TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND CYTOTOXIC
ACTIVITIES OF *Citrus hystrix* LEAF EXTRACTS

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TOTAL PHENOLIC CONTENT, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *Citrus hystrix* LEAF EXTRACTS

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A dissertation submitted in partial fulfilment of the requirements for the award of the degree of
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I would like to dedicate this thesis to my dearest uncle, MR Ravichandran Ramasamy. Without him, none of this would have happened. Thank you for everything.
WAYS AND MEANINGS OF PEACE IN THE UNITED NATIONS

Chapter 1

Introduction

The United Nations (UN) is an international organization founded in 1945 and headquartered in New York City. It was established after World War II with the intention of promoting peace, security, development, and cooperation among nations. The UN consists of 193 member states, each with a voice in its General Assembly, which is the main deliberative and representative body.

The UN operates through various bodies, including the Security Council, the Economic and Social Council, and the International Court of Justice. Its primary roles include maintaining international peace and security, promoting human rights, ensuring social and economic development, and protecting the environment. The UN also helps to address global challenges such as climate change, pandemic response, and conflict resolution.

The structure and processes of the UN have evolved over time, adapting to changing global circumstances. In 1995, the UN established the World Summit for Social Development, which led to the adoption of the United Nations International Decade for Natural Disaster Reduction and the International Year of Disasters Reduction. The UN also plays a crucial role in responding to crises, such as the global COVID-19 pandemic and the humanitarian crises in Syria and Yemen.

This chapter provides an overview of the UN's history, its structure, and its role in promoting peace and security. It also highlights the significance of the UN in addressing global challenges and its work towards achieving the Sustainable Development Goals (SDGs).

Chapter 2

Peacekeeping

The United Nations Peacekeeping Operations (UNPKOs) are a key component of the UN's efforts to maintain international peace and security. UNPKOs are deployed by the UN to help prevent conflicts, mitigate existing conflicts, and promote peace and stability in regions affected by conflict.

UNPKOs are complex and require a multidisciplinary approach. They involve military, political, and civilian components working together to achieve their objectives. The UN's Peacekeeping Department (DPKO) coordinates peacekeeping efforts, ensuring that they are consistent with the UN Charter and other relevant international legal frameworks.

UNPKOs can be categorized into three types: primary mandates, integrated mandates, and special political missions. Primary mandates are temporary operations aimed at a specific objective, such as the stabilization of a peace agreement. Integrated mandates are more long-term and have a broad mandate to support peace and development. Special political missions are established to address specific situations, such as elections or political transitions.

This chapter provides an overview of UNPKOs, their history, and the principles that guide their operations. It also discusses the challenges faced by UNPKOs and the role of the UN in promoting peace and security through these operations.
ABSTRACT

*Citrus hystrix (C.hystrix)* locally known as *Limau purut* is a culinary flavouring that is common in Malaysia and South East Asian cuisine as well as in herbal preparations. The aim of this study is to evaluate the antiproliferative, antioxidant and total phenolic content of ethanolic and aqueous extract of *C.hystrix* leaves. Qualitative phytochemical screening was also conducted to detect the presence of phytochemical compounds. The cytotoxic effect of the extracts on colon cancer cells (HT29) were determined by MTT assay. For the antioxidant activity, 2, 2, diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay was used. Total phenolic content (TPC) was evaluated by using Folin-Ciocalteu method. Qualitative phytochemical screening was carried using standard procedures. The ethanolic extract of *C.hystrix* exhibited higher percentage yield of 22.05 % compared to 19.22 % of aqueous during the solvent extraction method. The cytotoxic activity of the extracts showed IC$_{50}$ values of 0.25 and 3.25 mg/mL for ethanolic and aqueous extract respectively indicating stronger cytotoxic effects on HT29 cells in ethanolic extract. The TPC was also higher in ethanolic extract compared to aqueous which was 0.12 and 0.104 mg of GA/ g of extract respectively. For the antioxidant activity, both the ethanolic and aqueous extract showed IC$_{50}$ values of > 8 mg/mL in comparison of 0.03 mg/mL of the standard ascorbic acid, indicating low antioxidant activity. Both the ethanolic and aqueous extract of *C.hystrix* exhibited a weak, negative correlation between TPC and percentage inhibition of DPPH with R$^2$ value of 0.2278 and 0.434 respectively. The phytochemical screening of the extracts reveals the presence of several phytochemical compounds namely alkaloid, saponin, flavonoid and fixed oil and fats with maximum presence of phytochemicals in ethanolic compared to aqueous extract of *C.hystrix*. Relatively, the present result shows that the ethanolic extract of *C.hystrix*, which exhibited higher percentage yield, antiproliferative activity, TPC content and maximum presence of phytochemicals can be used as a potential source of anticancer agent but not a good source of natural antioxidants.
ABSTRAK

