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PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL ACTIVITY OF LYGODIUM MICROPHYLLUM EXTRACT

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Abstract

Lygodium microphyllum is a fern which has been used traditionally for controlling dysentery, treating swellings and skin diseases, curing hiccups as well as controlling high fever and typhoid fever. The aim of the study was to evaluate the antibacterial activities of five solvent fractions obtained from crude methanol extract of *L. microphyllum* leaves by liquid-liquid extraction of hexane, chloroform, ethyl acetate, n-butanol and water. Phytochemical analysis via Folin-Ciocalteu's method and aluminium chloride colorimetric method showed that ethyl acetate fraction obtained the highest total phenolic content of 11.59 ± 1.02 mg gallic acid equivalents (GAE)/g sample, whereas hexane fraction displayed the highest total flavonoid content of 60.27 ± 3.53 mg quercetin equivalents (QE)/g sample. The results also revealed that other phytochemical compounds such as alkaloid, glycosides, flavonoids, quinones, saponin and steroid were found in hexane and ethyl acetate fractions, whilst tannins were found only in the ethyl acetate fraction. The antibacterial study assessed via *in vitro* antibacterial test using broth microdilution method showed that ethyl acetate and hexane fractions displayed potent antibacterial activity against all tested clinically resistant enteric bacteria, namely, *Escherichia coli*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Salmonella typhi and Shigella dysenteriae* as well as the control strain isolate *Escherichia coli* (ATCC 25922) with minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of 6.25 mg/mL and 12.5 mg/mL respectively.

Keywords: L. microphyllum, phytochemical, antibacteria, solvent fraction, fern.

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1. Introduction

1.1. Lygodium microphyllum

Lygodium microphyllum (Lygodiaceae) is one of Malaysian's medicinal ferns which has important contributions for prevention and treatment of various human ailments. Alternatively known in Malay as "ribu-ribu kecil, selada, kapai alus'', *L.microphyllum* is a large and distinctive ground-to-crown climbing fern species. It has long climbing, stem-like fronds and wiry brown rhizomes. Fronds spread along the ground, overgrown shrubs, and also climb up and overgrow trees and other structures by twining around them (Figure 01). The rhizomes can accumulate to form dense mats on the ground. The leaflets (pinnules) are of two types (a) non-reproductive leaflets are unlobed along their margins, and oblong to lanceolate (pointed), and (b) reproductive leaflets (have tiny lobes along their margins covering the sporangia (Lott, Volin, Pemberton, & Austin, 2003).

