

## Research

# Anti-microbial Mechanism of *Lonicera japonica* and *Andrographis paniculata* Extract in Inhibiting *Pseudomonas* sp.

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### ABSTRACT

Antibiotic resistance reported in *Pseudomonas* sp. is associated with various opportunistic infections and is a concern in the public health system. The present study investigated the inhibitory effects of ethanol extracts of *Lonicera japonica* and *Andrographis paniculata* on *Pseudomonas* sp. The total phenolic compound (TPC) calculated in gallic acid equivalent (GAE) was 508.6±117.96 mg GAE/100 g dried weight (DW) in *L. japonica* while *A. paniculata* contained 129.0 ± 21.00 mg GAE/100 g DW. Both herbs significantly reduced the swarming zone diameters of *Pseudomonas* sp., even at a concentration as low as 5 mg/mL when compared to the controls. The anti-swarming effect of *L. japonica* and *A. paniculata* may be one of the mutual mechanisms underlying their anti-microbial properties. However, only the extract of *L. japonica* showed cell anti-adhesion activity, which can prevent biofilm formation. *Andrographis paniculata* extract on the other hand shows the highest activity in disrupting the cell membrane of *Pseudomonas* sp.

**Key words:** Biofilm, ethanol extract, membrane disruption, phenolic compound, swarming

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### INTRODUCTION

*Pseudomonas* sp. is a pathogenic agent of diseases due to its resistance to antibiotics (Subramanian *et al.*, 2009), which poses a serious problem to human health because it allows the bacteria to spread to other places easily and causes serious infection (Fujii *et al.*, 2014). The bacteria are motile and use coordinate swarming movement (quorum sensing) to form a biofilm (Gukui *et al.*, 2021).

Since antibiotic-resistant strains are on the rise, alternative strategies using medicinal plants have been explored for this purpose (Chaves *et al.*, 2014). Plants' extracts are reported to have antimicrobial activity (Geetha & Catherine, 2017) due to high phenolic compounds (Ugurlu *et al.*, 2016). In traditional Chinese medicine, *Lonicera japonica* (*L. japonica*) has been used to treat inflammation and respiratory tract infection (Shang *et al.*, 2018) whilst *Andrographis paniculata* (*A. paniculata*) is commonly used to treat fever (Jarukamjorn & Nemoto, 2008), diseases that can be traced to *Pseudomonas* sp. (Moore *et al.*, 2016). Despite some of the antimicrobial mechanisms such as elimination of resistant plasmid exerted by *L. japonica* (Wang *et al.*, 2000) and anti-biofilm formation by *A. paniculata* (Banerjee *et al.*, 2017) have been reported on *Pseudomonas* sp., the question of whether the total phenolic content (TPC) of the herbs exerted different antimicrobial mechanism is less known. This aspect is important because the herbs are usually consumed as a whole, whereas most reported studies focus on a particular individual phenolic compound. Thus, in the present study, the TPC content of *L. japonica* and *A. paniculata* was evaluated to see whether it influences the anti-swarming, anti-adhesion, and membrane destabilization of *Pseudomonas* sp.

## MATERIALS AND METHODS

### Revival of bacteria from stock culture

Glycerol stock of the *Pseudomonas* sp. (ATCC 27853) obtained from the Biotechnology Laboratory collection (INTI International University) was streaked on a nutrient agar plate and incubated for one day at room temperature (Jayanthi *et al.*, 2016). A single colony was selected from the culture agar plate inoculated into the fresh nutrient broth and incubated in a rotatory shaker (100 × g, room temperature) for 24 h (Tong *et al.*, 2022). After the incubation, the cultures were centrifuged (3000 × g, 15 min at room temperature) and the pellet was rinsed twice using deionized water. This is followed by resuspending the pellet in 0.85% of NaCl to acquire a spectrophotometric reading at OD600 nm ≈ 0.5 which contained an estimated number of ×10<sup>8</sup> colony-forming units (CFU/mL) of bacteria (Kok-Kee *et al.*, 2015). This was used as the starting inoculum for the subsequent experiments.

### Ethanolic extracts of *L. japonica* and *A. paniculata*

Ethanolic plant extraction was performed according to Rol *et al.* (2022) with modification whereby instead of 95% (v/v), 80% (v/v) of ethanol was used. *L. japonica* and *A. paniculata* powders were obtained from the Traditional Chinese Medicine Clinic, TCM INTI. The herbs powder was mixed with 50 mL of 80% (v/v) ethanol and stirred for 30 minutes. The mixture was left to stand for 24 h at room temperature before filtration using Whatman filter paper (No.1). At this stage the tentative concentration (biomass per solvent volume) of the herbs was 200 mg/mL. This was further diluted with 80% ethanol to obtain 5, 10, 50, and 100 mg/mL of *Lonicera japonica* and *A. paniculata* extracts, respectively.

