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PROTEIN PROFILING OF DIFFERENT PLANT TISSUES FROM HERB *PHYLLANTHUS NIRURI*

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Abstract

Herb *Phyllanthus niruri (P. niruri)* is known to have various pharmacological functions including anticancer, antibacterial, antioxidant, anti-hypertensive and also anti-diabetic properties. In this research, the proteomic part of *P. niruri* was studied to determine the bioactive peptides that responsible for specific characteristics. Total soluble proteins from different plant parts of freshly collected *P. niruri* were extracted using TCA/acetone method and then quantified using Bradford assay. Fruits part was found to have a significantly higher amount of proteins (4.91µg/µl ± 0.21) compared to leaves (4.18µg/µl ± 0.15). To determine the quality of proteins in the crude extract, SDS-Page was carried out which separates proteins in the basis of molecular weight. Proteins extracted from leaves were widely distributed between the range of 3.5 kDa to 160 kDA. Meanwhile, proteins in fruits mainly distributed within the range of 15 kDa to 80 kDa. The most highly expressed protein band was found in fruit, located in between 30 to 40 kDa. The protein extracts were then further analyzed based on the molecular weight and isoelectric points using two-dimensional gel electrophoresis (2D-GE) approach. Based on the profile pattern obtained from 2D-GE analysis, protein extract from fruits seems to express more protein spots compared to protein extract from leaves. Protein spots from fruit are seen to be intensely resolved within pH 4 to 10 at molecular weight between 10 kDa to 80 kDa. On the other hand, protein spots from leaves were moderately resolved at pH 4 to 10 at molecular weight within 10 kDa to 50 kDa.

Keywords: *Phyllanthus niruri*, bioactive peptides, proteomics

1.0 INTRODUCTION

Phyllanthus niruri (*P. niruri*), locally known as "dukung anak" is a herbaceous plant belongs to Euphorbiaceae family. It is originated from India and widely distributed in tropical climate. This plant grows well in moist, shady and sunny area [1]. It is well known for having a great potential of nutraceutical and medicinal values. In Malaysia, *P. niruri* is traditionally used as hepato-protective, anti-hypertensive and diuretic medicinal plant to treat jaundice, genital urinary infections and diarrhea. A lot of researches have been done on *P. niruri* and proven that extract from different plant parts of this plant including leaves, fruits and roots contains various type of bioactive compounds such as flavonoids, alkaloids, terpenoids, lignin, coumrin and tannin which each of them serve for a particular pharmacological properties like antimicrobial, antibacterial, antioxidant and also anti-diabetic properties. Hence, making *P. niruri* is a potential material for biomedical industries. However, little study has been done focusing on the proteomic of *P. niruri* and information about bioactive peptides or proteins from this medicinal plant is yet to be established. In the relation to the plant possesses various properties, it is believed that there might be

some kind of proteins that contribute to the particular characteristic. Thus, the discoveries of various types of proteins responsible for each property would be interesting findings. Study on bioactive peptides from *P. niruri* was conducted by comparing the protein profile of *P. niruri* from different plant parts using one-dimensional gel electrophoresis and followed by two-dimensional gel electrophoresis. It is anticipated that the bioactive peptides or proteins from *P. niruri* responsible for various desired properties could be identified.

2.0 EXPERIMENTAL

2.1 Medicinal Plant Collection

The whole plant of *Phyllanthus niruri* was freshly collected from Taman Pertanian Jubli Perak Sultan Ahmad Shah, Kuantan. The plant was rinsed and separated into different plant parts (leaves and fruits). Each plant part was analyzed in triplicate.

2.2 Bioactive Peptide Extraction

Total soluble proteins from different plant parts of *P. niruri* was extracted according to trichloroacetic acid (TCA)/acetone method which proposed by [15]. Each plant part (~1 g) of *P. niruri* was ground in liquid nitrogen to a fine powder using mortar and pestle and then incubated in acetone containing 10% TCA (w/v) and 1% dithiothreitol (DTT) (w/v) for overnight at -20°C. The mixture was then centrifuged at 25, 000 x g at 4°C for 20 minutes. The pellet obtained was then washed three times by suspension in acetone containing 1% (w/v) DTT and stored for 1 hour at -20°C. After that, the mixture was centrifuged at 25, 000 x g at 4°C for 20 minutes and the pellet obtained was vacuum dried. Then, iso-electric focusing (IEF) or rehydration buffer comprising 8 M urea, 20 mM DTT, 4% (w/v) CHAPS and 2% (v/v) ampholyte (pH 3-10) was directly added to the vacuum dried pellet. The mixture was vortexed briefly and then stored at -20°C for 1 hour before being centrifuged at 25, 000 x g at 4°C for 20 minutes. The supernatant was collected and the pellet was re-suspended with the IEF buffer and further centrifuged. The pellet was discarded and the collected supernatant (crude protein extract) was combined and then stored at -80°C for further proteomic analysis. The total protein content of the extracted bioactive peptides of *P. niruri* was then quantified spectrophotometrically according to [3] using Bovine Serum Albumin (BSA) (Sigma-Aldrich USA) as the standard. Fruits part was found to have a significantly higher amount of proteins (4.91µg/µl $\pm$ 0.21) compared to leaves (4.18µg/µl $\pm$ 0.15).

