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Original Article

Lygodium microphyllum lyophilizied extract: characterization and pharmacological activities evaluation of antifungal and antidiabetic

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ABSTRACT

Lygodium microphyllum (LLM) has long been a cornerstone of traditional medicine for treating diverse physiological disorders; however, its pharmacological transition into modern therapeutics remains incomplete. This study addresses this gap by investigating the physicochemical profile of LLM water extract processed through lyophilization (freeze-drying) and evaluating its dual-functional potential as both an antifungal and antidiabetic agent. Analytical profiling via LC-HRMS revealed a robust yield of highly polar secondary metabolites, identifying key compounds. Physicochemically, the lyophilized extract demonstrated optimal water solubility and a near-neutral pH, enhancing its suitability for pharmaceutical formulation. Pharmacological assays confirmed potent antifungal activity, evidenced by significant inhibition zones against *Aspergillus niger, Candida albicans*, and *Malassezia furfur*. Furthermore, in vivo evaluations identified 300 mg/kg BW as the optimal antidiabetic dose, demonstrating maximum glucose-lowering efficacy ($p = 0.749$ relative to controls). These findings substantiate the novelty of lyophilized *Lygodium microphyllum* as a high-yield source of bioactive polar metabolites. By bridging the gap between traditional knowledge and rigorous analytical validation, this research provides a standardized foundation for the development of multifaceted botanical drugs targeting metabolic and fungal pathologies simultaneously.

Keywords: *L. microphyllum*, LC-HRMS, Antifungal, Antidiabetic

Introduction

Lygodium microphyllum (Cav.) R. Br., a perennial fern belonging to the family Lygodiaceae, is internationally known as the Old World climbing fern [1-4]. This species is characterized by extensive creeping rhizomes and climbing fronds that can reach heights of up to 30 meters, often forming dense vegetative mats in moist habitats [1, 3]. Although classified as an aggressive invasive weed in regions such as Florida, United States, the plant is naturally and widely distributed throughout tropical and subtropical areas of Asia (including Indonesia and Malaysia), Africa, Australia, and the Pacific Islands [1-5]. In Indonesia, particularly in Kalimantan, this plant is locally known as "ribu-ribu" or "kerokot" [6-8].

From an ethnopharmacological perspective, *Lygodium microphyllum* has long been utilized in traditional medicine systems by various ethnic groups to treat a wide range of health disorders [1, 5, 8-20]. The Murut ethnic group in Sabah

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traditionally uses a decoction of the leaves to treat dysentery and skin diseases [21]. In Indonesia, this plant is widely used as a traditional remedy for fever, muscle pain, and kidney stones [6, 22]. Other reported traditional applications include the treatment of cough, hepatitis, and contraception; wound healing; as well as asthma and digestive disorders [14]. The plant parts most frequently utilized are the leaves and roots, which are commonly prepared as decoctions or pastes [11].

Despite the known medicinal potential of *Lygodium microphyllum*, there is a significant lack of data regarding the physicochemical properties of its extract when processed via freeze-drying (lyophilization). Therefore, this study aims to bridge this gap by characterizing the lyophilized Lygodium extract and evaluating its dual therapeutic potential as both an antifungal and antidiabetic agent.

Materials and Methods

Plant collection

Specimens of *Lygodium microphyllum* were collected from Lempake Village, Samarinda, East Kalimantan. Taxonomic verification and voucher registration (No. 102/UN17.4.08/LL/2023) were completed at Mulawarman University's Laboratory of Tropical Forest Ecology and Biodiversity Conservation. Following collection, 1.5 kg of plant material was washed, sectioned, and air-dried until a constant mass was reached. The dried herb was then pulverized into a fine powder for further analysis.

Chemical and instruments

The study utilized various analytical reagents, including distilled water, formic acid, acetonitrile, technical-grade 70% ethanol, ethyl acetate, $FeCl_3$, H_2SO_4, HCl, chloroform, methanol, 0.9% NaCl, NaOH, Orange G, paraffin, n-hexane, magnesium powder, and potato dextrose agar, all sourced from Merck (Germany). Additional substances included Dragendorff, Liebermann-Burchard, Mayer, and Wagner reagents, along with Sigma-Aldrich's ketoconazole, alloxan, and glibenclamide. Biological materials comprised *Candida albicans*, *Malassezia furfur*, and *Aspergillus niger* (ATCC), alongside male *Mus musculus* mice, OneMed alcohol swabs, and 10% formalin (Smartlab-Indonesia). Glucose levels were monitored via EasyTouch strips and meters. Laboratory instrumentation featured a GEA autoclave, Pyrex glassware, Buchi freeze dryer, IKA oven, and Precisia analytical balances. Chemical analysis employed a UHPLC system with a Binary Pump (Germering, Germany), utilizing a Thermo Scientific™ Hypersil GOLD™ analytical column (150 mm × 2.1 mm, 1.9 µm), coupled with a Thermo Scientific™ Orbitrap™ Exploris 240 HRMS containing an Optamax™ NG H-ESI source. Procedural tools included Dragonlab micropipettes, Gilson microtips, laminar airflow, Taffware vernier calipers, ObsidiMedica surgical instruments, stainless steel blocks, GEA freeze boxes, Leica light microscopes, Onemed syringes, GEA mercury thermometers, and Tanita animal scales. All experimental procedures were strictly maintained under standardized protocols to ensure high-quality reproducibility and robust scientific data throughout the research duration.

