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Studies on the phytochemical properties and antimicrobial activities of *in vitro* cultured *Lygodium microphyllum* (CAV.) R. BR.

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Abstract

Lygodium microphyllum, a perennial climbing fern from the *Lygodiaceae* family, is known for its various pharmacological benefits, mainly playing a role in enhanced digestion, increased energy levels, and boosted immunity due to its rich phytoconstituents. In phytochemical analysis, the UV-Vis analysis of *in vitro* cultured *Lygodium microphyllum* in ethanolic extract revealed the peaks at 238, 319, 398, 500, and 665 nm, indicating absorptions of 3.850, 4.000, 2.346, 0.176, and 0.411, respectively. These peaks signify the presence of carbonyl and nitroso groups within organic chromophores in the plant extract. Furthermore, the FTIR revealed the presence of carbonyls, alkynes, phenols, dialkyl groups, aromatic ethers, and aliphatic fluoro compounds. Analysis of GC-MS identified a total of 30 compounds. Regarding antibacterial activity, chloroform extract of *in vitro* cultured *Lygodium microphyllum* exhibited the highest inhibition rate recorded against the *Bacillus cereus* (19.23 ±2.12mm). Next to this the ethyl acetate extract of the fern shows significant inhibition against *Klebsiella pneumoniae* (19.00 ±1.48mm). However, in terms of antifungal activity, all tested fungal strains displayed minimal zone of inhibition.

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Introduction

Pteridophytes, which include ferns and fern-allies, represent the earliest vascular plants to have emerged on Earth, emerging in the midst of the Paleozoic era during the Silurian period approximately 438 million years ago. These plants marked a significant milestone in the evolution of vascular systems, possessing both xylem for water transportation and phloem for nutrient transport, earning them the designation of 'vascular cryptogams' (Dudani *et al.*, 2014). While recent studies in ethnobotany, phytochemistry, and pharmacology have highlighted the medicinal and pharmaceutical potential of numerous pteridophyte species, there remains a gap in the evaluation of certain species utilized by indigenous tribes for their pharmaceutical properties and the isolation of their active compounds (Pradeep Parihar and Leena Parihar, 2006).

Nowadays natural resources are facing serious threats; it is mainly because of the habitat loss, habitat degradation and increasing level of exploitation. Globally, 30% of the plant life faces threats. (Raven, 1997). A huge quantity of epiphytic and lithophytic ferns that have been destroyed. Because of the different types of deforestation activities in Western Ghats. In the Western Ghats, there are 44 fern species at risk of extinction, posing a significant conservation challenge for biologists (Manickam, 1995). *In vitro* propagation is a dynamic method of conservation, since the evolution of species continues in the same place in which the plant grows (Maxted *et al.*, 1997).

The researchers are developing more antimicrobial agents from the secondary metabolites of plants to avoid side effects, when correlate to its synthetic chemical counterparts (Rathee *et al.*, 2016). In worldwide many of the medicinally important plants have been screened for the potential of antimicrobial agents. The new diseases by microbes and the swift in the resistance to the drugs by various microbial strains prompted the researchers to create new drugs with the qualities to overcome these defenses. The pteridophytes possess the huge diversity in Indian medicinal plants and it shows that the plant extract for antibacterial activity is more useful for humans and plants diseases (Pradeep *et al.*, 2010). Considering these aspects, the objective of this study is to explore the phytochemical characteristics and antimicrobial efficiency of *in vitro* cultured *Lygodium microphyllum*.

Materials and methods

Phytochemical analysis of plant extracts

UV-Vis spectroscopy analysis

The *in vitro* cultured extracts of *Lygodium microphyllum* underwent UV-Vis spectroscopy analysis using a Perkin-Elmer Lambda 35 spectrophotometer to detect absorbance peaks. The spectrum was conducted within the range of 200-800nm.

Fourier Transform Infrared Spectroscopy (FTIR) analysis

The *in vitro* cultured extracts of *Lygodium microphyllum* underwent FTIR analysis using a Perkin-Elmer Spectrum Two instrument located in Germany. The wavelength of light absorbed is indicative of the chemical bonds present, as illustrated in the annotated spectrum. Interpreting the infrared absorption spectrum allows for the determination of chemical bonds within a molecule. The spectrum was conducted within the range of 4000 cm^-1 to 400 cm^-1 to assess the presence or absence of functional groups in the test sample.