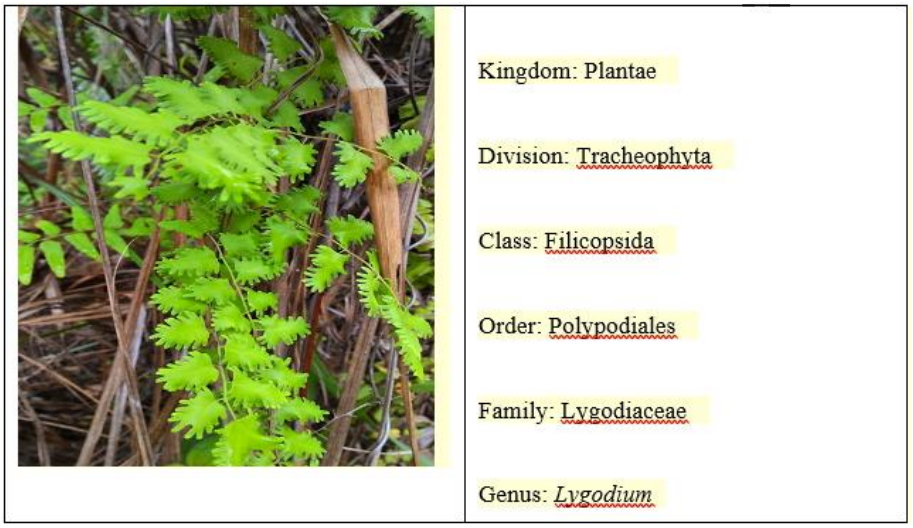

Figure 01. Taxonomic classification of Lygodium microphyllum

1.2. Phytochemicals

Phytochemicals or secondary metabolites from plants are produced to strengthen the plants in order for them to adapt to their environment, and also to defend themselves from predators and pathogens. Each species of the plant kingdom contains a great number of secondary metabolites. The presence of plant secondary metabolites has however fluctuated due to the environmental factors surrounding the plants of the same species including biotic (invaded by pathogens causing stress to plants) and abiotic (water stress, salinity stress and temperature stress) factors. Consequently, the plants of the same species may have different concentrations of a certain secondary metabolite, thereby affecting the effectiveness of certain pharmacology activity due to the lack of corresponding bioactive compound (Verma & Shukla, 2015).

Exploration of the *L. microphyllum* for the development of new compounds is beneficial for the production of target drugs and is a good solution to overcome problems caused by antibiotic-resistant gram-negative bacteria. Secondary metabolites such as phenolics, alkaloids, tannins and terpenoids which are synthesized by ferns to defend themselves from abiotic and biotic stresses have been responsible to

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provide a lot of therapeutic effects for clinical use. Therefore, the secondary metabolites have remained as the essential components and are always being explored when developing new drugs.

1.3. Antibacterial activity

In the 21st century, the spread of drug-resistant bacteria is a growing worldwide problem. Infectious diseases are difficult to treat because of the antibacterial resistance due to intensive use and misuse of available antibiotics. Common mechanisms of resistant bacteria to survive doses of antibiotics that would previously exhibit bactericidal effects are due to the production of enzymes which inactivate or modify antibiotic, modify the target site and changes in the bacterial cell membrane, thereby preventing the uptake of antibiotic (Wilson et al., 2002).

Lygodium microphyllum, a fern that has been reported to have therapeutic effects on diarrhoea or dysentery problem; and controlling high fever and typhoid fever (Benniamin, 2011) will be tested to find the potential constituents that promote antibacterial activity. So far, there is no comparative study specifically done on this fern species.

2. Problem Statement

Lygodium microphyllum (Schizaeaceae) is one of the ferns which has important contributions for prevention and treatment of various human ailments. Studies of the fern leaf decoction have revealed its effects on the control of dysentery, as poultices for skin diseases and swellings, and curing of hiccups (Benniamin, 2011). The Malay old folks have been using these fern leaves to control high fever and typhoid fever. Unfortunately to date, no comparative studies have been reported on the pharmacological effects of this fern, primarily in Malaysia.

In addition to these, the bioactive activities of the crude methanol *L.microphyllum* extract and its fractions may give us ideas for better understanding the correlation of the chemical compounds in exhibiting their pharmacological effects.

3. Research Questions

Ferns are selected for their special compounds and have become a major source for new therapeutic agents.

- Does the crude methanol *L.microphyllum* extract and its fractions has the essential phytochemical components that could attribute to antibacterial action?
- Does the crude methanol *L.microphyllum* extract and its fractions has the antibacterial activity against enteric bacteria?

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4. Purpose of the Study

The present study is aimed to evaluate the antibacterial potential of *L. microphyllum* extracts and its fractions against drug-resistant gram-negative bacteria, and to explore their phytochemical constituents using quantitative and qualitative tests associated with antibacterial effects on the several enteric bacteria.

5. Research Methods

Fresh leaves of *L. microphyllum* (Schizaeaceae) were collected from Kampung Matang Jelutong, Bagan Serai, Perak, Malaysia. The plant species was authenticated and deposited with voucher specimen (No. 11539) at the Herbarium Unit, School of Biological Sciences, Universiti Sains Malaysia. The plant was washed to discard any debris that may affect the results during the tests and air-dried for one to two weeks. The dried leaves were then milled into a fine powder and stored in the dark prior to extraction.