Experimental animal

The experimental subjects were adult mice, aged 6–8 weeks and weighing between 20 and 30 g, obtained from the Pharmaceutical Research and Development Laboratory at the Faculty of Pharmacy, Mulawarman University. Animals were housed in polypropylene cages in groups of five under controlled conditions: a temperature of 22 ± 3°C and a consistent 12-hour light/dark cycle. Mice had ad libitum access to standard feed and water, excluding designated fasting intervals. All animal care and experimental procedures strictly adhered to international ethical standards for laboratory research. The study protocol received formal authorization from the Faculty of Pharmacy, Mulawarman University, under the ethical exemption reference number 107/KEPK-FF/UNMUL/EC/EXE/08/2024, ensuring full compliance with institutional and global welfare guidelines throughout the investigation.

Extraction and lyophilization

The powdered sample was extracted using the decoction method, first weighing 340 grams of sample powder and measuring 3.4 liters of distilled water with a ratio of 1:10 [21]. It began with heating the pan set using a hot plate containing water, inserting a porcelain cup containing the sample powder, and waiting for 30 minutes after the temperature reached 90°C. Briefly, following filtration and cooling for 1 hour, the sample obtained the results of the water extract and was then frozen as kerokot water extract in the refrigerator. Then, continued with a lyophilization process using freeze-drying for 3x24 hours and kept at -20°C for further analysis.

Measurement of pH from lyophilized extract

pH testing is performed using a pH meter. The meter is dipped into the sample and then waits for an indicator number to appear on the meter's display, indicating the sample's pH value. pH testing is performed by measuring the acidity or alkalinity of the extract using a pH meter that has been calibrated with a standard buffer solution at a specific pH. The pH of the extract sample is measured using the meter [23].

Extract solubility testing

The extract solubility test was conducted using UV-Vis spectrophotometry (Double-beam UV-VIS Spectrophotometer UV-1800, Shimadzu, Japan) and was assessed from the resulting absorbance value. In the initial stage, the lyophilized extract was dissolved in distilled water as the original solvent. After the solution was formed, the absorption spectrum of each sample was measured at a wavelength of 440 nm using the solvent as a

blank. Next, a cross-test was performed, namely, the ethanol extract was dissolved in distilled water, and the lyophilized extract was dissolved in ethanol. The measurement results were compared based on the magnitude of the absorbance value, where solutions with stable and relatively high absorbance values indicated better solubility, while low or unreadable absorbance values indicated that the extract was less soluble in the solvent [23].

Untargeted profiling with LC-HRMS

Secondary metabolite compound analysis was performed using the LC-HRMS (Liquid Chromatography–High Resolution Mass Spectrometry) method. Before injection into the instrument, the sample was first prepared. A 10 mg sample was weighed and dissolved in 1 mL of HPLC-grade methanol. The solution was homogenized using a vortex for 2 minutes to ensure thorough mixing. The solution was then filtered through a 0.2 µm nylon filter to remove insoluble particles. The resulting filtrate was then transferred to an LC vial as a sample ready for analysis. The liquid chromatography process was carried out using a Thermo Scientific™ Vanquish™ Horizon UHPLC equipped with a Hypersil GOLD™ analytical column (150 mm × 2.1 mm, 1.9 µm particle size) [24-26].

Compound separation was carried out using a gradient with two solvents: mobile phase A, water containing 0.1% formic acid, and mobile phase B, acetonitrile containing 0.1% formic acid. Phase A is polar, while phase B is semi-polar. The flow rate used was 0.2 mL/min, the column temperature was maintained at 35°C, and the injection volume was 5 µL. The gradient program lasted for 23 minutes, starting at 0% B, then gradually increasing to 100%, and returning to the initial conditions for column re-equilibration. The separated eluate was then analyzed using a Thermo Scientific™ Orbitrap™ Exploris 240 HRMS in Full MS/dd-MS² mode with positive and negative polarity switching. The full MS acquisition resolution was set at 60,000 FWHM with a mass range of m/z 70–1000 and a mass tolerance of 5 ppm [24-26].

The ion fragmentation process was performed at a resolution of 22,500 FWHM with normalized collision energies (NCEs) of 30, 50, and 80 using nitrogen as the collision gas. Ionization was carried out using the Heated Electrospray Ionization (H-ESI) technique with a spray voltage of +3500 V in positive mode and -2500 V in negative mode, an ion transfer tube temperature of 275 °C, and a vaporizer temperature of 320 °C. The data obtained were then processed using Thermo Scientific Compound Discoverer 3.3 software. Identification of metabolite compounds was carried out by matching the mzCloud library, Arita Lab flavonoid database, endogenous metabolites database, natural products atlas, lipid maps structure database, and ChemSpider databases (BioCyc, ChEBI, ChEMBL, KEGG, Phenol-Explorer, etc.) [24-26].