Gas Chromatography Mass Spectroscopy (GC-MS) analysis

The *in vitro* cultured *Lygodium microphyllum* of ethanolic extract was analyzed using GC-MS to identify its chemical constituents. The instrument used was the GCMS-QP-2010 plus by SHIMADZU, Japan. Experimental conditions for the GCMS system were as follows: a capillary column Rtx-5MS with a length of 30.0 m, a thickness of 0.50 µm, and a diameter of 0.32 mm. The sample injection was conducted in split mode with a split ratio of 5.0. Helium served as the carrier gas with a total flow rate of 8.9 ml/min and a column flow rate of 0.99 ml/min.

The column oven temperature program began at 60˚C and ramped up to 250˚C with a 1-minute hold time. A 1µl volume of sample was injected at a temperature of 250˚C and a pressure of 56.7 KPa. The GC inlet ion source temperature was set to 230°C, while the interface temperature was set to 280°C. Solvent cut time was set at 3 minutes with a threshold level of 1000. The MS program conditions included a mass scan range from 50 to 800 m/z and a time duration of 3 to 48 minutes for mass spectrum detection. The results were compared using the Wiley8 and NIST11 spectral libraries, while Dr. Duke's Phytochemical and Ethanobotanical database was utilized for compound prediction.

Therapeutically studies of in vitro cultured fern extracts

Antimicrobial activity – Disc diffusion method

The antimicrobial activity of the test samples was assessed using method of disc diffusion. Specifically, the efficacy of the *in vitro* cultured fern extracts of *Lygodium microphyllum* was evaluated in this investigation. Microbial strains were procured from Joseph's Microbial Culture Collection (JMCC) at St. Joseph's College, Tiruchirappalli.

Antibacterial activity (Kora and Rastogi, 2013)

To assess the antibacterial activity, Nutrient agar medium was utilized. Gram-positive bacteria, including *Staphylococcus lentus* and *Bacillus cereus*, as well as gram-negative bacteria, such as *Escherichia coli* and *Klebsiella pneumoniae*, were employed as the test organisms. Discs with a diameter of 0.6 cm were impregnated with the crude plant extract for a duration of 3 days. For the antibacterial assay, 10 ml of sterile Nutrient Agar medium were poured into each petri plates, which were then maintained under aseptic conditions for solidification. Subsequently, the bacterial test strains were inoculated onto the surface of the medium using sterile cotton swabs. Following inoculation, the soaked discs were placed on the medium surface. The plates were then incubated at 37˚C for 24 hours. After the incubation period, the zones of inhibition were measured. Gentamicin readymade discs served as positive control.

Antifungal activity (Parveen et al., 2012)

To assess the antifungal activity, Potato Dextrose Agar medium was employed. Four different fungal strains, including *Aspergillus flavus*, *Candida albicans*, and *Mucor* sps. were utilized to test the antifungal properties of the plant extracts. The fungal test strains were inoculated onto the surface of the medium using sterile cotton swabs. Subsequently, discs impregnated with the plant extract were placed onto the medium surface. Following this, the plates were further incubated at 27°C for an additional 48 hours. Upon completion of the incubation period, the zones of inhibition were measured. Nystatin was used as a positive control.

Results and discussion

UV-Vis analysis of Lygodium microphyllum

The UV-Vis spectrum was analysed to identify phytoconstituents in the ethanolic extract of *in vitro* cultured *Lygodium microphyllum*. The examination covered a range of 200 – 800 nm, revealing peaks at 238, 319, 398, 500, and 665 nm with corresponding absorptions of 3.850, 4.000, 2.346, 0.176, and 0.411. The spectrum indicates the presence of carbonyl and nitroso group of organic chromophores in the tested plant extract. A qualitative UV-VIS profile of the methanolic extract of *Mentha spicata* was carried out across wavelengths ranging from 300 nm to 800 nm. The profile revealed peaks at 353, 407, 504, 535, 609, and 665 nm, accompanied by absorptions of 1.309, 1.463, 0.1, 0.066, 0.108, and 0.625, respectively, as documented by (Jain *et al.*, 2016).

