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# Evaluation of antibacterial activities of *Aquilaria malaccensis*, *Aquilaria subintegra* and *Aquilaria sinensis* in different solvents extraction

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*Aquilaria* is a plant from the family Thymeleceae. It is origin to Southwest Asia and has been used traditionally to treat various diseases. The objective of the present study is to evaluate the antibacterial activities of *Aquilaria malaccensis*, *Aquilaria sinensis* and *Aquilaria subintegra* leave extracts against *Staphylococcus aureus* (ATCC33591) and *Escherichia coli* (ATCC35218) in different drying methods and different solvents. To achieve this aim, disc diffusion tests were used. The random sampling technique in which a mix of young and old leaves was applied before being air-dried (AD) at room temperature or oven-dried (OD) at 40ºC. All the samples were subjected to four different solvents; water, 100% ethanol (EtOH), 70% ethanol (EtOH) and hexane. In term of total yield, OD samples have higher than AD samples. The highest yield was 70% EtOH while the lowest yield was hexane. *A. malaccensis* exhibited antimicrobial agents in hexane, 100% EtOH and 70% EtOH. Although the hexane extracts in both AD and OD method showed the ability to inhibit the growth of tested bacterial strains, we found out that the higher inhibition zone was when we tested the 70% EtOH extract in *S. aureus*. Basically, data analysis showed the crude extract of three *Aquilaria* sp. tested possess considerable antibacterial activities regardless of any extracting solvents used but the best solvent extract to inhibit the growth of tested microorganisms was in non-polar solvents, hexane. In conclusion, among the *Aquilaria* sp. tested, we found out *A. malaccensis* possess the best antibacterial activity.

**Keywords: Antibacterial activity, *Aquilaria* sp., *Staphylococcus aureus*, *Escherichia coli*, diffusion test, solvent extraction**

## INTRODUCTION

Karas, also known as gaharu locally or agarwood, aloes wood and eaglewood elsewhere are from the genus, *Aquilaria* Lam. *Aquilaria* is a genus in the family *Thymeleceae* and class *Magnoliopsida*. It is the origin to Southwest Asia. This tree usually found in the rain forest of Indonesia, Thailand, Cambodia, Laos, Malaysia, Northern India, Philippines and Borneo (Ng et al., 1997). Gaharu will produce fragrant resin when an injured or wounded tree is attacked by a fungus or certain insects. *Aquilaria* sp. serves as source material for the production of many aromatic medicinal products, stimulants and tonics. The essential oil extracted from the wood also serves as constituents for perfumes and cosmetics while the scented wood is highly sorted for incense (Faridah-Hanum et al., 2009).

Secondary metabolites of *Aquilaria* serve as natural medicines. Several factors involved in the production of secondary metabolites in plants, including leave age (Achakzai et al., 2009). The

distribution of secondary metabolites is affected by the type of organ and the growth stage of plants. A study on the secondary metabolites of agarwood leaves revealed that the leaves contained alkaloids, flavonoids, triterpenoids, steroids and saponins (Hashim et al., 2016). The phenolics, anthocyanins and flavonoids in plants have highly related to the antioxidant activity. Thus, it is most likely that the high efficacy of agarwood leaves as herbal drinks is closely related to its antioxidant activity. Furthermore, these compounds together with saponins and alkaloids have a proven record as antibacterial agents in many other plants (Hendra et al., 2016).

The objectives of this study are to evaluate the antibacterial activities of *A. malaccensis*, *A. sinensis* and *A. subintegra* against *S. aureus* and *E. coli* in different solvents; water, 100% EtOH, 70% EtOH, hexane and in different drying methods; air-dried (AD) and oven-dried (OD).

