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Research Article

Investigation of an *in vitro* antimicrobial activity of *Lygodium micophyllum (Cav.) R.Br* fronds

T.D. De Silva, G.H.C.M. Hettiarachchi

Department of Chemistry, Faculty of Science, University of Colombo, Sri Lanka

Address for Correspondence:
Dr. GHCM Hettiarachchi
E-mail: chamarih@chem.cmb.ac.lk

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ABSTRACT

Lygodium microphyllum(Cav.) R.Br. is a popular ethno-medicinal herb among ancestral people in Sri Lanka which cures different types of infectious diseases. However, no studies have been conducted to check the bioactivity of *L. microphyllum*. The present study was conducted to evaluate *in vitro* antimicrobial properties of the fronds of *L. microphyllum* and to identify the antimicrobial compound(s). Five different crude extracts were prepared via serial exhaustive extraction method using chloroform, dichloromethane, acetone, ethanol and methanol. Antibacterial and antifungal activities of all the crude extracts were tested using standard disk diffusion method for three bacterial species; *Streptococcus faecalis*, *Staphylococcus aureus*, *Escherichia coli*, and two fungal species *Penicillium notatum* and *Aspergillus niger*. Among all bacterial species, only *E. coli* showed the antibacterial activity with a Minimum Inhibitory Concentration (MIC) of 25µg/µl. None of the crude extracts could inhibit the growth of *P. notatum.* However, except the ethanol extract, all other crude extracts inhibited the growth of *A. niger*. The MIC values for chloroform, dichloromethane, acetone and methanol extracts were 10 µg/µl, 50 µg/µl, 10 µg/µl and 25 µg/µl respectively. Acetone crude extract showed the highest inhibitory activity against *A. niger* and it was separated to constituents by using Thin Layer Chromatography followed by Column chromatography. The fraction with highest anti-*A. niger* activity was identified by disk diffusion method and the fractions were analyzed by Infrared Spectroscopy. Further spectroscopic studies have to be carried out to confirm the structure of the antimicrobial compound(s) present in these extracts.

Key words: *Lygodium microphyllum;* Antibacterial activity; Antifungal activity; Agar disk diffusion.

INTRODUCTION

Since ancient time, plants and plant extracts have been used on variety of infectious diseases and the value of medicinal plants to human livelihood is infinite. Plants produce different types of secondary metabolites with potential antimicrobial value. Hence, the screening of plants and plant extracts for antimicrobial activity is beneficial and the active compounds may be useful in the preparation of novel drug formulations. A significant number of studies have been carried out to test the antimicrobial compounds of higher plants but very little attention has been given towards the lower plants, especially on ferns (Pteridophytes).

Pteridophytes and their allies have been survived from Paleozoic times to present by adapting to many changes in the environment[1]. Hence, Pteridophytes may have evolved with different types of secondary metabolites such as alkaloids, flavonoids, steroids, triterpenoid, phenols, varieties of amino acids and fatty acids etc[2]. Pteridophytes have lived with human beings for a long time and influenced millions of human lives as traditional medicinal cures or treatments for diarrhea, ascarid disease, cold, trauma bleeding, burn and more of them in many countries.[3-5] It was reported that Pteridophytes used in medicine with various bioactive properties, such as antioxidant,[6] antimicrobial,[7] antiviral,[8] anti-inflammatory,[9] antitumor[10] and anti-HIV[11] etc.

Sri Lanka is considered as a biodiversity hotspot due to its richness in flora and fauna. The country comprises about 343 species of Pteridophytes and 58 species of them are endemic[12]. A study was conducted to evaluate the uses of Pteridopytes by local healers, local people and tribal people in two selected areas, Bulathsinhala in Kaluthara District and Mahiyanganaya in Badulla District in Sri Lanka. Thirty-Five Pteridophytes were collected that have been used as remedies for many different infectious diseases and 27 were identified and authenticated. Out of them, *L. microphyllum* was selected for further investigation of the antimicrobial activity as of its heavy used in infectious diseases by tribal people in Sri Lanka. Fronds is the most common part of this plant in folk medicine. It has been used in folk medicine to treat skin ailments, swelling, dysentry, hiccough and cough. It is used as one of the ingredients in many lotions and the ground paste with turmeric is applied over the affected places to heal wounds. However, all these uses are according to the folk medicine, and there is no scientific base to validate such claims.