Data analysis

Extract pH data was obtained by measuring the pH of the ethanol extract and the lyophilized extract using a pH meter. Solubility test data was obtained using a UV-Vis spectrophotometer by observing the absorbance values of both extracts.

LC-HRMS data were analyzed in several stages. First, total ion chromatogram (TIC) extraction was performed to determine peak distribution and compound retention times. Second, mass spectra were deconvolved to separate compound signals from noise. Third, compound identification was performed by matching accurate mass (m/z) values and fragmentation patterns with a metabolite database (mzCloud). Next, relative quantification was based on peak area (ion intensity), allowing for comparison between samples. The final result was a list of dominant compounds along with their RT, m/z, and intensity, which served as the basis for interpretation [24-26].

Antifungal activities

PDA medium was placed in a petri dish and allowed to solidify. Inoculate the test fungus into the agar medium. Five wells were made using a 7 mm diameter stylus, then each control (positive control, negative control, and test control) was placed in each well. The incubator was then incubated for 3x24 hours at 37°C, followed by observations by measuring the resulting inhibition zone [27, 28].

Antidiabetic activities

Blood sugar level measurement

The research received ethical approval from the Faculty of Pharmacy at Mulawarman University, registered under protocol number 107/KEPK-FFUNMUL/EC/EXE/08/2024, specifically for studies utilizing murine models. Experimental procedures commenced with a seven-day acclimatization period, followed by an eighteen-hour fasting duration. Baseline glycemic levels were documented prior to the induction process. To establish a diabetic state, mice received an intraperitoneal injection of alloxan at a dosage of 200 mg/kg body weight, dissolved in a 0.9% NaCl solution. Hyperglycemia was confirmed three days post-induction if blood glucose concentrations reached or surpassed 200 mg/dL. Subsequently, subjects underwent various treatment regimens across all study groups for fourteen days. Monitoring of blood glucose levels occurred systematically on days 0, 3, 7, and 14. These collected measurements served as the primary dataset, which was then subjected to comprehensive statistical analysis to evaluate the physiological changes occurring both before and after the application of treatments [13, 29, 30].

Histological observation of the pancreas

Histological observations of the mice's pancreas began with neck dislocation to reduce pain in the experimental animals. A dissection was performed from the thorax to the pubis, and the pancreas was removed and divided into two sections to expedite

the fixation process. The pancreas was then washed with 0.9% NaCl. The next step was fixation with 10% formalin for 10 hours. The pancreatic tissue was embedded in a stainless steel paraffin block and covered with a plastic filter. Paraffin was then added to the cut line and allowed to solidify. The hardened paraffin block was then trimmed on a microtome. Once the sections were clearly visible, the sections were placed in a water bath and removed with a glass slide. The remaining paraffin was then removed from the tissue preparation using xylene for 60 minutes. Then, redehydration was carried out using alcohol at the highest to lowest concentration for 5 minutes, excluding the lowest concentration for 10 minutes, which aims at the coloring process, where the compounds from the dye easily enter. After that, the preparation was stained using hematoxylin dye for 5 minutes, and the preparation was washed with running water for 3 minutes. Then, the dehydration process was carried out with alcohol using a low to high concentration. Then, the preparation was soaked again with orange G for 3 minutes and put in 96% alcohol 8 times. Next, the preparation was dried and covered with a cover glass, after which microscopic observation was carried out. Thus, the results obtained from the microscope were documented as research data [13, 29, 30].

Data analysis

To investigate reductions in glucose levels following experimental treatment, this study utilized a one-way ANOVA framework via SPSS 27, presenting the findings through comprehensive descriptive analysis. Data regarding glycemic decline over time were systematically tabulated and processed; conclusions were subsequently derived from these descriptions to determine if statistically significant variations existed between treatment cohorts. In cases where significant differences were identified, post-hoc testing was performed to isolate the most effective intervention. Furthermore, pancreatic histopathology was evaluated by capturing images of preparations from each experimental group. These images were then examined using ImageJ software to assess the structural integrity and diameter of the islets of Langerhans, with the resulting observations documented in a descriptive manner to characterize cellular changes across all samples, ensuring a robust interpretation of the underlying biological responses observed during the research process [13, 29, 30].

Results and Discussion

pH evaluation of extracts

pH is a critical physicochemical parameter influencing extract stability, solubility, and biological activity. The pH of plant extracts affects metabolite stability and reactivity during further assays and formulation processes. pH measurements were performed using a calibrated digital pH meter (buffers pH 4.0 and 7.0), with triplicate measurements (n = 3)**(Table 1)** [31].

