Antibacterial activity of ethanolic leaf extract of *Aquilaria malaccensis* against multidrug-resistant Gram-negative pathogen

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Abstract

The rapid emergence of resistant Gram-negative bacteria and the limited discovery of novel antibiotic is a global healthcare challenge. Many medicinal plants with potent bioactivities have been developed for the treatment of bacterial infections. *Aquilaria malaccensis* exhibits wide applications from perfumes and aromatic foods ingredients and great potential in medicines. In this study, crude leaf extract of *A. malaccensis* was evaluated for its antibacterial activity against several pathogenic Gram-negative bacteria. The leaves were processed and extracted by Soxhlet method using ethanol as the solvent. The antibacterial activity of the crude extract was tested by disc diffusion method, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against *Acinetobacter baumannii* (ATCC 19606), *Klebsiella pneumoniae* (ATCC 10031 and ATCC 700603) and *Escherichia coli* (ATCC 1129). Using the optimized method, the Soxhlet extract produced a yield of 178.41 mg/g. Treatment of the extract at 200 mg/mL displayed the largest inhibition zones of 14.0 mm and 9.7 mm against *A. baumannii* and *K. pneumoniae* ATCC 10031, respectively. In contrast, against *E. coli* and *K. pneumoniae* ATCC 700603, smaller zones of inhibitions of 3.3 mm were demonstrated. The MIC values of the extract were 32 mg/mL against *A. baumannii* and *K. pneumoniae* ATCC 10031 and 64 mg/mL against *E. coli* and *K. pneumoniae* ATCC 700603. The MBC values of the extract were consistent with the MIC values for all the bacteria investigated. Overall, this study was the first to show antibacterial activity of *A. malaccensis* leaves extract particularly against *A. baumannii* and *K. pneumoniae* and potentially develop for the treatment of resistant bacteria.

1. Introduction

Tropical rainforest is a treasure house of many medicinal plant species. Historically, these medicinal plants or herbs have been used as folklore medicine for treating various infectious diseases and ailments. Herbal medicines constitute the Traditional and Complementary Medicine (T and CM) Products where the users are mainly from Europe, Africa, Asia, Australia and North America (WHO, 2013). Therapeutic effects from the medicinal plants are conferred by their constituent of bioactive compounds originated from the plant’s secondary metabolism generally grouped as carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulfur compounds (Cowan, 1999). Over the past few decades, much more attention has been placed on medicinal plants which have become a new promising source of antibacterial agents (Arifullah *et al.*, 2014).

The rising incidence of bacterial infections due to multidrug-resistant (MDR) pathogens is a critical health issue globally. Worryingly, a particular concern is associated with the emergence of MDR Gram-negative bacteria that are resilient to almost all antibiotics (CDC, 2013). In addition, the dry discovery and development pipeline of new antibiotics further lead to catastrophic consequences due to the lack of effective treatment schemes observed in patients (Schäberle and Hack, 2014). The threat is projected to persist and further rise by 2050 which would potentially lead to a mortality rate of 10 million people every year (O’Neill, 2014). Studies have reported on the increase in the prevalence of resistant bacteria, particularly of Gram-negative group including, *Escherichia coli* and *Klebsiella pneumoniae*...
(Guh et al., 2015; Zhang et al., 2018) as well as Acinetobacter baumannii (Almasaudi, 2018) that contributed to high mortality rates of more than 30% (Gutiérrez-Gutiérrez et al., 2017). Multiple factors and mechanisms of antimicrobial resistance in Gram-negative bacteria constitute a great challenge which requires serious attention and proactive action to counter the problem.

A considerable number of local medicinal plants have been reported to exhibit antibacterial properties against both Gram-negative and Gram-positive bacteria. Uyub et al. (2010) evaluated the in-vitro antibacterial activity of more than thirty selected Malaysian ethnobotanical plants and found that all the extract possessed antibacterial activity against several common pathogenic bacteria including E. coli, Pseudomonas aeruginosa and Staphylococcus aureus. A recent study revealed that 21 plant extracts of commonly used traditional medicine in Indonesia showed an inhibition effect on Enterococcus faecalis, P. aeruginosa and S. aureus (Romulo et al., 2018). Medicinal plants are generally considered as safe, effective and possess minimal undesirable side effects (Rafieian-kopaei, 2012). Their actions are complex and tend to have several broad actions such as complementary or synergistic actions on physiological systems at the same time (Nasri and Shirzad, 2013). From this point, the development of new antibacterial agents incorporating plants as a means of therapy has gained significant interest as it produces a vast range of pharmacologically active compound (Jamshidi-Kia et al., 2018).

