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Research Article

Evaluation of Anti-Bacterial and Anti-Biofilm Efficacy of *Lygodium flexuosum* (L.) Sw.: An *in vitro* Study

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ABSTRACT

Aim: The present study explores preliminary phytochemical analysis, anti-bacterial, and anti-biofilm activities of the leaf methanolic extract of *Lygodium flexuosum*. **Materials and Methods:** Soxhlet extraction was done on the powdered leaves of *L. flexuosum* using methanol as solvent. The secondary metabolites in this extracts was checked following standard protocols. Further, antibacterial activity was assessed against both gram positive and negative bacteria through disc diffusion methods. The activity of methanolic extract against biofilms of the pathogens with the best anti-bacterial activity was done using a modified crystal violet staining method. **Results:** The preliminary phytochemical study revealed the presence of secondary metabolites like alkaloids, flavonoids, tannins etc which has immense therapeutic applications. The results of anti-bacterial study showed that, the maximum zone of inhibition in methanolic leaf extract of *L. flexuosum* was shown against the gram-negative bacteria *Xanthomonas campestris* and in the gram-positive bacteria *Listeria monocytogenes*. Further, these two strains were used to assess the anti-biofilm assay. The activity was best shown against the biofilm of bacteria *X. campestris* in comparison with *L. monocytogenes*. The best reduction in biofilm of these bacteria was observed in higher concentrations of extract that is, 100 and 200 mg/mL. **Conclusion:** These results suggest that the methanolic extract of *Lygodium flexuosum* possesses strong antibacterial and anti-biofilm properties, attributed to its diverse phytochemicals. The study highlights the potential of this fern as a natural source of bioactive compounds that could serve as alternative treatments for managing bacterial infections and biofilm-associated complications.

Keywords: Antibacterial activity, Anti-Biofilm activity, *Lygodium flexuosum*, Methanolic Extract, Natural antimicrobials, Phytochemical analysis.

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INTRODUCTION

Since ancient times, medicinal plants have been integral to human health and healing practices. Studies on higher plants like angiosperms are mostly conducted for medicinal, ethnobotanical, economical, and agricultural properties. However, the medicinal significance of angiosperms far surpasses that of pteridophytes due to their chemical diversity, historical usage and extensive research. The disparity between medicinal uses of angiosperms and pteridophytes can be attributed to several factors, including the greater diversity of chemical compounds in angiosperms, their easier cultivation, and more extensive research and documentation of their therapeutic effects. As a result, angiosperms have become the cornerstone of complementary and allopathic medicine, whereas medicinal potential of pteridophytes remains relatively underexplored and less impactful in comparison. Healing potential of several pteridophytes are mentioned in various ancient literatures.[1] Tribal people worldwide use them to cure ailments such as dysentery, malaria, stomach aches, urinary disorders, burns, etc. They are also used in medicinal preparations such as Ayurveda, Homeopathy, and Unani.

The rise in anti-microbial resistant bacteria is a global problem of the current century and the medication of harmful infectious diseases is difficult at this point. Bacteria develop resistance to antibiotics through several mechanisms, including the production of enzymes that inactivate or alter the antibiotics, modifications to the target sites, and alterations in the cell membrane that block antibiotic uptake, allowing them to survive doses that were once lethal.[2] Microbial biofilms consist of sticky Extracellular Polymeric Substances (EPS) that enable microorganisms to adhere to biotic surfaces, such as host cells. These biofilm producing microorganisms cause repeated infections that can be fatal to biological systems.[3] To prevent these effects several plants including pteridophytes are being tested to find antibiofilm activity. *Lygodium flexuosum* (L.) Sw. belonging to the family *Schizaeaceae* is one such pteridophyte with profound medicinal uses including antibacterial activity.[4] The rhizomes and roots of this plant are widely used as ethnomedicines among the tribal

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communities of India.[5] The roots and leaf paste are useful in the treatment of jaundice and stomach ache. The rhizome of *L. flexuosum* is used to cure Sexually Transmitted Diseases (STDs) like gonorrhea, while the ash of the plant is used to treat herpes. The plant is also reported to have anti-fertility, analgesic, anti-proliferative and apoptotic activity in cancer cells.[6] Apart from this, *L. flexuosum* is also used as food and forage resources in Western Chitwan. All these properties are attributed to the immense phytoconstituents present in the plant. In the present work an attempt has been made to validate the anti-bacterial and anti-biofilm activity from the leaf methanolic extract of *L. flexuosum*.

