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ORIGINAL ARTICLE

Identification of Potential Anticancer Protein Targets in Cytotoxicity Mediated by Tropical Medicinal Fern Extracts

Siok-Thing Tan[1], Hean-Chooi Ong[2], Tsun-Thai Chai[1,3], Fai-Chu Wong[1,3]

[3]Centre for Biodiversity Research, Universiti Tunku Abdul Rahman, [1]Biochemistry Program, Department of Chemical Science, Faculty of Science, Universiti Tunku Abdul Rahman, 31900 Kampar, Perak, [2]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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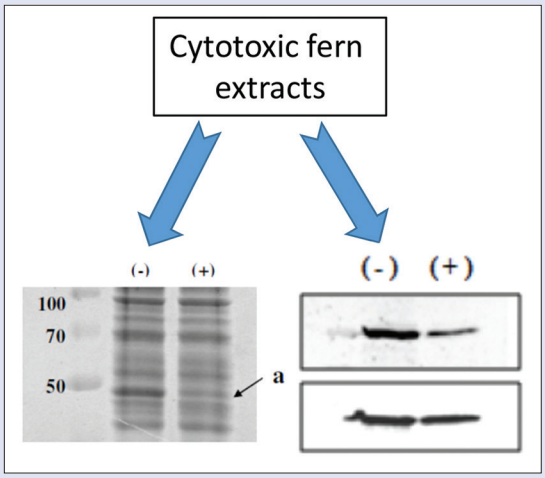
ABSTRACT

Background: Medicinal fern species represent a potentially important source for both food and medicinal applications. Previously, two underutilized tropical fern species (Blechnum orientale and Phymatopteris triloba) were reported with cytotoxic activities against selected cancer cell lines. However, the exact mechanism remains elusive. **Objective:** In this paper, we reported the identification of six differentially expressed proteins isolated from cancer cells, following exposure to the cytotoxic fern extracts. **Materials and Methods:** The identities of these cancer proteins were determined by matrix-assisted laser desorption ionization time-of-flight protein sequencing. **Results:** The cancer proteins were identified as follows: elongation factor 1-γ, glyceraldehydes-3-phosphate dehydrogenase, heat shock protein 90-β, heterogeneous nuclear ribonucleoprotein-A2/B1, truncated nucleolar phosphoprotein B23, and tubulin-β chain. To the best of our knowledge, this paper represents the first time these cancer proteins are being reported, following exposure to the aforementioned cytotoxic fern extracts. **Conclusion:** It is hoped that further efforts in this direction could lead to the identification and development of target-specific chemotherapeutic agents.

Key words: Anticancer, *Blechnum orientale*, medicinal fern, *Phymatopteris triloba*, protein expression

SUMMARY

- Cytotoxic fern extracts were tested in anti-cancer proteomic works.
- Six differentially-expressed cancer proteins were identified.
- Potential anti-cancer protein targets were reported.



Abbreviations used: EF: Elongation factor; HRP: Horseradish peroxidase; HSP: Heat shock protein; MALDI: Matrix-assisted laser desorption/ionization.

Correspondence:

Dr. Fai-Chu Wong,
Centre for Biodiversity Research, Universiti Tunku Abdul Rahman, Jalan Universiti, Bandar Barat, 31900, Kampar, Petaling Jaya, Perak, Malaysia.
E-mail: wongfc@utar.edu.my

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INTRODUCTION

Since ancient age, plant species represent important sources of food, treasured for their nutritional and medicinal values. In modern pharmaceutical science, plants with their rich secondary metabolites represent a reservoir of diverse phytochemicals waiting for medicinal discoveries. Notorious examples include vinblastine and vincristine, chemotherapeutic drugs which were originally derived from the alkaloid compounds in *Catharanthus roseus* (Madagascar periwinkle), a plant endemic to Madagascar.[1] Likewise, paclitaxel (a commercial antitumor drug) was originated from the bark extract of *Taxus brevifolia* (Pacific yew tree).[2]

Among the different plant species, ferns species represent a group of underutilized food and medicinal sources. Examples include *Selaginella willdenowii* which is reportedly used to treat wounds[3] and for tonic medicine,[4] as well as consumed as vegetables[5] in the tropical areas. Likewise, the young fronds of *Stenochlaena palustris* are harvested from the wild and consumed as vegetable in Southeast Asia.[6] Although fern species have been consumed by different ethnic groups across the world for both culinary and medicinal purposes, the full extent of their potentials has never been thoroughly investigated.