Aquilaria malaccensis (Thymelaeaceae Family) is a precious plant species which produces a valuable non-timber fragrant wood “resin” (Swee, 2008). The plant widely spread across the forest of Malaysia and Indonesia (known as gaharu and karas) as well as other regions including Bangladesh, India Myanmar, Philippines, Singapore and Thailand (Begum, 2016). The uses of agarwood are not restricted to incense and perfumery. The leaves can be processed into dietary supplements and consumed in food and beverage industry such as herbal tea, biscuits and instant noodle (Chen, 2013). Currently, the applications of agarwood have been increasingly explored for pharmaceuticals purpose obtained from other parts of the tree (Wang et al., 2018). Despite its abundance of supply, research on the leaf from the non-inoculated plant, the one without the introduction of pathogen or antigen to induce its resin are still infrequent (Zainurin, Hashim, Mohamed Azmin et al., 2020). Studies indicated that leaves from the tree contained great potential of bioactivities such as antioxidant (Begum, 2016), anti-inflammatory (Eissa et al., 2018), anti-hyperglycemic (Pranakonh et al., 2011) as well as antibacterial (Hendra et al., 2016). In a phytochemical screening study by Khalil et al. (2013), alkaloids, saponins, flavonoids, tannins and terpenoids were found in both non-inoculated and inoculated sample of A. malaccensis leaves and this could be exploited for the development of therapeutic agents. The present study was performed to demonstrate the in vitro antibacterial activity of crude A. malaccensis leaves extracts against Gram-negative bacteria including E. coli, K. pneumoniae and A. baumannii.

2. Materials and methods

2.1 Plant material and bacterial strains

A. malaccensis leaves were obtained from the private agarwood plantation, Global Root Resources located in Kajang, Selangor, Malaysia freshly collected in January 2020. Gram-negative bacteria of laboratory (reference) strains from American Type Culture Collection (ATCC) of E. coli ATCC 1129, A. baumannii ATCC 19606, K. pneumoniae ATCC 10031 and ATCC 700603 were investigated. A stock solution of the crude extract was prepared by dissolving the extract with Dimethylsulfoxide (DMSO) (Chemiz, 100%, Malaysia) to produce an initial concentration of 300 mg/mL. The stock solution was then further diluted to certain specific concentrations for further use.

2.2 Ethanolic leaves extraction

The fresh leaves of A. malaccensis (1 kg) were watery washed, rinsed with distilled water and dried in the oven at 50°C for 24 hrs. The dried leaves were grounded into a fine powder and kept at room temperature in a closed container. The extraction of leaves powder was in reference to the previous method with modification (Zainurin, Samsudin, Hashim et al., 2020). Leaves powder of 20 g were extracted with 300 mL ethanol (HmbG, 99%, Germany) at its boiling point 78°C in Soxhlet extractor for 18 hrs until the colour of solvent in the thimble becomes colourless. The extraction was performed in dark, at 1:15 of leaves-to-solvent ratio and in triplicates. The filtrate of ethanol solvent was evaporated by the rotary evaporator (Heidolph, Instruments GmbH and CO, Schwabach, Germany) at 45°C under 100 mbar and kept at 4°C until further use. The yield of crude extract was calculated as the percentage yield.

2.3 Preparation of bacteria

Bacterial isolates of E. coli ATCC 1129, A. baumannii ATCC 19606, K. pneumoniae ATCC 10031 and ATCC 700603 from the -20°C frozen stocks were sub-cultured onto a nutrient agar plate and incubated for 16 hrs to 18 hrs at 37°C. A single colony of the
respective Gram-negative bacteria were inoculated into 10 mL Mueller Hinton Broth media and grown for 16 hrs to 18 hrs at 37°C. The overnight cultures of the bacterial isolates were diluted using sterile normal saline to give an inoculum size of about 10^6 CFU/mL with reference to the McFarland turbidity standard.

