J. Mycopathol. Res. ***59(1)*** *: 37-45, 2021;* (ISSN 0971-3719)
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Evaluation of antimicrobial activity and HPLC analysis of phenolics present in *Nephrolepis cordifolia* (l.) C. (Persl)

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Received : 09.01.2021 Accepted : 29.01.2021 Published : 29.03.2021

Pteridophytes, being one of the important part of the biodiversity of the blue green planet form the potential source of herbal medicine due to the presence of various bioactive compounds. In this regard, present investigation was carried out to assess the antimicrobial activities of the locally available fern of Darjeeling Himalayas, *Nephrolepis cordifolia* along with the HPLC analysis of phenolics. Phytochemical analysis showed the presence of various bioactive compounds. The hot water and methanol (50%) extract was used against two gram positive (*Bacillus cereus* and *Bacillus megaterium*), two gram negative (*Burkholderia symbiont* and *Serratia marcescens*) bacterial strains and three fungal species (*Alternaria alternata, Curvularia lunuta* and *Fusarium oxysporum*) to measure its antimicrobial efficacy. Both the extracts were efficient in inhibiting the bacterial growth with the inhibition zone.Additionally, the extracts were also effective against the fungal strains used. The growth inhibition percentage ranged between 73.08 - 46.67%. *A. alternata* was found to be the most susceptible among all the tested fungi. Several phenolics detected were phloroglucinol, gallic acid, dihydrobenzoic acid, catechol, catechin, caffeic, vanillic, ferulic and salicylic acid. Bioactive compounds are known to have an advantageous role in human health. Thus, *N. cordifolia* enriched with numerous biologically active compounds may be considered as the potential natural source of antimicrobial and biocontrol agents as it exhibited broad spectrum antimicrobial activities against plant pathogens.

Key words: *Nephrolepis cordifolia,* phytochemical, antibacterial, antifungal, HPLC, phenolics

INTRODUCTION

According to The World Health Organization (WHO) 80% of the world's population (with large number of tribal and rural people across the globe) rely on traditional medicines for meeting their health care needs (Gurib-Fakim, 2006)).

Plant scientists have been dynamically involved in discovering and rediscovering the medicinal efficacies of plants and also isolating a range of therapeutic components responsible in treating various diseases (Shanmugam *et al*. 2009; Anuraja and Shanmugam, 2010). Recent years has witnessed rapid emergence in the search of natural antimicrobial compounds from medicinal plants because of the increased reports of drug resistant pathogenic microorganisms from across the globe and innumerable side effects of synthetic drugs.

As folk medicine, pteridophytes have been in use for over 2000 years and are well documented in ancient literature. Pteridophytes being one of the important part of the biodiversity of our planet, forms the potential source of herbal medicine due to the presence of various bioactive compounds. India, being one of the richest sources of medicinal ferns, much work has been done for screening their antimicrobial potential (Dalli et al. 2007; Maridoss, 2009; Singh *et al.* 2008).

Nephrolepis cordifolia (L.) C. Persl belonging to the family Nephrolepidaceae has been used ethnomedicinally to treat ailments like stomach ulcer, jaundice, respiratory troubles, liver, intestinal and renal disorders and as an antifungal and antitussive agents (Rout *et al.* 2009). Additionally, young fronds of this fern are known to be consumed as vegetables by some Indian mountain tribals. However, obscure information about the antimicrobial activity and the phytocompounds of *Nephrolepis cordifolia* growing in Darjeeling

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Himalayas has led to investigate the potentials of this fern. Hence, the objective of the present study was to evaluate the antimicrobial property of locally available fern of Darjeeling Himalayas, *N. cordifolia* along with the phytochemical screening and HPLC analysis of phenolics.

MATERIALS AND METHODS

Plant material

N. cordifolia healthy fronds were collected from the hills of Darjeeling (26º 58' 39.1440'' N 88º 21' 45.543" E), West Bengal, India. Sample collected was identified at the Herbarium, Department of Botany, University of North Bengal and deposited in North Bengal University Herbarium with accession no. 09874.

