



UNIVERSITI PUTRA MALAYSIA

***ANTI-LEUKAEMIC AND CHEMOTHERAPY SUPPORTIVE EFFECTS OF
BERBERIS VULGARIS L. FRUIT CRUDE EXTRACT ON IN VITRO AND
IN VIVO MODELS***

TAYEBEH AZAM SAEDI

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By

TAYEBEH AZAM SAEDI

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in
Fulfillment of Requirements for the Degree of Doctor of Philosophy**

August 2015

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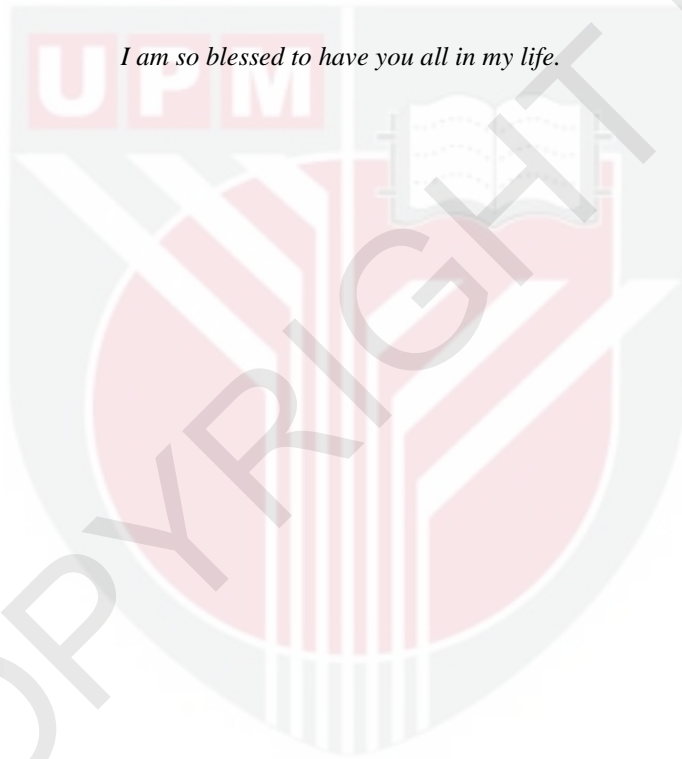
To my country,

To my beloved parents for all their endless love, invaluable care, unfaltering support, patience, and believe in me; they are the strongest inspiration in my life;

To my lovely brother and his wife, for their love, encouragement and understanding;

To my dear husband, for his endless emotional support throughout the thesis writing

I am so blessed to have you all in my life.



Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the Degree of Doctor of Philosophy

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August 2015

Chairman: Prof. Fauziah Othman, PhD
Faculty: Institute of Bioscience

Cancer is one of the most deadly diseases affecting human, and the second cause of death accounting for about 12.8% worldwide. Among the prominently worldwide cancer-affecting people is leukaemia. The disease is characterized by increasing of immature white blood cells called "blasts", and is estimated to account for about 3% of incident cases for all kind of cancer worldwide. There are different kinds of treatment methods for leukaemia including chemotherapy, which is known to have side effects. However, herbal therapy is considered to have fewer side effects. Hence, the used of *Berberis vulgaris* crude extract (BVFCE) in treating leukaemia is highlighted. Barberry is an herb derived from *Berberidaceae* shrub and is known as treat cancer. Total flavonoid and phenolic content (TFC and TPC) and DPPH assay were analyzed by spectrophotometer to determine the antioxidant activity of the Barberry. BVFCE was evaluated for its anti-leukaemic activities against two leukaemic cell lines; Human Promyelocytic (HL-60) and Mouse Myelomonocytic (WEHI-3) versus the normal mouse fibroblast cell (3T3) in both *in vitro* and *in vivo* leukaemic model. For the *in vitro* method, cytotoxic effects of BVFCE were evaluated using MTT assay to determine the IC₅₀ values and compare with non-leukaemic cell line (The 3T3 cell). Observation of the morphological changes in the treated leukaemic cell lines with BVFCE and Doxorubicin (Doxo) was examined using inverted microscope. Moreover, real time PCR was used to amplify mRNA and determine the expression of some specific related genes in leukaemia. Whereas, for the *in vivo* model, leukaemia was induced in male BALB/c mice using mouse leukaemic cell line (WEHI-3) and treatment was carried out using different concentrations of BVFCE and chemotherapy drug, separately for comparison. After the treatment period (5 weeks), the mice were fasted overnight and sacrificed for biochemical, haematological and histological analyses of their liver, kidney, heart and spleen. All data were analyzed using one-way ANOVA followed by Duncan's multiple post hoc tests. Differences between groups were considered significantly when the *p value* is less than 0.05. Results showed that BV100 has a higher level of total phenolic and flavonoids contents as well as antioxidant activity as compared with other concentrations (*p*<0.05). Biochemical assay on alkaline phosphatase (ALP), aspartate aminotransferase (AST), and alanine aminotransferase (ALT) showed significantly lower level of these markers in control and treated groups as compared to cancer group (*p*<0.05). Leukaemic group showed