2.4 Antimicrobial susceptibility test

The crude *A. malaccensis* extract was tested for its antibacterial activity against the *E. coli* ATCC 1129, *A. baumannii* ATCC 19606, *K. pneumoniae* ATCC 10031 and ATCC 700603 using disc diffusion method according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (Matuschek et al., 2014). Bacterial culture of 0.5 mL of 10^6 CFU/mL was aseptically dispensed and streaked on the surface of the solid nutrient agar plate. A sterile, blank disc of 6 mm in diameter was mounted on the agar plate. The extract (5 µL) of different concentrations at 100 mg/mL and 200 mg/mL were impregnated onto the discs and allowed to dry. To measure the effect of plant extract, Tetracycline (5 mg/mL) and DMSO (Chemiz, 100%, Malaysia) were used as the positive and negative controls, respectively. The plates were prepared in triplicates and incubated at 37°C for 16 hrs to 18 hrs. Antibacterial activity of the extract was evaluated by measuring the diameter of the inhibition zone around the discs (Matuschek et al., 2014).

2.5 Minimum inhibitory concentrations of crude extracts

The minimum inhibitory concentration (MIC) of *A. malaccensis* crude extract was determined using broth microdilution method (Wiegand et al., 2008). A volume of 100 µL of different concentrations of crude extract (2-fold serial dilution) was loaded into the sterile 96-well plates. The initial bacterial inoculum of 100 µL of ~10^6 CFU/mL was then added into each well. The wells without antibiotic and crude extract served as growth controls. The assay plate was incubated at 37°C for 16 hrs to 18 hrs. The MIC was taken from the well with the lowest concentration that resulted in no growth of a single bacterial colony on a nutrient agar plate.

2.6 Minimum bactericidal concentrations of crude extracts

The minimum bactericidal concentration (MBC) of the crude leave extract was determined by plating 100 µL of the bacterial culture from each well of the broth microdilution from the MIC assay onto the nutrient agar plate (Balouiri et al., 2016). The plate was incubated at 37°C for 16 hrs to 18 hrs. The MBC is defined as the lowest concentration of the extract that resulted in no growth of a single bacterial colony on a nutrient agar plate.

3. Results

3.1 Yield of crude leaf extract

The final product produced was in a dark green and waxy thick with a final yield of 178.41 mg/g, which was approximately 17.84% yield.

3.2 Antimicrobial susceptibility test

The antibacterial activity of the crude ethanolic extracts of *A. malaccensis* leaves was evaluated using disc diffusion method. The crude extract exhibited antibacterial activities against the Gram-negative bacteria of *E. coli* ATCC 1129, *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 10031 and 700603 as indicated by the varying degree of zones of inhibitions (Table 1). Compared to other bacteria tested, for *A. baumannii* ATCC 19606, the largest inhibition zone diameters of 14.0 mm and 9.0 mm treated at 200 mg/mL and 100 mg/mL, respectively, were demonstrated. In addition, the crude leaf extract showed good antibacterial activities against *K. pneumoniae* ATCC 10031 with 9.7 mm and 6.7 mm zones of inhibitions tested at 200 mg/mL and 100 mg/mL, respectively. As for *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603, the diameters of zones of inhibitions were relatively small which were 4.0 mm to 3.0 mm at 200 mg/mL and 100 mg/mL, respectively. The standard antibiotic tetracycline (5 mg/mL) which served as the positive control significantly exhibited the highest zone of inhibitions in all the Gram-negative bacteria tested ranging from 6.7 mm to 21.0 mm. On the other hand, negative control showed no inhibition zones in all

<p>| Table 1. Antibacterial activity of ethanolic extract of <em>A. malaccensis</em> by disc diffusion method |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Extract/Antibiotic</th>
<th><em>E. coli</em> ATCC 1129</th>
<th><em>K. pneumoniae</em> ATCC 10031</th>
<th><em>K. pneumoniae</em> ATCC 10031</th>
<th><em>A. baumannii</em> ATCC 19606</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/mL</td>
<td>3.0±0.0</td>
<td>3.0±0.0</td>
<td>6.7±1.1</td>
<td>9.0±1.0</td>
</tr>
<tr>
<td>200 mg/mL</td>
<td>3.3±0.6</td>
<td>3.3±0.6</td>
<td>9.7±0.6</td>
<td>14.0±0</td>
</tr>
<tr>
<td>^2Tetracycline</td>
<td>20.7±0.6</td>
<td>6.7±1.1</td>
<td>21.0±1.0</td>
<td>21.0±1.0</td>
</tr>
</tbody>
</table>

^1Inhibition Zone Diameter (mm±SD) ^2Inhibition zone excluding diameter disk (6 mm)

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the bacteria evaluated in this study.

