Phenolic compounds characterization and *in vitro* antioxidant activities of selected ferns of Darjeeling district of North Bengal region, India

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Abstract: In the present study, *in vitro* antioxidant activity of total phenolics of eight ferns collected from different places of Darjeeling, India, were evaluated. The content of phenolics ranged from 6.77 to 60.066mg FAE/g dry weight. The DPPH antioxidant activity expressed as IC_{50} values revealed *Nephrolepis cordifolia* and *Microsorum punctatum* to exhibit highest and lowest antioxidative activity respectively. Moderate correlation (R^2=0.547) was observed between the total phenolics content and antioxidant activity. HPLC analysis of phenolics from all the investigated plants revealed that ferulic acid, caffeic acid and salicylic acid were present while the other phenolics such as phloroglucinol, gallic acid, pyrogallol, 3,4- dihydroxybenzoic acid, catechol, catechin, chlorogenic acid, caffeine, vanillic acid and cinnamic acid were not uniformly present in all the plants. The phenolic contents values showed wide variation among

themselves, as well as within different plants. These ferns with considerable amount of phenolics can be the potential source of natural antioxidants.

Keywords: Ferns, antioxidant, total phenolics, DPPH radical scavenging activity, HPLC

Introduction

Phenolic compounds, such as simple phenols, hydroxybenzoic acid, coumarins, tannins and flavonoids are ubiquitously present in plants. These compounds are known to possess various pharmacological properties including antioxidant activity [1]. Ferns have been reported to be the potential source of bioactive constituents with myriad biological efficacies [2]. Rapid increase in the search for natural alternative to the synthetic drugs has led researchers across the globe to explore all available plant resources to identify and isolate the potential bioactive compounds from the plants. The phytochemicals of ferns are known to mainly belong to the families of phenolics, terpenoid and alkaloids [2]. Ferns have been recently explored for various biological activities for eg, *Dicranopteris linearis* has been extensively studied for its antioxidant, anti-inflammatory, chemopreventive and hepatoprotective activity [3,4]. Likewise, antibacterial, antipyretic, antihelmintic and anti-dermatophytic properties have been reported in D*rynaria* quercifolia [5,6]. *Pteris vittata* has been reported to exhibit potent antitumor and antidiabetic activity besides its potential as an antibacterial agent [7,8]. *Pteris biaurita* and *Nephrolepis cordifolia* are known to possess antibacterial property [9,10]. Presence of various bioactive constituents, mainly the polyphenolic compounds are known to contribute towards the various biological activities of plants and are helpful in mitigating the harmful effects of oxidative stress related diseases [11].

In recent days, antioxidant research focusing mainly on natural antioxidant is one of the important topics in medical and food industries as bioactive constituents have shown linear

positive correlation with their antioxidant activities [12]. Thus, in this present study eight locally available ferns of North Bengal region, West Bengal, India, namely *Nephrolepis cordifolia*, , *Cyclosorus dentatus Drynaria quercifolia*, *Phymatosorus cuspidatus*, *Dicranopteris linearis*, *Pteris biaurita*, *Pteris vittata* and *Microsorum punctatum* were evaluated for their antioxidant activity, total phenolic content and their identification by HPLC.

Materials and methods

Chemicals and materials- The chemicals and reagents 2,2-Diphenyl-1-picrylhydrazyl(H), methanol(M), L-ascorbate acid, Folin-Ciocalteau reagent (M), sodium carbonate(H) and standard phenols used in this study were obtained from Merck (M), Himedia (H) India and Sigma-Aldrich, India.

Preparation of plant extract- The fresh and mature fronds of *Nephrolepis cordifolia*, , *Cyclosorus dentatus Drynaria quercifolia*, *Phymatosorus cuspidatus*, *Dicranopteris linearis*, *Pteris biaurita*, *Pteris vittata* and *Microsorum punctatum* from Darjeeling district of North Bengal, West Bengal extending between 27.05°N and 88.263°E. Plant sample was identified using literature and confirmed at North Bengal University Herbarium, where specimen herbarium sheets have been deposited. The samples were shade dried and finely ground into powder using grinder (Jaipan, Super Deluxe, India), sieved and stored in air tight container at 4ºC for further use. The extract was prepared according to the method of Okwori *et al.* (2006) [13] with minor changes. The dried powdered sample and solvent in the ratio of 1:10 (sample:solvent) was mixed vigorously for 5minutes and then kept for 72h at room temperature while stirring at an interval of 24 hour. Whatman No.1 filter paper was used for filtration of mixture. The supernatant obtained was concentrated at 40ºC using rotary evaporator and lyophilized using Eyela Freeze Dryer (Japan).

