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LC-MS/MS and GC-MS Analysis for the Identification of Bioactive Metabolites Responsible for the Antioxidant and Antibacterial Activities of *Lygodium microphyllum* (Cav.) R. Br.

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Abstract: Natural products serve as a valuable source of antioxidants with potential health benefits for various conditions. *Lygodium microphyllum* (Cav.) R. Br., also known as Old World climbing fern, is an invasive climbing fern native to Southeast Asia, Africa, South America, Australia, and Melanesia. It has been reported to possess interesting pharmacological properties including hepatoprotective and anti-inflammatory mechanisms. This study analyzed the potential bioactive metabolites that contribute to the antioxidant and antimicrobial effects of *L. microphyllum* (LM) by profiling the crude extract using high-resolution LC-MS/MS and GC-MS systems. Several classes of compounds such as phenolics, flavonoids, terpenoids, steroids, macrolides, vitamins, lipids, and other hydrocarbons were found in the crude extract of LM through non-targeted analysis. A total of 74 compounds were detected in LC-MS/MS, whereas a total of nine compounds were identified in GC-MS. Out of the 74 compounds detected in LC-MS/MS, 34 compounds, primarily quercetin, kaempferol, trifolin, pyroglutamic acid, arachidonic acid, and rutin were reported with antioxidant, antimicrobial, anti-inflammatory, and hepatoprotective activities. The presence of phenolic and flavonoid compounds with reported bioactivities in the crude extract of LM evidence its pharmacological properties.

Keywords: flavonoids; antioxidants; LC-MS/MS; antibacterial; anti-inflammatory; hepatoprotective; *Lygodium microphyllum* (Cav.) R. Br.

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1. Introduction

Medicinal plants have many important roles in the pharmaceutical and food industries. The phytochemical compounds obtained from plants have been developed into commercial medicine and have always been a source for the discovery of new medicinal drugs. Bioactive compounds of plant origin are known to contribute to human health improvement, especially in treating diseases related to oxidative stress. Flavonoids are the largest group of polyphenolic compounds that are present in high concentrations in medicinal plants [1]. These compounds majorly contribute to the proclaimed pharmacological properties of the plants and have also been widely reported to possess therapeutic effects as individual compounds. Numerous polyphenols have been studied for their therapeutic values, and most of the compounds have been developed into commercial drugs for various diseases [2]. The

ability to scavenge free radicals is the fundamental requirement for a bioactive compound to exert pharmacological effects.

Free radicals are formed during the body's normal physiological activities but can be controlled under normal conditions due to the presence of an antioxidant defense system comprising reduced glutathione and antioxidant enzymes [3]. Excessive free radical formation is caused by unfavorable conditions such as ingestion of toxicants or exposure to radiation, thus inflicting an imbalance in the body's antioxidant defense system, which leads to oxidative stress. Numerous free radicals in the body at the cellular level cause an increase in malondialdehyde (MDA) formation, which is also a result of lipid peroxidation at the cell membrane. This formation triggers a cascade of events that could lead to tissue damage and eventually organ failures. Lipid peroxidation at the cellular level implies the activation of inflammatory and apoptosis events [4]. Oxidative stress and inflammation remain pathophysiological factors for the development of many ailments, including cancer, liver damage, diabetes, brain disorders, and heart problems [5]. Therefore, bioactive compounds from plants are among the most suitable drug candidates to counter oxidative stress-related diseases by exerting antioxidant effects. External supplementation of antioxidants has been proven to accelerate the endogenous antioxidant defense mechanism as these antioxidant compounds have the tendency to pass the gastrointestinal barrier as cytochrome P-450 enzymes cannot alter the compounds and cause them to be excreted out of the body. Bioactive compounds that are detected as xenobiotics in the liver could alter the mechanism of cytochrome P-450 by escaping biotransformation and hence could be present as an active compound in the same chemical configuration to reach the target site [6]. Crude extracts of medicinal plants tend to express synergistic effects in regard to antioxidant activities with minimal or no toxicity, especially in preclinical assessments. Bioactive compounds, especially flavonoids, tend to be commercialized into therapeutic drugs, but the bioavailability of the individual compounds in *in vivo* trials remains a hurdle. Therefore, the administration of crude extracts or a mixture of bioactive compounds could be regarded as a suitable measure to overcome the problem of bioavailability.

