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RESEARCH ARTICLE

# ANTIBACTERIAL ACTIVITY OF COMPOUNDS FROM *Azolla pinnata* EXTRACTED USING SOXHLET AND SUPERCRITICAL FLUID (SFE) METHODS

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**ABSTRACT**

The propagation, extraction, and antibacterial studies of *Azolla pinnata* were carried out in this study. The propagation involved two fertilizers, which were chicken manure and inorganic AB fertilizer. The dry yield was extracted using two methods, which were Soxhlet and supercritical fluid extraction (SFE). Methanolic extracts were obtained and subjected to several antibacterial tests, which include the disk diffusion assay, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests, against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. The results showed that AB fertilizer produced more dry yield compared to chicken manure. The extract yield from Soxhlet exhibited a higher yield than that of SFE, recording its highest at 21.20%. The findings of antibacterial tests revealed that all extracts inhibited the growth of *B. subtilis* and *S. aureus*, but none showed inhibition against *E. coli* and *P. aeruginosa*. The largest diameter of inhibition zone of 2.67 ± 1.53 mm was obtained by E2 (CM-SFE), with a MIC value of 0.125 mg/mL against *B. subtilis*. High MBC values further confirm that the mechanism of inhibition against *B. subtilis* and *S. aureus* were due to the bacteriostatic properties of the extracts tested.

**KEYWORDS**

*Azolla pinnata,* Soxhlet, supercritical fluid extraction, antibacterial activity.

## 1. INTRODUCTION

The misuse of antibiotics, as well as poor infection prevention and control, have given birth to antibiotics-resistant bacteria. In 2017, the Institute for Medical Research Malaysia reported the increase in the resistance of several bacterial isolates from local hospitals against antibiotics (Institute for Medical Research, 2019). This has led researchers to go on a quest for alternatives of antibiotics, which includes compounds extracted from plants. Plants produce a variety of compounds that have different purposes for their benefit and survival in the ecosystem. For instance, *Vaccinium* spp. contains fructose which could fight infections caused by *E. coli*, and *Eucalyptus globulus* comprises of tannin which acts antagonistically against bacteria and virus (Cowan, 1999).

*Azolla* is a small aquatic fern that is usually found floating on stagnant water and naturally grows in drains, canals, ponds, rivers and marshy lands. It is commonly known as mosquito fern and can be recognised by its thick layer of green leaves growing on the water's surface, which prevents mosquito from breeding (Nordiah et al., 2012). It forms a symbiotic relationship with the nitrogen-fixing cyanobacterium, *Anabaena azollae*, which offers large amounts of fixed nitrogen to *Azolla* that contributes to its fast growth, as well as its ability to grow in habitats where N is lacking (Rai and Rai, 2003). In this current study, *Azolla pinnata* was investigated and evaluated through parameters that may influence its antibacterial activity. Different fertilizer types and extraction methods were used to explore the effects of these parameters on the antibacterial activity of the plant.

## 2. MATERIAL AND METHODS

### 2.1 Preparation of plant sample

The plant was propagated in plastic containers filled with dechlorinated tap water for one week using chicken manure as growth fertilizer to obtain a sufficient amount of plant materials for the next procedures. Next, two types of fertilizers were applied, which were organic (chicken manure) and inorganic (AB fertilizer). AB fertilizer consisted of macronutrients and micronutrients needed for plant growth, while the chicken manure contained lower levels of nitrogen (N), potassium (K) and phosphorus (P).

### 2.2 Extraction of phytochemicals

Dried powdered samples were extracted using Soxhlet and supercritical fluid methods. Soxhlet extraction was performed using a Soxhlet apparatus with 11.0 g of dried sample powder inserted into the paper thimble. Next, some cotton wool was placed on the upper part of the extraction flask to prevent the sample from flowing onto other apparatus parts. Approximately 250 mL of methanol in a round-bottom flask was positioned on the heating mantle. The solvent was heated at a temperature

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of not more than 64°C, for approximately 12 hours until the solvent in the siphon arm became clear. On the other hand, supercritical fluid extraction was conducted at fixed conditions using pure liquefied carbon dioxide at a pressure range of 3500 – 5500 psi, using 20% (v/v) of absolute methanol as co-solvent. 12.0 g of dried sample powder was placed into the extractor vessel, and the temperature was kept at 60°C. The mode used for the extraction was a static extraction mode for 3 hours. After sample extraction by each method was complete, the extracts were dried in the rotary evaporator with 337 vapour pressure and water bath temperature of 60°C to obtain the crude extract. Finally, the crude extracts were weighed and stored at -4°C until further use.

