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Original Article

Andrographis paniculata as a potential sensitizing agent for antibiotics

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ABSTRACT

Antibiotic resistance is the ability of microbes to resist antibiotic action despite increasing concentration, and plant extracts have shown potential as antibiotic-sensitizing agents that reduce the dosage of antibiotic use. Thus, in this study, ethanolic extract of *Andrographis paniculata* was screened for antimicrobial property using modified Kirby Bauer disc diffusion assay. The results showed significant growth inhibition ($p<0.05$) of *Pseudomonas* sp., exhibited through the formation of zone of inhibition. Subsequently the minimum inhibitory concentration (MIC) for the ethanolic extract of *A. paniculata* was determined to be 25 mg/mL, which was the minimum extract concentration that significantly inhibited ($p<0.05$) the growth of *Pseudomonas* sp. This MIC of *A. paniculata* was used to determine if it can prevent bacterial cell adhesion. The results showed no significant ($p>0.05$) inhibition on bacterial cell adhesion, thus suggesting no inhibition on the biofilm formation by *Pseudomonas* sp. However, the *A. paniculata* ethanolic extract can destabilize the outer membrane of the *Pseudomonas* sp. Thus, the overall results suggest that *A. paniculata* can still be a good candidate as a sensitizing agent for antibiotics due to the strong antimicrobial and membrane destabilizing activities.

KEYWORDS: anti-adhesion, biofilm, ethanolic extract, membrane destabilization, *Pseudomonas* sp.

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INTRODUCTION

According to Centers for Disease Control and Prevention [1], at least 2 million people are infected with antibiotic-resistant bacteria each year in United States and approximately 23000 people die because of this infection. In the year 2017, WHO has listed some of the antibiotic-resistant pathogens that pose a threat to humanity such as methicillin resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* that is carbapenem resistant, *Neisseria gonorrhoeae* that is cephalosporin and fluoroquinolone resistant and many more (World Health Organization) [2]. Thus, due to the rise of antibiotic resistance crisis, natural products are now been used as an alternative for antibiotics.

Natural products from plants particularly received a lot of attention since there are numerous plants that exhibit antimicrobial activity and are widely available. One of the examples is *Andrographis paniculata. A. paniculata* has been used in traditional Asian medicine due to its multiple medicinal properties such as hepatoprotective effect, antimicrobial effect, antiparasitic effect and many more [3, 4, 5]. One study even showed that andrographolide, which is one of the active compounds presents in *A. paniculata* exhibits anticancer property and might be applicable in future cancer treatment [6].

Research findings have demonstrated that *A. paniculata* exhibits antimicrobial activity [7, 8]. It was reported that 100 μg/mL of ethanolic extract of *A. paniculata* was able to inhibit the growth of Gram-positive bacteria such as *Staphylococcus aureus*,

Bacillus licheniformis and also Gram-negative bacteria such as *Escherichia coli*, *Vibrio cholera*, *Salmonella typhi*, comparable to 100 µg/mL of ciprofloxacin [9].

Antimicrobial activities can be attributed to different modes of action, for example, inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of nucleic acid synthesis, alteration of cell membranes and so on [10]. In the case of antibiotics, the most common mechanism for antimicrobial action is inhibition of peptidoglycan synthesis that disrupts bacterial cell wall structure [11]. However, plants might have different ways of preventing bacteria growth. One of the researchers had shown that *A. paniculata* exhibits antimicrobial activity by preventing the formation of biofilm [12]. According to [13] *Mahonia aquifolium*, another traditional herb used for treating chronic skin conditions, contains a bioactive compound known as berberine that can prevent bacterial growth by intercalating with DNA and at the same time inhibit protein biosynthesis. Aside from traditional herbs, the common garlic, *Allium sativum* was shown to contain allicin, that interferes with RNA and lipid synthesis and, thus affecting the protein production and formation of phospholipid bilayer which are crucial for bacterial growth [14]. To date, no research had been carried out to determine the mode of antimicrobial action of *A. paniculata*. Hence, this paper reports on the antimicrobial and anti-biofilm formation properties of the ethanolic extract from *A. paniculata.*

MATERIALS AND METHODS

Ethanolic extraction of *Andrographis paniculata*

Approximately 20 g of *Andrographis paniculata* powder (TCM lab, INTI) was transferred into a flask followed by the addition of 100 mL of 80% ethanol. The mixture was stirred for 30 minutes and allowed to stand for 24 hours. The mixture was then filtered using Whatman filter paper (No. 1). The filtrate was centrifuged at 10 000 x g at room temperature for 20 minutes [15, 16]. The bioactive compounds from *A. paniculata* extracted into the ethanol solvent yielded a tentative concentration of 100 mg/mL (biomass per solvent volume).

