
**UNIVERSITI PUTRA MALAYSIA**

# *PREVENTIVE EFFECTS OF CONSUMING NUTRITIONAL SUPPLEMENT OF Morinda citrifolia L. ON BAX AND BCL-2 IN EARLY LEUKEMIC RATS*

**NURSYUHADA HARON**

**FPV 2012 7**

# PREVENTIVE EFFECTS OF CONSUMING NUTRITIONAL SUPPLEMENT OF *Morinda citrifolia* L. ON BAX AND BCL-2 IN EARLY LEUKEMIC RATS

**By**

**NURSYUHADA HARON**



**Thesis submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Master of Science**

**May 2012**

*This thesis is dedicated with love and gratitude to my parents,*

*Haron Marjunid and Sa'diah Saiman;*

*my brothers, Mohd Syukri Syafiq Haron and Mohd Fahrurrazi Haron;*

*my sister in law, Noor Hazilah Sukri;*

*and my dearest nephew, Muaz Mohd Syukri Syafiq*

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

**PREVENTIVE EFFECTS OF CONSUMING NUTRITIONAL SUPPLEMENT OF *Morinda citrifolia* L. ON BAX AND BCL-2 IN EARLY LEUKEMIC RATS**

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**May 2012**

**Chairman : Hazilawati Hamzah, PhD**

**Faculty : Veterinary Medicine**

The most common cancer in Malaysia includes leukaemia and lymphoma. Cancer has been linked with the failure of apoptosis process regulated by Bcl-2 family genes, consisting of pro apoptotic genes such as Bax and anti apoptotic genes such as Bcl-2. The ratio of Bcl-2:Bax determines susceptibility towards apoptosis. Meanwhile, *Morinda citrifolia* fruit is known to exhibit anticancer properties. Thus, the objectives of this study were to determine the anticarcinogenic and preventive effects of *M. citrifolia* fruit in rats with early leukaemia/lymphoma induced by *N*-Methyl-*N*-Nitrosourea (MNU). Male Sprague Dawley rats were divided into four groups; Group 1 (control group), Group 2 (*M. citrifolia* fruit treated group), Group 3 (MNU treated group) and Group 4 (leukaemia/lymphoma prevention group treated with *M. citrifolia* fruit). The rats in the Group 3 and Group 4 were injected with four intraperitoneal injections of the

MNU mixture, at a dose of 60 mg/kg of body weight per injection, twice a week for two consecutive weeks with a total dose of 240 mg/kg of body weight. Rats in Group 1 and Group 2 were injected with normal saline following the same procedure. Ground dried *M. citrifolia* fruit at the dose of 3000 mg/kg of body weight were supplemented daily to the rats in Groups 2 and 4 for 12 weeks. Blood samples were taken at week 0, week 4 and week 8 of experimental study via cardiac puncture. At week 12, all rats were sacrificed and blood samples were taken via posterior vena cava. RNA were extracted from the blood and the relative transcription of Bcl-2 and Bax were quantified using Eva Green quantitative real time reverse transcription-PCR. For haemogram and serum biochemical analyses, blood samples were taken at week 4, week 8 and week 12 of the experimental study and analysed using automated blood analysers. For histopathological examination, organs were taken after sacrifice at the end of the experimental period. The organs were spleen, lymph nodes, liver, kidneys, lung and heart. The organs were processed using standard methods and stained with hematoxylin and eosin (H&E). Quantitative real time RT-PCR assays (qRT-PCR) for quantification of Bax and Bcl-2 genes which were successfully developed using an Eva Green dye showed that the ratio of Bcl-2:Bax in the rats injected with MNU was significantly higher compared to other groups. Significant reduction of Bcl-2:Bax ratio in Group 4 at week 12 showed that *M. citrifolia* fruit limits the progression of leukaemia/lymphoma through regulation of apoptosis gene transcription. Intraperitoneal injections of MNU at a total dose of 240 mg/kg of body weight successfully induced leukaemia without lymphocytosis in Group 3 at 50% and 75% after 8 weeks and 12 weeks of injection, respectively. Meanwhile, supplementation of dried *M. citrifolia* fruit did not significantly reduce the percentages of leukaemia without lymphocytosis in Group 4 at

