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ORIGINAL ARTICLE

Protein Expression Patterns in HEK-Blue™ - Cells Treated with *Clinacanthus nutans* Extracts

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ABSTRACT

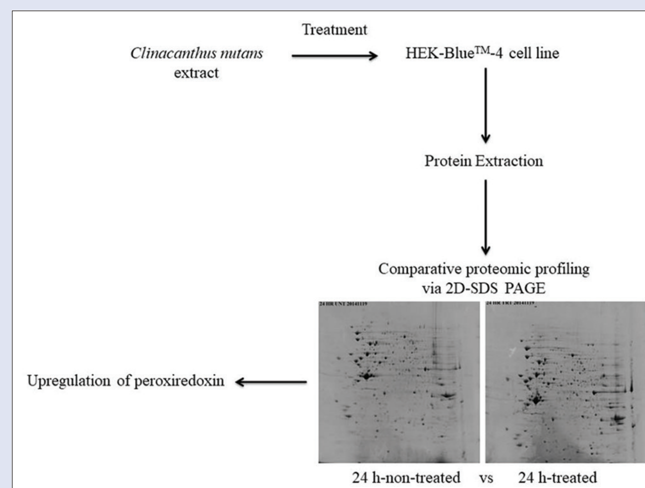
Background: *Clinacanthus nutans* (CN) is a small shrub native to tropical Asia known for their anti-oxidant, anti-inflammation, anti-cancer, and anti-viral activities. **Objectives:** This study aimed to investigate the effect of CN extract on human embryonic kidney cell line (HEK-Blue™-4) in a proteomic perspective. **Materials and Methods:** Comparative proteomic profiling through two-dimensional sodium dodecyl sulfate gel electrophoresis was performed on HEK-Blue™-4 treated with CN leaf polar extract. **Results:** We successfully identified seven upregulated proteins, of which five promoted the growth of the HEK-Blue™-4 cells. Interestingly, a potent antioxidant enzyme which neutralizes reactive oxygen or nitrogen species, peroxiredoxin-1 was also upregulated in HEK-Blue™-4 cell lines after treatment with CN leaf polar extract. **Conclusion:** CN leaf polar extract promotes the growth of HEK-Blue™-4 cells and induced the expression of peroxiredoxin-1, which protects the cells from reactive oxygen species during the inflammation process.

Key words: Anti-inflammatory, *Clinacanthus nutans*, comparative proteomic, HEK-Blue™-4 cell line, two-dimensional sodium dodecyl sulfate gel electrophoresis

SUMMARY

- *Clinacanthus nutans* (CN) is a small shrub native to tropical Asia known for their anti-oxidant, anti-inflammation, anti-cancer, and anti-viral activities. This study aimed to investigate the effect of CN extract on human embryonic kidney cell line (HEK-Blue™-4) in a proteomic perspective. CN leaves were collected at an Orchard in Temerloh, Pahang, Malaysia, followed by polar solvent extraction. Comparative proteomic profiling through two-dimensional sodium dodecyl sulfate gel electrophoresis was then performed on HEK-Blue™-4 treated with CN leave polar (CN-LP) extract. We successfully identified seven upregulated proteins, of which five promoted the growth of the HEK-Blue™-4 cells. Interestingly, a potent antioxidant enzyme which neutralizes reactive oxygen or nitrogen species, peroxiredoxin-1 was also upregulated in HEK-Blue™-4 cell line after treatment with CN-LP extract. CN-LP extract promotes the growth of HEK-Blue™-4 cells and induced the expression of peroxiredoxin-1, which protects the cells from reactive oxygen species during inflammation process.

Abbreviations used: 2D-SDS PAGE: Two-dimensional sodium dodecyl sulfate gel electrophoresis; ACN: Acetonitrile; CHAPS: 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate; CN: *Clinacanthus nutans*; CN-LP: *Clinacanthus nutans*-leave polar; DMEM: Dulbecco's Modified Eagle Medium; DTT: Dithiothreitol; IAA: Iodoacetamide; IEF: Isoelectric focusing; IPG: Immobilized pH gradient; LPS: Lipopolysaccharide; MALDI-TOF/TOF MS: Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PBS: Phosphate-buffered saline; TLR: Toll-like receptor.