significantly higher total serum than other groups as well ($p < 0.05$), which indicated the possible beneficial effect of BVFCE treatment on the above named markers.

From the haematological finding, the total white blood cell (WBC) count in the cancer group was high. In contrast, the amount of WBC decreased dramatically in mice treated with BVFCE, and doxorubicin compared to the mice group without treatment, which showed a high number of leukaemic cells. Liver, kidney, heart and spleen were stained with haematoxylin and eosin (H&E) and viewed under a light microscope. Spleen and liver weights were significantly low ($p < 0.05$) in the mice treated groups compared to the untreated group, indicating a significant splenomegaly and hepatomegaly. From the results above, it can be seen that the BVFCE has high anti-leukaemic activity, which might be due to its antioxidant. In conclusion, daily intake of this fruit by groups suffering from leukaemia could suppress or decrease cancer development with no or fewer side effect than the normal chemotherapeutic drug.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

**ANTI-LEUKEMIA DAN KESAN-KESAN SOKONGAN KEMOTERAPI
DALAM EKSTRAK MENTAH BERBERIS VULGARIS KE ATAS MODEL *IN
VITRO* DAN *IN VIVO***

Oleh

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Kanser adalah salah satu penyakit yang paling berbahaya yang memberi kesan kepada manusia, dan punca kematian kedua kira-kira 12.8% di seluruh dunia. Antara kanser yang memberi kesan ketara kepada manusia di seluruh dunia adalah leukemia. Penyakit ini dicirikan dengan peningkatan sel-sel darah putih yang tidak matang dikenali sebagai "blasts", dan dianggarkan menyumbang kepada kira-kira 3% daripada kes-kes untuk semua jenis kanser di seluruh dunia. Terdapat pelbagai jenis kaedah rawatan bagi leukemia termasuk kemoterapi, yang diketahui mempunyai kesan sampingan. Walau bagaimanapun, terapi herba dianggap mempunyai kesan sampingan yang kurang. Oleh itu, ekstrak mentah *Berberis vulgaris* (BVFCE) digunakan dalam merawat leukemia diserlahkan. Berry merupakan herba yang berasal dari pokok renek Berberidaceae dan dikenali sebagai rawatan kanser (Leukemia). Jumlah kandungan flavonoid dan fenolik (TFC dan TPC) dan ujian DPPH telah dijalankan menggunakan spektrofotometer untuk menentukan aktiviti antioksidan Berry. BVFCE, telah dinilai untuk aktiviti anti-leukemia menggunakan dua cell lines leukemia; Promyelocytic Manusia (HL-60) dan Myelomonocytic mencit (WEHI-3) berbanding sel normal fibroblast tikus (3T3) dalam model *in vitro* dan *in vivo* leukemia. Bagi kaedah *in vitro*, kesan sitotoksik BVFCE ditentukan menggunakan ujian MTT untuk menentukan nilai IC50 dan membandingkannya dengan cell line bukan leukemia (Sel 3T3). Pemerhatian perubahan morfologi dalam sel terawat leukemia dengan BVFCE dan Doxorubicin (Doxo) diperiksa menggunakan mikroskop terbalik. Selain itu, masa nyata PCR digunakan untuk mengandakan mRNA dan menentukan ekspresi beberapa gen berkaitan apoptotik khusus dalam leukemia. Manakala, bagi model *in vivo*, leukemia dirangsang dalam tikus BALB/c jantan menggunakan sel leukemia mencit (WEHI-3) dan rawatan dijalankan dengan menggunakan kepekatan BVFCE yang berbeza dan ubat kemoterapi secara berasingan bagi perbandingan. Selepas 5 minggu rawatan, tikus dipuasakan semalaman dan dibunuh untuk analisis biokimia, hematologi dan histologi hati, buah pinggang, hati dan limpa. Semua data dianalisis menggunakan ANOVA satu hala diikuti dengan ujian pelbagai post hoc Duncan. Perbezaan antara kumpulan dianggap signifikan apabila nilai p kurang daripada 0.05. Keputusan menunjukkan BV100 mempunyai kandungan jumlah fenolik dan flavonoid serta aktiviti antioksidan lebih tinggi berbanding kepekatan lain ($p < 0.05$). Ujian biokimia ke atas alkaline