The present study was conducted to investigate the antimicrobial properties of *L. microphyllum* crude extracts against two Gram positive bacteria, *S. aureus* and *S. faecalis*, a Gram-negative bacterium, *E. coli* and two fungal species *P. notatum* and *A. niger*. The results showed the highest inhibitory activity against the *A. niger*. Compared to antifungal activity, *L. microphyllum* showed very low antibacterial activity only against *E. coli*. Taking together the results suggested that *L. microphyllum* could be a potential plant source of strong occurrence of antifungal compounds.

MATERIALS AND METHODS

Preparation of plant materials:

Fresh fronds of *L. microphyllum* were washed 2-3 times with tap water followed by distilled water and shade dried at room temperature for a period of 2 weeks. A weight of about 100 g of the samples was freeze dried and used for the solvent extraction. *L. microphyllum* plants were authenticated by National Herbarium, Peradeniya, Sri Lanka and the authenticatation number is 6/01/H/501.

Preparation of crude extract:

Freeze dried samples of fronds of *L. microphyllum* was mixed with chloroform, dichloromethane, acetone, ethanol and methanol in the ratio of 1:10 (w/v) separately for the crude extract preparation. Each mixture was filtered through the Whatman No: 1 filter paper and extraction procedure was done twice for complete extraction of the bioactive compounds. The obtained filtrates were concentrated at 35 ^{0}C using rotary evaporator and the serial exhaustive extraction method was followed to obtain the fractions of each solvent[13]. The concentrated extract was completely dried by passing dry nitrogen and the weight of each residue was recorded. Each residue was dissolved in the respective solvent to prepare the concentration of 100 mg/mL stock solutions and stored in a refrigerator.

Test organisms:

A total of three bacterial strains; *E. coli* (ATCC 35218), *S. aureus* (ATCC 25928), *S. faecalis* (ATCC 9790) and two fungal strains, *A. niger*, (ATCC 8740) and *P. notatum* (ATCC 9478) were used for the present investigation. All microbial strains were bought from Industrial Technology Institute, Sri Lanka. Identification and confirmation of strains, maintaining stock culture, quantification and growth of microorganisms were carried out according to the method described in Jayathissa et al, 2011[14].

Antibacterial assay:

The bacterial cultures were maintained on nutrient agar slants at 4°C and each of the bacterial strain was reactivated prior to susceptibility testing. The isolated bacteria were re-suspended in saline solution 0.85 % NaCl and adjusted to 1.0×10^8–1.0×10^9 CFU/ml at 600 nm. Antibacterial assay was carried out by disk diffusion method[15] and the antibacterial activity were determined by measuring the diameter of zone of inhibition. The experiment was carried out three times and the results were the mean of three replicates. Gentamycin (10 µg/µl) was used as positive control and respective pure solvent was used as the negative control.

Minimum Inhibitory Concentrations (MIC) of the crude sample for bacteria:

MIC of *L. microphyllum* crude extract was carried out against all three bacterial strains using standard disk diffusion method[15]. The concentration series of crude of 10, 25 and 50 µg/µl were prepared for the assay and the experiment was performed in three replicates for each concentration. For each bacterial strain antibacterial activity was expressed in terms of the mean of diameter of zone of inhibition (mm).

Antifungal Assay:

Fungal cultures were maintained on Potato Dextrose Agar (PDA) slants at 4 ^{0}C. A few single sclerotia of the fungus were obtained from the stock cultures of *A. niger, P. notatum* and inoculated separately on the sterilized PDA plates. The plates were incubated at room temperature for 5-7 days[16]. All crude extracts mentioned in antibacterial assay (ethanol, methanol, dichloromethane and acetone extracts of fronds) were used in antifungal assay too. Fungal spore suspension was prepared from the fully grown fungal cultures and compared with 0.5 McFarland standards. 200 µl of each fungal spore suspension was spreaded on PDA plates and plates were incubated for 1-2 hours in room temperature. Antifungal assay was carried out using disk diffusion method[15] and the antifungal activity of the test organisms were determined by measuring the diameter of zone of inhibition and expressed in millimeter. The experiment was carried out three times and the results were the mean of three replicates. Fluconazole (10 µg/µl) was used as the positive control and respective pure solvent was used as the negative control.

