UNIVERSITI PUTRA MALAYSIA

BIOLOGICAL ACTIVITIES OF PINEAPPLE (*Ananas comosus* L. MERR. VAR. JOSAPHINE) VINEGAR *In Vitro* AND *In Vivo*

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FBSB 2016 20

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By

NURUL ELYANI BT MOHAMAD

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

June 2016

DEDICATION

I would like to dedicate this thesis to my beloved parents, Mr. Mohamad Othman and Mrs. Muhaini Ahmad for their love, patience and endless support. Also to my siblings and best friends who are always there through my thick and thin. Thank you for the unconditional support and courage. I would not have been able to face all the challenges without motivation and support from each of you. Thank you.

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

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Chairman : Assoc. Prof. Noorjahan Banu Alitheen, PhD
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Improper handling of work stress, diet and lifestyle cause imbalance to the biological system in our body and led to various diseases nowadays. The number of patients with chronic diseases such as cardiovascular disease and cancer have increased greatly and becoming a major public health concern. The current treatment approaches are unfavorable due to the unwanted side effects observed. Natural resources such as fruits and vegetables contain anti-oxidants, minerals and vitamins that may help to prevent diseases and enhance the immune system. Vinegar produced from carbohydrate sources, such as fruits and grains, contains not only acetic acid, but also other bioactive compounds such as polyphenolics, volatile compounds, and organic acids. The present study was carried out to investigate several biological activities such as immunomodulation, hepatoprotective, anti-tumor and anti-obesity activities of pineapple vinegar using *in vitro* and *in vivo* model. Assays such as anti-oxidant, real-time PCR, proteome, western blot and metagenomic analyses revealed the underlying mechanism of pineapple vinegar in all *in vivo* study. Through the *in vivo* toxicity study, it was shown that the tested concentrations of pineapple vinegar were not only safe to be taken continuously but they also helped to increase the antioxidant levels (FRAP and SOD) and immune response (CD4, CD8, IFNγ and IL2) in normal mice model. Meanwhile, *in vivo* hepatoprotective study showed that pineapple vinegar was able to promote the recovery of paracetamol induced liver inflammation in mice due to its antioxidant activities. Through several *in vitro* cancer assays such as MTT, flow cytometry cell cycle analysis, Annexin V analysis and scratch assay, the anti-cancer effect of pineapple vinegar was also revealed. Further investigation on the chemopreventive effect using murine model was done to support the results of *in vitro* study. Results showed that the treatment by high concentration pineapple vinegar has significantly reduced the tumor weight through its anti-metastasis and anti-inflammatory activities. Additionally, *in vivo* anti-obesity study indicated the potential of pineapple vinegar as anti-obesity agent through the reduction of the body weight, regulation of several obesity related genes and proteins and its ability to alter the gut micobiota. In

conclusion, oral administration of tested concentrations in all the *in vivo* studies proved clearly that pineapple vinegar confers hepatoprotective, anti-tumor and anti-obesity effects in dose dependent manner. This study signifies the potential of pineapple vinegar as a functional food for therapeutic purposes.

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

PENILAIAN KE ATAS AKTIVITI-AKTIVITI BIOLOGI CUKA NANAS (*Ananas comosus* L. MERR. VAR. JOSAPHINE) SECARA *In Vitro* DAN *In Vivo*

