Antioxidative, Tyrosinase Inhibiting and Antibacterial Activities of Leaf Extracts from Medicinal Ferns

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Leaf extracts of five medicinal ferns, Acrostichum auremum L. (Pteridaceae), Asplenium nidus L. (Aspleniaceae), Blechnum orientale L. (Blechnaceae), Cibotium barometz (L.) J. Sm. (Cyatheaceae) and Dicranopteris linearis (Burm.) underwood var. linearis (Gleicheniaceae), were investigated for their total phenolic content (TPC), and antioxidative, tyrosinase inhibiting and antibacterial activities. The antioxidative activity was measured by assays for radical scavenging against 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric ion reducing power (FRP), beta-carotene bleaching (BCB) and ferrous ion chelating (FIC). The results revealed B. orientale to possess the highest amount of total polyphenols and strongest potential as a natural antioxidative, tyrosinase inhibiting and antibacterial agent as demonstrated by its strong activities in all related bioassays. The other ferns with antioxidative potential were C. barometz and D. linearis. Except for A. aureum, all ferns showed antibacterial activity which may justify their usage in traditional medicines.

Key words: antioxidative; antimicrobial; tyrosinase inhibition; fern; polyphenol

The search for rich sources for bioactivity, namely antioxidative, tyrosinase inhibiting and antimicrobial property, has been escalating in recent years due to the increasing awareness of preventive healthcare and the rise of drug-resistant bacterial strains. The consumption of antioxidants has been associated with a reduced risk of the incidence of many oxidative diseases ranging from cardiovascular disorders, diabetes mellitus, cancer, rheumatic arthritis to aging.1) The use of synthetic antioxidants such as butylated hydroxytoluene, butylated hydroxyanisole, tert-butyldihydroquinone and propyl gallate has been negatively perceived by consumers due to safety and health effects.2) Tyrosinase inhibitors have important usage for the treatment of skin hyperpigmentation, as whitening agents in cosmetics and for preventing the browning process in the food industry.3,4) Synthetic inhibitors used by the food industry such as sulfites have been banned by FDA due to safety reasons.5) In recent years, a number of antibiotics have lost their effectiveness due to the development of resistant strains. In addition to this problem, antibiotics are sometimes associated with adverse effects including hypersensitivity, immune-suppression and allergic reactions.6) Hence, this has led to the need to develop new antibiotics from natural sources such as plants.

While it is estimated that less than half of 1 percent of the 265,000 flowering species on the globe has been exhaustively studied for their medicinal value,7) much less work has been done on non-flowering plants such as ferns. Ferns play an important role in folklore medicine. There have been numerous reports on the ethnomedical uses of ferns.8–10) However, comprehensive studies on the bioactivities of ferns have been scarce.

In this study, our main objective was to investigate the potential use of five medicinal ferns (listed in Table 1) as natural sources of antioxidants, tyrosinase inhibitors and antimicrobial agents. Despite varied uses in traditional medicine, there is no published report on the antioxidative activity, tyrosinase inhibiting and antimicrobial properties of these ferns. The results of this study may provide the scientific bases to justify their use in traditional medicines.

Materials and Methods

Chemicals and reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), trichloroacetic acid, β-carotene, quercetin, mushroom tyrosinase, and L-DOPA were purchased from Sigma. Folin-Ciocalteu’s phenol reagent, Tween-40, linoleic acid and gallic acid were obtained from Fluka. t-ascorbic acid was purchased from Merck. Ferrozine and potassium ferricyanide were purchased from Acros Organics. Iron (III) chloride-6-hydrate and chloroform (HPLC grade) were obtained from Fischer Scientific. Paper discs (6 mm), Mueller-Hinton agar, nutrient broth and streptomycin susceptibility discs were obtained from Oxoid. All other chemicals and solvents were of analytical grade.

Plant materials. Blechnum orientale L. and Cibotium barometz (L.) J. Sm. were obtained from Putrajaya Botanical Garden, Kuala Lumpur. Acrostichum aureum L., Asplenium nidus L. and Dicranopteris linearis (Burm.) underwood var. linearis were collected from their natural habitats in the vicinity of Kuala Lumpur. All collected ferns were authenticated by S. Anthonysamy, a plant taxonomist formerly from Universiti Putra Malaysia and currently with the landscape consulting firm, Aroma Tropic Limited, Kuala Lumpur. The voucher specimens labeled in Table 1 have been deposited in the herbarium Monash University Sunway Campus.

