FRACTIONATION OF ROSMARINIC ACID RICH EXTRACT FROM
ORTHOSIPHON STAMINEUS FOR ENZYMATIC ANTI-DIABETIC ACTIVITY

NGO YI LEI

A thesis submitted in fulfilment of the
requirements for the award of the degree of
Master of Philosophy

Faculty of Chemical and Energy Engineering
Universiti Teknologi Malaysia

JUNE 2018
To my beloved family, supervisor and friends
ACKNOWLEDGEMENTS

For my accomplishment, I would like to express my deepest appreciation and sincerest gratitude to those who supported me throughout my Master degree studies. I would foremost to thank my supervisor, Assoc. Prof. Dr. Chua Lee Suan for her helpful guidance and advice throughout my research project. Her kind motivation, constructive suggestions, and immense knowledge have made my research works completed successfully.

I would also like to extend many thanks to the post-graduate seniors of Institute of Bioproduct Development, Universiti Teknologi Malaysia, for their invaluable guidance and excellent assistance throughout the conduction of bioprocessing works in the laboratory.

Lastly but not least, my special thanks to my family and friends for their constant moral support and encouragement from the beginning until the end of the project. Without their continuous assistances and patience, this study would not have succeeded on time.
Orthosiphon stamineus (O. stamineus) is one of the herbal plants containing high amount of rosmarinic acid. However, there is no standard guideline, specifically for rosmarinic acid rich extract from O. stamineus. Therefore, this study was focused on the fractionation of rosmarinic acid from the O. stamineus crude extract using chromatographic methods, namely thin layer chromatography integrated with column chromatography (TLC-CC) and liquid-liquid extraction integrated with solid phase extraction (LLE-SPE). Both fractionation methods were compared, and it was found that TLC-CC performed better than LLE-SPE to recover rosmarinic acid from the O. stamineus crude extract. The purity of the rosmarinic acid rich extract was 100% (fraction 8) which was greater than sub-fraction 1-3 (75%) prepared by LLE-SPE. TLC-CC increased the rosmarinic acid content from 3.6% w/w in crude extract to 100.0% w/w in the fraction 8, while LLE-SPE only increased the content of rosmarinic acid up to 75.0% w/w in the combined sub-fraction 1 to 3. The increase of rosmarinic acid content also simultaneously increased enzymatic anti-diabetic activities compared to the crude extract. The fraction of 100% rosmarinic acid from TLC-CC significantly (P < 0.05) inhibited the activity of α-amylase and α-glucosidase with IC$_{50}$ values of 2.31 mg/mL and 0.34 mg/mL, respectively. The IC$_{50}$ values were comparable to those results of standard drug, acarbose which showed 1.03 mg/mL and 1.66 mg/mL for α-amylase and α-glucosidase, respectively. Kinetic studies revealed that the 100% rosmarinic acid fraction inhibited α-amylase competitively, but non-competitively for α-glucosidase inhibition. The introduction of rosmarinic acid as an inhibitor slow down the digestion rate of starch by α-amylases, and reduce the performance of α-glucosidase by decreasing its V$_{\text{max}}$ value to 0.05 mM/min. Understanding the kinetic information of the enzymatic reaction is important to predict in vivo metabolism of rosmarinic acid for drug design. In conclusion, high anti-diabetic property rosmarinic acid rich extract from O. stamineus can be obtained using column chromatography technology.
**ABSTRAK**