2.3 Sodium Dedocyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins in the crude extract was separated based on the molecular weight by using one dimensional discontinuous SDS-PAGE following method described by [9]. The protein separation was conducted on 4% stacking to 12% resolving gel. A total 10 µg of protein was loaded into the well of designated lanes on the gel. A set of protein marker which comprises of 12 precisely sized proteins (3.5, 10, 15, 20, 30, 40, 50, 60, 80, 110, 160 and 260 kDa) was used to determine the molecular weight of the protein in the samples. The electrophoresis was then carried out at constant voltage 200V for 45 minutes using Mini-PROTEAN® Tetra System (Bio-Rad). The gel was then stained with 0.1% (w/v) Coomasie® Brilliant Blue R-250 (AMRESCO®, USA) in a fixative 10% (v/v) acetic acid (SYSTERM®, USA) and 40% (v/v) methanol (SYSTERM®, USA) for 1 hour. The background stain was removed from the gel using first de-stain solution containing 30% (v/v) acetic acid and 40% (v/v) methanol for 10 minutes and followed by second de-stain solution containing 7% (v/v) acetic acid and 5% (v/v) methanol overnight with gentle shaking using Orbital Shaking UNIMAX 2010 (Heidolph Instruments). The gel was scanned using Molecular Imager GS-800 USB Calibrated Densitometer (Bio-Rad, Hercules, USA) to examine the difference in protein expression between various bioactive peptides from different plant parts.

2.3 Two Dimensional Gel Electrophoresis (2DGE)

In 2DGE, proteins in the crude extract was sorted based on the isoelectric point (pI) and followed by the molecular weight using 2D GE as introduced by [11]. For the first dimension, a 7 cm long, pH 3-10 ReadyStrip™ IPG Strips (Bio-Rad, Hercules, USA) was rehydrated by a passive in-gel rehydration method using rehydration buffer containing 0.002% (w/v) bromophenol blue, 7 M urea (Merck, Malaysia), 2 M thiourea (Merck, Malaysia), 40 mM tris-base, 50 mM DTT, 1% (v/v) Pharmalyte (GE Healthcare Life Sciencess, Uppsala, Sweden) and 2% (w/v) CHAPS (AMRESCO®, USA) mixed with a total of 250 µg protein for 12 hours prior to isoelectric focusing step. The rehydrated protein samples were then subjected to IEF at 20°C by the following protocols; 100 V for 20 minutes, 350 V for 1 hour, 3500 V for 2 hours, 10000 V for 1 hour and finally hold at 100 V. For the second dimension, the isoelectric focused IPG strip was incubated with the first equilibration buffer containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris-HCl, pH 8.8 and 2% (w/v) DTT and followed by incubation with the second equilibrium buffer containing 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 50 nM Tris-HCl, pH 8.8 and 2.5% iodoacetamide (IAA) for 15 minutes each. The equilibrated IPG strip was then resolved using 12% resolving gel. The electrophoresis was carried out in 1x electrode buffer at constant voltage 200 V for 35 minutes, until the bromophenol blue moved out of the gel. After that the gel was stained with 0.1% (w/v) Coomasie® Brilliant Blue R-250 (AMRESCO®, USA in a fixative containing 10% (v/v) acetic acid (SYSTERM®, USA) and 40% (v/v) methanol (SYSTERM®, USA) for 1 hour

with gentle shaking. The background stain was removed from the gel using first de-stain solution containing 30% (v/v) acetic acid and 40% (v/v) methanol for 10 minutes and followed by second de-stain solution containing 7% (v/v) acetic acid and 5% (v/v) methanol overnight with gentle shaking. The resolved protein spots on the gel were scanned using the Bio-Rad Molecular Imager GS-800 USB Calibrated Densitometer. The protein spots on the gel from different plant parts was compared and analyzed using the PDQuest analysis software (Bio-Rad, Hercules, USA).