## MATERIALS AND METHODS

### Preparation of Samples

### Collection of Plant Materials

The leaves samples of *A. malaccencis* were collected from Forest Research Institute Malaysia (FRIM), Forest Reserve in Merchang, Terengganu, while the leaf sample of *A. subintegra* was collected from a nursery at Alor Gajah, Melaka and the leaf sample of *A. sinensis*, were collected from the nursery at Sri Kembangan, Selangor. The random sampling technique was applied in which the young and old leaves from different trees were mixed together. The leaves were washed to remove the impurities before being air-dried (AD) at room temperature (22 to 23ºC) and oven-dried (OD) in a laboratory oven (40ºC) for 18 hours or until weight constant. Then, the leaves were ground coarsely using warring blander into powder form and kept in the plastic bags and stored at -80°C chiller prior to analysis.

### Extracts Preparation

The extraction process was carried out with four different solvents (water, 100% EtOH, 70% EtOH and hexane). The powdered material was macerated in the solvents with ratio sample to solvent (1:50) at room temperature (Amalina, 2015). The samples were soaked for 24 hours. The mixture was sonicated for 20 minutes and then filtered through using Whatman Filter No.1. The extraction steps were repeated three times for 3 days. The extracts, then were evaporated to dryness using a rotary evaporator (Heidoph Germany).

### Determination of Antibacterial Test

### Preparation of Bacterial Suspension

The antibacterial activity of the leaf extracts were assessed against two bacteria species: *Staphylococcus aureus* (ATCC33591) and *Escherichia coli* (ATCC35218). The bacterial suspensions were prepared by mixing each isolates *S. aureus* and *E. coli* in 50 mL Mueller-Hinton Broth (MHB). The bacterial turbidity was adjusted to have a similar absorbance with 0.5 Mc-Farland solutions at 0.1 OD λ 625 nm to reach 108 cfu/mL of bacterial suspension (Faridah-Hanum et al. 2009). All the bacterial stocks were obtained from the collection of Microbiology Laboratories, Faculty Bioresources and Food Industry, Universiti Sultan Zainal Abidin (UniSZA).

### Paper Disc Diffusion Test

A sterile petri dish containing 15 mL of Meuller Hinton Agar (MHA) media was inoculated with 200 µL of the bacterial suspension and incubated for 24 hours at 37°C. Eight crude extracts which were AD-water, OD-water, AD-70% EtOH, OD-70% EtOH, AD-100% EtOH, OD-100% EtOH, AD-hexane and OD-hexane, positive control and negative control were evaluated in the series concentration of 150, and 300 mg/mL. Each paper disc (Whatman no. 1) with a diameter of 5 mm were placed onto the media after previously pipetted out with the corresponding extract and dried at room temperature. The positive controls of 20 µg/mL ampicillin and 30 µg cefoxitin FOX were used while the negative controls were 10% DMSO (v/v) and 100% DMSO (v/v) solutions. The antibacterial activity of the tested extracts was shown by a clear zone of inhibition around the application point (Faridah-Hanum et al., 2009).

### Data Analysis

All experiments were conducted in triplicate. The diameter of the inhibition zone was observed and compared with the diameter zone of inhibition of the standard antibiotic or positive control. The one-way Analysis of Variance (ANOVA) was used for the data analysis for presented ± standard deviation (SD). Significant different based on p-value where $p<0.05$.

## RESULTS AND DISCUSSION

**Extraction Yields of *Aquilaria* sp.**

The yield of leaf extracts as a result of extracting with different solvents extracts are summarized in Table 1.