20% and 60% after 8 weeks and 12 weeks of injection, respectively. All blood results obtained from the automated analysers in rats injected with MNU were similar to the control. All organs were macroscopically normal. Histopathology results showed that the rats in Group 3 developed 100%, 50% and 25% early lymphoma lesions in the spleen, mesentric lymph nodes and other lymph nodes respectively. Meanwhile, histopathology results showed that the rats in Group 4 developed 100%, 80% and 20% early lymphoma lesions in the spleen, mesentric lymph nodes and other lymph nodes respectively. Lesion scoring results showed that supplementation of *M. citrifolia* fruit at the dose of 3000 mg/kg of body weight did not significantly reduced the incidence of leukaemia and lymphoma Group 4.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

**KESAN PENCEGAHAN PEMAKANAN TAMBAHAN *Morinda citrifolia* L. KE ATAS BAX DAN BCL-2 DALAM TIKUS LEUKEMIA AWAL**

Oleh

**NURSYUHADA HARON**

**Mei 2012**

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Antara kanser yang selalu terdapat di Malaysia termasuklah leukemia dan limfoma. Kanser telah dikaitkan dengan kegagalan proses apoptosis yang di kawal atur oleh kumpulan gen-gen Bcl-2, terdiri daripada gen pro apoptotik seperti Bax dan anti apoptotik seperti Bcl-2. Kadar Bcl-2:Bax menentukan kecenderungan kepada apoptosis. Manakala buah *Morinda citrifolia* diketahui mempunyai ciri-ciri antikanser. Maka, objektif kajian ini adalah untuk menentukan kesan-kesan antikarsinogenik dan pencegahan buah *M. citrifolia* dalam tikus dengan leukemia/limfoma awal yang diaruh oleh *N*-Methyl-*N*-Nitrosourea (MNU). Tikus Sprague Dawley jantan telah dibahagikan kepada empat kumpulan; kumpulan 1 (kumpulan kawalan), kumpulan 2 (kumpulan rawatan *M. citrifolia*), kumpulan 3 (kumpulan rawatan MNU) dan kumpulan 4 (kumpulan pencegahan leukemia/limfoma dan rawatan *M. citrifolia*). Kumpulan 3 dan kumpulan 4 telah disuntik dengan empat suntikan campuran MNU secara

intraperitoneum, pada dos 60 mg/kg berat badan setiap suntikan, dua kali seminggu untuk dua minggu berturutan dengan jumlah dos 240 mg/kg berat badan. Tikus dalam kumpulan 1 dan kumpulan 2 disuntik dengan normal saline mengikut prosedur yang sama. Buah *M. citrifolia* yang kering pada dos 3000 mg/kg berat badan diberi makan kepada kumpulan 2 dan 4 sehingga akhir eksperimen. Sampel darah diambil pada minggu 0, minggu ke 4 dan minggu ke 8 kajian melalui tusukan jantung. Pada minggu ke 12, semua tikus dikorbankan dan sampel darah diambil melalui vena kava posterior. RNA diekstrak daripada darah dan transkripsi relatif Bcl-2 dan Bax ditentukan mengunakan PCR masa nyata. Untuk hemogram dan analisis biokimia serum, sampel darah diambil pada minggu ke 4, minggu ke 8 dan minggu ke 12 dan dianalisa menggunakan penganalisa darah automatik. Untuk pemeriksaan histopatologikal, organ-organ diambil setelah dikorbankan pada akhir tempoh eksperimen. Organ-organ yang diambil adalah limpa, nodus limfa, hati, buah pinggang, paru-paru dan jantung. Organ-organ diproses mengikut kaedah standard dan diwarnakan dengan hematoksilin dan eosin (H&E). Asai kuantitatif PCR masa nyata (qRT-PCR) untuk pengkuantitian gen Bax dan Bcl-2 yang telah berjaya dibangunkan menggunakan pewarna Eva Green menunjukkan bahawa kadar Bcl-2:Bax dalam tikus yang disuntik MNU adalah lebih tinggi ketara berbanding kumpulan lain. Penurunan ketara kadar Bcl-2:Bax dalam kumpulan 4 pada minggu ke 12 menunjukkan bahawa buah *M. citrifolia* yang kering menghalang progresi leukemia/limfoma melalui regulasi proses transkripsi gen apoptosis. Suntikan intraperitoneum MNU pada jumlah dos 240 mg/kg berat badan berjaya mengaruh leukemia tanpa limfositosis pada 50% dan 75% setelah 8 minggu dan 12 minggu menerima suntikan, masing-masing. Manakala, pemakanan tambahan buah *M. citrifolia* kering tidak meberikan penurunan ketara pada peratusan leukemia tanpa