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Ketidak kecekapan dalam menguruskan tekanan kerja, pemakanan dan cara hidup menyebabkan ketidakseimbangan kepada sistem biologi badan dan seterusnya membawa kepada pelbagai penyakit dalam dunia harini. Bilangan pesakit kronik seperti pesakit kardiovaskular dan kanser telah meningkat dengan tinggi dan menjadi isu utama dalam kesihatan di kalangan masyarakat. Kaedah rawatan yang digunakan kini kurang sesuai berdasarkan kesan sampingan yang dilihat. Sumber semulajadi seperti buah-buahan dan sayur-sayuran mengandungi anti-oksidan, mineral dan vitamin yang dapat membantu mencegah pelbagai penyakit dan meningkatkan imun sistem. Cuka yang dihasilkan menggunakan sumber karbohidrat dari buah-buhanan dan bijirin bukan sahaja mengandungi asid asetik, tetapi juga pelbagai sebatian bioaktif lain seperti polifenolik, sebatian meruap dan asid organik. Penyelidikan ini dijalankan untuk mengkaji beberapa aktiviti biologi cuka nanas ke atas aktiviti imunisasi, perlindungan hati, anti-tumor dan anti-kegemukan menggunakan model *in vitro* dan *in vivo*. Beberapa eksperimen seperti analisis anti-oksidan, analisis real-time PCR, analisis proteome, analisis Western blot dan analisis metagenomik menunjukkan mekanisma secara menyeluruh cuka nanas dalam semua ujian *in vivo* yang dijalankan. Melalui ujian toksik yang dijalankan melalui ujian *in vivo* menunjukkan kesemua kepekatan cuka nanas yang digunakan bukan sahaja selamat jika diambil berterusan, malah, cuka nanas juga dapat membantu meningkatkan tahap anti-oksidan (FRAP dan SOD) dan juga tahap imunisasi (CD4, CD8, IFNγ and IL2) dalam mencit normal. Selain itu, ujian perlindungan hati yang dijalankan secara *in vivo* menunjukkan keupayaan cuka nanas dalam mempercepatkan penyembuhan pada pembengkakkan hati yang disebabkan oleh paracetamol dalam tikus adalah dibantu oleh aktiviti-aktiviti anti-oksidan cuka nanas. Melalui pelbagai ujian kanser yang dijalankan secara *in vitro* seperti ujian MTT, analisis kitaran sel menggunakan flow cytometry, analisis Annexin V dan analisis ujian goresan sel, aktiviti anti-kanser cuka nanas telah dapat dibuktikan. Ujian secara menyeluruh ke atas kesan perlindungan cuka nanas terhadap kanser menggunakan model murin telah dijalankan untuk menyokong dan

membuktikan keputusan dari ujian *in vitro* yang telah diajalankan. Dari ujian yang dijalankan, keputusan menunjukkan rawatan menggunakan kepekatan tertinggi cuka nanas telah berjaya mengurangkan berat tumor melalui aktiviti anti-metastasis dan anti-radang. Di samping itu, ujian anti-kegemukan yang dijalankan melalui ujian *in vivo* juga menunjukkan potensi cuka nanas sebagai agen anti-kegemukan melalui pengurangan dalam berat badan mencit, perubahan kitaran dalam beberapa gene dan protein berkaitan kegemukan dan kebolehan cuka nanas dalam mempengaruhi pembiakan microb di dalam gut. Secara kesimpulanya, kesemua kepekatan cuka nanas yang diberi secara oral dalam kesemua ujian *in vivo* yang dijalankan membuktikan kebolehan cuka nanas dalam melindungi hati, sebagai anti-tumor dan anti-kegemukan dan kesannya bergantung kepada kepekatan yang digunakan. Penyelidikan kali ini membuktikan cuka nanas boleh digunakan sebagai makanan tambahan untuk kegunaan terapeutik.

ACKNOWLEDGEMENTS

First and foremost, *Alhamdulillah*, all thanks to Allah the Almighty for giving me the strength to complete my study. I would like to highlight my appreciation and gratitude to my supervisor, Associate Professor Dr.Noorjahan Banu Mohamed Alitheen for her perceptiveness, advices and encouragement throughout the completion of my thesis. I would also like to extend my appreciation to my co-supervisory committee members Dr. Kamariah Long (MARDI) and Dr.Nik Afizan Nik Abd. Rahman for their valuable advice and assistance for the accomplishment of this project.

Next, my deepest gratitude goes to Dr. Yeap Swee Keong and Mr. Shaiful Adzni Sharifuddin (MARDI) for their guidance, constructive suggestion and review of my works. Also, many thanks to all my fellow Animal Tissue Culture Lab (ATCL) members; Mrs. *Roszaimah* Muhammad *Sapah*, Mrs. Hamidah Yusuf, Dr. Nadiah Abu, Mr. Firdaus Romli, Ms. Nur Rizi Mohd Zamberi, Mrs. Noraini Nordin, Dr. Ho Wan Yong, Ms. Kristeen Teo Ye Wen, Ms Norlaily Mohd Ali and Dr. Ng Wei Lun for their continuous support and generous help during the time of my study. Special thanks to Mr. Beh Boon Kee, Dr. Hyunh Ky, Dr. Lim Kian Lam, Mrs. Nancy Liew and Dr. Tan Sheu Wei for their assistance and guidance. My humbly appreciation also goes to Mr. *Zainuddin* Ibrahim and Kamarzaman Ahmad from Department of Veterinary Pathology & Microbiology, University Putra Malaysia and Mr. Andrew Leong Kum Loong from Monash University Malaysia for supplying the mice and assistance throughout my *in vivo* study.

