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SHORT COMMUNICATION

CYTOTOXICITY AND ANTIGLUCOSIDASE POTENTIAL OF SIX SELECTED EDIBLE AND MEDICINAL FERNS

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Globally, bioactive phytochemicals are gaining popularity among consumers, as evidenced by the steady rise in the demand for botanical dietary supplements. One driving force behind this is the perception of herbal medicine being a safe and relatively inexpensive alternative in the management of human diseases (1, 2). There is currently intensive effort worldwide to search for bioactive phytochemicals for potential applications in the development of novel or alternative nutraceutical, cosmetic and pharmaceutical products (3, 4). Phytochemicals with cytotoxic and antiglucosidase properties, for example, can be exploited in the formulation of chemopreventive and antidiabetic drugs (5, 6) as well as in the development of nutraceuticals and functional food (7-9).

Ferns are rich sources of natural products with diverse bioactivities, including anticancer, antibacterial, antioxidant, and antiinflammatory activities (7). Nevertheless, less emphasis has been given to ferns than to other plant groups in bioprospecting research aiming at discovering natural therapeutic or bioactive agents. Many ferns have been traditionally used as remedies for human diseases as well as being consumed as vegetables. Thus, ferns are potential candidates for the discovery of bioactive constituents and bioactivity which can be exploited for the development of nutraceutical, cosmetic and pharmaceutical products (7, 8).

This study focused on six medicinal and edible ferns, namely *Christella arida*, *Christella dentata*, *Cyclosorus interruptus*, *Microsorum punctatum*, *Nephrolepis acutifolia* and *Pleocnemia irregularis*. The leaf and root of *C. arida* are traditionally used to treat dysentery and skin diseases (10). *C. dentata* is an edible fern (11), which is also a folk remedy for skin diseases (12). *C. interruptus* is a medicinal plant used for treating sores, burns, liver diseases, gonorrhea, cough, and malaria (13). *C. interruptus*-derived coumarin derivatives are cytotoxic to human nasopharyngeal carcinoma (KB) cell line and exhibited antibacterial activity (14). Juice extracted from the fronds of *M. punctatum* is used as purgative, diuretic, and wound healing agents (15). *N. acutifolia* and *P. irregularis* are both edible ferns (16, 17). *P. irregularis* is also used to treat diarrhea, skin diseases (18) and weak muscles (19). At present, the anticancer potential of these six ferns, except *C. interruptus*, has not been reported. Neither has the antiglucosidase activity of any of the six ferns been investigated. Information on the polyphenol, hydroxycinnamic acid, flavonoid, and proanthocyanidin contents of the six selected ferns is scarce. Such phytoconstituent classes are known to have important health-promoting and therapeutic effects (7, 9, 20). Hence, our goals were to fill in current gaps of knowledge about the bioactivities of these ferns, in addition to identifying a promising fern species which can be used in the future investigations for the isolation of active compounds. The specific objective of this study was two-fold: first, to assess the cytotoxicity and antiglucosidase activity of the aqueous extracts of the six selected ferns; second, to determine whether such bioactivities can be

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attributed to the contents of phenolics, hydroxycinnamic acids, flavonoids, and proanthocyanidins in the extracts.

MATERIALS AND METHODS

Plant samples

Healthy specimens of six ferns, namely *Christella arida* (D. Don) Holtt (Thelypteridaceae), *Christella dentata* (Forsk.) Brownsey & Jermy (Thelypteridaceae), *Cyclosorus interruptus* (Willd.) H. Ito (Thelypteridaceae), *Microsorum punctatum* (L.) Copel. (Polypodiaceae), *Nephrolepis acutifolia* (Desv.) Christ. (Nephrolepidaceae), and *Pleocnemia irregularis* (C. Presl.) Holtt. (Tectariaceae) were gathered from the countryside of Bidor town, Malaysia, by T.-T. Chai and F.-C. Wong in February 2013. The species of the ferns were authenticated by H.-C. Ong.

