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Original Research Article

Evaluation of *Salvinia molesta* D.S.Mitch (Salviniaceae) for Antioxidant and Antibacterial Properties

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ABSTRACT

Salvinia molesta D.S.Mitch, identified as a troublesome invasive aquatic plant in Malaysia and recognized as the world's most invasive alien species in the Global Invasive Species Database (GISD) since 2013, underwent a comprehensive study to explore its phytochemical composition, antioxidant potential, and antibacterial properties. The leaves were meticulously extracted using both distilled water and ethanol, and subsequent quantification involved assessing phenolic, flavonoid, tannin, alkaloid, and saponin contents using standard methods. Additionally, the antioxidant activity of the extracts was evaluated through DPPH, ABTS, and FRAP assays. The ethanol extract stood out with notably higher phytochemical content, particularly in tannin (275.98 ± 3.70 mg TAE/g), followed by phenolic, saponin, flavonoid, and alkaloids. Impressively, the ethanol extract exhibited substantial antioxidant capacity, as evidenced by IC50 values of 4.90 ± 0.25 µg/mL (DPPH), 15.22 ± 1.91 µg/mL (ABTS), and a FRAP value of 298.27 ± 4.04 mg TE/g. Moving beyond antioxidant properties, the ethanol extract showcased robust antibacterial activity, notably against *Bacillus cereus*, followed by *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* in disc-diffusion tests. Further analysis through minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays underscored the ethanol extract's capacity to inhibit growth and effectively eliminate all tested bacterial strains. In summary, this research highlights the ethanol extract of *Salvinia molesta* as a promising source of phytochemicals with substantial antioxidant and antibacterial properties, surpassing the aqueous extract in efficacy.

Keywords: *Salvinia molesta*, phytochemical, antioxidant activity, antibacterial properties, invasive species

Introduction

People have used plants as sources for medicines since the ancient times. According to the World Health Organization (WHO), medicinal plants have played a significant role in modern culture as well as in the health care of ancient civilizations for thousands of years due to their therapeutic potential. Majority of consumers rely on herbal medications due to the higher cost and frequently dangerous side effects of alternative treatment options.[1] Knowledge of plant chemical components is desirable for the identification of therapeutic agents as well as the discovery of new sources of profitable phytocompounds for the synthesis of complex chemical substances and the actual significance of folklore medicines.[2]

Invasive alien aquatic plants (IAAPs), on the other hand, are non-native aquatic plant species that grow faster than native species, even in habitats with low resources.[3]

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Salvinia molesta D.S. Mitch is a worldwide aquatic invader, named after Antonio Maria Salvini (1633 – 1729), belonging to the family Salviniaceae (Table 1).[4, 5] One genus, *Salvinia*, which contains about 12 species that are all indigenous to South America, makes up the Salviniaceae family.[6, 7] The name of the species, *molesta*, was derived from the Latin word *molestus*, emphasizes its weediness as it invades both natural and artificial impoundments all over the world.[8–10]

Fan & Marston[11] suggested, instead of focusing on rare or extinct species, research on the phytochemicals of common invasive species will utilize biomass as a source of potentially essential phytochemical compounds. Research related to the isolation of secondary metabolites of *S. molesta* has been carried out using HP-LC and GC-MS analysis. The HP-LC analysis of butanol extract showed the presence of 6'-O-(3,4-dihydroxyl benzoyl)-β-D-glucopyranosyl ester, 4-O-β-D-glucopyranoside-3-hydroxy methyl benzoate, paeoniflorin, and pikuroside, while methyl benzoate, hypogallic acid, and caffeic acid were found in the ethyl acetate extract.[12] On the other hand, GC-MS analysis of ethanol extract showed the presence of apiol, methyl palmita, hexadecatrienoic acid, methyl ester, and methyl isostearate.[13] Most of these compounds were mentioned to have antioxidant and antimicrobial activity.

In Malaysia, research and documentation on the phytochemicals and medicinal values of *S. molesta* remain sparse and insufficient. So far, there has been no study on the antioxidant and antibacterial activity of aqueous and ethanol extracts of the *S. molesta* have been carried out together. In this study, *S. molesta* extracts, aqueous and ethanol, were examined to quantify the presence of phytochemicals and analyse the medicinal properties, particularly the antioxidant and antibacterial

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activity. Understanding and assessing the advantages of *S. molesta* extracts could be the first step in controlling their invasive growth in Malaysia and promote their utilization for useful purposes.[14]

Table 1: Taxonomy of *Salvinia molesta*

Kingdom	Plantae
Division	Pteridophyta
Class	Liliopsida
Order	Hydropteridales
Family	Salviniaceae
Genus	*Salvinia*
Species	*Salvinia molesta* D.S.Mitch

Materials and Methods

Sample authentication
The comprehensive identification of the aquatic plant *S. molesta* necessitated the examination of its entire structure, encompassing both leaves and distinctive root-like filaments. These filaments played a crucial role in differentiating *S. molesta* from other species within the same genus. The identification process was conducted and confirmed by a qualified botanist, and the corresponding herbarium number assigned to the specimen is 11887.

