ORIGINAL ARTICLE

The Effect of Water Extracts of *Andrographis Paniculata* and *Lonicera Japonica* Against Infectious Methicillin-Resistant *Staphylococcus Epidermidis*

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ABSTRACT

Introduction: Methicillin-resistant *Staphylococcus epidermidis* (MRSE) causes various infectious diseases, ranging from minor skin infections to life-threatening bacteremia, posing a public health risk. Antibiotic misuse has increased challenges in the treatment of MRSE infections. An alternative is to investigate the pharmacological bioactivity of aqueous extracts of *Andrographis paniculata* and *Lonicera japonica* against MRSE. **Materials and Methods:** The total phenolic and flavonoid contents in the water extracts of *A. paniculata* and *L. japonica* were quantified using the Folin-Ciocalteu and aluminum chloride colorimetric methods, respectively. Antimicrobial and anti-biofilm activities against MRSE were assessed via disc diffusion and anti-adhesion assays, and statistical analysis was performed using Analysis of Variance (ANOVA) ($p < 0.05$). **Results:** The flavonoid content of *A. paniculata* was 25.85 µg/mL, which was threefold higher than that of *L. japonica* (8.95 µg/mL). While, the phenolic acid content of *L. japonica* at 12.91 µg/mL was 54 times higher than that of *A. paniculata* (0.24 µg/mL). In the disc diffusion assay, anti-MRSE activity was observed for the positive control (vancomycin) with a zone of inhibition of 31.67 ± 1.53 mm and *L. japonica* extract with 9.97± 2.08 mm. *A. paniculata* extract showed no anti-MRSE activity ($p>0.05$) compared to the negative control (distilled water) with 0 mm inhibition. In the anti-adhesion assay, showed higher anti-biofilm formation activity ($p<0.05$), not observed with *A. paniculata* extract compared to the negative control. **Conclusion:** Based on disc diffusion and anti-adhesion assays, *L. japonica* extract inhibited MRSE growth by preventing biofilm formation. This suggests that phenolic acids, rather than flavonoids, exhibit anti-MRSE activity owing to their anti-biofilm formation properties.
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Keywords: *Andrographis paniculata*, *Lonicera japonica*, methicillin-resistant *Staphylococcus epidermidis* (MRSE), phenolic compound

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INTRODUCTION

The antibiotic resistance crisis is becoming increasingly aggravated, with more bacteria developing resistance to antibiotics, resulting in millions of deaths each year [1]. Among all the known bacteria, *Staphylococcus epidermidis*, a coagulase-negative staphylococci, is responsible for 40-90% of hospital-acquired infections with medical implants such as catheters and prosthetic joints [2]. These infections are increasingly challenging to treat owing to the emergence of methicillin-resistant *Staphylococcus epidermidis* (MRSE). MRSE accounts for 5.14% of multidrug resistance recorded in most hospitals [3]; however, there is hardly any research information available on alternative strategies to address this issue.

A promising alternative is the use of traditional Chinese medicinal (TCM) herbs to prevent the spread of antibiotic resistance in bacteria. Numerous studies on TCM herbs have shown that such herbal extracts can eliminate resistant plasmids, increase drug permeability into bacterial cells, inhibit efflux pumps, modify antibiotic molecules, and alter drug targets [4]. However, one of the antibiotic resistance mechanisms of MRSE is via biofilm formation, which decreases the bioavailability of drugs to bacteria [3], which has not been explored. Hence, two TCM herbs, *Andrographis paniculata* and *Lonicera japonica*, which have known antibacterial characteristics and contain various secondary metabolites such as phenolic acids and flavonoids

[5], were selected to assess their ability to prevent biofilm formation. Therefore, the mode of action of pharmacologically active compounds in *A. paniculata* and *L. japonica* in inhibiting biofilm formation can be utilized in the treatment of MRSE infections.

MATERIALS AND METHODS

Quantification of the Total Phenolic Acid Content in The Water Extract of *A. paniculata* and *L. japonica*

Water was used to extract the pharmacologically active compounds from *A. paniculata* and *L. japonica* from the traditional herbal drug preparations as closely as possible. The dried herbs (stem and leaves), grounded into powder were purchased from Traditional Chinese Medicine (TCM) Clinic Centre, INTI International University, Nilai. The extraction was performed using 20 g of *A. paniculata* or *L. japonica* powder boiled in 100 mL of distilled water for 15-20 minutes [6], left to cool, and allowed to infuse for 24 hours in a shaking incubator at 100 xg [7]. The extract was then filtered using Whatman filter paper No.4.

