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Phytochemical Screening and Prophylactic Antibacterial Effects of *Andrographis Paniculata* Extracts from Kemaman, Malaysia

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Abstract
Higher plants, as source of medicinal compounds, have continue to play a significant role in the maintenance of health for centuries, especially in developing countries. Plant such as *Andrographis paniculata* extracts have been utilised for the treatment of many diseases including diarrhoea and upper respiratory tract infections. The aim of this study was to screen the phytochemical constituents and prophylactic antibacterial anti-bacterial effect of methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous extracts on *Staphylococcus aureus, Staphylococcus typhemorium, Staphylococcus epidermidis* and *Escherichia coli* respectively. The highest zone of growth inhibition (14 and 10 mm) observed by ethyl acetate and methanol extacts at the lowest concentration of 0.625 mg/mL, against *S.aureus ATCC* 9144, respectively. The ethanol: water (1:1 v/v) and aqueous exhibited no activity against the tested pathogenes as compared with the standard ampicillin (2.9 mm) at lowest concentration. Both ethyl acetate and methanol extracts indicated a lower MIC value of 1.25, and 0.625 mg/mL respectively against gram-negative bacteria (*E.coli*), while higher MIC values of 2.5 mg/ml against gram-positive bacteria (*S.aureus and S.typhii*) was observed. The methanol and aqueous extracts showed lower MIC values of 0.625 mg/mL against *E. coli* (indicating better activity) than ethanol: water (1:1 v/v) against both gram -ve bacteria (*S. epidermidis* and *E. coli*). However, all the extracts have lower MIC values of 0.625 mg to 1.25 mg against gram -ve bacteria (*S.epidermidis* and *E.coli*). The result showed a better activity of the extracts against gram -ve bacteria, which are significantly important in causing common problems of bacterial infections in hospitals causing many diseases such as respiratory tract infections.
Keywords: key words, Bacteria, minimum inhibitory concentration, zone of growth inhibition, *Andrographis paniculata*

1. Introduction

Infectious diseases due to microbes such as *Staphylococcus aureus, Staphylococcus typhemorium, Staphylococcus epidermidis* and *Escherichia coli* are extremely prevalent and responsible for mortality around the world (Zhang *et al*., 2013). In the feature the mortality rate may continue to increase due to the following. First, non-availability of anti-microbial agents to tackle antibiotic resistance strains of pathogens and the second is lack of sufficient supply of antibiotics in developing countries. Globally, medicinal plants have received significant attentions as new agents or source of anti-microbial agents to address these challenges. In this respect, it is important to note that more than 90 % of higher plants are not analysed for their bioactivity and hence enormous potentials of these plants are not fully utilised (Zhang *et al*., 2013). The synergistic effects of conventional antibiotics with plant-based flavonoids is proving to be the important new direction in complementary medicine research (Daglia, 2012). Medicinal plants may be defined as any plant that has medicinal use such as foxglove, opium, garlic, etc. Plants may be an important source of potentially useful structures for the development of new therapeutic agents (Mishra et al., 2013).

Plants are alternative for development of common resistance antibiotics because they are cheaper, and are readily available locally. Plants are been rediscovered because of growing awareness of unwanted side effects, drug resistance and other aspects of allopathic medicine (Alagesaboopathi, 2009). Therefore, there is growing interest in the herbal remedies because of their effectiveness, minimal side effects in clinical experiences and relatively low cost. Herbal drugs or their extracts are prescribed widely; even when their biological active compounds are unknown as they are traditionally used (Kamaraj *et al.*, 2012). Higher plants, as a source of medicinal compounds, have continued to play a significant role in the maintenance of health for centuries, especially in developing countries (Mishra *et al*., 2013).