*Orthosiphon stamineus* (*O. stamineus*) merupakan salah satu herba yang mengandungi asid rosmarinik yang banyak. Walau bagaimanapun, tiada garis panduan khusus bagi penyediaan asid rosmarinik dari *O. stamineus*. Oleh itu, kajian ini telah tertumpu kepada pemeringkatan asid rosmarinik daripada ekstrak mentah *O. stamineus* dengan menggunakan kaedah kromatografi, iaitu kromatografi lapisan nipis bersepadu dengan kromatografi turus (KLN-KT) dan pengekstrakan ceair-ceair bergabung dengan pengekstrakan fasa pepejal (PCC-PFP). Kedua-dua kaedah pemeringkatan ini telah dibandingkan dan didapati bahawa KLN-KT menghasilkan lebih banyak asid rosmarinik berbanding PCC-PFP daripada ekstrak mentah *O. stamineus*. Ketulenan ekstrak kaya asik rosmarinik adalah 100% (pecahan 8) dan ianya lebih tulen berbanding sub-pecahan 1-3 (75%) yang dihasilkan melalui kaedah PCC-PFP. KLN-KT meningkatkan kandungan asid rosmarinik daripada 3.6% berat dalam ekstrak mentah kepada 100% berat dalam pecahan 8, manakala PCC-PFP hanya meningkatkan kandungan asid rosmarinik kepada 75% berat dalam gabungan sub-pecahan 1 hingga 3. Peningkatan kandungan asid rosmarinik juga meningkatkan aktiviti perencatan enzim kencing manis berbanding dengan ekstrak mentah. Pecahan 100% asid rosmarinik dari KLN-KT merencat aktiviti α-amilase dan α-glukosidase secara ketara (*P < 0.05*) dengan nilai IC$_{50}$ masing-masing ialah 2.31 dan 0.34 mg/mL. Nilai IC$_{50}$ ini setanding dengan ubat piawai, acarbose yang menunjukkan nilainya masing-masing pada 1.03 dan 1.66 mg/mL bagi α-amilase dan α-glukosidase. Kajian kinetik menunjukkan bahawa pecahan 100% asik rosmarinik merencat α-amilase secara kompetitif, tetapi tidak bagi α-glukosidase. Penggunaan asid rosmarinik sebagai perencat dapat melambatkan kadar penghadaman kanji oleh α-amilases, dan mengurangkan prestasi α-glukosidase dengan menurunkan nilai $V_{max}$ kepada 0.05 mM/min. Pemahaman tentang kinetik enzim tersebut adalah penting untuk meramalkan metabolisma asid rosmarinik secara *in vivo* dalam rekaan ubat. Kesimpulannya, pecahan asid rosmarinik yang mempunyai potensi tinggi untuk mengurangi gejala kencing manis boleh diperolehi daripada *O. stamineus* melalui teknologi kromatografi turus.
## TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>DECLARATION</td>
<td></td>
<td>ii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td></td>
<td>iii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td></td>
<td>iv</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td></td>
<td>v</td>
</tr>
<tr>
<td>ABSTRAK</td>
<td></td>
<td>vi</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td></td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td></td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>xii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td></td>
<td>xv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td></td>
<td>xvi</td>
</tr>
</tbody>
</table>

### 1 INTRODUCTION

1.1 Research Background  
1.2 Research Problem  
1.3 Research Objectives  
1.4 Research Scopes  
1.5 Research Significance  

### 2 LITERATURE REVIEW

2.1 Background of *Orthosiphon stamineus*  
2.1.1 Phytochemicals in *Orthosiphon Stamineus*
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1.2 Medicinal Uses of <em>Orthosiphon Stamineus</em></td>
<td>14</td>
</tr>
<tr>
<td>2.2 Rosmarinic Acid and Its Pharmacological Activities</td>
<td>15</td>
</tr>
<tr>
<td>2.3 Extraction Techniques for Herbal Plant</td>
<td>19</td>
</tr>
<tr>
<td>2.4 Fractionation of Crude Extract</td>
<td>22</td>
</tr>
<tr>
<td>2.4.1 Liquid-Liquid Extraction</td>
<td>23</td>
</tr>
<tr>
<td>2.4.2 Solid-Liquid Extraction</td>
<td>24</td>
</tr>
<tr>
<td>2.5 Chromatographic Analysis of Herbal Extract and Fractions</td>
<td>32</td>
</tr>
<tr>
<td>2.5.1 Thin Layer Chromatography</td>
<td>32</td>
</tr>
<tr>
<td>2.5.2 High Performance Liquid Chromatography</td>
<td>35</td>
</tr>
<tr>
<td>2.5.3 Liquid Chromatography-Tandem Mass Spectrometer</td>
<td>39</td>
</tr>
<tr>
<td>2.6 Pathophysiology of Diabetes Mellitus</td>
<td>42</td>
</tr>
<tr>
<td>2.7 Types of Diabetic Mellitus</td>
<td>45</td>
</tr>
<tr>
<td>2.8 Anti-diabetic Agents</td>
<td>47</td>
</tr>
<tr>
<td>2.9 Techniques of Anti-Diabetic Evaluation</td>
<td>50</td>
</tr>
<tr>
<td>2.9.1 Enzymatic α-amylase Inhibitory Assay</td>
<td>51</td>
</tr>
<tr>
<td>2.9.2 Enzymatic α-glucosidase Inhibitory Assay</td>
<td>53</td>
</tr>
<tr>
<td>2.9.3 Kinetics of Enzyme Inhibition</td>
<td>54</td>
</tr>
<tr>
<td>2.10 Chapter Summary</td>
<td>60</td>
</tr>
</tbody>
</table>