Quantification of total phenolic compounds and determination of DPPH radical scavenging activity

Total phenolic compounds of the plant samples were determined using the method of Bray and Thorpe (1954) [14]. The ability of the methanolic extracts of the plants to scavenge DPPH was determined using the method described by Lim & Quah (2007) [15] with slight modification. Briefly, 1mL of the sample and the reference sample (L-ascorbic acid) of different concentration was mixed with 1mL of DPPH methanolic solution (100µM) and incubated for 30 minutes in dark condition. The absorbance was measured at 517nm against proper blank solution. Ascorbic acid was used as a positive control.

Inhibition was estimated as:

% inhibition= (A0-A1/A0)*100

Where, A0 is the absorbance of the control and A1 is the absorbance of the extract/standard.

HPLC pertaining to phenolics and statistical analysis.

HPLC investigations pertaining to phenolics was performed as per the scheme proposed by Pari and colleagues (2007) [16] with minor modifications. Statistical analysis was performed employing SPSS and KyPlot (v2beta15) software packages.

Results and discussion

Total phenolics- The total phenol content of *N.cordifolia* and *C.dentatus* was found to vary significantly from all the other samples studied. The phenolic content among the samples ranged from 6.77 to 60.066 mgFAE/g dry weight sample (Table 1).The phenolic content of fertile frond of *D.quercifolia* was lower than those reported by Anuja *et al.* (2014) [17] while that of *P. vittata* and *P.biaurita* was in concordance with Gracelin *et al.* (2013) [18]. *D.linearis* and *D.quercifolia*

showed lower phenolic contents than that reported by Lai & Lim (2011) [19] while that of *M.punctatum* was more or less similar with the previous report.

Table 1. Quantification of total phenolic contents in the crude powdered plant samples

Plant sample	Phenol(mgFAE/gdw)*
N.cordifolia	60.066 ± 5.333^{a}
C.dentatus	32.336 ± 1.308^{b}
D.quercifolia	13.168 ± 1.036^{c}
P.cuspidatus	$12.787\pm0.148^{c,d}$
P.biaurita	10.399 ± 0.052^{e}
D.linearis	$10.399\pm1.427^{e,f}$
P.vittata	$10.017\pm1.156^{e,f,g}$
M.punctatum	6.77 ± 0.97^{h}
LSD value	1.936

Values are presented as mean±SD (n=10). *Values are expressed as mg Ferulic acid equivalent /g dry weight sample. Values in the same column that are followed by different superscript letters ($^{a-h}$) are significantly different ($p<0.05$), as determined using the Fisher's LSD test.

DPPH radical scavenging activity- The antioxidant ability of the extracts was evaluated using DPPH radical scavenging activity method and was expressed as IC_{50} values (concentration of the samples/antioxidant required to scavenge 50% of the initial DPPH radicals) (Table 2). Lower the IC_{50} values, higher the activity. The activity of *N cordifolia* was insignificantly higher than that of *C.dentatus* while significantly higher than all the other extracts over the range of concentrations tested (Table 2). However, ascorbic acid had the lowest IC_{50} and highest percentage of inihibition (96.075%) as compared with all the extracts. The percentage inhibition values ranged between 71.579% *(M.punctatum)* to 91.886% (*N.cordifolia*). The percentage of DPPH inhibited by the extracts at varying concentrations was depicted in Fig. 1. The DPPH activity of *P. vittata*, *D.linearis* and *D.quercifolia* was lower than that reported by Lai & Lim