The total phenolic content of the aqueous extract of both TCM herbs was analyzed using the Folin-Ciocalteu reagent. Briefly, the water extract (0.5 mL) was mixed with the Folin-Ciocalteu reagent (0.5 mL). The solution was kept at 25°C for 5-8 minutes, followed by the addition of 2 mL 7.5% sodium carbonate solution. The total volume was adjusted to 8 mL by adding water. The absorbance was measured at OD725 nm. The reading obtained was then compared against the standard gallic acid curve (0.2, 0.4, 0.6, 0.8 and 1.0 mg/mL) that was prepared using gallic acid powder (Sigma Aldrich, Germany) [8]. The total phenolic content was expressed as µg gallic acid equivalents (GAE) per mL extract (µg/mL).

Quantification of the Total Flavonoid Content in The Water Extract of *A. paniculata* and *L. japonica*

Total flavonoid content was quantified using the aluminum chloride colorimetric method [9]. In triplicate, 1 mL of the water extract was added to 4 mL of distilled water followed by 0.3 mL of 5% $NaNO_2$. After 5 minutes of incubation at room temperature, 0.3 mL $AlCl_3$ was added, followed by 2 mL of 1M NaOH and 3.4 mL distilled water [10]. A UV spectrophotometer at a wavelength of 510nm was used to measure the absorbance of the water extract. The reading was then compared against a standard curve plotted following the same method, using quercetin to represent the total flavonoids [9]. The concentration of total flavonoids in the water extract was expressed as µg quercetin equivalents (QE) per mL extract (µg/mL).

Preparation of Standard MRSE Inoculum

The MRSE stock culture, a gift from the Institute of Medical Research Malaysia (IMR), was streaked onto nutrient agar plates aseptically and incubated for 24 hours at 37°C [11]. A single colony was picked from the nutrient agar, inoculated into 50 mL of Luria-Bertani (LB) broth, and incubated in a shaking incubator (100 xg, 37°C for 24 hours). After 24 hours, the culture was centrifuged at 3000 x g (Megafuge 16R ThermoScientific, USA) for 10 minutes, and the pellet obtained was resuspended twice using 0.85% saline solution [12]. This was followed by resuspension in saline phosphate buffer to obtain OD 600 nm ≈ 0.5, which has an estimated number of x108 colony-forming units (CFU/mL) of bacteria, as suggested in the established spread-plated technique [13]. This suspension was used as the standard MRSE inoculum for the disc diffusion and anti-adhesion assays.

Determination of Antimicrobial Activity Using Disc Diffusion Assay

LB agar plates were divided into quadrants and lawned with 10 µL of standard inoculum MRSE. The first two quadrants were placed on a filter paper disc (6 mm in diameter) soaked in either *A. paniculata* or *L. japonica*. A 30 µg/mL vancomycin disc was placed in the third quadrant as a positive control. Filter paper soaked in distilled water was placed in the fourth quadrant as a negative control. The experiment was performed in triplicate and the plates were incubated for 24 hours at 37°C [6]. After incubation, the diameter of the inhibition zone was measured (mm).

Determination of The Anti-Biofilm Formation Activity Using Anti-Adhesion Assay

In triplicate, each well of a microtiter plate was filled with 100 µL of the water extract or 100 µL of distilled water as a control. The microtiter plate was left to air dry overnight before 100 µL of MRSE suspension was transferred into the wells and incubated for 24 hours at room temperature [14]. After incubation, the wells were emptied and rinsed three times with phosphate buffered saline (PBS) to remove MRSE cells that failed to adhere to the microtiter plate wall [14]. For visualization, 100 µL of 2% methylene blue dye was added for 15 min to fix the MRSE cells attached to the walls of the microtiter plate. The contents of the wells were then emptied and rinsed with distilled water to remove the excess dye. This was followed by the addition of 100 µL of 33% acetic acid to the wells to re-dissolve the dye for 10 minutes. A microplate reader was used to measure the absorbance of the dye at 600 nm [14].