Preparation of aqueous extracts

Fern samples were oven-dried at 45°C for 72 h and then ground to powder with a Waring blender. Aqueous extracts were prepared by suspending the fern powder in autoclaved, deionized water at a ratio of 1 : 20 (dry weight : volume). The mixture was incubated in a 90°C water bath for 1 h. The extract was then vacuum-filtered and the filtrate obtained was centrifuged at 7830 rpm at 4°C for 5 min. Next, the supernatant collected was freeze-dried to constant weight. The freeze-dried extract was dissolved in deionized water to prepare aliquots of 50 mg/mL, which were then stored at -20°C until further use.

Cytotoxicity and antiglucosidase activity

The cytotoxic activity of the fern extracts was assessed by conducting a methylthiazol tetrazolium (MTT) assay using a human chronic myelogenous leukemia cell line (K562) as previously described (21). Among the six selected ferns, the cytotoxicity of only *C. interruptus* was previously investigated (14). The cytotoxic effects of these ferns on a leukemia cell line have never been reported in the literature. More importantly, this choice of cell line was in line with our long-term research interest to identify cytotoxic compounds from ferns which are useful for the development of therapeutic agents against leukemia. 5-Fluorouracil (5FU), an anticancer drug, was used as the positive control. EC_{50} value, defined as the extract concentration required for achieving 50% cytotoxic activity, was determined by using linear regression analysis.

Glucosidase inhibitory activity was determined as described in (22). Myricetin and acarbose were used as the positive controls. The effectiveness of myricetin as an inhibitor of yeast and mammalian α-glucosidases has been established (23). Acarbose is an oral antihyperglycemic drug with antiglucosidase activity (24). EC_{50} value, defined as the extract concentration required for achieving 50% antiglucosidase activity, was computed by using linear regression analysis.

Phytochemical contents

Total phenolic (TP) content of the fern extracts was determined by using a Folin-Ciocalteu colorimetric assay (25). TP content was expressed as mg gallic acid equivalents (GAE)/g extract, calculated from a standard curve prepared with 0-100 µg/mL gallic acid. Total hydroxycinnamic acid (THC) content was determined by using the Arnow's reagent (26). THC content was expressed as mg caffeic acid equivalents (CAE)/g extract, computed from a standard curve prepared with 0-0.2 mg/mL caffeic acid. Total flavonoid (TF) content was determined by using an aluminum chloride colorimetric assay (27). TF content was expressed as mg quercetin equivalents (QE)/g extract, calculated from a standard curve prepared with 0-500 µg/mL of quercetin. Total proanthocyanidin (TPro) content was assessed by the acid-butanol assay (28). TPro content was calculated with the assumption that effective $E^{1\%,\ 1\ cm,\ 550\ nm}$ of leucocyanidin is 460 (28) and expressed as mg leucocyanidin equivalents (LE)/g extract.

Data analysis

All experiments were carried out in triplicates. Statistical analyses were performed using SAS (Version 9.2). Data were analyzed by one-way ANOVA test and means of significant differences were separated using Fisher's Least Significant Difference (LSD) test or Student's *t* test at $\alpha = 0.05$. Linear regression and correlation analyses were carried out by using Microsoft Office Excel 2007.

RESULTS

All fern extracts investigated in this study were cytotoxic toward K562 cells. Statistical analysis revealed that the EC_{50} values for cytotoxic activity of *C. dentata*, *N. acutifolia*, and *P. irregularis* extracts were not significantly different ($p > 0.05$) from that of 5-fluorouracil (Table 1). *C. arida* was the fern species with the highest EC_{50} value, which was 2.2-fold greater compared with 5-fluorouracil. Concentration-dependent increase in antiglucosidase activity was observed in all fern extracts, except *C. interruptus*, at 200-1000 mg/mL (data not

shown). The EC_{50} values for antiglucosidase activity among the fern extracts, ranked in ascending order, were: *C. dentata* < *P. irregularis* < *N. acutifolia* < *C. arida* < *M. punctatum* (Table 1). The EC_{50} values of *C. dentata* and *M. punctatum* extracts were 1.6- and 25-fold higher than that of myricetin, respectively. *C. interruptus* exhibited α-glucosidase stimulatory activity; hence its EC_{50} value was not determined. The EC_{50} value for acarbose was 2750 µg/mL (data not shown), which was markedly higher than those of myricetin and the fern extracts examined.