(2011)[19] while only slight difference in the activity of *M.punctatum* was observed. Likewise, other polypodiales such as *Drynaria fortunei* and *Pseudodrynaria coronas* showed higher activity than the *D.quercifolia* (Chang *et al.* 2007) [20]. Besides, the geographical differences, the reason for variation in the biological activity could be due to the differences in the extraction solvent used, as different solvent may extract different bioactive components eventually leading to the differences in the antioxidant activity. Chang *et al.* (2007) [20] reported higher antioxidant potentials in water extracts than ethanol extracts while methanolic extract was revealed to exhibit higher DPPH activity than ethanol and hot water (Karimi *et al.* 2012) [21]. Phenolics are considered to be one of the most important phytochemical contributing towards the antioxidant activity of the extracts. Zakaria *et al.* (2011) [22] reported methanolic extract of *D.linearis* containing high phenolic content to exhibit higher antioxidant activity. However, the finding of the present study was in contrast to the reports of Zakaria *et al.* (2011) [22] and Lai & Lim (2011) [19] as only moderate correlation (R^2=0.547) was observed between the phenolic content and antioxidant activity of the plant extracts (Fig. 2). Inspite of the moderate correlation, good antioxidative ability was shown by the plant extracts which may be because of the fact that besides phenolics, other bioactive compounds like, glycosides, vitamin E &C, and tannins may have synergistically contributed towards antioxidant activities of the plant.

Table 2. IC_{50} values of DPPH radical scavenging activity exhibited by the plant extracts

Samples	IC_{50} values (mg/mL)
N.cordifolia	0.822±0.069
C.dentatus	1.218±0.143
D.quercifolia	1.413±0.093
P.cuspidatus	1.455±0.035
D.linearis	1.479±0.035
P.biaurita	1.840±0.013
P.vittata	2.127±0.149
M.punctatum	2.446±0.043

Ascorbic acid	0.142±0.041
LSD value	0.735

Values are expressed as the mean±SD (n=3) and significance at $p<0.05$ was determined using Fisher's LSD test.