*Citrus hystrix* (*C. hystrix*) atau dikenali sebagai *Limau purut* digunakan dalam masakan Malaysia dan Asia Tenggara serta dalam perubatan tradisional. Tujuan kajian ini dilakukan adalah untuk mengesan aktiviti anti-kanser, anti-oksida, jumlah kandungan fenolik serta penyaringan sebatian fitokimia di dalam ekstrak ethanol dan aqueous daun *C. hystrix*. Aktiviti anti-kanser ekstrak daun limau purut di atas sel kanser kolon (HT29) dianalisis menggunakan kaedah MTT. Aktiviti anti-oksidan dianalisis menggunakan kaedah DPPH. Jumlah kandungan fenolik ditentukan dengan menggunakan ujian penyaringan bahan fitokimia. Hasil kajian mendapati ekstrak ethanol daun limau purut mempunyai jumlah berat yang lebih tinggi iaitu 22.05 % berbanding ekstrak aqueous iaitu 19.22 %. Ekstrak ethanol juga menunjukkan nilai IC₅₀ yang lebih rendah iaitu 0.25 mg/mL berbanding ekstrak aqueous iaitu 3.25 mg/mL dalam ujian anti-kanser terhadap sel kanser kolon. Bagi ujian jumlah kandungan fenolik, ekstrak ethanol menunjukkan nilai lebih tinggi iaitu 0.12 mg GA/g dibandingkan dengan ekstrak aqueous iaitu 0.104 mg GA/g. Bagi aktiviti anti-oksidan kedua-dua ekstrak ethanol dan aqueous menunjukkan nilai IC₅₀ > 8 mg/mL dibandingkan dengan nilai peratus inhibisi ascorbic acid iaitu 0.03 mg/mL yang menunjukkan aktiviti anti-oksida kedua-dua ekstrak daun limau purut adalah rendah. Ujian penyaringan fitokimia telah mengesan beberapa bahan fitokimia iaitu alkaloid, saponin, flavonoid, dan minyak/lemak. Berbanding dengan ekstrak aqueous, lebih banyak bahan fitokimia dikesan di dalam ekstrak ethanol. Kedua-dua ekstrak ethanol dan aqueous daun limau purut menunjukkan polarisasi negatif di antara jumlah kandungan fenolik dan peratus (%) inhibisi DPPH di mana nilai R² adalah 0.2278 bagi ekstrak ethanol dan 0.4344 bagi ekstrak aqueous. Secara keseluruhan, didapati ekstrak ethanol *C. hystrix* mempunyai jumlah ekstrak tinggi serta mempunyai aktiviti anti – kanser, kandungan fitokimia dan fenolik yang lebih tinggi. *C. hystrix* berpotensi untuk digunakan sebagai agen anti-kanser namun bukan sebagai anti-oksidan semula jadi.
# 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>ACKNOWLEDGEMENTS</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>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td></td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS/SYMBOLS</td>
<td></td>
<td>xiii</td>
</tr>
<tr>
<td>LIST OF APPENDICES</td>
<td></td>
<td>xv</td>
</tr>
</tbody>
</table>