Phytochemical analysis found *P. irregularis* extract to be the richest in THC, TF and TPro contents (Table 2). Notably, TF, expressed as quercetin equivalents, accounted for about 37% of *P. irregularis* extract by weight. By contrast, *M. punctatum* extract had the lowest abundance of TP, THC and TF among the six extracts studied. TPro was not detectable in *M. punctatum* extract. *N. acutifolia* extract had the highest TP content, which was about 3.3-fold higher than that of *C. interruptus* and *M. punctatum* extracts. *C. interruptus* extract had the lowest, detectable TPro content, which was 56-fold lower compared with *P. irregularis*.

Only TP contents of the ferns significantly correlated with EC_{50} values for antiglucosidase activity ($R^2 = 0.82$, $p < 0.05$). No statistically significant correlations were detected between phytochemical contents of the fern extracts and EC_{50} values for cytotoxic activity.

DISCUSSION

This study demonstrated for the first time the cytotoxicity of *C. arida*, *C. dentata*, *N. acutifolia*, *M.*

Table 1. EC_{50} values of the cytotoxic and antiglucosidase activities of fern extracts, compared with 5-fluorouracil and myricetin, respectively.

Species	EC_{50} values	
	Cytotoxic activity (µg/mL)	Antiglucosidase activity (µg/mL)
C. arida	478.62 ± 39.11*	559.87 ± 21.04*
C. dentata	194.50 ± 14.74	87.48 ± 7.45*
C. interruptus	314.52 ± 6.34*	nd
M. punctatum	399.68 ± 48.60*	1345.73 ± 129.44*
N. acutifolia	190.82 ± 5.52	249.57 ± 2.67*
P. irregularis	253.85 ± 22.82	112.68 ± 1.72*
Positive control	212.86 ± 7.89 (5-Fluorouracil)	53.21 ± 0.91 (Myricetin)

Data are the mean ± standard errors (n = 3). The asterisks (*) denote values that are significantly different ($p < 0.05$) compared with the positive control, as determined by using Student's *t* test. nd = not determined.

Table 2. Phytochemical contents of fern extracts.

Species	TP (mg GAE/g)	THC (mg CAE/g)	TF (mg QE/g)	TPro (mg LE/g)
C. arida	97.21 ± 1.78^a	100.75 ± 1.28^a	324.24 ± 3.69^a	9.55 ± 0.16^a
C. dentata	107.80 ± 3.52^a	64.83 ± 1.08^b	191.52 ± 4.59^b	18.57 ± 1.79^b
C. interruptus	43.90 ± 0.62^b	9.10 ± 0.19^c	59.82 ± 1.91^c	0.44 ± 0.06^c
M. punctatum	42.57 ± 0.55^b	3.57 ± 0.04^d	15.73 ± 1.55^d	nd
N. acutifolia	143.79 ± 5.19^c	67.42 ± 0.92^b	246.67 ± 1.32^e	4.14 ± 0.25^d
P. irregularis	136.38 ± 6.65^c	119.75 ± 2.08^e	367.88 ± 2.89^f	24.71 ± 1.51^e

Data are the mean ± standard errors (n = 3). Values in the same column that are followed by different superscript letters are significantly different ($p < 0.05$), as determined by using Fisher's LSD test. TP = total phenolics; THC = total hydroxycinnamic acids; TF = total flavonoids; TPro = total proanthocyanidins;. nd = not detectable.