Lygodium microphyllum (Cav.) R. Br. (LM) is an invasive fern species native to American and Asian countries. Although this invasive plant is considered a threat to the forest as it grows rapidly, it contains important phytochemical compounds that are useful for the treatment of diseases. The native community of Sabah claimed that this plant could be used for the treatment of skin ailments and dysentery through the consumption of aqueous decoctions. Our previous study on LM reported the hepatoprotective and immunosuppressive effects and effective antioxidant properties in the aqueous extracts of the leaves [7]. The antioxidant properties of LM in an aqueous extract were noteworthy, with the total phenolic content of 206.38 $\pm$ 9.62 mg/g gallic acid equivalent (GAE) and the IC_{50} value for the 2,2-diphenylpicrylhydrazine (DPPH) study of 65 µg/mL. Other reports on the anthelmintic, antipyretic, anti-inflammatory, and antioxidant activities and the qualitative phytochemical analysis of LM support the medicinal claims made about this plant [8]. This study was conducted to screen the potential bioactive compounds present in an aqueous extract of LM using liquid chromatography–high resolution tandem mass spectrometry (LC-MS/MS) and gas chromatography–mass spectrometry (GC-MS) and to evaluate its antibacterial properties on several bacterial strains.

2. Materials and Methods

2.1. Plant Collection and Extraction

The fresh plant specimen was collected from the area of Papar, Kota Kinabalu, Sabah, and a voucher specimen was deposited at Universiti Malaysia Sabah (voucher number CG005). The plant specimen was verified by a field botanist of the same institute upon collection from the wild. The mature leaves (1 kg) were cleaned, air-dried for 5 days, homogenized using a heavy-duty blender, and subjected to aqueous extraction as described previously in a ratio of 1:10 in distilled water [6]. Briefly, 100 g of LM was boiled in 1 L

of distilled water for 10 min, followed by cooling for 1 h and filtration. The extracts were lyophilized and kept at −20 °C for further analysis.

2.2. LC-MS/MS Analysis

High-resolution MS/MS analysis was performed as described by Haron et al. [8] using the Thermo Scientific Q Exactive HF Orbitrap mass spectrometry system (Thermo Fisher Scientific, Waltham, MA, USA). Prior to the analysis, metabolite separation was performed with the Dionex UltiMate 3000 ultra-high-performance liquid chromatography system (Thermo Fisher Scientific, Waltham, MA, USA) coupled with a Thermo Syncronis C18 column (2.1 mm × 100 mm × 1.7 µm; Thermo Fisher Scientific, Waltham, MA, USA). The column was maintained at 55 °C at a flow rate of 450 µL/min during analysis. All instrumental settings, elution gradients, and calibration were performed as described previously [9,10]. The mobile phases were prepared with HPLC-grade deionized water with formic acid 0.1% (Solvent A) and acetonitrile with formic acid 0.1% (Solvent B). The elution gradient program was started with 0.5% of Solvent B for 1 min, followed by 0.5% gradually to 95.5% of Solvent B for 15 min which was maintained for 4 min. The injection volume of the sample was set at 2 µL. The column was later conditioned for the next injection through flushing for 2 min as the initial cycle.

The acquired data were processed and analyzed using the Thermo Scientific Compound Discoverer 3.3 SP1 software (Thermo Fisher Scientific, Waltham, MA, USA) with minor adjustments on the default settings for the natural product workflow. Briefly, the workflow included background subtraction with blank data, retention time alignment, feature detection, elemental composition determination, library matching, and fragment ion search (FISh) scoring. The identification of compounds was primarily based on the matching of MS/MS data against the mzCloud database. Identification of unmatched signals was re-attempted on the ChemSpider database [11] using MS data and supported with a FISh scoring of above 50.

2.3. GC-MS Analysis

LM aqueous extract was diluted in methanol, and the sample was injected (1 µL) into a GC-MS system consisting of a gas chromatography system (Agilent 7890A) coupled with a mass spectrometry detector (Agilent 5975C). An HP-5MS (30 m × 0.25 mm) capillary column was used with 0.25 µm film thickness of coated material. The injector temperature (250 °C) was set; the temperature program was as follows: starting at 40 °C, hold for 3 min, from 40 to 300 °C (3 °C/min), and hold for 3 min. A post-run at 300 °C for 5 min was performed to prepare for the next injection. Gas chromatography was performed in spitless mode using helium gas as a carrier at a constant flow rate of 1 mL/min. Compounds were identified with reference to the NIST 11 library, and compositions were computed with reference to the abundance of compounds in the chromatogram. The complete analysis was performed in triplicate, together with a blank solvent.