### The total phenolic content in the ethanolic extracts of *L. japonica* and *A. paniculata*

The total phenolic content of the extract was determined using the Folin–Ciocalteu method (Kaur & Kapoor, 2002). Briefly, 200 µL of ethanolic extract was made up to 3 mL using distilled water and mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min. This was followed by 2 mL of 20% (w/v) sodium carbonate and the mixture was allowed to stand for 60 min in the dark. The absorbance measured at 650 nm was then taken. The total phenolic content (mg gallic acid equivalent/100 g dried weight) was calculated from the calibration curve using gallic acid as standard (0-1.0 mg/mL).

To compare the phenolic content in another herbal extract, the formula below was used to standardize the unit used by other studies (Abdelhady *et al.*, 2011).

$$T = C \times \frac{V}{M}$$

T- Total phenolic content in mg/g of the extracts as Gallic acid equivalent (GAE)

C- Concentration of gallic acid established from the calibration curve in mg/mL

V- Volume of the extract solution in mL

M- Weight of the extract in g

### Measurement of anti-swarming activity

Following the protocol of Kwil *et al.* (2013), the inoculum was prepared by mixing an equal volume of bacterial culture with distilled water (negative control), ethanol (positive control), or herbal extracts (sample) (Table1). 5 µL of each set of inoculums was spotted at the center of Luria-Bertani (LB) agar plates containing 1.5% agar. The droplet was allowed to dry from the surface of the agar medium and incubated at 37 °C for 24 h (At the end of the incubation period, the mean diameters (mm) of swarming zones were measured in three different directions. All experiments were performed in triplicates of each different concentration (100 mg/mL, 50 mg/mL, 10 mg/mL, and 5 mg/mL for both extracts).

**Table 1.** Preparation of negative control, control with ethanol and sample (ethanolic extract of herbs)

Set	Volume (µL)			
	Standard inoculum	Ethanol	<i>L. japonica</i> / <i>A. paniculata</i> extract	Distilled water
Negative control	5	0	0	5
Control with ethanol	5	5	0	0
Sample	5	0	5	0

### Measurement of anti-biofilm formation activity using anti-adhesion assay

The assay was performed based on the method of Rufino *et al.* (2011). 100 µL of the aqueous extract of *L. japonica* as well as *A. paniculata* was transferred to a 96-well microtiter plate each and 100

$\mu\text{L}$  distilled water was added to a third column in the microtiter plate as a control. After drying the plate overnight, 100  $\mu\text{L}$  of the starting inoculum was added and incubated (24 h at room temperature). After the incubation, the wells were rinsed with phosphate-buffered saline (PBS). Bacterial cells attached to the walls of the microtiter plate were incubated with 100  $\mu\text{L}$  of 2% methylene blue dye for 15 min. The contents were then rinsed with distilled water to remove the excess dye. 100  $\mu\text{L}$  of 33% acetic acid was then added to re-dissolve the dye. A microtiter plate reader was then used to measure the absorbance at OD 600 nm (Rufino *et al.*, 2011). If more bacterial cells are adhering to the microtiter plate and forming biofilm, the OD600 nm readings will be higher.

### Cell membrane disruption assay

Following the protocol by Clementi *et al.* (2014), the bis-oxonol dye DiBAC4(3) and the cell-impermeant dye propidium iodide were used to measure membrane depolarization and rupture, respectively. Briefly, 1 mL of the bacterial cells from the starting inoculum of *Pseudomonas* sp. were aliquoted into a microcentrifuge tube. This was followed by 5  $\mu\text{L}$  of 50  $\mu\text{M}$  of DiBAC4 (3) stock and 10  $\mu\text{L}$  of the 2 mg/mL propidium iodide stock. This mixture was then aliquoted into 96 well plates that contained media containing 100 mg/mL of the herb extract. A control was prepared by replacing the herb extract with PBS media. The plate was then placed in a pre-warmed (37 °C) fluorescence detection plate reader. The fluorescence intensities of the wells were determined using an excitation wavelength of 492 nm and an emission wavelength of 518 nm (HITACHI 4500 fluorescence spectrophotometer). Fluorescence reading over time was plotted to evaluate membrane depolarization and rupture. Increasing fluorescence of DiBAC and propidium iodide indicated that depolarization and rupture occurred within the bacterial cells (Clementi *et al.*, 2014).

### Statistical analysis

All the experiments were performed at least three times on independent days. All the data was expressed as mean  $\pm$  standard deviation. The statistical significance of the diameter of the swarming zone was assessed by performing a Student *t*-test with SPSS PASW Statistic 17 software (SSPS, Chicago, IL).  $p$ -value < 0.05 was considered as significant.