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INTRODUCTION

Clinacanthus nutans (CN) is a small shrub native to tropical Asia, especially Indonesia and Malaysia, with a high medicinal value. CN had been prescribed as a traditional medicine in treating ailment related to kidney and bladder.[1] Previous reports have also documented antioxidant, anti-inflammation, anti-cancer, and anti-viral activities exhibited by CN extracts.[2-5] For instance, Sarega *et al.* showed antioxidant activity of

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polar and semi-polar extracts from CN in rats, whereby extract-treated rats had higher level of antioxidant enzyme activity against oxidative stress.[6] Study by Tu *et al*. also suggest anti-inflammatory activity of methanol and ethanol extracts of CN, assessed through neutrophil elastase release and superoxide anion generation. Tu *et al.* showed 10 μg/mL of ethanol extract inhibited 68% of elastase release and 30% of superoxide formation.[7] Anti-cancer activity of CN extract had also been documented by numerous researchers. For instance, Ng *et al*. showed that hexane and chloroform extracts from CN had anti-cancer activity against CNE, A549, and HepG2 cancer cell line at 300 μg/mL, respectively.[8] Kunsorn *et al*. had reported anti-viral activity from hexane and methanol extracts of CN against HSV-1 virus when compared to the dichloromethane extract.[9]

In our previous study, we found that CN-leave polar (CN-LP) extract produced a significant, concentration-dependent reduction in the lipopolysaccharide (LPS)-induced toll-like receptor-4 (TLR-4) activation in HEK –Blue™-4 cells.[10] In additional, we also found that CN-LP extracts inhibited LPS-induced TLR-4 inflammatory proteins, one of the key pro-inflammatory signaling receptors against acute and chronic inflammation, producing a potent anti-inflammatory effect.[5,10] However, the underlying mechanism of CN-LP extracts in inhibiting TLR-4 required further elucidation before application in the clinical setting as an anti-inflammatory agent. Therefore, this study was performed to identify and profile differentially expressed proteins using two-dimensional sodium dodecyl sulfate gel electrophoresis (2D-SDS PAGE) and mass spectrometry in the HEK-Blue™-4 cells treated with CN-LP extract.

MATERIALS AND METHODS

Cell line and culture conditions

HEK-Blue™-4 cells are human embryonic kidney cells obtained from InvivoGen (San Diego, CA, USA). The cells were cultured in Dulbecco's Modified Eagle Medium supplemented with 4.5 g/L glucose and L-glutamine, 10% heat inactivate fetal bovine serum, 1% penicillin-streptomycin, Normocin™ (InvivoGen, San Diego, CA, USA), and HEK-Blue™ selection medium (InvivoGen, San Diego, CA, USA).

Clinacanthus nutans-leave polar treatment and protein extraction

HEK-Blue™-4 cells of 70% confluency were treated with 100 μg/mL CN-LP extract for 24, 48, and 72 h. Cells were harvested and washed with cold phosphate-buffered saline buffer for three times. The cells were resuspended with lysis buffer (7M urea, 2M thiourea, 4% [w/v] 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate [CHAPS], 2% [v/v] pharmalyte [pH 3-10], 40 mM dithiothreitol [DTT], and protease inhibitor cocktail [Merck, Kenilworth, NJ, USA]) and lysed through sonication with a probe sonicator for 5 min on ice. The lysate was subjected to centrifugation at 40,000 g for 30 min, and the resulting supernatants were collected and quantified through Bradford assay.

Comparative proteomic analysis through two-dimensional sodium dodecyl sulfate gel electrophoresis

2D-SDS PAGE was performed as detailed in Teh *et al*.[11] A total of 600 μg of the protein samples was rehydrated with rehydration buffer (8 M urea, 4% [w/v] CHAPS, 0.01% [w/v] bromophenol blue, 0.4% [w/v] DTT) for 12 h at 20°C with immobilized pH gradient strips (13 cm with a non-linear gradient pH of 3–10). Isoelectric focusing was carried out immediately after rehydration; 500 V for 1 h, followed by gradient mode at 1000 V for 1 h and 8000 V for 2.30 h, and finally with 8000 V for 30 min. Focused strips were equilibrated with two equilibration buffers: reducing buffer (10 mM DTT, 100 mM ammonium bicarbonate) for 15 min and alkylating buffer (55 mM IAA, 100 mM ammonium bicarbonate) for another 15 min. Equilibrated gel strips were loaded onto 12% w/v acrylamide Laemmli gels, and electrophoresis was performed at 10 mA per gel for 15 min, followed by 20 mA per gel until indicator dye front reached the gel bottom. The electrophoresis gels were stained with PhastGel Blue R-350 (GE Healthcare, Chicago, IL, USA). 2D-SDS PAGE was performed in duplicates. Gel images were then acquired with GS800 calibrated imaging densitometer (BioRad, Hercules, CA, USA) and processed through Progenesis SameSpots software (Nonlinear Dynamics, Garth Heads, Newcastle, UK). Proteins considered differentially expressed were those that displayed at least a 2-fold significant change in percentage volume with ANOVA test ($P < 0.05$). These protein spots were picked manually for further analysis in mass spectrometry.

