DETERMINATION OF ISOELECTRIC FOCUSING METHOD FOR PROTEOMIC ANALYSIS OF PURPLE VARIETY OF *Orthosiphon stamineus*

ZETTY AMIRAH BINTI ZULKIFLI

A dissertation submitted in partial fulfilment of the
requirements for the award of the degree of
Master of Science (Biotechnology)

Faculty of Biosciences and Medical Engineering
Universiti Teknologi Malaysia

JULY 2017

ACKNOWLEDGEMENT

Though only my name appears on the cover of this dissertation, great many peoples have contributed to its production. I owe my gratitude to all those people who have made this dissertation possible.

My deepest appreciation to Dr Zaidah bt Rahmat, Faculty of Biosciences and Medical Engineering who has been my supervisor for dissertation project. She provided me with many helpful suggestions, important advice and constant encouragement throughout of my work. One simply could not wish for a better supervisor.

Sincere gratitude to my fellow seniors, lab-mates, and staff in Faculty of Biosciences and Medical Engineering, who made valuable discussions, essential guidance and endless support during the course of this work. All of the hardwork we shared will never be forgotten.

Special attribution goes to my beloved parent, Zulkifli bin Ali and Latifah Abd Jalil. They gave me my name, they lead me my amibition, and everything else in between. They are the reason I did this. Their pride is my main goal in life as the only thing I aspire for is when they say, "I am proud of my only daughter."

Thanks to my family and friends for coming into my life and giving me joy, thank you for having me around. Thank you for the memories I will cherish forever.

Finally, bottomless gratitude to my husband and daughter, Mohd Saifudin bin Othman and Laiqa Maira bt Mohd Saifudin for endless supports, countless helps and precious time, the most thoughtful gift of all. Thank you for the good times, the days you filled with pleasure. Thank you for those fond memories and for feelings I'll always treasure.

ABSTRACT

An ancient medicinal plant known as *Orthosiphon stamineus* purple variety exhibiting multiple remedial effects by the bioactive compound had been reported. However, no study on its protein separation work was documented. Indeed, two-dimensional (2D) electrophoresis systems have few major issues such as complex time-consuming procedure and poor reproducibility result. Therefore, this proteomic research can be utilized as preliminary platform in identification of protein responsible for the production of bioactive compound and its application as remedies. Different sample require different voltage of isoelectric focusing (IEF) to separate the protein due to its isoelectric point (pI) hence this study was conducted in order to determine the best IEF method producing reproducible protein spots and to determine the protein spot distribution and pattern from 2D proteomics of *O. stamineus*. Initially, protein extraction utilizing unmodified and modified (1 mL of 80% methanol in 0.1M ammonium acetate, 0.6 mL of each phenol and SDS buffer in 5 % ß-mercaptoethanol, and 0.15mL lysis buffer) phenol extraction with three preliminary washes were performed. The extracted protein was then quantified by Bradford Assay whereas its quality was checked via Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE). The modified extraction method resulted in higher spots distribution compared to the unmodified method. In this study, three different IEF method of method 1 (2000V), method 2 (4000V), and method 3 (8000V) were applied before the protein spots pattern and distribution were analysed further. It was found that method 2 was the best IEF method for this plant as 2-fold higher reproducible protein spots were produced. In fact, 15 proteins were expected to be associated with the protein spots obtained that may have high relation with photosynthesis and antioxidant activity. The findings suggested that numerous valuable proteins are expressed in *O. stamineus*. Hence, the proteins mechanism and properties should be revealed to serve the medical sector in the future.