5.1. Extraction and fractionation of crude methanol *L. microphyllum* extract

Dried powder leaves (200 g) was macerated with 2 L of methanol solvent (1:10, w/v) (Perumal & Mahmud, 2013) at room temperature (27± 3 °C) for three days. The extract was filtered and the residue solvent eliminated by concentrating it using a rotary evaporator (Rotavapor® R-200, Buchi, Switzerland) under reduced pressure of 40 °C until dryness, which was then oven-dried (40 °C) to afford 58.13 g of dried extract. The crude extract was stored in a refrigerator at 4 °C until further use.

Crude methanol extract was fractionated via liquid-liquid extraction using a separatory funnel. The extracted organic solvents from polar to non-polar solvents were used (hexane, chloroform, ethyl acetate and n-butanol). The extract was dissolved in distilled water first, and then mix with hexane (1: 3, v/v). The mixture was then gently shaken and allowed to settle down overnight until two layers of solution were formed. The hexane layer was identified (supernatant layer), decanted and replaced with fresh hexane. This process was repeated until the hexane layer becomes colourless. The pool of decanted solution was concentrated using a rotary evaporator and dried in the fume cupboard to obtain hexane fraction. The residue (aqueous layer) was further mixed with other extracting solvents and the respective chloroform, ethyl acetate and n- butanol fractions were obtained. Lastly, the water fraction was obtained by concentrating using rotary evaporator and oven-dried (Hot-air drying 80 °C) the aqueous residue (Ngoc et al., 2015). These fractions were stored in refrigerator at 4 °C prior to further use.

5.2. Quantitaive and qualitative phytochemical analysis

The phytochemical compounds of phenolic and flavonoids of crude methanol *L. microphyllum* extract and its fractions were determined and quantified by standard procedures.

The qualitative analysis of secondary metabolites present in the crude methanol of *L. microphyllum* extract and its fractions (hexane, chloroform, ethyl acetate, n-butanol and water) were conducted and analyzed following the standard procedures of Edeoga, Okwu, and Mbaebie (2005), Chanda, Parekh, and Karathia (2006), Onwukaeme, Ikuegbvweha, and Asonye, (2007), Parekh and Chanda (2007) and Kumar et al. (2007) with some modifications.

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The stock concentration (1 mg/mL) of all the extracts was prepared and dissolved in methanol prior to use.

5.3. Determination of antibacterial activity

Antibacterial activities of crude methanol *L. microphyllum* extract and its fractions (hexane, chloroform, ethyl acetate, n-butanol and water) were determined by using broth micro-dilution assay according to the protocols described by Wiegand, Hilpert, and Hancock (2008), following the guideline of Clinical and Laboratory Standard Institute (CLSI, 2015). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of *L. microphyllum* extracts were obtained against resistant clinical isolates of enteric bacteria and the American Type Culture Collection (ATCC) standard bacterial strain.

6. Findings

6.1. Quantitative phytochemical analysis

Total phenolic content (TPC) values of the samples (crude methanol *L. microphyllum* extract and its fractions) were determined by using an equation obtained from the standard gallic acid calibration curve and expressed as mg gallic acid equivalents (GAE)/g samples. The order of the decreasing TPC was as follows : ethyl acetate fraction (11.59 ± 0.59) > chloroform fraction (6.53 ± 0.10) > hexane fraction (5.62 ± 0.09) > crude methanol extract (4.51 ± 0.47) > n-butanol fraction (2.44 ± 0.23) > water fraction (1.15 ± 0.15) (Table 01). The TPC value of the ethyl acetate fraction was 2.6 times higher than the crude methanol extract.