### 3 MATERIALS AND METHODOLOGY

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0 Introduction to Overall Study</td>
<td>62</td>
</tr>
<tr>
<td>3.1 Chemicals and Reagents</td>
<td>64</td>
</tr>
<tr>
<td>3.2 Reflux Technique for Crude Extract Preparation</td>
<td>64</td>
</tr>
<tr>
<td>3.3 Fractionation Based on Column Chromatography</td>
<td>65</td>
</tr>
</tbody>
</table>
3.3.1 Solvent System Development by Thin Layer Chromatography 65
3.3.2 Optimization of Solvent System in Column Chromatography 66
3.3.3 Optimization of Packed Column Height 67
3.3.4 Stability of Absorbent 68

3.4 Two-Dimensional Fractionation Based on Liquid-Liquid Extraction and Solid Phase Extraction 68
  3.4.1 Sample Partitioning by Liquid-Liquid Extraction 68
  3.4.2 Second Dimension of Solid Phase Extraction for Rosmarinic Acid Rich Fraction 69

3.5 Quantification of Rosmarinic Acid by LC-DAD-MS/MS 70

3.6 Anti-diabetic Enzymatic Activity 70
  3.6.1 α-amylase Inhibitory Assay 71
  3.6.2 α-glucosidase Inhibitory Assay 72
  3.6.3 Mode of α-amylase Inhibition 72
  3.6.4 Mode of α-glucosidase Inhibition 73
  3.6.5 Calculation of 50% Inhibitory Concentration 74

3.7 Design of Experiments 74
3.8 Statistical Analysis 74

4 RESULTS AND DISCUSSION 75

4.1 Reflux Extraction of Orthosiphon Stamineus Crude Extract 75

4.2 Fractionation of Rosmarinic Acid Based on Thin Layer Chromatography Integrated with Column Chromatography 77
  4.2.1 Development of Solvent System from Thin Layer Chromatography 78
4.2.2 Effect of Solvent Systems on Rosmarinic Acid Recovery from Column Chromatography