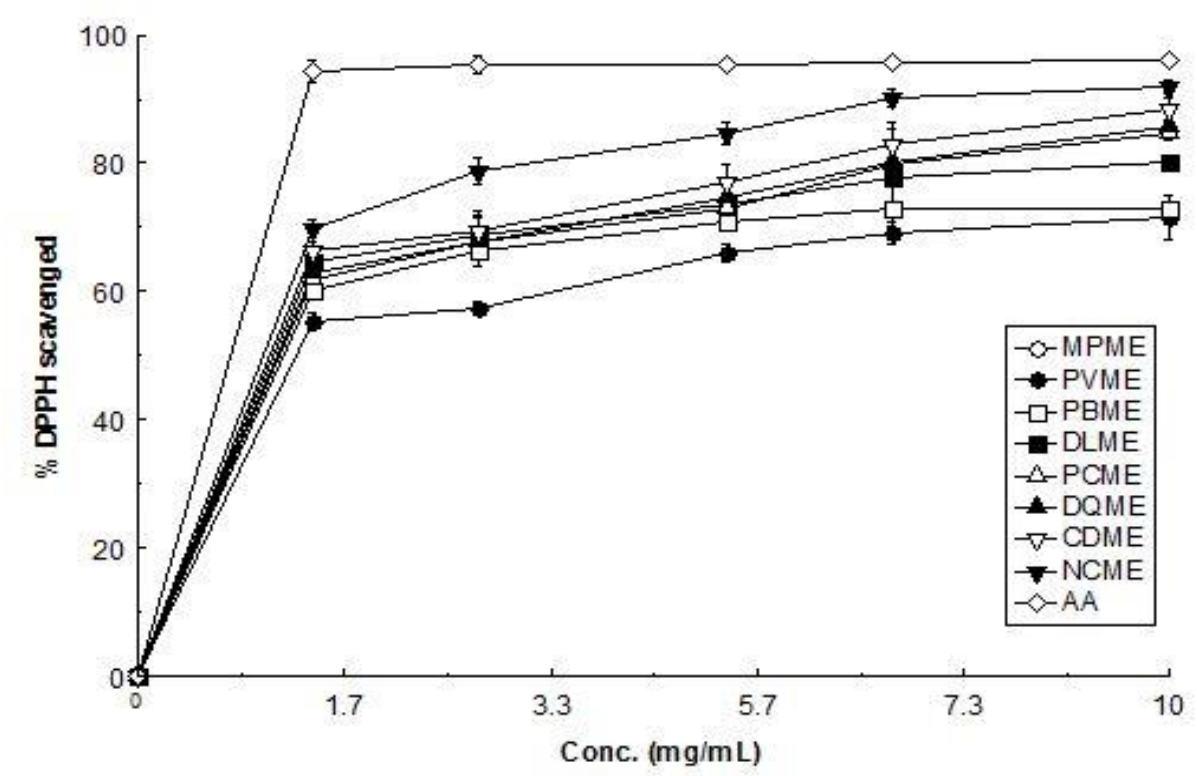

Fig.1- DPPH scavenging activity of methanolic extracts of eight different ferns. Ascorbic acid was used as positive control. AA: Ascorbic acid, NCME: *Nephrolepis cordifolia* methanolic extract, CDME: *Cyclosorus dentatus* methanolic extract, DQME: *Drynaria quercifolia* methanolic extract, PCME: *Phymatosorus cuspidatus* methanolic extract, DLME: *Dicranopteris linearis* methanolic extract, PBME: *Pteris biaurita* methanolic extract, *Pteris vittata* methanolic extract, MPME: *Microsorum punctatum* methanolic extract.

Identification and quantification of phenolic compounds in the plant extracts by HPLC

Phenolic compounds are one of the largest complex groups of phytochemicals present in the natural mixtures. The phenolic contents varied significantly among the samples studied. Diverse

group of phenolics were identified in the extracts with caffeic acid, ferulic acid and salicyclic acid being universally present in all the samples with the values ranging between 0.978-31.945, 0.896-44.953 and 4.594-140.608 mg/100g dry sample respectively (Table 3). Highest content of caffeic acid was found in *N.cordifolia* (31.945±0.026mg/100g dry sample) while ferulic acid (44.953± 0.017mg/ 100g dry sample) and salicyclic acid (140.608±0.038mg/100g dry sample) was highest in *C.dentatus.* Higher value of ferulic acid (76mg/100g dw) and caffeic acid (858 mg/100gdw) was reported by Wojdyło *et al.* (2007) [23] in some of the 32 different spices studied while lower values of ferulic acid (1.52 mg/100 g dry sample) and caffeic acid (0.93 mg/100g dry sample) was reported by Proestos *et al.* (2006) [24] than in *C.dentatus* and *N.cordifolia* respectively. Ferulic and caffeic acid has been detected in *Polypodium leucotomos* [25].

Gallic acid was detected in *N.cordifolia, C.dentatus* and *M.punctatum* amongst the other plant samples. The values ranged between 1.500-9.990mg/100g dry sample with the highest being in *N.cordifolia* and lowest in *C.dentatus.* The values were higher than that of the greek aromatic plants (1.5–2.6 mg/100 g dry sample) as reported by Proestos and Komaitis (2013) [26] and by Vesna *et al.* (2004) [27] in *Teucrium montanum* L.

Table 3: Phenolic contents in the methanolic extract of the plant samples

Compounds	Phenolics content in plant extracts (mg/100g dry sample)							
	NC	CD	DQ	PC	DL	PB	PV	MP
PhlognL	^{a}173.50 ±0.09	ND	ND	ND	ND	ND	^{b}62.40 ±0.09	ND
Gallic acid	^{a}9.99 ±0.01	^{c}1.50 ±0.01	ND	ND	ND	ND	ND	^{b}5.69 ±0.01
Pyrogallol	ND	ND	ND	ND	ND	ND	^{a}17.62 ±0.01	ND
DHBA	^{b}11.09 ±0.02	ND	ND	ND	^{a}12.02 ±0.02	^{d}0.33 ±0.001	ND	^{c}1.90 ±0.01
Resorcinol	ND	ND	ND	ND	ND	ND	ND	ND
Catechol	^{b}5.85 ±0.01	ND	^{a}32.19 ±0.02	^{d}4.18 ±0.01	ND	^{e}1.15 ±0.003	ND	^{c}4.25 ±0.01
Catechin	^{a}104.75 ±0.04	ND	^{f}5.59 ±0.01	^{b}63.68 ±0.01	^{c}18.64 ±0.01	^{g}0.78 ±0.004	^{d}9.79 ±0.01	^{e}7.58 ±0.004
CgcA	ND	ND	ND	ND	ND	^{a}5.67 ±0.01	^{b}3.23 ±0.01	ND
Caffeine	ND	^{b}3.64 ±0.01	^{a}3.77 ±0.02	ND	ND	^{d}1.42 ±0.01	^{c}3.61 ±0.003	ND
Caffeic acid	^{a}31.96 ±0.03	^{f}2.09 ±0.01	^{c}3.19 ±0.01	^{g}1.23 ±0.01	^{e}2.22 ±0.01	^{b}3.75 ±0.01	^{d}2.69 ±0.012	^{h}0.98 ±0.031
Vanillic acid	^{b}2.93 ±0.01	ND	^{d}1.49 ±0.01	^{c}2.13 ±0.004	^{a}3.64 ±0.01	^{f}0.62 ±0.003	^{e}1.38 ±0.01	ND
Ferulic acid	^{b}42.62 ±0.01	^{a}44.95 ±0.02	^{f}10.37 ±0.01	^{d}17.12 ±0.03	^{g}4.67 ±0.01	^{e}14.07 ±0.01	^{c}19.06 ±0.02	^{h}0.89 ±0.01
Salicylic acid	^{d}17.17	^{a}140.61	^{f}7.65	^{e}8.45	^{b}83.29	^{g}7.55	^{c}43.11	^{h}4.59