Preparation of bacterial inoculum

From a glycerol stock, the gram-negative *Pseudomonas* sp. was inoculated into fresh nutrient broth and incubated inside a rotatory shaker at room temperature for 24 hours [17]. After the overnight incubation, the culture was centrifuged (3000 × g, 15 minutes) and the resulting pellet was rinsed twice using saline phosphate buffer. This was followed by a resuspension in saline phosphate buffer to obtain OD 600nm ≈ 0.5 which have an estimated number of $x10^8$ colony-forming units (CFU/mL) of bacteria as suggested in the established spread plated technique [18]. This became the starting inoculum used for the following experiment.

Screening of antimicrobial activity using disc diffusion assay

A sterilized cotton swab was dipped into *Pseudomonas* sp. starting culture and swabbed onto the surface of a nutrient agar plate to obtain a uniform bacterial lawn. The plate was divided into three quadrants and labelled as *A. paniculata* ethanolic extract, 80% ethanol, and ciprofloxacin. A sterilized Whatman antibiotic assay disc (6 mm) was soaked in the ethanolic extract of *A. paniculata* (100 mg/mL) and transferred onto the first quadrant. The second disc was soaked in 80% ethanol and transferred onto the second quadrant, to serve as negative control. The third disc was soaked in ciprofloxacin (10 µg/mL) and transferred onto the third quadrant, to serve as the positive control. The plates were incubated at 37 °C for 24 hours and the formation of inhibition zone around the disc was measured [19].

Determination of the minimum inhibitory concentration

The antimicrobial effect of ethanolic extract of *Andrographis paniculata* on *Pseudomonas* sp. was determined using microdilution, carried out on a 96-well microtitre plate and in triplicates [20]. 125 µL of sterile nutrient broth medium was added into first three wells from the first column to the eighth column (except for seventh column) as triplicates were prepared. After that, 125 µL of *A. paniculata* ethanolic extract (200 mg/mL) was pipetted into wells in the first column thus yielding a concentration of 100 mg/mL. By using a multichannel pipette, serial dilution was performed by transferring 125 µL of the mixture from first column into second column and the process was repeated from second column to third column and so on. The 125 µL of the mixture from the sixth column was discarded to

ensure that the final volume for all the wells from the first column to the sixth column was 125 µL. This resulted in a 2-fold serial dilution of the *A. paniculata* ethanolic extract yielding a concentration ranging from 100 mg/mL to 3.125 mg/mL.

For eighth column, 125 µL of 80% ethanol was added to serve as a negative control. All the wells from the first column to the eighth column (except for seventh column) were then inoculated with 2.5 µL of bacteria culture (OD600nm ≈ 0.5) and incubated at 37 °C for 24 hours [21, 22]. At the end of the incubation period, minimum inhibitory concentration (MIC) was determined by comparing the OD 595 nm reading of *A. paniculata* ethanolic extract with the negative control using a microtiter plate reader. The lowest concentration of *A. paniculata* ethanolic extract that showed a significantly lower ($p<0.05$) OD 595 nm reading than the negative control was chosen as the MIC.

Anti-adhesion assay

Using the MIC determined earlier, 125 µL of this concentration was transferred to 96-well microtitre plate. For the negative control, 125 µL of phosphate buffer saline (PBS) was transferred instead of *A. paniculata* ethanolic extract. The plate was air-dried overnight followed by rinsing twice with PBS (Rufino *et al.*, 2011). 125 µL of bacterial suspension (OD 600nm ≈ 0.5) was transferred into the wells and incubated at room temperature for 24 hours. After the incubation period, the contents of the wells were pipetted out, and the wells were rinsed thrice with PBS to remove any unattached bacteria. 125 µL of 2% crystal violet dye was then added into each well to allow the dye to fix onto the bacteria cells adhered to the wall of the wells. After 15 minutes, the contents inside the wells were pipetted out and rinsed with running tap water to remove any excessive stain. 125 µL of 33% acetic acid was pipetted into the wells and left for 10 minutes to re-dissolve the dye, before reading at OD 595 nm using a microtiter plate reader. The experiment was carried out using triplicates [22].