## RESULTS

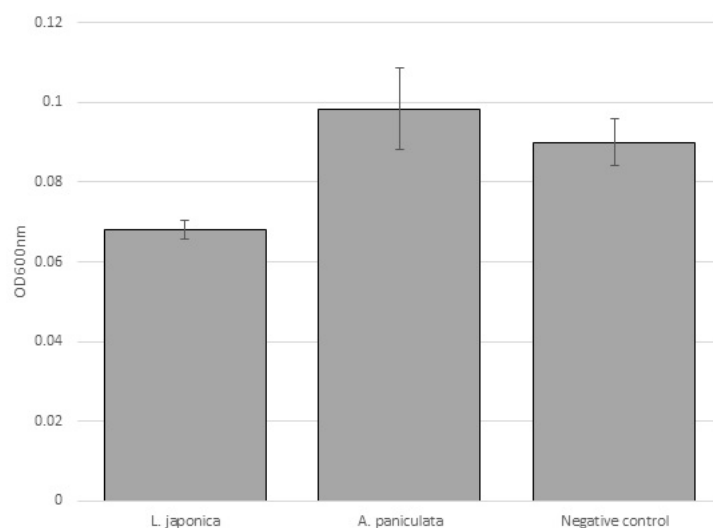
The concentration of total phenolic compound in *L. japonica* was calculated to be  $508.6 \pm 117.96$  mg GAE/100 g dried weight (DW) while *A. paniculata* contained  $129.0 \pm 21.00$  mg GAE/100 g DW. Both herb extracts were shown to inhibit the *Pseudomonas* sp. from swarming on agar as summarized in Table 2. All the agar plates with 10, 50, and 100 mg/mL of *L. japonica* and *A. paniculata* extract did not show any swarming zone, demonstrating that at these concentrations, both herbs were able to completely inhibit the swarming activity of *Pseudomonas* sp. The agar plates with 5 mg/mL of *L. japonica* and *A. paniculata* extract showed a 6 to 10 times smaller swarming zone of *Pseudomonas* sp. compared to the negative control plate ( $p < 0.05$ ). Both negative and positive control consisted of distilled water and ethanol, respectively did not have TPC that can exert anti-swarming activity, thus allowing the *Pseudomonas* sp. to swarm freely.

**Table 2.** Diameter of the swarming zone of *Pseudomonas* sp.

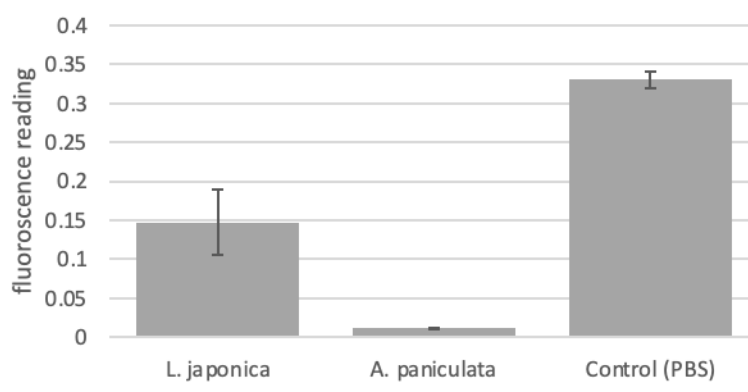
Sample (mg /mL)	Swarming diameter (mm)	
	<i>L. japonica</i>	<i>A. paniculata</i>
Negative control	$83.7 \pm 1.35$	$84.6 \pm 0.77$
Ethanol Control	$20.0 \pm 2.52$	$19.1 \pm 6.66$
100	$0.0 \pm 0.0$	$0.0 \pm 0.0$
50	$0.0 \pm 0.0$	$0.0 \pm 0.0$
10	$0.0 \pm 0.0$	$0.0 \pm 0.0$
5	$7.7 \pm 4.81$	$14.1 \pm 15.00$

Figure 1 shows how *Pseudomonas* sp. treated with *L. japonica* has the lowest bacterial cell adhesion on the microtiter plate. In comparison, the *A. paniculata* did not have any effect in preventing the bacterial cells from adhering to the microtiter plate, since the OD600 nm reading was similar to the control.

Figure 2 shows that *Pseudomonas* sp. treated with *A. paniculata* extract and *L. japonica* extract showed 30-fold and 2-fold lower ( $p < 0.05$ ) absorbance readings of DiBAC4 (3) fluorescent dye, compared to untreated bacterial cells, respectively. This indicated a change in the membrane potential of *Pseudomonas* sp. when treated with the two herbs extract.