4.2.3 Effect of Packed Column Height on Rosmarinic Acid Content

4.2.4 Stability of Adsorbent

4.3 Two-dimensional Fractionation of Rosmarinic Acid Based on Liquid-Liquid Extraction and Solid Phase Extraction

4.3.1 Sample Partitioning by Liquid-Liquid Extraction

4.3.2 Second Dimension of Solid Phase Extraction for Rosmarinic Acid Rich Fraction

4.4 Enzymatic Anti-diabetic Activity

4.4.1 In vitro α-amylase Inhibition Study

4.4.2 In vitro α-glucosidase Inhibition Study

5 CONCLUSION

5.1 Conclusion

5.2 Recommendations

REFERENCES

Appendices A-C
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Plant taxonomy of <em>Orthosiphon stamineus</em></td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Phytochemicals in <em>Orthosiphon stamineus</em></td>
<td>12</td>
</tr>
<tr>
<td>2.3</td>
<td>Chemical and physical properties of rosmarinic acid</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>Rosmarinic acid content in different plants</td>
<td>17</td>
</tr>
<tr>
<td>3.1</td>
<td>Different solvent ratios of mobile phases</td>
<td>66</td>
</tr>
<tr>
<td>4.1</td>
<td>Recovery and concentration of rosmarinic acid from each sub-fraction in solid phase extraction</td>
<td>96</td>
</tr>
<tr>
<td>4.2</td>
<td>IC$_{50}$ of samples for $\alpha$-amylase inhibitory activity</td>
<td>101</td>
</tr>
<tr>
<td>4.3</td>
<td>IC$_{50}$ of samples for $\alpha$-glucosidase inhibitory activity</td>
<td>104</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE NO.</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Orthosiphon stamineus in Malaysia</td>
<td>9</td>
</tr>
<tr>
<td>2.2</td>
<td>Leaf morphological observation of (a) white and (b) purple varieties of Orthosiphon stamineus</td>
<td>10</td>
</tr>
<tr>
<td>2.3</td>
<td>Chemical structure of rosmarinic acid</td>
<td>16</td>
</tr>
<tr>
<td>2.4</td>
<td>Hydrogen donation mechanism of rosmarinic acid</td>
<td>18</td>
</tr>
<tr>
<td>2.5</td>
<td>Column chromatography involves a mobile phase flowing over a stationary phase</td>
<td>25</td>
</tr>
<tr>
<td>2.6</td>
<td>Column chromatography, (a) before fractionation and (b) separation of compound bands after a period of fractionation</td>
<td>26</td>
</tr>
<tr>
<td>2.7</td>
<td>Relative polarity of various solvents</td>
<td>29</td>
</tr>
<tr>
<td>2.8</td>
<td>A developed TLC plate with spots visualized and R&lt;sub&gt;f&lt;/sub&gt; values determined</td>
<td>34</td>
</tr>
<tr>
<td>2.9</td>
<td>Chromatogram of Orthosiphon stamineus leaves extracts</td>
<td>37</td>
</tr>
<tr>
<td>2.10</td>
<td>Ultraviolet-visible spectroscopy of rosmarinic acid from (a) Orthosiphon stamineus and (b) Rosmarinus officinalis</td>
<td>38</td>
</tr>
<tr>
<td>2.11</td>
<td>Principle of tandem mass spectrometry</td>
<td>40</td>
</tr>
<tr>
<td>2.12</td>
<td>ESI-MS/MS spectrum of rosmarinic acid at m/z 359.10</td>
<td>41</td>
</tr>
<tr>
<td>2.13</td>
<td>Glycogenolysis pathway</td>
<td>44</td>
</tr>
<tr>
<td>2.14</td>
<td>Gluconeogenesis pathway</td>
<td>45</td>
</tr>
<tr>
<td>2.15</td>
<td>Use of rosmarinic acid in inhibiting the starch digestion process by α-amylase and α-glucosidase</td>
<td>51</td>
</tr>
</tbody>
</table>
2.16 Determining the initial velocity of an enzyme reaction from the slope of the graph at the beginning of a reaction 56
2.17 Michaelis-Menten kinetics of an enzyme reaction 56
2.18 Double reciprocal Lineweaver-Burk lines of competitive inhibition 58
2.19 Double reciprocal Lineweaver-Burk lines of uncompetitive inhibition 59
2.20 Double reciprocal Lineweaver-Burk lines of non-competitive inhibition 60
3.1 Flowchart of the overall study 63
4.1 Chromatogram of *Orthosiphon stamineus* crude extract at 254 nm 77
4.2 TLC images of *Orthosiphon stamineus* crude extract using the mobile phase of ethyl acetate-ethanol (70:30 % v/v) with (a) and without (b) 0.1 % formic acid as additive 78
4.3 Thin layer chromatogram for standard rosmarinic acid and *Orthosiphon stamineus* crude extract at different ratios of solvent systems (ethyl acetate and ethanol) with 0.1% v/v formic acid 80
4.4 *R*\(_f\) values of standard rosmarinic acid and rosmarinic acid in crude extract at different concentrations of ethyl acetate 81
4.5 Eluent volume of different solvent systems required for complete rosmarinic acid elution by packed column fractionator 83
4.6 Recovery and purity of each fraction at different solvent systems of ethyl acetate and ethanol with 0.1% v/v formic acid 84
4.7 Comparison of recovery and purity of rosmarinic acid at the first rosmarinic acid containing fraction from all solvent systems 86
4.8 Chromatograms of the first rosmarinic acid containing fraction at different ethyl acetate concentrations 87
4.9 Eluent volume of different packed bed heights required for the collection of first and subsequent fractions containing rosmarinic acid 89
4.10 The effect of packed bed height on recovery and purity of rosmarinic acid content 90
<table>
<thead>
<tr>
<th>Section</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.11</td>
<td>Comparison of recovery and purity of first rosmarinic acid containing fraction (fraction 8) for each cycle</td>
</tr>
<tr>
<td>4.12</td>
<td>Liquid-liquid extraction of <em>Orthosiphon stamineus</em> crude extract using different solvent systems to recover rosmarinic acid</td>
</tr>
<tr>
<td>4.13</td>
<td>Chromatograms of diethyl ether, ethyl acetate and water fractions of <em>Orthosiphon stamineus</em> crude extract after liquid-liquid extraction</td>
</tr>
<tr>
<td>4.15</td>
<td>Chromatograms of the rosmarinic acid fraction after solid phase extraction with water-ethanol solvent system</td>
</tr>
<tr>
<td>4.16</td>
<td>Inhibitory activity of crude extract and its rosmarinic acid rich fractions on α-amylase</td>
</tr>
<tr>
<td>4.17</td>
<td>IC₅₀ of samples on α-amylase inhibitory activity</td>
</tr>
<tr>
<td>4.18</td>
<td>Mode of competitive inhibition for α-amylase by 100% rosmarinic acid fraction from <em>Orthosiphon stamineus</em></td>
</tr>
<tr>
<td>4.19</td>
<td>Inhibitory activity of each sample on α-glucosidase inhibition <em>in vitro</em></td>
</tr>
<tr>
<td>4.20</td>
<td>IC₅₀ of samples on α-glucosidase inhibitory activity</td>
</tr>
<tr>
<td>4.21</td>
<td>Mode of non-competitive inhibition for α-glucosidase by 100% rosmarinic acid from <em>Orthosiphon stamineus</em></td>
</tr>
</tbody>
</table>
# LIST OF APPENDICES