2.4. Disc Diffusion Assay for Antibacterial Activity

Staphylococcus aureus (ATCC 33862), *Bacillus cereus* (ATCC 14579), *Escherichia coli* (ATCC 25922), and *Salmonella* sp. (ATCC 29890) were used as test bacteria to assess the antibacterial activity of LM. All test bacteria were obtained from UniKL-RCMP and cultured using Mueller–Hinton agar (MHA). The antibacterial activity of LM was evaluated using the disc diffusion method [12]. First, test bacteria were suspended in 0.9% saline solution. The optical density (OD) of bacterial suspension was adjusted to match the 0.5 McFarland standard (0.08 to 0.12 at 625 nm). Then, 100 µL of the bacterial suspension was pipetted onto the MHA plate to prepare bacterial lawn using the spread plate technique. LM aqueous extract was dissolved in 0.9% saline solution into two different concentrations (500 mg/mL and 100 mg/mL) to test for antibacterial activity. Then, 20 µL of the sample were pipetted onto sterile filter paper discs (Whatman No. 3; 6 mm diameter) and placed on the surface of the agar. A 10 µg gentamicin antibiotic disc (Oxoid) was used as a positive

control, while a filter paper disc with 0.9% saline solution added acted as a negative control. The plates were incubated at 37 °C for 18 h. The diameter of the inhibition zone (mm) was measured, and the experiment was performed in triplicates.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

The broth microdilution method was used to determine MIC [12]. LM water extract was dissolved in Mueller–Hinton broth to achieve 12.5 mg/mL. One hundred microliters of 12.5 mg/mL water extract was loaded into each well of a 96-well plate and 2-fold serially diluted using Mueller–Hinton broth (50 µL in each well as diluent). Then, *Staphylococcus aureus* suspension was prepared with an OD of 0.08 to 0.12 at 625 nm wavelength. This bacterial suspension was diluted 150 times using Mueller–Hinton broth to obtain an inoculum at 10^6 CFU/mL. Fifty microliters of inoculum was added into each well containing the extract. The 96-well plate was incubated for 18 h at 37 °C. The MIC was determined based on the lowest water extract concentration with the absence of turbidity. Gentamicin was used as positive control, whereas Mueller–Hinton broth was used as negative control.

2.6. Statistical Analysis

All data were analyzed using SPSS 25.0 (IBM, Armonk, NY, USA) Windows statistical package. Results are shown as mean ± standard deviation of triplicate measurements.

3. Results and Discussion

3.1. LM Extraction Yield and Antioxidant Properties

The extraction yield of LM was 18.3% for aqueous extract, which is considered sufficient for water extracts. In our previous study, we performed the extraction of LM with different solvents in various polarities, i.e., methanol, hexane, ethyl acetate, chloroform, and butanol [13]. Total phenolic content (TPC), total flavonoid content (TFC), and DPPH antioxidant tests were performed for all the extracts. The TPC was high in hexane (354.38 ± 0.57 mg/g GAE) and ethyl acetate (347.18 ± 0.28 mg/g GAE) extracts but was also comparable in the other extracts. The aqueous extract of LM had a TFC of 20.68 ± 3.67 mg/g catechin equivalent (CE), which was slightly lower than the TFC of the methanol extract (39.36 ± 2.73 mg/g CE), the highest TFC among the extracts. The DPPH results were convincing, with effective antioxidant effects shown in all the extracts of LM. IC_{50} values for the solvent extracts, i.e., methanol (60 µg/mL), ethyl acetate (52 µg/mL), hexane (61 µg/mL), butanol (81 µg/mL), and chloroform (76 µg/mL), were also comparable with that of the aqueous extract (65 µg/mL). The reason for choosing aqueous extract for this study is to validate the medicinal claim made by the indigenous people of Sabah that an aqueous decoction of the leaves of LM can treat several ailments. Moreover, we had previously published results showing the hepatoprotective effect of LM aqueous extract against carbon tetrachloride (CCl_4)-induced liver damage in rats [6]. The metabolites responsible for hepatoprotective and immunosuppressive effects were not elucidated in our previous study. These results indicate that the aqueous extract of LM had adequate phenolic and flavonoid contents although organic solvents had slightly higher TPC and TFC contents. The DPPH results indicate the ability of the aqueous extract to demonstrate excellent antioxidant effects by scavenging free radicals. This property is essential for therapeutic interventions, especially for oxidative stress-based diseases. It is suggested that organic solvent extracts of LM such as hexane, ethyl acetate, and methanol with higher TPC and TFC values should be evaluated for their pharmacological properties and the bioactive compounds responsible for the activities should be elucidated.