Sample collection and extraction
Healthy plant samples with deep green color of *S. molesta* (Figure 1) were collected from Bukit Belah Lake in Taman Botani Johor, Batu Pahat (1.9794º N, 102.9605º E) from September until November 2020. The preparation of plant samples was followed the method from previous literature works with minor modifications.[15, 16] The leaves were detached from the root-like filaments and washed thoroughly with running water. The leaves were dried using an oven (Labec, Australia) for 48 hours at 40ºC. The dried leaves were then grinded into powder. Extractions of *S. molesta* leaf powder was conducted by maceration method (1:20, w/v) using distilled water and 95% ethanol (HmbG Chemicals, Germany) for 72 hours at room temperature with constant shaking in the incubator shaker (KS 4000 i control, IKA, Germany). After that, both aqueous and ethanol extracts were evaporated to paste under vacuum rotary evaporator (N-1300, Eyela, United States) at 45ºC and further dried in the oven (Labec, Australia) overnight at 40ºC. The crude extracts were transferred into sterile bottles individually and kept at < 4ºC prior to analysis.[17, 18]

Yield (%) of plant extracts
The crude extracts of aqueous and ethanol of *S. molesta* were weighed on the measuring balance and the constant weight obtained was recorded as the final weight. The percentage yield for both extracts of *S. molesta* was calculated using Equation 1:[19]

$$\%\ \text{yield} = \frac{\text{weight of crude}}{\text{weight of plant sample}} \times 100 \qquad \text{Equation 1}$$

Quantitative analysis of phytochemicals
Total phenolic content (TPC)
The total phenolic content in the leaf extracts of *S. molesta* was determined by the Folin–Ciocalteau colorimetric method.[20] Initially, an aliquot of the sample in amount of 0.5 mL was added to 0.5 mL of Folin–Ciocalteau reagent (0.5 N) (Merck, Germany), and the flask content was then mixed thoroughly. Later, Later, 2.5 mL of 2% sodium carbonate (R & M Chemicals, Malaysia) was added, and the mixture was allowed to stand for 30 minutes after mixing. The absorbance was measured at 760 nm in a UV–visible spectrophotometer (T60, PG Instrument, United Kingdom). Gallic acid (Sigma Aldrich, USA) was used as standard control; thus, the TPC is expressed as mg gallic acid equivalents (GAE)/g extract. The phenolic content was determined using the following Equation 2.

$$\text{Total phenolic content (mg GAE/g)} = \frac{c \times V}{m} \qquad \text{Equation 2}$$

where,
c: concentration of gallic acid obtained from the standard curve
V: volume of sample solution
m: dry weight of sample

Total flavonoid content (TFC)
The total flavonoid content in the leaf extracts of *S. molesta* was determined by the aluminium chloride colorimetric method.[21] Initially, 0.5 mL of *S. molesta* extracts at a concentration of 1 mg/mL were taken and the volume was made up to 3 mL with methanol (HmbG Chemicals, Germany). Subsequently, 0.1 mL of aluminium chloride$_3$ (10%), 0.1 mL of potassium acetate (Friendemann Schmidt, Australia), and 2.8 mL distilled water were added sequentially, and the test solution was vigorously shaken. Absorbance was recorded at 415 nm after 30 minutes of incubation. A standard calibration plot was also generated at 415 nm using known concentrations of quercetin (Sigma Aldrich, USA). The concentrations of flavonoids in the test samples were calculated from the calibration plot and expressed as mg quercetin equivalent (QE)/g of sample. The flavonoid content was determined using the following Equation 3:

$$\text{Total flavonoid content (mg QE/g)} = \frac{c \times V}{m} \qquad \text{Equation 3}$$

where,
c: concentration of quercetin obtained from the standard curve
V: volume of sample solution
m: dry weight of sample

Total tannin content (TTC)
The standard method was used to determine the content of tannins in *S. molesta* leaf extracts.[22] The leaf extracts (1 mL) were mixed with Folin–Ciocalteau's reagent (0.5 mL) (Merck, Germany), followed by the addition of saturated sodium carbonate (R & M Chemicals, Malaysia) solution (1 mL) and distilled water (8 mL). The reaction mixture was allowed to stand for 30 minutes at room temperature. The supernatant was obtained by centrifugation and absorbance was recorded at 725 nm using a UV-visible spectrophotometer (T60, PG Instrument, United Kingdom). Different concentrations of standard tannic acid (HmbG Chemicals, Germany) were prepared, and the absorbance of various tannic acid concentrations was plotted for a standard graph. The tannin content is expressed as µg tannic acid equivalent (TAE)/g of the sample. The tannin content was determined using the following Equation 4:

$$\text{Total tannin content (mg TAE/g)} = \frac{c \times V}{m} \qquad \text{Equation 4}$$

where,
c: concentration of tannic acid obtained from the standard curve
V: volume of sample solution
m: dry weight of sample

Figure 1: Whole plant of *Salvinia molesta* in Taman Botani Johor, Batu Pahat.

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Total saponin content (TSC)
The determination of total saponin was conducted per the standard method with minor modifications.[23] The crude extract in amount of 1 g was added to 100 mL of 20% aqueous ethanol and kept in a flask on a stirrer for a half-hour before being heated over for 4 hours at 45°C with mixing. The mixture was filtered using Whatman No. 1 filter paper and the residue was again extracted with another 100 mL of 25% aqueous ethanol. The combined extracts were concentrated using a rotary evaporator (N-1300, Eyela, United States) at 40°C to obtain 40 mL approximately. The concentrate was transferred into a separator funnel and extracted twice with 20 mL diethyl ether (HmbG Chemicals, Germany). The ether layer was discarded while the aqueous layer was kept and then re-extracted with 30 mL n-butanol (HmbG Chemicals, Germany) that has been added. The n-butanol extracts were washed twice with 10 mL of 5% aqueous sodium chloride and the remaining solution was evaporated. After evaporation, the samples were dried in the oven (Labec, Australia) at 40°C to a constant weight and the saponin content was calculated using the following Equation 5:

$$\text{Percentage of saponin} = \frac{\text{final weight of sample}}{\text{initial weight of extract}} \times 100 \qquad \text{Equation 5}$$