Sample preparation

The plant sample collected was initially washed with tap water followed by double distilled water and dried under shade for 7 days. The dry samples were finely ground and stored in plastic bottle at 4°C till further use. For antimicrobial assay, powdered sample was further extracted in hot water and 50% metanol using standard method (Coban and Konoklugil, 2005). Extracts were filtered and evaporated using rotary evaporator under reduced pressure and further lyophilized for complete solvent removal. It was then stored in sterilized glass vials at -20°C until further analysis. In order to avoid any contamination and alterations of chemical constituents, the extracts were used within 2-3 days of preparation (Singh *et al.*2012).

Qualitative screening of phytochemicals

Small fraction of the dried powder was used for the detection of phytochemicals using various standard methods as follows:

Test for phenols

About 200mg powdered sample was dissolved in 10mL distilled water, mixed thoroughly and then filtered using Whatman filter paper. 1mL of 1% $FeCl_3$ was added to 1mL of the filtrate. Appearance of blue or green color indicates presence of phenols.

Test for flavonoids

The powdered sample was mixed thoroughly with 10mL of acetone which was allowed to evaporate completely. Further, it was extracted using 10mL of warm double distilled water and filtered while hot. The filtrate was allowed to cool at room temperature. 5mL of the filtrate was added with 5mL of 20% NaOH. Presence of flavonoid was indicated by the appearance of yellow coloured solution.

Test for alkaloids

Methanolic filtrate of sample (2mL) was mixed with equal volume of 1% HCl and kept over steam for 5mins. Mayer's/Wagner's reagent was added to 1mL of the mixture. Creamish/Brown/Red/Orange precipitate was indicates the presence of alkaloid.

Test for tannins

Powdered sample was mixed with 10mL distilled water for 10 minutes and then filtered. To the filtrate, 5% $FeCl_3$ (1mL) was added. Formation of yellow brown precipitate indicates the presence of tannins.

Test for saponins

Aqueous filtrate of sample (0.5mL) and 5mL distilled water was mixed vigorously by shaking for about 30 seconds. Formation and persistence of froth indicates the presence of saponins.

Test for terpenoid

Methanolic filtrate (2mL) was mixed with chloroform (5mL) and acetic anhydride (2mL) to which 1mL of concentrated H_2SO_4 and was added carefully. Reddish brown coloration at the interface indicates the presence of terpenoid.

Test for cardiac glycosides

2mL methanolic filtrate was mixed with 1mL glacial acetic acid and 3-4 drops of 5% $FeCl_3$. Further, 1mL of concentrated H_2SO_4 was added carefully to the mixture. Brown ring at the interface indicates presence of cardiac glycosides. A violet colour may also appear below the brown ring.

Test for Steroid

Methanol extract (5mL) was treated with 0.5mL of anhydrous CH_3COOH and was allowed to cool for 15minutes on an ice bath. To the mixture, 0.5mL

(chloroform) and 1mL concentrated H_2SO_4 was added. Reddish-brown ring at the junction of two liquid phase indicates the presence of steroid.

Test for Anthraquinone

0.5g powdered sample was mixed with 5mL (chloroform), mixed properly and filtered. 3mL of filtrate was shaken with 3mL (10% ammonia solution). Pink/red/violet color in the aqueous layer after shaking indicates the presence of free anthraquinone.

Antibacterial sensitivity test

The antibacterial activity of plant extracts both hot water extracts (HWE) and 50% methanolic extracts (ME) were evaluated following the agar disc-diffusion method (35). Bacterial strains used were two gram-positive and two gram-negative bacteria namely, *Bacillus cereus* (MTCC 10665), *Bacillus megaterium* (TRS7) MTCC 1684- NCBI GENBANK acc no.JX312687, *Burkholderia symbiont* (previously grouped in *Pseudomonas)* NAIMCC–B 01489, NCBI GENBANK acc no. JQ765578 and *Serratia marcescens* (TRS1)-NCBI GENBANK acc no. JN020963. All the strains were obtained from the culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal.