In-gel digestion

Protein plugs with at least 2-fold significant changes were excised, destained, and dehydrated (50% acetonitrile, 50 mM ammonium bicarbonate) followed by reduction (10 mM DTT, 100 mM ammonium bicarbonate) for 30 min at 60°C and alkylation (55 mM IAA, 100 mM ammonium bicarbonate) for 20 min in the dark at room temperature. The processed protein samples were then washed with 50% ACN in 50 mM ammonium bicarbonate for three times, 20 min each and dried completely with SpeedVac concentrator. Dried protein samples were incubated with 25 μL of 6 ng/μL trypsin in 50 mM ammonium bicarbonate for overnight at 37°C. The digestion was terminated by addition of 50% ACN for 15 min. Digested peptides were reconstituted with 0.1% formic acid and desalted using μC18 ZipTips (Millipore, Billerica, MA, USA) as detailed in the supplier's instructions.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Digested protein samples were identified through matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF MS) using ultrafleXtreme MALDI-TOF/TOF Analyzer (Bruker Daltonics, Hamburg, Germany) as detailed by Teh *et al*.[11] The peptides were ionized with a smart beam laser at 355 nm, using a delayed extraction approach and accelerated with 25 kV injection pulse for TOF analysis. Each spectrum was the cumulative average of 5000 laser shots. The MS/MS was performed in 1 kV-positive reflector mode with fragments generated by postsource decay. The monoisotopic peptide masses obtained were analyzed with Bruker Daltonics flexAnalysis. All spectra obtained were identified using Mascot search engine with the following parameters: carbamidomethylation of cysteine and oxidation of methionine were included as modifications, peptide tolerance of ±0.2 Da, MS/MS tolerance of 0.5 Da, and peptide charges of +1, +2, and +3. Trypsin was set as proteolytic enzyme and allowed only one missed cleavage per peptide. For confident identification, only proteins with a minimum of two matched peptides and extensive homology ($P < 0.05$) were considered.

RESULTS AND DISCUSSION

As shown in Figure 1, we successfully identified seven proteins spots which were significantly upregulated following the exposure of HEK-Blue™-4 cells to CN-LP extract at different time points of 24, 48, and 72 h [Supplemental Table 1]. Protein spots were excised and then identified through mass spectrometry detailed in Table 1. As shown in Mai *et al*., CN-LP extract showed no cytotoxicity toward HEK-Blue™-4 cell line in viability assay. This was reflected in our proteomic analysis, whereby only seven proteins, namely protein disulfide isomerase-related

Table 1: Differentially expressed proteins identified from human embryonic kidney-Blue™-4 cells treated with *Clinacanthus nutans*-leaf polar extract after 24, 48, and 72 h

Spot number	Protein name	GI accession number	Theoretical molecular weight (kDa)	MS/MS search score	Fold change	*P*	Biological processes
24 h							
5	Protein disulfide isomerase-related protein 5	GI1710248	46.512	40	2.7	0.007	Protein modification
9	Pyruvate dehydrogenase beta subunit	GI189754	39.566	97	5.3	0.002	Tricarboxylic acid cycle
48 h							
14	L-lactate dehydrogenase B chain	GI4557032	36.900	69	1.9	0.026	Carbohydrate metabolism
16	Proteasome subunit alpha Type-5	GI7106387	26.565	41	5.0	0.006	Protein catabolism
23	Nucleoside diphosphate kinase A isoform A	GI38045913	19.869	38	1.8	0.041	Nucleotide metabolism
24	Peroxiredoxin-1	GI4505591	22.324	79	2.1	0.010	Oxidative stress response
72 h							
26	Phosphoglycerate mutase 1	GI4505753	28.900	56	1.7	0.025	Glycolysis

MS: Mass spectrometry; Spot no.: spot with differential expressed proteins, as indicated in Figure 1; GI accession: GenInfo Identifier accession number.

