

SHORT COMMUNICATION

Comparison of well diffusion, disc diffusion and broth dilution methods for antimicrobial activity of *Andrographis paniculata* herbal gel against acne-associated pathogen

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ABSTRACT

Aims: Acne is a common skin disease among teenagers and also affects other ages. It occurs when the oil and dead skin cells plug into the hair follicles and causing pimples or whitehead. Although antibiotics have been used for many years in treating acne, the widespread use of it has led to the development of bacterial resistant, which resulted in unsuccessful treatment. Thus, in this study, *Andrographis paniculata* (AP) herbal formulation gel is proposed in order to determine its effectiveness in treating acne. Three different methodologies were used to compare the antimicrobial effect of *A. paniculata* herbal gel against acne-associated pathogens.

Methodology and results: Well diffusion, disc diffusion and broth dilution methods were applied to evaluate the antimicrobial effect of AP herbal gel at concentrations of 1.5% (w/w), 2.5% (w/w) and 5.0% (w/w) onto selected pathogens associated with acne which consisted of *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Propionibacterium acnes* and *Candida albicans*. Among the three methods, broth dilution showed the best antimicrobial effect towards all microorganisms used. AP herbal gel at concentration 2.5% (w/w) showed the optimum antimicrobial effect of *S. aureus* and *C. albicans*, while 5.0% (w/w) exhibited the best antimicrobial activities for *P. acnes* and *S. epidermidis*.

Conclusion, significance and impact of study: Broth dilution method appears to be a reliable method for the determination of antimicrobial effects for the pathogens tested. In addition, AP herbal formulation gel has great potential to treat acne effectively.

Keywords: Acne, *Andrographis paniculata*, well diffusion, disc diffusion, broth dilution

INTRODUCTION

Acne usually happens when oil and dead skin cells plug into the hair follicles and the consequence. This situation commonly causes a lot of symptoms such as pimples and whiteheads. The pimples can occur on many parts of the body including face, shoulders, upper back, chest and forehead (Tan *et al.*, 2017). The most common pathogen associated with acne are *Staphylococcus epidermidis* and *Propionibacterium acnes*. There was also evidence which suggested the infections of *Staphylococcus aureus* and

Candida albicans associated with acne (Khorvash *et al.*, 2012).

The acne development is a process of excretion of sebum and followed by the changes of microbial flora which associated with the pathogens' infections. Generally, antibiotic therapy is a common management of acne since many years ago. However, the widespread use of antibiotics has led to the antimicrobial resistance among microorganisms (García-Sosa *et al.*, 2011).

The resistant of *P. acnes* had been reported, but the antibiotic resistant is different among different countries.

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This might happen due to the difference of clinician antibiotic prescribing habit and the use of topical agents such as retinoids, benzoyl peroxide, erythromycin, tetracycline, doxycycline and trimethoprim. The different populations also isolated different strain of *P. acnes* and therefore the resistant pattern were various among different countries (Dessinioti and Katsambas, 2017). The prolonged use of topical clindamycin was also associated with antibiotic resistance of this antibiotic onto *P. acnes* (Leyden and Lev, 2001).

Staphylococcus aureus recorded the increasing trend of resistance to tetracycline, erythromycin and clindamycin which is consistent to other researchers' reports (Ashkenazi *et al.*, 2003). A study conducted by Eladli *et al.* (2019) also revealed the antibiotic resistance of fosfomycin and erythromycin onto *S. epidermidis*. As the subject of the study included patient and healthy people, the study concluded that multi-resistant *S. epidermidis* strains are widespread among them.

The resistance of *C. albicans* play a role of recurrence with acne after it is completely treated. The factors such as changing seasons and changing habits are among the risk of antifungal resistance. Most of resistance *C. albicans* cases are the major factors of defeated sequential treatment which most of them are resistant to common antifungal drugs, itraconazole, fluconazole and ketoconazole (Omran and Mansori, 2018).

In order to search for alternative medicine, medicinal plants have served as new resources for antimicrobial therapy. *Andrographis paniculata* (AP) is one of a potential antimicrobial agent. It is a herb used traditionally to treat various diseases such as, respiratory infections, influenza and dyspepsia (Okhuarobo *et al.*, 2014). In Malaysia, it is locally known as "hempedu bumi". Since time long ago, the antimicrobial effects of this plant have been discovered. It has shown several pharmacological activities such as anti-typhoid, anti-fungal, anti-bacterial, anti-malarial, anti-hepatitic, and anti-inflammatory activities (Asai *et al.*, 2012; Benoy *et al.*, 2012; Darestani *et al.*, 2014). The major chemical compounds presence in this plant are diterpene andrographolide and neo andrographolide, which contributed to the medicinal effects of the plant (Siripong *et al.*, 1992). In order to treat microbial infections, AP was also used traditionally by topical application.