Fourier Transform Infrared (FTIR) spectroscopy analysis of Lygodium microphyllum

FTIR analysis performed to study the presence of functional groups in the plant material. FTIR spectrum for the ethanolic extract of *in vitro* cultured *Lygodium microphyllum* shows the peak at 3409.53 cm^{-1} represents the O-H stretch of alcohol. 2132.04 and 1923.98 cm^{-1} were assigned to have C≡C and M-C stretch in the vibration of alkyne &carbonyls respectively. The peak at 1923 cm^{-1} shows the

combination bond or transition metal carbonys. The peaks 1648 & 1451 cm^{-1} were assigned to have C=C and C-H stretch of alkenyl and methylene groups. The peak at 1385 and 1332 cm^{-1} represent the phenol and dialkyl groups. The peak in the regions of 1393 & 1383 cm^{-1} shows OH bend of alcoholic group. The peaks observed in 1267, 1078 and 1049 cm^{-1} were assigned to have the stretches of aryl-O, C-O and C-F with the functional group of aromatic ether, ethers and aliphatic fluoro compounds repectively. 880, 688 and 437 cm^{-1} were represent the C-O-O, C-Br and S-S stretches respectively.

Table 1. UltraViolet – Visible (UV-Vis) Spectrum of ethanolic extract of *Lygodium microphyllum*.

S.No	Wave length	Absorbance
1	238	3.850
2	319	4.000
3	398	2.346
4	500	0.176
5	665	0.411

Table 2. FTIR Spectrum of ethanolic extract of *Lygodium microphyllum*.

Frequency in cm−1 (Intensity*)	Bond	Functional Group
3409.53	O-H	Alcohol
2976.35	N-H	Amine
2901.52	C-H	Alkane
2132.04	C≡C	Alkyne
1923.98	M-C	Carbonyls
1648.05	C=C	Alkenyl
1451.10	C-H	Methylene
1385.86	O-H	Phenol
1332.09	S-O	Dialkyl
1267.79	aryl-O	Aromatic ethers
1078.49	C-O	Ethers
1049.47	C-F	Aliphatic fluoro compounds
880.57	C-O-O	Peroxides
688.99	C-Br	Aliphatic bromo compounds
437.38	S-S	Aryl disulfides

GC-MS analysis of Lygodium microphyllum

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was performed to identify the chemical constituents within the plant extract. The results table of the GC-MS analysis includes information such as the retention time (RT) of the compound, molecular formula, molecular weight (MW), height (%), peak area (%), and the name of the compound.

The GC-MS spectrum of in vitro cultured Lygodium microphyllum reveals peaks in descending order from the highest to lowest with various retention times. The identified compounds were Benzoic Acid, 2,4-Bis (Trimethylsiloxy)-, Trimethylsilyl Ester, etramethyl hexadec -1-En-3-Ol, 6(E),9(Z),13(E) - Pendectriene, Ethyl Ester Of 6-Hydroxyhexanoic acid, Cyclononasiloxane, Octadecamethyl, Cosamethyl cyclodecasiloxane, Icosa methyl cyclodecasiloxane, Octadecanoic Acid, 2-Hydroxy-1- (Hydroxymethyl) Ethyl Ester, Cyclononasiloxane, Octadecamethyl, Cyclononasiloxane, Octadecamethyl, 3 - [6'-[(Trimethylsilyl) Ethynyl] Bicyclo [2.2.1] Hepten-5'-Yl] Cyclopentanone, 1.Alpha., 4.Alpha., 4a.Alpha., 10a.Beta, 1, 4, 4a, 5, 6, 7, 8, 9, 10, 10a Decahydro -'1, 4, 11, 11- Tetramethyl-1, 4-Methanocycloocta [D] Pyridaz, Phenanthrene, 9- (2-Phenylethyl),

(3r*,1's*,4'r*,5'r*,6'r*) -3- [6'- [(Trimethylsilyl) Ethynyl] Bicyclo [2.2.1] Hepten-5'-Yl] Cyclopentanone, Cyclononasiloxane, Octadecamethyl and they were observed at 40.00 minutes of retention time. Table 3 provides details about the compounds present in *Lygodium microphyllum* extracts.