Overall, OD samples showed higher percentage yield as compared to AD samples (Table 1). In this experiment, OD-70% EtOH produced the highest percentage yield (30.82%), followed by OD-70% EtOH (22.35%) and OD-70% EtOH (21.32%). Hexane in both OD and AD samples gave minimal percentage yield with 0.82-2.39%. This result was in agreement with the study by Wan-Nadilah et al. (2019) towards *A. malaccensis* showed that the highest percentage yield (OD-ethanol with 26.94%) and the lowest percentage yield (AD-hexane with 1.48%). In this study, the higher percentage yield were obtained from OD samples in all *Aquilaria* sp. used. According to Doymaz (2005), William (2013) and Sabarez (2016), the drying technique is a very common preservation method used in food study and food development. The reduction of water activity by moisture removal leads to preserve the phytochemicals (Vongsak et al., 2013) and importantly can alter biological and chemical properties of foods (Okos et al. 1992). Whereas, fresh sample using AD method are fragile and tend to deteriorate faster than dried sample (Azwanida, 2015). This is due to more water content in leaves sample may cause contamination at unstable temperature (Arabhosseini et al. 2007; Harbourne et al., 2009; Suvarnakuta et al., 2011; Mediani et al., 2014).

In the study, the leaves of *Aquilaria* sp. were dried whether at room temperature or in the oven at the temperature 40ºC before were ground to powder. The drying may cause losses in volatilities or formation of new volatilities as a result of oxidation reactions, esterification reactions (Diaz-Maroto et al., 2002). The powder form of *Aquilaria* samples will increase the surface area, thereby allowing the maximum contact of samples with the extraction solvents.

As shown in Table 1, the 70% EtOH and water extracts yielded more than 10% of total yield in all *Aquilaria* sp. tested in this study while hexane extract gave the lowest yield. Overall, the increasing order for extract yield was hexane > water > 100% EtOH > 70% EtOH. It clearly showed that polar solvents were able to extract more polar compound compared to those non-polar. This finding supported by the previous studies that used more organic solvent, particularly the EtOH:water (80:20 and 60:40) extracts, has been found to be superior in extracting higher yield of antioxidant, anti-cancer, anti-diabetic compounds from a wide range of plants (Chatha et al., 2011). This result can be compared with previous studies by Wan-Nadilah et al. (2019), which reported that high percentage yields were on solvent polar (ethanol and water extract) while low percentage yield was from hexane.

**Antibacterial Activities**

The antibacterial activity of *Aquilaria* sp. extracts were tested against *S. aureus* and *E. coli* at 300 mg/mL and 150 mg/mL for AD and OD drying method for *Aquilaria* sp. are presented in Figure 1 (A,B,C). The *Aquilaria* sp. leaves extracts had antibacterial activities on *S. aureus* and *E. coli* but differ in strength as mentioned by Hendra et al., (2016).

**Table 1; Percentage yield of air-dried and oven-dried of different *Aquilaria* sp. leaf extracts in different solvent extractions.**

| ***Aquilaria* sp.** | **Drying method** | **Percentage yield (%)** | | | |
|---|---|---|---|---|---|
| | | **Water** | **100% EtOH** | **70% EtOH** | **Hexane** |
| *A. malaccensis* | AD | 11.24 | 8.59 | 16.93 | 1.18 |
| | OD | 13.50 | 14.66 | 21.32 | 1.30 |
| *A.sinensis* | AD | 13.83 | 5.29 | 10.51 | 1.04 |
| | OD | 17.72 | 13.00 | 22.35 | 2.39 |
| *A.subintegra* | AD | 12.71 | 18.53 | 14.70 | 0.82 |
| | OD | 16.21 | 18.54 | 30.82 | 1.42 |