Total alkaloid content (TAC)
The quantification of alkaloid determination was performed by standard methods.[24] Initially, 100 mL of 10% acetic acid in ethanol was added to 1 g of crude extract of *S. molesta* and the extracts were then covered and allowed to stand for 4 hours. Subsequently, the extracts were filtered and concentrated in a water bath to 25 mL of the original volume. The droplets of concentrated ammonium hydroxide (R & M Chemicals, Malaysia) were added to the extract until the whole solution was allowed to settle. Next, the precipitates were washed with dilute ammonium hydroxide and then filtered using Whatman No. 1 filter paper. Finally, the residue was dried in the oven (Labec, Australia) at 40°C and weighed. The alkaloid content was determined using the following Equation 6:

$$\text{Percentage of tannin} = \frac{\text{final weight of sample}}{\text{initial weight of extract}} \times 100 \qquad \text{Equation 6}$$

Antioxidant activity
DPPH radical scavenging activity
DPPH free radical scavenging assay was modified to evaluate the antioxidant activity of one hundred pure chemical compounds.[25] The DPPH reagent constitutes DPPH (8 mg) dissolved in MeOH (100 mL) (EAM, Elite Advanced Materials, Malaysia) for a solution concentration of 80 µL/mL. Additionally, 100 µL of DPPH reagent (Sigma Aldrich, USA) was mixed with 100 µL of sample extract in the 96-well microplate and incubated at room temperature for 30 minutes. After incubation, the sample absorbance was measured at 514 nm using an ELISA reader (TECAN, Groding, Austria), and 100% methanol was used as a control. The antioxidant activity of the sample was then compared with the known synthetic standard of ascorbic acid (Sigma Aldrich, USA). Free radical scavenging activity was calculated using the following Equation 7:

$$\text{DPPH radical scavenging } (\%) = \frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}} \times 100 \qquad \text{Equation 7}$$

The antioxidant activity of the plant extracts was expressed as IC_{50}, which is defined as the concentration of the extract required to inhibit the formation of DPPH radicals by 50%. The IC_{50} values were obtained through extrapolation from the regression analysis.

ABTS radical scavenging activity
This assay was carried out by following the method proposed with modifications.[26] The ABTS radical reagent was generated by reacting an equal volume of 2.45 mM of potassium persulfate and 7 mM ABTS solution (Roche, Germany) prepared in methanol (EAM, Malaysia). The reagent solution was kept in the dark for 12-18 hours at room temperature and further diluted with methanol in a 1:50 ratio until an absorbance of 0.700 ± 0.0003 was attained at 734 nm. Equal volumes of sample extracts and synthetic standards of different concentrations were reacted with $ABTS^+$ (1:1 v/v), left in the dark for 6 minutes, and absorbance was taken at 734 nm. The percentage $ABTS^+$ scavenging activity of the extracts and standard (ascorbic acid; Sigma Aldrich, USA) was calculated using the formula Equation 8:

$$\text{ABTS radical scavenging } (\%) = \frac{\text{Abscontrol} - \text{Abssample}}{\text{Abscontrol}} \times 100 \qquad \text{Equation 8}$$

Ferric reducing antioxidant power (FRAP) activity
Ferric reducing ability assay was implemented following the method outlined with slight modifications.[25] In order to dissolve the sample extracts, 80% of methanol (Sigma Aldrich, USA) was added and the solutions were centrifuged (Mikro 22R Refrigerated Centrifuge, Hettich, Germany) at 7500 rpm for 10 minutes at 4°C. Next, 100 µL of supernatant was added to 250 µL of 0.2 M phosphate buffer (pH 6.6) and 250 µL of 1% potassium ferricyanide (Sigma Aldrich, USA). The mixture was then incubated at 50°C for 20 minutes. After that, 250 µL of 10% trichloroacetic acid (Sigma Aldrich, USA) was added to the reaction mixture, followed by centrifugation at 3000 rpm for 10 minutes (25°C). Subsequently, 400 µL of supernatant was mixed with 400 µL of distilled water and 80 µL of 0.1% ferric chloride (Sigma Aldrich, USA). After 10 minutes, the absorbance of the solution was measured at 700 nm using a spectrophotometer (Synergy H1, BioTek, USA). Trolox (Sigma Aldrich, USA) was used as a standard for this assay.

Bacterial strains and culture
The antibacterial activity of aqueous and ethanol extracts of *S. molesta* was evaluated against four bacterial strains of which two bacterial strains were Gram-positive: *Staphylococcus aureus* ATCC 25923 and *Bacillus cereus* ATCC 11778, and the other two were Gram-negative: *Escherichia coli* ATCC 8739 and *Pseudomonas aeruginosa* ATCC 10145. All the bacterial strains were obtained from culture collections of the Food Microbiology Laboratory, UTHM, Pagoh Campus. The bacteria were cultivated in Mueller–Hinton agar (MHA) and incubated at 37ºC for 24 hours. All the strains underwent regular subcultures and all tests involving bacteria were carried out in Biosafety Cabinet (Class II, ESCO Lifesciences, Singapore).