Plant such as *Andrographis paniculata*, belonging to the family Acanthaceae has been used for centuries in Asia to treat several diseases due to its known biological activities (Sattayasai *et al*., 2010). In Malaysia and other countries it is reported to have multiple clinical applications. It's an important cold property herb, used in fevers to remove toxins from the body (Jayakumar *et al*., 2013). Traditional approaches such as ayurvedic system of medicine recommends the use of herbal combination and extracts to improve health, as well

as to prevent microbial infections (Mishra *et al*., 2013; Tambekar & Dahikar, 2010). Therefore, the present investigation evaluate the phytochemical compounds and prophylactic antibacterial effects of *A. paniculata* extracts (methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous) against Gram-positive (*S. aureus, and S. typhii*) and Gram-negative (*S. epidermidis and E. coli*) bacteria respectively.

2. Materials and method

2.1 Collection of plant material

Andrographis paniculata plant was obtained in September from Kemaman in Terengganu, Malaysia. The plant was identified, authenticated by Norhaslinda Haron from Faculty of Agriculture and Animal Sciences, Universiti Sultan Zainal Abidin, and deposited at the herbarium unit with specimen voucher number 00266.

2.2 Test organisms

The bacterial strains of *Staphylococcus aureus* ATCC 9144, *Staphylococcus typhemorium* ATCC 41221, *Staphylococcus epidermidis* ATCC 14990 and *Escherichia coli* ATCC 85218, were used in this study. Antibiotic sensitivity pattern of these test organisms was tested by using FDA-recommended antibiotic and standard methodology

2.3 Preparation of plant extracts

The plant was washed with distilled water, dried at 40^{o}C and then grinded into powdered form. The powdered plant was weighed and soaked in different solvents (methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous) in a ratio of 1:10 respectively. The extracts were then decanted and filtered through filter paper. The filtrate obtained was concentrated using rotary evaporator at temperature 40^{o}C. Extracts were then dried at 50^{o}C, and kept in a freeze at 4^{o}C till use.

2.4 Antibacterial assays

The antibacterial assay of *A.paniculata* was investigated as follows by adopting the method of Wen *et al*., 2014 with slight modifications.

2.4.1 Bacterial preparation

A few single bacterial colonies from overnight culture on on Muller-Hilton-Agar (MHA) were inoculated into nutrient broth (NB) to achieve a turbidity of 0.5 McFarland (1 x 10^{8} CFU/mL) standard. The bacterial suspension were further diluted with NB until a final concentration of the inoculum was 5 x 10^{5} CFU/mL, using a spectrophotometer to get the optical density range (0.01-0.8) of McFarland standard.

2.4.2 Zone of inhibition

The antibacterial assay was performed by agar well diffusion method. Different volumes of the (20-50 µL) of the extracts dissolved in NB to make different concentrations of the extract (10, 5, 2.5, 1,25 and 0.625 mg respectively). The extracts were directly applied to the wells made on the surface of MHA containing bacterial lawn. Control wells received only dimethyl sulfoxide (DMSO). Positive control wells received ampicillin at different concentrations (1000, 500, 250, 125, and 62.5 mg/mL) respectively. After difussion, plates were incubated at 37^{o}C for 18 hours and zone of growth inhibition were measured. Antibacterial activity of the plant extracts was tested in direct proportion method.

2.4.3 Determination of minimalinhibitory concentration (MIC)

The minimum inhibitory concentration of the extracts was determined by broth dilution method using test-tubes. Nutrient broth (NB) medium was prepared according to prescription, sterilised using autoclave at 121^{o}C. 1 mL of the extracts were poured into the test-tubes containing 1 mL of NB to get different concentrations (10, 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mg/ml respectively). 20 µL of the bacterium suspension was then added to each tube and the mixtures were then incubated at 37^{o}C for 24 hours. The lowest concentration of the extracts that prevented the growth of each microorganism , as detected by lack of visual turbidity or no apparent increase compared to a negative control (nutrient broth without extracts and bacteria), was recorded as the MIC. All extracts were freshly prepared before each assay.

2.4.4 Determination of minimum bacteriacidal concentration (MBC)

The minimum bactericidal concentration (MBC) was determined by taking a loop full of the culture medium from each test-tube (from the MIC assay) that showed no apparent growth and sub-culturing on fresh MHA plates. After incubation at 37^{o}C for 24 h, the MBC was read as the least concentration showing no growth on the MHA plates.