Minimum Inhibitory Concentration of the crude samples for fungi:

The crude samples which could inhibit the activity of fungi were selected to check the MIC. The concentrations of 10, 25, and 50 µg/µl were prepared and 10 µl from each solution was aseptically transferred onto different sterilized filter paper disks. The disks were placed on each PDA plate that was already inoculated with fugal spore suspension and incubated in room temperature for 5 to 6 days. The experiment was performed in three replicates for each concentration level and for each fungal strain, antifungal activity was expressed in terms of the mean of diameter of zone of inhibition (mm).

Statistical analysis of bacteria and fungi bioassays:

All the experimental data were performed in triplicate and the results were expressed as means ± SE using Minitab Release 15 software program.

Thin Layer Chromatography (TLC):

The crude samples which showed the highest antifungal activity was used in TLC. Stock solution of the particular sample was applied on silica coated TLC plates (20×20 cm TLC silica gel 60-F_{254} aluminum sheet, Merck, Germany). The spotted TLC plates were allowed to separate by keeping them in saturated chambers of different solvent systems at room temperature. The solvent systems were made by using acetone, chloroform, dichloromethane, ethanol, methanol and hexane in different combinations and different ratios. After drying, TLC plates were examined under UV light (254 nm) and followed by dipping in an iodine chamber.

Separation of compounds by column chromatograph:

The crude product which gave the maximum inhibition was subjected to normal phase column chromatographic separation at room temperature and pressure. Around 1 g of the dried sample was carefully placed on top of the column (15 cm x 2.5 cm, inside diameter) which was already packed with silica. The mobile phase was prepared using the chemicals identified in the solvent system. Gradient elution was carried out by gradually increasing the polarity of the eluting solvent by mixing the polar solvent in the solvent system.

Identification of the compound containing anti-fungal activity:

Eighteen fractions collected were pooled together based on the results of TLC and five fractions were prepared. Antifungal bioassay was carried out to for these five fractions as described above in order to observe which fraction contains the antifungal activity.

Infrared Radiation (IR) spectrum analysis:

The fraction that showed highest antifungal activity was subjected to IR analysis[18]. The dried fraction was dissolved in 1 ml of hexane and an IR spectrum was obtained using sodium chloride disks. Band positions of the spectrum were compared with the standard data.

RESULTS AND DISCUSSION

Antibacterial activity of L. microphyllum fronds:

L. microphyllum fronds crude extracts were prepared using different solvent systems. The selection of solvent systems in extraction procedures largely depends on the specific nature of the bioactive compounds being targeted[17]. The extraction of hydrophilic compounds uses polar solvents such as methanol or ethanol. Most of the identified antimicrobial components from plants are aromatic or saturated organic compounds, they are often obtained through ethanol or methanol extraction. To extract, more lipophilic compounds, dichloromethane or a mixture of dichloromethane and methanol is used. Tannins and terpenoids are more often obtained by treatment with less polar solvents[17]. Considering all the above facts a series of solvents with different polarities were selected in the study. They were chloroform, dichloromethane, acetone, ethanol and methanol. In order to make the extraction more effective, it was started from the least polar solvent and gradually the polarity of the solvent was increased. Five crude extracts prepared from serial extraction of five different solvent types were checked for antibacterial activity against Gram positive bacteria *S. faecalis* and *S. aureus* and Gram negative *E. coli.*

According to the results shown in Table 1, the highest antibacterial activity was seen with *E. coli* when tested against methanol extracts. Both Gram positive strains did not show any activity. To determine the minimum concentration required for the inhibition of *E. coli*, MIC assay was conducted and the results showed 25 µg /µl methanol crude extract is enough to inhibit the growth of *E. coli*. Also these results concluded antibacterial activity of *L. microphyllum* is only against *E.coli* from the tested bacterial strains. Further studies have to be conducted to confirm the claim.