It is reported that *A. paniculata* plant has the enzyme induction mechanism which contributed to the therapeutic value of antimicrobial effect. It is useful in treating diseases such as dysentery, cholera, fever, diabetes, sore throat, tonsillitis, arthralgia, piles and gonorrhoea (Kumar *et al.*, 2019). Antimicrobial effects of AP were recorded for various organism including *Salmonella typhi* and *Helminthosporium sativum*. Besides that, its shoot extract was effective against *Micrococcus pyogenes* var *aureus*, while diluted sulphuric acid extract inhibited *Escherichia coli* (Okhuarobo *et al.*, 2014). Since long ago, the plant decoction was consumed to treat high blood pressure and jaundice (Wiar, 2000).

Due to the potential of microbial effects of AP, in this study, AP ethanol extract was formulated into gel form

and tested onto selected acne-associated pathogens: *S. epidermidis*, *S. aureus*, *P. acnes* and *C. albicans*. Owing to the lack of international standard methods for antimicrobial activities of herbal formulation, three methodologies has been used in this study. The three methodologies include; well diffusion, disc diffusion and broth dilution methods. This study was conducted to compare the methods for screening the antimicrobial effect of AP herbal gel onto the acne-associated pathogens.

MATERIALS AND METHODS

Plant materials and preparation of AP ethanol extract

Andrographis paniculata fresh plant was collected from Kampung Machang-Limbat, Kota Bharu, Kelantan and cleaned using tap water. After that, it was dried and blended into powder form. An amount of 100 g AP powder was then soaked and stirred into one liter of ethanol at room temperature (25 °C to 32 °C) and kept overnight. After that, it was filtered and evaporated using rotary evaporator. The concentrated plant extract was then placed in universal bottle and kept in freezer at -20 °C before used.

Preparation of AP gel

For the preparation of AP gel, AP ethanol extract was added into petroleum jelly (Vaseline, Malaysia) and mixed together. After that, it was stirred using homogenizer to obtain AP gel concentrations at 1.5% (w/w), 2.5% (w/w), and 5.0% (w/w). They were melted together at approximately 75 °C using double boiler technique for about ten minutes. The freshly prepared AP gel was used every time conducted new experiment to maintain its' sterility.

Primary microbial isolates

Microbial stock cultures were collected from the Mycology Laboratory, Department of Medical Microbiology and Parasitology, School of Medical Sciences, Universiti Sains Malaysia. The microbes used in this study included *S. epidermidis*, *S. aureus*, *P. acnes* and *C. albicans*. The microorganisms were isolated using standard laboratory methods by inoculating *S. epidermidis*, *S. aureus* and *P. acnes* onto blood agar plates, while *C. albicans* onto Sabouraud dextrose agar plates. All the microorganisms were incubated at 35 °C in aerobic conditions, except for *S. epidermidis* and *P. acnes*, which were incubated in anaerobic conditions at the same temperature. After the organisms' growth was established, the colonies were used for further studies.

For the preparation of microbial inoculum, each colony was transferred to a tube with 5 mL of Mueller Hinton broth (MHB) to obtain a concentration of approximately 0.5 McFarland turbidity standard.

Preparations of AP formulation disc

The antimicrobial susceptibility test disc (Oxoid, United Kingdom) with 6.3 mm diameter was used as the test discs. The discs were impregnated with 10 mg of AP ethanol extract gel at concentrations of 1.5% (w/w)/disc, 2.5% (w/w)/disc, and 5.0% (w/w)/disc. After that, they were left to dry at room temperature (25 °C to 32 °C).

Disc diffusion method

The agar plate surface was spread with microbial inoculum for each microbial isolate. The Mueller Hinton agar plate was used for *S. epidermidis* and *S. aureus*, whereas the Mueller Hinton blood agar plate was used for *P. acnes*. On the other hand, the Sabouraud dextrose agar plate was used for *C. albicans*. The culture was inoculated by spreading the inoculum over the entire agar surface. After that, the AP formulation discs were placed onto the surface of the agar. There were three agar replicates for each microorganism tested. The culture was incubated under suitable conditions as the primary microbial isolates. The principle of this method is that the antimicrobial agent will diffuse into the agar and inhibits the growth of microorganism, and finally the growth inhibition zones were measured.