MATERIALS AND METHODS

Collection and authentication of plant material

For the present study, clean and healthy aerial parts of *Lygodium flexuosum* were procured from Vattappara, Thiruvananthapuram, Kerala (8.588360 ^{0}N, 76.949738 ^{0}E). The identification and authentication of specimens were done by the Curator, Department of Botany, University of Kerala, Kariavattom.

Preparation of plant extract

Fresh leaves of *L. flexuosum* were procured, washed under tap water, further by distilled water and made into small pieces. These were shade dried and grounded to coarse powder using a mechanical grinder. The plant powder was kept in airtight bags, away from light, heat, moisture and with proper labelling for further analysis.

Preliminary phytochemical analysis

The phytochemical extraction was performed using polar solvent, methanol. For preparing methanolic plant extract. About 10 g of shade dried and coarse powdered plant material was extracted for a day with methanol in soxhlet apparatus. The extract was filtered using Whatman No.1 filter paper to remove all undissolved matter and allowed to evaporate. This concentrated extract was used for further analysis. The residue weighed and the percentage yield was calculated using the formula.

$$\text{Percentage yield} = \frac{\text{Weight of residue obtained}}{\text{Weight of ground sample}} \times 100$$

Qualitative analysis of Phytoconstituents

Preliminary phytochemical testing for the presence of various compounds was analysed using standard protocols of Harborne.[7]

Test for Alkaloids

Mayer`s Test: Few drops of Mayer`s reagent ($KgHgI_2$) was added to 100 µL of extract.

Test for Flavonoids

Alkaline reagent Test: To 200 µL of extract about 500 µL of 2% sodium hydroxide solution was added. It was then followed by the addition of 500 µL of diluted sulphuric acid.

Test for Steroids

Salkowski Test: Dissolve about 200 µL of plant extract in equal volume of concentrated sulphuric acid, added gently through the sides of the test tube.

Test for Phenol

Lead Acetate Test: To about 100 µL of extract 500 µL of 10% Lead Acetate solution was added.

Test for Carbohydrates

Molisch Test: 100 µL of extract was mixed with a few drops of Molisch reagent (alpha naphthol). Concentrated sulphuric acid was added along the side of the test tube.

Test for Saponins

Foam Test: 1 mL of distilled water was added to 200 µL of extract and shaken well for few min.

Anti-bacterial activity

Three species of gram-negative bacteria viz., *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (ATCC 27853), and *Xanthomonas campestris* (S3) and gram-positive bacteria viz., *Listeria monocytogenes* (S2), *Staphylococcus aureus* (MTCC 96), and *Bacillus subtilis* (MTCC 2511) were used to analyze antibacterial activity. The cultures were obtained from standard culture collections maintained at the Department of Biotechnology, University of Kerala, Thiruvananthapuram. The above-mentioned bacterial strains were grown in nutrient agar and nutrient broth media (HIMEDIA Laboratory Pvt. Ltd., Mumbai). The methanolic extract of *L. flexuosum* was tested for antibacterial activity by following disc diffusion method.[8] The antibiotic disc streptomycin (0.125 mg/mL) was used as positive control. The antimicrobial plates were incubated at 37°C for 24 hr without agitation. The zone of inhibition marking the antibacterial activity was noted in mm diameter.

Anti-biofilm activity

The potential of the plant extract to prevent initial bacterial cell attachment was investigated through the biofilm inhibition assay. Briefly, 20 µL aliquot of standardized concentration of cultures was added to 96-well microtiter plates and incubated at 37°C for 4 hr. Then the plates were removed from the incubator and 100 µL (varying concentrations) aliquots of test extracts were added. Make up the volume to 220 µL with nutrient broth in replicates into the wells of 96-well microtiter plates and then incubate further at 37°C for 24 hr without agitation. The organism containing 20

µL culture and 200 µL nutrient broth was used as the positive control. The biomass was quantified using crystal violet staining method.

Crystal violet staining assay

The 96-well microtiter plates were washed five times with sterile distilled water and allowed to air dry. Next, 220 µL of 1% crystal violet was added to each well, and the plates were incubated at room temperature for 15 min. After incubation, the wells were washed three times with sterile distilled water to remove any excess stain. Biofilm formation was visible as purple rings along the walls of the wells. To semi-quantitatively assess biofilm production, 220 µL of DMSO was added to dissolve the stain. A 100 µL portion of the resulting solution was transferred to a fresh sterile plate, and the absorbance was measured, and the percentage inhibition of biofilm was determined using the equation:

$$\text{Percentage (\%) Inhibition} = \frac{\text{OD Positive control} - \text{OD Experimental}}{\text{OD Negative control}} \times 100$$

Table 1: Results of the preliminary phytochemical analysis in leaf extract of *Lygodium flexuosum*.