phosphatase (ALP), aminotransferase aspartate (AST), dan alanina aminotransferase (ALT) menunjukkan kadar lebih rendah signifikan

dalam kumpulan kawalan dan terawat berbanding kumpulan kanser ($p < 0.05$). Kumpulan leukemia menunjukkan jumlah serum lebih tinggi daripada kumpulan yang lain ($p < 0.05$), yang menunjukkan kebarangkalian kesan baik pada rawatan BVFCE di penanda dinamakan di atas. Dari hasil kajian hematologi, jumlah sel darah putih (WBC) dalam kumpulan kanser adalah tinggi. Sebaliknya, jumlah WBC menurun mendadak pada tikus yang dirawat dengan BVFCE, dan doxorubicin berbanding dengan kumpulan tikus tanpa rawatan, yang menunjukkan jumlah sel-sel leukemia yang tinggi. Hati, buah pinggang, jantung dan limpa diwarnakan dengan haematoxylin dan eosin (H&E) dan dilihat di bawah mikroskop cahaya. Berat limpa dan hati lebih rendah ($p < 0.05$) di kumpulan tikus yang dirawat berbanding kumpulan yang tidak dirawat, menunjukkan signifikan splenomegaly dan hepatomegaly. Dari keputusan di atas, dapat dilihat bahawa BVFCE mempunyai aktiviti anti-leukemia yang tinggi, mungkin disebabkan oleh antioksidan. Oleh itu, disimpulkan bahawa, pengambilan harian buah ini oleh golongan yang menderita leukemia dapat menyekat atau mengurangkan perkembangan kanser tanpa atau kurang kesan sampingan berbanding ubat kemoterapi yang biasa.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

3T3	Mouse Spleen Lymphocyte
ALL	Acute Lymphoblastic Leukaemia
ALP	Alkaline Phosphates
ALT	Alanine Aminotransferase
AML	Acute Myeloblastic Leukaemia
ATTC	American Type Culture Collection
ANOVA	Analyze of Variance
BVFCE	<i>Berberis vulgaris</i> Fruit Crude Extract
CLL	Chronic Lymphocytic Leukaemia
CML	Chronic Myelocytic Leukaemia
DNA	Deoxyribonucleicacid
DPX	Di-N-Butyle Phthalate in Xylene
GST	Glutathion-S-Transferase
HL-60	Promyelocytic Leukaemia
H&E	Haematoxylin and Eosin
MTT	3-(4,5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide
NCBI	National Center for Biotechnology Information
OD	Optical Density
PCR	Polymerase Chain Reaction
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
RNA	Ribonucleic Acid
RT	Reverse transcriptase
RBC	Red Blood Cell
rpm	Revolution Per Minute
SEM	Scanning Electron Microscopy