3.2. Identified Bioactive Compounds in LC-MS/MS

A total of 74 compounds were identified (59 in positive mode and 15 in negative mode) in the aqueous extract of LM using the MS/MS spectra (Tables 1 and 2). A few important classes of bioactive compounds such as flavonoids, phenolics, terpenoids, steroids, alkaloids, and vitamin B were detected in the extract of LM. Out of the 74 compounds, 13

flavonoids, 12 phenolic, and 6 terpenoids were detected as major contributors to the antioxidant activity and pharmacological functions of LM. The remaining classes of compounds such as fatty acids, aromatic compounds, amino acids, lactones, and heterocyclic ketones also partly contributed to the medicinal value of LM since some of the compounds were reported to possess pharmacological properties [7].

Table 1. Compounds identified in LM aqueous extract by positive mode of analysis.

Name	R. Time (min)	Formula	Mass Error (ppm)	Calc. Molecular Mass	Database	Matching Score (MzCloud)/ FISh Score (Chemspider)	Class
Pyroglutamic acid	1.04	$C_5H_7NO_3$	0.56	129.0427	mzCloud	95.6	Amino acid
Succinylproline	4.79	$C_9H_{13}NO_5$	0.45	215.0795	ChemSpider	50.0	Amino acid
3-Indoleacrylic acid	6.35	$C_{11}H_9NO_2$	0.15	187.0634	mzCloud	94.3	Aromatic
Adicardin	4.88	$C_{20}H_{24}O_{12}$	0.26	456.1269	mzCloud	91.7	Aromatic
Phenylpropiolic acid	5.64	$C_9H_6O_2$	−0.86	146.0367	ChemSpider	50.0	Aromatic
9S,13R-12-Oxophytodienoic acid	8.13	$C_{18}H_{28}O_3$	−0.84	292.2036	mzCloud	92.1	Cyclic ketone
Jasmonic acid	8.51	$C_{12}H_{18}O_3$	−0.28	210.1255	ChemSpider	66.3	Cyclic ketone
Maltol	3.74	$C_6H_6O_3$	0.85	126.0318	mzCloud	99.3	Cyclic ketone
Vomifoliol	4.97	$C_{13}H_{20}O_3$	−0.44	224.1412	ChemSpider	69.9	Cyclic ketone
(12S)-12-Hydroxy-16-heptadecynoic acid	10.81	$C_{17}H_{30}O_3$	−0.10	282.2195	ChemSpider	53.9	Fatty acyl
(2E)-6-Hydroxy-2,6-dimethyl-2,7-octadienoic acid	5.09	$C_{10}H_{16}O_3$	0.48	184.1100	ChemSpider	54.6	Fatty acyl
1-[(2-Hydroxyethyl)amino]-2-dodecanol	21.44	$C_{14}H_{31}NO_2$	−0.33	245.2354	ChemSpider	55.6	Fatty acyl
11-Methoxy-3,7,11-trimethyl-2,4-dodecadienoic acid	8.43	$C_{16}H_{28}O_3$	−0.12	268.2038	ChemSpider	65.9	Fatty acyl
13-Hydroxy-9,11,15-octadecatrienoic acid	8.52	$C_{18}H_{30}O_3$	−0.40	294.2194	mzCloud	85.4	Fatty acyl
4-Oxo-dodecanedioic acid	6.44	$C_{12}H_{20}O_5$	−0.52	244.1310	mzCloud	80.2	Fatty acyl
Arachidonic acid	7.01	$C_{20}H_{32}O_2$	−0.98	304.2399	ChemSpider	90.5	Fatty acyl
Levulinic acid	1.10	$C_5H_8O_3$	1.96	116.0476	ChemSpider	62.5	Fatty acyl
Palmitoleyl oleate	20.98	$C_{34}H_{64}O_2$	−0.03	504.4906	ChemSpider	85.8	Fatty acyl
Parinaric acid	11.45	$C_{18}H_{28}O_2$	−0.74	276.2087	ChemSpider	85.6	Fatty acyl
Traumatin	5.76	$C_{12}H_{20}O_3$	−0.11	212.1412	ChemSpider	56.4	Fatty acyl
2-(3,4-Dihydroxyphenyl)-5,7-dihydroxy-4-oxo-4H-chromen-3-yl 6-O-(carboxyacetyl)-β-D-threo-hexopyranoside	5.84	$C_{24}H_{22}O_{15}$	0.78	550.0963	mzCloud	99.0	Flavonoid
Kaempferol	5.35	$C_{15}H_{10}O_6$	−1.04	286.0474	mzCloud	98.2	Flavonoid