Petriplates were prepared by mixing a final inoculum of 100µL suspension containing 10^8CFU/mL of each bacterium with the sterilized nutrient agar media and allowed to solidify in the laminar air flow. Crude extracts were sterilized in disposable Millipore filter (0.22 µm pores) prior to use. After 15min, the plates were impregnated with sterile Whatman No.1 filter paper dics (6mm) containing desired concentrations viz. 500 mg/mL, 250 mg/mL and 100mg/mL of the hot water extract (HWE) and methanolic extract (ME) of the sample. Solvents were completely evaporated from the disc in the laminar air flow before aseptically placing it on the agar surface. Negative control (sterile hot water), solvent control (50% MeOH) and positive control plates were also prepared. Positive control discs (7mm) of Chloramphenicol (C_{25}) (25mcg), Kanamycin (K) (30mcg) and Ampicillin (A_{25}) (25mcg) were used in the study. The plates were then incubated at 37˚C for 24 h after which the diameter of inhibition zones was noted. All the assays were performed in triplicates.

Antifungal study
Spore germination bioassay

Spore germination bioassay was performed following the method of Trivedi and Sinha (1976). Fungal species used were *Alternaria alternata* (NCBI ACC NO. KT818507), *Curvularia lunuta* (NCBI ACC NO. KT697995) and *Fusarium oxysporum* (NCBI ACC NO. KF952602) obtained from Immuno-Phytopathology Laboratory. Different concentrations (500, 200 and 100mg/mL) of plant extracts were used to study the germination inhibitory effect. Extracts was placed at the two ends of clean, grease free slide and allowed to dry inside a laminar air flow after which spore suspension of test fungus was placed on top of the dried extract spots. Similarly, the slides for positive control (Griseofulvin 1mg/mL), solvent control (50% MeOH) and negative control (sterile distilled water/sdH_2O) were prepared and incubated for 24h. Precautions were taken to avoid the drying of the spores. Following the incubation, the spores (or the spots) were stained with lactophenol cotton blue, fixed and observed under the microscope. The microscopic observation was done from about 5-8 microscopic fields and a total of 500 spores were counted for each case. Further, the percentage of germination was calculated using the formulae:

$$\text{Percentage (\%) of spore germination} = \frac{\text{Number of spores germinated}}{\text{Total number of spores counted}} \text{ X } 100$$

Radial growth bioassay

Food poisoning technique was also employed to study the antifungal activity of the extract (37). Small fraction of plant extract was re-dissolved in respective solvents to make the stock solution of 2000 mg/mL. Prior to use, the extracts were sterilized in disposable Millipore filter (0.22 µm pores) and mixed with sterile potato dextrose agar medium (PDA) to obtain the final concentration of 500 mg/mL of each plant extract and then poured in sterile petridishes. Control plates were mixed with sdH_2O (negative control), 50% methanol (solvent control) and griseofulvin (1mg/mL). The agar block (discs) of about 7mm diameter of phytopathogenic fungi were cut from the periphery of 7 days old cultures and inoculated aseptically to the centre of the poured petridishes of treatment and control sets. The plates were then incubated at 25 ± 2°C for 3 days and the zone of inhibition were noted. All the assays were performed in triplicates. Fungal colony diameter of treatments and control sets were

measured and percentage of mycelia inhibition was calculated using the following formula.

$$\text{Percentage of mycelial inhibition} = \frac{(C - T)}{C} X\, 100$$

where, C and T are the growth diameter (mm) in control and treatment respectively.

HPLC analysis of phenolics

HPLC analysis of total phenols was done following the method described by Pari *et al.*(2007). The flow rate of 1mL/min and injection volume of 20 *ì*L was applied. The analysis of phenolic compounds were carried out using binary gradient of acetonitrile–water–acetic acid (5:93:2, v/v/v) [solvent A] and acetonitrile–water–acetic acid (40:58:2, v/v/v) [solvent B], starting with solvent B from 0 to 100% over a period of 50 mins. The separation of compounds was monitored at 280 nm. Caffeic acid, caffeine, catechin, catechol, chlorogenic acid, cinnamic acid, 3,4-dihydroxybenzoic acid, ferulic acid, gallic acid, phloroglucinol, pyrogallol, resorcinol, salicylic acid and vanillic acid were used as standards for identification and quantification.