<table>
<thead>
<tr>
<th>APPENDIX</th>
<th>TITLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Results of thin layer chromatography</td>
<td>131</td>
</tr>
<tr>
<td>B</td>
<td>Results of anti-diabetic activity assay</td>
<td>132</td>
</tr>
<tr>
<td>C</td>
<td>Calibration curves of reference standard</td>
<td>135</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg120</td>
<td>Arginine 120</td>
</tr>
<tr>
<td>CC</td>
<td>Column chromatography</td>
</tr>
<tr>
<td>DAD</td>
<td>Diode array detector</td>
</tr>
<tr>
<td>DE</td>
<td>Diethyl acetate</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>DMH</td>
<td>1,2-dimethylhydrazine</td>
</tr>
<tr>
<td>DNS</td>
<td>3,5, dinitrosalicylic acid</td>
</tr>
<tr>
<td>EA</td>
<td>Ethyl acetate</td>
</tr>
<tr>
<td>EC50</td>
<td>Half maximal effective concentration</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Glucose transporter type 4</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HFD</td>
<td>High-fat diet</td>
</tr>
<tr>
<td>HPLC</td>
<td>High-performance liquid chromatography</td>
</tr>
<tr>
<td>HSCCC</td>
<td>High-speed counter-current chromatography</td>
</tr>
<tr>
<td>IC50</td>
<td>Half maximal inhibitory concentration</td>
</tr>
<tr>
<td>Km</td>
<td>Michaelis constant</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid-liquid extraction</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass-to-charge ratio</td>
</tr>
<tr>
<td>MAE</td>
<td>Microwave-assisted extraction</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometer</td>
</tr>
<tr>
<td>PE</td>
<td>Petroleum ether</td>
</tr>
<tr>
<td>p-NPG</td>
<td>4-nitrophenyl-α-D-glucopyranoside</td>
</tr>
<tr>
<td>R</td>
<td>Recovery</td>
</tr>
<tr>
<td>RA</td>
<td>Rosmarinic acid</td>
</tr>
<tr>
<td>Rf</td>
<td>Retention factor</td>
</tr>
<tr>
<td>Ser353</td>
<td>Serine 353</td>
</tr>
<tr>
<td>SGLT1</td>
<td>Sodium-glucose transport proteins</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>SSL</td>
<td>Spent sulphite liquor</td>
</tr>
<tr>
<td>STZ</td>
<td>Streptozocin</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>UAE</td>
<td>Ultrasound assisted extraction</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum reaction velocity</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Research Background

