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CHEMO-PROFILING AND ASSESSMENT OF IN VITRO ANTIOXIDANT EFFICACY OF EIGHT FERNS OF DARJEELING HIMALAYAS, INDIA

Botany

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ABSTRACT

AIM: The present study was performed to profile some phenolics and explore the antioxidant effect of eight locally available ferns collected from different places of Darjeeling Himalayas, India.

Methods: The antioxidant activities of methanol (MeOH) extract was evaluated by DPPH free radical scavenging activity. Qualitative analysis of phenol was done using standard methods. Further, characterization of phenolics was done using High performance liquid chromatography.

Result: 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 the presence of caffeic acid, ferulic acid and salicylic acid while the other phenolics such as phloroglucinol, gallic acid, pyrogallol, 3,4-dihy droxybenzoic 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, cinnamic acid derivatives, flavonoids, coumarins and tannins are ubiquitously present in all the plants and 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]. *Pteris vittata* has been reported to exhibit potent antitumor and antidiabetic activity besides its potential as an antibacterial agent[5,6].

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[7]. Presence of unique metabolites has further intrigued to explore the potential therapeutic prospects of ferns. Current information on the pharmacological properties of ferns, particularly available at higher altitude, is inadequate.

Thus, the present study was undertaken to evaluate an antioxidant activity, phenolics content and its profiling of eight locally available ferns of Darjeeling Himalayas, West Bengal, India, namely *Nephrolepis cordifolia*, , *Cyclosorus dentatus Drynaria quercifolia*, *Phymatosorus cuspidatus*, *Dicranopteris linearis*, *Pteris biaurita*, *Pteris vittata* and *Microsorum punctatum.*

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 of analytical grade obtained from Merck (M), Himedia (H) India and Sigma-Aldrich, India.

Preparation of plant extract-

The fresh and mature fronds of *N. cordifolia*, , *C. dentatus D. quercifolia*, *P. cuspidatus*, *D. linearis*, *P. biaurita*, *P. vittata* and *M. punctatum* from Darjeeling district of North Bengal, West Bengal extending between 27.05°N and 88.263°E was collected, identified and the herbarium was deposited at North Bengal University Herbarium, Department of Botany. The collected plant materials were washed thoroughly, shade dried, finely ground, 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)[8] 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. The mixture was then filtered with Whatman No.1 filter paper. The supernatant obtained was concentrated at 40ºC using rotary evaporator and lyophilized using Eyela Freeze Dryer (Japan).

Quantification Of Total Phenolic Compounds

Total phenolic compounds of the plant samples were determined using the method of Bray and Thorpe (1954)[9]. Briefly, 1mL of extract, 1mL of 1N Folin ciocalteau's phenol reagent and 2ml of 20% Na_2CO_3 solution was mixed thoroughly and boiled in water bath for 1min. The reaction mixture was cooled under running tap water and then diluted with distilled water to make the final volume up to 25ml. The absorbance was recorded at 650nm in a colorimeter against appropriate blank solution and expressed as mg Ferulic acid (FAE) equivalents/ g dry weight sample.

Determination Of DPPH Radical Scavenging Activity

The ability of the methanolic extracts of the plants to scavenge DPPH was determined using the method described by Lim & Quah (2007)[10] 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.

The percentage inhibition was calculated according to the formula:
% inhibition= (A0-A1/A0)*100

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

HPLC Analysis Of Phenolics

HPLC analysis of total phenolics present in the sample was done using High performance liquid chromatograph (Shimadzu) equipped with HPLC pumps (model LC 10ATVP), UV-Vis detector (model SPD-10AVP) and C18 column, following the method described by Pari *et al.* (2007)[11] with minor modification. 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 flow rate of 1mL/min and injection

volume of 20 μL was applied. The separation of compounds was monitored at 280 nm. Standards such as 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 for identification and quantification.

Statistical Analysis

All analyses were carried out in triplicate. Data are expressed as mean ± standard deviation (SD). Differences were estimated by one-way analysis of variance (ANOVA) followed by Fisher's least significance difference (LSD) test. Probability values of less than 0.05 were considered statistically significant. All statistical analyses were performed using KyPlot (v2beta15) software.