Table 1. pH Comparison between ELM and LLM

Extract type	pH (Mean ± SD)
ELM	5,54 ± 0,06
LLM	6, 56 ± 0,02

Description:
ELM = EtOH 70 % Extract *L. microphyllum*
LLM = Lyophilization Extract *L. microphyllum*

The lyophilized extract exhibited a near-neutral pH, whereas the ethanol extract was moderately acidic. The preservation of near-neutral pH in LLM suggests that lyophilization minimally alters the acid–base properties of the extract, likely due to reduced thermal and oxidative stress during processing. Neutral pH is advantageous for formulation stability and biological compatibility.

Solubility assessment

The solubility of the lyophilized extract was evaluated using UV–Visible spectrophotometry at 440 nm. Absorbance values were recorded in triplicate, with higher absorbance indicating greater solubility of bioactive compounds **(Table 2)**.

Table 2. Solubility test results of ELM and LLM

Type of Extract	Blanko	$\bar{x}$ Absorbance
ELM	Ethanol	0, 986
LLM	Aquadest	0, 939
ELM	Aquadest	0, 777
LLM	Ethanol	0, 399

Description:
ELM = EtOH 70 % Extract *L. microphyllum*
LLM = Lyophilization Extract *L. microphyllum*

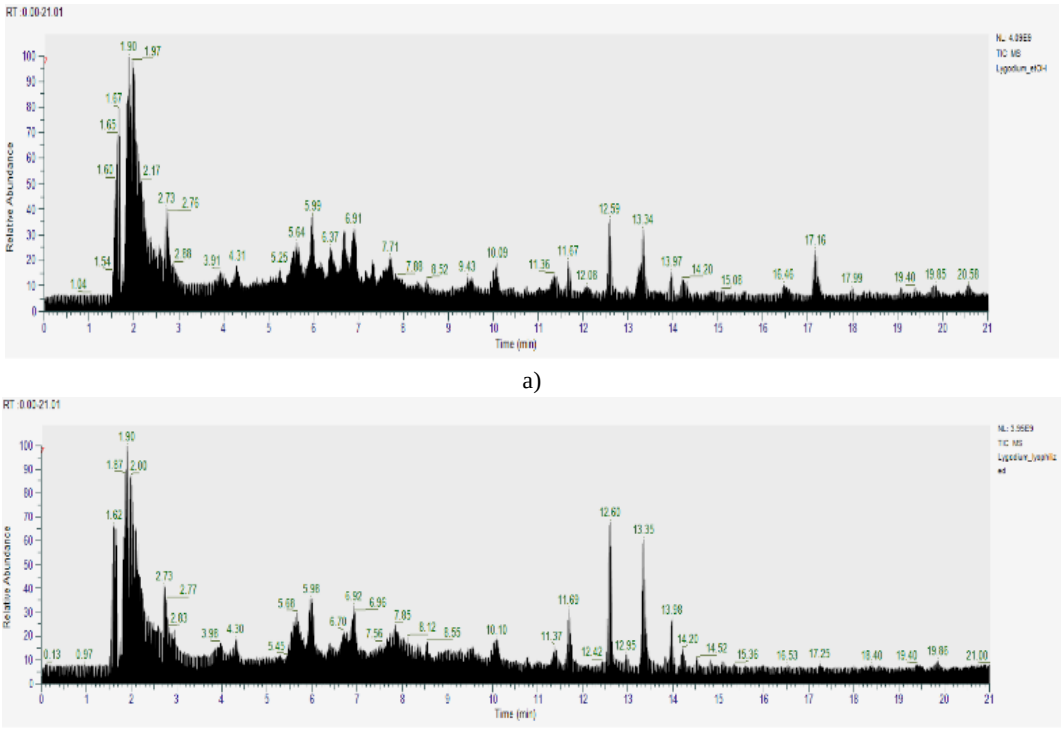
The highest solubility was observed when extracts were dissolved in their original extraction solvent, consistent with polarity principles. LLM demonstrated optimal solubility in water, while its solubility in ethanol was markedly reduced, indicating structural or crystallinity changes during freeze-drying that may affect re-dissolution.

LC–HRMS untargeted metabolomic profiling

High-resolution liquid chromatography–mass spectrometry (LC–HRMS) was employed to comprehensively identify metabolites in both extracts using untargeted profiling. This technique provides high mass accuracy and resolution, allowing detection of metabolites at trace concentrations.

The analytical workflow included chromatographic separation, electrospray ionization, high-resolution mass detection, total ion chromatogram (TIC) extraction, spectral deconvolution, and database-based compound annotation. Numerous compounds belonging to organic acids, flavonoids, phenolic glycosides, amino acids, sugar derivatives, and bioactive secondary metabolites were identified in both ELM and LLM extracts. Most detected compounds showed mass accuracy within ±5 ppm, confirming high-confidence identification. LLM extracts demonstrated richer polar metabolite profiles, including

gluconic acid, 1-O-coumaroyl-β-D-glucose, daldiniside B, isoferulic acid, and 2,5-anhydro-1-deoxy-mannitol, which are associated with antioxidant, antimicrobial, and metabolic regulatory activities. The total ion chromatograms (TIC) of both extracts are presented in **Figure 1**.