Table 3. GC-MS spectrum of ethanolic extract of *Lygodium microphyllum*.

Peak#	R.Time	Compound Name	Molecular formula	Molecular weight (MW)	Area%	Height %
1	6.77	Acetophenone, 2-(allyloxy)-	$C_{11}H_{12}O_2$	176.21	0.39	27.58
2	6.846	1-methylbutyl nitrite	$C_5H_{11}NO_2$	117.14	0.59	5.38
3	8.409	Ethyl benzoyl pyruvate	$C_{12}H_{12}O_4$		2.17	5.04
4	9.168	Disulfide, dioctyl	$C_{16}H_{34}S_2$	290.57	0.6	7.7
5	13.902	Cyclohexasiloxane, dodecamethyl-	$C_{12}H_{36}O_6Si_6$	444.92	5.7	4.69
6	17.514	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris(trimethylsiloxy)tetrasiloxane	$C_{18}H_{52}O_7Si_7$	577.2	6.49	6.1
7	19.839	1,2-benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	222.24	8.23	4.38
8	19.923	1,2-benzenedicarboxylic acid, diethyl ester	$C_{12}H_{14}O_4$	222.24	17.88	3.26
9	20.75	Benzoic acid, 2,4-bis(trimethylsiloxy)-, trimethylsilyl ester	$C_{14}H_{24}O_4Si_2$	312.51	6.05	11.24
10	20.922	1-butanol, 3-(1-ethoxyethoxy)-, benzoate, (r*,s*)-(.+-.)-	$C_9H_{20}O_3$	176.25	0.93	3.54
11	23.547	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy)tetrasiloxane	$C_{16}H_{48}O_6Si_7$	533.15	4.63	4.24
12	24.501	Neophytadiene	$C_{20}H_{38}$	278.5	2.52	5.7
13	26.036	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-icosamethylcyclodecasiloxane	$C_{20}H_{60}O_{10}Si_{10}$	741.53	3.22	4.29
14	26.467	Furo[2,3-c]pyridine, 2,3-dihydro-2,7-dimethyl-	$C_9H_{11}NO$	149.19	0.35	3.58
15	27.096	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284.47	7.64	3.28
16	28.308	Benzoic acid, 2,4-bis(trimethylsiloxy)-, trimethylsilyl ester	$C_{14}H_{24}O_4Si_2$	312.51	3.46	27.58
17	28.921	3,7,11,15-tetramethylhexadec-1-en-3-ol	$C_{20}H_{40}O$	296.5	0.95	5.38
18	29.764	6(e),9(z),13(e)-pendectriene	$C_{15}H_{26}$	-	2.09	5.04
19	30.172	Ethyl ester of 6-hydroxyhexanoic acid	$C_8H_{16}O_3$	160.21	0.48	7.7
20	30.37	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.38	4.06	4.69
21	32.275	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-icosamethylcyclodecasiloxane	$C_{20}H_{60}O_{10}Si_{10}$	741.53	3.58	6.1
22	34.07	2,2,4,4,6,6,8,8,10,10,12,12,14,14,16,16,18,18,20,20-icosamethylcyclodecasiloxane	$C_{20}H_{60}O_{10}Si_{10}$	741.53	4.48	4.38
23	34.538	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	$C_{21}H_{40}O_4$	356.5	0.57	3.26
24	35.763	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.38	3.82	11.24
25	37.344	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.38	3.27	3.54
26	38.317	(3r*,1's*,4'r*,5'r*,6'r*)-3-[6'-[(trimethylsilyl)ethynyl]bicyclo[2.2.1]hepten-5'-yl]cyclopentanone	$C_{10}H_{18}$	138.25	0.89	4.24
27	38.375	(1.alpha.,4.alpha.,4a.alpha.,10a.beta.)-1,4,4a,5,6,7,8,9,10,10a-decahydro-'1,4,11,11-tetramethyl-1,4-methanocycloocta[d]pyridaz	$C_{15}H_{26}N_2$	234.39	0.5	5.7
28	38.435	Phenanthrene, 9-(2-phenylethyl)	$C_{22}H_{16}$	280.36	0.23	4.29
29	38.465	(3r*,1's*,4'r*,5'r*,6'r*)-3-[6'-[(trimethylsilyl)ethynyl]bicyclo[2.2.1]hepten-5'-yl]cyclopentanone	$C_{10}H_{18}$	138.25	0.98	3.58
30	38.846	Cyclononasiloxane, octadecamethyl-	$C_{18}H_{54}O_9Si_9$	667.38	3.25	3.28