Determination of cell membrane disruption

1.0 mM membrane potential-sensitive fluorescence probe DiBAC4 (3) was added into the starting culture and incubated for 30 min. The stained cells were aliquoted into wells with PBS media only (control) and wells with media containing 100 mg/mL of *A. paniculata* ethanolic extract. The fluorescence intensities were determined using an excitation wavelength of 492 nm and an emission wavelength of 518 nm (HITACHI 4500 fluorescence spectrophotometer) [23].

Statistical analysis

The data collected were analyzed using analysis of variance (ANOVA) with 95% confidence level. Results were reported in ± standard deviation (n=3) [24].

RESULTS

In the preliminary antimicrobial screening, zone of inhibition was observed in all discs on nutrient agar lawned with *Pseudomonas* sp. Ciprofloxacin (control positive) showed the highest zone of inhibition followed by *Andrographis paniculata* ethanolic extract and lastly the 80% ethanol (control negative) (Table 1). Statistical analysis showed that there was no significant difference ($p>0.05$) between diameter of the zone of inhibition caused by ciprofloxacin and *A. paniculata* ethanolic extract. This indicated that *A. paniculata* ethanolic extract was equally effective in inhibiting the growth of *Pseudomonas* sp. as ciprofloxacin. Compared to negative control of 80% ethanol, *A. paniculata* ethanolic extract significantly increased ($p<0.05$) the zone of inhibition by approximately 24%. This shows that it was not the ethanol that solely inhibited the bacterial growth, instead the antimicrobial properties found in *A. paniculata* contributed to the growth inhibition.

From the serial dilution carried out on 200 mg/mL of *A. paniculata* ethanolic extract, it was determined that the minimum inhibitory concentration (MIC) was 25 mg/mL, the minimum concentration that was able to show a significant difference ($p<0.05$) in inhibiting the growth of *Pseudomonas* sp. compared to 80% ethanol which was the negative control in this experiment (Table 2).

The anti-adhesion assay was performed using the MIC of *A. paniculata* ethanolic extract to assess whether the extract can inhibit biofilm formation in *Pseudomonas* sp. Although the mean absorbance of 595 nm (Table 3) showed that the number of bacterial cells that adhere to the plate treated with *A. paniculata* was lower at 0.146 ± 0.134, the

reading is not statistically different ($p>0.05$) from that obtained from the negative control plate treated with PBS (0.213 ± 0.102). This indicated that *A. paniculata* extract at 25 mg/mL did not exert antibiofilm formation activity on *Pseudomonas* sp.

On the other hand, *Pseudomonas* sp. treated with *A. paniculata* extract showed 30-fold lower absorbance reading of DiBAC4 (3) fluorescent dye, compared to untreated bacterial cells. This indicated a change in the membrane potential of *Pseudomonas* sp. treated with *A. paniculata* extract (Table 4).

Table 1. Mean of diameter of zone of inhibition by different types of disc.

Type of disc	Diameter (cm)
Ciprofloxacin (10 μg/mL)	1.60 ± 0.200
A. paniculata ethanolic extract	1.40 ± 0.100
80% ethanol	1.13 ± 0.208

DISCUSSION

The *Andrographis paniculata* ethanolic extract exhibited antimicrobial properties comparable to ciprofloxacin, as demonstrated by the disc zone inhibition of *Pseudomonas* sp. The minimum inhibitory concentration (MIC) determined for *A. paniculata* ethanolic extract was 25 mg/mL indicative of the lowest concentration tested that significantly inhibited ($p<0.05$) growth of Gram negative *Pseudomonas* sp. This shows that the extract of *A. paniculata* exerted a strong antimicrobial property since the MIC for the ethanolic extract of *Iresine herbstii* was reported to be 10 times higher at 256 mg/mL [25]. In order to better understand how the extract of *A. paniculata* can inhibit the growth of *Pseudomonas* sp., two modes of actions were tested.

The anti-adhesion assay using absorbance readings recorded at 595 nm shows no significant difference ($p>0.05$) between negative control (PBS + *Pseudomonas* sp.) and *Pseudomonas* sp. incubated with *A. paniculata* ethanolic extract, indicating that

Table 2. Mean of absorbance at 595 nm for the determination of minimum inhibitory concentration of *A. paniculata* ethanolic extract.