*Orthosiphon stamineus* belongs to the family Lamiaceae, and this plant is also known as “Misai Kucing” which is literally meant cat’s whisker in Malaysia. It is a perennial herb which can grow well in temperate and tropical areas up to 0.3–1.0 m with 4-angled, poorly ramified and ascending stem (Wiart, 2000). It has been traditionally used as folk medicine for illnesses such as rheumatoid diseases, diabetes, hypertension and renal calculus (Awale et al., 2003). Owing to the promising medicinal values, *O. stamineus* has been formulated into many commercial products in the forms of tea sachets, powdered herb, tablets and capsules (Indubala and Ng, 2000). The extract of *O. stamineus* leaves and stems were found to have more than 20 phenolic compounds including rosmarinic acid, eupatorin, 3’-hydroxy-5, 6, 7, 4’-tetramethoxyflavone and sinensetin (Indubala and Ng, 2000).

Nowadays, entrepreneurs prefer to have herbal extract rich in bioactive compound instead of plant crude extract. The demand of rosmarinic acid rich extract is increasing in herbal products market nowadays (Shekarchi *et al*., 2012). Therefore, this study was aimed to prepare the rosmarinic acid rich extract from *O. stamineus*. 
Plant crude extract is very complex because it contains thousands of phytochemicals with diverse chemical properties. Therefore, a fractionation process is necessary to separate the target compound from the crude extract. Fractionation is a separation process to segregate a complex mixture into smaller fractions with higher quantity of desired compounds according to a gradient of the solvent system (WHO, 2017). The quality of the herbal extract could be enhanced by fractionating them according to their chemical characteristics, usually based on solvent property (WHO, 2017).

In the present study, the fractionation of *O. stamineus* crude extract was conducted by thin layer chromatography integrated with column chromatography (TLC-CC), and liquid-liquid extraction integrated with solid phase extraction (LLE-SPE). Both methods were evaluated and compared in the term of recovery and purity of rosmarinic acid. TLC is used to optimize the conditions of column chromatography by selecting suitable mobile and solid phases (Liu, 2011), while LLE acts as a pre-treatment step before SPE to separate the crude extract based on the different distribution and solubility of the compounds between two immiscible liquid solvents (Houghton and Raman, 1998).

CC and SPE are simple fractionation techniques that are widely used for purification and separation of target compounds from the complex mixture which involves the sorption of solutes from a liquid medium into a solid adsorbent. The selection of adsorbent and solvent system are dominant factors affecting the recovery and purity of target compound (Williamson and Masters, 2016). Somehow, the option of impurity retention and target compound collection would always be the method of choice for time saving and effective solvent consumption. Liquid chromatography with diode array detector and tandem mass spectrometer (LC-DAD-MS/MS) was used to identify and quantify the rosmarinic acid.