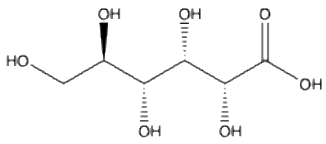
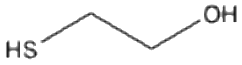
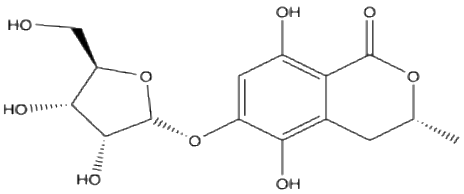
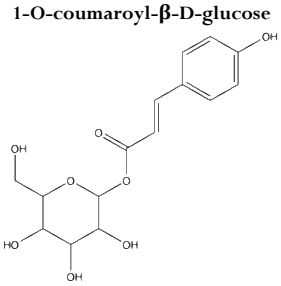


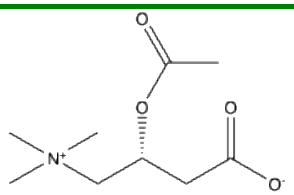
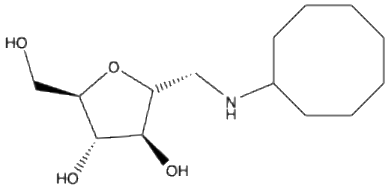
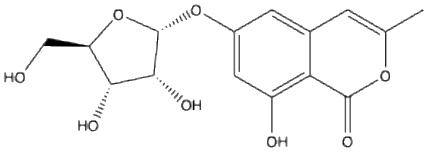
a)

b)

Figure 1. Chromatogram Graph of 70% Ethanol Extract (ELM) (a) and Lyophilized Extract of Kerokot Herb (*Lygodium Microphyllum*)(LLM) (b)

Table 3. Comparison of Bioactive Compounds of ELM and LLM

Compound name		ELM			LLM		
	Label	RT (Minute)	m/z	Area	RT (Minute)	m/z	Area
Gluconic acid 	4.1	1.855	195.05077	8.× 10^9	1.846	195.05074	6.1 × 10^9
Mercaptoethanol 	4.	2.726	79.02102	7.2 × 10^9	-	-	-
Flavoroseoside 		5.974	341.08704	6.8 × 10^9	6.702	341.0871	1.5 × 10^9
1-O-coumaroyl-β-D-glucose 		6.896	325.09259	6.5 × 10^9	6.918	325.09256	5.0 × 10^9
Acetyl-L carnitine		1.978	325.09259	6.3 × 10^9	-	-	-

 **2,5-Anhydro-1-deoxy-mannitol**	4.6	2.164	236.14886	$1,2 \times 10^9$	1.974	236.14893	5.0×10^9
 **Daldiniside B**		5.636	325.09143	4.6×10^9	8.552	325.09146	$9,4 \times 10^8$
 **Isoferulic acid**		13.245	193.05048	3.6×10^9	13. 265	193.05045	$7,8 \times 10^8$

LC–HRMS analysis of kerokot herb (*Lygodium microphyllum*) extracts successfully revealed eight dominant compounds characterizing the phytochemical profile of the lyophilized extract.

Overall, LC–HRMS profiling of *Lygodium microphyllum* herb extracts successfully identified eight dominant metabolites **(Table 3)**, namely gluconic acid, mercaptoethanol, flavoroseoside, 1-O-coumaroyl-β-D-glucose, acetyl-L-carnitine, 2,5-anhydro-1-deoxy-mannitol, daldiniside B, and isoferulic acid, highlighting the chemical diversity and pharmacological potential of this medicinal fern.

Antifungal activity assay LLM extract against aspergillus niger, candida albicans, and malassezia furfur

The findings of this study demonstrate that the LLM extract exhibits notable antifungal activity. This effect was evidenced by the formation of clear inhibition zones against Aspergillus niger, Candida albicans, and Malassezia furfur. These results highlight the potential of the LLM extract as a promising source of natural antifungal agents. An antifungal activity assay of the LLM extract was conducted against several fungal species. The extract was tested at varying concentrations of 20%, 30%, and 40%. The assay results showed measurable inhibition zone diameters for Aspergillus niger, Candida albicans, and Malassezia furfur. The antifungal activity test of the LLM extract was performed using the agar well diffusion method. Potato Dextrose Agar (PDA) was used as both the cultivation and test medium. A fungal suspension (0.2 mL) and 15 mL of molten PDA medium were poured into sterile Petri dishes. Wells were prepared using a sterile cork borer, into which the test extract of LLM Extract, a positive control (ketoconazole), and a negative control (distilled water) were introduced. The plates were incubated at 37 °C for 72 hours (3 × 24 h). After incubation, the diameter of the inhibition zones formed around each well was measured using a digital vernier caliper. The results of the antifungal activity of the lyophilized *Lygodium microphyllum* herb extract are presented in **Table 4**.