Table 1. *Cont.*

Name	R. Time (min)	Formula	Mass Error (ppm)	Calc. Molecular Mass	Database	Matching Score (MzCloud)/ FISh Score (Chemspider)	Class
Luteolin 7-O-malonylglucoside	6.22	$C_{24}H_{22}O_{14}$	0.31	534.1011	ChemSpider	62.5	Flavonoid
Nicotiflorin	5.85	$C_{27}H_{30}O_{15}$	0.45	594.1587	mzCloud	99.4	Flavonoid
Quercetin	5.07	$C_{15}H_{10}O_7$	−1.22	302.0423	mzCloud	99.4	Flavonoid
Quercetin 3-O-rhamnoside-7-O-glucoside	5.54	$C_{27}H_{30}O_{16}$	−0.33	610.1532	mzCloud	98.2	Flavonoid
Quercetin-3β-D-glucoside	5.67	$C_{21}H_{20}O_{12}$	−0.33	464.0953	mzCloud	96.8	Flavonoid
Robinin	5.48	$C_{33}H_{40}O_{19}$	0.40	740.2167	mzCloud	97.2	Flavonoid
Trifolin	5.99	$C_{21}H_{20}O_{11}$	−0.55	448.1003	mzCloud	98.2	Flavonoid
Adenine	2.15	$C_5H_5N_5$	0.08	135.0545	mzCloud	89.3	Heterocyclic
Adenosine	2.15	$C_{10}H_{13}N_5O_4$	−0.21	267.0967	mzCloud	99.8	Heterocyclic
Coniine	21.44	$C_8H_{17}N$	−0.04	127.1361	ChemSpider	52.9	Heterocyclic
Guanine	21.56	$C_5H_5N_5O$	−0.30	151.0494	mzCloud	95.4	Heterocyclic
(3S,4R,5R,6R)-6-[(4R)-2,2-Dimethyl-1,3-dioxolan-4-yl]-3,4-dihydroxy-5-methyltetrahydro-2H-pyran-2-one	2.94	$C_{11}H_{18}O_6$	−1.05	246.1101	ChemSpider	52.4	Lactone
Massoilactone	5.89	$C_{10}H_{16}O_2$	−0.41	168.1150	ChemSpider	65.0	Lactone
Albocyclin	6.53	$C_{18}H_{28}O_4$	−0.53	308.1986	ChemSpider	61.1	Macrolide
Rustmicin	6.48	$C_{21}H_{32}O_6$	−1.59	380.2193	ChemSpider	52.2	Macrolide
1-(4-Hydroxy-3-methoxyphenyl)-5-methoxy-3-decanone	6.77	$C_{18}H_{28}O_4$	−1.03	308.1984	ChemSpider	69.1	Phenolic
1-(4-Hydroxyphenyl)-1-heptanone	4.71	$C_{13}H_{18}O_2$	0.21	206.1307	ChemSpider	52.6	Phenolic
1-Caffeoyl-β-D-glucose	2.22	$C_{15}H_{18}O_9$	−1.26	342.0947	ChemSpider	76.2	Phenolic
3,4-Dihydroxybenzaldehyde	3.98	$C_7H_6O_3$	−0.44	138.0316	mzCloud	84.6	Phenolic
Caffeic acid	4.10	$C_9H_8O_4$	−0.62	180.0422	mzCloud	99.2	Phenolic
Coniferol	5.08	$C_{10}H_{12}O_3$	−0.16	180.0786	ChemSpider	53.8	Phenolic
Conocarpin	4.90	$C_{15}H_{16}O_8$	−1.03	324.0842	ChemSpider	75.0	Phenolic
Demethoxycurcumin	6.55	$C_{20}H_{18}O_5$	−1.50	338.1149	ChemSpider	62.9	Phenolic
Esculin	3.56	$C_{15}H_{16}O_9$	−0.83	340.0792	mzCloud	87.1	Phenolic
Paradol	9.85	$C_{17}H_{26}O_3$	−0.24	278.1881	ChemSpider	59.5	Phenolic
Shogaol	9.59	$C_{17}H_{24}O_3$	−1.81	276.1720	ChemSpider	64.8	Phenolic
Steroidal compound	19.77	$C_{29}H_{46}O$	−0.65	410.3546	ChemSpider	62.3	Steroid
Steroidal compound	8.07	$C_{18}H_{26}O_2$	−0.98	274.1930	mzCloud	93.8	Steroid
(3R)-Hydroxy-beta-ionone	4.78	$C_{13}H_{20}O_2$	−0.06	208.1463	ChemSpider	76.9	Terpenoid
Caryophyllene oxide	6.69	$C_{15}H_{24}O$	−0.85	220.1825	mzCloud	80.2	Terpenoid
Costunolide	5.99	$C_{15}H_{20}O_2$	−1.17	232.1461	mzCloud	85.3	Terpenoid