Agar well diffusion method

The procedure for agar well diffusion is almost similar to the disc diffusion as mentioned above. Briefly, 100 µL of microbial inoculum was spread onto the surface of the agar. After that, 6 mm hole was punched into the agar using aseptic technique and 10 mg of AP gel was introduced into each well using sterile micropipette. Each plate contained four wells which consisted the control gel (base gel only), 1.5% (w/w), 2.5% (w/w), and 5.0% (w/w) of AP gel. Finally, the agar plates were incubated at suitable conditions as in primary microbial isolates. Three agar replicates were used for each microorganism.

Broth dilution method

In broth dilution method, various dilution was prepared by mixing 13 g of the AP gel and bringing the volume up to 26 mL using MHB. The AP gel used were 1.5%, 2.5% and 5.0% (w/w). The final concentrations of AP gel and MHB at 5, 10, 15, 20 and 25% (w/v) were tested against each type of microorganisms by inoculating with a standard inoculum of *S. epidermidis*, *S. aureus*, *P. acnes* and *C. albicans* which were adjusted to 0.5 McFarland turbidity standard. All tests were performed in triplicates in order to obtain reliable results.

After overnight aerobic incubation for *S. epidermidis*, *S. aureus*, and *Candida albicans* at 35 °C, and anaerobic incubation for *P. acne*, the optical density (OD) of the suspensions was measured using spectrophotometer at 620 nm. The AP gel that prevented the growth of each microorganism, as detected by lack of visual turbidity compared to negative control, was recorded as the

percentage of microbial inhibition. The higher percentage indicates the higher inhibition and the lower percentage indicates the lower inhibition, while the negative values indicates the growth promotion of the microorganism. The percentage of microbial inhibition was calculated as the formula adapted from Campbell (2011).

Percentage (%) of microbial inhibition =

$$1 - \frac{OD_t - OD_{Nc}}{OD_{Vc} - OD_{Bo}} \times 100$$

where, t: test well (wells containing MHB, AP gel and microorganisms' inoculum); Nc: negative control well (wells containing MHB and AP gel only); Vc: viability control well (wells containing MHB and microorganisms' inoculum only); Bo: broth only well (wells containing MHB only)

RESULTS AND DISCUSSION

In agar well diffusion and disc diffusion method, all of the tested microorganisms were managed to grow in all AP gel concentrations tested from 1.5% (w/w) to 5.0% (w/w). Therefore, both of the methods showed no zone of inhibition around the tested discs and well (Figures 1 and 2). However, positive results were recorded using broth dilution method which showed microbial inhibition of certain microorganisms tested.

Negative findings of agar disc diffusion and well diffusion method might be due to the reason that the microorganisms were not fully exposed to the whole AP gel tested. Certain antimicrobial compounds are problematic and not diffused well into the agar plate as the physiochemical properties of the AP gel is not suitable for disc diffusion. An antimicrobial study by Agyare *et al.* (2013) of *Justicia flava* and *Lansea welwitschii* extracts onto bacteria had showed that the extracts were less active when the well diffusion method was used. This result is comparable with the previous study which suggested the possible reason of non-active AP gel. This is because of the extract which contained active principal compounds could not diffuse into the agar medium and demonstrated the effects. Fernández-Torres *et al.* (2006) had reported that disc diffusion method is not recommended to use on azoles as these drugs diffused poorly in the agar. Other than azoles, vancomycin, colistin, and clarithromycin have higher molecular weights and therefore diffuse very slow in agar. These findings are in agreement with the present study which suggested the higher molecular weight of AP gel had the limited diffusion and poorly resolved into the disc and resulted in the ambiguous result.

Table 1 shows the percentage of inhibition of AP gel towards acne causing pathogen by broth dilution method. At 2.5% (w/w) and 5.0% (w/w) of AP gel concentrations, most of the microorganisms showed inhibition growth. Certain microorganisms showed negative results which promoted the organisms' growth at 1.5% (w/w) AP gel,