Table 4. Inhibition Zone Diameter of LLM

Fungi	Experimental	Inhibition Zone Diameter (mm)				Conclusion*
		R1	R2	R3	Average ±SD	
Aspergillus niger	Positive control (Ketokonazol)	15,70	15,95	16,50	16,06±0,40	Strong
	Negative control (Aquadest)	0,00	0,00	0,00	0	Negative
	20%	13,80	12,10	11,00	12,3±1,41	Strong
	30%	14,60	13,80	12,65	13,68±0,98	Strong
	40%	15,70	15,20	15,60	15,5±0,26	Strong
Candida albicans	Positive control (Ketokonazol)	20,75	21,85	22,85	21,81±1,05	Strongest
	Negative control (Aquadest)	0,00	0,00	0,00	0	Negative

	20%	11,35	12,30	11,05	11,56±0,65	Strong
	30%	12,85	13,90	13,10	13,28±0,54	Strong
	40%	15,00	16,20	15,20	15,46±0,64	Strong
	Positive control (Ketokonazol)	21,00	19,10	21,65	20,58±1,32	Strongest
	Negative control (Aquadest)	0,00	0,00	0,00	0	Negative
Malassezia furfur	20%	9,50	9,60	9,20	9,43±0,20	Moderate
	30%	11,50	11,20	11,30	11,33±0,15	Strong
	40%	13,60	13,20	12,60	13,13±0,50	Strong

References [20]

The results presented in **Table 4** demonstrate that the LLM extract exhibited antifungal activity against *A. niger, C. albicans,* and *M. furfur.* Antifungal activity was confirmed by the presence of inhibition zones surrounding the agar wells, which were measured using a digital vernier caliper. The negative control (distilled water) showed no antifungal activity, indicating that distilled water had no effect on fungal growth inhibition. Therefore, the observed inhibition zones can be attributed solely to the bioactive constituents of the LLM extract. The extract concentrations tested (20%, 30%, and 40%) produced varying inhibition zone diameters. For *A. niger* and *C. albicans,* all concentrations resulted in strong antifungal activity. In contrast, against *M. furfur*, the 20% concentration produced a moderate inhibition zone, whereas the 30% and 40% concentrations resulted in strong antifungal activity. Data from **Table 4** indicate that increasing extract concentration led to a corresponding increase in the diameter of the inhibition zones. This trend suggests that higher extract concentrations enhance antifungal efficacy, as extract concentration is directly proportional to the magnitude of fungal growth inhibition [21, 22]. The antifungal activity data were subsequently subjected to statistical analysis, beginning with tests of normality and homogeneity. For all three fungi, the data were normally distributed based on the Shapiro–Wilk test and demonstrated homogeneity of variance using the Levene test. Consequently, a parametric one-way ANOVA was performed. The ANOVA results showed a significant difference among treatment groups (Sig. < 0.05), with a reported significance value of 0.00, indicating statistically significant differences in mean inhibition zone diameters across concentrations [32].

Based on the experimental findings, the LLM exhibits notable antifungal activity against *A. niger, C. albicans*, and *M. furfur*. The extract concentrations of 20%, 30%, and 40% produced substantial inhibition zones, demonstrating a clear concentration-dependent relationship with antifungal efficacy. Increasing extract concentration resulted in larger inhibition zones, indicating enhanced antifungal potency. Moreover, the inhibitory effect of the extract was comparable to that of the positive control. Future studies are recommended to proceed toward formulation development to further explore the therapeutic potential of this extract.

Antidiabetic activity of LLM

The antihyperglycemic evaluation demonstrated that the lyophilized extract of *Lygodium microphyllum* produced a significant reduction in fasting blood glucose levels in alloxan-induced diabetic mice, with the effect being clearly dose-dependent. Collectively, these results substantiate the therapeutic potential of LLM extract as an antidiabetic agent and indicate that appropriate dose optimization is critical to achieving maximal pharmacological efficacy. The reduction in blood glucose levels observed during the treatment period is presented in **Table 5**, which summarizes the mean blood glucose levels across all experimental groups.