ABSTRAK

Pokok perubatan purba iaitu *Orthosiphon stamineus* berwarna ungu mempunyai pelbagai kesan pemulihan kesan daripada sebatian bioaktif telah dilaporkan sebelum ini. Tetapi, tiada sebarang kajian tentang pemisahan protein pokok ini didokumenkan. Sistem elektroforesis dua dimensi (2D) mempunyai beberapa kelemahan iaitu prosedur yang rumit dan kadar kebolehulangan yang rendah. Oleh itu, kajian proteomik terhadap pokok ini dapat digunakan sebagai platform kerja awal dalam mengenal pasti protein yang bertanggungjawab menghasilkan sebatian bioaktif dan aplikasinya sebagai penawar. Voltan isoelectric focusing" (IEF) yang diperlukan untuk memisahkan protein berbeza bagi setiap sampel kerana "isoelectric point" (pI). Kajian ini dijalankan bagi mengenal pasti kaedah IEF terbaik yang menghasilkan titik protein berulang dan mengenal pasti corak dan taburan titik protein bagi pokok ini.menggunakan kaedah proteomik 2D. Pada mulanya, protein diekstrak dengan kaedah pengekstrakan menggunakan fenol yang asal dan yang diubah suai (1 mL 80% methanol dalam 0.1M ammonium acetate, 0.6 mL phenol dan SDS buffer dalam 5 % ß-mercaptoethanol, dan 0.15mL lysis buffer) sebelum kuantiti dan kualiti protein dikenal pasti menggunakan Bradford assay dan sodium dodecyl sulfate-poliakrilamid gel elektroforesis (SDS-PAGE). Kaedah pengekstrakan menggunakan fenol yang telah diubah suai menghasilkan lebih banyak bilangan titik protein berbanding kaedah yang asal. Selain itu, tiga kaedah IEF yang digunakan dalam kajian ini ialah kaedah 1(2000V), kaedah 2(4000V) dan kaedah 3(8000V). Kaedah 2 menghasilkan corak titik protein dua kali ganda lebih tinggi berbanding kaedah IEF lain. Seperkara lagi, 15 protein yang dijangka mempunyai hubungkait dengan titik protein dari elektroforesis 2D telah dikenal pasti dan mungkin mempunyai kaitan dengan proses fotosintesis dan aktiviti antioksidan pokok ini. Kesimpulannya, terdapat pelbagai protein berharga dihasilkan oleh pokok ini. Oleh itu, sifat dan mekanisma protein ini harus dikaji agar dapat dimanfaatkan dalam bidang perubatan di masa hadapan.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

1D	-	One dimensional
2-DE	-	Two dimensional electrophoresis
APS	-	Ammonium persulfate
BSA	-	Bovine serum albumin
CHAPS	-	3-(3-Cholamidopropyl)dimethylammonia)-1-Propanesulfonic acid
CBB	-	Coomassie Brilliant Blue
cm	-	Centimeter
DNA	-	Deoxyribonucleic acid
DTT	-	Dithiothreitol
EDTA		Ethylenediaminetetraacetic acid
et al.	-	And friends
HCl	-	Hydrochloric acid
IAA	-	Indole-3-acetic acid
IEF	-	Isoelectric focusing
IPG	-	Immobilised pH gradient
KPO_4		Potassium phosphate
QB	-	Quenching buffer
RNA	-	Ribonucleic acid
Rpm	-	Rotary per minute
SDS	-	Sodium Dodecyl Sulfate
SDS-PAGE	-	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	-	Standard Error Mean
TCA	-	Trichroloacetic acid
TEMED	-	Tetramethylethylenediamine

LIST OF SYMBOLS

A_{595}	-	Absorbance at 595 nanometer
β	-	Beta
°C	-	Degree celcius
G	-	Gram
kDa	-	Kilo Dalton
µg	-	Microgram
µg/mL	-	Microgram per milliliter
µg/g	-	Microgram per gram
µL	-	Microliter
mg	-	Milligram
mL	-	Milliliter
mM	-	Milli Molar
M	-	Molar
%	-	Percent
V	-	Voltage
v/v	-	Volume per volume
w/v	-	Weight per volume

LIST OF APPENDICES

CHAPTER 1

INTRODUCTION

1.1 Background of study

Orthosiphon stamineus Benth or Cat's Whiskers or "misai kucing" is from the family of Lamiaceae. *O. stamineus* is an ancient medicinal herb that had been widely used since many centuries before (Wiart, 2000). This herbal tea is a well-known herbal drink consumed to cure critical human disease (Basheer and Majid, 2010). It is commonly found in South East Asia used for treatment of various disorders and ailments including urinary lithiasis, edema, eruptive fever, influenza, rheumatism, hepatitis, jaundice and biliary lithiasis (Tran, 1990).

