Anti-Proliferative, Antioxidant and Iron-Chelating Properties of the Tropical Highland Fern, *Phymatopteris triloba* (Houtt) Pichi Serm (Family Polypodiaceae)

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**Abstract**

**Purpose:** To determine the phenolic constituents as well as anti-proliferative, antioxidant and iron-chelating activities of the leaf and rhizome extracts of *Phymatopteris triloba*.

**Methods:** Concentrations of selected hydroxybenzoic acids, hydroxycinnamic acids and flavonoids in aqueous extracts were quantified using high performance liquid chromatography (HPLC). Anti-proliferative activity was assessed on human cervix cancer cell line (HeLa) and human chronic myelogenous leukemia cell line (K562). Superoxide and nitric oxide scavenging activities as well as iron-chelating activity were determined colorimetrically.

**Results:** Protocatechuic acid content of the rhizome extract (154.7 µg/g dry matter (DM)) was 1.9-fold higher than in the leaf extract. p-Hydroxybenzoic acid (34.6 µg/g DM) and gallic acid (18.9 µg/g DM), which were only detected in the rhizome extract. Sinapic acid (6.6 µg/g DM) was detected in the leaf extract only. Myricetin content of leaf extract (98.5 µg/g DM) was 3.7-fold higher compared with the rhizome extract. At 500 µg DM/ml, both extracts produced about 40 and 30 % anti-proliferative activity on HeLa cells and K562 cells, respectively. Both extracts had moderate nitric oxide-scavenging and iron-chelating activities. The leaf extract half-maximal effective concentration (EC₅₀) value of 0.85 mg/ml (scavenging of superoxide radicals) was higher than that of ascorbic acid.

**Conclusion:** *P. triloba* is a potential source of anti-proliferative, antioxidant and iron-chelating agents. Its bioactivities may be attributed to the presence of phenolic constituents.

**Keywords:** Bioactivity, Fern, Phenolics, Anti-proliferative, Anti-oxidant, Iron-chelating, *Phymatopteris triloba*

INTRODUCTION

Fern species growing at high altitudes have attracted little attention in bioprospecting research aimed at discovering novel plant-derived drugs. Highland ferns may be a rich source of bioactive natural products, although evidence substantiating this proposal is still limited.

*Phymatopteris triloba* (Houtt.) Pichi Serm. (Family Polypodiaceae) is a common epiphytic...
fem of tropical mountain forests [1]. The genus *Phymatopteris* is understudied with regards to its bioactive potential. Nevertheless, at least two members of the genus *Phymatopteris* are used in traditional medicine. *P. hastata* (Thunb.) Pichi Serm. is used as a traditional remedy in China for diarrhea, bronchitis, influenza, and other diseases [2]. Antioxidant activities of *P. hastata* extracts and antioxidant phenolic compounds isolated from *P. hastata* were recently reported [2, 3]. On the other hand, *P. quasidivaricata* (Hayata) Pichi Serm. is used in Nepal as a traditional treatment for musculo-skeletal problems and dermatological infections [4]. The therapeutic properties of related species *P. hastata* and *P. quasidivaricata* suggest that *P. triloba* may also have therapeutically relevant bioactivities.

At present, there is no report in the literature regarding the anti-cancer and metal-chelating activities of the genus *Phymatopteris*. Similarly, the superoxide and nitric oxide scavenging potential of the genus *Phymatopteris* is unknown. Moreover, except for *P. hastata* [2,3], nothing is known about the phenolic profiles of other species in the genus *Phymatopteris*. Hence, to fill in gaps in current knowledge about the bioactivity of the genus *Phymatopteris*, we had two objectives in this study; first, to determine the phenolic acid and flavonoid constituents of *P. triloba* leaf and rhizome extracts; second, to assess the anti-proliferative, radical scavenging and iron-chelating activities of the extracts.

**EXPERIMENTAL**

**Plant material**

Specimens of *P. triloba* were collected from Cameron Highlands, Malaysia (site elevation 1495 m) in January 2012. The species of the fern was authenticated by H.-C. Ong of Institute of Biological Sciences, University of Malaya, Malaysia. A voucher specimen (no. TTC01/2012(4)) was kept in a herbarium at the Department of Chemical Science, Universiti Tunku Abdul Rahman.