Antifungal activity of L. microphyllum

In vitro antifungal activity of *L. microphyllum* crude extracts were determined by standard disk diffusion method[15] against *P. notatum* and *A. niger*. An antifungal activity was expressed as the mean diameters of zones of inhibition are shown in Table 3. Among tested fungal strains, *A. niger* showed the best activity against all crude extracts except ethanol extract. As per the results given in Table 3 all five different extracts could not inhibit the growth of *P. notatum*. So, *L. microphyllum* might not be having compounds that could inhibit *P. notatum*.

When *A. niger* was used, out of five different extracts, chloroform, dichloromethane, acetone and methanol were capable of inhibiting the growth of *A. niger*. MIC values of chloroform, dichloromethane, acetone and methanol extracts of *L. microphyllum* were 10 µg/µl, 50 µg/µl, 10 µg/µl and 25 µg/µl respectively. However, the highest antifungal activity was showed by acetone extracts of *L. microphyllum* frond sample (Figure 2a). This is an indication of the effectiveness of the bioactive constituents of the plant extracts against the *A. niger* which could be heavily isolated by using acetone. Hence, acetone extracted crude sample was used in normal phase TLC studies.

Table 1: The mean values of inhibitory zone diameter (mm) with different crude extractions against bacteria when 100 µg/µL samples were used

Test organism	Sample	The diameter of the inhibitory zone against bacterial growth (mm) when 100 µg/µl samples were used				
		Chloroform	Dichloro-methane	Acetone	Ethanol	Methanol
E. coli	Crude	-	-	-	-	3.83±2.00
	Positive	10.16±0.33	7.67±1.40	13.67±0.21	9.74±0.76	12.33±1.85
	Negative	-	-	-	-	-
S. faecalis	Crude	-	-	-	-	-
	Positive	13.00± 0	13.00± 0	20.96±0.44	12.50±0.42	14.00±0.36
	Negative	-	-	-	-	-
S. aureus	Crude	-	-	-	-	-
	Positive	9.34±1.46	9.67±0.85	13.50±0.36	14.00±0.00	13.50±0.34
	Negative	-	-	-	-	-

Table 2: The mean values of inhibitory zone diameter (mm) with methanol extracted crude concentrations against *E. coli*.

Solvent	The diameter of the inhibitory zone against *E. coli* growth (mm)				
	Positive 10 µg/ µl	Negative	50 µg/µl	25 µg/µl	10 µg/µl
Methanol	12.66±1.85	-	2.78±0.87	1.00±0.36	-

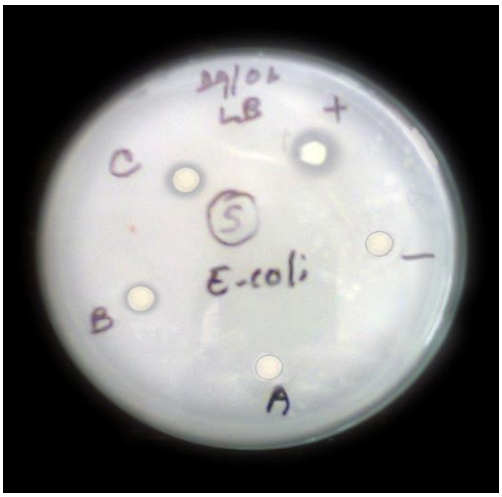

Figure 1: Antibacterial activity of E. coli against methanol extracts

Antibacterial activity against (B) 25 µg/µl (C) 50 µg/µl of the methanol extracted samples and inhibition absent for 10 µg/µl (A).

Table 3: Mean values of inhibitory zone diameter (mm) with different crude extracts against fungi when 100 µg/µl samples were used.