3. Results and discussion

3.1 Preliminary phytochemical screening

Table 1: Qualitative preliminary phytochemical screening of *A. paniculata* constituents

TEST	Methanol	Ethyl acetate	Ethanol:water (1:1v/v)	Aqueous
Saponins	-	-	++	+++
Phenols	+	+	+++	+++
Glycosides	+	+	++	+
Flavonoids	+	+	+	+
Alkaloids	+++	+++	++	+
Tannins	-	-	++	++
Terpenoids	+	-	-	++

+ indicates presence of phyto constituents, - indicates absence of phyto constituents

Preliminary qualitative phytochemical test of *A.paniculata* extract indicates the presence of saponins, phenols, glycosides, alkaloids, tanins, and flavonoids in aqueous and ethanol: water (1:1 v/v) while terpenoids tested negative in ethanol: water respectively. Also, methanol and ethyl acetate tested positive for all the constituents except saponins, and tannins that tested negative. Moreover, terpenoids tested negative in ethyl acetate extract (Table 1).

3.2 Qualitative bacterial assay

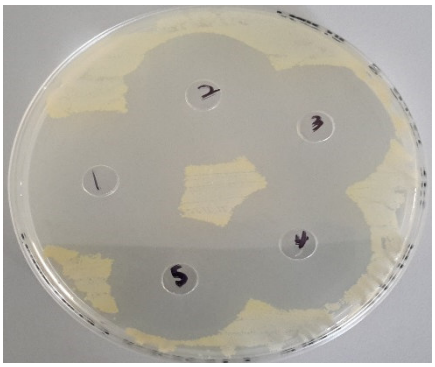
3.2.1 Zone of inhibition

Table 1: Antibacterial activity of *A.paniculata* extracts and the standard (ampicillin) by agar well diffusion

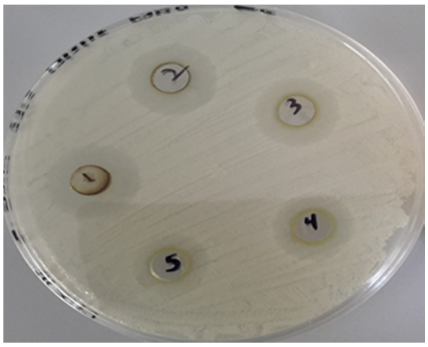
Extracts (mg/ml)	Zone of inhibition (mm)			
	S. aureus	*S. typhii*	*E. coli*	*S. epidermidis*
Methanol				
10	18	-	-	-
5	11	-	-	-
2.5	12	-	-	-
1.25	12	-	-	-
0.625	10	-	-	-
Ethyl acetate				
10	18	-	-	-
5	17	-	-	-
2.5	16	-	-	-
1.25	16	-	-	-
0.625	14	-	-	-
Ethanol: water				
10	-	-	-	-
5	-	-	-	-
2.5	-	-	-	-
1.25	-	-	-	-
0.625	-	-	-	-
Aqueous				
10	-	-	-	-
5	-	-	-	-
2.5	-	-	-	-
1.25	-	-	-	-
0.625	-	-	-	-
Ampicillin				
10	36	32	31	30
5	35	34	39	31
2.5	31	29	30	26
1.25	29	21	19	19
0.625	21	17	15	13

Key terms: ND = not detected

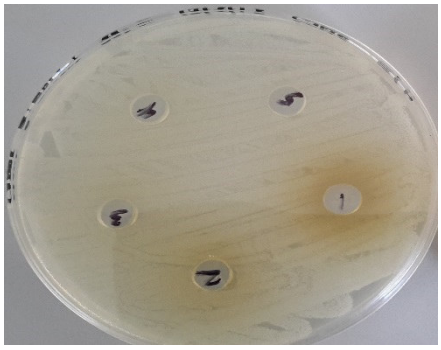
Antibiotic resistance against bacteria prevails certainty of evolving resistance. Advancement in medical sciences results in more patients is critical, thus creating a perpetual need for new antibiotics (Mishra *et al*., 2013). Figure 1, indicates the primary screening test of *A.paniculata* extracts revealed that highest zone of growth inhibition (14 and 10 mm) was observed on ethyl acetate and methanol extracts at lowest concentration of 0.625 mg/ml respectively, against *S.aureus* ATCC 9144, while ethanol: water (1:1 v/v) and aqueous exhibited no activity against the tested pathogens (Table 1). This may be attributed to the high amount of alkaloids present in ethyl acetate and methanol extracts while little value of the alkaloids was observed in ethanol: water (1:1 v/v) and aqueous respectively