Blechnum orientale and *Phymatopteris triloba* are two fern species found in the tropical regions. The leaves of *B. orientale* were reportedly used to treat blister, sores, stomach pain, and urinary bladder-related complications,[7] while *P. triloba* was found in the tropical mountain forest. No much information is available on *P. triloba*'s medicinal bioactivity; however, two fern members in this same genus Phymatopteris (*Phymatopteris hastata* and *Phymatopteris quasidivaricata*) were reportedly used in traditional medicines.[8] *P. hastata* was used to treat diarrhea and bronchitis-related diseases,[9] while *P. quasidivaricata* was applied to treat musculoskeletal related problems.[10]

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Previously, both *B. orientale* and *P. triloba* had been reported with anticancer bioactivities;[7,8] however, their exact cytotoxic mechanism remains elusive. It is not clear that which cancer signaling pathways or cancer enzymes are being targeted by these cytotoxic fern extracts. For instance, it is curious to determine whether the observed fern-induced cytotoxicity is exerted through cell cycle arrest, through microtubule interference, or by inhibiting the DNA synthesis pathway in cancer cells.[11] In this paper, selected cancer cell lines were exposed to the cytotoxic *B. orientale* and *P. triloba* fern extracts. Proteomic works were performed to identify the differentially expressed cancer proteins implicated in the fern-induced cytotoxic mechanism. The identities of these cancer proteins were then confirmed by matrix-assisted laser desorption ionization time-of-flight (MALDI TOF-TOF) protein sequencing. Through these efforts, we hope to contribute toward the understanding of fern-induced cytotoxic mechanism and potential anticancer protein targets.

MATERIAL AND METHODS

Preparation of fern extracts

Tropical fern species were collected from Perak and Pahang states (Malaysia) from July to November of 2013. The fern species were authenticated by Professor Dr. Hean-Chooi Ong at the Institute of Biological Sciences, University of Malaya, Malaysia. Following harvesting and rinsing with distilled water, the ferns were dried in oven at 40°C, until constant weights were achieved. The dried ferns were then pulverized using a warring blender, followed by solvent extraction. The extracted supernatants were filtered, concentrated, and stored in −20°C. The typical fern extract yields were in the range of 4%–9%.

Solation of proteins from cancer cells for SDS-PAGE analysis

Human cervical cancer cells (HeLa) and human myelogenous leukemia cancer cells (K562) were cultured in RPMI 1640 medium, supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Both cancer cell lines were maintained in a humidified incubator with 5% CO_2 at 37°C. Subculturing was performed when the cultured cancer cells reached 75% confluency, and cultured cells in the exponential phase were used in experiments. Cancer cell cultures were treated with optimal concentrations of the filtered fern extracts (0.1–1 mg/ml), and sterile water was used as the negative control. The cytoplasmic and nuclear proteins of cancer cells were extracted using NE-PER nuclear and cytoplasmic protein extraction kit (Thermo Scientific).

SDS-PAGE gel analysis and identification of differentially expressed cancer cellular proteins

SDS-PAGE gel electrophoresis was performed as previously described.[12] Gel electrophoresis was performed with constant electric current of 135 mV for approximately 90 min. The protein gels were then stained with Coomassie Brilliant Blue R-250 (Fisher Scientific). Stained protein bands were visualized under densitometer (Bio-Rad), and the protein band densities were determined with Image Lab software (Bio-Rad). Differentially expressed cancer proteins were excised with sterile razor blades and subjected to analysis using MALDI TOF-TOF mass spectrometry (4800 Proteomics Analyzer, AB Sciex) (Proteomics International, Perth, Australia). The spectra were analyzed using Mascot sequence matching software (Matrix Science) with Ludwig NR Database to identify the proteins of interest.

Western blotting

Extracted cancer proteins were resolved using 12% SDS-PAGE gel and then transferred onto a nitrocellulose membrane. After blocking the nitrocellulose membrane with Tris-buffered saline containing 1% Tween-20 (TBST) and 5% bovine serum albumin for an hour, the membrane was probed overnight with anti-heat shock protein 90 (HSP90) primary antibody and anti-β-actin antibody (as control) at 4°C, followed by incubation with horseradish peroxidase-conjugated secondary antibody (Thermo Scientific). After washing the nitrocellulose membrane with TBST, the protein bands were detected using enhanced chemiluminescence kit (Thermo Scientific) and visualized by exposing the membrane to X-ray film. Band intensities were determined using Image Lab software (Bio-Rad).

RESULTS AND DISCUSSION

In this paper, a total of six differentially expressed proteins were pinpointed and isolated from cancer cells, following exposure to cytotoxic fern extracts [Figure 1]. The identities of these differentially expressed proteins were then determined by MALDI TOF-TOF protein sequencing, and their characteristics were summarized in Table 1.