Extracts of *O. stamineus* leaves contain wide variety of compounds, including flavonoids, terpenoids or organic acids such as rosmarinic or caffeic acid (Tezuka *et al*., 2000). The polyphenols for instance are able to provide enzyme inhibition and antioxidant activity which lead to therapeutic effect to its consumer (Hossain and Rahmanjung, 2011). The various remedial effects of this herb has attracted researcher's interest to further investigate its valuable components and mechanism of action. Proteomic analysis approach is a great option to complement previous studies as it enables the study of protein properties extracted from the valuable herbs. Indeed, the study of the plant proteomes wholly contributes to its medicinal value mainly for human sake.

Proteomics was traditionally used for protein separation utilizing the phenomenal two-dimensional gel electrophoresis technology (Celis and Bravo, 1984). This enables quantitative analysis of protein amounts available in the complex extracts according to the protein's isoelectric point and molecular weight*et al*, 1989). The fundamentals and principles of two dimensional (2D) proteomics have been established and practiced in numerous successful studies (Doleckova *et al*., 2012; Barrabes *et al.*, 2010; Hartinger *et al*., 1996; Tang *et al*., 1997). Hence, its advantages and limitations are widely found in reviews and articles (Rogowska *et al*., 2013).

1.2 Problem statement

O. stamineus is well known for its medicinal values contributed by the bioactive compound which attracts researchers' interest. Still, no study on its protein separation work was reported due to the complexity of the protein and the proteomic protocol itself. In fact, this plant specifically require more starting material to extract its protein sample compared to other plant since it contain high impurities. The most commonly used protein separation alternative is the 2D proteomics methods; combination of IEF protocol and molecular weight (Rabilloud *et al*., 2010). However, 2D electrophoresis systems still have few major issues that need to be addressed, such as complex time-consuming experimental procedure and poor reproducibility of the result (Barrabes *et al*., 2010). In this study, the experiment was conducted in order to determine the best IEF protocol producing reproducible protein spot of *Orthosiphon stamineus* thus saving time. The findings of the study can be further utilised for identification of protein responsible in producing valuable bioactive compound and application of the protein in medical area.

1.3 Objectives

The objectives of this study are as follows:

i. To determine the best IEF method producing reproducible protein spots from purple variety of *Orthosiphon stamineus.*
ii. To determine the protein spot distribution and pattern from 2D proteomics of purple *Orthosiphon stamineus.*

1.4 Significance of work

Various medicinal value that *O. stamineus* offers since decades ago has attracted the researchers' interest (Tran, 1990). Numerous study were done on its pharmacological and phytochemical properties. On the other hand, fewer study related to the herb's protein content was established. The findings of this project are beneficial as the best IEF method producing reproducible spots of extracted protein from *O. stamineus* was determined thus saving time consumed in performing IEF. The 2D proteomic analysis of this herbal plant can be utilized as preliminary work platform for further protein identification especially related to its medicinal potential. Hence, those valuables protein will be able to serve the mankind in the pharmaceutical industry and the medical sector according to its medicinal properties.

1.5 Scope of work

The purple variety of *O. stamineus* was chosen in this study since it is pigmented hence it was expected to contain higher protein content compared to the white variety. In order to achieve the objectives of the study, there are 3 main procedures involved. These procedures are, protein extraction from the *O. stamineus* leaves using phenol extraction with 3 solvent wash, protein quantification utilizing

Bradford assay, and protein separation technique which focuses on the 2D gel electrophoresis. The 2D gel electrophoresis involves the isoelectric focusing (IEF) and SDS-PAGE principles which separate extracted protein according to its pI and molecular weight respectively. In fact, different protein sample require different voltage of IEF method in order to separate and focus the protein spots according to its pI. In this study, three IEF methods of method 1 (2000V), method 2 (4000V), and method 3 (8000V) were applied to compare its protein spot distribution and pattern.

REFERENCES

Adam, Y., Somchit, N. N., Sulaiman, M.R., Nasaruddin, A. A., Zuraini, A., Bustamam, A. A., and Zakaria, Z. A. (2009). Diuretic properties of *Orthosiphon stamineus* Benth.. *Journal of Ethnopharmacology*. 124, 154–158.

Almatar, M., Rahmat, Z., and Salleh, F. M. (2013). Preliminary morphological and anatomical study of *O. stamineus*. *Indian Journal of Pharmaceutical and Biological.Research*. 1(4), 1-6.

