UNIVERSITI PUTRA MALAYSIA

EFFECTS OF HARUAN PRESSURIZED IN WATER EXTRACT ON SK-N-SH HUMAN NEUROBLASTOMA CELL LINE IN VITRO

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By

BUHARI IBRAHIM

Thesis submitted to the School of Graduate Studies, University Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

June 2015

DEDICATION

This work is dedicated to:

Almighty Allah who made this research work possible,
My parents,
My wife and son, and
My Supervisor Prof. Dr. Abdul Manan Mat Jais
For the efficient support they gave me throughout the time of the study.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

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June 2015

Chairman: **Professor Abdul Manan Mat Jais, PhD**
Faculty: **Medicine and Health Sciences**

Effects of haruan pressurized in water extract (HPIWE) on SK-N-SH neuroblastoma cell line was studied *in vitro*. MTT assay, phase contrast microscopy and flow cytometry were employed to evaluate the effects of HPIWE on SK-N-SH cells. To start with, MTT assay was run to observe the effect of the haruan extract on cell viability of SK-N-SH human neuroblastoma cells. The cells were treated with various concentrations of the HPIWE to undertake a first step in screening the extract for its cytotoxic effects. The result showed the cell viability of 68.8 % at the highest concentration of 100 µg/m, indicating that HPIWE possesses no cytotoxic effect because no IC_{50} could be determined. Another MTT assay was run with 250 µg/ml of the extract as highest concentration to observe if increasing the concentration will produce better result. However, the result indicates about 72% cell viability of the SK-N-SH cells. Therefore, since 100 µg/ml produces less viability,it was adopted for subsequent experiments. This concentration was then used to study the effects HPIWE on cellular morphology of the SK-N-SH cells using phase contrast microscope. No alteration of cellular morphology was observed on SK-N-SH cells as compared to the control cells which did not receive treatment. Based on the MTT assay and the morphological studies by phase contrast, there were no observable effects of the HPIWE on SK-N-SH cells. Our study further search for the apoptotic effects of the extract using ApoBrdU *in situ* DNA fragmentation assay kit followed by quantification of the DNA strand break using flow cytometry. One-way ANOVA test was used to compare the difference between the treated cells and the control cells (untreated cancer cells). The analysis showed that the HPIWE induced apoptosis in a time dependent manner and significant value ($*p < 0.05$) was obtained when compared with the control. The result confirmed that the HPIWE induced apoptosis on the SK-N-SH human neuroblastoma cancer cells. However, the amount of cells that underwent apoptosis were less than 50% of the total cells, meaning that even from the flow cytometry result, no IC_{50} could be generated. Therefore, the result of flow cytometry could be correlated with the MTT assay result, because in both assays, over 30% of cell death was recorded. However, the overall result of the study showed that HPIWE was not an effective anti-proliferative agent on SK-N-SH cells. Therefore, further studies should target the use of non-polar haruan extract for cancer related studies. Also angiogenesis and cell migration assay should be used to demonstrate the effect of HPIWE on brain cancer and or other cancer cell lines.