**Extract preparation**

The sterile fronds and rhizomes of *P. triloba* were cleaned and oven-dried at 45 °C for 72 h. Aqueous extracts were prepared from pulverized oven-dried samples with autoclaved deionized water at a 1:20 (dry weight: volume) ratio. The mixtures were heated at 90 °C for 60 min. The extracts were then clarified by vacuum-filtration and centrifugation at 9000 rpm and 4 °C for 10 min. The supernatant obtained, taken as 50 mg dry matter (DM) of plant powder/ml, was immediately aliquoted (1 ml each) and stored at -20 °C until used.

**Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis**

RP-HPLC analysis was performed with the Shimadzu HPLC system (Shimadzu Co., Kyoto, Japan) equipped with a Shimadzu Prominence SPD-20A UV/Vis Detector. Chromatographic separations were performed using a Phenomenex Gemini 5U C-18 110A column (150 × 4.6 mm)(Phenomenex, Torrance, CA, USA). The binary solvent system described by Kaisoon et al. [5] was used with some modifications. The mobile phase consisted of deionized water with acetic acid (pH 2.8) as solvent A and acetonitrile as solvent B. The gradient elution program was as follows: 0 – 5 min, 5 – 9 % B; 5 – 15 min, 9 % B; 15 – 22 min, 9 – 11 % B; 22 – 38 min, 11 – 18 % B; 38 – 43 min, 18 – 23 % B; 43 – 44 min, 23 – 90 % B; 44 – 45 min, 90 – 95 % B; 45 – 50 min, 95 – 100 % B; 55 – 60 min, 100 – 5 % B. Between individual runs, the column was equilibrated using 5 % solvent B for 20 min. The operating conditions were column temperature of 38 °C and injection volume of 20 μl. UV/Vis detection was carried out at 280 nm for hydroxybenzoic acids, 320 nm for hydroxycinnamic acids and 370 nm for flavonoids at a flow rate of 0.8 ml/min. Identification and quantification of phenolic compounds in the samples was carried out by comparing their retention times and peak areas with those of pure standard compounds using an external standard method.

**Determination of anti-proliferative activity**

Human cervix cancer cell line (HeLa) and human chronic myelogenous leukemia cell line (K562) were cultured in RPMI-1640 medium supplemented with 10 % fetal bovine serum and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere of 5 % CO₂. The anti-proliferative effects of the extracts were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [6]. Cells were plated in 96-well plates at a concentration of 1x10⁴ cells per well in 200 μl of medium. The cells were treated with different concentrations of extracts and 5-fluorouracil (125, 250 and 500 μg/ml). Following a 72-h incubation period, 20 μl of 5 mg/ml MTT solution was added and the mixture was incubated for 4 h. The supernatant was aspirated and MTT formazan crystals in the wells were dissolved in 100 μl of dimethyl sulfoxide. Absorbance was measured at 550 nm by using a microplate.
reader. Anti-proliferative activity (P) was calculated as in Eq 1.

\[ P(\%) = \{1 - (A_s / A_c)\} \times 100 \]  

where \(A_c\) is the absorbance of the control well (untreated cells) and \(A_s\) is the absorbance of the experimental well (cells treated with an extract).

**Determination of superoxide anion radical scavenging activity**

Superoxide anion radical scavenging activity was determined as described in [7] with modifications. A reaction mixture containing 0.8 ml of potassium phosphate buffer (100 mM, pH 7.4), 0.1 ml of extract, 0.1 ml of nitroblue tetrazolium (0.78 mM), 0.1 ml of NADH (2.34 mM), and 50 \(\mu\)l of phenazine methosulfate (60 \(\mu\)M) was incubated for 20 min in darkness. Then, the absorbance of the mixture was read at 560 nm. A reaction blank was prepared for each measurement by replacing NADH with water. Ascorbic acid was used as the positive control. Superoxide scavenging activity (S) was calculated as in Eq 2.

\[ S(\%) = \{1 - (A_s / A_c)\} \times 100 \]  

where \(A_c\) is the absorbance of control reaction (without extract) and \(A_s\) is the absorbance in the presence of an extract. EC\(_{50}\), defined as extract or positive control concentration needed to achieve 50% scavenging activity, was calculated by means of linear regression analysis.