punctatum and *P. irregularis*. The cytotoxicity of *C. interruptus*-derived coumarins against human nasopharyngeal carcinoma (KB) cell line was previously reported (14); however, this is the first account of the fern's cytotoxicity toward K562 cell line. Different cancer cell lines respond differently even to treatment with the same cytotoxic agents (29). Hence, our study has added valuable information to current knowledge of the anticancer potential of *C. interruptus*. Hot water extracts of all six ferns analyzed in this study were cytotoxic to K562 cancer cell line. Thus, our findings suggest that these six ferns are promising sources of water-soluble and heat-stable cytotoxic agents. *C. dentata*, *N. acutifolia*, and *P. irregularis* are consumed as vegetables in Malaysia and India (16, 17, 30). Considering current interests to discover anticancer agents of food origin, future research to isolate and identify cytotoxic constituents from the three ferns will be of great value in the context of therapeutic agent development, especially for leukemia treatment.

We report for the first time the *in vitro* antiglucosidase activity of *C. arida*, *C. dentata*, *M. punctatum*, *N. acutifolia*, and *P. irregularis*. Notably, edible ferns *C. dentata*, *N. acutifolia*, and *P. irregularis* demonstrated stronger antiglucosidase activity than the other ferns. In line with current interests to search for food-derived antidiabetic natural products and management of diabetes by dietary intervention (31-34), the three edible ferns deserve more attention in future research. These ferns are not traditionally used as antidiabetic remedies. Nevertheless, owing to their α-glucosidase inhibitory activity, these ferns, when consumed, may be beneficial to the diets of diabetic patients. The water-soluble and thermally-stable nature of the α-glucosidase inhibitors in the three ferns imply that such constituents could be easily extracted with water and that their activity is likely retained after cooking with heat. In this study, we evaluated the antiglucosidase activity of the fern extracts by using yeast α-glucosidase. Yeast α-glucosidase is commercially available in pure form and has been routinely used as a model for investigating antiglucosidase potential of natural products (22, 35-37). Previous work has shown that plant extracts which inhibited the activity of yeast α-glucosidase also inhibited mammalian α-glucosidase. In addition, antiglucosidase plant extracts can significantly dampen postprandial hyperglycemia in streptozocin-induced diabetic mice (37, 38).

The presence of TP, THC, TF, and TPro in all the fern extracts, except for the absence of TPro in *M. punctatum*, implies that the cytotoxic and antiglucosidase activities of the extracts may be partly attributable to their phenolic constituents. *P. irregularis* extract, which was enriched in TF (37% by weight), showed potent cytotoxicity and concurrently exhibited relatively high antiglucosidase activity compared with the other ferns examined. Thus flavonoids may be a key group of bioactive constituents in *P. irregularis*. In this study, antiglucosidase activity of the ferns correlated with TP content, also implying that antiglucosidase activities of the ferns may be attributable to their phenolic constituents. No correlations were detected between the four phytochemical parameters measured and the cytotoxic activities of the fern extracts. It is likely that the fern extracts contained phenolic constituents that varied considerably in their efficacies or specific activity per unit mass as cytotoxic agents. Consequently, their cytotoxic activities cannot be directly predicted from their phenolic contents.

In conclusion, the cytotoxic effects of six selected edible and medicinal ferns toward K562 cell line were demonstrated for the first time. The water extracts of *C. dentata*, *N. acutifolia*, and *P. irregularis* were strong cytotoxic agents, with potency comparable to that of anticancer drug 5-fluorouracil. Antiglucosidase activity was also detected in five of these ferns for the first time. *C. dentata* had the strongest antiglucosidase activity among the ferns, which was stronger than acarbose but weaker than myricetin. This study has provided preliminary evidence that *C. dentata* is a promising source of cytotoxic and antiglucosidase agents, which warrant more in-depth investigations. To attest to their potential application as anticancer drugs, the effects of cytotoxic constituents isolated from *C. dentata* on normal cells should be confirmed. Correlation analysis suggests that antiglucosidase activity of the fern extracts was attributable to their phenolic contents. Hence, future research can consider bioassay-guided isolation of glucosidase inhibitors from phenolic extracts of the ferns, especially *C. dentata*.

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