limfositosis dalam kumpulan 4 pada 50% dan 75% setelah 8 minggu dan 12 minggu menerima suntikan, masing-masing. Keputusan analisa darah yang didapati daripada penganalisa automatik dalam kumpulan MNU tidak berbeza tererti dengan kumpulan kawalan. Tikus dalam kumpulan 3 membentuk 100% limfoma pada limpa, 50% limfoma pada limfa nodus mesentrik dan 25% limfoma pada nodus limfa yang lain. Tikus dalam kumpulan 4 pula membentuk 100% limfoma pada limpa, 80% limfoma pada limfa nodus mesentrik dan 20% limfoma pada nodus limfa yang lain. Keputusan menunjukkan bahawa penambahan buah *M. citrifolia* yang kering pada dos 3000 mg/kg berat badan tidak berjaya menurunkan ketara insiden leukemia dan limfoma.

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I certify that a Thesis Examination Committee has met on 31 May 2012 to conduct the final examination of Nursyuhada binti Haron on her thesis entitled "Preventive Effects of Consuming Nutritional Supplement of *Morinda citrifolia* L. on Bax and Bcl-2 in Early Leukemic rats" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the degree of Master of Science.

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The thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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## DECLARATION

I declare that the thesis is my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been and is not concurrently, submitted for any other degree at Universiti Putra Malaysia or at any other institution.

**NURSYUHADA HARON**

Date: 31 May 2012

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| | |
|---|---|
| % | percentage |
| °C | degree Celcius |
| Alb | albumin |
| ALL | Acute lymphoid leukaemia |
| ALP | alkaline phosphatase |
| ALT | alanine aminotransferase |
| AML | acute myeloid leukaemia |
| AST | alanine aminotransferase |
| bp | base pair |
| CAM | complementary and alternative medicine |
| CBC | complete blood count |
| cDNA | complementary deoxyribonucleic acid |
| CK | creatinine kinase |
| CL | chloride |
| CLL | chronic lymphocytic leukaemia |
| CML | chronic myeloid leukaemia |
| $dH_2O$ | distilled water |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotides |
| dsDNA | double stranded DNA |
| EDTA | ethylenediaminetetraacetic acid |
| HL | Hodgkin's lymphoma |

| | |
|---|---|
| IL | interleukin |
| K | potassium |
| L | litre |
| LDH | lactic dehydrogenase |
| M | molar |
| mA | miliampere |
| MC | *Morinda citrifolia* |
| MCH | mean corpuscular haemoglobin |
| MCHC | mean corpuscular haemoglobin |
| MCV | mean corpuscular volume |
| mg | miligram |
| MNU | *N*-Methyl-*N*-nitrosourea |
| mRNA | messenger ribonucleic acid |
| MTP | mitochondrial transmembrane potential |
| Na | sodium |
| NHL | non-Hodgkin's lymphoma |
| NK | natural killer |
| OD | optical density |
| PAHs | polycyclic aromatic hydrocarbons |
| PBS | phosphate buffered saline |
| PCR | polymerase chain reaction |
| PCR | polymerase chain reaction |
| PCV | packed-cell volume |
| pH | *puissance hydrogen* |

| | |
|---|---|
| PTCL | peripheral T-cell lymphoma |
| qPCR | quantitative real-time PCR |
| qRT-PCR | quantitative real time reverse transcriptase-polymerase chain reaction |
| RBC | red blood cells |
| RNA | ribonucleic acid |
| RNAase | ribonuclease |
| rpm | revolutions per minute |
| RS | Reed-Sternberg |
| TAE | Tris-acetate-EDTA-buffer |
| Taq | *Thermus aquaticus* |
| TNJ | Tahitian Noni Juice Liquid Dietary Supplement |
| ug | microgram |
| ul | microlitre |
| uM | micromolar |
| v/v | volume/volume |
| w/v | weight/volume |
| WBC | white blood cells |
| WHO | World Health Organization |