Test organism	Sample	The diameter of the inhibitory zone against fungal growth (mm) when 100 µg/µL samples were used				
		Chloroform	Dichloro-methane	Acetone	Ethanol	Methanol
P. notatum	Crude	-	-	-	-	-
	Positive	21.90±0.22	21.06±0.21	21.06±0.21	21.06±0.21	21.06±0.21
	Negative	-	-	-	-	-
A. niger	Crude	13.06±0.33	12.63±0.33	12.30±0.25	-	11.96±0.33
	Positive	21.73±0.55	20.80±0.56	21.80±0.22	22.40± 0	22.30± 0
	Negative	-	-	-	-	-

Table 4: The mean values of inhibitory zone diameter (mm) with different crude extractions against *A. niger*.

Solvent	Positive control (mm)	Negative control (mm)	The diameter of the inhibitory zone against *A. niger* growth (mm)		
			50 µg/µl	25 µg/µl	10 µg/µl
Chloroform	16.33±0.55	-	2.33±0.66	1.16±0.47	0.83±0.30
Dichloromethane	14.50±0.56	-	3.00±0.25	-	-
Acetone	15.50±0.22	-	8.33±0.55	6.16±0.47	5.16±0.47
Methanol	16.00±0	-	1.66±0.33	0.50±0.34	-

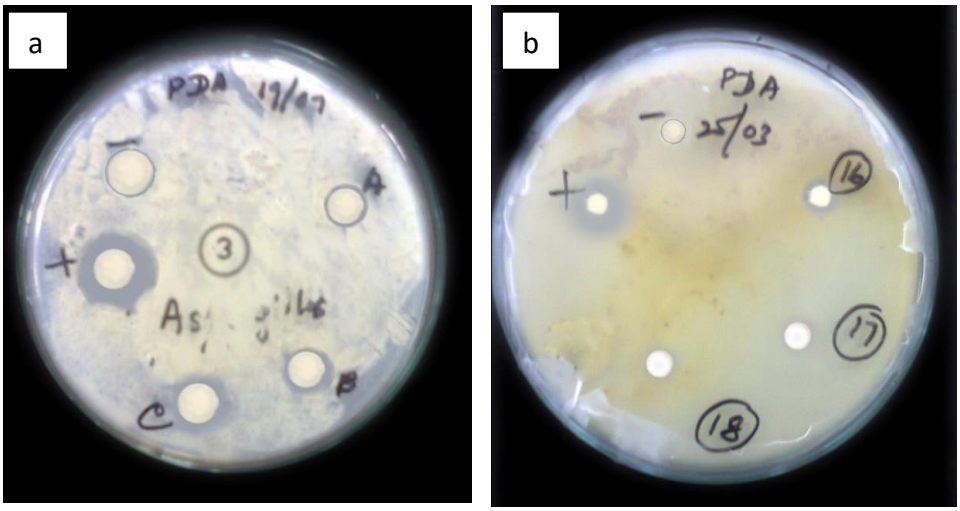

Figure 2: Antifungal activity of *A. niger* with acetone extracted samples

a - (A) 10 µg/µL (B) 25 µg/µL (C) 50 µg/µL of the acetone extracted samples
b- *A. niger* culture showing inhibitions with fractions 16, 17 and 18 of acetone extracted samples.

Identification of bioactive compounds in acetone crude extract:

Acetone extract of *L. microphyllum* were subjected to TLC and TLC plate was observed in the iodine chamber. Five spots were identified, three under naked eye and two when those compounds were allowed to interact with iodine vapour.

Column chromatography technique[17] was used starting from dichloromethane and gradually increasing the polarity by adding ethanol. The eluent was collected in to eighteen samples and they were mixed together and to make five mixtures in order to do the anti-fungal bioassay against *A. niger*. Mixture V was identified as anti-*A. niger*. When fractions 16, 17 and 18 which were the components of mixture V were used separately for the antifungal assay against *A. niger* fraction 16 showed a remarkable inhibition zone (Figure 2b).

The presence of compounds, especially such as flavonoids, alkaloids, tannins, saponins, terpens, steroids and essential oils, or any other type of secondary metabolite(s) in *L. microphyllum* crude extracts, may be collectively or individually responsible for the observed antifungal activity.[19] Nevertheless, the various constituents of plant extracts with the essential oils may act synergistically to bring the overall antifungal activity.