Alves, C. T., Ferreira, I. C. F. R., Barros, L., Silva, S., Azeredo, J., and Henriques, M. (2014). Antifungal activity of phenolic compounds identified in flowers from North Eastern Portugal against Candida species. *Future Microbiology*. 9(2), 139–146.

Ameer, O. Z., Salman, I. M., Asmawi, M. Z., Ibraheem, Z. O., and Yam, M. F. (2012). *Orthosiphon stamineus*: traditional uses, phytochemistry, pharmacology, and toxicology. *Journal of Medicinal Food*. 15, 678–690.

Amzad, H. M., Ismail, Z., Rahman, A., and Kang, S. C. (2008). Chemical composition and anti-fungal properties of the essential oils and crude extracts of *Orthosiphon stamineus* Benth industrial crops and products. 27, 328–334.

Ansari, P., Stoppacher, N., and Baumgartner, S. (2012). Marker peptide selection for the determination of hazelnut by LC–MS/MS and occurrence in other nuts. *Analytical and Bioanalytical Chemistry*. 402(8), 2607–2615.

Arafat, O. M., Tham, S. Y., Sadikun, A., Zhari, I., Haughton, (2008). Effects of *Orthosiphon stamineus* methanol extracts in rats. *Journal of Ethnopharmacology*. 13, 118(3), 354-60.

Awale, S., Tezuka, Y., Banskota, A. H., Adnyana, I. K., and Kadota, S. (2003). Highly oxygenated isopimarane-type diterpenes from *Orthosiphon stamineus* of

Indonesia and their nitric oxide inhibitory activity. *Chemical and Pharmaceutical Bulletin*. 51, 268–275.

Barrabes, S., Sarrats, A., Fort, E., De Llorens, R., Rudd, P. M., and Peracaula, R. (2010). Effect of sialic acid content on glycoprotein pI analyzed by two-dimensional electrophoresis. *Electrophoresis*. 31, 2903–2912.

Basheer, A. M. and Majid, A. A. (2010). Medicinal Potentials of *Orthosiphon Stamineus* Benth. *WebmedCentral CANCER*. 1(12), WMC001361.

Bauw, G., Van Damme, J., Puype M., Vandekerckhove, J., Gesser, B., Ratz, G. P., *et al*. (1989). Protein-electroblotting and microsequencing strategies in generating protein data bases from two-dimensional gels. *Proceedings of the National Academy of Sciences* USA. 86, 7701–7705.

Beaux, D., Fleurentin, J., and Mortier, F. (1999). Effect of extracts of *Orthosiphon stamineus* Benth, *Hieracium pilosella L., Sambucus nigra L.* and *Arctostaphylos uva-ursi* (L.) Spreng. In Rats. *Phytotherapy Research*. 13, 222–225.

Bjellqvist, B. K., Ek, P.G., Righetti, E., Gianazza, A., Gorg, R., Westermeier, and Postel, W. (1982). Isoelectric focusing in immobilized pH gradients: principle, methodology and some applications. *Journal of Biochemical and. Biophysical Methods.* 6, 317-339.

Bojórquez-Velázquez, E., Lino-López, G. J., Huerta-Ocampo, J. A., Barrera-Pacheco, A., de la Rosa, A. P., Moreno, A., Mancilla-Margalli, N. A., and Osuna-Castro, J. A., (2016). Purification and biochemical characterization of 11S globulin from chan (*Hyptissuaveolens L. Poit*) seeds. *Food chemistry*. 1(192), 203-211.

Carter, B. L. and Basile, J., (2005). Development of Diabetes with Thiazide Diuretics: The Potassium Issue. *The Journal of Clinical Hypertension*. 7 (11), 638-640.

Caverzan, A., Passaia, G., Rosa, S. B., Ribeiro, C. W., Lazzarotto, F., and Margis-Pinheiro, M. (2012). Plant responses to stresses: Role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology*. 35(4), 1011–1019.

Chan, L. K., and Loo, P. S., (2006). Morphological Similarities and Differences between the Two Varieties of Cat`s Whiskers (*Orthosiphon stamineus* Benth.) grown in Malaysia. *International Journal of Botany.* 2, 1-6.

Celis, J. E. and Bravo. R. (1984). Two-dimensional Gel Electrophoresis of Proteins: Methods and Applications. *Academic Press New York*.

Celis, J. E., Carter, N., Hunter, T., Shotton, D., Simons, K., and Small, J. V., (1997). Cell Biology: A laboratory Handbook. *New York: Academic Press*. 4(2).