Statistical analysis

All analyses were carried out in triplicates. Data are expressed as mean ± standard error. Student's t-test of significance of was evaluated using SPSS software package version 21.0.

RESULTS AND DISCUSSION

Qualitative analysis of the sample

Phytochemical analysis revealed the presence of diverse compounds such as flavonoid, phenol, tannin, saponin, terpene and steroid (Table 1).This was in accordance to the finding of Xavier *et al.*(2016).In their study, they could detect the presence of alkaloids and cardiac glycosides which was in contrast to the present study. The differences may be because of the differences in the age of plant, percentage humidity of the harvested material, time and situation of harvest, extraction procedures and the amount of the samples used.

Moreover, importance of phytocompounds to human health is well documented. Plant phenolics possess anticancerous, antioxidative, antidiabetic, antimutagenic activity besides being effective vasodilators and anticancerous agents. Further, its preventive roles in various neurodegenerative diseases are encouraging (Zhang *et al.* 2011; Mohanlal *et al*. 2013; Jaishee and Chakraborty 2014). Likewise, flavonoids and tannins are known to possess antiviral, antibacterial, antifungal, anticancer, anti-allergic, antiparasitic, anti inflammatory, antiulcer and antioxidant activity (Montro *et al*. 2005).

Table 1: Phytochemical compounds of *N. cordifolia*

Bioactive constituents	Present/Absent
Phenol	+
Flavonoid	+
Tannin	+
Alkaloid	-
Cardiac glycosides	-
Carbohydrates	+
Reducing sugar	+
Protein	+
Saponin	+
Terpenoid	+
Steroid	+
Anthraquinone	-

"+"= Present, "-"= Absent

Antibacterial activity

Both gram positive and gram negative bacterial growth was observed to be significantly inhibited by the extracts (Table 2). Methanolic extracts was noticed to show higher inhibition zones than hot water extract. Nonetheless, irrespective of the concentrations used the zone of inhibition against *B. cereus* by the water extract of *N. cordifolia* was similar to our findings. The differences in the activity may be attributed to the geographical variation, age of the plant and extraction procedures used. In the present study, inhibition zones for gram positive bacteria (*B. cereus* and *B. megaterium*) ranged between 24.03 to 22.73 mm for methanolic extract and between 20.00 to 19.50 for hot water extract.

Similarly, the inhibition zone by methanolic extract ranged between 24 and 21 mm and by hot water extract between 23.00 and 19.00 against gram negative bacteria. Antibacterial efficacy of ferns has been previously reported by many authors

Table 2. Antibacterial activity of the Hot water and methanol extract of *N. cordifolia*

Bacteria	Extract	Conc. (mg/mL)	Inhibition zone (mm)			
				Positive controls		
				C_{25}	K_{30}	A_{25}
	ME	500	24.03±0.27^{a}	21.53±0.20	15.03±0.15	-
Bc		250	21.43±0.34^{b}			
		100	18.03±0.20^{c}			
	HWE	500	19.50±0.23^{a}			
		250	18.10±0.24^{b}			
		100	15.00±0.29^{c}			
	ME	500	22.73±0.38^{a}	12.00±0.28	16.00 ±0.26	-
Bm		250	20.03±0.28^{a,b}			
		100	16.00±0.37^{c}			
	HWE	500	20.00±0.37^{a}			
		250	18.00±0.33^{ab}			
		100	14.00±0.22^{c}			
	ME	500	21.00±0.23^{a}	18.00±0.31	19.50±0.15	10.00±0.26
Bus		250	18.00±0.37^{b}			
		100	15.00±0.37^{c}			
	HWE	500	19.00±0.37^{a}			
		250	17.00±0.26^{b}			
		100	12.00±0.35^{c}			
	ME	500	24.00±0.40^{a}	29.00±0.37	16.00±0.24	-
Sm		250	22.50±0.35^{b}			
		100	20.20±0.29^{c}			
	HWE	500	23.00±0.29^{a}			
		250	21.00±0.38^{b}			
		100	19.00±0.33^{c}			