Phytochemicals	Results
Alkaloids	+++
Steroids	+++
Carbohydrates	+++
Phenols	+++
Flavonoids	+++
Saponins	– – –

+++ Presence, --- Absence.

RESULTS

Preliminary phytochemical study

Qualitative tests were done to identify the presence of secondary metabolites. It was observed that steroids, phenols, flavonoids, alkaloids and carbohydrates were present in the leaf extract, but saponins were absent. The percentage yield was calculated as 15.1 µg/mL. The results of qualitative analysis of methanol leaf extract of *Lygodium flexuosum* are shown in Table 1.

Anti-bacterial activity

The anti-bacterial efficiency of leaf extracts of *Lygodium flexuosum* was tested using disc diffusion method. The test revealed that inhibitory effects were found in all bacterial strains used. The results of the antibacterial activity of methanol leaf extract of *Lygodium flexuosum* are summarised in Table 2. Among the bacteria tested, the maximum zone of inhibition in methanolic leaf extract of *L. flexuosum* was shown against the gram-negative bacteria *Xanthomonas campestris* and in the gram-positive bacteria *Listeria monocytogenes*. In gram positive bacteria *L. monocytogenes*, the methanolic leaf extract showed the zone of inhibition of 10 mm for 25 mg/mL, 12 mm for 50 mg/mL and 14 mm for 100 mg/mL (Figure 1a). In gram positive bacteria *B. subtilis*, the extract showed zone of inhibition of 7 mm for 25 mg/mL, 9 mm for 50 mg/mL and 12 mm for 100 mg/