Inoculum preparation
In the beginning of the preparation, 500 mL of sterilized Mueller-Hinton broth (MHB; HiMedia, India) was inoculated with a loopful of isolated colonies to create a pure bacterial culture of each strain. The bacterial culture was then incubated for 24 hours at 37ºC with continuous shaking in an incubator shaker (KS 4000 i control, IKA, Germany). Subsequently, the bacteria were collected using centrifugation and rinsed twice with a sterile 0.9% saline solution (Centrifuge 5804 R, Eppendorf, Germany). The density of the cell suspension was standardized to 0.5 McFarland turbidity standard using sterile saline solution to produce approximately 1×10^8 colony forming units (CFU) per mL.[17] The optical density was measured using a spectrophotometer (T60, PG Instrument, United Kingdom) ranging from 0.8-1.2 at 625 nm.

Antibacterial susceptibility test
The sensitivity of various bacterial strains to different *S. molesta* extracts was determined using the modified Kirby-Bauer disc diffusion method in terms of zone of inhibition (mm), as described by Santhosh *et al.*[27] Mueller-Hinton agar (MHA; HiMedia, India) was sterilized in flasks and allowed to cool to 45ºC before being poured into sterile Petri dishes. After that, 50 µL of suspension was spread on the solid media plates using a sterile L-shaped cell spreader, and the plates were then allowed to air-dry for approximately 10 minutes before the discs were put on the agar. Subsequently, 20 mg of aqueous and ethanol crude extracts of *S. molesta* were re-dissolved in 1 mL of each solvent to obtain the final concentration of 20 mg/mL stock solution. For screening purposes, sterile filter paper discs (6 mm in diameter) were impregnated with 50 µL of the extracts and placed on the inoculated MHA plates. Sterile distilled water and ethanol were used as negative control corresponding to extracts tested, while ciprofloxacin (100

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µg/mL) was used as a positive control. The plates were then incubated at 37ºC for 24 hours and the zone of inhibition (diameter) was measured in millimetres (mm). The presence of antibacterial activity was finally indicated by clear inhibition zones surrounding the discs. All antibacterial activity data entail the mean of three independent analyses.

Determinantion of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The minimum inhibitory concentration (MIC) values were determined by the broth microdilution method in 96-well sterile microtiter plates as described with some modifications.[28, 29] Briefly, 100 µL of Mueller-Hinton broth (MHB; HiMedia, India) was added to a 96-well plate and 100 µL of *S. molesta* extracts were then added. Both aqueous and ethanol extracts were initially prepared at 20 mg/mL. The ethanol extract was added into the second column of the second, third, and fourth rows (2B, 2C, and 2D). Besides, the aqueous extract was added into the second column of the fifth, sixth, and seventh rows (2E, 2F, and 2G). The extracts of *S. molesta* were then serially diluted twofold to final concentrations of 20.00, 10.00, 5.00, 2.50, 1.25, 0.63, and 0.31 mg/mL. Besides, a bacterial inoculum of 1×10^8 CFU/mL was prepared in MHB and 100 µL was introduced into the well. Wells of 9B until 9G served as the negative control containing broth and bacteria culture only. The 10B until 10G wells were added with antibiotic (ciprofloxacin) and bacterial culture (positive control), while the 11B until 11G wells were added with only broth (sterility control) without bacterial culture or extract. Next, the plates were sealed with parafilm tape and incubated at 37ºC for 24 hours. On the next day, the MIC of aqueous and ethanol extracts were detected following the addition of 50 µL of 0.5 mg/mL p-iodonitrotetrazolium chloride (INT) (Sigma Aldrich, USA) solution and incubated at 37ºC for 30 minutes. Viable bacteria reduced the yellow dye of INT to pink colour. MIC corresponded to the well-performed concentration whereby no colour change will be observed, indicating no growth of the corresponding microbe.

As for the determination of MBC, 50 µL from each well that showed no change in colour was plated on Mueller-Hinton agar (MHA) and further incubated for 24 hours at 37ºC. Consequently, the MBC was determined to be the lowest concentration that yielded no growth after the sub-culturing.[29]

Statistical analysis

All experiments were carried out in triplicates. The data recorded and obtained from the study of the extracts of *S. molesta* were presented as mean ± standard deviation (n= 3). All the statistical analyses were performed using GraphPad Prism software (version 9.3). Data were analysed by ANOVA test at the 0.05 level of probability.

Results and Discussion

Yield (%) of plant extracts

The percentage yield of different extracts obtained from the powdered leaves of *S. molesta* is shown in Figure 2. Among the extraction solvents, the aqueous extract showed a higher percentage of extraction yield at 42%, followed by the ethanol extract (24.16%). The extraction technique and the extraction solvent influence the extraction yield and biological activity of the resulting extracts.[30, 31] Based on Figure 2, the number of extracts in the yield produced using various solvents varies due to the capacity of each solvent to dissolve compounds according to their polarity.

Ethanol extraction was done by using 95% ethanol while distilled water was utilized in aqueous extraction. Both solvents used in this study were polar solvents, which are better for the extraction of bioactive compounds.[32] The higher value in aqueous extraction indicates that compounds in *S. molesta* are mostly high in polarity and soluble in water. The selectivity of the solvent in extracting the target compound from plant material is related to the polarity compatibility of the metabolites.[33] Polar solvents are expected to have high extractive values because most active phytochemical ingredients are polar.[34]

Quantitative analysis of phytochemicals

Total phenolic content (TPC)

The concentrations of bioactive compounds (phenolics, flavonoids, tannins, alkaloids, and saponins) varied among the extracts in accordance with the extraction yields. The value of the total phenolic (TPC) is expressed as mg gallic acid equaivalent (GAE)/g using the standard curve equation: $y = 0.0074x + 0.6878$, $R^2 = 0.9892$. The TPC in the ethanol extract of *S. molesta* was higher than the aqueous extract, with 183.41 ± 0.81 mg GAE/g and 167.99 ± 1.02 mg GAE/g (Figure 3), respectively. The study showed no statistically significant difference in the phenolic content of *S. molesta* extracts at $p > 0.05$.