AD means air dried, OD means oven dried

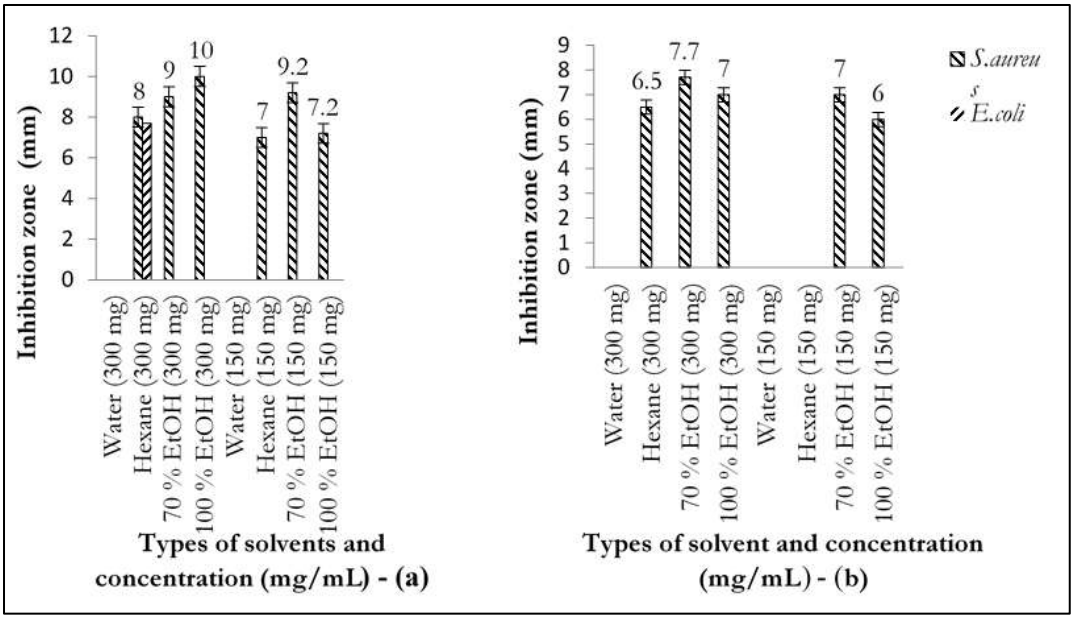


**Figure 1(A); Antibacterial activities of *A. malaccensis* in (a) AD and (b) OD sample against 300 mg/mL and 150 mg/mL.**