### 2.3 Antibacterial assay

Mueller Hinton (MH) agar and broth were prepared by dissolving 34 g of MHA powder, and 21 g of MHB powder into 1 L of distilled water, respectively. The mixtures were then sterilized at 121°C for 15 minutes. Then the agar medium was poured into sterilized petri dishes and allowed to solidify at room temperature. The plates and broths were kept at 4°C until further use. The 0.5 McFarland standard was prepared by mixing 0.5 mL of 1.175% barium chloride dihydrate ($BaCl.2H_2O$) with 99.5 mL of 1% sulphuric acid. The optical density (OD) of 0.5 McFarland standard was measured at a range of 600 – 625 nm by using an ultraviolet-visible (UV-Vis) spectrophotometer. Two Gram-positive bacteria, *B. subtilis* and *S. aureus*, as well as two Gram-negative bacteria, *E. coli*, and *P. aeruginosa,* were tested in this study. All the bacteria were cultured on MH agar using the streaking method until single and pure colonies were obtained.

The bacteria were incubated at 37°C for 18 - 24 hours to enable them to grow. Then, the single and pure colonies of the bacteria were transferred into MH broth and incubated for 18 – 24 hours before their OD were measured using a UV-Vis spectrophotometer. The density of bacterial culture was standardized by comparing their OD with that of the 0.5 McFarland standard. The reading was adjusted by adding a bacterial culture or sterile broth until the acceptable OD reading was achieved. For the test samples, four extracts from the extraction were used. 100 mg of extract was dissolved in 1 mL methanol, making a stock concentration of 100 mg/mL. Streptomycin was used as the positive control, while methanol as the negative control. Then, the disk diffusion assay was conducted in a laminar air flow cabinet, and aseptic techniques were applied. First, the agar surface was inoculated with 50 µL of the standardized bacterial suspension. The suspension was made to spread evenly on the agar surface using a hockey stick. Next, in an empty petri dish, sterilized disks were impregnated with 10 µL of extract samples, positive and negative controls.

The disks were then placed on the pre-inoculated agar surface. Finally, the plates were sealed with parafilm and incubated at 37°C for 24 hours. After the incubation period, the plates were observed, and the diameters of inhibition zones (in mm) were measured and recorded. Each bacterial plate had two replicates for consistency. Following the disk diffusion assay, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) tests were performed. In the MIC test, the microbroth dilution method using 96-well microplate was conducted, and concentrations ranging from 1 to 0.0156 mg/mL for the test compound were prepared by performing two-fold dilution throughout the wells. The plates were incubated for 24 hours at 37°C before the observation was taken, by visually observing turbidity of mixtures in each well. Next, the MBC test was carried out by selecting two lowest concentrations that produced clear solutions from the MIC test and streaking it on MH agar plates. The plates were then incubated for 24 hours at 37°C. After the incubation period, the plates were observed. The lowest concentration of the tested compound to completely kill the bacteria was determined by observing the plates that did not show any growth of bacteria colony.

## 3. Results and Discussion

The two methods used for extracting compounds from the samples were Soxhlet (SOX) and SFE. Crude extracts were dried using the rotary evaporator and weighed using an electronic balance. Table 1 shows the percentage yield of the extracts based on the weight of dry sample used for extraction. Extracts 1 and 2 represent the samples treated with chicken manure (CM), while extracts 3 and 4 were treated with fertilizer AB (AB). Extracts 1 and 3 were obtained through SOX, while extracts 2 and 4 utilised SFE method.

**Table 1:** Percentage yield (%) of extract samples from Soxhlet and supercritical fluid extraction methods

| Extract sample | | Initial weight of sample (g) | Weight of extract sample (g) | Percentage yield (%) |
|---|---|---|---|---|
| SOX | 3 | 11.00 | 2.33 | 21.20 |
| | 1 | 11.00 | 2.23 | 20.29 |
| SFE | 2 | 12.00 | 2.28 | 19.00 |
| | 4 | 12.00 | 1.56 | 13.00 |

1: CM-SOX; 2: CM-SFE; 3: AB-SOX; 4: AB-SFE

Based on Table 1, the yield of Extract 3 is the highest among all other extracts, recording 21.20%. This extract was obtained from the AB-SOX portion. On the other hand, the lowest yield is from Extract 4, which was obtained from the AB-SFE portion, with a value of 13.00%. Both the extracts obtained from Soxhlet showed higher percentage yield than those from SFE. However, t-test analysis comparing the mean values from both extraction methods had a p-value > 0.05, which indicates that the two methods are not significantly different from each other. The results of this study showed that yields from Soxhlet portions are higher than those of SFE portions. This occurrence might be due to the selectivity of the extraction methods. The Soxhlet extraction produced a yield containing volatile compounds together with high-molecular-mass compounds, that indicates low selectivity with respect to volatile compound (Herzi et al., 2013).