Preparation of the plant extracts. For the total phenolic content and antioxidant tests, 1 g of fresh ferns was pulverized in liquid nitrogen and extracted with 50 ml of methanol continuously for an hour in a rotary orbital shaker. The extract was filtered and stored at −20 °C to

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Abbreviations: AEAC, ascorbic acid equivalent antioxidative capacity; BCB, beta-carotene bleaching; DMSO, dimethylsulfoxide; DOPA, 3,4-dihydroxyphenylalanine; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DR, degradation rate; FIC, ferrous ion chelating; FRP, ferric ion reducing power; IC₅₀, concentration for 50% inhibition; GAE, gallic acid equivalent; MID, minimum inhibitory dose; ROS, reactive oxygen species; TPC, total phenolic content

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Table 1. Medicinal Ferns Investigated

<table>
<thead>
<tr>
<th>Fern (family)</th>
<th>Voucher no.</th>
<th>Common name</th>
<th>Traditional use</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrostichum aureum (Pteridaceae)</td>
<td>LAA020</td>
<td>Golden leather fern</td>
<td>Ulcers, boils, wounds, worms and bladder complaints$^8$</td>
</tr>
<tr>
<td>Asplenium nidus (Aspleniaceae)</td>
<td>LAA018</td>
<td>Bird’s nest fern</td>
<td>Depurative, sedative, easing labor and chest pain$^9$</td>
</tr>
<tr>
<td>Blechnum orientale (Blechnaceae)</td>
<td>LAA007</td>
<td>Centipede fern</td>
<td>Antihelminthic, boils, blisters, abscesses and sores$^9$</td>
</tr>
<tr>
<td>Cibotium barometz (Cyatheaeeae)</td>
<td>LAA009</td>
<td>Scythian lamb</td>
<td>Curing fainting, wounds and ulcers, coughs, anti-rheumatic, kidney and liver tonic$^9$</td>
</tr>
<tr>
<td>Dicranopteris linearis (Gleicheniaceae)</td>
<td>LAA003</td>
<td>Old world forked fern</td>
<td>Controlling fever, wounds and ulcers, anti-helminthic, treating asthma$^{10}$</td>
</tr>
</tbody>
</table>

be used within one week. For the tyrosinase inhibition and antimicrobial tests, 10.00 to 15.00 g of leaves was used. After being pulverized in liquid nitrogen, 100 ml of methanol was added and the mixture was shaken for an hour. Each extract was filtered. The residue was repeatedly extracted until the filtrate was light coloured. The filtrates were pooled and the solvent evaporated. The extract was then freeze-dried.

Total phenolic content (TPC). TPC in each extract was determined by the procedure described by Lim et al.$^{11}$ The extract solution (0.3 ml, in triplicate) was mixed with 1.5 ml of 10% Folin-Ciocalteau’s reagent and 1.2 ml of 7.5% (w/v) sodium carbonate. The mixture was kept in the dark for 30 min, before absorbance was measured at 765 nm. The gallic acid standard curve used was $y = 0.01078x$ ($R^2 = 0.9996$), where $y$ is the absorbance at 765 nm and $x$ is the concentration of gallic acid in mg/l. TPC is expressed as mg of gallic acid equivalent (GAE)/100 g of fresh leaves.

DPPH radical scavenging activity. The method described previously was employed.$^{11}$ Various dilutions of the extract solution (1.0 ml, in triplicate) were added to 2.0 ml of DPPH (5.9 mg/100 ml methanol). The mixture was left in the dark for 30 min, before reading the absorbance at 517 nm, with methanol as blank. The percentage radical scavenging activity was calculated as follows:

$$\text{scavenging} \% = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100$$

where $A_{\text{control}}$ is the observed absorbance and $A_{\text{sample}}$ is the concentration of gallic acid in mg/l. TPC is expressed as mg of gallic acid equivalent (GAE)/100 g of fresh leaves.