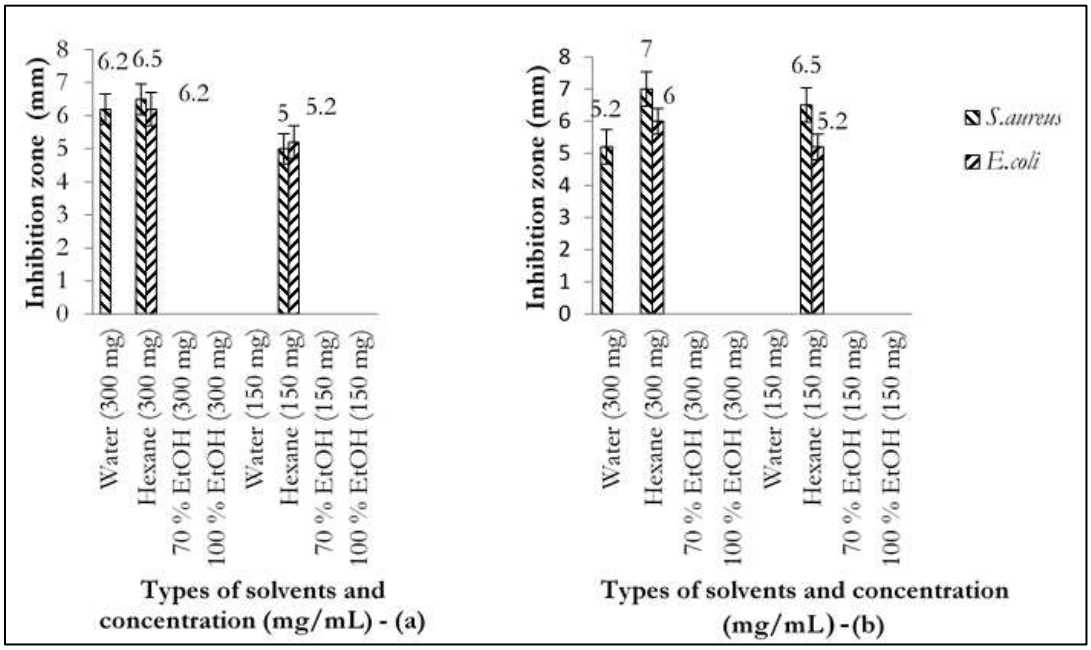


**Figure 1(B); Antibacterial activities of *A. sinensis* in (a) AD and (b) OD sample.**

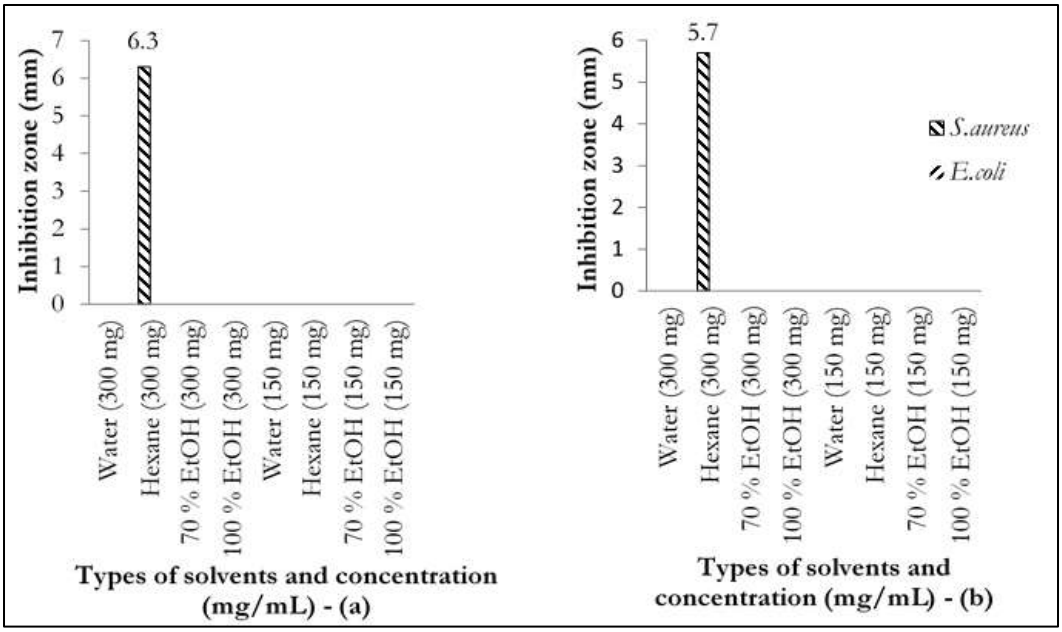


**Figure 1(C); Antibacterial activities of *A. subintegra* in (a) AD and (b) OD sample.**

Furthermore, the optimum inhibition has been shown by *A. malaccensis* hexane extract, then concentration of 75 mg/mL was added as presented in Table 2.

**Table 2; Antibacterial activity of *Aquilaria* sp. against *S. aureus* and *E. coli* in hexane extract under different drying methods.**

| Extract | Drying method | Concentration (mg/mL) | Diameter of inhibition zone (mm) | |
|---|---|---|---|---|
| | | | *S. aureus* | *E. coli* |
| *A.malaccensis* | AD | 300 | 8.0 | 7.8 |
| | | 150 | 7.0 | NA |
| | | 75 | 6.0 | NA |
| | OD | 300 | 6.5 | NA |
| | | 150 | NA | NA |
| | | 75 | 5.5 | NA |
| *A.sinensis* | AD | 300 | 6.5 | 6.2 |
| | | 150 | 5.0 | 5.2 |
| | OD | 300 | 7.0 | 6.0 |
| | | 150 | 5.7 | 5.2 |
| *A.subintegra* | AD | 300 | 6.3 | NA |
| | | 150 | NA | NA |
| | OD | 300 | 5.7 | NA |
| | | 150 | NA | NA |