Plant bioactive substances are generally known for their wide range of functions, including antioxidant, antimicrobial, and immunomodulatory activities, while phytotherapy is thought to be the first known form of medical treatment.[35] Most medicinal plants are high in phenolic compounds, which have been shown to possess biological and pharmacological characteristics such as antibacterial, antiviral, antioxidant, anti-carcinogenic, anti-inflammatory, anti-mutagenic, and cytotoxic activities.[36, 37] Moreover, these compounds also have antibacterial activity because they have been shown to have a high effect on a variety of bacteria and cause structural or functional damage to bacterial cell membranes.[38]

Total flavonoid content (TFC)

As a basis for quantitative determination, the total flavonoid content (TFC) results were expressed as mg/g equivalent to quercetin (QE) using the standard curve equation: $y = 0.0062x + 0.0509$, $R^2 = 0.9865$. Evidently, the overall TFC was 36.84 ± 1.09 mg QE/g and 36.20 ± 0.49 mg QE/g in the ethanol and aqueous extracts (Figure 3), respectively. In addition, the present study also found no statistically significant difference in the content of flavonoids in *S. molesta* extracts ($p > 0.05$).

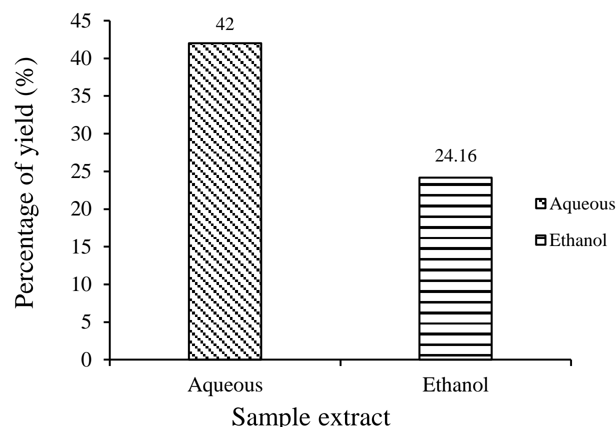

Figure 2: Percentage yield of aqueous and ethanol extracts of *Salvinia molesta*.

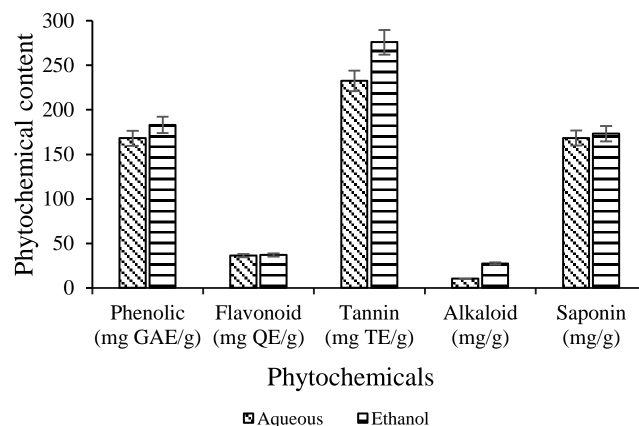

Figure 3: Phytochemicals content of aqueous and ethanol extracts of *Salvinia molesta*.

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Flavonoids tend to be well-known regarding their antioxidant nature and these substances are very good scavengers of the most oxidizing molecules such as singlet oxygen and various free radicals that are linked to several diseases.[39] According to Jasmine *et al.*,[40] plants produce flavonoids to prevent microbial infections; hence, they are a powerful antibacterial substance against many infectious agents. They are also biochemical transformers that modify the body's biochemical reactivity to carcinogenic substances, viruses, and allergens. The anticancer, anti-inflammatory, antibacterial, and anti-allergy properties of various structural molecules of flavonoids[41] are also known as antioxidant and antiviral properties, as well as their ability to change the biological activity of cell systems and enzymes.[42]

Total tannin content (TTC)
Based on Figure 3, tannin content in both aqueous and ethanol extracts of *S. molesta* were found higher compared to the other phytochemicals. The TTC was expressed as mg equivalent to tannic acid/g using the standard curve equation: $y = 0.0033x + 1.2586$, $R^2 = 0.9764$. The tannin content was 275.98 ± 3.70 mg TAE/g in the ethanol and 232.55 ± 2.42 mg TAE/g in the aqueous extracts. It was reported no statistical significance in both *S. molesta* extracts ($p > 0.05$).
Tannin is one of the major active ingredients found in plant-based medicines.[43] Tannin has antiviral, antibacterial, and anticancer activities,[44] and has been shown to decrease HIV replication selectively.[45] In a prior study by Oladunmoye,[46] tannins were discovered to have a high level of immunomodulatory activity. Moreover, this secondary metabolite entails water-soluble polyphenols and precipitated proteins found in a variety of plant foods that have been shown to inhibit the growth of microorganisms by precipitating microbial protein.[47] Westendarp[48] also reported that tannin-containing plants are used to treat non-specific diarrhoea, mouth and throat inflammations, and mildly injured skin.