Chin, J. H., Abas, H. H., and Sabariah, I., (2008). Toxicity study of *Orthosiphon stamineus* Benth (Misai Kucing) on Sprague Dawley rats. *Tropical Biomedicine*. 25, 9–16.

Chun-Hoong, H., Ismail, N., Shaida-Fariza, S., and Ahmad, R. (2010). In vitro antibacterial and antioxidant activities of *Orthosiphon stamineus* Benth extracts against food-borne bacteria. *Food Chemistry*. 122, 1168–1172.

Costa, T.R., Fernandes, O.L.F., Santos, S.C., Oliveria, C.M.A., Liao, L.M., Ferri, P.H., Paulo, J.R., Ferreira, H.D., Sales, B.H.N., and Silva, M.R.R., (2000). Antifungal activity of volatile constituents of *Eugenia dysenterica* leaf oil. *Journal of Ethnopharmacology*. 72 (12), 111-117.

Doleckova, I., Rarova, L., Gruz, J., Vondrusova, M., Strnad, M., and Krystof, V. (2012). Antiproliferative and antiangiogenic effects of flavone eupatorin, an active constituent of chloroform extract of *Orthosiphon stamineus* leaves. *Fitoterapia*. 83, 1000–1007.

Eisai, P. T. (1995). Indonesia Medicinal Herb Index in Indonesia. *Dian Rakyat Publication*,Jakarta, Indonesia, 263.

Ernst, O., and Zor, T. (2010). Linearization of the Bradford Protein Assay. *Journal of Visualized Experiments : JoVE*, 38, 1918.

Faizul, F. M., Aminudin, N., Kadir, H. A., and Tayyab, S. (2009). Bilirubin lowering potential of *Orthosiphon stamineus* in temporarily jaundiced adult rats. *African Journal of Pharmacy and Pharmacology*. 3(7), 359-361.

Faurobert, M., Pelpoir, E., and Chaïb, J. (2007). Phenol extraction of proteins for proteomic studies of recalcitrant plant tissues. *Methods Molecular Biology*. 355, 9–14.

Gorg, A., Weiss, W., and Dunn, M. J. (2004). Current two-dimensional electrophoresis technology for proteomics. *Proteomics*. 4, 3665–3685.

Gorg, A. and Weiss, W. (2000). Two-dimensional electrophoresis with immobilized pH gradients. Rabilloud, T. (Ed.). *In Proteome Research: Two-dimensional Gel Electrophoresis and Identification Methods*. (57-106). German: Springer.

Haleem, J. I. and Timothy, D. V. (2008). Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. *BioTechniques*. 44, 697-700.

Haloui, M., Louedec, L., Michel, J. B., and Lyoussi, B., (2000). Experimental diuretic effects of *Rosmarinus officinalis* and *Centaurium erythraea*. *Journal of Ethnopharmacology*. 71, 465‑472.

Hartinger, J., Stenius, K., Hogemann, D., and Jahn, R., (1996). 16-BAC/SDS-PAGE: a two-dimensional gel electrophoresis system suitable for the separation of integral membrane proteins. *Analytical Biochemistry*. 240, 126–133.

Hew, C. S., and Gam, L. H. (2011). Proteome analysis of abundant proteins extracted from the leaf of *Gynura procumbens* (Lour.) Merr. *Applied Biochemistry and Biotechnology.* 165, 1577–1586.

Hossain, M. A., and Rahman, S. M. (2011). Isolation and characterisation of flavonoids from the leaves of medicinal plant *Orthosiphon stamineus*. *Arabian Journal of Chemistry.* Article in press.

Hsuan, K. (1986). Order dan Famili Tumbuhan Berbiji di Tanah Melayu. *Dewan Bahasa dan Pustaka,* Kuala Lumpur, Malyasia. 393–397.

Janine, K., Margarete, B., Frank, H., Uwe, K., Gary, H., Peter, S., and Karl-Josef, D. (2002). The plant-specific function of 2-Cys peroxiredoxin mediated detoxification of peroxides in the redox-hierarchy of photosynthetic electron flux. *Proceedings of the National Academy of Sciences*. 99(8), 5738–5743.