Values are expressed as mean±SE(n=3). Solvent controls: No inhibition. Values within the column for each solvent and each bacterium with a common superscript (a,b,c) are not significantly (pÂ0.05) different as determined by Fisher's LSD test. *Bc*: *Bacillus cereus*, *Bm*: *Bacillus megaterium*, *Bus*: *Burkholderia symbiont*, *Sm*: *Serratia marcescens,* HWE: Hot water extract, ME: Methanol extract, C: Chloramphenicol, K: Kanamycin, A: Ampicillin. "-": No inhibition. Inhibition zone values include the disc size of 7mm for positive controls and 6mm for Whatmann filter paper discs.

(Chai *et al*. 2013; Bahadori *et al*. 2015). Besides many secondary metabolites, phenolics and flavonoids are well established for their antimicrobial property (Pereira *et al*. 2014). *N. cordifolia* extract was able to inhibit the growth of both gram positive and gram negative bacteria and may be considered as a potential source of broad spectrum antibacterial agents. However, the mechanism as to how they control the activity is to be further examined.

Antifungal study

Spore germination assay in general provides a preliminary information regarding the effectiveness of the plant extracts against the fungi studied. Both, hot water and methanolic extract was observed to inhibit the spore germination of *A. alternata*, *C. lunuta* and *F. oxysporum*. However, among the three concentrations used 200 and 100mg/mL concentrations were ineffective against any of the fungi studied, thus, the result of percent germination (% inhibition) of only 500mg/mL has only been presented (Table 3,4).

Comparatively higher methanolic extract showed higher antifungal activity than hot water extract against the three fungal species used. These finding was further confirmed with radial growth bioassay (Fig. 1). Percentage of growth inhibition

Table 3. Spore germination bioassay of crude plant extracts and controls against different fungal species

Samples	Solvent	Conc. (mg/mL)	% spore germination		
			A. alternata	*C. lunata*	*F. oxysporum*
Nc	ME	500	27.60±0.46^{b} (71.95)	30.40±0.67^{b} (69.35)	37.80±0.64^{b} (61.51)
	HWE	500	38.00±0.41^{b} (62.00)	43.12±0.70^{b} (56.53)	56.80±0.46^{b} (43.20)
Positive control	G	1mg/mL	23.60±0.50^{b} (76.40)	0.00±0.00^{b} (100)	1.80±0.62^{b} (68.20)
Sovlent control	MeOH	-	98.40±1.13^{a}	99.20±0.60^{a}	98.20±1.50^{a}
Negative control	HW	-	100±0.00^{a}	99.20±0.55^{a}	100±0.00^{a}

Values are expressed as mean±SE of 500 spores. Means within the column with different superscript differ significantly ($p<0.05$) with respect to each solvent used, as determined using Students't'-test. *Nc*: *Nephrolepis cordifolia* HWE: Hot water extract ME: Methanol extract G: Griseofulvin. Values inside the parentheses represent the percentage of inhibition in relation to respective controls.

Table 4. Radial growth bioassay of crude plant extracts and controls against different fungal species

Samples	Solvent	Conc. (mg/mL)	Diameter of radial growth (cm)		
			A.alternata	*C.lunata*	*F.oxysporum*
Nc	ME	500	1.4± 0.23^{b} (73.08)	1.4±0.24^{b} (72.55)	1.7±0.16^{b} (62.22)
	HWE	500	2.0±0.23^{b} (61.54)	2.1±0.24^{b} (58.82)	2.4±0.25^{b} (46.67)
Positive control	G	1 (mg/mL)	1.1±0.11^{b} (78.85)	0.00±0.00^{b} (100)	1.5±0.19^{b} (66.67)
Solvent control	ME	-	5.1±0.29^{a} (0)	5.1±0.32^{a} (0)	4.5±0.24^{a} (0)
Negative control	HW	-	5.2±0.14^{a} (0)	5.1±0.29^{a} (0)	4.5±0.29^{a} (0)