# CHAPTER 1

## INTRODUCTION

*Morinda citrifolia*, or locally known as mengkudu, is believed to originate from Polynesia. Apart from growing widely in Pacific Islands such as Tahiti and Hawaai (McClatchey, 2002), this plant is also found in other tropical countries such as Malaysia and Indonesia. Nowadays, *M. citrifolia* has been grown commercially as it offered a variety of interesting medicinal values. *M. citrifolia* is used regularly as folk medicine to treat common illnesses among the Polynesians. It was reported that the fruit juice is able to stimulate significant body's immune responses and therefore was mainly consumed for health maintenance (Wang *et al*., 2002).

Cancer is one of the major killer diseases in human, caused by an abnormal proliferation of cells in the body. The abnormal cells can block the circulations and invade other tissues thus causing devastating damage to normal organ functions which may lead to death (Bozzone, 2009). There are many types of cancer, usually named from the type of organs or cells which were involved. According to National Cancer Registry, 67,792 new cases of cancer were reported among Malaysians in Peninsular Malaysia from the year of 2003 to 2005 (Lim *et al*., 2008).

Leukaemia, cancer of the blood cells, is one of the threatening cancers that attacked not only adult human being but the children. Leukaemia falls at the fifth place for the ten most frequent cancers among Malaysians in Peninsular Malaysia from the year of 2003

to 2005.The disease was most frequent in both males and females at the age of 0 to 14 years old with 47.6% and 45.4% of incidence respectively (Lim *et al*., 2008). Generally, leukaemia can be divided into acute and chronic leukaemia based on the time taken for the disease to progress. Leukaemia is also further divided into lymphocytic or myeloid leukaemia based on the progenitor of the blood cells, either myeloid lineages or lymphoid lineages (Bain, 2005).

Lymphoma is a cancer of the lymphatic system that starts from the lymphocytes. In the case of lymphoma, blood cells called lymphocytes grow abnormally and proliferate continuously without control. Lymphoma usually begins in a lymph node, but it can also begin in the stomach, intestines, skin or any other organ (Chua *et al*., 2009). According to the World Health Organization (WHO), there are three major categories of lymphoid neoplasms; B cell neoplasms, T cell and natural killer (NK) cell neoplasms and Hodgkin's lymphoma (Jaffe *et al*., 2001). A study done by Chai *et al*. (1999) shown that the spectrum of lymphoma seen in East Malaysia was similar to West Malaysia except for the very low prevalence of peripheral T-cell lymphoma (PTCL) in Sarawak (3.3%). Another recent statistical data shown that lymphoma, along with leukaemia, is listed in the ten most frequent cancers that occur among males and females in peninsular Malaysia from the year 2003 to 2005 (Lim *et al*., 2008).

One of the leading causes of cancer is exposure to carcinogens. Carcinogens can be defined as a group of substances that able to alter the genetic structure of the cells, thus causing the cells to multiply uncontrollably and becomes malignant cells. Epidemiological studies indicate that approximately 80% of human cancer is caused by

exposure to chemical carcinogens in tobacco smoke, in the diet, and in the work place (Srivastava, 1999). Benzene, ionizing radiation, aromatic hydrocarbons and organic solvents, electromagnetic fields, infectious agents and pesticides were suspected to induce the carcinogenesis in human (Descatha *et al*., 2005).