Next, I would also like to acknowledge and thank myPhD from the Ministry of Higher Education and Graduate Research Fellowship (GRF) from School of Graduate Studies, Universiti Putra Malaysia for the financial support all the way through my study and also RMK10, MARDI for funding this project.

Last but not least, thanks to everyone who had helped me directly and indirectly in making this project a success. Thanks for the knowledge, encouragements and enthusiasm. Without everyone assistance and support, it is impossible for me to completed my PhD project.

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LIST OF ABBREVIATIONS

%	Percentage
γ	Gamma
μ	Micro
m	Milli
ACTB	Beta Actin
ALP	Alkaline Phosphatase
ALT	Alanine aminotransferase
ANOVA	Analysis of variance
AST	Aspartate aminotransferase
ATCC	American Tissue Culture Collection
BHT	Butylated hydroxytoulene
BSA	Bovine serum albumin
cDNA	Complementary DNA
COX-2	Cyclooxygenase 2
CO_2	Carbon dioxide
DMSO	Dimethyl sulfoxide
DPPH	1,1-diphenyl-2-picryl-hydrazil
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing anti-oxidant power

g	Gram
g	Centrifugal force
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GLUT 4	Glucose Transporter 4
h	Hour
HCl	Hydrochloric acid
H&E	Hematoxylin and eosin
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HRP	Horseradish peroxidase
IC50	Inhibitory Concentration at 50%
ICAM-1	Intercellular Adhesion Molecule
IL	Interleukin
IFN	Interferon
INOS	Inducible nitric oxide synthase
L	Liter
LD_{50}	Lethal dose that cause 50% of death in animal
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
M	Molar
MARDI	Malaysian Agriculture Research Development Institute
MDA	Melondialdehyde
MMP 9	Matrix metalloproteinase 9
MRNA	Messenger RNA
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
n	nano

Na_2HPO_4	Disodium hydrogen phosphate anhydrous
NaCl	Sodium chloride
NAOH	Sodium hydroxide
NBT	Nitro blue tetrazolium
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
NO	Nitric oxide
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PS	Phosphatidylserine
QPCR	Quantitative PCR
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase PCR
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SOD	Superoxide dismutase
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
TG	Triglycerides
TMB	3,3',5,5'-Tetramethylbenzidine
TPC	Total phenolic content
TPTZ	2,4,6-tripyridyl-s-triazine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end

	labeling
v	volume
VEGF	Vascular endothelial growth factor
w	weight

CHAPTER 1

INTRODUCTION

Modernization has brought upon positive impacts on human being but at the same time, it is undeniable that modernization leads to unhealthy lifestyle as well (Hidaka, 2012). Work pressure, imbalanced diet and stressful environment disrupt the biological system and can subsequently lead to various diseases (Chakravarti & Little, 2003; Kivimäki *et al.*, 2002; Mera, 1994). To date, cardiovascular disease and cancer are the major causes of death worldwide (WHO, 2014). Researchers have suggested that a change in the current lifestyle may be one of the most effective measures to prevent the occurrence of many diseases (Bækgaard & Schmidt, 2015; Moritani, 2015). For years, functional food has been consumed extensively as it is believed that the consumption can benefit the body and certain functional foods have been proven scientifically in treating some diseases (Ryan *et al.*, 2015; Wong *et al.*, 2015). A food is considered to be under the functional food category if it is beneficial to the health and supplies additional nutrition as needed by the body (Roberfroid, 2000). Functional food contains certain bioactive compounds, which is one of the major contributors to strengthen the immune system and protecting the body to fight against various diseases including cardiovascular and cancer (Canene-Adams *et al.*, 2005; Kris-Etherton *et al.*, 2002; Lordan *et al.*, 2011).