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

KESAN ESTRAK HARUAN BERTEKANAN DALAM AIR KE ATAS GARIS SEL NEUROBLASTOMA SK-N-SH MANUSIA IN VITRO

Oleh

BUHARI IBRAHIM

Jun 2015

Pengerusi: **Profesor Abdul Manan Mat Jais, PhD**
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Kesan haruan bertekanan dalam ekstrak air (HPIWE) pada SK-N-SH garis sel neuroblastoma dikaji in vitro. MTT assay, mikroskopik sebaliknya fasa dan sitometri aliran telah digunakan untuk menilai kesan HPIWE pada sel-sel SK-N-SH. Sebagai permulaan, analisis MTT telah dijalankan untuk melihat kesan ekstrak haruan pada daya maju sel-sel neuroblastoma manusia, SK-N-SH. Sel-sel telah dirawat dengan pelbagai kepekatan HPIWE untuk dilaksanakan sebagai langkah pertama dalam saringan ekstrak untuk kesan sitotoksik itu. Keputusan menunjukkan kebolehan sel 68.8% pada kepekatan tertinggi 100 µg / m, menunjukkan bahawa HPIWE tidak mempunyai kesan sitotoksik kerana tiada IC_{50} dapat dikenalpasti. Satu lagi analisis MTT telah dijalankan dengan ekstrak 250 µg / ml sebagai kepekatan tertinggi untuk melihat jika peningkatan kepekatan yang akan menghasilkan keputusan yang lebih baik. Walau bagaimanapun, keputusan kajian menunjukkan kira-kira 72% daya maju sel-sel SK-N-SH. Oleh itu, 100 µg / ml menghasilkan kurang daya maju, ia telah diterima pakai untuk eksperimen berikutnya. Kepekatan ini telah digunakan untuk mengkaji kesan HPIWE pada morfologi sel sel SK-N-SH menggunakan mikroskop Sebaliknya fasa. Tiada perubahan morfologi sel diperhatikan pada sel-sel SK-N-SH berbanding dengan sel kawalan yang tidak menerima rawatan. Berdasarkan analisis MTT dan kajian morfologi sebaliknya fasa, tiada perubahan HPIWE dikesan pada sel-sel SK-N-SH. Kajian kami untuk mencari kesan apoptotic ekstrak dengan menggunakan ApoBrdU in situ pemecahan kit analisis DNA diikuti dengan kuantifikasi rehat strand DNA menggunakan pengalir sitometri. Sehala ujian ANOVA telah digunakan untuk membandingkan perbezaan di antara sel-sel dirawat dan sel-sel kawalan (sel-sel kanser yang tidak dirawat). Hasil analisis menunjukkan bahawa HPIWE dirangsang apoptosis dengan cara yang bergantung kepada masa dan nilai signifikan (* $p < 0.05$) telah diperolehi berbanding dengan kawalan. Hasilnya mengesahkan bahawa HPIWE teraruh apoptosis di SK-N-SH sel-sel kanser neuroblastoma manusia. Walau bagaimanapun, jumlah sel-sel yang menjalani apoptosis kurang daripada 50% daripada jumlah sel-sel, yang bererti bahawa walaupun dari hasil sitometri aliran, tiada IC_{50} boleh dihasilkan. Oleh itu, hasil daripada aliran sitometri boleh dikaitkan dengan hasil analisis MTT, kerana dalam kedua-dua tujuan esei, lebih 30% daripada kematian sel direkodkan. Walau bagaimanapun, keputusan keseluruhan kajian menunjukkan HPIWE bukan agen anti proliferatif berkesan ke atas sel-sel SK-N-SH. Oleh itu, kajian lanjut perlu menyasarkan penggunaan ekstrak haruan bukan kutub untuk kajian kanser yang berkaitan. Juga angiogenesis dan analisis

penghijrahan sel boleh digunakan untuk menunjukkan kesan HPIWE kepada kanser otak dan atau garis sel kanser lain.

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Master of Science. The members of the Supervisory committee are as follows:

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LIST OF ABBREVIATIONS

%	Percent
°C	Degree Celsius
ABU	Ahmadu Bello University
AECS	Aqueous extract of *Channa striatus*
ANOVA	Analysis of variance
AO	Acridin Orange
ATCC	American Type Culture Collection
BrdUTP	Bromodeoxyuridine triphosphate
Cm	Centimetre
CO2	Carbon dioxide
COX-2	Cyclo-oxygenase
DDH_{20}	Deionized distilled water
DHA	Deicosahexaenoic acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleotide acid
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
EPA	Ecosapentaenoic acid
FA	Fatty acid
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
HL-60	promyelocytic leukaemia
PHIWE	Haruan Pressurized in water extract
H	Hours
HT-29	human colon adenocarcinoma
IC_{50}	Inhibition concentration for 50% of cell population
INRG	International neuroblastoma risk group
KCl	Potassium chloride
KH2PO4	Potassium dihydrogen phosphate
LECS	Lipid extract of *Channa striatus*
MEM	Eagle's Minimum essential Medium
mL	Millilitre
mm	Millimetre
MTT	Microculture tetrazolium
NFKB	Nuclear Factor Kappa Beta
NYSC	National youth service corps
OD	Optical density
PBS	Phosphate buffer saline
PDECGF	Platelet-Derived Endothelial Cell Growth Factor
PDGF	Platelete drive growth factor
PGE2	Prostaglandin-E2
pH	Hydrogen ion concentration
PhD	Doctor of philosophy
PUFA	Polyunsaturated fatty acids
RNase	Ribonuclease
Rpm	Rotation per minute
SD	Standard deviation
TdT	Terminal deoxynucleotidyl Transferase

TUNEL	Terminal deoxyribonucleotide Transferase-mediated dUTP Nick-End Labelling
UPM	Universiti Putra Malaysia
VEGF	Endothelial growth factor
w/v	Weight per volume
WHO	World Health Organization
µL	Microlitre

CHAPTER 1

INTRODUCTION

Cancer is one of the major causes of death globally. The World Health Organization (WHO) estimated the sum of 14 million new cancer cases and about 8.2 million deaths due to cancer in the year 2012, and it was said the death record could hit 13.1 million by the year 2030. About 60% of total cancer cases in the world occur in Asia, Africa, and Central and South America. These regions were said to account for 70% of total cancer death in the world (World Cancer Report, 2014). According to the International Agency for Research on Cancer (IARC) Globocan of the World Health Organisation (WHO), in 2012, the number of cancer incidence and cancer death stood at 37,000 and 21,700 respectively.