Ferrous ion chelating (FIC) activity. The procedure has been described previously.$^{11}$ Various dilutions of the extract solution (1.0 ml, in triplicate) were added to 1.0 ml of 0.1 mM FeSO$_4$ and 1.0 ml of 0.25 mM ferrozine. The tubes were shaken well and left to stand for 10 min. The absorbance was measured at 562 nm, against a blank containing water in place of ferrozine. The control consisted of water in place of the extract. The ability of a sample to chelate ferrous ion was calculated as follows: chelating effect (％) = (A_{control} - A_{sample})/A_{control} × 100

Tyrosinase inhibition activity. The method employed by Masuda et al. was followed with a slight modification.$^{11}$ A 96-well microtitre plate was used. Each well consisted of 40 μl of test sample (2.5 mg/ml, dissolved in 50% dimethylsulfoxide), 80 μl of a 0.1 m potassium phosphate buffer at pH 6.8, 40 μl of 0.02 mg/ml of tyrosinase and 40 μl of 2.5 mM of l-DOPA. After 30 min, the absorbance was read with Biotek PowerWave XS microplate scanning spectrophotometer at 492 nm, with the reference wavelength at 700 nm. Each sample had a blank consisting of all components except l-DOPA. Results were compared with a control consisting of 50% DMSO in place of the sample. The percentage tyrosinase inhibition was calculated as follows: tyrosinase inhibition (％) = (A_{control} - A_{sample})/A_{control} × 100. Kojic acid and quercetin were used as standards. The standard curve for kojic acid has the equation $y = 1277.1x$ ($R^2 = 0.9932$), while that using quercetin has the equation $y = 137.45x$ ($R^2 = 0.9813$), where $y$ is the observed absorbance and $x$ is the concentration of the standard in mg/ml. Results were expressed as mg of kojic acid/g of extract and as mg of quercetin/g of extract.
Results and Discussion

Total phenolic content

The total polyphenol content (TPC) was determined by using the Folin-Ciocalteu reagent. An initial test to determine the efficiency of TPC extraction using methanol was carried out on two of the ferns (D. linearis and A. nidus) with 100% methanol, 70% methanol and 50% methanol. Methanol has been reported to be a suitable solvent for the extraction of polyphenols from fresh plants due to its ability to inhibit the action of polyphenol oxidases which could affect the antioxidative activity and to its ease of evaporation.13) There was no significant difference in TPC of the extracts when different ratios of methanol was used. In addition, the first extraction using 100% methanol revealed a good recovery of 83% ± 2% TPC. Hence 100% methanol was used as the solvent for extraction of all the ferns investigated.

As listed in Table 2, the order of the medicinal ferns in decreasing TPC (in mg of GAE/100 g) was B. orientale (2095) > D. linearis (2023) > C. barometz (1589) > A. aureum (945) > A. nidus (305). Phenolic compounds are generated by plants in response to environmental stress. It has been reported that light stimulated the synthesis of flavonoids, especially anthocyanins and flavones, via phenylalanine ammonia lyase,13) and phenolics are thought to provide a means of protection against UV-B damage and subsequent cell death by protecting DNA from dimerization and breakage.14) Therefore, plants in high-mountain areas which are exposed to stress factors such as low air temperature, decreased partial O2 pressure, increased UV radiation and unfavorable water regime generally have an increased accumulation of polyphenols.15) This partially explains the exceptionally high TPC values for B. orientale, C. barometz and D. linearis as they grow well in habitats of exposed sunlight and on slopes or in mountain regions with an altitude up to 1500–1700 m. Salinity is the primary environmental stress factor for the mangrove plant, and hence high TPC was expected for A. aureum. However, A. aureum showed moderate TPC (945 mg of GAE) which could have been due to the shade-tolerant nature of this fern which helps to reduce the rate of evaporation and hence the salt stress encountered by A. aureum.16) A similar trend was observed with A. nidus, an epiphyte, which showed very low TPC due to its natural habitat on trees, and hence a lower stress factor.