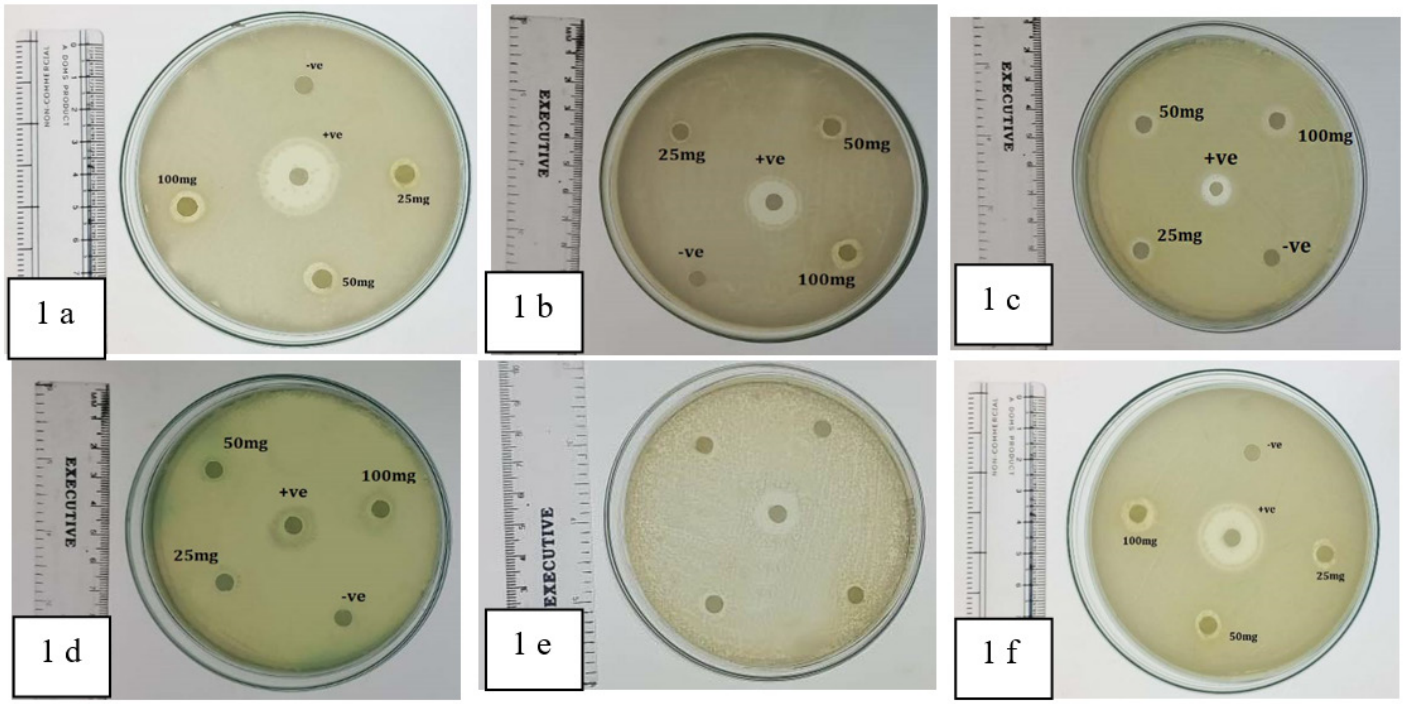

Figure 1: Diameter of zone of inhibition observed against different test bacteria of methanolic extract of *Lygodium flexuosum*. (1 a) *L. monocytogenes* (1 b) *B. subtilis* (1 c) *S. aureus* (1 d) *X. campestris* (1 e) *E. coli* (1 f) *P. aeruginosa*.

Table 2: Zone of inhibition of gram positive and gram-negative bacteria using different concentrations of methanolic leaf extract.

Concentration of extract	Zone of inhibition (mm in diameter)					
	L. monocytogenes	*X. campestris*	*E. coli*	*B. subtilis*	*P. aeruginosa*	*S. aureus*
Streptomycin (+ve control)	21	22	14	15	14	14
25 mg	10	11	8	7	7	7
50 mg	12	13	10	9	9	9
100 mg	14	15	12	12	12	12
0.05% DMSO (-ve control)	No Activity					

Table 3: Table showing the percentage inhibition of anti-biofilm activity of the *L. flexuosum*.

Concentration of extract	Percentage Inhibition by *X. campestris*	Percentage Inhibition by *L. monocytogenes*
12.5	7.52	3.44
25	17.26	9.57
50	22.12	18.18
100	33.63	29.19
200	36.72	33.49

mL (Figure 1b) and in *S. aureus*, the leaf extract showed zone of inhibition of 7 mm for 25 mg/mL, 9 mm for 50 mg/mL and 12 mm for 100 mg/mL (Figure 1c). In the gram-negative bacteria taken, *X. campestris,* the methanol leaf extract showed the zone of inhibition of 11 mm for 25 mg/mL, 13 mm for 50 mg/mL and 15 mm for 100 mg/mL (Figure 1d). In gram-negative bacteria *E. coli*, the extract showed zone of inhibition of 8 mm for 25 mg/mL, 10 mm for 50 mg/mL and 12 mm for 100 mg/mL (Figure 1e) and in *P. aeruginosa,* the leaf extract showed zone of inhibition of 7 mm for 25 mg/mL, 9 mm for 50 mg/mL and 12 mm for 100 mg/mL (Figure 1f). It was observed that the inhibitory action of *L. flexuosum* extract against gram-negative bacteria is more than that to gram positive bacteria and thus, the antibacterial activity of extract is more in gram negative bacteria.

Anti-biofilm activity

The anti-biofilm activity of methanol leaf extract of *L. flexuosum* was tested using microtiter plate biofilm assay. Gram-positive bacteria *Listeria monocytogenes* and gram-negative bacteria *Xanthomonas campestris* cultures were selected for testing the biofilm inhibition since the extract showed maximum antibacterial activity with these two bacteria (Table 3). Absorbance of test sample at 12.5, 25, 50, 100 and 200 mg/mL of extract in *X. campestris* bacterial culture was obtained ranging from 0.209 to 0.143. And the absorbance of test sample at 12.5, 25, 50, 100 and 200 mg/mL extract in *L. monocytogenes* bacterial culture was obtained ranging from 0.199 to 0.139. Biofilm formation inhibition results by addition of different concentrations of *Lygodium flexuosum* extract against gram-negative bacteria *Xanthomonas campestris* and gram-positive bacteria *Listeria monocytogenes* showed that the obtained effect was dose dependent. The anti-biofilm activity revealed that the inhibitory effect was found in both bacterial strains used. The activity was best shown against the biofilm of bacteria *X. campestris* in comparison with *L. monocytogenes*. The best reduction in biofilm of these bacteria was observed in higher concentrations of extract that is, 100 and 200 mg/mL. From this test results *Lygodium flexuosum* has the property for inhibiting biofilm formation.