Brain cancer is the second most popular cancer among children after leukaemia (Siegel *et al*.., 2013) . Brain tumour may be categorised into primary and secondary tumours. Primary tumour originated from the brain tissue, whereas secondary tumour metastasizes from other tissues (Villarreal-Garza. *et al*., 2013). Even though brain cancer was termed the third most cause of death in adult under the age of 34 years, more of these tumours show up after the age of 50 years (Castro *et al.,* 2003).

Neuroblastoma is the prevailing solid malignant tumour in infancy and childhood. Two-thirds of the tumour metastasize from other organs (Modak and Cheung, 2010). Despite the fact that neuroblastoma accounts for only 8% of all childhood malignant tumours, 15% of childhood cancer deaths are due to it (Castel *et al*., 2013). Treatment of neuroblastoma includes induction of high dose radiotherapy, chemotherapy, stem-cell transplantation and surgical removal of the tumour (Navarra *et al.,* 2014). Although, some level of improvement was accomplished using the earlier mention methods of treating brain malignancy, a number of patients with high risk of neuroblastoma do not respond to the conventional therapy, or the symptoms appear to come back later (Castel *et al.,* 2013). Therefore, screening of natural products that can cure this disease may give an alternative treatment with no or less side effects.

Natural products derived from marine animals such as, coral, sponges, microorganisms and fishes was shown to possess anti-inflammatory, anti-viral and anti-cancer effects. It was reported that 74.8% of currently used anti-cancer drugs such as taxanes, vinca alkaloids and camptothecin class of compounds are sourced from natural products (Newman and Cragg, 2012).

Haruan fish, found in many tropical countries like Malaysia, Indonesia, and Thailand is widely known as a source of food and traditional medicinal products since pre-historic time (Abdul Manan, 2007). Preliminary bioactive component (Zakaria *et al*., 2007) include proteins, peptides, glycoproteins, fat, and minerals (Abdul Manan, 2007). Amino acids; histidine, lysine, aspertate and arginine are reported to produce antioxidant effect (Dahlan-daud *et al*., 2010; Frankel, 1998). The amino acids as well as the fatty acids content of the fish could give a tremendous effect on cancer treatment. An earlier report by Nur Syamsiah (2012) suggested the cytotoxic and anti-proliferative effects different fractions of haruan traditional extract (HTE) on colon-rectal and Leukaemia cancers. Also, antioxidant effects of aqueous extract of *Channa*

striatus (AECS) was demonstrated using 2,2-diphenyl-picrylhydrazyl (DPPH) assay, azino-bis(3 ethylbenzothiazoline-6 sulphonic acid (ABTS) and ferric reducing ability of power (FRAP) (Radzak *et al*., 2014). The authors were able to correlate the antioxidant effects of AECS with cytotoxicity assay on in human liver hepatoma HepG2 cells by the MTT assay.

Therefore, this study evaluated the anti-proliferative effect of HPIWE on SK-N-SH neuroblastoma, brain cancer cell *in vitro*.

1.1 Problem Statement

Neuroblastoma is a solid brain tumour that accounts for more than 15% of childhood cancer death. However, conventional treatment of the tumour using chemotherapy, radiotherapy and surgery is associated with toxicity, drug resistance and screening effect of blood brain barrier. Search for potential agent from natural product with less side effects will be a welcome development.

1.2 Justification of the study

HPIWE showed the anti-proliferative effects on human colon adenocarcinoma (HT-29) and Human promyelocytic leukaemia (HL-60). The inhibitory concentration (IC_{50}) value of 78.0 ± 0.28 µg/ml and 44.0 ± 0.37 µg/ml was found in the two cells respectively. However, the potential of the HPIWE on SK-N-SH neuroblastoma cells have not been studied. Because our interest was on cancer that affects neuronal cells, SK-N-SH cell was used throughout the study.

1.3 Research Questions

a) Could HPIWE inhibit proliferation of SK-N-SH neuroblastoma cell line *in vitro*?
b) Will there be any change in the morphological appearance of SK-N-SH when treated with HPIWE?
c) Could HPIWE induce apoptosis on SK-N-SH neuroblastoma cells when observed under Flow cytometry?

1.4 Hypothesis

HPIWE inhibit proliferation of SK-N-SH neuroblastoma via apoptosis.

1.5 Main Objectives

To determine the effects of the HPIWE on human neuroblastoma cell line.

1.5.1 Specific Objectives

1. To determine the anti-proliferative effects of the HPIWE on SK-N-SH neuroblastoma cells *in vitro* using MTT assay.
2. To study the morphological features of SK-N-SH neuroblastoma cells treated with HPIWE using phase contrast microscope
3. To determine the apoptotic effect of the HPIWE on SK-N-SH neuroblastoma cells by flow cytometry.

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