Total flavonoid contents (TFC) of the samples (crude methanol *L. microphyllum* extract and its fractions) were calculated from the equation obtained from the standard quercetin calibration curve and expressed as mg quercetin equivalents (QE)/g samples. The highest content of total flavonoids was found in hexane fraction (60.27 ± 2.03) followed by chloroform fraction (58.37 ± 1.46), ethyl acetate fraction (16.22 ± 1.94), crude methanol extract (15.66 ± 0.23), n-butanol fraction (5.32 ± 0.34) and water fraction (3.29 ± 0.15). The TFC value of hexane fraction was recorded 3.8 higher than the crude methanol extract.

Table 01. Total phenolic and flavonoid contents of crude methanol *L. microphyllum* extract and its fractions

Samples	**Total phenols[a] (mg GAE[b]/g extract)**	**Total flavonoids[a] (mg QE[c]/g extract)**
L. micropyllum crude methanol	4.51 ± 0.47	15.66 ± 0.23
Hexane Fraction	5.62 ± 0.09	60.27 ± 2.03
Chloroform Fraction	6.53 ± 0.10	58.37 ± 1.46
Ethyl Acetate Fraction	11.59 ± 0.59	16.22 ± 1.94
n-Butanol Fraction	2.44 ± 0.23	5.32 ± 0.34
Water Fraction	1.15 ± 0.15	3.29 ± 0.15

Notes: [a] Values were presented as mean ± SEM (n = 3); [b] GAE, gallic acid equivalents; [c] QE, Quercetin equivalents

The crude methanol *L. microphyllum* extract which was fractionated in less polar solvent (n-butanol fraction) and the polar solvent (water fraction) showed the lowest phenolic and flavonoid contents compared to the intermediate polar and non-polar solvents.

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In these assays, ethyl acetate fraction (intermediate polarity solvent) and hexane fraction (non-polar solvent) exhibited higher value of TPC and TFC respectively, compared to crude methanol. This may be due to the presence of complex mixtures of secondary metabolites in the crude methanol that interfered and inhibited these activities. As was previously noted by Tiwari, Kumar, Mandeep, Kaur, and Kaur (2011), the crude extracts are believed to have complex mixtures of various classes of bioactive compounds and need to subject to further separation to obtain an active portion for bioassay studies.

6.2. Qualitative phytochemical analysis

The qualitative analysis of the crude methanol *L. microphyllum* extract and its fractions showed the presence of secondary metabolite compounds namely alkaloid, cardiac glycosides, flavonoids, quinones, saponin and steroid (Table 02). All extracts however showed the absence of anthraquinones. Tannins were only present in the intermediate polarity ethyl acetate fraction. Meanwhile, terpenoid was found in the crude methanol *L. microphyllum* extract and less polar butanol and the polar water fractions. Terpenoids constitute the largest chemical group found in ferns including mainly triterpenoids, diterpenoids, and sesquiterpenoids having various biological interests. Occasionally terpenoids will be found in the aqueous phase, but they are more often obtained by treatment with less polar solvents (Tiwari et al., 2011).

Table 02. Phytochemical analysis of crude methanol *L. microphyllum* extract and its fractions

Secondary metabolite[a]	***L. microphyllum* extracts**					
	CM	HF	CF	EAF	nBF	WF
Alkaloid	+	+	+	+	+	+
Anthraquinones	-	-	-	-	-	-
Cardiac glycosides	+	+	+	+	+	+
Flavanoids	+	+	+	+	+	+
Quinones	+	+	+	+	+	+
Saponin	+	+	+	+	+	+
Steroid	+	+	+	+	+	+
Tannins	-	-	-	+	-	-
Terpenoids	+	-	-	-	+	+

Notes: (CM); crude methanol *L. microphyllum* extract, (HF); hexane fraction, (CF); chloroform fraction, (EAF); ethyl acetate fraction, (nBF); n-butanol fraction, (WF); water fraction; [a] (+); Present, (-); Absent.