3.0 RESULTS AND DISCUSSION

3.1 SDS-Page

Based on the results of SDS-PAGE, it can be seen that most of the protein bands in both samples were distinctly separated across the polyacrylamide gels with minimal smears can be observed. This results could be related to the efficiency of extraction process which is important to ensure good quality of proteins obtained before proceed to further protein analysis. Gel image in Figure 1 shows the protein profiles of different plant parts of *P. niruri* (leaves and fruits) of which the differences can be seen based on the absence or presence of bands and their intensities. There are approximately 20 bands from leaves which are evenly distributed within the range of 3.5 to 160 kDa meanwhile proteins from fruits show about 10 bands located in between 15 to 60 kDa. Bands labelled 1, 2, 3, 4 and 5 shows proteins that are strongly expressed in leaves sample. Protein band in leaves located in between 50 to 60 kDa, labelled as 1 is expected to be large subunit of Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco). According to [7 and 17], rubisco is the predominant protein and exists in abundant in plants. It consists of eight large subunits that are encoded in nuclear genome. The expected size large subunit of rubisco protein is approximately 53 kDa [6 and 17]. Rubisco is an enzyme that play role in catalysis of carbon dioxide fixation in photosynthesis and also oxygen fixation in photorespiration in plants [4]. On the other hand, protein bands labelled 6, 7 and 8 are intensely expressed in fruits sample. Protein band in both leaves and fruits located in the range of 30 to 40 kDa labelled as 3 and 6 is expected to be antioxidant protein. Based on the research done by [13], *P. niruri* possesses a 35 kDa antioxidant protein which was successfully isolated, purified and characterized. From the SDS results, it can be seen that a protein of this size seems to be expressed more in fruits compared to leaves suggesting that the fruits tissue of *P. niruri* contains higher amount of antioxidant proteins compared to the leaves tissue. *P. niruri* is known to have antioxidant activity but the mechanisms that contribute to this property are yet to be defined. The antioxidant component in *P. niruri* is known to play hepatoprotective role [8] and useful in diabetes mellitus (DM) treatment [14] by acting at cellular level in suppressing the oxidative stress through scavenging of the free radicals and rejuvenate antioxidative defense mechanism of the cells [2].

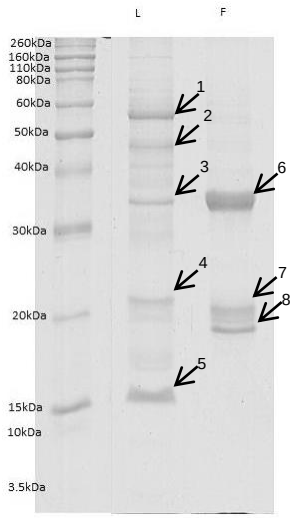

Figure 1 Protein profiles of leaves (L) and fruits (F) tissues of *P. niruri* resolved in 12% polyacrylamide gel. M, broad range protein marker

3.2 2DGE

Figure 2(a) and 2(b) show the results of two-dimensional gel electrophoresis which were carried out for protein extract from leaves and fruits respectively.

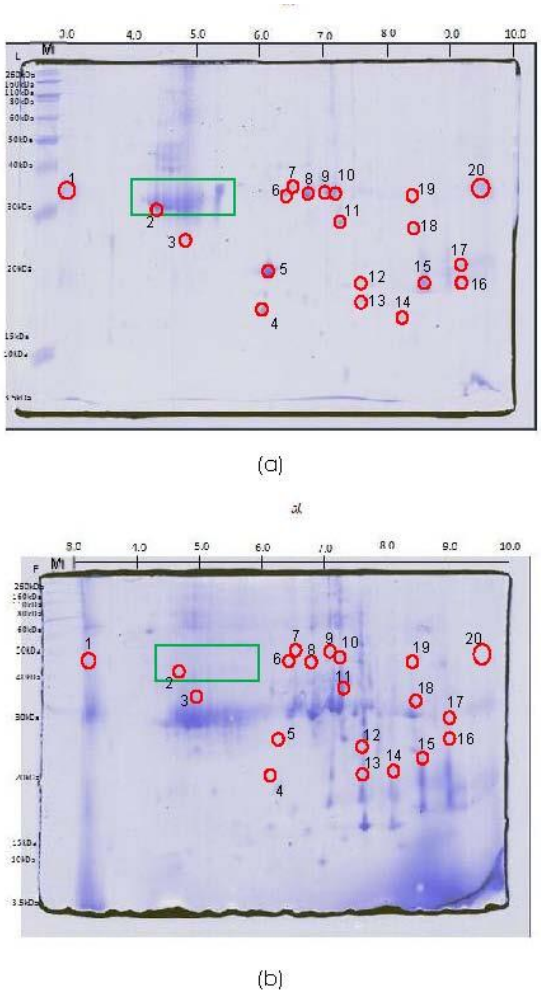

Figure 2 Protein profiles of leaves, L (a) and fruits, F (b) tissues of *P. niruri* from two dimensional gel electrophoresis. M, broad range protein marker