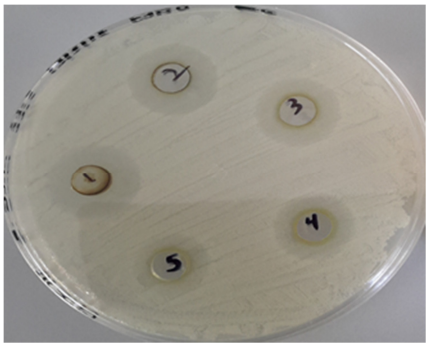
Ampicillin (standard drug)



Aqueous extract



Ethanol: water (1:1 v/v) extract



Ethyl acetate extract

Figure 1: Zone of inhibition of *A. paniculata* extracts on *S.aureus*

3.2.2 Minimum inhibitory concentration

Methanol and ethyl acetate extracts showed lower MIC values of 0.625 mg against *S. epidermidis* compared to ethanol: water and aqueous extracts (1.25 mg) indicating better activity of methanol extracts. Also methanol and aqueous extracts showed lower MIC values against *E. coli* (indicating better activity) as compared to ethanol: water (1:1 v/v) against both gram -ve bacteria (*S. epidermidis* and *E. coli*) as shown in table 4.8. Also, all the extracts have lower MIC values (0.625 mg to 1.25 mg) against gram -ve bacteria (*S. epidermidis* and *E. coli*), compared to higher MIC values (1.25 mg to 2.5 mg) against gram-positive bacteria (gram +ve).

In addition, all the extracts (methanol, ethyl acetate, ethanol: water (1:1 v/v) and aqueous) have same MIC values 2.5 mg and 1.25 mg against *Staph aureus* and *S.typhi* respectively, indicating less activity against Gram +ve bacteria compared to gram –ve bacteria. Therefore, methanol extracts was found to have better activity against both gram –ve bacteria followed by ethyl acetate, aqueous and ethanol: water (1:1 v/v). Low concentrations of the samples that is needed to kill 99.9 % of the bacteria indicate a better activity of the respected samples against different bacteria. Methanol extract indicated lower MIC than the other three extracts against gram –ve bacteria. Previous studies by (Souza *et al*., 2011) reported that terpenoids and flavonoids have antimicrobial properties. Therefore, the antimicrobial effect of *A. paniculata* might be attributed to its flavonoids and terpenoids.

Table 2: Minimum inhibitory concentrations of *A. paniculata* extracts in mg/mL

No	Microorganisms	MEOH	EA	E:H(1:1 v/v)	A
1	*S.aureus*	2.5	2.5	2.5	2.5
2	*S.typhii*	1.25	1.25	1.25	2.5
3	*S.epidermidis*	0.625	0.625	1.25	1.25
4	*E.coli*	0.625	1.25	1.25	0.625

Key terms: MEOH = methanol, EA = ethyl acetate, E: H = 50 % ethanol: water & A =water

The methanol extracts was found to have better activity against both gram –ve bacteria followed by, aqueous, ethyl acetate and ethanol: water (1:1 v/v). This might be attributed to the high amount of terpenoids present in both aqueous and methanol extracts, while ethyl acetate extracts showed a high amount of alkaloids respectively. (Daglia, 2012; Lima *et al*., 2011; Rapp, 2004) reported that the antimicrobial potentials of higher plants might be due to its alkaloid, flavonoid, and terpenoids contents as this strongly support that plants with high polyphenols contents display anti-microbial properties. This further support the anti-microbial potentials of *A. paniculata* might likely to be due to high amount of alkaloid, flavonoids and terpenoids present in the plant.