The phytochemicals present in the crude methanol *L. microphyllum* extract and its fractions also play important roles for the evident antibacterial activity; since, phenolic and polyphenols (such as quinones, flavonoids and tannins), terpenoids and alkaloids possess the beneficial mechanism of actions to combat bacteria such as binds to adhesins, complex with cell wall, inactivates enzymes, substrate deprivation and membrane disruption which are responsible to exhibit antibacterial activity (Tiwari et al., 2011). These findings from quantitative and qualitative phytochemical analysis showed that intermediate polarity ethyl acetate fraction (which exhibited higher value of TPC and present of tannin) and non-polar hexane fraction (which exhibited higher value of TFC) have the potentials to promote antibacterial activity.

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6.3. Antibacterial activities of crude methanol *L. microphyllum* extract and its fractions

The results from the *in vitro* antibacterial susceptibility test revealed that the crude methanol extract of *L. microphyllum* in non-polar solvent (hexane fraction) and intermediate polar solvent (ethyl acetate fraction) showed strong inhibitory effects (MIC and MBC values of 6.25 mg/mL and 12.5 mg/mL, respectively) compared to the crude methanol extract in less polar and polar (n-butanol and water fractions) solvent systems against all tested gram-negative bacteria including *E. coli* as standard strain. This could have resulted from the presence of phenolic, flavonoid, alkaloid, glycoside, quinones, saponin and tannins (only presence in ethyl acetate) compounds that are known to possess antibacterial activity (Cowan, 1996). Thus, all the resistant clinical isolates which consist of enteric bacteria were susceptible to hexane and ethyl acetate fractions.

Table 03. Antibacterial activity (MIC) of crude methanol *L. microphyllum* extract and its fractions

Bacteria	***L. microphyllum* extracts (mg/mL)**						
	CM	HF	CF	EAF	nBF	WF	Tetracycline[c]
Escherichia coli[a]	12.5	6.25	12.5	6.25	25	50	1.0×10^{-3}
Klebsiella pneumonia[a]	12.5	6.25	12.5	6.25	25	50	0.5×10^{-3}
Proteus mirabilis[a]	25	6.25	6.25	6.25	25	50	3.9×10^{-3}
Salmonella typhi[a]	12.5	6.25	6.25	6.25	25	25	0.5×10^{-3}
Shigella dysenteriae[a]	25	6.25	6.25	6.25	25	25	3.9×10^{-3}
Escherichia coli (ATCC 25922)[b]	25	6.25	6.25	6.25	25	25	1.0×10^{-3}

Notes: (CM); crude methanol *L. microphyllum* extract, (HF); hexane fraction, (CF); chloroform fraction, (EAF); ethyl acetate fraction, (nBF); n-butanol fraction, (WF); water fraction; [a] Clinical isolate (drug-resistant bacteria); [b] Standard strain (American Type Culture Collection, ATCC); [c] Antibiotic

Crude methanol *L. microphyllum* extract and its butanol and water fractions (extracted in less polar and the polar solvent system, respectively) displayed weak inhibitory effects against all bacterial isolates with the MIC values varied from 12.5–50 mg/mL (Table 03). The crude methanol extract, butanol fraction and water fraction are rich in terpenoid compound which is listed as a useful antibacterial compound. However, these extracts exhibited poor inhibitory effects compared to other fractions. Thus, this clearly showed that the antibacterial activity of the extract did not depend solely on the presence of a certain type of bioactive secondary metabolite. The expression of additive, synergistic or antagonistic effects of various bioactive compounds present in the crude methanol extract, butanol and water fractions may affect the bacterial growth inhibitory activity.

Tetracycline, an inhibitor of bacterial protein synthesis was found to possess the strongest inhibitory activity against all tested gram-negative bacteria.