By using gel with protein profiles from leaves as the master gel, PDQuest analysis software had detected approximately 236 spots in leaves and 386 protein spots in fruits sample. Both gels were then overlapped to get the matching spots. Based on the analysis, there were 83 matched spots recognized with 21% match rate. Protein spots from leaves were moderately resolved at pH 4 to 10 at molecular weight within 10 kDa to 50 kDa. On the other hand, protein spots from fruit extract were seen to be resolved within pH 4 to 10 at molecular weight between 10 kDa to 80 kDa. A large cluster of proteins was detected in both leaves and fruits sample at pH 4 to 6 with molecular weight approximately 30 kDa which denoted by the green box. The separation of these protein spots was quite poor since the space between the spots were too close [5]. This condition could be improved in future by using longer IPG strips so that the spots can be resolved better. Red circles numbered 1 to 20 (figure 2) indicates the similar spots that present in both leaves and fruits part. Comparison of two gels showed that spots labeled 4 and 5 have higher intensity in leaves compared to in fruits. Meanwhile, spots labeled 1, 2, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 and 20 demonstrate higher intensity in fruits part. On the other hand, spot labeled 3 shows similar intensity in both sample. The gels were then compared and cross-referenced with 2D-PAGE database of model plant, *Arabidopsis thaliana (A. thaliana)* to determine the possible identity of the protein spots. The protein profile of *A. thaliana* in the database only previews the expressed proteins within the range of 4 to 9 [14]. Thus, only protein spots that fall within the range were possible to be analyzed. Among all, there were four protein spots that were likely to be matched with the database of *A. thaliana* based on the molecular weight and also pI value which are listed in Table 1 below.

Table 1 List of proteins identified from leaves and fruits part of P. niruri through comparison with *A. thaliana* database

Label	Protein	pI	MW	Accession no
2	Ribonucleoprotein	4.66	29181	Q92UU4
4	Ribulose bisphosphate carboxylase small chain 3B	6.07	17609	P99057
5	Nucleoside diphosphate kinase 1	6.19	19999	P39207
6	Vegatative storage protein	6.47	29849	082122

Spot labeled 2, ribolucleoprotein refers to protein that attached to ribonucleic acid (RNA) to form complex that take part in various cellular process including in pre-mRNA processing and also act as signal recognition particles [8]. Meanwhile, ribulose bisphosphate carboxylase small chain 3B or known as small subunit of rubisco is major protein in plants that play role in simultaneously catalyzing the carboxylation of D-ribulose 1, 5- bisphosphate (RuBP) in Calvin cycle and oxygenation of RuBP in photorespiration [17]. On the other hand, nucleoside diphosphate kinase 1(NDK 1) is an enzyme that catalyzes the exchange of phosphate group mainly from nucleoside triphosphates to nucleoside diphosphates-generating nucleoside triphosphates. The activity of NDK 1 helps in maintaining the balance of nucleoside triphosphate in the system. For vegetative storage protein (VSP), it has been identified in many other plants including soybean and potato and can accumulate in plant up to 50% of total soluble proteins [10]. VSP store amino acids temporarily and helps in buffering the availability of nitrogen and other nutrients [10]. Meanwhile, spots labeled 3, 7, 8, 9, 10, 11, 12 and 13 were considered

as hypothetical or unknown protein which refer to proteins that are expressed in the database of *A. thaliana* but not being yet defined [12].

4.0 CONCLUSION

The protein profile of different plant parts of *P. niruri* were successfully compared using one-dimensional and two-dimensional gel electrophoresis. Several expressed protein spots were cross-referenced with *A. thaliana* database and expected to be ribolucleoprotein, ribulose bisphosphate carboxylase small chain 3B, nucleoside diphosphate kinase 1 and vegetative storage protein which play roles at transcripts level, photosynthesis, energy-generation and amino acid storage respectively. For future confirmation of the identity of each protein, the spots on gel could be excised and subjected to MALDI-TOF analysis. The protein profile of bioactive peptides or proteins will give information about the biological characteristic and functions of the protein compound. The discovery of *P. niruri* as the natural source of bioactive compounds may be an economically viable and therapeutically superior alternative to the current generation of drugs and medicines.

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