Table 4. Antibacterial Activity of *Lygodium microphyllum*.

Organism	Diameter of inhibition zone* (mm)			
	Ethyl acetate extract	Chloroform extract	Ethanolic extract	Control#
Gram-positive bacteria:				
Bacillus cereus	05.00±1.32	19.23±2.12	04.00±1.14	31
Staphylococcus lentus	10.33±2.13	09.00±2.35	09.66±1.14	37
Gram-negative bacteria:				
Escherichia coli	04.00±2.26	07.33±1.24	05.00±1.23	30
Klebsiella pneumoniae	19.00±1.48	17.00±2.13	07.66±2.32	39

* Mean of triplicate
± Standard Deviation
Gentamycin.

Therapeutical studies of in vitro cultured fern extracts

Antibacterial Activity of in vitro cultured Lygodium microphyllum

For the antibacterial activity assessment, Nutrient agar was employed to prepare the medium. The antibacterial efficacy of *in vitro* cultured *Lygodium microphyllum* exhibited a higher inhibition rate compared to chloroform extracts. The maximum inhibition rate was observed with *Bacillus cereus* (19.23±2.12mm) in the chloroform extract. Following this, the ethyl acetate extract (19.00±1.48mm) displayed the highest inhibition against *Klebsiella pneumoniae*.

Table 5. Antifungal Activity of *Lygodium microphyllum*.

Organism	Diameter of inhibition zone* (mm)			
	Ethyl acetate extract	Chloroform extract	Ethanolic extract	Control#
Candida albicans	04.66±1.12	02.33±1.15	02.66±2.22	27
Aspergillus flavus	-	-	-	25
Mucor sps	02.33±1.21	03.00±1.14	05.33±1.24	27

* Mean of triplicate

± Standard Deviation

Nystatin

The chloroform extract of *in vitro* cultured fern exhibited the maximum zone of inhibition against all tested strains. Conversely, the ethanolic extract of *in vitro* cultured fern displayed the lowest zone of inhibition against *Bacillus cereus*. According to Jeeshna (2017), the chloroform extract of the sporophyll type showed bacterial activity against *Klebsiella pneumoniae* (8.2 mm).