NA means no activity

Overall, hexane extracts were found to be more effective on microorganisms in comparison to the other extract with 5.0-8.0 mm of zone inhibition, (Table 2). The results suggest that antibacterial principle lies in non-polar solvent extract as compared to polar extracts. There are a number of reports of hexane extracts of plant possessing antibacterial activity. The hexane solvent are chosen based on the polarity of the solute of interest. The polarity, from least polar to most polar, of a few common solvents is as follows: Hexane < Chloroform < Ethyl acetate < Acetone < Methanol < Water (Altemimi et al., 2017). In this study, hexane, ethanol and water were selected as a solvent extraction with similar polarity to the solute will properly dissolve the solute. In fact, a study conducted by Singh et al. (2012), also found out that the hexane extract showed the ability to inhibit the growth of bacteria.

Hashim et al., (2012) also showed the positive result of *A. subintegra* extract. Particularly, the highest zone of inhibition was observed when using AD-Hexane extract at 300 mg/mL which were tested to *S. aureus* and *E. coli* with 8.0 mm and 7.8 mm, respectively. This result was consistent with the previous studies conducted by Yusoff et al., (2015) and Wan-Nadilah et al. (2019), which suggested that as the concentration of extract increased, the antibacterial activity of the plants were also increased significantly. Unfortunately, OD-Hexane extract at 150 mg/mL of *A. malaccensis* was resistant to the tested microorganisms even though both of the solvents from *A. malaccensis* samples were able to inhibit the growth of bacteria. A possible explanation for such a difference is that a conditions of sample that were used either in a good condition, vice versa. Another distinction that can be explained is the handling or sterilization technique during the experiment.

However, the highest diameter of inhibition zone are in AD-100% EtOH with 10 mm on *S. aureus* for *A. malaccensis* leaves extracts (Figure 2a-A). This results in the present work might be due to the plant extract contain different active compounds where each of them functions differently for inhibition of microorganisms and a mixture of them will cause the synergism activity which sometimes makes compounds became more optimum (Wariska et al. 2014; Yusoff et al. 2015; Wan-Nadilah et al. 2019).

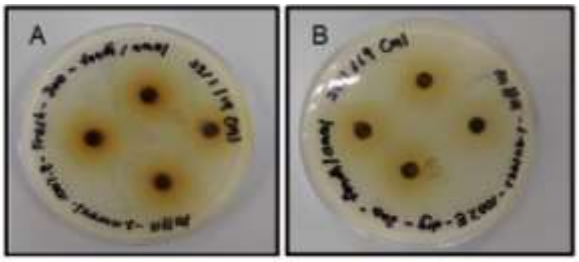


**Figure 2(a); Zone inhibition of *A. malaccensis* 100% EtOH extract in 300 mg/mL (A) AD and (B) OD drying method.**

The current study also found that antibacterial activity for *A. malaccensis* extracts are greater in AD samples compared to the OD samples. The AD-100% EtOH extract was able to inhibit better compare to the AD-hexane extract and AD-70% EtOH extracts when tested on *S. aureus*. While neither the aqueous AD nor OD extracts were resistant against tested microorganisms. Furthermore, this study did not found any significant inhibition zone for 70% EtOH extracts at the increasing concentration and this might be due to the poor dissolving capability of 70% EtOH extracts. Thus, it is crucial to make sure that the extracts dissolve well and can be done by increasing the water concentration in the mixture of DMSO followed by actions such as vortexing and sonicating to make sure the extract dissolve well. Previous study by Yavuz et al., (2017) used 25% DMSO (v/v) to properly dissolve the extracts.

As shown in Figure 2b, *A. sinensis* showed that OD method has a greater ability to inhibit the growth of *E. coli* and S*. aureus*. This may be due to the ability of non-polar secondary compounds from hexane extract to diffuse into the layer peptidoglycan of the Gram-negative bacteria, weakening the peptidoglycan scaffold within the bacterial wall and compromising the structural integrity (Yuan et al., 2017). Unfortunately, 100% EtOH and 70% EtOH extracts of *A. sinensis* were resistant to the tested microorganisms even though both of the solvents from *A. malaccensis* samples were able to inhibit the growth of bacteria.