Table 1. *Cont.*

Name	R. Time (min)	Formula	Mass Error (ppm)	Calc. Molecular Mass	Database	Matching Score (MzCloud)/ FISh Score (Chemspider)	Class
Fencibutirol	8.12	$C_{16}H_{22}O_3$	−0.76	262.1567	ChemSpider	70.9	Terpenoid
Nootkatone	10.09	$C_{15}H_{22}O$	0.09	218.1671	mzCloud	81.3	Terpenoid
Perillic acid	5.56	$C_{10}H_{14}O_2$	−0.54	166.0993	ChemSpider	54.0	Terpenoid
Nicotinamide	1.05	$C_6H_6N_2O$	1.13	122.0482	mzCloud	97.4	Vitamin B
Pantothenic acid	3.50	$C_9H_{17}NO_5$	−0.06	219.1107	ChemSpider	95.2	Vitamin B
Pyridoxine	1.01	$C_8H_{11}NO_3$	−0.14	169.0739	mzCloud	96.2	Vitamin B

Table 2. Compounds identified in LM aqueous extract by negative mode of analysis.

Name	R. Time (min)	Formula	Mass Error (ppm)	Calc. Molecular Mass	Database	Matching Score (MzCloud)/ FISh Score (Chemspider)	Class
Melilotoside	4.71	$C_{15}H_{18}O_8$	0.65	326.1004	ChemSpider	65.4	Aromatic
N-Acetyl-L-phenylalanine	5.20	$C_{11}H_{13}NO_3$	−1.73	207.0892	mzCloud	87.4	Aromatic
(15Z)-9,12,13-Trihydroxy-15-octadecenoic acid	8.53	$C_{18}H_{34}O_5$	0.53	330.2408	mzCloud	88.3	Fatty acyl
12,13-Dihydroxyoctadec-9-enoic acid	10.75	$C_{18}H_{34}O_4$	0.20	314.2458	mzCloud	90.0	Fatty acyl
13-Hydroxy-9,11,15-octadecatrienoic acid	11.46	$C_{18}H_{30}O_3$	0.83	294.2197	mzCloud	89.4	Fatty acyl
13-Hydroxy-9,11-octadecadienoic acid	12.06	$C_{18}H_{32}O_3$	0.33	296.2352	mzCloud	85.5	Fatty acyl
16-Hydroxyhexadecanoic acid	14.12	$C_{16}H_{32}O_3$	0.36	272.2352	mzCloud	87.5	Fatty acyl
9-Hydroperoxy-10,12-octadecadienoic acid	10.22	$C_{18}H_{32}O_4$	0.67	312.2303	mzCloud	87.8	Fatty acyl
Corchorifatty acid F	8.09	$C_{18}H_{32}O_5$	0.70	328.2252	mzCloud	97.9	Fatty acyl
Dodecanedioic acid	6.60	$C_{12}H_{22}O_4$	−0.67	230.1517	mzCloud	95.7	Fatty acyl
5,7-Dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-3-yl 6-O-(6-deoxyhexopyranosyl) hexopyranoside	5.86	$C_{27}H_{30}O_{15}$	0.70	594.1589	mzCloud	88.0	Flavonoid
Astragalin	6.15	$C_{21}H_{20}O_{11}$	0.56	448.1008	mzCloud	96.4	Flavonoid
Rutin	5.56	$C_{27}H_{30}O_{16}$	0.72	610.1538	mzCloud	83.4	Flavonoid
Trifolin	6.00	$C_{21}H_{20}O_{11}$	0.90	448.1010	mzCloud	95.4	Flavonoid
1-Caffeoyl-β-D-glucose	4.30	$C_{15}H_{18}O_9$	0.08	342.0951	ChemSpider	69.8	Phenolic