Statistical Analysis

All results obtained from the quantification of the total phenolic acid content, total flavonoid content, diameter of the zone of inhibition, and anti-adhesion assay were analyzed using analysis of variance (ANOVA) with a 95% confidence level. Results are reported as mean ± standard deviation (n=3).

RESULTS

Quantification of the Total Phenolic Acid Content

and Flavonoid Content in the Water Extract of *A. paniculata* and *L. japonica*

Table I shows the total phenolic acid and flavonoid contents in the water extracts of *Andrographis paniculata* and *Lonicera japonica*. The water extract of *L. japonica* contained 54 times more phenolic acid ($p < 0.05$) than *A. paniculata*. However, the water extract of *A. paniculata* contained three times higher flavonoid content ($p<0.05$) than that of *L. japonica*.

Table I: The total phenolic acid and flavonoid content in the water extract of *Andrographis paniculata* and *Lonicera japonica*

Water Extract	Mean Concentration (µg/ mL)	
	Total phenolic acid gallic acid equivalents (GAE) mg/g sample	Total flavonoid quercetin equivalents (QE) mg/g sample
A. paniculata	0.24 ± 0.01	25. 85 ± 0.51
L. japonica	12.91 ± 0.47	8.94 ± 0.44

Determination of Antimicrobial Activity Using Disc Diffusion Assay

The potential of the water extracts of *A. paniculata* and *L. japonica* to inhibit MRSE growth was assessed using a disc diffusion assay. The mean diameter of the zone of inhibition, measured in triplicate, was calculated (Figure 1). The positive control with vancomycin had the highest antibacterial activity ($p< 0.05$) with the largest Zone of inhibition (ZOI) of 31.67 ± 1.53 mm compared to the negative control with a ZOI of 0 mm. *L. japonica* also has an antibacterial activity against MRSE ($p<0.05$) with a recorded ZOI of 9.97± 2.08, whilst *A. paniculata* with recorded ZOI of 0.67± 1.54 mm ($p> 0.05$) exerted no inhibition zone against MRSE. Between the two herbal extracts, *L. japonica* had higher antibacterial activity($p<0.05$) than *A. paniculata*.

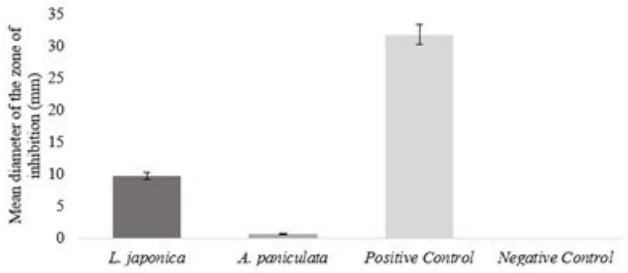

Fig. 1: Mean diameter of the zone of inhibition (mm) recorded with error bars indicates the standard deviation.

Determination of the Anti-Biofilm Formation Activity Using Anti-Adhesion Assay

An anti-adhesion assay was used to investigate the anti-biofilm properties of the water extract (Figure 2). MRSE cells incubated with *L. japonica* showed the lowest absorbance reading (0.078 ± 0.01, $p<0.05$) compared to *A. paniculata* and distilled water (negative control), with absorbance readings of 0.091 ± 0.01, and 0.089 ± 0.04, respectively. A lower absorbance reading indicated that fewer MRSE cells adhered to the wall of the microtiter plate.

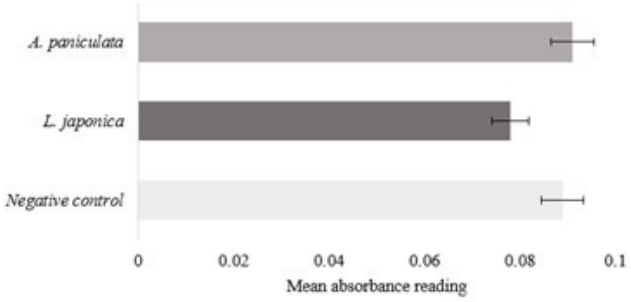

Fig. 2: Mean absorbance readings recorded with error bars indicate standard deviation.