Total alkaloid content (TAC)
The total alkaloid content (TAC) was 27.33 ± 1.53 mg/g and 10.33 ± 1.53 mg/g in the ethanol and aqueous extracts (Figure 3), respectively. However, it was found no statistical difference in the alkaloid content between *S. molesta* extracts at $p < 0.05$. According to Sodipo, Akinniyi, & Ogunbameru,[49] most phytochemicals are natural antibiotics that help the body to fight illnesses and microbial invasions. For example, even though alkaloids are chemical compounds that mostly contain basic nitrogen atoms found naturally in plants, they may also be created by bacteria, fungi, and mammals. Alkaloids exhibit a wide range of pharmacological activities, including antimalarial, anticancer,[50] antibacterial,[51] and antihyperglycemic properties.[52] The value of alkaloids in traditional pharmacological use has also been utilized. For instance, other alkaloids have been utilized as recreational drugs owing to their psychotropic and stimulant properties.[52]

Total saponin content (TSC)
The total saponin content (TSC) in the ethanol extract (173.13 ± 0.24 mg/g) was higher than the aqueous extract (168.22 ± 0.38 mg/g) (Figure 3). However, the study found no statistically significant difference in the saponin content between *S. molesta* extracts ($p < 0.05$). Since they are found widely in plants, saponins have long been thought of as phytochemicals that help plants fend off pathogens[53] and they are also useful for treating yeast and fungal infection.[54] Moreover, saponins are widely used in veterinary vaccinations due to their ability to function as an adjuvant and aid in the enhancement of immune response. Many of them may be used for intracellular histochemistry labelling, which allows antibodies to reach intracellular protein molecules.[55, 56]

Antioxidant activity
DPPH and ABTS radical scavenging activities
Based on Figure 4 and 5, the ability of aqueous and ethanol extracts of *S. molesta* to scavenge the radical activity of DPPH and ABTS were found increased in a concentration–dependent manner over the range of concentrations tested. The scavenging activities of extracts were observed over nine varying concentrations ranging from 0.9766 to 250 µg/mL, which demonstrated different percentages of inhibition. The aqueous and ethanol extracts showed the highest antioxidant activity at a concentration of 250 µg/mL based on the two methods. For DPPH, the ethanol extract (99.0947 ± 0.49%) significantly ($p > 0.05$) recorded higher inhibition followed by the aqueous extract (86.4752 ± 0.27%). Additionally, the ethanol extract also had a higher inhibition activity than the aqueous extract, 98.1528 ± 0.37% and 95.5669 ± 0.08%, for ABTS, respectively.

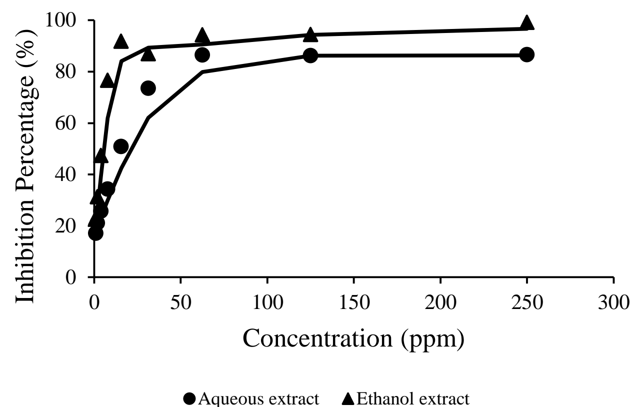

Figure 4: Radical scavenging activity represented by the inhibition percentage (%) of aqueous and ethanol extracts of *Salvinia molesta* by DPPH assay.

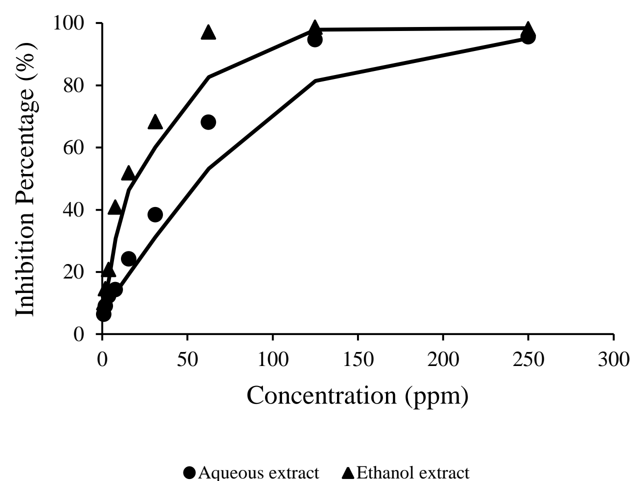

Figure 5: Radical scavenging activity represented by the inhibition percentage (%) of aqueous and ethanol extracts of *Salvinia molesta* by ABTS assay.

Table 2: IC_{50} values of DPPH and ABTS radical scavenging activities of aqueous and ethanol extracts of *Salvinia molesta*

Sample extracts	DPPH	ABTS
	IC_{50} values (µg/mL)	
Aqueous	15.79 ± 0.37	38.41 ± 1.70
Ethanol	4.90 ± 0.25*	15.22 ± 1.91*
Ascorbic acid	4.49 ± 0.19	2.19 ± 0.41

Values presented are mean ± SD (n = 3). Values with * indicate a significant difference at $p < 0.05$, as determined using the ANOVA test.