Our previous study on the hepatoprotective and immunosuppressive effects of LM aqueous extract reported the ability of LM to reverse the effect of CCl_4 administration in rats and demonstrated the immunosuppressive effect on proinflammatory cytokines and oxidative stress markers [6]. The bioactive compounds identified in LM aqueous extract that could potentially be responsible for the hepatoprotective and anti-inflammatory effects are jasmonic acid [14], maltol [15,16], kaempferol [17,18], luteolin 7-O-malonylglucoside [19], nicotiflorin [20,21], quercetin [22,23], robinin [24], trifolin [25], adenine [26], adenosine [27,28], coniine [29], guanine [30], massoilactone [31], caffeic acid [32,33], coniferol [34], demethoxycurcumin [35], esculin [36,37], paradol [38], shogaol [39,40], (3R)-hydroxy-beta-ionone [41], caryophyllene oxide [42,43], costunolide [44,45], nootkatone [46,47], nicotinamide [48,49], pantothenic acid [50], pyridoxine [51,52], astragalin [53,54], and rutin [55,56].

Two unknown steroidal compounds were also detected in the aqueous extract of LM but were not identified since the identification of underivatized steroidal compounds via tandem mass spectrometry is impossible and any putative identity could be misleading due to their stable four-ring skeleton and diverse stereoisomerisms [57]. Kuncoro et al. [58] reported two new steroidal compounds in a methanol extract of LM, stigmast-5 (6)-en-3β-ol and stigmast-4-en-3-one, identified using NMR spectroscopy. Hence, the two unidentified steroidal compounds detected in MS/MS analysis could be the same as those reported earlier or their derivatives, as the suggested molecular formulas of the compounds are almost the same.

3.3. Identification of Metabolites in GC-MS

The aqueous extract of LM was subjected to GC-MS analysis to screen volatile bioactive compounds that might also be responsible for the pharmacological properties of this plant. The compounds identified are listed in Table 3. The identified bioactive compounds from different classes such as cyclic aldehyde (5-hydroxymethylfurfural), phenolic acid (p-coumaric acid), and fatty acyls (E-15-heptadecenal, n-hexadecanoic acid) were reported to possess pharmacological properties such as anti-inflammatory, anticancer, and antimicrobial effects [59–62]. The detected volatile compounds could have exerted synergistic effects to mitigate oxidative stress by enhancing the antioxidant and immunosuppressive properties of LM. GC-MS analysis was performed to identify the volatile compounds that could have been missed in the LC-MS/MS analysis. LM leaves exerted a strong aroma while being boiled for extraction, indicating the presence of essential oils and other volatile aromatic compounds in the leaves. Hence, the leaves of LM can be subjected to essential oil extraction and organic solvent extracts for a future direction to elucidate the volatile compounds present using GC-MS analysis since most of the thermolabile compounds would be lost due to high temperature during boiling for aqueous extract.

Table 3. Compounds identified in the aqueous extract of LM by GC-MS.

