commercial mouthwashes, such as Listerine and ZAK. This is due to the phenol-conjugated essential oil (thymol, menthol, methyl salicylate in up to 26% alcohol) contained in Colgate Plax mouthwash which was found to be destructive to the bacterial cell by inhibiting the bacterial enzyme.

This present work was supported by the finding by Zhang *et al*. (2019) confirmed the remarkable antibacterial activity of bioactive compound; Andrographolide found in the AP plant. In direct bacteriostatic action, Andrographolide can prevent the formation of bacterial biofilms, the synthesis of virulence factors, bacterial adhesion, and the loss of

Table 1. Diameter of inhibition zone (mm) of AP crude extract and Colgate Plax mouthwash against selected oral pathogens using disc diffusion method

Tested Organisms	Control	Dilution of AP crude extract	AP crude extract concentration (g/mL)			
	Colgate Plax		1.0	0.5	0.25	0.125
S. mutans	11.91 ± 0.83	1	NA	10.67 ± 1.15	6.77 ± 5.86	7.90 ± 6.87*
		1:2	7.20 ± 6.25	11.43 ± 1.25	10.75 ± 0.43*	12.27 ± 0.40
		1:4	9.57 ± 1.25*	11.50 ± 1.82	3.67 ± 6.35	4.17 ± 7.22
		1:8	11.10 ± 1.82	4.33 ± 7.51	NA	NA
		1:16	12.37 ± 0.91	NA	NA	NA
S. sobrinus	11.52 ± 1.00	1	NA	8.67 ± 7.51	8.33 ± 7.23	9.60 ± 0.66
		1:2	NA	11.33 ±1.53	12.50 ± 0.50*	12.82 ± 0.85
		1:4	NA	9.67 ± 1.15*	10.75 ± 1.09	12.35 ± 0.30*
		1:8	NA	5.43 ± 4.75	9.77 ± 0.46*	11.77 ± 1.12
		1:16	NA	NA	9.90 ± 0.30*	6.67 ± 5.77
A. viscosus	13.53 ± 1.72	1	13.37 ± 0.57	7.10 ± 6.17	9.00 ± 0.35*	7.55 ± 0.48*
		1:2	11.88 ± 2.01	9.87 ± 1.33*	8.43 ± 0.64*	8.87 ± 0.99*
		1:4	9.83 ± 0.92*	8.90 ± 0.17*	8.07 ± 0.55*	8.08 ± 1.07*
		1:8	8.70 ± 0.36*	8.00 ± 0.00*	7.93± 0.75*	8.70 ± 0.00*
		1:16	2.93 ± 5.08*	7.90 ± 1.73*	7.57 ± 0.55*	6.77 ± 0.38*

[#] Data expressed as mean ± standard deviation, NA is no activity, * denotes significant when the value of p<0.05 compared to control.
Note: No inhibition zone for *S. aureus* and *P. gingivalis*.

Table 2. Diameter of inhibition zone (mm) of AP crude extract and Colgate Plax mouthwash against selected oral pathogens using well diffusion method

Tested Organisms	Control	Dilution of AP crude extract	AP crude extract concentration (g/mL)			
	Colgate Plax		1.0	0.5	0.25	0.125
S. mutans	13.19 ± 0.62	1	11.33 ± 1.15*	9.33 ± 0.58*	8.20 ± 0.35*	7.40 ± 0.00*
		1:2	9.77 ± 0.25*	8.50 ± 0.00*	7.33 ± 0.29*	NA
		1:4	8.83 ± 0.76*	7.37 ± 0.18*	NA	NA
		1:8	7.63 ± 0.12*	NA	NA	NA
		1:16	NA	NA	NA	NA
S. sobrinus	13.06 ± 0.64	1	8.87 ± 0.47*	10.38 ± 0.23*	8.27 ± 0.46*	9.10 ± 0.00*
		1:2	9.58 ± 0.72*	9.27 ± 0.14*	7.50 ± 0.00*	7.00 ± 0.00*
		1:4	9.13 ± 0.75*	9.00 ± 0.00*	NA	NA
		1:8	NA	NA	NA	NA
		1:16	NA	NA	NA	NA
A. viscosus	16.04 ± 2.27	1	10.67 ± 0.58*	9.90 ± 0.35*	8.10 ± 0.00*	7.70 ± 0.00*
		1:2	9.45 ± 0.26*	8.50 ± 0.35 *	NA	NA
		1:4	7.83 ± 0.29*	NA	NA	NA
		1:8	7.20 ± 0.00*	NA	NA	NA
		1:16	NA	NA	NA	NA
P. gingivalis	18.91 ± 0.00	1	14.37 ± 0.83*	11.00 ±1.00*	10.53 ± 0.55*	NA
		1:2	13.20 ± 0.69*	6.57 ± 5.69*	8.60 ± 0.65*	NA
		1:4	10.83 ± 0.58*	2.85 ± 4.94*	6.02 ± 5.22*	NA
		1:8	8.95 ± 0.33*	NA	2.83 ± 4.91*	NA
		1:16	7.40 ± 0.00*	NA	NA	NA