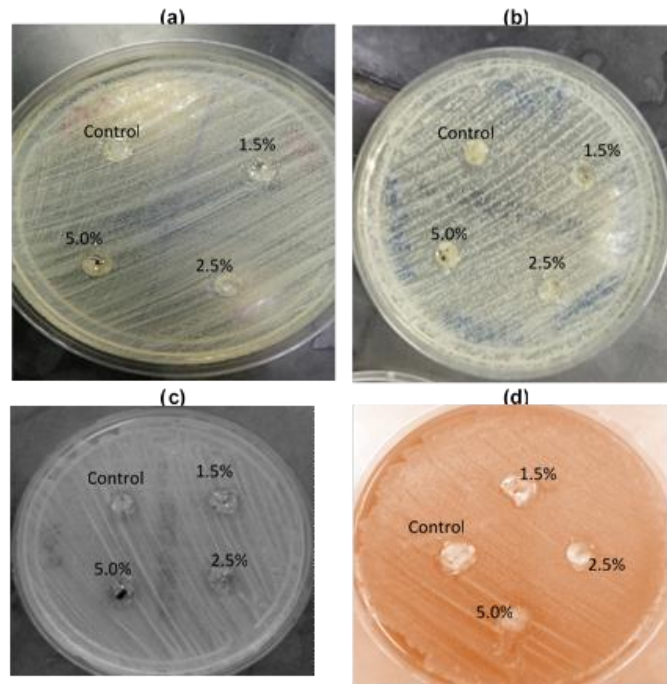


Figure 1: The antimicrobial study of microorganisms using well diffusion method; a) *S. aureus*, b) *C. albicans*, c) *S. epidermidis*, and d) *P. acnes*. Note: 1.5%, 2.5% and 5.0% (w/w) are the AP gel concentrations, while control is the base gel.

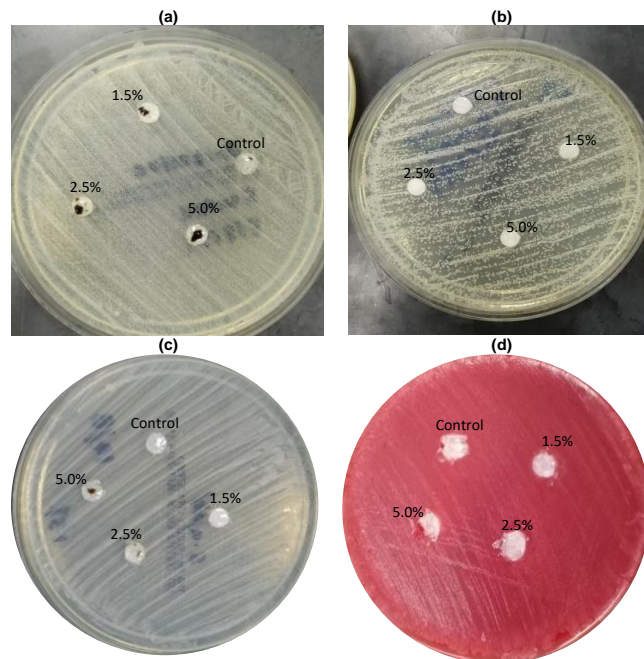


Figure 2: The antimicrobial study of microorganisms using disc diffusion method; a) *S. aureus*, b) *C. albicans*, c) *S. epidermidis*, and d) *P. acnes*. Note: 1.5%, 2.5% and 5.0% (w/w) are the AP gel concentrations, while control is the base gel.

Table 1: The effect of AP gel onto percentage of microbial inhibition using broth dilution method towards acne causing pathogen.

AP gel concentration (%) (w/w)		Microbial inhibition (%)											
		<i>S. aureus</i>			<i>C. albicans</i>			<i>S. epidermidis</i>			<i>P. acnes</i>		
AP gel/ MHB concentration (%) (w/v)	AP gel concentration (%) (w/w)	1.5	2.5	5.0	1.5	2.5	5.0	1.5	2.5	5.0	1.5	2.5	5.0
		5		24.28 ± 0.12	60.51 ± 0.20	13.56 ± 0.01	56.58 ± 0.14	41.67 ± 0.32	25.88 ± 0.19	40.00 ± 0.04	73.03 ± 0.01	54.04 ± 0.01	-14.40 ± 0.02
10		-27.59 ± 0.07	75.14 ± 0.16	76.32 ± 0.01	25.00 ± 0.28	38.74 ± 0.16	35.53 ± 0.10	73.20 ± 0.02	67.60 ± 0.02	72.70 ± 0.12	24.77 ± 0.03	10.29 ± 0.07	145.10 ± 0.05
15		-3.01 ± 0.15	2.73 ± 0.01	6.05 ± 0.20	66.23 ± 0.22	44.01 ± 0.08	33.04 ± 0.83	76.80 ± 0.01	85.10 ± 0.02	95.70 ± 0.02	9.19 ± 0.21	74.84 ± 0.04	94.66 ± 0.08
20		-17.09 ± 0.03	3.89 ± 0.03	52.72 ± 0.09	46.49 ± 0.16	55.26 ± 0.11	79.39 ± 0.65	70.80 ± 0.03	76.00 ± 0.02	75.80 ± 0.06	1.93 ± 0.08	42.20 ± 0.02	65.30 ± 0.17
25		-14.06 ± 0.09	13.84 ± 0.14	30.63 ± 0.09	46.36 ± 0.06	38.45 ± 0.03	41.23 ± 0.58	89.40 ± 0.05	79.50 ± 0.02	68.20 ± 0.05	22.95 ± 0.69	77.05 ± 0.13	59.80 ± 0.10

such as *S. aureus* at 5 to 25 % (w/v) and *P. acnes* at 5% (w/v).