No.	Compound Name	Molecular Formula	Molecular Weight	Area (%)	RT
1.	Furfural	$C_5H_4O_2$	96.08	0.72	7.4391
2.	2-furanmethanol	$C_5H_6O_2$	98.09	0.82	7.9820
3.	2-furancarboxaldehyde, 5-methyl-	$C_6H_6O_2$	110.11	0.16	11.4392
4.	Benzyl alcohol	C_7H_8O	108.14	0.28	14.6725
5.	Acetic acid, phenylmethyl ester	$C_9H_{10}O_2$	150.17	0.20	20.7917
6.	5-hydroxymethylfurfural	$C_6H_6O_3$	126.11	8.24	24.4822
7.	p-Coumaric acid	$C_9H_8O_3$	164.16	0.34	46.3873
8.	E-15-heptadecenal	$C_{17}H_{32}O$	252.44	0.08	50.2349
9.	n-Hexadecanoic acid	$C_{16}H_{32}O_2$	256.42	0.18	51.7683

3.4. Antibacterial Effect of LM

LM was tested against two Gram-positive bacteria (*S. aureus* and *B. cereus*) and two Gram-negative bacteria (*E. coli* and *Salmonella* sp.). LM showed antibacterial activity at high and low concentrations against *S. aureus* with 15 mm and 13 mm inhibition zones, respectively, but did not show activity against *B. cereus* (Table 4). This suggested certain Gram-positive bacteria species are susceptible to the phytochemicals present in the aqueous extract. LM was not able to inhibit the growth of the tested Gram-negative bacteria. There is a distinct difference between Gram-positive and Gram-negative bacterial cell walls. Gram-negative bacteria have three cell wall layers (outer membrane, peptidoglycan, and inner membrane), while Gram-positive bacteria have two cell wall layers (peptidoglycan and inner membrane) only. The extra outer membrane of Gram-negative bacteria is the main reason for their lower sensitivity to many antibacterial compounds [63]. The antibacterial effect of LM aqueous extract was further determined to estimate the MIC against *S. aureus* using the broth microdilution method. The results indicated LM required more than 6.25 mg/mL to achieve the MIC value. This value is considered low as compared to the positive control gentamicin that exhibited MIC at 2.5 µg/mL. The broth microdilution method used for the determination of MIC in LM aqueous extract showed the cloudy and intense color of the plant extract, which interfered with the MIC evaluation. Therefore, a disc diffusion test on the aqueous extract of LM at low concentration should be performed to determine the exact MIC value. However, the MIC value of LM was not strong enough to motivate the continuation of the experiment. Compounds with antioxidant properties are known to possess antimicrobial effects [64,65]. The bioactive compounds that could have contributed to the antibacterial activities of LM, apart from flavonoids, phenolics, and terpenoids, are fatty acyl groups and amino acids such as pyroglutamic acid [66], 3-indoleacrylic acid [67], trifolin [68], and arachidonic acid [69].

Table 4. Inhibition zone produced by LM against test bacteria.

		Inhibition Zone (mm)			
Samples	**Concentration (mg/mL)**	***S. aureus***	***B. cereus***	***E. coli***	***Salmonella* sp.**
LM	500	15.0 ± 3.1	-	-	-
	100	13.0 ± 1.7	-	-	-
Gentamicin	10 µg/disc	23.0 ± 0.6	25.0 ± 1.5	24.0 ± 1.0	28.0 ± 0.6

The negative control did not show a growth inhibition effect. Values are given as mean ± SD of three replicate samples.

4. Conclusions

Screening of bioactive compounds in the aqueous extract of LM using LC-MS/MS and GC-MS resulted in the successful identification of flavonoids, phenolics, terpenoids, amino acids, cyclic ketones, lactones, amino acids, fatty acyls, and aromatic compounds that contributed to the antioxidant, hepatoprotective, anti-inflammatory, and antibacterial activities of the plant. Almost half of the list of compounds identified were reported to have pharmacological properties. The high presence of flavonoids such as quercetin, kaempferol, trifolin, and rutin could be attributed to the efficient antioxidant, free radical scavenging, and antibacterial effects of LM. Therefore, the presence of important phytochemicals could be responsible for the medicinal properties of LM against diseases prevailing from oxidative stress. It can be suggested that organic solvent extracts of LM such as methanol, ethyl acetate, and hexane could potentially have additional bioactive compounds; therefore, studies should be directed to identify the metabolites in the extracts as well to evaluate their pharmacological properties on various diseases relating to oxidative stress.

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