1 INTRODUCTION
1.1 Background of Study 1
1.2 Statements of Problem 3
1.3 Objectives of Study 4
1.4 Scope of Study 4

2 LITERATURE REVIEW 6
2.1 Citrus hystrix 6
2.2 Antioxidant 9
2.3 Colon cancer 10
   2.3.1 HT-29 Colon cancer cells 12
2.4 Phytochemicals 12
   2.4.1 Phenolic 13
   2.4.2 Flavonoids 15
   2.4.3 Alkaloids 16
   2.4.4 Terpenoids 17
   2.4.5 Tannins 18
   2.4.6 Saponin 19

3 MATERIALS AND METHODS
3.1 Chemicals and reagents 21
3.2 Experimental design 22
3.3 Methods 23
   3.3.1 Culture of HT-29 Colon cancer cells 23
   3.3.2 Preparation of crude plant extract 23
      3.3.2.1 Ethanol crude extract 23
      3.3.2.2 Aqueous crude extract 24
3.4 Cytotoxicity (MTT) assay 24
3.5 Antioxidant assay 25
   (DPPH radical scavenging activity)
3.6 Total Phenolic Content (TPC) 26
3.7 Qualitative phytochemical screening 27
   3.7.1 Screening for phenolic compound 27
      3.7.1.1 Ferric chloride test 27
   3.7.2 Tannin test 27
   3.7.3 Saponin/ Froth test 28
   3.7.4 Fats and fixed oil test 28
3.7.5 Terpenoid (Salkowski) tests 28
3.7.6 Flavonoid test 28
3.7.7 Alkaloid test 29
3.8 Statistical Analysis 29

4 RESULT AND DISCUSSIONS 30
4.1 Extraction of C. hystrix leaves 30
4.2 Cytotoxicity (MTT assay) 31
4.3 Total Phenolic Content (TPC) assay 33
4.4 DPPH radical scavenging activity 36
4.5 Correlation between TPC and DPPH radical scavenging activity 39
4.6 Phytochemical screening 41
  4.6.1 Test for phenolic compounds 42
  4.6.2 Tannin test 43
  4.6.3 Saponin (Froth) test 44
  4.6.4 Fixed oils and fats test 45
  4.6.5 Flavonoids test 46
  4.6.6 Terpenoids test 47
  4.6.7 Alkaloid test 48