3.3 Minimum inhibitory concentrations and minimum bactericidal concentrations

Based on the visual investigation of the bacterial culture, the MIC value of the crude leave extract of *A. malaccensis* against the *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 10031 was at 32 mg/mL (Table 2). In turn, the crude extract of *A. malaccensis* exhibited a higher MIC value of 64 mg/mL against the *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603 (Table 2). Furthermore, the results indicated that the MBC values of the crude extract were similar to the MIC for all the bacteria tested (Table 2).

Table 2. MIC and MBC values of the ethanolic crude extract of *A. malaccensis* leaves against different Gram-negative bacteria isolates.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>MIC (mg/mL)</th>
<th>MBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>A. baumannii</em> ATCC 19606</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 10031</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> ATCC 700603</td>
<td>64</td>
<td>64</td>
</tr>
<tr>
<td><em>E. coli</em> ATCC 1129</td>
<td>64</td>
<td>64</td>
</tr>
</tbody>
</table>

4. Discussion

Soxhlet extraction of the *A. malaccensis* leaves using ethanol as the solvent was performed according to the previously optimized method with slight modifications (Zainurin, Samsudin, Hashim et al., 2020). The yield produced approximately 17.84% which was consistent with the previous report that employed a similar method for *A. malaccensis* leaves with 18.45% yield (Zainurin, Samsudin, Hashim et al., 2020). Various studies have shown that the types of solvents used for the extraction of plant materials significantly affect the amount and the effectiveness of the extracts obtained (Koffi et al., 2010; Anokwuru et al., 2011). Compared to other extraction solvents, polar solvents such as ethanol and methanol provide a better capability in extracting secondary metabolites such as flavonoids, phenolic acids and alkaloids (Šibul et al., 2016). Ethanol has been known as an effective and safe polar organic solvent with its wide solubility properties that can extract low molecular weight molecule and moderately polar substances (FDA, 2012). It has been reported in a previous study that ethanolic extracts of *A. malaccensis* leaf gave higher yield (121.34±31.6 mg/g) as compared to hexane extract (34.38±1.0 mg/g) (Hashim et al., 2019).

Crude ethanolic leaves extract of *A. malaccensis* demonstrated a varying degree of antibacterial activity against Gram-negative bacteria including *E. coli, A. baumannii, and K. pneumoniae*. The results demonstrated the presence of clear zones of inhibition around the disc mounted on the nutrient agar plate at the concentrations of 100 mg/mL and 200 mg/mL of the *A. malaccensis* leaves crude extract (Figure 1). The *A. malaccensis* extract showed a strong antibacterial activity against *A. baumannii* ATCC 19606 (9.0 mm – 14.0 mm) and *K. pneumoniae* ATCC 10031 (6.7 mm – 9.7 mm) (Figure 1). Compared to other Gram-negative bacterial strains, the crude extract showed moderate antibacterial activity on *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603 (~ 3.0 mm). Previous studies have been reported on the chloroform extract of agarwood which showed good antibacterial activity against *E. coli, P. aeruginosa*, and *S. aureus* with inhibition zones between 16-17 mm at 50 mg/mL of concentration (Begum, 2016; Hendra et al., 2016). A recent study by Singh et al. (2019) reported on the sensitivity of *A. baumannii, K. pneumoniae* and *E. coli* on commercialized agarwood oil which varied from 30% to 70%. To the best of our knowledge, our present work is the first to report on the antibacterial activity of ethanolic crude leave extract of *A. malaccensis* against *A. baumannii* and *K. pneumoniae*.