From Table 1, among all the tested AP gel concentrations, 5.0% (w/w) of AP gel exhibited the highest inhibition against all microorganisms tested. The AP gel concentration is considered as effective if the microbial inhibition is more than 90% (Akinduti *et al.*, 2019). By applying 5.0% (w/w) of AP gel, it was able to inhibit *P. acnes* at 10% (w/v) (145.1%) and 15% (w/v) (94.66%) of AP gel-MHB. Similarly, 5.0% (w/w) AP gel also able to inhibit the growth of *S. epidermidis* at 15% (w/v) (95.7%) and 20% (w/v) (75.8%) of AP gel-MHB. The growth of *S. aureus* and *C. albicans* were also inhibited by 5.0% (w/w) AP gel, at 10% (w/v) (76.32%) and 20% (w/v) (79.39%) of AP gel-MHB respectively.

As 1.5% (w/w) AP gel tested on *S. aureus* showed growth promotion but not inhibition, in general, this concentration is not suitable to be used for the treatment of acne associated infection. Therefore, by excluding the 1.5% (w/w) AP gel concentrations, the minimum inhibitory concentration for antimicrobial effect of the acne associated pathogen tested is considered at 2.5% (w/w) AP gel, and the best antimicrobial effect is at 5.0% (w/w).

Most of the tested microorganisms showed the increasing trend of inhibition when the concentrations of AP gel were increased. However, the dilution of AP gel and Muller Hinton broth didn't seem to decrease the microbial inhibition as there were various trend of percentage of microbial inhibition among all organisms tested. These could happen due to the reason that Muller Hinton broth could contain certain agent that is chemically antagonistic to the AP gel which made the actual concentration in the broth to be less or more than the value defined. The phenomenon of antagonistic effect commonly occurred in natural product extracts which resulted in conflicting results (Caesar and Cech, 2019).

Broth dilution is commonly used for antimicrobial assay against various microorganism. However, this method has the high risk of bacterial contamination (Luber *et al.*, 2003). Among the three methodologies tested in this study, broth dilution is the best method to assess the antimicrobial effect of AP gel.

Meyer *et al.* (2011) had studied the comparison of broth microdilution and disk diffusion method onto *Yersinia enterocolitica* 4/O:3 strains and found that disk diffusion produced high rates of very major errors for ampicillin and a high frequency of minor errors for sulfamethoxazole, compared to broth microdilution method. Another study conducted by Inneke *et al.* (2007) comparing the disc diffusion and broth microdilution method of *P. aeruginosa* showed a major error rates of susceptibility when comparing polymyxin B disc diffusion with the broth microdilution, however for disc diffusion, susceptibility of colistin did not report major errors. These showed that different psychochemical of antimicrobial agents tested showed the various susceptibility result when the different assay was carried out.

Although the Clinical and Laboratory Standards Institute (CLSI) recommended broth dilution as a standard method, sometimes it is associated with technical

drawbacks. For example, the poor end point precision associated with antifungal agent such as azole and fluconazole (Kumar *et al.*, 2010). The molecule size also plays a role in determining whether it is poor or well diffused into agar plate. Certain herbal properties have big molecular compound and therefore decomposition reaction is suggested to make them to smaller molecular compound using gas chromatography analysis (Willow, 2011). These statement supports our results which suggested that AP gel might have the higher molecular weight and not diffused well into agar plate and therefore well diffusion and disc diffusion method are not recommended for susceptibility study of AP gel.

CONCLUSION

After comparing the three methodologies, the usefulness of broth dilution method for determination of the antimicrobial effect of AP gel onto acne associated pathogens has been confirmed. As the method is fast and reasonably priced, the standardization procedure for antimicrobial susceptibility testing of AP gel using broth dilution method can be easily set up.

Besides, this study has showed the potential antimicrobial effect of AP ethanol extract gel. It was found that the best concentration of AP gel was 5.0% (w/w) to obtain a standard medicinal treatment for acne infection. The herbal gel should be further explored for its clinical use in the management of anti-acne treatment. In addition, AP active compounds should be identified to embark the positive effect of AP gel for future commercialization of AP gel formulations.

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