Fruits and vegetables which contain high antioxidants and minerals are the examples of natural resources of functional foods (Rodríguez *et al.*, 2006; Schieber *et al.*, 2001; Shui & Leong, 2006). Studies have shown that the consumption of fruit markedly decreased the risk of cardiovascular disease and cancer, which might be contributed to the presence of high level of antioxidants and nutrients in the fruit (Stahl & Sies, 2005). The benefits conferred on the body by the consumption of fruits are at its peak when it is consumed fresh. However, fruits rot quickly in the tropical climate especially when the supply exceeds the demand (Nunes *et al.*, 2009; Parfitt *et al.*, 2010). To overcome this problem, fruits can be kept in several forms such as jams (García-Martínez *et al.*, 2002), concentrated juice (Ashurst, 2008), canned fruit (Rickman *et al.*, 2007), dried fruit (George *et al.*, 2004) and also fermented fruit (Di Cagno *et al.*, 2013). Pineapple is one of the local fruits and is in abundance in Malaysia. It contains high vitamin C content and other antioxidants, which helps to enhance the immunity and protect the body from various diseases (Gardner *et al.*, 2000). During the canning process, only part of the pineapple fruit will be cut and used while remaining will be discarded. Since pineapple contains lots of nutrients, vitamin and possess several bioactivities, it is a huge waste to discard the remaining part of the fruits. By utilizing this discarded part and convert it into vinegar, it can avoid this wastage and generate a zero waste product.

Vinegar, which mainly consists of water and acetic acid, is one of the valuable end-products of the fermentation process. It is produced through two-step

fermentation, starting with an alcoholic fermentation and followed by acetification. The preservation of fruits through fermentation process into a final product such as vinegar is not only economically viable and environmentally friendly but it also enhances the nutritional value of the product (Motarjemi; 2002). During fermentation, several bacteria produce bioactive by-products such as vitamins and antioxidants (Blandino *et al.*, 2003).

To date, there have been many studies done on several types of vinegar which show that these vinegars possess several bioactivities such as anti-cholesterol, anti-obesity, anti-cancer, anti-hypertensive and anti-diabetic activities (Budak *et al.*, 2014; Kondo *et al.*, 2001a; Nanda *et al.*, 2004; Seo *et al.*, 2014; Setorki *et al.*, 2010; Shishehbor *et al.*, 2008). Nevertheless, there are no experimental evidences about the health-promoting effectiveness of pineapple vinegar in conferring beneficial effect such as hepatoprotective, anti-inflammation, anti-tumor and anti-obesity has been reported so far. Thus, this study was aimed to determine the *in vitro* and *in vivo* bioactivities of pineapple vinegar.

The specific objectives of this study are:

1. To determine the anti-oxidant activities, immunomudulatory effects and the maximum dose of pineapple vinegar which is safe to be consumed through *in vivo* acute and sub-chronic toxicity studies.
2. To investigate the effect of pineapple vinegar in reversing paracetamol-induced liver damage and anti-inflammatory activity in mice.
3. To determine the anti-tumor effect of pineapple vinegar *in vitro* and *in vivo*.
4. To evaluate the anti-obesity effect of pineapple vinegar in diet induced obese mice.

The hypotheses of this study are:

1. Pineapple vinegar has antioxidant and immunomodulatory effects and safe to be consumed at all concentrations tested.
2. Pineapple vinegar helps to protect the paracetamol induced liver injury and shows anti-obesity effect in diet induced mice
3. Pineapple vinegar possess anti-tumor effect *in vivo* and *in vitro*.
4. Pineapple vinegar shows anti-obesity effect in diet induced mice.

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BIODATA OF STUDENT

The student, Nurul Elyani bt Mohamad, was born on 16th July 1987, in Seremban, Negeri Sembilan. She went through her primary education at several schools in different state including Malacca, Selangor, Perak and Kedah, following her father who worked as an army officer. After UPSR, she furthered her secondary education at Sekolah Menengah Kebangsaan Agama Kuala Abang, Terengganu before move to MRSM Pengkalan Chepa, Kelantan until SPM. After finishing schooling, she pursued her first degree in Bachelor of Biochemical-Biotechnology Engineering (Hons) at International Islamic University Malaysia (IIUM) and completed her degree in 2011. After graduation, she worked as a research assistant at IIUM before pursued her Master of Science at Faculty of Biotechnology and Science Biomolecule, University Putra Malaysia in 2012 under a collaborating project with Malaysian Agricultural Research and Development Institute (MARDI). In 2014, she was converted to the doctoral degree under the same project.

LIST OF PUBLICATIONS

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