Figure 1. Visible zone of inhibitions produced by *A. malaccensis* extracts against Gram-negative bacteria (A) *A. baumannii* ATCC 19606, (B) *K. pneumoniae* ATCC 10031, (C) *K. pneumonia* ATCC 700603 and (D) *E. coli* ATCC 1129. 1 = tetracycline as positive control (5 mg/mL); 2 = DMSO as negative control; 3 = 100 mg/mL; 4 = 200 mg/mL.

The presence of antibacterial activity observed from the crude extract of *A. malaccensis* leaves on *A. baumannii* and *K. pneumonia* was most likely contributed by the constituents of active biological compounds. Qualitative screening of phytochemical compounds of methanolic *A. malaccensis* leave extract revealed the presence of alkaloids, and phenolic compounds (flavonoids, terpenoids, and tannins) that likely to contribute to its antibacterial properties (Khalil et al., 2013). The results were supported by Hendra et al.
(2016) where reported on the antibacterial activity of chloroform fraction of *A. malaccensis* leave extract on *S. aureus* and *E. coli* tested at concentration 300 mg/mL potentially due to the presence of alkaloid and terpenoid (Hendra et al., 2016). Analysis by gas chromatography-mass spectroscopy (GC-MS) revealed that the methanolic and ethanolic extract of agarwood leaves contain hexadecanoic acid which is potentially a major compound contributing to its strong antibacterial activity against Gram-negative bacteria (Khalil et al., 2013; Zainurin, Hashim, Mohamed Azmin et al., 2020). Hexadecanoic acid is one of the most common saturated fatty acids found in animals and plants and has been reported widely to possess antibacterial property (Aparna et al., 2012).

The results of disk diffusion test on *A. baumannii* ATCC 19606 and *K. pneumonia* ATCC 10031 showed notable antibacterial activity with a concentration-dependent effect as indicated by the increased diameter of the zone of inhibition as the concentration increased from 100 mg/mL to 200 mg/mL (Figure 1 and Table 2). Similarly, the results are consistent with a previous study by Hendra et al. (2016) that showed the increasing diameter of the inhibition zone by the increasing concentrations of extract in *E. coli* and *S. aureus*. In addition, Alimon et al. (2011) found that the increased in inhibition zones of *Aquilaria crassna* methanolic leaf extract occurred as the concentration of the extracts increased from 4 mg/mL to 10 mg/mL when tested against *Bacillus spizienii*, *S. aureus*, *Shigella flexneri* and *P. aeruginosa*. Nevertheless, antibacterial activity for the *K. pneumonia* ATCC 700603 and *E. coli* ATCC 1129 showed no significant effects when treated at different extract concentrations of 100 mg/mL and 200 mg/mL.

In the present study, the results showed that the MIC values of *A. malaccensis* crude leave extracts were 32 mg/mL against *A. baumannii* ATCC 19606 and *K. pneumonia* ATCC 10031 which were lower than the MIC values for *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603 (64 mg/mL). The results highlight the susceptibility of the different Gram-negative bacteria strains that showed *A. baumannii* ATCC 19606 and *K. pneumonia* ATCC 10031 were more susceptible towards the crude extract of *A. malaccensis* leaves as compared to the *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603. The results highlight on the antibacterial activity of crude extract and we expect a stronger activity from a purified fraction of *A. malaccensis* leaves likely due to the presence of hexadecanoic acid, alkaloid and terpenoid. Notably, the same MBC value and the MIC values were obtained against all the bacteria tested. This demonstrates that the concentrations of extract needed to inhibit the growth of organisms were sufficient to kill the organisms and this may give an extract a positive vibe as a bactericidal agent against Gram-negative bacteria.

5. Conclusion

The ethanolic extract of *A. malaccensis* leaves showed good antibacterial activity against different strains of Gram-negative pathogens as tested at two different concentrations. The strongest antibacterial activity was observed against *A. baumannii* ATCC 19606 and *K. pneumoniae* ATCC 10031 with MIC and MBC value of 32 mg/mL, to a lesser extent against *E. coli* ATCC 1129 and *K. pneumoniae* ATCC 700603. Overall, the results highlight the potential of *A. malaccensis* leaves as a novel source of antibacterial agents in the fight against the problem of antimicrobial resistance in the Gram-negative bacterial infections.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgement

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