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The IC_{50} value was evaluated to determine the concentration of the sample required to inhibit at 50% of radical. The lower the IC_{50} value, the higher the samples' antioxidant activity.[57] In Table 2, the observed IC_{50} values for DPPH and ABTS showed that the ethanol extract exhibited the highest antioxidant activity with 4.90 ± 0.25 µg/mL and 15.22 ± 1.91 µg/mL, respectively ($p < 0.05$), followed by the aqueous extract with 15.79 ± 0.37 µg/mL (DPPH) and 38.41 ± 1.70 µg/mL (ABTS). Remarkably, the IC_{50} value of the ethanol extract for DPPH is near the IC_{50} value of the standard (ascorbic acid) with 4.49 ± 0.19 µg/mL. This study revealed that both ethanol and aqueous extracts of *S. molesta* possessed very strong antioxidant activity based on the IC_{50} values of ABTS scavenging activity. This is because the IC_{50} values recorded were lower than 50 µg/mL, as mentioned by Mardawati *et al.*[58]

Ferric reducing antioxidant power (FRAP) activity

The reducing power test is a technique for enhancing the findings of the DPPH assay for measuring antioxidant activity. The FRAP values of aqueous and ethanol extracts of *S. molesta* are summarized in Table 3. Trolox was used as a standard and the FRAP values are expressed as mg/g equivalent to Trolox by using the standard curve equation: $y = 0.0025x + 0.0175$, $R^2 = 0.9995$. Both extracts examined in the current study demonstrated good antioxidant potential due to their ability to reduce ferrous ions. However, the ethanol extract showed a higher FRAP value (298.27 ± 4.04 mg/g TE) with a $p < 0.005$ significance than the aqueous extract (218.80 ± 2.99 mg/g TE).

In brief, this study demonstrates that both the aqueous and ethanol extracts of *S. molesta* are rich in bioactive compounds that are effective antioxidants when tested using the DPPH, ABTS, and FRAP assays representing various levels of antioxidant action.[59] This shows that the leaves of *S. molesta* are rich in antioxidants and can be explored for their ability to eliminate free radicals. This study also suggests that the ethanol extract of *S. molesta* is more successful than the aqueous extract regarding scavenging the radical activity, which may be due to the higher concentration of bioactive compounds of the ethanol extract with antioxidant properties.

Table 3: FRAP activity values of aqueous and ethanol extracts of *Salvinia molesta*

Sample extracts	FRAP (mg TE/g)
Aqueous	218.80 ± 2.99
Ethanol	298.27 ± 4.04*

Values presented are mean ± SD (n = 3). Values with * indicate a significant difference at $p < 0.05$, as determined using the ANOVA test.

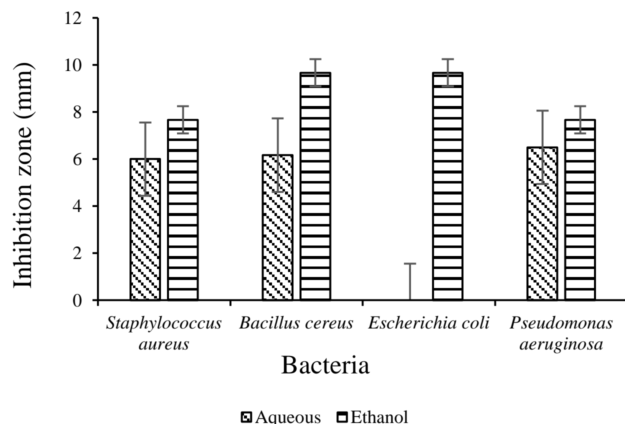

Figure 6: Antibacterial activity of aqueous and ethanol extracts of *Salvinia molesta* against selected bacteria.

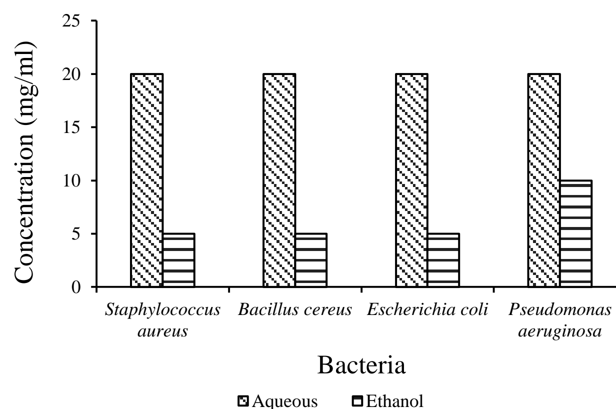

Figure 7: Minimum inhibition concentration of aqueous and ethanol extracts of *Salvinia molesta* against selected bacteria.

Antibacterial activity

Diameter of inhibition zone of Salvinia molesta extracts

The study revealed that the examined *S. molesta* leaf extracts of both solvents, has significant antibacterial activity against four bacterial strains, including two Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and two Gram-negative bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) (Figure 6). Both aqueous and ethanol extracts of *S. molesta* were observed with more activity against the Gram-positive strains compared to the Gram-negative strains. In the study, the aqueous extract of *S. molesta* showed antibacterial activity at 20 mg/mL against three bacterial strains, except *E. coli*. The highest diameter inhibition zone (mm) was observed against *P. aeruginosa* with 6.50 ± 0.50 mm, followed by *B. cereus* and *S. aureus* with 6.17 ± 0.29 and 6.00 ± 0.50, respectively. Meanwhile, the ethanol extract showed activity against all the bacterial strains tested at 20 mg/mL. The highest diameter inhibition zone was noted against *B. cereus* (9.67 ± 2.08 mm) and *E. coli* (9.67 ± 0.58 mm), which was significant at $p < 0.05$, and the lowest against *S. aureus* (7.67 ± 0.58 mm) with a $p < 0.05$ significance and *P. aeruginosa* (7.67 ± 0.28 mm).