Jiaman, S., Junfan, F., and Rujun, Z. (2014). Proteomic analysis of differentially expressed proteins induced by salicylic acid in suspension-cultured ginseng cells. *Journal of Biological Sciences*. 21(2), 185–190.

Jung, M., Park, M., Lee, H. C., Kang, Y. H., Kang, E. S., and Kim, S. K. (2006). Antidiabetic agents from medicinal plants. *Current Medicinal Chemistry*. 13, 1203-1218.

Jungblut, P., Otto, A., Zeindl-Eberhart, E., Plessner, K. P., Knecht, M., RegitzZagrosek, V., Fleck, E., Wittmann-Liebold, B., (1994). Protein composition of the human heart: the construction of a myocardial two-dimensional electrophoresis database. *Electrophoresis.* 15, 685-707.

Klose, J. and Kobalz, U. (1995). Two-dimensional electrophoresis of proteins: An updated protocol and implications for a functional analysis of the genome. *Electrophoresis.* 16, 1034-1059.

Kolin, A. (1954). Separation and concentration of proteins in a pH field combined with an electric field. *Journal of Chemical Physics*. 22, 1628-1629.

Kreydiyyeh, S. I., and Usta, J., (2002). Diuretic effect and mechanism of action of parsley. *Journal of Ethnopharmacology*. 79, 353‑357.

Ku, H. K., Lim, H. M., Oh, K. H., Yang, H. J., Jeong, J. S., and Kim, S. K. (2013). Interpretation of protein quantitation using the Bradford assay: comparison with two calculation models. *Analytical Biochemistry*. 434, 178-180.

L´opez, J. L. (2007). Two-dimensional electrophoresis in proteome expression analysis. *Journal of Chromatography B*. 849, 190–202.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. *227*, 680-685.

Lee, K. S. (2015). *Gel-Based Proteome Analysis of Ocimum sanctum*. Bachelor of Science, Universiti Teknologi Malaysia, Skudai.

Lee, W. L. (2014). *Micropropagation and cell culture of misai kucing (Orthosiphon stamineus Benth) and detection of rosmarinic acid in the in vitro cultures*. Master of Science, Universiti Sains Malaysia, Penang, Malaysia.

Li, Q., Li, Y., Wang, Y., Cui, Z., Gong, L., Qu, Z., Zhong, Y., Zhou, J., Zhou, Y., Gao, Y., and Li, Y. (2016). Quantitative proteomic study of human prostate cancer cells with different metastatic potentials. *International journal of oncology*. 48(4), 1437-1446.

Lu, Y. X., Zhang, Q., Li, J., Sun, Y. X., Wang, L.Y., Cheng, W. M., *et al.*(2010). Antidiabetic effects of total flavonoids from *Litsea coreana* leave on fat-fed, streptozotocin-induced type 2 diabetic rats. *The American Journal of Chinese Medicine*. 38, 713-725.

Mariam, A., Asmawi, M. Z., and Sadikun, A. (1996). Hypoglycaemic activity of the aqueous extract of *Orthosiphon stamineus. Fitoterapia*. 67, 465-468.

Mohamed, E. A. H., Yam, M. F., Ang, L. F., Mohamed, A. J., Mohamed, A. J., and Asmawi, M. Z. (2013). Antidiabetic Properties and Mechanism of Action of *Orthosiphon stamineus* Benth Bioactive Sub-fraction in Streptozotocin-induced Diabetic Rats. *Journal of Acupuncture and Meridian Studies.* 6(1), 31-40.

Nail, A. M. and Zin, N. H. M. (2015). Protein profiling of different plant tissues from herb *Phyllanthus niruri*. *Jurnal Teknologi*. 77(24), 95-99.

Natarajan, S., Xu, C., Caperna, T. J., and Garrett, W. M. (2005). Comparison of protein solubilisation method suitable for proteomic analysis of soybean seed proteins. *Analytical Biochemistry*. 342, 214-220.

Ng, M. L. (2016). *Gel-based Proteome Analysis of Orthosiphon stamineus.* Bachelor of Science, Universiti Teknologi Malaysia, Skudai.

O'Farrell, P. H. J. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry*. 250 (10), 4007-4021.