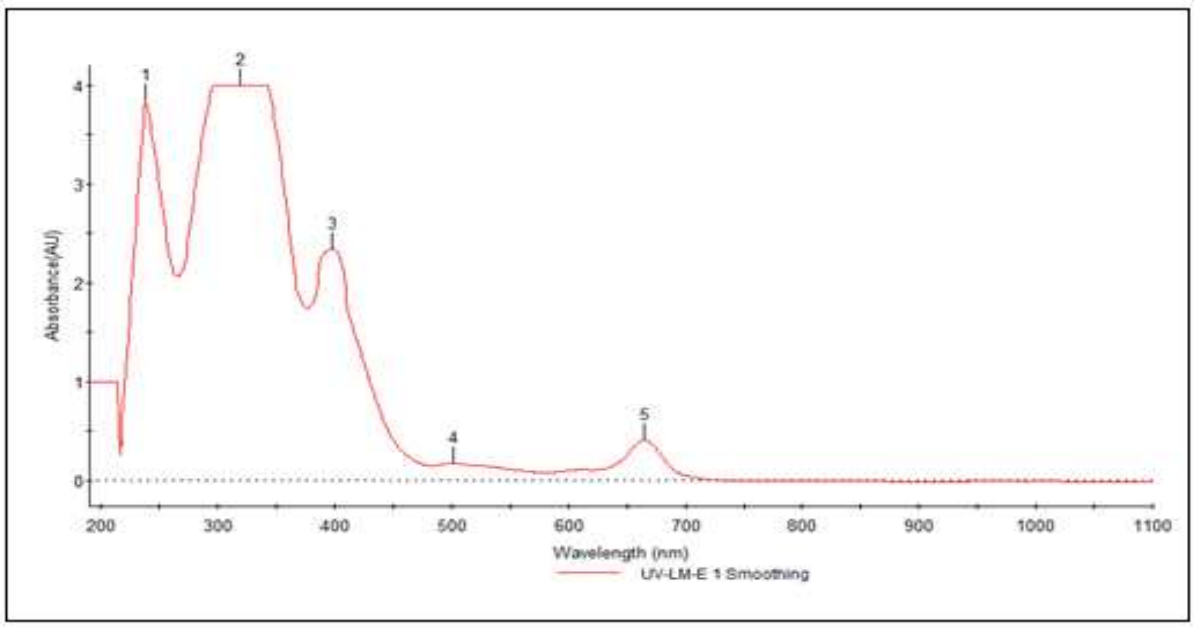

Fig. 1. UltraViolet – Visible (UV-Vis) Spectrum of ethanolic extract of *microphyllum*.

Antifungal activity of in vitro cultured Lygodium microphyllum

In the antifungal activity assessment, the *in vitro* cultured fern extracts of *Lygodium microphyllum* exhibited minimal zone of inhibition against all tested fungal strains. Among them, the ethanolic extract (05.33±1.24mm) demonstrated the highest inhibition of growth against *Mucor* spp. Following this, the ethyl

acetate extract exhibited the maximum level of inhibition zone against *Candida albicans* (04.66±1.12mm). However, none of the three extracts from *in vitro* cultured *Lygodium microphyllum* showed any zone of inhibition against *Aspergillus flavus*.