	±0.02	±0.04	±0.01	±0.01	±0.09	±0.01	±0.01	±0.004
Cinnamic acid	ND	^{c}2.68 ±0.01	^{b}4.74 ±0.01	ND	^{a}7.03 ±0.01	ND	ND	ND

Values within the row followed by different superscript $^{(a-h)}$ are significantly different at $p<0.05$, determined using Fisher's LSD test. **PhlognL:** Phloroglucinol, **DHBA:** 3, 4- dihydroxybenzoic acid, **CgcA:** Chlorogenic acid. **NC:** *Nephrolepis cordifolia*, **CD:** *Cyclosorus dentatus*, **DQ:** *Drynaria quercifolia*, **PC:** *Phymatosorus cuspidatus*, **DL:** *Dicranopteris linearis*, **PB:** *Pteris biaurita*, **PV:** *Pteris vittata*, **MP:** *Microsorum punctatum*. **ND:** Not detected.

Vanillic acid was detected in all the samples except for *C.dentatus* and *M.punctatum.* It has been reported even in *Polypodium leucotomos* (Garcia *et al.* 2006) [25]. Highest content was in *D.linearis* (3.638±0.009mg/100g dry sample) which was higher than in *Origanum vulgare* L. (1.00±0.02mg/100g dry sample) (Proestos and Komaitis (2013) [26] and lower than in water extract of *T. montanum* L.) [27]. Except for *P.biaurita* and *P.vittata*, none of the samples revealed the presence of chlorogenic acid with the values 5.666±0.009 and 3.226±0.010mg/100g dry sample respectively which was lesser than that reported by Vesna *et al.* (2004) [27]. Likewise, five chlorogenic acid isomers were detected in *Polypodium leucotomos* (Garcia *et al.* 2006) [25].

Another phenolic compound catechin was detected in all the investigated plants except in *C.dentatus.* The values ranged between 0.782-104.752mg/100g dry sample which was higher than those that reported by (Proestos and Komaitis (2013) [26] in *Origanum vulgare* L. (0.5-22.1mg/ 100g dry sample).