DISCUSSION

In this study, two traditional Chinese medicinal herbs, *Andrographis paniculata* and *Lonicera japonica*, were assessed for their potential to inhibit the growth of methicillin-resistant *Staphylococcus epidermidis* (MRSE). The disc diffusion assay results showed that the water extract of *A. paniculata* was unable to inhibit MRSE growth, whereas the water extract of *L. japonica* was able to inhibit MRSE growth. However, *L. japonica* exerted lower growth inhibition on MRSE than the antibiotic vancomycin. This can be attributed to vancomycin being a pure antibiotic, whereas the water extract of *L. japonica* contains a mixture of various compounds, including phenolic acids and flavonoids, which may or may not inhibit the growth of MRSE [6]. Interestingly, the water extract of *L. japonica* also prevented MRSE cells from adhering to the microtiter plate, thus showing that the extract contained higher anti-adhesion activity compared to *A. paniculata*.

Taken together, these results suggest that one of the main mechanisms by which *L. japonica* inhibits the growth of MRSE is by inhibiting the MRSE cells from adhering to each other to form a biofilm. It has been reported that the ability of S. epidermidis to form biofilms composed of sulfated polysaccharides can aid the bacteria to resist antibiotics, leading to more serious infections [15]. The multilayer biofilm structure and thickness prevented or delayed the diffusion of antibiotics into the bacterial cells, making it difficult to effectively clear S. epidermidis infections using antibiotics [16], resulting in increased pathogenicity [17]. In addition, the matrix of the biofilm structure acts as a physical barrier that prevents phagocytic activity of the host immune system [16]. Bacterial infection is reported to be closely related to quorum sensing-mediated biofilms, whereby the quorum sensing signaling molecules are crucial in the formation, maturation, and regulation of bacterial biofilms [18]. Therefore, the results of this study suggest that the water extract of *L. japonica* contains bioactive compounds that can inhibit the by quorum-sensing process of MRSE.

The higher total phenolic acid content found in the water extract of *L. japonica* might have contributed to the growth inhibition and anti-adhesion properties of MRSE.

The total phenolic acid content of *L. japonica* was more than 50 times higher than that of *A. paniculata*. The structure of phenolic acids with hydroxyl groups and benzene rings destabilizes the cytoplasmic membrane, leading to membrane rupture and bacterial cell death [19]. Furthermore, lipophilic phenolic acids have been reported to disrupt specialized transport membrane proteins required to transport signaling molecules involved in quorum sensing [20]. With the disruption, the cell-to-cell communication system required for biofilm formation collapsed [21]. This anti-quorum sensing mechanism of phenolic acids reduces bacterial motility, decreases superficial adhesion, and prevents the expression of virulence factors that contribute to pathogenic characteristics in bacteria, such as MRSE [22].

Interestingly, the water extract of *A. paniculata* contained three times as many flavonoids as *L. japonica*, but did not show antibacterial growth or anti-adhesion properties. Although various studies have indicated that flavonoids possess antibacterial properties [23-26], the different flavonoid sub-groups that might have been present in the aqueous extract of *A. paniculata* were not determined. Flavonoid sub-groups with different structures due to different patterns of methylation, hydroxylation, or conjugation with saccharides have different pharmacological effects. Among the sub-groups, flavonols and flavanols have the highest antibacterial activity because of their ability to inhibit the production of virulence factors, disrupt the cytoplasmic membrane, and suppress biofilm formation [24]. It is possible that these two subgroups were either absent or present at very low concentrations in the water extract of *A. paniculata*.

The limitation of this study was only to identify the identification of a group of bioactive compounds present in the water extracts of *A. paniculata* and *L. japonica*, but neither the ratio nor the exact identity of the sub-group was identified. Further studies to isolate, purify, and identify the sub-group or individual bioactive molecules can better elucidate the function of the different modes of action of each compound and their roles in inhibiting the growth and anti-adhesion activity against MRSE.

CONCLUSION

Between the two, only the water extract of *L. japonica* was found to inhibit the growth of MRSE and showed anti-adhesion properties, whereas *A. paniculata* did not, despite the latter having a higher concentration of flavonoids. *L. japonica* with the higher phenolic acid content indicates that phenolic acid rather than flavonoid, plays a vital role in exerting anti-MRSE growth due to its anti-adhesion properties.

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