**Data Analysis**

All experiments were carried out in triplicate and data are presented as mean ± standard error. Data were analyzed using Microsoft Excel 2003. Comparison of mean pair values was performed using Student’s t-test at 0.05 level of probability.

**RESULTS**

HPLC profiles of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids

The abundance of three selected hydroxybenzoic acids, four hydroxycinnamic acids, and three flavonoids in the aqueous extracts of *P. triloba* leaves and rhizomes were assessed. The phenolic compounds in the extracts were identified by comparing the retention times of their peaks in the chromatograms with those of pure commercial standards. Among the hydroxybenzoic acids, protocatechuic, p-hydroxybenzoic and gallic acids were all detected in rhizome extract (Table 1). By contrast, only protocatechuic acid was found in the leaf extract. Notably, rhizome extract had 1.9-fold more protocatechuic acid (\(p<0.05\)) than the leaf extract. Among the four hydroxycinnamic acids, caffeic, p-coumaric, and ferulic acids were not detected in the leaf or rhizome extracts. Sinapic acid was detected in the leaf extract but not in rhizome extract. Among the flavonoids analyzed, only myricetin was detected in leaf and rhizome extracts. Notably, myricetin content of the leaf extract was 3.7-fold greater than that in the rhizome extract (\(p<0.05\)).
Table 1: Contents of selected hydroxybenzoic acids, hydroxycinnamic acids, and flavonoids in leaf and rhizome extracts

<table>
<thead>
<tr>
<th>Extract</th>
<th>Hydroxybenzoic acids (µg/g DM)</th>
<th>Hydroxycinnamic acids (µg/g DM)</th>
<th>Flavonoids (µg/g DM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protocatechuic acid</td>
<td>p-hydroxybenzoic acid</td>
<td>Gallic acid</td>
</tr>
<tr>
<td>Leaf</td>
<td>83.1 ± 3.5</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Rhizome</td>
<td>154.7 ± 12.6</td>
<td>34.6 ± 5.4</td>
<td>18.9 ± 10.0</td>
</tr>
</tbody>
</table>

n.d., not detected. Data are presented as mean ± standard errors (n = 3).

Anti-proliferative activity

The leaf and rhizome extracts exerted concentration-dependent anti-proliferative activity against HeLa (Fig 1A) and K562 (Fig 1B) cell lines. Treatment of HeLa cells with 500 µg DM/ml of leaf and rhizome extracts produced 42% and 39% anti-proliferative activity respectively, comparable to that of 5-fluorouracil. By contrast, both extracts produced about 30% anti-proliferative activity, which was about half of that of 5-fluorouracil, on K562 cells. As the anti-proliferative activities of the extracts did not exceed 50%, we did not compute the EC<sub>50</sub> values for the extracts.

Superoxide and nitric oxide scavenging activity

The leaf and rhizome extracts exhibited superior or similar superoxide scavenging activities in comparison with ascorbic acid (Fig 2). Over the range of extract concentrations tested, the leaf extract also acted as a stronger superoxide scavenger than rhizome extract. At 1.5 mg DM/ml, scavenging activities of the leaf and rhizome extracts were 1.8-fold and 1.4-fold higher (p < 0.05), respectively, compared with ascorbic acid. The EC<sub>50</sub> of leaf extract, rhizome extract and ascorbic acid were 0.85, 1.43 and 1.63 mg/ml, respectively.

Iron-chelating activity

The leaf extract (EC<sub>50</sub> 15.8 mg/ml) was less potent than the rhizome extract (EC<sub>50</sub> 9.4 mg/ml) with respect to iron-chelating activity. At 5 mg DM/ml, the iron-chelating activity of rhizome extract was 2.8-fold higher (p<0.05) than that of leaf extract (Fig 4). At 20 mg DM/ml, the iron chelating activity of the rhizome extract was 1.6-
fold higher \((p<0.05)\) compared with leaf extract. However, the iron-chelating activities of leaf and rhizome extracts were lower compared with EDTA, the positive control \((EC_{50} \, 0.01 \, mg/ml)\).