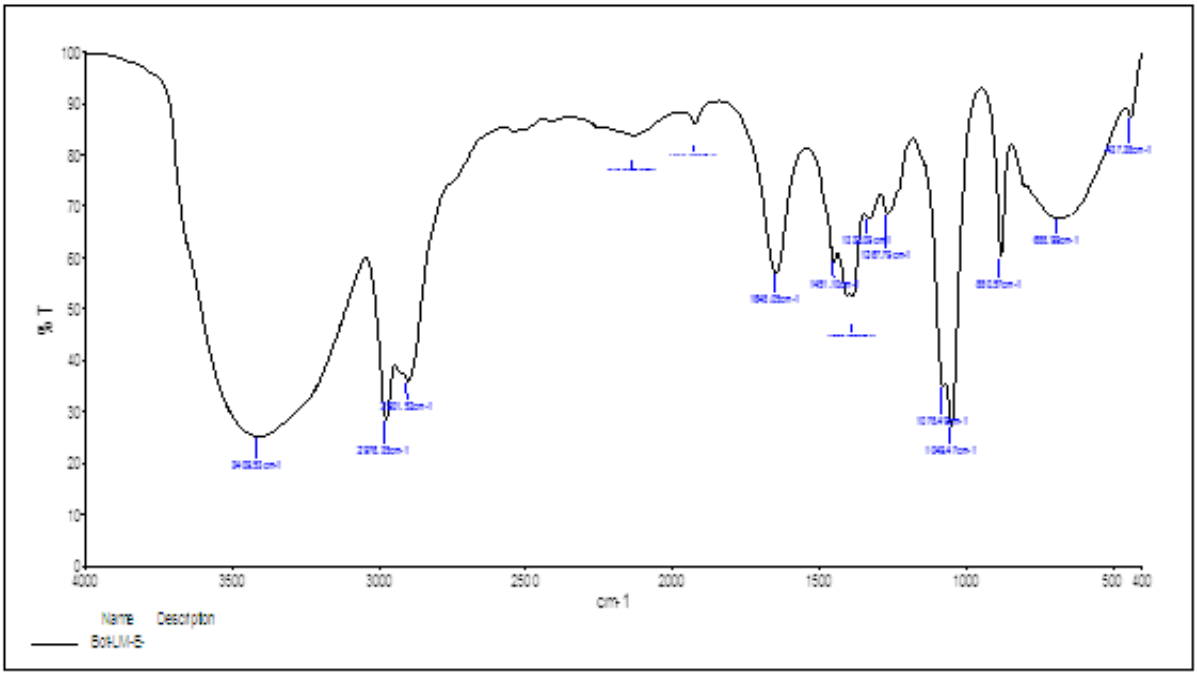

Fig. 2. FTIR Spectrum of ethanolic extract of *Lygodium microphyllum*.

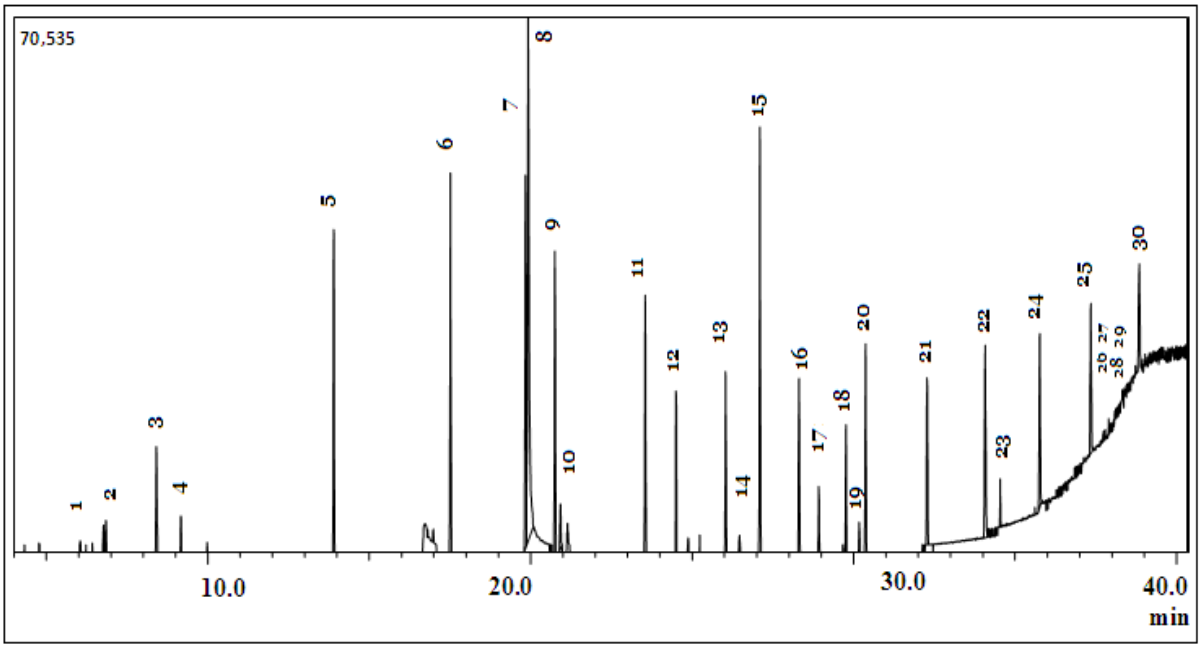

Fig. 3. GC-MS Spectrum of ethanolic extract of *Lygodium microphyllum*.

Conclusion

The phytochemical analysis revealed the presence of numerous medically significant phytoconstituents in the tested *in vitro* cultured *Lygodium microphyllum* plant extract. The tested plant, *Lygodium microphyllum*, exhibited a broad spectrum of inhibitory activity against all tested pathogens, with the exception of *Aspergillus flavus*. These findings suggest that *Lygodium microphyllum* have proved that it has the potential to develop new antimicrobial

agents. The potential of creating antimicrobials derived from lower group of plants seems promising, as it could result in the development of phytomedicine, which is effective against pathogenic microbes. Plant-based antimicrobials offer substantial therapeutic potential, presenting an option with fewer side effects compared to those commonly linked with synthetic antimicrobials.

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