SD	Standard Deviation
TEM	Transmission Electron Microscopy
UPM	Universiti Putra Malaysia
U/L	Units per Liter
V	Volume
W	Weight
WBC	White Blood Cells
WEHI-3	Mouse Myelomonocytic Leukaemia
WHO	World Health Organization
FMHS	Faculty of Medicine and Health Sciences
BHD	Butylated Hydroxytoluene

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer as a well-known term, is the second cause of death accounting for about 12.8% worldwide (WHO, 2004). The term cancer has been associated with despair, agony, and dreadful death. Cancer can be defined as a broad group of various diseases, all involving unregulated cell growth in which a group of abnormal cells grow uncontrollably by disregarding the normal rules of cell division, which is medically known as a malignant neoplasm (Moscow & Cowan, 2011). In Malaysia, the incidence of various kinds of cancer has been estimated to be around 30,000 annually where the cancer was the fourth leading cause of death (Ezat *et al.*, 2012; Lim, 2002).

Haematological malignancies consist of a group of cancers that arise from malignant transformation of various blood cells derived from peripheral blood, lymphatic system or bone marrow. These diseases include the acute and chronic leukaemias, Hodgkin's disease also known as (Hodgkin's lymphoma), non-Hodgkin's lymphoma, and multiple myeloma. The heterogeneity seen in these cancers collection reflects the complexity of the normal haematopoietic and immune systems.

Individually, these types of cancers are less common than some solid tumors. However, leukaemia, lymphoma, and myeloma accounted for 118,310 new cancer cases in 2006 (9% of cancer cases diagnosed in the United States) and 53,920 cancer deaths.

Leukaemia is estimated to account for about 3% of incident cases of cancer worldwide and, in Malaysia leukaemia is the 4th main cause of death (Lim, 2001). It is characterized by increasing number of immature white blood cells called "blast cells". There are two common types of leukaemia; acute and chronic. An abnormal number of immature and mature white cells are characterized in the acute and chronic leukaemia respectively. Leukaemia can also be classified based on their cell origin-myeloid and lymphoid. Hence, different types of leukaemia are named as in acute lymphocytic, acute myeloid, chronic lymphocytic and chronic myeloid leukaemia (Hughes-Jones & Wickramasinghe, 1996).

Established causes of leukaemia include, occupational exposure to ionizing radiation , certain drugs used in the treatment of cancer and some chemicals (most notably benzene) used largely in industrial settings . Because of an increased in the morbidity and mortality of human leukaemia in recent years, control of human leukaemia through intervention is highly desirable (Gao *et al.*, 2009). Blood diseases such as leukaemia expected to be increasing with years, as there is an increased in morbidity and mortality of leukaemia recently. Therefore, the need for control of leukaemia through chemoprevention or intervention with fewer side effects is highly required.

Epidemiologic studies have indicated that consumption of a fruit and vegetable-based diet reduces the risk of various cancers (Vecchia, 2004). These observations had led to

the latest global strategy on the cancer prevention which recommends consumption of colorful fruits and vegetables (Heber, 2004). Consequently, the focus of cancer research in recent years has been shifting toward the isolation, and characterization of potential chemo-preventive agents present in some fruits and vegetables (Cooke *et al.*, 2005).