Data expressed as mean ± standard deviation, NA is no activity, * denotes significance when the value of $p<0.05$ compared to control.
Note: *S. aureus* only showed inhibition at 1.0 g/mL AP extract (8.80 ± 0.22*).

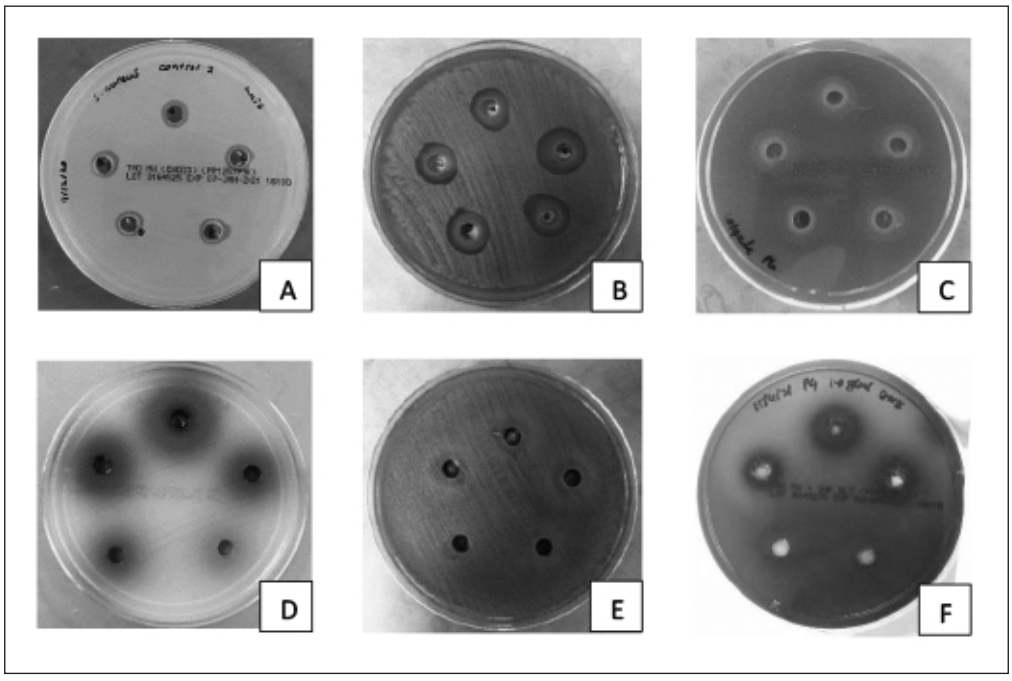

Fig. 1. Antimicrobial activities by well diffusion method; (A-C) using Colgate Plax mouthwash against; (A) *S. aureus*, (B) *S. mutans*, and (C) *P. gingivalis*. (D-F) using 1.0 g/mL AP crude extract against; (D) *S. aureus*, (E) *S. mutans*, and (F) *P. gingivalis*.