Antioxidative activities

The antioxidative activities were assessed by four different methods: DPPH radical scavenging, ferric ion reducing power (FRP), beta-carotene bleaching (BCB) and ferrous ion chelating (FIC). The results of the DPPH radical scavenging and ferric ion reducing power (FRP) assays are summarized in Table 2. The order of the fern extracts in these antioxidative activities was similar to that for TPC, i.e., B. orientale > D. linearis > C. barometz > A. aureum > A. nidus. Strong correlations of TPC with AEAC (R2 = 0.9731) and FRP values (R2 = 0.9468) indicate that the scavenging and reducing power of the plant extracts were due to the phenolic compounds. This trend is in agreement with those reported in earlier studies.2,17) The antioxidative activity in terms of its ability to inhibit lipid peroxidation was measured by using a BCB assay. Figure 1 shows that, at the highest concentration, the BCB antioxidative activity decreased in the order D. linearis (99%) > B. orientale (73%) > C. barometz (69%) > A. aureum (51%) > A. nidus (47%). The poor correlation between TPC and BCB antioxidative activity (R2 = 0.4989) has also been previously reported.11,17) The variation of the BCB activity as compared to the DPPH and FRP activities may be associated with different amounts of lipophilic antioxidants in the plants. The lipophilic antioxidant molecules would partition more into the oily droplets of the β-carotene/linoleic acid system as measured in the BCB assay.11) The results of the chelating activity assay are summarised in Fig. 2. This assay measured the ability of an extract to act as a secondary antioxidant which helps to prevent the generation of the hydroxyl radical via Fenton’s reaction. In contrast with the trend observed in the previous antioxidative assays, all ferns showed very low chelating activity (<22%), except for A. aureum (58% at a concentration of 6.7 mg/ml). No correlation was found between TPC and FIC (R2 = 0.2228). A similar trend was observed in a previous study.17) The results imply that the phenolic compounds in B. orientale and D. linearis were poor ion chelators.
between TPC and tyrosinase inhibition (strong antioxidative activity. Poor correlation was seen activity (35% and 23%, respectively) despite possessing barometz and medicinal plants (8.3–56.8%). It has been indicated that strong antioxidants might have strong antioxidative, tyrosinase inhibiting and antibacterial activities in ferns although they are strong radical scavengers, reductants and inhibitors of lipid peroxidation.

Tyrosinase inhibition
Tyrosinase inhibition activity was studied by using the dopachrome test, with quercetin and kojic acid as standards. Amongst the ferns, only B. orientale possessed good tyrosinase inhibition activity (51%, equivalent to 143 mg of quercetin/g of extract and 15 mg of kojic acid/g of extract; Table 3). The activity reported is comparable to that found with the leaf extracts of some tropical plants (2–77%), seashore plants (6.8–60.8%) and medicinal plants (8.3–56.8%). It has been indicated that strong antioxidants might have strong tyrosinase inhibition activity. However, our findings for C. barometz and D. linearis showed weak tyrosinase activity (35% and 23%, respectively) despite possessing strong antioxidative activity. Poor correlation was seen between TPC and tyrosinase inhibition (R² = 0.2412). An analysis of the relationship between the tyrosinase inhibition activity and antioxidant data showed moderate correlation (0.4349) only with FRP. This may suggest a link between tyrosinase inhibitors and antioxidants possessing reducing capacity.

Table 3. Tyrosinase Inhibition Activity

<table>
<thead>
<tr>
<th>Fern</th>
<th>Inhibition (%)</th>
<th>mg of quercetin/g</th>
<th>mg of kojic acid/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. aureum</td>
<td>33.3 ± 4.4</td>
<td>91.3 ± 7.2</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>A. nidus</td>
<td>21.9 ± 6.3</td>
<td>63.7 ± 18.0</td>
<td>6.8 ± 1.9</td>
</tr>
<tr>
<td>B. orientale</td>
<td>50.7 ± 6.0</td>
<td>143.2 ± 21.9</td>
<td>15.4 ± 2.4</td>
</tr>
<tr>
<td>C. barometz</td>
<td>35.0 ± 1.3</td>
<td>102.0 ± 3.7</td>
<td>11.0 ± 0.4</td>
</tr>
<tr>
<td>D. linearis</td>
<td>23.2 ± 1.3</td>
<td>67.4 ± 3.9</td>
<td>7.2 ± 0.4</td>
</tr>
</tbody>
</table>

Table 4. Antimicrobial Activity: Inhibition Zone Diameter, Percentage Inhibition and Minimum Inhibitory Dose

<table>
<thead>
<tr>
<th>Fern</th>
<th>Inhibition zone diameter in mm²/1 mg per disc (%) inhibition compared with streptomycin</th>
<th>Minimum inhibitory dose (MID) (µg per disc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BC⁺</td>
<td>ML⁺</td>
</tr>
<tr>
<td>A. aureum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. nidus</td>
<td>7.3 (49)</td>
<td>—</td>
</tr>
<tr>
<td>B. orientale</td>
<td>8.5 (57)</td>
<td>11.0 (48)</td>
</tr>
<tr>
<td>C. barometz</td>
<td>8.0 (53)</td>
<td>—</td>
</tr>
<tr>
<td>D. linearis</td>
<td>8.7 (58)</td>
<td>10.0 (43)</td>
</tr>
<tr>
<td>Streptomycin¹</td>
<td>15.0</td>
<td>23.0</td>
</tr>
</tbody>
</table>

¹Microorganisms: BC, Bacillus cereus; ML, Micrococcus luteus; SA, Staphylococcus aureus; EC, Escherichia coli; PA, Pseudomonas aeruginosa; SC, Salmonella cholerasuis; EA, Enterobacter aerogenes; KP, Kluyverella pneumoniae.