The potential metabolite in those extracts which contribute to that interested biological activity might be not present in *A. sinensis* due to many factors such as plant varieties, geographical origin, developmental stage and environmental conditions (Maulidiani et al., 2012). Increasing the concentration of hexane extracts would increase the diameter zone of inhibition against both Gram positive and Gram-negative bacteria tested (Figure 2b) with 6.2 mm and 5.0 mm, respectively.

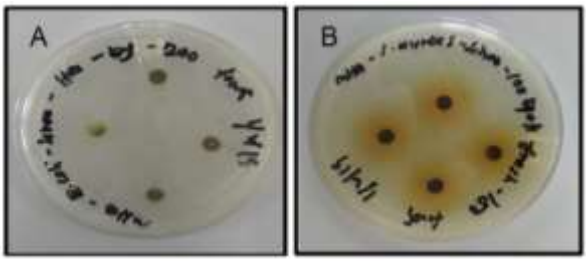


**Figure 2(b); Zone inhibition of *A. sinensis* hexane extract (A) OD toward *E. coli* in 300 mg/mL and (B) AD towards *S. aureus* in 150 mg/mL.**

However, only AD and OD-hexane extracts showed inhibition of *E. coli* growth (Table 2). The findings of the present study are consistent with Chen et al., (2012) that reported an agarwood essential oil derived from *A. sinensis*, regardless of whether it originated from artificial or natural agarwood, had inhibitive activities towards several

microorganisms (i.e *Bacillus subtilis* and *Staphylococcus aureus*).

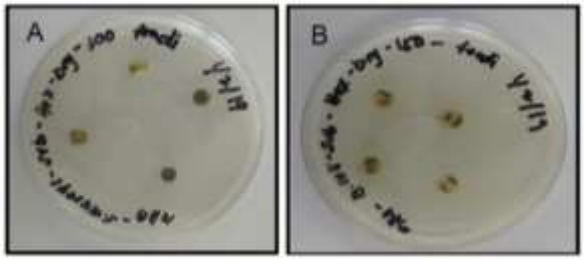


**Figure 2(c); Zone inhibition of *A. subintegra* OD-hexane extract in (A) 300 mg/mL towards *S. aureus* and (B) 150 mg/mL towards *E. coli.***

For *A. subintegra* extracts, the antibacterial properties showed the weakest activity compared to the other *Aquilaria* sp. used in this study (Table 2). Only AD-hexane and OD-hexane extracts showed promising inhibition against *S. aureus* at the highest concentration tested (300 mg/mL) (Figure 1C) Whereas, Figure 2(c) showed the antibacterial activity of *A. subintegra* against *S. aureus* at the 300 mg/mL and 150 mg/mL against *E. coli* with 5.7nm and no inhibition reported, respectively.

However, even tested at the highest concentration, the zone of inhibition was actually smaller compared to the other species. The present results show that *A. subintegra* extract was expected do not contain many potential metabolites that influence antibacterial activity. However, this result was disagreeing with the study by Hashim et al. (2012), that shown the inhibition zone against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* for acetone and hexane extract. There could be several reasons for the negative result. One of the factors may be because the chemical components of agarwood are diverse and complex (Chen et al., 2012). Thereby, contributing to the diverse in the bioactivity and pharmacology effects. As reported before, *A. subintegra* can be used as natural AChe inhibitory effect that related on Alzheimer disease (Bahrani et al. 2014), antidiabetic agent (Yunus et al., 2015) and laxative for weight reducing (Ibrahim et al., 2018).

As the results were shown, the dilution concentration was made from 300 mg/mL to 150 mg/mL and 75 mg/mL in *A. malaccensis* for hexane extracts due to higher inhibition zone than other species. The result gives hexane extract can inhibit on *S. aureus* in both AD and OD samples with 6.0 mm and 5.5 mm, respectively in low concentration (Table 2).