RESULTS AND DISCUSSION

Total phenol- 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)[12] while that of *P. vittata* and *P.biaurita* was in concordance with Gracelin *et al.* (2013)[13]. *D.linearis* and *D.quercifolia* showed lower phenolic contents than that reported by Lai & Lim (2011)[14] while that of *M.punctatum* was more or less similar with the previous report. The variations may be attributed to several factors like age of the plant, time and percentage of humidity of the harvested material besides the differences in the areas of the plants grown.

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±0.148^{c,d}
P.biaurita	10.399±0.052^{e}
D.linearis	10.399±1.427^{e,f}
P.vittata	10.017±1.156^{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).

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.

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.

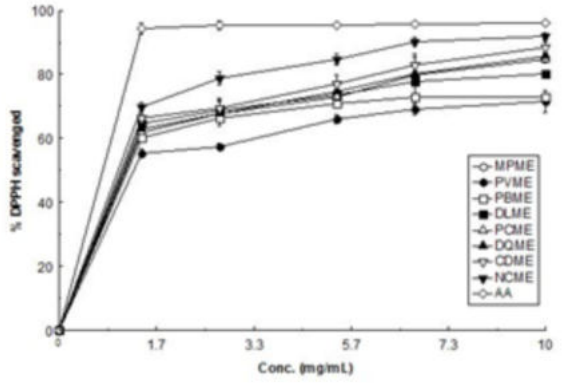

Fig.1- DPPH scavenging activity of methanolic extracts of eight different ferns. Ascorbic acid was used as positive control. AA: Ascorbic acid, NCME: *Nephrolepis cordifolia* methanolic extract, CDME: *Cyclosorus dentatus* methanolic extract, DQME: *Drynaria quercifolia* methanolic extract, PCME: *Phymatosorus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

The DPPH activity of *P. vittata*, *D.linearis* and *D.quercifolia* was lower than that reported by Lai & Lim (2011)[14] 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)[15]. 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)[15] 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)[16]. Phenolics are considered to be one of the most important phytochemical contributing towards the antioxidant activity of the extracts. Zakaria *et al.* (2011)[17] reported positive correlation between phenolic content and antioxidant activity in their study. However, our finding was in contrast to the reports of Zakaria *et al.* (2011)[17] and Lai & Lim (2011)[14] as only moderate correlation (R^2=0.547) was observed between the phenolic content and antioxidant activity of the plant extracts (Fig. 2).

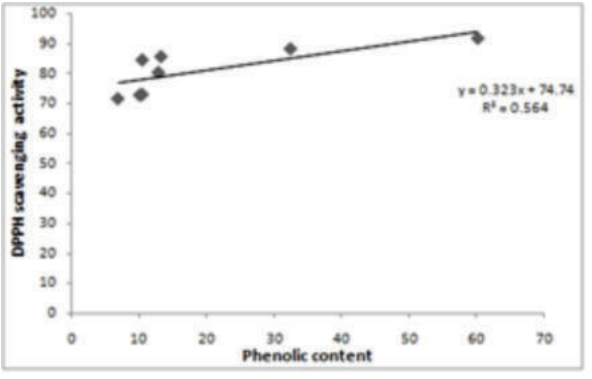

Fig. 2- Relationship Between Total Phenol Content And Antioxidant Activity

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.

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)[18] 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)[19] than in *C. dentatus* and *N. cordifolia* respectively. Ferulic and caffeic acid has been detected in *Polypodium leucotomos*[20].

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.* 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)[20]. Highest content was in *D. linearis* (3.638±0.009mg/100g dry sample). 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. Likewise, five chlorogenic acid isomers were detected in *Polypodium leucotomos* (Garcia *et al.* 2006)[20].

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. 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. 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)[21] while highest caffeine content of (4.5%) was reported in white tea by Komes *et al*. (2009)[22].

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)[23] in poplar tree (0.132mg/g). Presence of 3,4-dihydroxybenzoic acid (DHBA)was reported by Garcia *et al.* (2006)[20] 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.*

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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