Chemotherapy is a main curative approach to treat leukaemia. Most, anti-cancer drugs destroy cancer cells by inhibiting their growth rate or multiplying at one or more points in their cell cycles. Some chemotherapy is characterized by relatively low inactivity and high toxicity for patients. Recently, besides conventional methods such as surgery, radiotherapy and chemotherapy, herbal therapy has been distinguished as a safe way for cancer treatment (Saiful, 2003). Although, chemotherapy drug are powerful enough to kill cancer cells and also may be used as a medications to prolong life period when a cure is impossible, it has notable side effects such as nausea and vomiting, hair loss, early menopause, fatigue, infections, mouth and throat sores, gain weight, nail weakness and memory problems (chemo-brain) (George, 2014; National Cancer Institute, 2012). In this research introducing BVFCE as a complementary treatment to over come to side effect of chemotherapy is highlighted.

The importance of herbal therapy does not limit itself to local Malaysian's plants, but also overspreads of foreign plants. It leads to a study of non-native plant called *Berberis vulgaris* is which predominantly found in Asia, Europe, the British Isles and in North America (Arayne *et al.*, 2007). To date, there is no literature report on the mechanism of *Berberis vulgaris* fruit crude extract (BVFCE) and its effects on leukaemia. Hence, the effect of BVFCE as anti-leukaemic was investigated in both *in vitro* and *in vivo* models of experiments.

The determination of nutritional compounds in barberry (*Berberis vulgaris*) fruits is very important as it is a part of the daily diet for certain people. It is an herb derived from the Berberidaceae shrub family. Hanchi and her colleagues reported that *Berberis vulgaris* exhibited varying degree of antioxidant properties (Hanachi *et al.*, 2006).

Berberine, a natural isoquinoline alkaloid, has been found in many clinically important plants like *Berberis vulgaris* (Barberry) (Piyanch *et al.*, 2007). The most promising actions of berberine are its inhibition of cell growth, and induction of apoptosis in many human cancer cells (Mantena *et al.*, 2006), but the mechanism behind these actions need to be elucidated.

This research resulted to determine the herbal therapy and antioxidant effect of BVFCE on leukaemia as an herbal treatment. As compared to chemotherapy, herbal therapy has shown lower toxicity, easier availability and better acceptability, thus patients with cancer prefer to use complementary and alternative medicine (Gao *et al.*, 2009). Therefore, this study also aims to determine the use of *Berberis vulgaris* fruit crude extract (BVFCE) as a co-treatment in leukaemia cases.

1.2 Research Hypothesis

Based on the current explanation, it is hypothesized that the *Berberis vulgaris* fruit crude extract has an anti-leukaemic effect in both the *in vitro* and *in vivo* experiments. It seems as a complementary agent with no side effect, to the current chemotherapy drugs which are known with adverse side effects. BVFCE with the high antioxidant activity could be

a novel anti-leukaemic mediator in the medicinal world by suppressing of cancer cells development in leukaemic groups.

1.3 Research Objectives

1.3.1 General Objective:

To evaluate the anti-leukaemic properties of the *Berberis vulgaris* fruit crude extract using both the *in-vitro* and *in-vivo* experimental models.

1.3.2 Specific Objectives:

The specific objectives for the *in vitro* and *in vivo* experiments are as follows:

1. To obtain the crude extract of *Berberis vulgaris* fruit (B.v).
2. To evaluate the total phenolic, total flavonoid, and total antioxidant content of *Berberis vulgaris* crude extract (BVFCE).
3. To determined specific compound of *Berberis vulgaris* fruit (B.v) by HPLC.
4. To detect the presence of toxic metal elements in BVFCE.
5. To determine the cytotoxic effect of BVFCE on murine leukaemia (WEHI-3) cells and human promyelocytic leukaemia cells (HL-60) compared with the normal fibroblast cell line (3T3).
6. To examine the expression level of genes (p53, Bcl2, MAPK-1 and Cox2) using quantitative RT-PCR in both the treated, and untreated leukaemic cell lines.
7. To determine physical appearance of BALB/c mice after induction by WEHI-3 (Gross body weight, organomegaly, bleeding tendency and hair loss).
8. To observe histological changes of tissues stained with Haematoxyline and Eosine.
9. To detect haematological parameters (WBC, PLT, Hb level and blast count percentage).
10. To measure biochemical properties (liver and kidney functional tests)

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