Values are expressed as mean±SE (n=3). Means within the column for each plant with different superscript differ significantly ($p<0.05$) with respect to each solvent used, determined using Students 't'-test. *Values compared with the solvent control. *¹Values compared with the negative control.*Nc*: *Nephrolepis cordifolia* HWE: Hot water extract ME: Methanol extract G: Griseofulvin. Growth diameter includes the inoculum agar block size of 6mm. Values inside the parentheses represent the percentage of inhibition

by methanolic extracts ranged between 73.08 - 62.00 while by hot water extract ranged between 61.5 - 46.67%. *A.alternata* was found to be most susceptible than the other two fungi. Antifungal activities of pteridophytes have been reported by many workers (Patric *et al.*2012). Presence of various bioactive compounds which would have been better extracted in methanol may have contributed towards the antimicrobial activity. The broad spectrum of *in vitro* antimicrobial activity of *N. cordifolia* may be due to the presence of some bioactive principles in the extract which may have acted alone or in combination to inhibit the microbial growth.

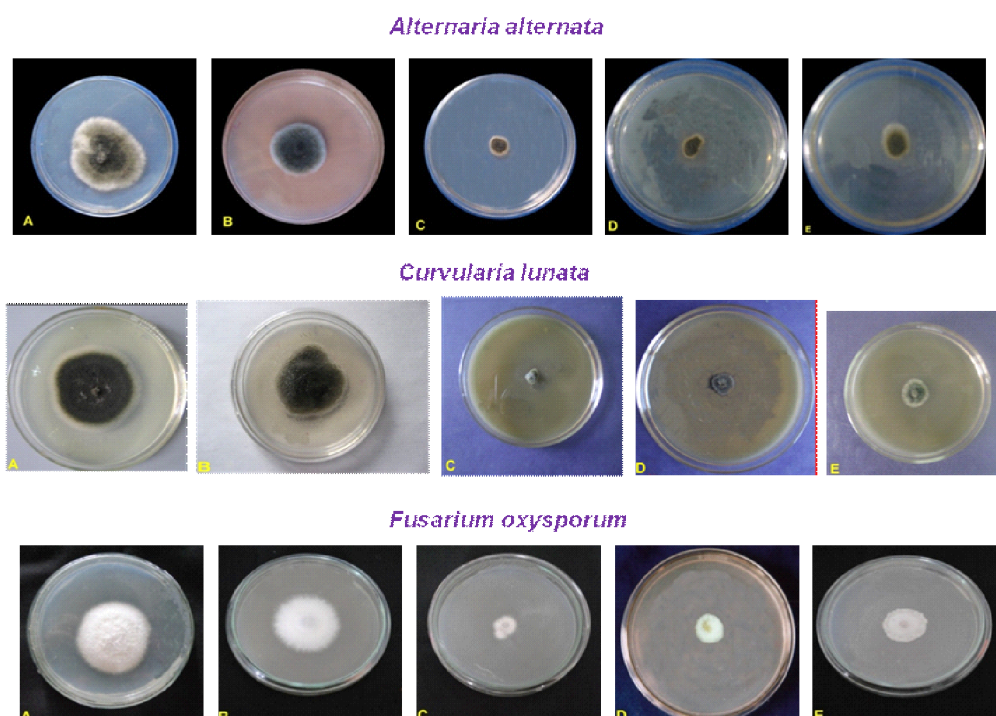

Figure 1: Antifungal activity of plant extract using radial growth bioassay

A: Negative control (dH_20) **B:** Solvent control (Methanol) **C:** Positive control (Griseofulvin) **D:** *Nephrolepis cordifolia* (*Nc*) extract (Methanol) **E:** *Nc* extract (HW)