5 CONCLUSION AND RECOMMENDATIONS 51

LIST OF REFERENCES 53
APPENDICES 67
### LIST OF TABLE

<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>C. hystrix</em></td>
<td>7</td>
</tr>
<tr>
<td>4.1</td>
<td>The percentage yield of the plant using ethanolic and aqueous extraction</td>
<td>31</td>
</tr>
<tr>
<td>4.2</td>
<td>Percentage viability and inhibition of HT-29 colon cancer cells tested against different concentrations of ethanolic and aqueous extract of <em>C. hystrix</em> leaves</td>
<td>32</td>
</tr>
<tr>
<td>4.3</td>
<td>IC$_{50}$ values of ethanolic and aqueous extract of <em>C. hystrix</em> leaves</td>
<td>33</td>
</tr>
<tr>
<td>4.4</td>
<td>Total phenolic contents in the ethanolic and aqueous extract of <em>C. hystrix</em> leaves expressed in terms of gallic acid equivalent</td>
<td>36</td>
</tr>
<tr>
<td>4.5</td>
<td>Percentage inhibition of DPPH tested against different concentrations of ethanolic and aqueous extract of <em>C. hystrix</em> leaves</td>
<td>38</td>
</tr>
<tr>
<td>4.6</td>
<td>IC$_{50}$ values of ethanolic and aqueous extract of <em>C. hystrix</em> leaves</td>
<td>39</td>
</tr>
<tr>
<td>4.7</td>
<td>Qualitative analysis of phytochemical constituents of <em>C. hystrix</em> in ethanolic and aqueous extract</td>
<td>42</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><em>Citrus hystrix</em></td>
<td>6</td>
</tr>
<tr>
<td>2.2</td>
<td>Polyps in the colon</td>
<td>11</td>
</tr>
<tr>
<td>2.3</td>
<td>Pie chart representing the major groups of plant phytochemicals</td>
<td>13</td>
</tr>
<tr>
<td>2.4</td>
<td>Major classes of phenolic compounds in plants</td>
<td>14</td>
</tr>
<tr>
<td>2.5</td>
<td>The chemical structures of major classes of flavonoids</td>
<td>16</td>
</tr>
<tr>
<td>2.6</td>
<td>Mode of action of berberine, evodiamine, matrine, piperine, sanguinarine, and tetrandrine on cancer cells</td>
<td>17</td>
</tr>
<tr>
<td>2.7</td>
<td>Classifications of tannins</td>
<td>19</td>
</tr>
<tr>
<td>3.1</td>
<td>Experimental design of the overall scope of study</td>
<td>22</td>
</tr>
<tr>
<td>4.1</td>
<td>The graph of percentage inhibition of HT-29 colon cancer cell treated with different concentration of ethanolic and aqueous extract of <em>C.hystrix</em> leaves</td>
<td>33</td>
</tr>
<tr>
<td>4.2</td>
<td>Concentration of gallic acid (mg/mL) against absorption at 750 nm</td>
<td>35</td>
</tr>
<tr>
<td>4.3</td>
<td>Percentage inhibition of the ethanolic and aqueous extracts of <em>C.hystrix</em> leaves and standard on DPPH</td>
<td>39</td>
</tr>
<tr>
<td>4.4</td>
<td>Correlation between TPC and percentage inhibition of DPPH For ethanolic extract of <em>C.hystrix</em> leaves.</td>
<td>40</td>
</tr>
<tr>
<td>4.5</td>
<td>Correlation between TPC and percentage inhibition of DPPH for aqueous extract of <em>C.hystrix</em> leaves</td>
<td>41</td>
</tr>
<tr>
<td>4.6</td>
<td>Ferric chloride test of aqueous and ethanolic crude extracts of <em>C.hystrix</em> leaves</td>
<td>43</td>
</tr>
<tr>
<td>4.7</td>
<td>Gelatin test of aqueous and ethanolic crude extract of <em>C.hystrix</em></td>
<td>44</td>
</tr>
<tr>
<td>Section</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>Saponin/ froth test of aqueous and ethanolic extract of <em>C. hystrix</em> leaves</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>Fixed oil and fats test of aqueous and ethanolic crude extract of <em>C. hystrix</em> leaves</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>Flavonoid test of aqueous and ethanolic extract of <em>C. hystrix</em> leaves</td>
<td></td>
</tr>
<tr>
<td>4.11</td>
<td>Terpenoids test of aqueous and ethanolic extract of <em>C. hystrix</em> leaves</td>
<td></td>
</tr>
<tr>
<td>4.12</td>
<td>Alkaloid test of aqueous and ethanolic extract of <em>C. hystrix</em> leaves</td>
<td></td>
</tr>
</tbody>
</table>
**LIST OF ABBREVIATIONS/ SYMBOLS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATC</td>
<td>animal tissue culture</td>
</tr>
<tr>
<td>BHA</td>
<td>butylated hydroxyl anisole</td>
</tr>
<tr>
<td>BHT</td>
<td>butylated hydroxyl toluene</td>
</tr>
<tr>
<td>CHCl₃</td>
<td>chloroform</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2-diphenyl-1-picrylhydrazyl</td>
</tr>
<tr>
<td>H₂SO₄</td>
<td>sulphuric acid</td>
</tr>
<tr>
<td>GAE</td>
<td>gallic acid equivalent</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HT-29</td>
<td>human colorectal adenocarcinoma cell line</td>
</tr>
<tr>
<td>IC</td>
<td>inhibitory concentration</td>
</tr>
<tr>
<td>K</td>
<td>potassium</td>
</tr>
<tr>
<td>K562</td>
<td>human erythromyeloblastoid leukemia cell line</td>
</tr>
<tr>
<td>KB</td>
<td>human mouth epidermal carcinoma</td>
</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>Ml</td>
<td>milliliter</td>
</tr>
<tr>
<td>Mg</td>
<td>milligram</td>
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<td>MTT</td>
<td>3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide</td>
</tr>
<tr>
<td>Molt4</td>
<td>human leukemia cells</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>NCI</td>
<td>National Cancer Institute</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
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<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
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<tr>
<td>PG</td>
<td>propyl gallate</td>
</tr>
<tr>
<td>P388</td>
<td>murine leukemic cell lines</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RPM1-1640</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SPSS</td>
<td>Statistical Package for the Social Sciences</td>
</tr>
<tr>
<td>TPC</td>
<td>total phenolic content</td>
</tr>
<tr>
<td>TAC</td>
<td>total antioxidant capacity</td>
</tr>
<tr>
<td>TPA</td>
<td>12-Otetradecanoylphorbol 13-acetate</td>
</tr>
<tr>
<td>TBHQ</td>
<td>tertiarybutyl hydroquinone</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
</tr>
<tr>
<td>USDA</td>
<td>United States of Department of Agriculture</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
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<td>U937</td>
<td>human macrophage cell line</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>%</td>
<td>percentage</td>
</tr>
<tr>
<td>ºC</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>EW</td>
<td>extract weight</td>
</tr>
<tr>
<td>DW</td>
<td>dry weight</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 Background of Study