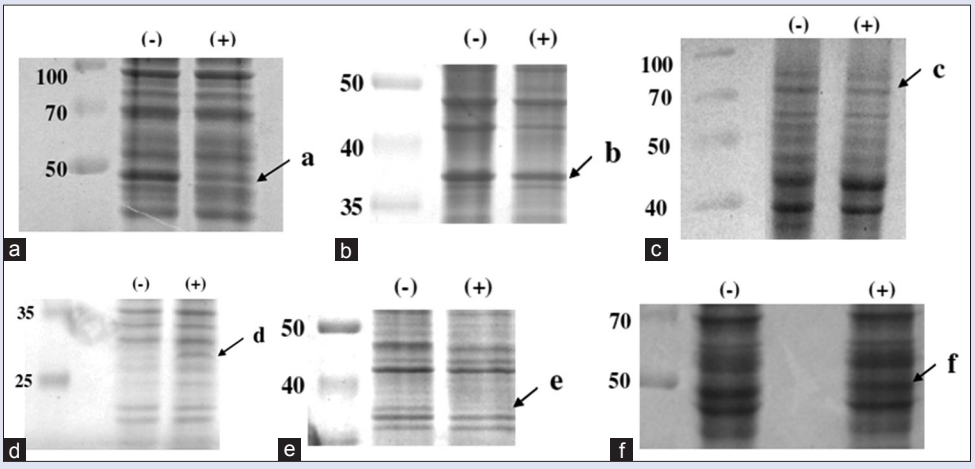

Figure 1: (a-d) Cancer protein expression profiles following exposure to *Blechnum orientale* extract (e-f). Cancer protein expression profiles following exposure to *Phymatopteris triloba* extract. (-) and (+) indicated the absence or presence of fern extract in the cancer cell culture medium, respectively. Labels (a-f) indicated differentially expressed cancer protein bands selected for matrix-assisted laser desorption ionization time-of-flight protein sequence analysis

Table 1: Summary of identified differentially expressed cancer proteins, using matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Protein band	Protein size (aa)	Number of peptide sequence identified	Sequence covered (%)	Annotation
a	487	6	13	Elongation factor 1-γ
b	335	8	26	Glyceraldehyde-3-phosphate dehydrogenase
c	724	12	19	Heat shock protein 90-β
d	353	4	16	Heterogeneous nuclear ribonucleoprotein A2/B1
e	274	2	12	Truncated nucleolar phosphoprotein B23
f	426	7	20	Tubulin-β chain

Protein bands (a to f) are labeled corresponding to those in Figure 1

In our works with *B. orientale* fern extract, four differentially expressed cancer cellular proteins were identified (Protein bands a to d) [Figure 1]. Based on MALDI-TOF-TOF protein sequencing results, protein bands a to d were determined as elongation factor 1-γ (EF1-γ), glycealdehyde-3-phosphate dehydrogenase (GAPDH), HSP90-β, and heterogeneous nuclear ribonucleoprotein A2/B1 (hnRNP-A2/B1), respectively. EF1-γ was previously reported with functional role in transporting aminoacyl-tRNA to ribosome, during the elongation stage of protein synthesis.[13] Earlier studies had also reported on the increased expression of EF1-γ in assorted cancer cells, compared to normal cells.[14,15] Interestingly, a separate study reported on the downregulation of EF1-γ in cervical cancer cells, following treatment with antitumor drugs (paclitaxel and cisplatin).[16] In our study, the expression of EF1-γ was downregulated, following exposure to *B. orientale* [Figure 1a]. It is tempting to speculate that *B. orientale* extract may exert its cytotoxic effect, partly by interfering with the cancer cell's protein synthesis pathway, through reduction of EF1-γ expression level. However, further analysis is needed to determine if this is indeed the case or not.

On the other hand, GAPDH was reported as an enzyme involved in glycolysis pathway,[17] the metabolic pathway that proliferating cancer cells rely on for energy generation. Previous studies had reported on increased glycolysis activities in rapidly growing tumor cells, together with the upregulated glycolysis-related genes, which include GAPDH.[17,18] On the other hand, methyl jasmonate (a plant-derived lipid derivative) was previously reported with anticancer and apoptosis-inducing effects, which correlating closely with its inhibition activities on components in the glycolytic pathway.[19,20] In our study, GAPDH was found to be downregulated, following exposure to *B. orientale* fern extract [Figure 1b]. The reduced expression of GAPDH could possibly be part of the reasons which contributed to the cytotoxic effect of *B. orientale* extract.