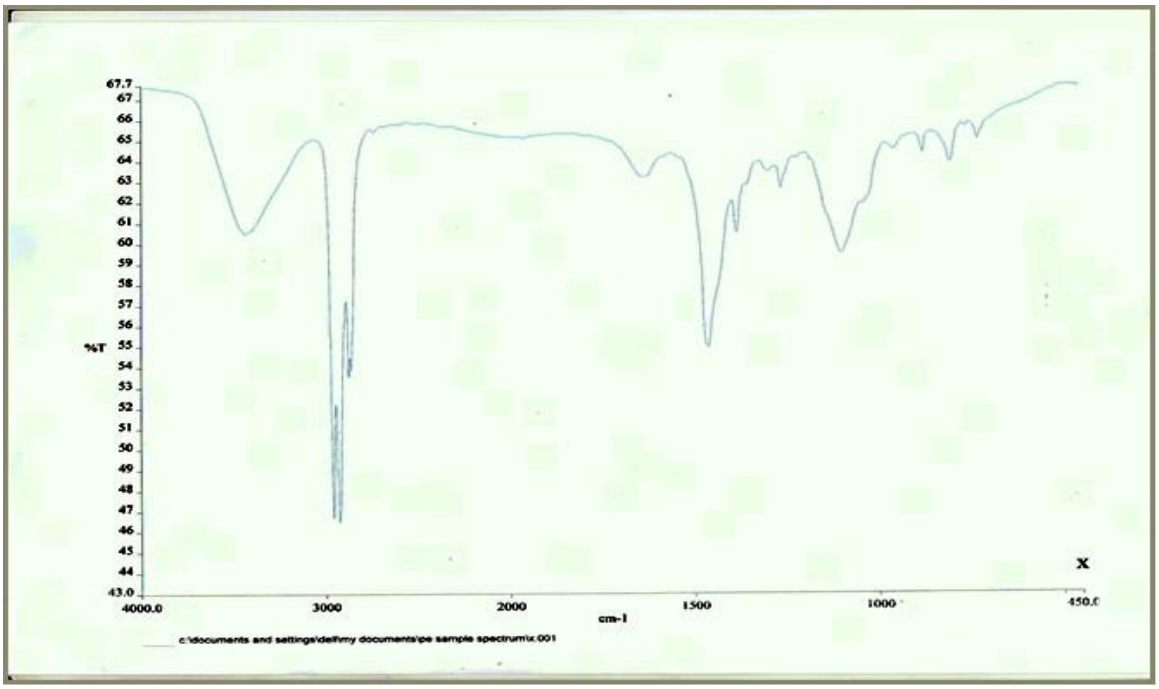

Figure 3: IR spectrum of fraction 16 of Acetone extracted sample

Several investigators have found eugenol to exert antifungal activity against *Aspergillus* spp. in various foods[20]. Cinnamaldehyde, eugenol and cinamic acid in addition to flavonoids, alkaloiks, tannins and saponins recognised by some investigators as antifungal especially against *A. niger*. Sometimes the compound related to anti-*A. niger* activity in the acetone extracted crude sample might be due to one of the above compounds present in the fronds of *L. microphyllum*.

IR spectrum (Figure 3) was obtained for the fraction which showed the highest activity in order to have an idea about the compound with anti-*A. niger* activity. After analyzing the spectrum, it was tallied with literature available. It might be an alkaloid compound which is capable of inhibiting the growth of *A. niger*[20]. However, further analyses are required to confirm the structure of the active compound against *A. niger*.

CONCLUSION

The antimicrobial properties of *L. microphyllum* fronds were tested in acetone, chloroform, dichloromethane, methanol and ethanol extracts. Except *E.coli* all other bacteria showed very poor antibacterial activity. The highest antifungal activity was observed in acetone crude extracts of *L. microphyllum* fronds against *A.niger*. 16th fraction obtained from Column Chromatography showed the highest anti *A.niger* activity and IR spectrum of 16th fraction showed the presence of alkaloids in it. Hence these activities might be due to the strong occurrence of antifungal compounds such as alkaloids.

