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Original Research Article

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), 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 (EC_{50}) 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*

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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

fern 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 ^{o}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 ^{o}C in a humidified atmosphere of 5 % CO_2. 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 $1x10^4$ 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.

$$P\ (\%) = \{1- (A_s /A_c)\}100 \ldots\ldots\ldots\ldots (1)$$

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 μl of phenazine methosulfate (60 μM) 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.

$$S\ (\%) = \{1- (A_s /A_c)\}100 \ldots\ldots\ldots\ldots (2)$$

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.

Determination of nitric oxide scavenging activity

Nitric oxide (NO) scavenging activity was determined as previously described [8] with modifications. A mixture of 0.8 ml of extract and 0.2 ml of sodium nitroprusside (5 mM in phosphate buffered saline, pH 7.4) was incubated at room temperature for 150 min under light source (24 W compact fluorescent light bulb). Then, 0.6 ml of the mixture was transferred to a new tube containing 0.6 ml of Griess reagent (1% sulphanilamide and 0.1% N-(1-naphthyl)ethylenediamine dihydrochloride in 5% phosphoric acid). This mixture was allowed to stand in darkness for 10 min before absorbance was read at 546 nm. A reaction blank was prepared for each measurement by replacing Griess reagent with water. Ascorbic acid was used as the positive control. NO scavenging activity was calculated as described for the superoxide scavenging assay above.

Determination of iron-chelating activity

Iron-chelating activity of the extract was evaluated based on its ability to interfere with the formation of ferrozine-Fe^{2+} complex. A previously described assay protocol [9] was followed, using disodium salt of EDTA as the positive control. Iron-chelating activity (R) was calculated as in Eq 3.

$$R\ (\%) = \{1- (A_s /A_c)\}100 \ldots\ldots\ldots\ldots (3)$$

where A_c is the absorbance of control reaction (without extract) and A_s is the absorbance in the presence of an extract. Disodium salt of EDTA was used as the positive control. EC_{50}, defined as extract or positive control concentration needed to achieve 50% chelating 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

Extract	Hydroxybenzoic acids (μg/g DM)		
	Protocatechuic acid	*p*-hydroxybenzoic acid	Gallic acid
Leaf	83.1 ± 3.5	n.d.	n.d.
Rhizome	154.7 ± 12.6	34.6 ± 5.4	18.9 ± 10.0

Extract	Hydroxycinnamic acids (μg/g DM)			
	Sinapic acid	Caffeic acid	*p*-coumaric acid	Ferulic acid
Leaf	6.6 ± 1.3	n.d.	n.d.	n.d.
Rhizome	n.d.	n.d.	n.d.	n.d.

Extract	Flavonoids (μg/g DM)		
	Myricetin	Rutin	Quercetin
Leaf	98.5 ± 25.0	n.d.	n.d.
Rhizome	26.7 ± 1.5	n.d.	n.d.

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

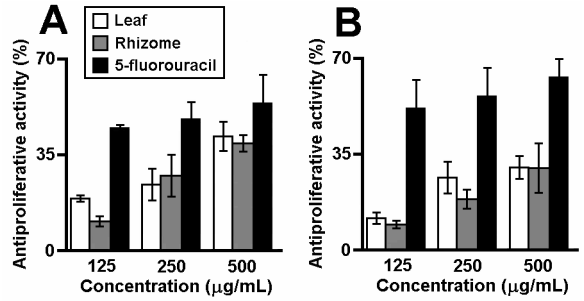

Fig 1: Anti-proliferative activity of leaf and rhizome extracts on HeLa (**A**) and K562 (**B**) cell lines, compared with anticancer drug 5-fluorouracil; data are mean ± SE (n = 3).

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_{50} 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_{50} of leaf extract, rhizome extract and ascorbic acid were 0.85, 1.43 and 1.63 mg/ml, respectively.

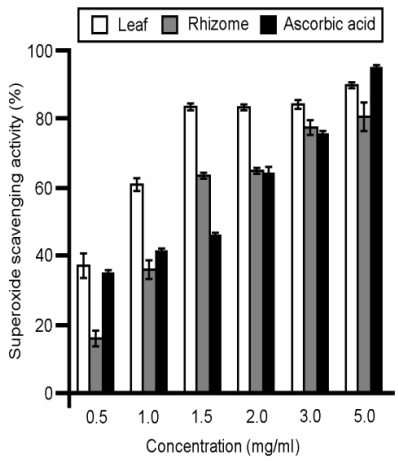

Fig 2: Superoxide anion radical scavenging activities of leaf and rhizome extracts, compared with ascorbic acid. Data are mean ± SE (n = 3).

NO scavenging activities of the leaf and rhizome extracts were similar over the range of extract concentrations tested, both lower compared with ascorbic acid (Fig 3). At 5 mg DM/ml, the NO scavenging activity of both extracts was about 30% lower than that of ascorbic acid ($p<0.05$). Due to a lack of strong linear trends in the extract data, EC_{50} values were not computed for NO scavenging activities.

Iron-chelating activity

The leaf extract (EC_{50} 15.8 mg/ml) was less potent than the rhizome extract (EC_{50} 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).

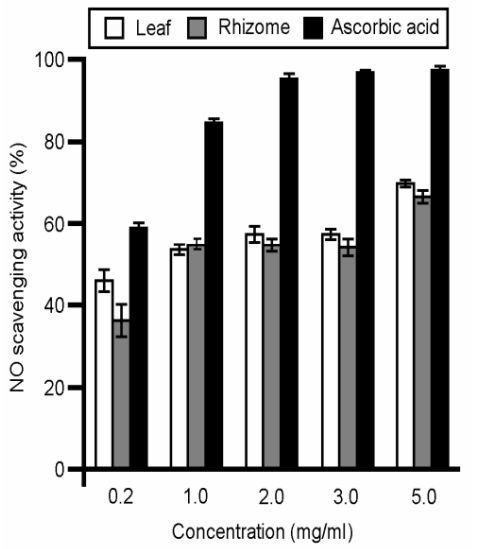

Fig 3: Nitric oxide (NO) scavenging activities of leaf and rhizome extracts, compared with ascorbic acid. Data are mean ± SE (n = 3).

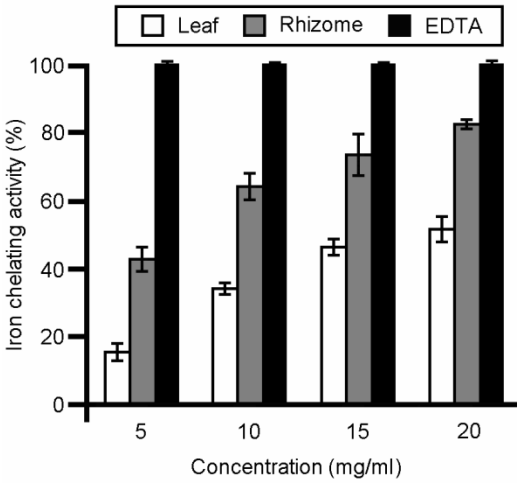

Fig 4: Iron-chelating activities of leaf and rhizome extracts, compared with EDTA. Data are mean ± SE (n = 3).

DISCUSSION

Our results show that the aqueous extracts of the leaves and rhizomes of *P. triloba* have quantitatively and qualitatively different phenolic profiles. Both extracts were similarly effective as anti-proliferative agents and NO scavengers, but the rhizome extract was superior to the leaf extract as an iron-chelating agent. Importantly, both extracts exhibited superior or similar superoxide scavenging activities when compared with ascorbic acid. To our knowledge, this is 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*.

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 inter-species 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

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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