**Fig. 1.** Mean absorbance reading of OD600 nm showing bacterial cells adhesion to the microtiter plate treated with *L. japonica* and *A. paniculata* compared to untreated control.



**Fig. 2.** Mean of absorbance of fluorescence detection of DiBAC4 (3) fluorescent dye in bacterial cells treated with *L. japonica* and *A. paniculata* compared to untreated control.

## DISCUSSION

The present study demonstrated the ethanolic extract of *L. japonica* and *A. paniculata* was able to prevent the *Pseudomonas* sp. from swarming, even at the lowest concentration tested which was 5 mg/mL. At higher concentrations of 10-100 mg/mL, both extracts completely inhibited the swarming activity of *Pseudomonas* sp. This suggests that both herbs contained bioactive compounds that have anti-swarming properties. The total phenolic compounds (TPC) in *L. japonica* extract were four times higher than in *A. paniculata*. This in turn suggests that TPC appears to be one of the contributing factors for the greater anti-swarming property exerted by *L. japonica* whereby the extract demonstrated approximately 50% reduction in the diameter of the swarming zone of *Pseudomonas* sp., compared to *A. paniculata*. Despite this, both herb extracts at concentrations as low as 5 mg/mL were able to inhibit the swarming in *Pseudomonas* sp.

Several individual phenolic compounds with anti-swarming properties have been reported to be found in both *L. japonica* and *A. paniculata* extracted using other organic solvents (Chen et al., 2010; Praveen et al., 2014). It was reported that phenolic compounds such as chlorogenic acid (Truchado et al., 2012) and salicylic acid can degrade quorum-sensing signaling molecules and prevent the coordination of swarming movement in *Pseudomonas* sp. (Bandara et al., 2006). Both *p*-coumaric acid and gallic acids were also reported to decrease the expression of the flagella gene (*flagA*) in *Pseudomonas* sp. (Myszk et al., 2015). The *p*-coumaric acid also deactivates the expression of bacterial virulence factors via inhibition of quorum-sensing receptors such as *LuxR*, *AhyR*, and *TraR* receptors (Bouyahya et al., 2017).

However, results from this study showed only *L. japonica* extract was able to prevent the *Pseudomonas* sp. from adhering to each other and other surfaces, a first step in biofilm formation. It is possible that both herbs, despite containing a high number of phenolic compounds, but the individual types varied. This is because only certain phenolic compounds are reported to contribute to anti-biofilm formation. Quercetin is one such phenolic compound, acting as a competitive inhibitor for the *N*-acyl homoserine lactone protein (AHL) receptor binding sites, resulting in the interruption of the initiation mechanism for biosurfactant production (Vasavi et al., 2014). The binding of AHL signal molecules to protein receptors regulates biosurfactant production, the first step toward biofilm formation. Although such individual compounds (chlorogenic acid, salicylic acid, quercetin) from the same phenolic family were not measured in the present study, it is an interesting possibility that such compounds are present in *L. japonica* with higher TPC content. Thus, this aspect will be explored in future studies to better assess the role of *L. japonica* as a sustainable alternative drug.

This suggests that *A. paniculata* might have another mechanism to inhibit *Pseudomonas* sp. from spreading. The cell membrane disruption assay using DiBAC4(3) showed both herb extracts were able to destabilize the outer membrane of *Pseudomonas* sp. Interestingly, of the two herbs, the extract of *A. paniculata* has the highest ( $p < 0.05$ ) ability to destabilize bacterial cell membranes. A diterpenoid, andrographolide, although it was not assayed in this study, can be found in *A. paniculata* and shown to reduce cell membrane integrity, (Zaid et al., 2015). Taken together, despite both herb extracts possessing anti-swarming activity, only *L. japonica* showed anti-adhesion activity with relatively lower cell membrane disruption activity compared to *A. paniculata*. This can be attributed to the higher TPC content in *L. japonica* found in this study, with a higher possibility of possessing a higher variety of phenolic compounds (eg. chlorogenic acid, salicylic acid, quercetin) as reported in other studies.

## CONCLUSION

The concentration of total phenolic compound (TPC) in *L. japonica* was calculated to be  $508.6 \pm 117.96$  mg GAE/100 g dried weight (DW) while *A. paniculata* contained  $129.0 \pm 21.00$  mg GAE/100 g DW. The total phenolic compounds at the lowest concentration tested 5 mg/mL were able to exert anti-swarming activity on *Pseudomonas* sp. The extract of *L. japonica* with higher TPC concentrations showed cell anti-adhesion activity, that can prevent biofilm formation. Whereas *A. paniculata* with lower TPC concentration shows the highest activity in destabilizing the cell membrane of *Pseudomonas* sp.

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## ETHICAL STATEMENT

Not applicable.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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