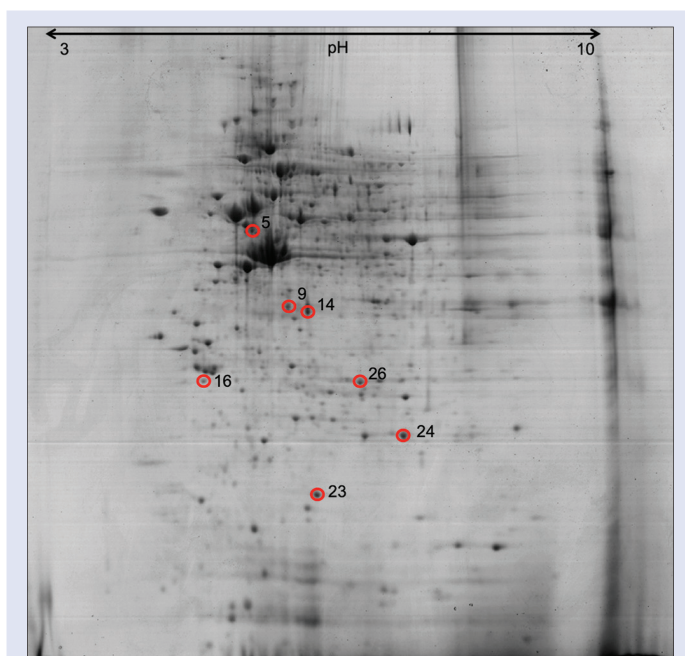

Figure 1: Reference proteome map of HEK-Blue™4 cells treated with *Clinacanthus nutans* leaf polar extract. Red circle indicates upregulated protein plugs that were excised and identified

protein 5, pyruvate dehydrogenase beta subunit, L-lactate dehydrogenase B chain, proteasome subunit alpha type-5, nucleoside diphosphate kinase A isoform A, peroxiredoxin-1, and phosphoglycerate mutase 1, are differentially expressed following prolonged exposure to CN-LP extract at 24, 48, and 72 h.[10] Protein disulfide isomerase-related protein 5 and pyruvate dehydrogenase beta subunit were upregulated after 24 h of exposure to CN-LP extract. Protein disulfide isomerase has been shown to play a role in protein modification and folding and had been found to promote the growth of cancer cells by suppressing apoptosis.[12,13] Pyruvate dehydrogenase, on the other hand, is mainly involved in the tricarboxylic acid cycle and serves as a rate-determining enzyme which converts pyruvate to acetyl-CoA, generating energy in the process.[14] Both of these identified upregulated proteins after 24 h and exposure indicated growth promoting effects onto the HEK-Blue™-4 cell line, as it is an immortalized cancer cell line.

At the 48-h interval, L-lactate dehydrogenase B chain, proteasome subunit alpha type-5, nucleoside diphosphate kinase A isoform A, and peroxiredoxin-1 were upregulated. L-lactate dehydrogenase is involved in carbohydrate metabolism, which converts lactate into pyruvate for use in the tricarboxylic acid cycle for energy synthesis, whereas nucleoside diphosphate kinase A isoform A is involved in nucleotide metabolism.[15] The upregulation of these two proteins indicates that CN-LP extract promotes cell growth and proliferation. Interestingly, peroxiredoxin-1, a potent antioxidant enzyme which neutralizes reactive oxygen, or nitrogen species was also upregulated in HEK-Blue™-4 cell line after 48 h of treatment with CN-LP extract.[16] The induced expression of peroxiredoxin-1 by CN-LP extracts might have facilitated the anti-inflammatory activity in HEK-Blue™-4 cell line. From our previous study, we showed that CN-LP extract inhibits the activity of LPS-induced TLR-4 inflammatory protein.[10] During inflammation, LPS induces the production of TLR-4 inflammatory proteins, which signals immune cells such as mast cell and leukocytes releases a large amount of reactive oxygen species.[17] The expression of peroxiredoxin-1 before inflammation prepared the cell to neutralize oxidative stress during inflammation, thus protecting the cells from the oxidative stress during inflammation. Last but not least, phosphoglycerate mutase 1 was upregulated after 72 h of treatment with CN-LP extract. Phosphoglycerate mutase is involved in glycolysis, which generates energy and also promotes cell proliferation.[18]

CONCLUSION

Comparative proteomic analysis through 2D-SDS PAGE coupled with MALDI-TOF/TOF MS revealed that CN-LP extract promotes the growth of HEK-Blue™-4 cells and induced the expression of peroxiredoxin-1, which protects the cells from reactive oxygen species during inflammation.

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Conflicts of interest

There are no conflicts of interest.

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