DISCUSSION

Plants are well-known for producing a wide range of secondary metabolites, used in traditional medicine for centuries to treat various diseases. These metabolites in plant products are responsible for numerous biological activities in living organisms. The antimicrobial properties of many plant extracts are often attributed to these secondary metabolites.[9] Recently, the pharmaceutical and scientific communities have turned their attention to medicinal plants, with numerous studies documenting the therapeutic value of natural compounds, thereby validating their claimed biological activities.[10] Pteridophytes constitute a minor part in earth's vegetation except in special environments which are particularly suited to their vegetative and reproductive structures. They are an important phytogenetic bridge between lower and higher plants. They have been used by humans for centuries as ornamental plants, food, in handicrafts and most importantly as medicinal herbs. Medicinal properties of fern species were reported because of the presence of secondary metabolites and different bioactive compounds that could potentially cure many diseases.[11]

Lygodium flexuosum is a fern reported to have several phytochemicals and bioactive compounds which increase its medicinal importance.[12] Its n-hexane extract has been reported to have hepatoprotective action. Antifertility, antiproliferative and apoptotic activity in cancer cells adds up to the list of promising activities shown by it. It is a rich source of alkaloids, flavonoids, steroids, phenols, carbohydrates and antheridiogens. The main constitute of the plant is lygodinolide which is mainly used in wound healing. The phytochemicals present in leaf

extracts of *L. flexuosum* were analysed through various chemical tests. Methanol was used as the solvent for extraction, due to its high polarity which could produce high extraction yields.[13] Occasionally terpenoids will be found, but they are more often obtained by treatment with less polar solvents.[14] The inhibitory activity was higher for extracts of high polar solvent like methanol compared to other solvents like acetone, chloroform, petroleum ether etc.

A substance that kills bacteria or stops them from growing and causing disease is known as antibacterials. A common strategy used by resistant bacteria to withstand antibiotic doses that would typically be effective involves producing enzymes that inactivate or alter the antibiotic, modifying the target site, and altering the bacterial cell membrane to block antibiotic uptake.[2] In this study, variations in antibacterial activity may be linked to differences in cell wall thickness, as gram-positive bacteria have a thicker cell wall compared to gram-negative bacteria due to its composition of peptidoglycan molecules. Additionally, compounds such as phenolics, flavonoids, alkaloids, glycosides, quinones, and tannins are recognized for their antibacterial properties.[10] Apart from the present study, *Lygodium microphyllum* was found to possess the strongest inhibitory activity against tetracycline. Crude extract of butanol and water fractions in *L. microphyllum* displayed weak inhibitory effects against all bacterial isolates with MIC values varied from 12.5-50 mg/mL.[15]

Plant extracts as a source of antibacterial components have effective activity against biofilm development. In the fern, *Adiantum philippense* crude extract on biofilms was performed and biofilms microbial strains were observed.[16] A biofilm is a collection of microbial cells attached to a surface and embedded within a matrix of extracellular polymeric substances. Bacteria within biofilms exhibit greater resistance to antibiotics and chemical agents compared to planktonic cells in suspension. This enhanced tolerance to antimicrobials makes the treatment of biofilm-associated infections less effective. Plant extracts as a source of antibacterial components have effective activity against biofilm development. Sometimes biofilms can be pathogenic. Most human infections are biofilm-associated. The biofilm-inhibiting activity demonstrated by the extract holds significant potential for developing new therapies to combat chronic and persistent infections involving biofilms and intracellular bacteria. Of the six bacteria tested, *Listeria monocytogenes* and *Xanthomonas campestris* showed maximum zone of inhibition. For the anti-biofilm activity, a positive control prepared by using the bacteria taken for biofilm screening and nutrient broth, sample extract of *Lygodium flexuosum* taken in different concentrations (12.5, 25, 50, 100 and 200 mg) along with bacteria and nutrient broth. The antibiofilim activity might be due to the specific metabolite present in plant extract. More *in vitro* and *in vivo* works are essential in determining the actual reason for this activity.

CONCLUSION

The present work demonstrated that *Lygodium flexuosum* exhibits significant antibacterial and anti-biofilm activities against various bacterial strains. The effectiveness of the extract against both gram-positive and gram-negative bacteria suggests the presence of potent bioactive compounds that disrupt bacterial growth and biofilm formation. These findings highlight the potential of *Lygodium* as a natural source of antibacterial agents that could be further developed into alternative therapeutic options, especially in combating antibiotic-resistant infections. Further research, including the isolation and characterization of specific active compounds, as well as *in vivo* studies, is necessary to fully understand the mechanisms of action and to explore the clinical applicability of these extracts. This study supports the broader use of plant-derived antimicrobials in addressing the growing challenge of biofilm-associated infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EPS: Exo Polymeric Substances; **ATCC:** American Type Culture Collection; **MTCC:** Microbial Type Culture Collection and Gene Bank; **OD:** Optical Density.

SUMMARY

The present study provides an insight into the ability of a lesser-known fern, *Lygodium flexuosum* as an anti-bacterial and anti-biofilm agent. This study might help in developing drugs that can aid therapeutic needs.

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