Identification of phenolics by HPLC

Phenolic compounds are one of the largest complex groups of chemicals present in plant kingdom and are well-known for various biological activities. In the present study,diverse group of phenolics were identified like caffeic acid, vanillic acid, ferulic acid, salicyclic acid,catechin etc while caffeine, cinnamic acid, pyrogallol etc was not detected in the sample(Table 5; Fig. 2).Diverse group of phenolics have been reported in plants by many authors (Proestas and Komaitis, 2013; Syafni *et al*.2012). Jaishee and Chakraborty (2015) reported that in *Dicranopteris linearis* and *Pteris vittata* the predominantly occurring phenolics identified by HPLC analysis in both the samples were catechin, caffeic, ferulic, salicylic and vanillic acid.

Numerous studies have reported that phenolics may have contributed to their biological activities like photo-peroxidation of linoleic acid by ferulic acid and antioxidant activity by caffeic and p-coumaric acid. Similarly, components like 3,4-dihydroxybenzoic acid,isolated terpenoid like Friedelin have been effective against both gram positive and gram negative bacteria (Chiozem *et al*., 2009; Khan *et al.,* 2007).

Table 5. HPLC analysis of phenolics in the methanol extract

Compounds	Plant sample *Nc*
PhlognL	+
Gallic acid	+
Pyrogallol	ND
DHBA	+
Resorcinol	ND
Catechol	+
Catechin	+
CgcA	ND
Caffeine	ND
Caffeic acid	+
Vanillic acid	+
Ferulic acid	+
Salicylic acid	+
Cinnamic acid	ND

'+' = detected, '-'= not detected. All the data are the mean of three replicates.

PhlognL: Phloroglucinol CgcA:Chlorogenic acid , DHBA: 3,4-dihydroxybenzoic acid

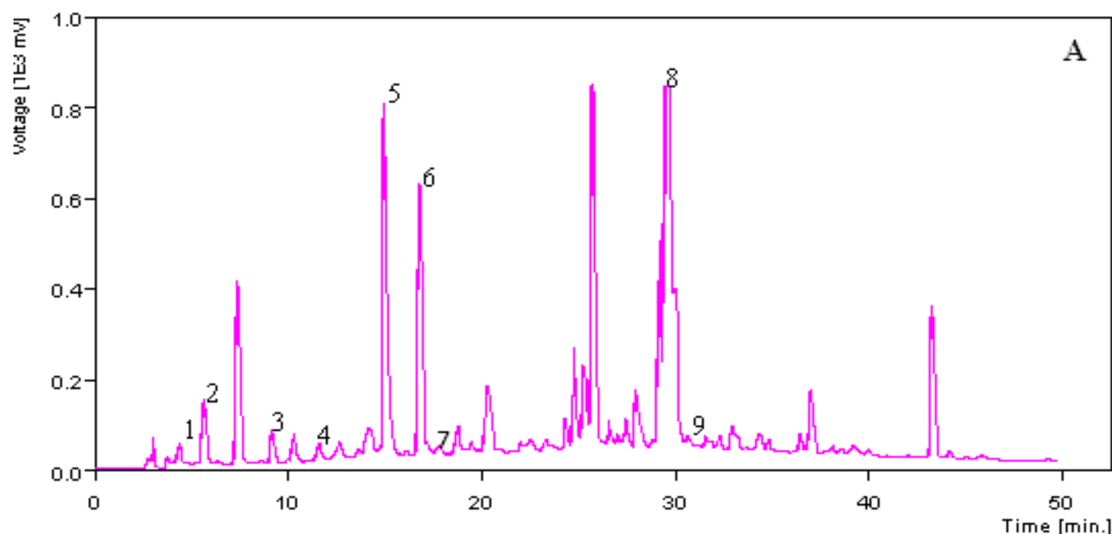

Figure 2: HPLC fingerprint of the phenolics present in the plant extract

A. *Nephrolepis cordifolia* – 1: Phloroglucinol 2: Gallic acid 3: 3,4- dihydroxybenzoic acid
4: Catechol 5: Catechin 6: Caffeic acid 7: Vanillic acid 8: Ferulic acid 9: Salicylic acid

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