Ong, B. H. (2017). *Heavy Metal Content and Protein Expression Profile of Melastoma malabathricum from Ex-mining Site.* Master of Science, Universiti Teknologi Malaysia, Skudai

Perry, L. M. (1980). Medicinal plants of east and south East Asia; attributed properties and uses. The Massachusetts Institute of Technology, Cambridge, MA, 190–191.

Rabilloud, T., Chevallet, M., Luche, S., and Lelong, C. (2010). Two-dimensional gel electrophoresis in proteomics: past, present, and future. *Journal of Proteomics.* 73, 2064–2077.

Rabilloud, T. and Charmont, S. (2000). Detection of proteins on two-dimensional gels. Rabilloud, T. (Ed.). *Proteome Research:Two-dimensional Gel Electrophoresis and Identification Methods.* (107–126). France: Springer

Ramly, N. A. (2016). *Evaluation of 1D and 2D gel-based proteome from fresh leaf of Moringa oleifera.* Master of Science, Universiti Teknologi Malaysia, Skudai.

Raunkjær, K., Hvitved-Jacobsen, T., and Nielsen, P. H. (1994). Measurement of Pools of Protein: Carbohydrate and Lipid in Domestic Wastewater. *Water Research*. 28(2), 251-262.

Rogowska-Wrzesinskaa, A., Bihana, M. C. L., Morten, T. A. and Roepstorff P. (2013). 2D gels still have a niche in proteomics. *Journal of proteomics*. 88, 4-13.

Saidan, N. H., Hamil, M. S. R., Memon, A. H., Abdelbari, M. M., Hamdan, M. R., Mohd, K. S., Ismail, Z. (2015). Selected metabolites profiling of *Orthosiphon stamineus* Benth leaves extracts combined with chemometrics analysis and correlation with biological activities. *BMC Complementary and Alternative Medicine*. *15*, 350.

Saravanan, R. S., and Rose, J. K. (2004). A critical evaluation of sample extraction techniques for enhanced proteomic analysis of recalcitrant plant tissues. *Proteomics*. 4, 2522–2532.

Shim, S. Y. and Gam, L. H. (2012). Analysis of *Piper sarmentosum* proteome using two dimensional gel electrophoresis and mass spectrometry. *Asia-Pacific Journal of Molecular Biology and Biotechnology*. 20(4), 124-139.

Shaw, M. M. and Riederer, B. M. (2003). Sample preparation for two-dimensional gel Electrophoresis. *Proteomics.* 3, 1408–1417.

Tang, W., Harrata, A. K., and Lee, C. S., (1997). Two-dimensional analysis of recombinant *E. coli* proteins using capillary isoelectric focusing electrospray ionization mass spectrometry. *Analytical Chemistry*. 69, 3177-3182.

Tezuka, Y., Stampoulis, P., Banskota, A. H., Awale, S., Tran, K. Q., Saiki, I., *et al*. (2000).Constituents of the Vietnamese medicinal plant *Orthosiphon stamineus*. *Chemical Pharmaceutical Bulletin* (Tokyo). 48, 1711–1719.

Tran, K. (1990), Medicinal Plants in Viet Nam. WHO Regional Office for the Western Pacific Manila and Institute of Material Medica Hanoi, Science and Technology Publishing House, Hanoi, Vietnam, 271.

Usman, K. (2014). *Protein Profiling of stamineus.* Master of Science, Universiti Teknologi Malaysia, Skudai.

Wang, W., Bignani, R., Scali, M., and Cresti, M. (2006). A Universal and Rapid Protocol and Protein Extraction from Recalcitrant Plant Tissues for Proteomic Analysis. *Electrophoresis.* 27(0). 2782-2786.

Wang, Y., Shan, Q., Hou, G., Zhang, J., Bai, J., Lv, X., Xie, Y., Zhu, H., Su, S., Li, Y., and Zi, J. (2016). Discovery of potential colorectal cancer serum biomarkers through quantitative proteomics on the colonic tissue interstitial fluids from the AOM–DSS mouse model. *Journal of Proteomics*. 132, 31-40.

Wiart, C., (2000). Medicinal plant of Southeast Asia. *Pelanduk Publication Sdn. Bhd*, Selangor, 37–45.

Wilkins, M. R., Williams, K. L., Appel, R. D., and Hochstrasser, D. F. (1997). Proteomic Research: New Frontiers in Functional Genomics (Principles and Practice). *Berlin: Springer Verlag*; 1997