Meanwhile, HSP90-β is a molecular chaperone with a mass of 90 KDa, reported with functional role to stabilize cellular proteins and enhance proper folding.[21] Previously, it was reported that HSPs were abundantly expressed in cancer cells, partly to enhance protein stabilization and to promote cellular proliferation.[16,22] In addition, other researchers have reported on antitumor agents which are targeting HSP90.[21,22] In our study, the expression of HSP90-β was found to be downregulated [Figure 1c], following exposure to the cytotoxic *B. orientale* extract. The downregulation of HSP90 was also verified by western blot study [Figure 2]. However, it remains to be determined whether *B. orientale* extract induced cytotoxicity by modulating the HSP90-β expression level, through similar mechanism observed in other HSP inhibitors.

The fourth identified differentially expressed cancer protein was hnRNP-A2/B1, which was previously reported as a multifunctional RNA-binding protein, associated with cell proliferation and carcinogenesis.[23] In addition, an earlier study documented the overexpression of hnRNP-A2/B1 in cancer cells.[24] Although the exact reason remains unclear, it was proposed that this may be linked to the functional roles of hnRNP proteins in telomere regulation.[24,25]

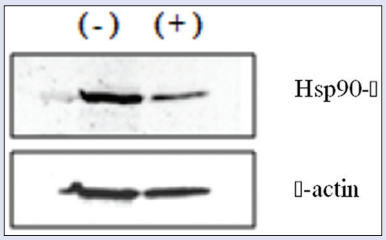

Figure 2: Western blot study of heat shock protein 90-β. (-) and (+) indicated the absence or presence of fern extract in the cancer cell culture medium, respectively. β-actin was used as the control

Furthermore, hnRNP-A2/B1 may also be regulated by the presence of other related hnRNP family members.[24] In our study, hnRNP was found to be upregulated, following exposure to *B. orientale* extract [Figure 1d]. This phenomenon may be due to the accelerated metabolic activities of cancer cells, in response to cytotoxic stress. However, we could not rule out the possibility that the cytotoxic *B. orientale* extract may stimulate the cancer cells to produce more hnRNP-A2/B1, to compensate for other downregulated hnRNP family members. Further detailed research is needed to distinguish which is the case.

In our works with *P. triloba* fern extract, two differentially expressed cancer proteins were isolated. The identities of these two proteins were determined through MALDI TOF-TOF protein sequencing as truncated nucleolar phosphoprotein B23 (nucleophosmin [NPM]) and tubulin beta chain (tubulin-β) [Figure 1e and f], respectively. NPM was previously reported as a multifunctional protein linked to cell proliferation and cancer pathogenesis.[26,27] Meanwhile, NPM was also found to overexpress in many types of malignant cells.[28] Interestingly, a recent study reported on the downregulation of NPM protein, in cancer cells treated with antitumor triterpenoid compounds (derived from Thai medicinal plant *Trichosanthes cucumerina*).[29] Collectively, these studies highlighted the potential of NPM as a chemotherapeutic target. In our study, the observed downregulation of NPM expression, following exposure to *P. triloba* extract, hinted the possible presence of bioactive compounds which may induce NPM-mediated cytotoxic activities.

On the other hand, literature search revealed tubulin-β as an important component of microtubules. In the cells, microtubules are the major constituents of cytoskeletons, which are involving in cell division, chromosome separation, as well as maintaining cellular structures.[30,31] Meanwhile, paclitaxel (a chemotherapeutic drug) functions by stabilizing the microtubule polymers, which leads to suppression of microtubule dynamics and subsequently preventing the metaphase spindle configuration in chromosome.[32] Recently, efforts to study synthetic small molecules (benzenesulfonamide derivatives) which demonstrated anticancer and tubulin-targeting activities were also reported.[33] Similarly, a related study reported on phenethyl isothiocyanate (a phytochemical found in many edible cruciferous vegetables), which reduced tubulin expression

and exerted cytotoxic effects in prostate cancer cells.[34] In our study, the tubulin-β expression was found to be downregulated, following exposure to the cytotoxic *P. triloba* extract [Figure 1f]. It is possible that the *P. triloba* extract may inhibit tubulin-β expression and subsequently lead to cytotoxicity in cancer cells. However, the detailed relationship between the reduced tubulin-β expression and the anticancer activity of *P. triloba* extract required further elucidation in the future study.

CONCLUSION

We reported in this paper the identification of six differentially expressed cancer proteins, following exposure to cytotoxic tropical fern extracts. These identified cancer proteins could further be classified into cancer cellular pathways pertaining to protein synthesis, glycolysis, chaperone-mediated protein stabilization, proliferative-related factors, as well as microtubule dynamics. It is hoped that further study in this direction could eventually lead to the identification of fern-derived, target-specific chemotherapeutic agents.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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