3.2.3 Minimum bactericidal concentration

The MBC values obtained in this study indicated the minimum concentration of 0.625 mg/mL of both methanol and aqueous extracts of *A. paniculata* needed to kill 99.9 % of bacteria. Although, indications for detecting bactericidal activity are rare and usually meant for serious infections, such as systemic infections or those infections difficult to reach at the site with available antibiotics, the MBC aqueous extracts of *A. paniculata* was remarkable against gram *E.coli* whereas methanol extract showed bactericidal potential against both *S. epidermidis* and *E.coli* as shown in table 3

Table 3: Minimum bactericidal concentrations of *A. paniculata* extracts in mg/mL

No	Microorganisms	MEOH	EA	E:H(1:1 v/v)	A
1	*S.aureus*	5	5	5	5
2	*S.typhii*	2.5	2.2	1.25	2.5
3	*S.epidermidis*	0.625	1.25	1.25	1.25
4	*E.coli*	0.625	1.25	1.25	0.625

Key terms: MEOH = methanol, EA = ethyl acetate, E: H = ethanol: water (1:1 v/v) & A = water

The MBC values obtained in this study indicated the minimum concentration (0.625 mg/mL) of both methanol and aqueous extracts of *A.paniculata* needed to kill 99.9 % of bacteria. Furthermore, the MBC of both methanol and aqueous extracts from *A.paniculata* was remarkable against gram –ve bacteria especially *E. coli.* The Methanol extract showed bactericidal potential activity against *S.epidermidis* (Table 3) and this is in agreement with the presence of terpenoids in both extracts. (Souza *et al*., 2011) revealed the importance of terpenoids and polyphenolic compounds in exhibiting anti-bacterial properties.

4. Conclusion

Plants produces several secondary metabolites that include flavonoids, phenols, flavones, flavonols, tannins, coumarins, alkaloids, lectins, polyphenols, and other compounds and these different classes of bioactive compounds support the plant defence against predators such as microorganisms, insects and herbivores (Cushnie & Lamb, 2005; Zhang *et al*., 2013). These compounds have also been confirmed to possess anti-oxidant, anti-inflammatory, and anti-infectious properties (Daglia, 2012; Lima *et al*., 2011). Plants with high concentrations of flavonoid, alkaloids, and terpenoids content have been established to possess anti-microbial activity (Lima *et al*., 2011).

Although, the flavonoid contents reported in this paper was little, *A. paniculata* showed a potential antimicrobial activity. It could be assumed that the anti-microbial activity might be due to synergistic effects of different compounds present in the plant. Hence, methanol and aqueous extracts should be considered as possible candidate for anti-microbial drug discovery against gram –ve bacteria due to lower MIC values compared to other extracts indicating better activity and a better solvent for extracting anti-microbial agents from *A. paniculata.* However, this is an improvement on (Mishra *et al*., 2013) report, where 75% methanol was employed for the extraction process and anti-bacterial assay and reported a higher zone of inhibition against Gram +ve bacteria (*S.aureus*). Moreover, it should be noted that anti-microbial activity of this plant has been reported by (Melchior *et al.*, 2000; Singha *et al*., 2003), but the potentiality of its anti-microbial property might be large influence by soil and environmental conditions which may postulate the content of its compounds.

The *in vitro* activity of the plant extracts suggest that it can be used to treat infections, inflammation and diabetic that lead to wide application of *A. paniculata* as a multiple treatment plant in Asia. The methanol and ethyl acetate extracts exhibited variable activities against typical gram +ve and gram –ve bacteria. The potency of methanol extract against certain bacteria suggests its potential to be used as an alternative therapeutic agent or in combination with other antibiotics for certain medical conditions particularly diabetic foot. Therefore, the synergistic effects of conventional antibiotics with plant-based flavonoids can be an important alternative for

improving complementary medicine.

Declaration
We declared that this manuscript is original and not published or communicated for publication elsewhere in part or full.

Conflict of interest
Authors declared no conflict of interest.

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