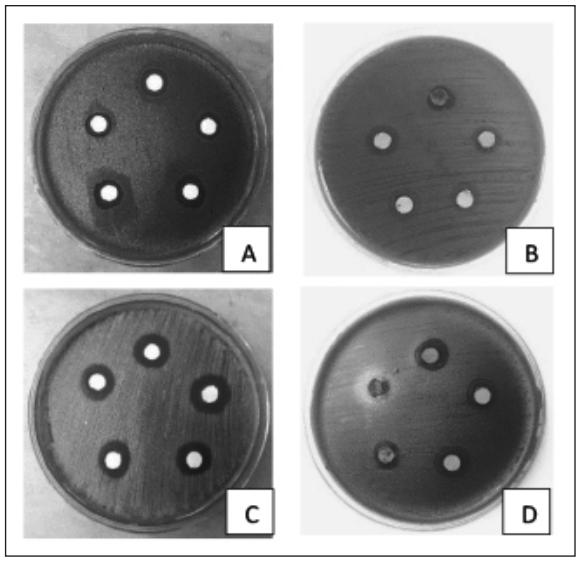

Fig. 2. Antimicrobial activities by disc diffusion method; (A-B) using Colgate Plax mouthwash against; (A) *S. sobrinus,* and (B) *A. viscosus*. (C-D) using 1.0 g/mL AP crude extract against; (C) *S. sobrinus*, and (D) *A. viscosus.*

bacterial integrity. As a result, this study recorded an increasing trend of inhibition when the concentrations of the AP crude extract were increased. For example, these trends were recorded in Table 1 at 1.0 g/mL AP extract for *S. mutans* (12.37 ± 0.91), while the lowest AP concentration (0.125 g/mL) showed a lower inhibition zone for these bacteria (4.17 ± 7.22). However, there was a slightly various trend in certainly tested microorganisms. The variation could be attributed to the synergistic effect of herbal extract, always occurring in herbal-based products (Caesar & Cech, 2019).

The AP crude extract showed maximum growth inhibition at the highest concentration of 1 g/mL (13.37 ± 0.57) against *A. viscosus* compared to other bacteria in the agar disc diffusion test. Otherwise, in the agar well diffusion test, *P. gingivalis* showed a maximum inhibition zone at a concentration of 1 g/mL (14.37 ± 0.83) compared to other bacteria. In contrast, the AP crude extract displayed the least inhibition against *S. aureus* in both tests. Furthermore, it did not display any inhibitory effect against *S. aureus* in agar disc diffusion. In agar well diffusion, it only exhibited inhibition on the highest concentration and undiluted AP crude extract (1.0 g/mL). The result is in contrast to a study by Aniel *et al*. (2016) that showed high antimicrobial activity of AP methanolic extract against *Staphylococcus aureus*. These findings evaluate that variation of extraction methods could have influenced some of the antimicrobial properties of the herbal compound.

A negative finding was also displayed against *P. gingivalis,* in which there was no sign of inhibition in the disc diffusion method. A possible explanation for the finding may be due to the differences in the outer layer membrane of gram-negative bacteria. *P. gingivalis,* which is gram-negative bacteria, possess a unique periplasmic space of the outer membrane. Therefore, the hydrophilic plasma membrane inhibits the AP crude extract accumulation; thus, lowering the efficacy (Polash *et al*., 2017).

The statistical analysis showed a significant difference between agar disc diffusion and agar well diffusion methods ($p<0.05$). Between the tested methods, agar well diffusion appeared to be more effective to inhibit the growth of the selected oral pathogens. The comparison among the methods can be explained by the fact that the AP crude extract has a relatively high molecular weight; thus, causing low migration of the AP crude extract from the disc into the agar medium. Another possible reason is that the difference in the AP crude extract's absorption capacity in the disc diffusion method compared to the maximum capacity of the AP crude extract impregnated into the well also supported the results, suggesting that the agar well diffusion method is more effective than the agar disc diffusion method for measuring inhibition zones (Tuan Kub *et al*., 2021).