Table 5. the mean blood glucose levels across all experimental groups

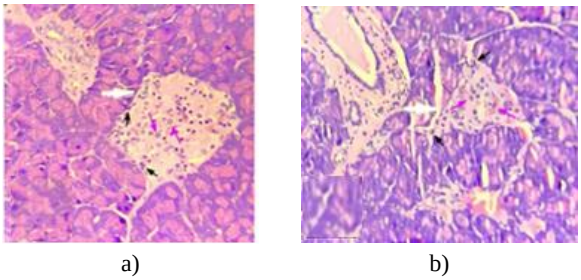
Group	Blood Glucose level (mg/dL)			
	H - 3	H 0	H+7	H+14
Normal	104.25±14.523	147.50±15.022	141.00±21.863	115.25±21.77
Negative control	88±10.360	557±32.568	527.50±40.079	557.75±32.613
Positive control	94±6.976	432.25±21.407	401.75±22.081	326.50±58.049
Dose 100 mg/Kg BW	119.25±12.987	498±39.710	288.75±109.524	174.75±73.898
Dose 300 mg/Kg BW	103.25±26.107	452.50±19.330	264.50±180.160	200.50±119.179
Dose 600 mg/kg BW	121.25±30.203	412.50±47.403	498±32.711	303±105.676

The present study demonstrates that administration of the test extract produced a significant antihyperglycemic effect in alloxan-induced diabetic animals, with the magnitude of glucose reduction being both dose- and time-dependent. A progressive

decrease in blood glucose levels was observed after treatment, indicating that repeated exposure to the extract enhances its pharmacological efficacy. Similar time-dependent antihyperglycemic patterns have been reported for various medicinal plant extracts in streptozotocin-induced diabetic models, suggesting that sustained modulation of glucose metabolism and pancreatic β-cell function may underlie this effect [24, 25]. Overall, the findings of this study are consistent with previous reports on plant-derived antidiabetic agents and support the therapeutic potential of the tested extract as a complementary strategy for diabetes management [33-39]. However, further studies focusing on bioactive compound isolation, molecular mechanism elucidation, and long-term toxicity evaluation are warranted before clinical translation [40-44].

The present study evaluated the antihyperglycemic activity of LLM extract in an alloxan-induced hyperglycemic mouse model. Prior to induction, all experimental animals were acclimatized and fasted to ensure stable baseline glucose levels and minimize dietary variability. Fasting is a widely accepted approach in metabolic studies, as it facilitates depletion of hepatic glycogen stores and enhances the uniformity of physiological conditions, thereby improving the reliability of pre- and post-treatment glucose measurements [30]. Baseline blood glucose levels recorded on day H−3 were within the normal range (<200 mg/dL), confirming the suitability of the animals for subsequent induction and treatment protocols. The combined action of these phytoconstituents likely underlies the significant antihyperglycemic effects observed at optimal extract doses [45]. Overall, the results indicate that LLM extract exhibits significant antihyperglycemic activity at doses of 100–300 mg/kgBW, with 300 mg/kgBW identified as the most effective dose. The diminished efficacy observed at higher doses highlights the importance of dose optimization and supports the concept of a therapeutic window for phytopharmaceutical development. Future studies should focus on dose standardization, long-term safety evaluation, molecular mechanism elucidation, and formulation development to advance LLM extract as a potential antidiabetic agent.

Pancreatic tissue repair in mice induced by lyophilized kerokot extract



a) b)

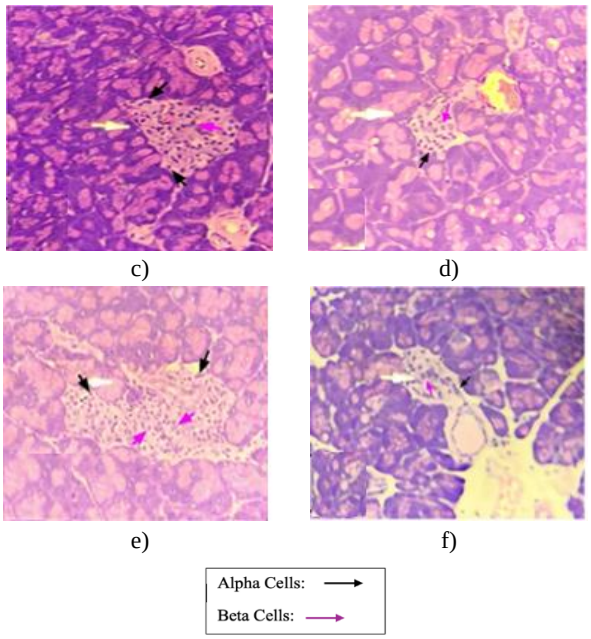


Figure 2. Histology and islet diameter measurement of the mouse pancreas at 200x magnification. Upper panels (a-f): cellular morphology of the islets of Langerhans (black arrows = alpha cells, magenta arrows = beta cells). Lower panels (a-f): measurement of the islet diameter.
Description a = Normal control, b = Negative control, c = Positive control, d = LLM extract group with a dose of 100 mg/kg BW, e = LLM extract group with a dose of 300 mg/kg BW, and f = LLM extract group with a dose of 600 mg/kg BW.

Histological analysis and diameter measurement of the islets of langerhans

Data analysis was conducted by measuring the diameter and evaluating the histology of the pancreatic islets across all treatment groups. The diameter of the islets of Langerhans (μm) was measured using ImageJ software to determine changes in islet size caused by diabetes induction and the administration of lyophilized *Lygodium microphyllum* (LLM) extract. Histological observations of the islets were presented descriptively based on photomicrographs of hematoxylin–eosin (HE)-stained pancreatic sections observed under a light microscope at 200× magnification. The principal parameters evaluated in this analysis were the structural integrity and cellular morphology of pancreatic cells within the islets of Langerhans. Representative photomicrographs and the corresponding islet diameter measurements for all treatment groups are presented in **Figure 2**.