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REFERENCES

1. Wallace RA, Sander GP, Ferl RJ. Biology: the science of life. Harper Collins: New York; 1991.
2. Zeng-fu LI, Huil H, Hang-yi Z, Jun-chen Z. Review on the extraction of flavonoids from fern. Journal of San University. 2008; 25: 22.
3. May LW. The economic uses and associated folklore of ferns and fern allies. The Botanical Review. 1978; 44(4): 491–528.
4. Wu CY. A compendium of new China herbal medicine. Volume 3. Shanghai Science and Technology Press: Shanghai; 1990.
5. Benjamin A, Manickam VS. Medicinal Pteridophytes from the Western Ghats. Indian Journal of Traditional Knowledge. 2007; 6(4): 611–618.
6. Garcia F, Pivel JP, Guerrero A, Brieva A, Martinez-Alcazar MP, Caamano-Somoza M, Gonzalez S. Phenolic components and antioxidant activity of Fernblock, an aqueous extract of the aerial parts of the fern *Polypodium leucotomos*. Methods Find Exp Clin Pharmacol. 2006; 28(3): 157–160.
7. Maruzzella JC. Antimicrobial substances from ferns. Nature. 1961; 191: 518.
8. McCutcheon AR, Roberts TE, Gibbons E, Ellis SM, Babiuk LA, Hancock RE, Towers GH. Antiviral screening of British Columbian medicinal plants. Journal of Ethnopharmacology. 1995; 49(2): 101–110.
9. Liu B, Diaz F, Bohlin L, Vasange M. Quantitative determination of anti-inflammatory principles in some *Polypodium* species as a basis for standardization. Phytomedicine. 1998; 5(3): 187–194.
10. Konoshima T, Takasaki M, Tokuda H, Masuda K, Arai Y, Shiojima K, Ageta H. Anti-tumor-promoting activities of triterpenoids from ferns. I. Biol Pharm Bull. 1996; 19(7): 962-965.
11. Mizushina Y, Watanabe I, Ohta K, Takemura M, Sahara H, Takahashi N, Gasa S, Sugawara F, Matsukage A, Yoshida S, Sakaguchi K. Studies on inhibitors of mammalian DNA polymerase α and β:: Sulfolipids from a pteridophyte, *Athyrium niponicum*. Biochem Pharmacol. 1998; 55(4): 537–541.
12. Fernando B. Ferns of Sri Lanka. The Fern Society of Sri Lanka. Katuneriya, Sri Lanka. 2002.
13. Das K, Tiwari RKS, Shrivastava DK. Techniques for evaluation of medicinal plant products as antimicrobial agent: Current method and future trends. Journal of Microbial Plant Research. 2010; 4(2): 104-111.
14. Jayathissa RN, Perera RP, Hettiarachchi CM, Weerawarna PM. In vitro antibacterial activity of 4 phenyl 1-1(2 phenylallyl)pyrinidium bromide: A noval class of pyrinidium based antimicrobial compounds. Indian Journal of Microbiology. 2012; 52(1): 83-87.
15. Bauer AW, Perry DM, Kirby WMM. Single disk antibiotic sensitivity testing of Staphylococci: An Analysis of Technique and Results. AMA Arch Intern Med. 1959; 104(2): 208–216.
16. Nilanthi SMLD, Wijayarathna CD, Hettiarachchi GHCM. Investigation of an Antimicrobial activity of *Angiopteris evecta* (Forst) Hoffm. Research & Review Journal of Chemistry. 2015; 4(3): 47-52
17. Sewell P, Clarke B. Chromatographic Separations. Wiley: New York; 1988.
18. Silverstein RM, Bassler GC, Morrill TC. Spectrometric Identification of Organic Compounds. 5th Edition. Wiley: New York; 1991.
19. Owolabi OJ, Omogbai EKI, Obasuyi, O. Antifungal and antibacterial activities of the ethanolic and aqueous extracts of *Kigelia africana* (Bignoniaceae) stem bark. African journal of Biotechnology. 2007; 6(14): 1677-1680.
20. Vazquez IB, Fente CM, Franco MJ. Inhibition effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. Int J Food Microbiol. 2001; 67: 157-163.