Content of well	Mean of absorbance at 595 nm
100 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.002 ± 0.001
50 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.009 ± 0.001
25 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.020 ± 0.012
12.5 mg/ mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.032 ± 0.009
6.25 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.041 ± 0.028
3.125 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.147 ± 0.020
80% ethanol + *Pseudomonas* sp.	0.040 ± 0.001

Table 3. Mean of absorbance at 595 nm for the assessment of antibiofilm formation ability of *A. paniculata*.

Content of wells	Mean of absorbance at 595 nm
25 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.146 ± 0.134
Phosphate buffer saline + *Pseudomonas* sp.	0.213 ± 0.102

Table 4. Mean of absorbance at 518 nm for the assessment of cell membrane disruption in *Pseudomonas* sp.

Content of wells	Mean of absorbance at 595 nm
25 mg/mL of *A. paniculata* ethanolic extract + *Pseudomonas* sp.	0.010 ± 0.001
Phosphate buffer saline + *Pseudomonas* sp.	0.314 ± 0.012

the extract was not able to inhibit the formation of biofilm in *Pseudomonas* sp. However, prior research has shown that the quorum sensing system, LuxI-LuxR found in *P. aeruginosa* which enabled the bacteria to develop biofilm [26] can be inhibited by a phytochemical called andrographolide at 50 µg/mL of [27]. This andrographolide is also reported to be found in *A. paniculata* [28]. It is possible that the crude extract of *A. paniculata* used in this experiment contains insufficient concentration of andrographolide to inhibit the biofilm formation, as opposed to the study by Banerjee *et al.* [27] that used pure andrographolide. Although the extract of *A. paniculata* did not exhibit significant anti-biofilm activity in this experiment, the andrographolide can be purified from the ethanolic extract and used as a sensitizing agent.

On the other hand, *Pseudomonas* sp. treated with *A. paniculata* extract showed a significant ($p<0.05$) decrease in the absorbance reading of DiBAC4 (3) fluorescent dye, compared to untreated bacterial cells. This indicated that the *A. paniculata* extract can destabilize the outer cell membrane of the *Pseudomonas* sp. bacteria. The outer membrane of the Gram-negative *Pseudomonas* sp. is protected by a relatively thin cell wall composed of a single layer of peptidoglycan, lipoproteins and lipopolysaccharides. The outer membrane acts as a molecular barrier that restricts the access of some drugs, particularly hydrophobic antibiotics [29]. Thus, the membrane integrity is a prerequisite, and its disruption can cause metabolic dysfunction and finally lead to bacterial death [30]. Flavonoid is known to inhibit such functionality of cytoplasmic membrane [31], either by partitioning the more non-polar compounds in the hydrophobic interior of the membrane, or by forming hydrogen bonds between the polar head groups of lipids in the membrane and the more hydrophilic flavonoids [32]. It was also reported that the nonspecific interactions between flavonoids and phospholipids can damage the structure of the membrane [33]. The phytoconstituents of *A. paniculata* were analysed and showed the presence of flavonoids and other phytochemicals such as alkaloid, glycoside, phenol, phytosterol and tannin [4, 34]. Hence, flavonoids present in *A. paniculata* ethanolic extract not only can inhibit the growth of *Pseudomonas* sp., but can act as a sensitizing agent for antibiotics by facilitating the antibiotic to penetrate through the outer membrane of *Pseudomonas* sp.

CONCLUSION

Andrographis paniculata ethanolic extract showed antimicrobial property by inhibiting the growth of *P. aeruginosa* as observed through the formation of zone of inhibition on nutrient agar. The minimum inhibitory concentration (MIC) of *A. paniculata* ethanolic extract was 25 mg/mL, the lowest concentration that significantly inhibited ($p<0.05$) the growth of *P. aeruginosa*, suggesting a strong antimicrobial effect. Although the *A. paniculata* ethanolic extract was not able to inhibit the formation of biofilm in *P. aeruginosa*, the extract was able to destabilize bacteria's cell membrane, thus playing a role of antibiotic sensitizing agent.

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CONFLICT OF INTEREST STATEMENT

There is no conflict of interest.

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