The islets of Langerhans constitute the endocrine component of the pancreas and are distributed throughout the organ, with considerable variability in islet size. Approximately 1–2% of the pancreatic tissue consists of islets, which are most abundant in the tail region of the pancreas. Several cell types are present within the islets, including PP cells, D1 cells, EC cells, and epsilon cells (minor cell populations). However, three major cell

types are classically identified using the Mallory–Azan staining method: α (alpha) cells, β (beta) cells, and δ (delta) cells [40].

These findings demonstrate that lyophilized *L. microphyllum* extract exhibits therapeutic potential in pancreatic tissue repair, as evidenced by increased islet diameter and improved histological features in diabetic mice following 14 days of treatment. The regenerative effect is attributed to flavonoid compounds present in *L. microphyllum*, which act as antioxidants by scavenging reactive oxygen and nitrogen species. Flavonoids may inhibit xanthine oxidase and lipid peroxidation, primarily through phenolic hydroxyl (–OH) groups that neutralize free radicals. This mechanism is consistent with previous studies reporting that flavonoids protect pancreatic beta cells from oxidative damage and promote gradual beta-cell repair and proliferation [18, 21].

In conclusion, a 14-day administration of lyophilized *L. microphyllum* extract at a dose of 300 mg/kg body weight effectively improved pancreatic morphology in diabetic mice, as demonstrated by restoration of islet diameter and regeneration of endocrine cells, outperforming both lower and higher dose regimens.

Potential of lyophilized kerokot extract in comparison with glibenclamide

The evaluation was based on comparative analyses of blood glucose levels and pancreatic histopathological findings between the treatment groups and the positive control group. Data were obtained from measurements of blood glucose reduction on days 7 and 14 of treatment, as well as from microscopic examination of pancreatic tissue collected from both the glibenclamide-treated group and the groups receiving the lyophilized kerokot extract. These data were subsequently compared to assess the relative antidiabetic efficacy of the optimal dose of the lyophilized extract in relation to glibenclamide. Blood glucose assessment was conducted by dividing the mice into six experimental groups. Group 1 served as the normal control, Group 2 as the negative control (distilled water), and Group 3 as the positive control (glibenclamide 5 mg). The remaining three groups were treatment groups receiving LLM extract at doses of 100, 300, and 600 mg/kg body weight, respectively. Diabetes was experimentally induced using the oral glucose tolerance method following intraperitoneal administration of alloxan at a dose of 200 mg/kg body weight, with the aim of inducing a diabetic state in the mice. Mice with blood glucose levels ≥ 200 mg/dL were classified as diabetic. Subsequently, the animals were fasted, fasting blood glucose levels were measured, and each group received the assigned treatment for a period of 14 days. The results comprise comparative analyses of mean blood glucose levels and statistical evaluations between the positive control group and LLM extract treatment groups.

Comparison of blood glucose levels between lyophilized extract and positive control.

A comparison of blood glucose outcomes was conducted between the lyophilized kerokot extract groups and the positive control group treated with glibenclamide at a dose of 5 mg/kg body weight (BW). These results indicate no significant differences between the groups. Based on these findings, the lyophilized kerokot extract at a dose of 300 mg/kg BW was identified as the optimal dose, as it demonstrated the highest antidiabetic activity ($p = 0.749$) compared with other dose levels and showed potential efficacy comparable to glibenclamide at 5 mg/kg BW.

Conclusion

The LLM extract exhibited a higher yield, near-neutral pH, optimal solubility in water, and was more effective at attracting highly polar compounds. LLM extracts demonstrated richer polar metabolite profiles, including gluconic acid, 1-O-coumaroyl-β-D-glucose, daldiniside B, isoferulic acid, and 2,5-anhydro-1-deoxy-mannitol, from untargeted profiling with LC-HRMS. The findings of this study demonstrate that the LLM extract exhibits notable antifungal activity. This effect was evidenced by the formation of clear inhibition zones against *Aspergillus niger, Candida albicans, and Malassezia furfur*. These results substantiate the therapeutic potential of LLM extract as an antidiabetic agent and indicate that appropriate dose optimization is critical to achieving maximal pharmacological efficacy. Based on these findings, the lyophilized kerokot extract at a dose of 300 mg/kg BW was identified as the optimal dose, as it demonstrated the highest antidiabetic activity ($p = 0.749$) compared with other dose levels and showed potential efficacy

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Conflict of interest: None

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Ethics statement: This study was performed in accordance with internationally accepted ethical guidelines for the use of laboratory animals, and the study protocol was approved by the Faculty of Pharmacy, Mulawarman University, with the description of ethical exemption No. 107/KEPK-FF/UNMUL/EC/EXE/08/2024.

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