As the AP crude extract exhibited antimicrobial activity towards most oral pathogen (5 out of 5 in well diffusion method & 3 out of 5 in disc diffusion method) and it is known that the AP plant is widely available in Malaysia, AP's potential is to be exploited in the oral hygiene industry. Moreover, this study will provide scientific data of recommended concentrations that can benefit the pharmaceutical industries to formulate new herbal products in absence of alcohol in developing alternative treatments of oral diseases. As far as the problem of existing mouthwash is concerned, detailed research is needed to assess the toxicity effect of the AP and its safe concentration for consumer use.

CONCLUSION

This study has proven the efficacy of the AP crude extract as a potential antimicrobial agent. The best concentration of the AP crude extract was at 1.0 g/mL without any serial dilution. Among the methods used, the effectiveness of agar well diffusion for measuring antimicrobial activity against selected oral pathogens has been confirmed.

ACKNOWLEDGEMENT

We would like to acknowledge Universiti Sains Malaysia for the financial support via a short-term grant (304/PPSP/6315341) for this study.

REFERENCES

Alash, S.A. & Mohammed, M.Q. 2019. Antibacterial activity of some mouthwash solutions against *Staphylococcus lentus* isolated from mouth infections. *Iraqi Journal of Science*, **60(12)**: 2583-2589.

Alexander, S.C.P. 2017. Antibacterial activity of *Andrographis paniculata* extracts. *The Pharma Innovation Journal*, **6(5)**: 01-04.

Aniel, K., Naidu, L. & Rao. 2010. *In vitro* antibacterial activity in the extrats of *Andrographis paniculata* Burm. F. *International Journal of Pharmtech Research*, **2(2)**: 1383-1385.

Azizah, M., Pripdeevech, P., Thongkongkaew, T., Mahidol, C., Ruchirawat, S. & Kittakoop, P. 2020. UHPLC-ESI-QTOF-MS/MS-based molecular networking guided isolation and dereplication of antibacterial and antifungal constituents of *Ventilago denticulata*. *Antibiotics*, **9(9)**: 606.

Balouiri, M., Sadiki, M. & Ibnsouda, S.K. 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of Pharmaceutical Analysis*, **6(2)**: 71-79.

Benahmed, A., Gasmi, A., Dadar, M., Arshad, M. & Bjorklund, G. 2021. The role of sugar-rich diet and salivary protein in dental plaque formation and oral health. *Journal of Oral Biosciences*, **63(2)**: 134-141.

Caesar, L.K. & Cech, N.B. 2019. Synergy and antagonism in natural product extracts: When 1 + 1 does not equal 2. *Natural Product Reports*, **36(6)**: 869-888.

Gahlot, M., Bhatt, P., Joshi, J., Fellow, S.R. & Pantnagar, T. 2018. Study on yield of plant extracts using different solvents and methods. *Bulletin of Environment, Pharmacology and Life Sciences*, **7(6)**: 65-67.

Nath, S. 2018. Syringe punch and delivery protocol for well diffusion test. *MOJ Toxicology*, **4(1)**: 23-24.

Polash, S.A., Saha, T., Hossain, M.S. & Sarker, S.R. 2017. Investigation of the phytochemicals, antioxidant, and antimicrobial activity of the *Andrographis paniculata* leaf and stem extracts. *Advances in Bioscience and Biotechnology*, **8(5)**: 149-162.

Sadhana, H.M, Suresh, J. & Hamsalakshmi. 2020. *Andrographis paniculata* – a review. *International Journal of Research in Pharmaceutical Sciences*, **11(4)**: 5395-5400.

Tuan Kub, T.N., Ab Manaf, N.A. & Che Ibrahim, A.S. 2021. Comparison of well diffusion, disc diffusion and broth dilution methods for antimicrobial activity of *Andrographis paniculata* herbal gel against acne-associated pathogens. *Malaysian Journal of Microbiology*, **17(1)**: 90-96.

Van Swaaij, B.W.M., Van Der Weijden, G.A., Bakker, E.W.P., Graziani, F. & Slot, D.E. 2020. Does chlorhexidine mouthwash, with an anti-discoloration system, reduce tooth surface discoloration without losing its efficacy? A systematic review and meta-analysis. *International Journal of Dental Hygiene*, **18(1)**: 27-43.