²Each value is the mean of triplicate measurements of the inhibition zone diameter (mm) including the diameter of disc (6 mm). A ‘dash’ indicates no inhibition.

³Positive control: Streptomycin, 10µg; negative control containing only the solvent did not show any activity.

Antimicrobial test
Ferns have been regarded as being particularly resistant to plant pathogens. Investigations on some ferns, particularly those commonly used for the treatment of skin ailments and wounds, have confirmed their antimicrobial properties. Table 4 shows the results of screening of the antibacterial activity of the fern extracts at a dose of 1 mg/disc. All ferns, except A. aureum, showed antibacterial activity against at least one test bacterium. The ranking of the antibacterial activity based on the number of test bacteria inhibited was A. nidus (4) > D. linearis (3) > B. orientale (3) > C. barometz (2). A. nidus showed a broad spectrum of antimicrobial activity against two Gram-positive (B. cereus and S. aureus) and two Gram-negative bacteria (E. coli and P. aeruginosa), with the strongest inhibition against P. aeruginosa (74% inhibition compared to streptomycin; MID 200µg/disc). It is apparent that Gram-negative bacteria were more resistant to the extracts of the ferns, except for A. nidus. The insensitivity of the fern extracts against Gram-negative bacteria is presumably due to the impermeable nature of the outer membrane of the bacteria. These findings are consistent with the observations of previous screening of medicinal plants, with most of the active plant extracts showing activity against Gram-positive strains only. D. linearis showed the strongest activity against S. aureus (73% inhibition, MID 125µg/disc) and B. cereus (58% inhibition, MID 125µg/disc). The highest activity against M. luteus was demonstrated by B. orientale (48% inhibition, MID 500µg/disc). MID reported here is comparable with the figures reported for the methanolic extracts of other medicinal plants: 1000µg/disc from Garcinia atroviridis leaves and 16 to >1000µg/disc from roots and rhizomes of some Zingiberaceae species. It should be noted that although the values for inhibition are lower than for streptomycin, these results nevertheless imply good antibacterial potential in the ferns, since they had been obtained with crude extracts and showed more than half the inhibition value of streptomycin.

Our results for the antibacterial assays justify and support the popular usage of the ferns. S. aureus and M. luteus are known to cause skin and soft tissue infections. These bacteria were susceptible to the fern extracts which justifies the traditional use of these ferns for the treatment of wounds, blisters, sores, ulcers and boils. The use of A. nidus to treat chest pain can be partially supported by the observation that the extract...
inhibited \textit{P. aeruginosa} which is known to cause diseases associated with respiratory infections, especially in immunocompromised patients.\textsuperscript{27}} In addition, the traditional use of \textit{A. nidus} to treat lice infection could be attributed to its broad spectrum of antibacterial activity. The wide medicinal uses of \textit{D. linearis} to relieve fever, to remove intestinal worms, and to alleviate indigestion, ulcers and appendicitis pain may be attributed to its high sensitivity in bacterial inhibition, as shown by its lowest MID values against all Gram-positive bacteria. The inability of \textit{A. aureum} to demonstrate any visible activity against any of the bacteria tested is, however, noteworthy and seems not to justify its traditional use in wound healing. This could be due to a difference in the concentration used. The concentration tested in this study was probably lower than that used by traditional herbal practitioners who may probably apply an extract with no upper limit to its concentration.\textsuperscript{28}

In conclusion, the leaf extracts of \textit{B. orientale} demonstrated the highest potential as a natural agent capable of use as an antioxidant, and for tyrosinase inhibition and antibacterial activity. Amongst the other ferns, \textit{C. barometz} and \textit{D. linearis} have potential as natural antioxidants and antibacterial agents. Further work involving the isolation of active compounds in these potent ferns would be necessary to elucidate the actual source of the observed bioactivities. Work is currently being undertaken for this.

**Acknowledgments**

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**References**