Moreover, in terms of positive control, the two current antibiotics ampicillin and cefoxitin FOX were introduced in order to evaluate the effectiveness of the antibiotics against antibiotics resistance (Table 3). Meanwhile Figure 3 showed the inhibition zone of cefoxitin FOX and negative control 100% DMSO.

**Table 3; Positive and Negative Control against *S.aurues* and *E.coli***

| Control | The diameter of inhibition zone (mm) | |
|---|---|---|
| | ***S. aureus*** | ***E. coli*** |
| 100% DMSO (v/v) | NA | NA |
| 10% DMSO (v/v) | NA | NA |
| Ampicillin (20 µg) | NA | NA |
| Cefoxitin FOX (30 µg) | 20 | 20 |

NA means no antibacterial activities

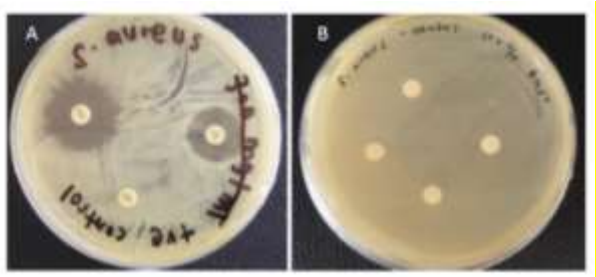


**Figure 3; (A) Positive control using cefoxitin FOX and (B) negative control using 100% DMSO with no inhibition.**

Dimethyl sulfoxide (DMSO) acted as the negative control in this study because this solvent was used to dissolve the crude extracts (Bakhtiar et al., 2015; Rahman et al., 2016; Khan et al., 2017). This study found that 10% DMSO (v/v) and 100% DMSO (v/v) did not inhibit the growth of bacteria tested. This result is in agreement with the previous work conducted by Yavuz et al. (2017), towards methanol extract of plant in Lamianeae family. This study showed no activity of DMSO reported as a negative control. Other studies by Hashim et al. (2012) and Ghosh et al. (2013) towards *A. subintegra* and *A. agallocha,* respectively shown the negative inhibiton of DMSO. Chen et al. (2012) also used DMSO as a negative control in *A. sinensis* extract. Thus, it can be concluded that the inhibition of bacteria was solely due to the potential antibacterial metabolites in the plant extracts.

## CONCLUSION

Among the three species of *Aquilaria* evaluated in this study, *A. malaccensis* exhibited

considerable antibacterial properties as it showed zone of inhibition even tested at the smallest concentration at 75 mg/mL. This study concluded that the best extraction solvent to extract the potential metabolite for antibacterial is hexane. Hexane extracts in both drying methods (AD and OD) for *A. malaccensis* were able to inhibit the growth of *S. aureus* compared to *E. coli*. This may due to the reason that the potential metabolite that contributes to the activity is not temperature sensitive. However, the extraction yields for hexane extracts were the lowest. Thus, the successful concentrations for this screening are at 75 mg/mL. As suggestion, further study should focus on a wide range of bacterial pathogens. *Staphylococcus aureus, Enterococcus* spp. or *Staphylococcus epidermidis* should be used to improve the results obtain and test minimum inhibitory concentration (MIC) of applied antibiotics for bacterial isolates. Furthermore, different types of phytochemical tests should be carried out to investigate the natural compound that present in plants.

## CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

## ACKNOWLEGEMENT

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## AUTHOR CONTRIBUTIONS

AR and WNAWM conceived and designed the experiment. AR and WNAWM performed the experiment. AR and WNAWM analyzed the data. AR and WNWA wrote the article. NHM, AMA, WNWA and SMY provided supervision and research facilities. AMA, WNWA and NHM provided intellectual inputs. All authors read and approved the final version.



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