*N*-Methyl-*N*-Nitrosourea (MNU) is a highly reliable carcinogen and mutagen. MNU is an alkylating agent, and exhibits its toxicity by transferring its methyl group to nucleobases in nucleic acids. Acute exposure to MNU in humans can result in skin and eye irritation, headache, nauseas, and vomiting. MNU is reasonably anticipated to be a human carcinogen based on sufficient evidence of carcinogenicity in experimental animals (IARC, 1972, 1978, 1987) and was shown to be found in food products (Fiddler, 1975).

Research had shown that cancer can be linked with the failure of cells to undergo a process called apoptosis which is a process where cells were eliminated from the system. The programmed cell death was triggered when cells reach some physiologic conditions in the biological systems (Hanahan and Weinberg, 2000). Among the functions of apoptosis were to remove the abnormal and defect cells during the process of development, differentiation and proliferation of cells (Fadeel *et al*., 1999), and to maintain the number of cells (Rathmell and Thompson, 2002). However, there are also other types of cell death that might be of importance but nevertheless, the evasion of cells from apoptosis is one of the important factors in the development of cancer.

Apoptosis is regulated by two sets of genes called pro apoptotic and anti apoptotic genes. Pro apoptotic genes such as Bax and Bak promote the process of apoptosis, while the anti apoptotic genes such as Bcl-2 and Bcl-xl inhibit the apoptosis process. The relative abundance of pro apoptotic and anti apoptotic proteins determines the susceptibility of the cell to programmed death. Research has shown that haematological malignancies such as leukaemia and lymphoma are the result of inactivation of the apoptotic genes and over expression of the pro apoptotic genes (Lowe and Lin, 2000).

Currently, the most common treatment for cancers includes surgery, chemotherapy and radiotherapy. However, some of the procedures can give unwanted side effects such as hair loss, nausea and vomiting. Thus many cancer patients decide on Complementary and Alternative Medicine (CAM) for the treatment of cancer. CAM refers to a term for medical products and practices that are not part of a standard care. Complimentary medicine is a non standard treatment used with standard treatments; meanwhile Alternative medicine refers to treatments being used instead of the standard treatments (Cassileth *et al*., 2001).

In 1997, 43% of Americans used CAM particularly among the elders (Astin *et al*., 2000; Flaherty *et al*., 2001). One of the commonly used treatments for CAM is medicinal herb that has been used traditionally for treatment of diseases. Current research has shown the significant of nutritive and non nutritive plant-based dietary factors that can prevent the process of carcinogenesis effectively.

*M. citrifolia* has many potential in acting as an anticancer agent. Hirazumi and Furusawa (1999) showed that a better survival time and curative effects occurred when Noni-ppt (derived from *M. citrifolia* fruits) was combined with sub optimal doses of the standard chemotherapeutic agents such as adriamycin (adria), 5-flourouracil (5-FU), cisplatin (CDDP) and vincristine (VCR) in tumour bearing mice. The findings supported the hypothesis that *M. citrifolia* can act as a potential therapeutic agent to improve the effects of anticancer drugs by incorporating the fruits of *M. citrifolia* in patient's diet.

This research seeks to address the effects of *M. citrifolia* in the regulation of apoptotic genes as the failure of cells to undergo apoptosis process which contribute to the development of leukaemia/lymphoma. Based on the current issues to reduce the incidence of cancer and search for a potential anti cancer agent, it is worth looking on natural sources especially from local source as a promising approach towards the prevention of cancer. Therefore, the present study hypothesizes that *M. citrifolia* fruit will prevent tumourigenesis induced by MNU via down regulating the transcription of anti apoptic gene, Bcl-2 and up regulating the transcription of pro apoptotic gene, Bax, thus decreasing the ratio of Bcl-2:Bax transcripts.

The anticarcinogenic effects of *M. citrifolia* fruit in rats with early leukaemia/lymphoma induced using MNU were investigated through these following objectives:

1. to evaluate the effects of *M. citrifolia* fruit in alleviating the carcinogenic effects of MNU in rats via haemogram, serum biochemistry and histopathological examination of the organs, and

2. to investigate the effects of *M. citrofolia* fruit in regulating the transcription of Bax and Bcl-2 in early leukaemic rats by determining the ratio of Bcl-2:Bax mRNA transcripts in the blood.

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