Standard isolate *Escherichia coli* (ATCC 25922) was used in order to verify the accuracy of the susceptibility results as recommended by Wiegand *et al.* (2008). Results are acceptable when the test values of MIC for control strains are within the suggested MICs range of antibiotics used. The MIC for routinely used tetracycline for quality control strain of *Escherichia coli* (ATCC 25922) was based on CLSI (2015) whereby the test value for this strain must be within 0.5×10^{-3} to 2.0×10^{-3} mg/mL. Hence, the MIC value of the studied *Escherichia coli* (ATCC 25922) was 1.0×10^{-3} mg/mL. Therefore, the results from this finding are acceptable. Furthermore, the susceptibility results of 5 isolates of clinically

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resistant enteric bacteria when tetracycline was used in this study were also acceptable since the MIC values of these bacterial isolates conformed with the suggested range for MIC determination of Enterobacteriaceae which is less than or equal to 4.0 x 10^{-3} mg/mL.

Findings from MBC determinations suggested that the hexane, chloroform, ethyl acetate and butanol fractions showed bactericidal effect; whilst the crude methanol of *L.microphyllum* extract and water fraction showed a bacteriostatic effect against tested enteric bacteria (Table 04).

Table 04. Antibacterial activity (MBC) of crude methanol *L. microphyllum* extract and its fractions

Bacteria	***L. microphyllum* extracts (mg/mL)**						
	CM	HF	CF	EAF	nBF	WF	Tetracycline[c]
Escherichia coli[a]	>100	12.5	25	12.5	50	>50	1.9 x 10^{-3}
Klebsiella pneumonia[a]	>100	12.5	25	12.5	50	>50	1.0 x 10^{-3}
Proteus mirabilis[a]	>100	12.5	12.5	12.5	50	>50	7.8 x 10^{-3}
Salmonella typhi[a]	>100	12.5	12.5	12.5	50	>50	1.0 x 10^{-3}
Shigella dysenteriae[a]	>100	12.5	12.5	12.5	50	>50	7.8 x 10^{-3}
Escherichia coli (ATCC 25922)[b]	>100	12.5	12.5	12.5	50	>50	1.9 x 10^{-3}

Notes: (CM); crude methanol *L. microphyllum* extract, (HF); hexane fraction, (CF); chloroform fraction, (EAF); ethyl acetate fraction, (nBF); n-butanol fraction, (WF); water fraction; [a] Clinical isolate (drug-resistant bacteria); [b] Standard strain (American Type Culture Collection, ATCC); [c] Antibiotic

The presence of secondary metabolite compounds in *L.microphyllum* were associated with the antibacterial effect. However, the antibacterial activity did not only depend on a single compound but a combination of different bioactive compounds may produce better expression of bacterial inhibition activity.

Thus, the present study confirmed the antibacterial activity of *L.microphylum* extracts, particularly hexane and ethyl acetate fractions which distinctly showed antibacterial activities against tested clinically resistant enteric bacteria.

7. Conclusion

Results of the quantitative and qualitative phytochemical analysis, MIC and MBC have highlighted an interesting antibacterial activity of *L.microphyllum* extracts. In comparison to all the extracts of *L.microphyllum* investigated, ethyl acetate fraction gave the highest total phenolic content (11.59 ± 1.02 mg GAE/g extract) whist hexane fraction gave the highest total flavonoid content (60.27 ± 3.53 mg QE/g extract). Hexane and ethyl acetate fractions have verified the presence of alkaloid, cardiac glycosides, flavonoids, quinones, saponin, steroid and tannins which could have attributed to the antibacterial action. In addition, the strongest antibacterial activity was found in ethyl acetate and hexane fractions against drug-resistant gram-negative bacteria with MIC and MBC values of 6.25 mg/mL and 12.5 mg/mL, respectively. Thus, these MIC and MBC results verified the antibacterial activity possessed by hexane and ethyl acetate fractions. Comprehensively, the present findings from this study seem to validate the rationale of *L.microphyllum* fern used as the treatment of enteric infections; thus, supports the potential use of *L.microphyllum* as an antibacterial agent.

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