Colon cancer, also known as the colorectal cancer is the fourth leading cause of cancer death worldwide (American Cancer Society, 2014). In Malaysia, a total of 2 866 cases were registered nationwide with the Malaysian National Cancer Registry in 2006 and represent 13.2% of all cancer cases registered (Kong et al., 2010). Patients diagnosed with colorectal cancer often undergoes treatments such as chemotherapy, radiation therapy and surgery (American Cancer Society, 2014). However, chemotherapy works by killing abnormally fast dividing cells and thus effects cells that divide rapidly under normal circumstances, which leads to unwanted side effects (Chueahongthong et al., 2011). Hence, naturally occurring plant compounds that have cancer inhibitory effects but have fewer side effects are a valuable alternative treatment for cancer (Jusoh et al., 2012).

Medicinal plants have played an important role in the treatment of various types of cancers (Valiyari et al., 2012). Numerous plant-derived compounds including taxol from *Taxus brevifolia*, camptothecin from *Camptotheca acuminata*, Decne, vinca alkaloids from *Catharanthus roseus* Don and podophyllotoxin from
Podophyllum peltatum are used in clinic all over the world (Valiyari et al., 2012; Chueahongthong et al., 2011).

In this project, the cytotoxic effects of ethanolic and aqueous crude extract of Citrus hystrix leaves on colon cancer cells (HT29) were examined. Citrus hystrix which is locally known as Limau purut or Kaffir lime is one of the traditional medicinal plant that has gain the interest of many researches over the decades due to their potential source of natural antioxidants (Almey et al., 2010; Jamilah et al., 1998; Nurain et al., 2013; Wong et al., 2006; Idris et al., 2008). Besides its leaves, the crude extract of C.hystrix such as peels, stems, and juice have shown potential antioxidant activity as well (Ghafar et al., 2010; Chowdhury et al., 2009). C.hystrix also exhibits anti-microbial (Chowdhury et al., 2012; Suri et al., 2002) activities.

In regard to cancer research, Citrus hystrix extracts have been shown to have anti-proliferative activity on KB (cervical cancer) and P388 (mouse leukemia) cell lines (Manosroi et al., 2006).Glyceroglycolipids in Citrus hystrix leaves could inhibit 12-Otetradecanoylphorbol 13-acetate (TPA) and skin carcinogen activities in mice (Murakami et al., 1995). According to research conducted by Ampasavate et al. 2010, C.hystrix leaves had strong cytotoxic effects on four leukemic cell lines (U937, K562, HL60, Molt 4).