In this study, selected hydroxybenzoic and hydroxycinnamic acids as well as flavonoids with therapeutic and/or nutritional potential were quantified in the leaf and rhizome extracts of \(P. \) triloba. Our results reveal that the rhizome extract is a richer source of protocatechuic, gallic and \(p\)-hydroxybenzoic acids than the leaf extract. Protocatechuic and gallic acids were previously detected in \(P. \) hastata, a related species used in Traditional Chinese Medicine [2,3]. Rutin was detected in organic solvent extracts of \(P. \) hastata by HPLC analysis [2], but we did not detect any rutin in the aqueous extracts of \(P. \) triloba leaves and rhizomes. Whether this reflects an interspecies difference or discrepancy resulting from different extraction methodologies is unclear and can be clarified when more data become available. Importantly, protocatechuic, \(p\)-hydroxybenzoic and gallic acids as well as myricetin, which we detected in \(P. \) triloba extracts, are also the main phenolic constituents found in medicinal plants with anticancer properties [10].

The leaf extract had a higher superoxide scavenging activity compared with rhizome extract. This correlates with the greater abundance of sinapic acid and myricetin in the leaf extract as the two phenolic constituents are capable of scavenging superoxide [11,12]. However, besides sinapic acid and myricetin, protocatechuic and gallic acids are also superoxide scavengers [13,14]. On the other hand, the relative iron-chelating ability between the leaf and rhizome extracts correlate with their relative abundance of protocatechuic, \(p\)-hydroxybenzoic and gallic acids. Among these three phenolic compounds, protocatechuic and gallic acids are capable of chelating metal irons [15]. However, sinapic acid [16] and myricetin [17] are also potent metal chelators. Hence, the possibility of multiple phenolic constituents detected in these extracts, including hydroxybenzoic and hydroxycinnamic acids as well as flavonoids, working in concert to scavenge superoxide radicals and chelating iron cannot be ruled out. Similar levels of anti-proliferative and NO scavenging activities of the leaf and rhizome extracts apparently did not correlate with the relative abundance of any single phenolic acid or flavonoid detected in the first report of the phenolic profiles of \(P. \) triloba as well as its multiple bioactivities as an anti-proliferative, radical scavenging and metal chelating agent. Notably, this is also the first report of anti-proliferative and iron-chelating potential as well as detection of \(p\)-hydroxybenzoic acid, myricetin and sinapic acid in the genus Phymatopteris.
extracts. Gallic, protocatechuic and sinapic acids as well as myricetin detected in the leaf and rhizome extracts are known to have anticancer properties [18].

Meanwhile, myricetin [19] and protocatechuic acid [20] are capable of scavenging NO in vitro. Hence, it is likely that the anti-proliferative and NO scavenging activities of both extracts are dependent on the additive or synergistic effects of multiple phenolic constituents. This possibility may be tested in future by exposing of the same cancer cell lines to a combination of pure compounds of the phenolic constituents detected in this study.

Our findings on the NO scavenging activity of the extracts have two implications. Firstly, it suggests that assessment of radical scavenging abilities of plant extracts should be based on multiple assays as such abilities may vary depending on the assay systems used. In this study, the superoxide scavenging assay indicates that the leaf extract was a better radical scavenger compared with rhizome extract. However, the NO scavenging assay found both extracts to be similarly effective as radical scavengers. Secondly, the ability of leaf and rhizome extracts to concurrently scavenge NO and superoxide radicals suggests that these extracts can be an inhibitor of peroxynitrite formation. Peroxynitrite radicals are products of reaction between superoxide and NO. Peroxynitrite formation in vivo is associated with pathological conditions such as stroke, myocardial infarction, and chronic heart failure. Our results therefore suggest that P. triloba is a potential source of therapeutic agents for the treatment of such peroxynitrite-associated pathological conditions.

CONCLUSION

The findings of this study show that P. triloba, a highland fern, is a potential source of superoxide and NO scavengers, iron chelators and anticancer agents. Although the chelating and anticancer properties of the extracts are inferior to the pure compounds used as positive controls, the extracts are considered potent as they were only crude aqueous extracts. Importantly, even as crude extracts, P. triloba extracts exhibited superior or similar superoxide-scavenging potential compared to ascorbic acid. Thus, these findings suggest that highland ferns may be promising sources of bioactive natural products. The bioactivity of highland ferns should, therefore, be further explored. In addition, the in vivo effects of the fern also need to be established.

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REFERENCES