Another deduction that could interpret the results obtained in this study is that the yield was affected by the shorter extraction time applied when extracting the compound using SFE, which was approximately 3 hours (Hasmida et al., 2014). Soxhlet extraction, on the other hand, was operated for about 16 hours. The yield from SFE might increase if the extraction time was longer. For the disk diffusion assay, the concentration of extract samples was fixed at 40 µL per disc. The assay was carried out in triplicates. After the overnight growth of bacteria, all the plates were carefully examined for any zone of inhibition. The diameters of inhibition zones (in mm) of each replicate were measured and recorded. The average values of the inhibition zones are displayed in Table 2.

**Table 2:** Diameter of inhibition zones (in mm) of methanolic extracts of A. pinnata

| No. | Inocula | Compound | Diameter (mm) |
|---|---|---|---|
| **1.** | *B. subtilis* | E1 | 2.33 ± 0.58 |
| | | E2 | 2.67 ± 1.53 |
| | | E3 | 0.67 ± 1.15 |
| | | E4 | 1.67 ± 0.58 |
| | | Positive control | 23.00 ± 6.25 |
| | | Negative control | NA |
| **2.** | *S. aureus* | E1 | 0.67 ± 1.15 |
| | | E2 | 1.00 ± 1.00 |
| | | E3 | 1.67 ± 2.89 |
| | | E4 | 1.33 ± 1.53 |
| | | Positive control | 13.7 ± 0.58 |
| | | Negative control | NA |
| **3.** | *E. coli* | E1 | NA |
| | | E2 | NA |
| | | E3 | NA |
| | | E4 | NA |
| | | Positive control | 19.00 ± 1.00 |
| | | Negative control | NA |
| **4.** | *P. aeruginosa* | E1 | NA |
| | | E2 | NA |

| | | | |
|---|---|---|---|
| | | E3 | NA |
| | | E4 | NA |
| | | Positive control | 12.33 ± 2.52 |
| | | Negative control | NA |

E1: CM-SOX; E2: CM-SFE; E3: AB-SOX; E4: AB-SFE
Positive control: Streptomycin
Negative control: Methanol
NA: not available

Among the four bacteria tested, only two produced positive results, which were both the Gram-positive bacteria. All the extract samples showed antibacterial properties against *B. subtilis* and *S. aureus*, but none of them did against *E. coli* and *P. aeruginosa*. The results corroborate with a study conducted which had discovered that the organic extracts from *A. filiculoides, A. caroliniana,* and *A. rubra* inhibited the growth of *B. subtilis* (Peveira et al., 2015). It is known that Gram-negative bacteria are much more resistant to antimicrobial agents, than Gram-positive bacteria (Zarrsnik et al., 2011). This is because Gram-negative bacteria have an outer membrane which acts as a selective barrier on the materials being exchanged across it. The highly hydrophobic lipid bilayer with pore-forming proteins of specific size-exclusion properties provides the bacteria an extra layer of protection (Delcour, 2009). Therefore, it is harder for compounds to penetrate through their membrane. Furthermore, the increased heat exposure towards plant materials during Soxhlet extraction might have caused the compounds responsible for anti-pseudomonas activity to degrade (Kothari et al., 2012). Hence, no inhibition zone was observed for *P. aeruginosa*.

Between the extract samples, E2 showed the largest inhibition zone against *B. subtilis*, recording 2.67 mm. Both the extracts obtained from samples cultivated with chicken manure showed higher values of inhibition against the bacteria. A group researhcers reported that low fertilization rates of organic fertilizer led to an accumulation of soluble sugar, which enhanced the levels of flavonoids and total phenolics, indirectly affecting the production of the antimicrobial compound (Ibrahim et al., 2013). The carbon nutrient balance hypothesis also states that plants growing in nitrogen-deficient soils will allocate unused carbon that is not used for growth to the synthesis of secondary metabolites (Ibrahim et al., 2011). Since chicken manure consisted of lower levels of macronutrients, this explains why extracts derived from it have shown higher values of inhibition in the disk diffusion assay. Furthermore, the inhibition zone produced by E2, which was obtained from the SFE method, was larger than E1, which was the sample extracted using Soxhlet. SFE most likely favoured the extraction of both polar and non-polar compounds due to the polarity of solvent $CO_2$ and methanol as co-solvent, and it operates on the selectivity theory to produce extracts which are pure and in high quality, when compared to Soxhlet (Sapkale et al., 2010). Hence, this shows that the extraction methods influence the efficacy of the extracts against *B. subtilis.*