Previous studies reported that rosmarinic acid showed significant antioxidant (Chen *et al*., 2014), anti-cancer (Venkatachalam *et al*., 2013), and anti-diabetic activities (Runtuwene *et al*., 2016). Rosmarinic acid is a phenolic compound
(C<sub>18</sub>H<sub>16</sub>O<sub>8</sub>) comprised of an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which is commonly found in many plant families including Lamiaceae and Boraginaceae (Bandoniene et al., 2005). Lau et al. (2014) reported that the <i>O. stamineus</i> contains higher amount of rosmarinic acid, approximately 4.1% w/w compared to other plants such as <i>Origanum vulgare</i> (0.94% w/w), <i>Rosmarinus officinalis</i> (0.2% w/w) and <i>Satureja macrostema</i> (0.06% w/w) (Alonso-Carrillo et al., 2017; Jacotet-Navarro et al., 2015; Baranauskaite et al., 2016).

Recently, rosmarinic acid has extensively been studied in the management of diabetic conditions, which indicates that rosmarinic acid could be used to reduce the diabetes-induced disorders and complications (Rao et al., 2014). Diabetes mellitus (DM) is a global health problem and the number of diabetic people is expected to increase to 366 million in year of 2030 and majority of them were type 2 diabetes (Sarah et al., 2004). Hyperglycemia in type 2 diabetic patients is caused by a sudden increase in the blood glucose levels due to starch hydrolysis by enzymes such as α-amylase and α-glucosidases in gastrointestinal tract (Gray, 1975). The pancreatic α-amylase enzymes convert starch to maltose and iso-maltose, which then travel to the small intestine where they are further digested to monosaccharides such as glucose and fructose by α-glucosidase. The glucose and fructose are transported by the intestinal sodium-glucose cotransporter, and thereby increasing blood glucose level (Xiao et al., 2013).

One of the commonly applied therapeutic approaches in the management of blood glucose level in type 2 DM is to control and decrease the postprandial hyperglycemia through inhibition of starch hydrolyzing enzymes such as α-amylase and α-glucosidases (Megh et al., 2008). There are fewer reports available on the activity of rosmarinic acid in inhibiting the α-amylase and α-glucosidases to regulate diabetes mellitus (Azevedo et al., 2011; McCue and Shetty, 2004; Runtuwene et al., 2016). The currently available therapeutic options for diabetes like oral hypoglycemic agents and insulin have limitations of their own, therefore many herbal medicines have been recommended for diabetes treatment (Sharma, 2012). Hence, the inhibitory
activity of crude extract and rosmarinic acid fraction from *O. stamineus* on α-amylase and α-glucosidase enzymes were also investigated.

1.2 Research Problem

*O. stamineus* crude extract is very complex in its chemical composition. Hence, the crude extract is needed to fractionate into several fractions to concentrate the rosmarinic acid content. Till to date, there is no standard guideline, specifically for rosmarinic acid rich extract from *O. stamineus* using thin layer chromatography integrated with column chromatography, and liquid-liquid extraction integrated with solid phase extraction. Furthermore, it is difficult to maintain the quality of herbal extract for product formulation due to the difference in phytochemical profile of the herbal extract from one batch to another batch of processing. Hence, it is important to study the optimal fractionation conditions for the highest recovery and purity, as well as quality control of rosmarinic acid.

Previously, the optimization of the fractionation of rosmarinic acid from *O. stamineus* crude extract using column chromatography was carried out based on single factor (solvent system), which only recovered 4.10 % w/w rosmarinic acid (81.31 ppm) in 80% v/v ethanol fraction with a large consumption of solvent (1000 to 1500 mL) (Chua and Lau, 2016). Another study using solid phase extraction also showed poor separation of rosmarinic acid from *O. stamineus* extract using the solvent system of methanol-acetonitrile as almost all the phenolic compounds were eluted out in fraction 1 (Lau *et al*., 2015). Therefore, this study was focused on the establishment of an optimal fractionation for the highest yield and recovery of rosmarinic acid.