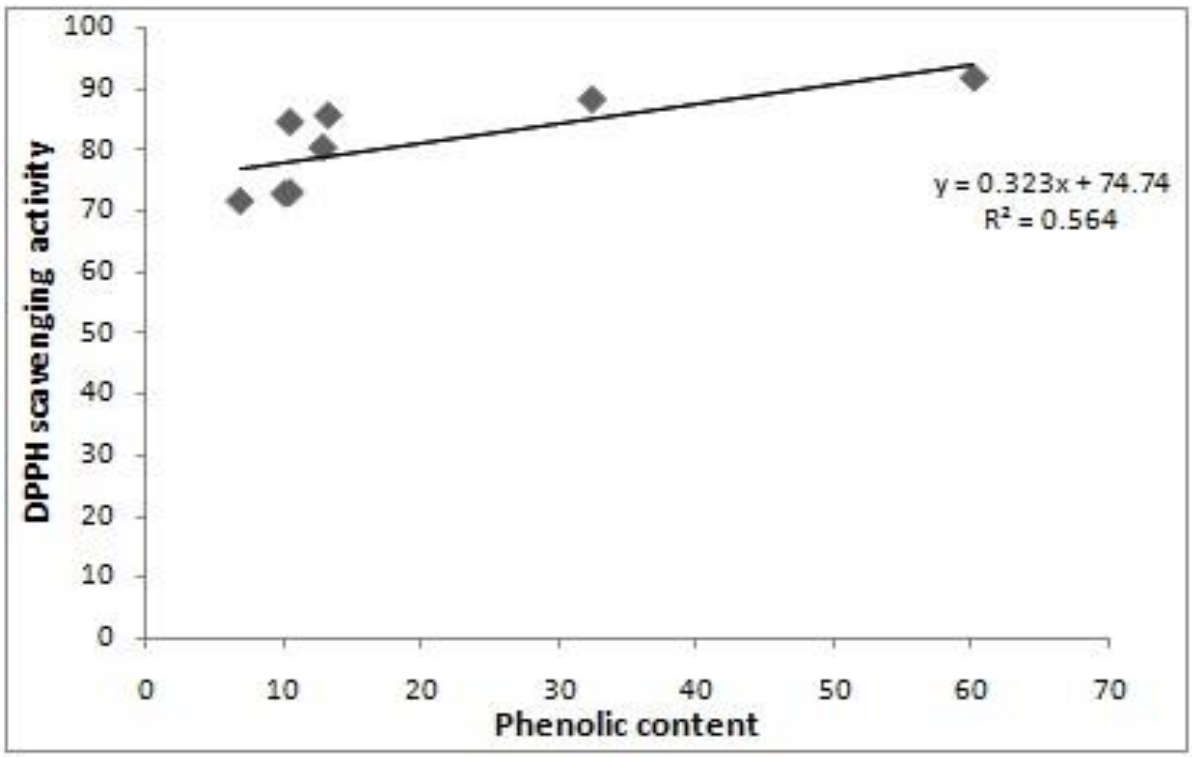

Fig. 2- Relationship between total phenol content and antioxidant activity

Resorcinol was not detected in any of the plant studied whereas pyrogallol was detected only in *P.vittata* with an amount of 17.615±0.005 mg/ 100g dry sample. The value was lower than that reported by Karimi & Jaafar (2011) [28] in *Labisia pumila* var.*alata* (1128.55µg/g dry sample) and in *Posidonia aceanica* (1.3mg/g dry weight) (Agostini *et al.* 1998) [29]. Amongst the investigated plants caffeine was detected only in *C.dentatus, D.quercifolia, P.biaurita and P.vittata.* Highest content was obtained in *D.quercifolia.* It has been detected and isolated from coffee and green tea leaves (Mohammed & Al-Bayati 2009) [30] while highest caffeine content of (4.5%) was reported in white tea by Komes *et al*. (2009) [31].

Catechol was detected in *N.cordifolia*, *D.quercifolia, P.cuspidatus*, *P.biaurita* and *M.punctatum* while 3,4- dihydroxybenzoic acid (DHBA) was detected in *N.cordifolia, D.linearis, P.biaurita* and *M.punctatum.* The highest catechol concentration was in *D.quercifolia* (32.186mg/100g dry

sample) which was higher than that reported by Haung *et al.* (2014) [32] in poplar tree (0.132mg/g). Presence of 3,4- dihydroxybenzoic acid (DHBA)was reported by Garcia *et al.* (2006) [25] in *Polypodium leucotomos* .

Cinnamic acid was identified only in *C.dentatus*, *D.quercifolia* and *D.linearis* with the highest content in *D.linearis* (7.026mg/100g dry sample) while phloroglucinol was detected only in *N.cordifolia* and *P.biaurita.* The cinnamic acid content was lower than that reported by Awad *et al.* (2014) [33] in *Cinnamon verum.*

Though, it is obvious that different plants accumulate different amount and types of phenolics, with the result obtained it can be further concluded that the accumulation of the phenolics may vary even within the genus level. The presence of considerable amount of phenolics in all the plant species may have contributed towards the antioxidant as determined using DPPH radical scavenging method. Moreover, these natural phenolics can be the potential alternatives to the synthetic antioxidants.

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