On the other hand, the largest inhibition zone against *S. aureus* was shown by E3, which was 1.67 mm. The extracts obtained from samples cultivated with AB fertilizer showed higher antibacterial activity. This observation might be related to the enhanced synthesis and accumulation of compounds resistant to *S. aureus*, which can be supported through a study by Osuagwu and Edeoga, which revealed that the application of inorganic fertilizer NPK had a positive effect on the antibacterial activity against the bacteria (Osuagwel and Edeoga, 2010). Furthermore, the inhibition zone produced by E3, which was obtained from Soxhlet, was larger than E4, which was extracted through SFE. The compounds present in E3 most probably favoured extraction of polar compounds, which was easily achieved via the Soxhlet method using methanol as solvent (Ravi et al., 2018). Therefore, it can be said that *S. aureus* was inhibited more effectively by the polar compounds present in E3. After the extract samples have shown zones of inhibition, they were subjected to MIC and MBC tests. Table 3 below shows the results obtained from the tests after incubation of 24 hours.

**Table 3:** Antibacterial activity of compounds by MIC and MBC analysis

| Bacteria | Compound | MIC (mg/mL) | MBC (mg/mL) |
|---|---|---|---|
| *B. subtilis* | E1 | 0.5 | 0.5 |
| | E2 | 0.125 | 0.5 |
| | Positive control | 0.016 | NA |
| | Negative control | 0.25 | NA |
| *S. aureus* | E1 | 0.125 | 0.5 |
| | E2 | 0.125 | 0.5 |
| | Positive control | 0.016 | NA |
| | Negative control | 0.25 | NA |

Positive control: Streptomycin
Negative control: Methanol
NA: Not available

The MIC values were determined by visually observing the turbidity in each well. Those with clear solutions were considered as positive results, meaning that the compound was able to inhibit the growth of bacteria in the well. Meanwhile, wells which showed cloudy solutions and the presence of a white 'button' on the bottom of the well were marked as negative observation, since it indicates that the compound did not inhibit the growth of bacteria. Based on the MIC test, E2 showed lower concentrations of inhibition than E1 against *B. subtilis*. It is noteworthy to mention that the negative control showed inhibition at a concentration lower than E1. This suggests that the MIC value of E1 is probably due to the inhibitory actions of solvent methanol. The lowest MIC value was obtained by streptomycin, which inhibited bacterial growth at 0.016 mg/mL. Meanwhile, the MBC test revealed that no bacterial growth was observed for E1, confirming the MIC results. The MBC value for E2, however, was determined at 0.5 mg/mL because the lower concentrations showed bacterial growth on the agar plates.

On the other hand, similar values were obtained for E1 and E2 concentrations against *S. aureus*. Streptomycin had the lowest inhibitory concentration of 0.016 mg/mL. MBC values of E1 and E2 were determined at a higher concentration of 0.5 mg/mL since the lower concentrations exhibited bacterial growth on agar plates. The results from MIC and MBC tests could be explained by the mechanism in which the compound inhibits bacterial growth. The compounds could either be bacteriostatic, i.e., prevent the growth of bacteria, or bactericidal, i.e., cause bacterial cell death (Pankey and Sabath, 2004). For *B. subtilis*, it appears that the inhibitory action by E1 was due to the solvent methanol, while E2 showed bacteriostatic characteristics. The same can be said for *S. aureus*; both E1 and E2 exhibited bacteriostatic characteristics.

## 4. Conclusion

The findings of this study indicate that the active fractions of *A. pinnata* extracts contain bioactive compounds that exhibit antibacterial activities, yet none can be used as a potential antibacterial agent since the bioactive compounds only showed bacteriostatic attribute. Both the fertilizer application and extraction methods showed an effect on the efficacy of extracts' antibacterial activity against Gram-positive bacteria. However, further research is required to establish a stronger conclusion regarding the effects of fertilizer application and extraction methods on the antibacterial activity of *A. pinnata*

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