Researchers have proven that the plants rich in phenolic compounds, especially rosmarinic acid could exhibit high antioxidant (Zheng and Wang, 2001), anti-allergic
(Ito et al., 1998), anti-hyperglycemic (Kumar et al., 2010), anti-inflammatory (Al-Sereiti et al., 1999), and antimicrobial (Nascimento et al., 2009) properties. The *O. stamineus* crude extract was found to have anti-diabetic activity (Rao et al., 2014). To the knowledge of researchers, there is no previous report on the *in vitro* inhibitory activity of rosmarinic acid from *O. stamineus* in α-amylase and α-glucosidases catalysed reaction, especially the kinetic study of the inhibitory mode caused by rosmarinic acid. Therefore, no clear understanding whether the inhibitory activity is contributed by either one or more compounds such as eupatorin and/or sinensetin in the *O. stamineus* crude extracts (McCue and Shetty, 2004). Possibly, the other compounds in the crude extract also contributed to the enzyme inhibitory activity. Therefore, this study was also determined the inhibitory potential of rosmarinic acid rich extract from *O. stamineus* on the activities of α-amylase and α-glucosidase concurrently.

### 1.3 Research Objectives

The followings are the objectives of this study:

i. To extract rosmarinic acid containing crude extract from *Orthosiphon stamineus* using reflux extraction method.

ii. To optimize the thin layer chromatography integrated with column chromatography technique in fractionating rosmarinic acid from *Orthosiphon stamineus* crude extract based on the solvent system, packed bed height, stability of adsorbent, and compare this system with liquid-liquid extraction integrated with solid phase extraction techniques.
iii. To determine the kinetics of inhibitory mode caused by rosmarinic acid rich extract in α-amylase and α-glucosidase catalysed anti-diabetic assays using Michaelis–Menten kinetic model.

1.4 Research Scopes

In order to achieve the objectives, the scopes of the study include:

i. Extraction of rosmarinic acid from *O. stamineus* using a reflux extraction system with 70% v/v ethanol at 78.4 °C for 1 hour.

ii. Fractionation of *O. stamineus* crude extract by a normal phase column chromatography using ethyl acetate-ethanol as the eluent system with formic acid as an additive to improve the elution of rosmarinic acid. Investigation of the stability of silica gel adsorbent (0.063-0.2 mm, 70-230 mesh) for rosmarinic acid fractionation by repeating the fractionation process using the same packed column.

iii. Extraction of rosmarinic acid from *O. stamineus* crude extract using liquid-liquid extraction as pre-treatment step before solid phase extraction. Recovery of rosmarinic acid by solid phase extraction using the solvent system of water-ethanol. Comparison of the performance of both method in term of recovery and purity of rosmarinic acid.

iv. Investigation of the anti-diabetic capacity of *O. stamineus* extract and rosmarinic acid rich extract based on the α-amylases and α-glucosidases
inhibitory activities. Determination of the inhibitory mode of rosmarinic acid rich extract in $\alpha$-amylases and $\alpha$-glucosidases catalysed reaction through Michaelis–Menten kinetic model to predict the mechanism of inhibition by rosmarinic acid in anti-hyperglycemic treatment.

1.5 Research Significance

In this study, the high yield of rosmarinic acid rich extract could be produced using column chromatography method. This method is considered as cost effective technique due to its minimal solvent consumption and high recovery of rosmarinic acid from the $O. stamineus$ crude extract. The optimized fractionation conditions in the column chromatography is also very useful for herbal industry as the optimized conditions can be used to produce high purity of rosmarinic acid rich extract satisfactory. The production of rosmarinic acid from $O. stamineus$ is more profitable, since it contains higher amount of rosmarinic acid (Akowuah et al., 2004; Shekarchi et al., 2012). The plant could be cultivated approximately after ten weeks of plantation (Zaharah, 2005).

Rosmarinic acid has been found to have potential health benefits, particularly on its anti-diabetic capacity. The rosmarinic acid rich extract from $O. stamineus$ obtained from CC was found to be the most effective in inhibiting the $\alpha$-amylases and $\alpha$-glucosidases. Understanding of the kinetic of inhibitory mode caused by rosmarinic acid rich extract is very important for the early phase of clinical study. The rosmarinic acid rich extract could be the lead source to be developed into a natural anti-diabetic drug replacing the commonly used strong allopathic drugs that possess several harmful side effects. This study brings benefits to the diabetic patients as herbal formulations are lower cost and lesser side effects, so that the low to middle income families can be affordable with the treatment.
REFERENCES