The leaves of C.hystrix are used in many Malaysian and South-East Asian regions cuisines, for example in the famous local dishes such as Tom yam, stews, curries, and sauces (Almey et al., 2010; Nurain et al., 2013). It is also well known for its medicinal properties in treating skin disorders (Aziman et al., 2012). Besides, it has also been used in aromatheraphy, nutraceutical, and personal care products (Almey et al., 2010). Due to its various ethnomedical properties, this plant was selected for this study (Almey et al., 2010; Aziman et al., 2012). Also, as the leaves of this plant have been used as a spice in many Thai and Malaysian cuisines, the safety of C.hystrix leaves are highly acceptable (Chueahongthong et al., 2011). Till date, little is known concerning the effect of crude extract of Citrus hystrix leaves on colon cancer (HT29) cells.
The aim of this project was to determine the cytotoxic effect of ethanolic and aqueous crude extracts of *C. hystrix* leaves on colon cancer cells (HT29), to determine the antioxidant activity and total phenolic content (TPC) of the crude extracts, as well as to determine the phytochemical compounds present in each extract by performing phytochemical screening.

1.2 Statements of Problem

The current medical treatment for colorectal cancer includes polypectomy (removing the polyp) or colon resection (colectomy) through surgery, chemotherapy and radiation therapy (American Cancer Society, 2014). However, some of these methods cause undesired side effects by the non-specific targeting of both normal and cancer cells (Chueahongthong *et al.*, 2011). Thus, research for cancer treatment by using natural products has been increasing rapidly (Valiyari, 2012). Among natural sources, medicinal plants have played an important role in the treatment of many forms of cancer. Numerous studies have identified medicinal plant extracts which not only displayed antioxidant activity, but also cytotoxicity to many forms of cancer (Valiyari, 2012; Chueahongthong *et al.*, 2011). Such compounds include curcumin (Sandur *et al.*, 2007), guava extract, and basil leaf extract (Manosroi *et al.*, 2006).

Moreover, synthetic antioxidants such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), tertiarybutyl hydroquinone (TBHQ) and propyl gallate (PG) are conventional food antioxidants which are being added to foods to prevent the oxidation process of foods during the exposure to environments and increase the shelf life (Jamilah *et al.*, 2011; Wong *et al.*, 2006). Due to increasing regulatory scrutiny, consumer concerns and safety issues concerning synthetic antioxidants, the possibility of natural antioxidants from plants, as an alternative to the synthetic antioxidants are also being actively studied (Almey *et al.*, 2010; Nurain *et al.*, 2013; Jamilah *et al.*, 2011). Due to their natural origin, antioxidant from plants,
does not exhibit side effects and safer for consumption in comparison to synthetic antioxidants (Nurain et al., 2013).

*Citrus hystrix* leaves have various medical and culinary uses in South East Asia (Almey et al., 2010; Aziman et al., 2012). As the plant is used mostly for cuisine purposes, the cytotoxic effect of the *Citrus hystrix* leaf crude extract on colon cancer cells are studied.

### 1.3 Research Objectives

Followings are the objectives of this research:

1.3.1 To investigate the cytotoxic effect of *C. hystrix* leaf extract on human colon cancer cells (HT29).

1.3.2 To determine the antioxidant properties of *C. hystrix* leaf extract.

1.3.3 To investigate the total phenolic content of *C. hystrix* leaf extract.

1.3.4 To identify the presence of phytochemical compounds present in *C. hystrix* leaf extract by performing phytochemical screening.

### 1.4 Scope of Research

This project was aimed to determine the cytotoxic effect of *Citrus hystrix* crude extracts on colon cancer cells, to determine the antioxidant and total phenolic content of *C. hystrix* leaves extracts as well as to identify phytochemical compounds present in the crude extract of the leaves. The leaves were extracted by using two
polar solvents namely aqueous and ethanol. Colon cancer cells, (HT-29) were used to test the cytotoxic effect of *C. hystrix* by using the MTT assay. 50% inhibition concentration (IC$_{50}$) of the active substances was determined as the lowest concentration which reduced cell growth by 50%. The antioxidant activity was measured by using the DPPH free radical scavenging assay and expressed as percentage of DPPH radical inhibition and IC$_{50}$ values. The total phenolic content was determined by using the Folin- Ciocalteu method and is expressed as mg of GA/g of extract. Following that, phytochemical screening was conducted to determine the presence of phytochemicals such as phenol, tannin, saponin, flavonoid, alkaloid, terpenoids and fixed oils.
REFERENCES