This freshwater fern has exhibited antibacterial activity against a variety of tested bacterial strains. The microorganisms examined, the types of chemicals present in the extract, and the mechanism of action all play a significant role in determining the antibacterial activity of a plant extract.[60] The presence of some bioactive compounds or secondary metabolites has been linked to the antibacterial activities of plant extracts. These secondary metabolites defend the plants themselves from bacterial, viral, and fungal infections.[61] According to phytochemical content results, ethanol extract contains tannins, flavonoids, phenolics, alkaloids, and saponins that have important antibacterial activity.[62]

Minimum inhibitory concentration (MIC) of Salvinia molesta extracts

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract that inhibits organism growth (Sen & Batra, 2012). Specifically, the MIC values of *S. molesta* extracts were obtained from antibacterial testing using the broth microdilution method with the addition of p-iodonitrotetrazolium chloride (INT). The presence of bacteria would reduce the colour of INT from yellow to pink. The concentration range used in this study ranges from 0.3125 to 20 mg/mL.

The most potent antibacterial activity was exhibited by the ethanol extract against all the bacterial strains at 5 mg/mL (*S. aureus*, *B. cereus*, and *E. coli*) and 10 mg/mL (*P. aeruginosa*) (Figure 7). However, the aqueous extract could only exhibit all the bacterial strains at the highest concentration of 20 mg/mL. Both extracts were chosen for further investigation to determine their effects on the inhibition and killing of *S. aureus*, *B. cereus*, *E. coli*, and *P. aeruginosa* on agar plates. According to Yisa,[63] antibacterial agents with low activity against an organism tend to have a high MIC, whereas antibacterial agents with high activity may have a low MIC.

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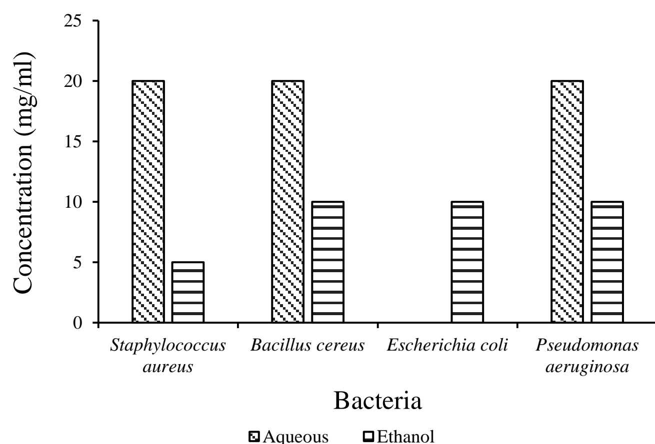

Figure 8: Minimum bactericidal concentration of aqueous and ethanol extracts of *Salvinia molesta* against selected bacteria.

Minimum bactericidal concentration (MBC) of Salvinia molesta extracts

In addition to minimum inhibitory concentration (MIC), the bactericidal effect of *S. molesta* extracts were investigated. Both aqueous and ethanol extracts, showed the ability to kill all the selected bacterial strains except for the aqueous against the *E. coli* strain. The bactericidal properties of the extracts were further confirmed when no re-growth occurred even after 24 hours of incubation. Additionally, the bactericidal activity of the aqueous extract was observed at 20 mg/mL for three bacteria, which are *S. aureus*, *B. cereus*, and *P. aeruginosa*, and no killing activity occurred against *E. coli*. In addition, the ethanol extract was highly likely to bactericide all the bacterial strains. The concentration of 10 mg/mL was needed to kill *B. cereus*, *E. coli*, and *P. aeruginosa*, and only 5 mg/mL to kill *S. aureus* (Figure 8). According to Omar *et al*.,[64] most of antibacterial properties in various plant part extractions also revealed MBC values that were nearly two-fold higher than their corresponding MICs.

The findings on MIC and MBC regarding the antibacterial activity of aqueous and ethanol extracts of *S. molesta* showed that the extracts had more effect on gram-positive bacteria compared to the gram-negative bacteria. This is because gram-negative bacteria form a more complicated barrier system against the permeation of foreign molecules. The gram-negative bacteria consist of specific cell wall structures, particularly the presence of the outer envelope, which results in the impermeability of these microorganisms to antibiotics and biocides, and sometimes, regulates and prevents their passage to the target region.[620] Additionally, the outer layer of the gram-negative membrane is made up of lipopolysaccharide molecules, which creates a hydrophilic environment against hydrophobic molecules.[65]

Both the aqueous and ethanol extracts of *S. molesta* seem to possess the antibacterial properties to inhibit the growth of the selected bacterial strains. However, the ethanol extract was more effective in inhibiting and killing all bacteria than the aqueous extract. This might be due to the higher concentration of bioactive compounds in the ethanol extract with antibacterial properties.

Conclusion

Based on the results, there is no significant difference in the phytochemical contents of ethanol and aqueous extracts. The highest phytochemical content that can be extracted from ethanol extract is tannin, followed by phenolic, saponin, flavonoid, and alkaloid. Aqueous extract was reported to have the same result as ethanol extract. Even so, according to the results of DPPH, ABTS, and FRAP assays, the antioxidant efficacy of ethanol extract was found to be higher than aqueous extract, which significance at $p < 0.05$. Additionally, antibacterial activity tests showed some antibacterial potency of the extracts even though they were very lower as compared to the standard antibiotic. Ethanol extract showed the highest antibacterial activity against *E. coli*, whereas aqueous extract against *P. aeruginosa*. In the MIC and MBC tests, however, ethanol extract showed its ability to inhibit and kill all the tested bacterial strains compared to aqueous extract. Overall, all the objectives of this study were met by the findings. Further studies involving in vivo practices are required to understand more about mechanisms and cellular sites of action, and therapeutic doses of these bioactive compounds.

Conflict of Interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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