ANTIOXIDANT ELEMENTS ANALYSIS AND ENZYME ACTIVITIES IN HEPATOCARCINOGENESIS INDUCED RATS TREATED WITH *STROBILANTHES CRISPUS*

By

YOGESPIRIYA A/P SHREERAMAN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, In Fulfilment of the Requirements for the Degree of Master of Science

November 2004

Specially dedicated to

My lovely parents,

MR. SHREERAMAN A/L RAMASAMY and

MRS. LALITHA A/P RAJAMANIKAM

My husband,

MR. SURESH A/L PALPANABAN

My brothers, sister in law and their daughter

MR. PUNITHAN
MRS. ALAMELU MANGAI
MR. AMITEN
MISS SRI SHAANKEREY

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

ANTIOXIDANT ELEMENTS ANALYSIS AND ENZYME ACTIVITIES IN HEPATOCARCINOGENESIS INDUCED RATS TREATED WITH *STROBILANTHES CRISPUS*

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Chairman: Associate Professor Fauziah Othman, Ph.D

Faculty: Medicine and Health Sciences

This study was conducted to investigate the effect of 5% (w/v) *Strobilanthes crispus* crude extract on rat liver during induced hepatocarcinogenesis. Elemental analysis, was done to support this study on all the liver tissues by energy filter transmission electron microscope (EFTEM) and variable pressure scanning electron microscope (VPSEM). Histological and gross changes of liver evaluation were conducted to observe the cellular and morphological changes during induced hepatocarcinogenesis in rats treated with *S.crispus*. Xenobiotics detoxification enzymes such as aniline hydroxylase (ANH) and glutathione S-transferase (GST) were assayed in liver tissues. Gross examination on liver of rats which were induced with diethylnitrosamine/acetylaminofluorene (DEN/AAF) showed the presence of nodules, haemorrhages and swelling on liver surface. The 5% (w/v) *Strobilanthes crispus* extract effectively reduced gross changes on liver during induced hepatocarcinogenesis. Meanwhile, histological evaluation revealed that a certain grade of inflammation or necrosis at portal and lobular region and stages of fibrosis

during induced hepatocarcinogenesis was successfully reduced after the administration of *Strobilanthes crispus* extract. Nevertheless, supplementation of *S. crispus* did not fully recover the liver to normal histological feature. This could be due to the short duration of this experiment. Elemental analysis by VPSEM showed that 5% w/v *Strobilanthes crispus* extract contained 63.52% of carbon, 16.56% of oxygen, 0.66% of sodium, 0.08% of magnesium, 11.78% of aluminium, 0.17% of phosphorus, 1.19% of sulphur, 1.66% of chloride, 0.95% of potassium, 0.19% of iron, 0.20% of copper, 2.30% of calcium, 0.39% silicon and 0.35% of argentum. The fresh liver tissue obtained from rats administered with DEN/AAF and treated with *Strobilanthes crispus* extract showed higher percentage distribution of antioxidant elements such as potassium and magnesium when compared to the liver from rats induced with DEN/AAF and untreated rats, DEN/AAF administered and treated with glycyrrhizin rats, normal without treatment rats, normal with *Strobilanthes crispus* supplemented rats and normal with glycyrrhizin supplemented rats. To detect the antioxidant elements such as potassium, calcium, magnesium and iron at ultrastructural level, EFTEM was used. By utilizing EFTEM, the distributions of these elements were also found to be higher in cancer with *S.crispus* group compared to other groups. This may suggest showed that tumor cells has high uptake of these elements from *S.crispus* extract. Meanwhile, DEN/AAF induced rats showed an increase activity of drug/carcinogen detoxification enzymes i.e. GST and ANH. However, 5% w/v *S.crispus* extract effectively inhibit the activity of these enzymes in DEN/AAF induced rats. *S.crispus* which is rich with antioxidant elements such as potassium, magnesium, calcium and iron play important role during carcinogenesis. Thus, *S.crispus* can be considered as potential chemopreventive agent

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

ANALISIS ELEMEN-ELEMEN ANTIOKSIDAN DAN AKTIVITI ENZIM SEMASA KARSINOGENESIS HEPAR DIARUH DALAM TIKUS DIRAWAT DENGAN *STROBILANTHES CRISPUS*

Oleh

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Kajian ini dijalankan untuk mengenalpasti kesan 5% ekstrak *Strobilanthes crispus* ke atas hepar tikus semasa karsinogenesis hepar diaruh. Bagi menyokong kajian ini, elemen-elemen di dalam semua tisu hepar tikus dianalisis dengan menggunakan "energy filter transmission electron microscope" (EFTEM) dan "variable pressure scanning electron microscope'' (VPSEM). Pemeriksaan makroskopik dan histologi pada hepar tikus dijalankan untuk memerhati perubahan pada sel dan morfologinya semasa karsinogenesis hepar diaruh dan dirawat dengan *Strobilanthes crispus*. Enzim-enzim yang boleh menyahtoksinkan drug atau karsinogen seperti aniline hydroxylase (ANH) dan glutathione s-transferase (GST) dalam tisu-tisu hepar tikus dikaji. Pemeriksaan mata kasar pada hepar tikus yang disuntik dengan diethilnitrosamin/acetilaminoflorin (DEN/AAF) menunjukkan kemunculan nodul-nodul, pendarahan dan pembengkakan pada hepar tikus. Rawatan dengan 5% ekstrak *Strobilanthes crispus* dapat mengurangkan perubahan makroskopik pada hepar tikus semasa karsinogenesis hepar diaruh. Sementara itu, penilaian histologi menunjukkan skor inflamasi atau necrosis pada bahagian portal,

lobular hepar dan peringkat fibrosis semasa karsinogenesis hepar diaruh berjaya dikurangkan setelah diberi 5% ekstrak *Strobilanthes crispus ke*pada tikus. Walaubagaimanapun*, Strobilanthes crispus* tidak dapat merawat perubahan ini ke tahap normal. Ini mungkin disebabkan tempoh eksperimen yang singkat. Elemen-elemen yang dianalisis dengan VPSEM menunjukkan bahawa 5% ekstrak *Strobilanthes crispus* terdiri daripada 63.52% karbon, 16.56% oksigen, 0.66% natrium , 0.08% magnesium, 11.78% aluminium, 0.17% fosforus, 1.19% sulfur, 1.66% klorin, 0.95% kalium, 0.19% besi, 0.20% kuprum, 2.3% kalsium, 0.39% silikon dan 0.35% argentum. Tisu hepar segar dari kumpulan tikus yang disuntik dengan DEN/AAF dan dirawat dengan *Strobilanthes crispus* menunjukkan peratus taburan elemen-elemen antioksidan seperti kalium dan magnesium yang tinggi jika dibandingkan dengan tisu hepar daripada tikus yang disuntik dengan DEN/AAF yang tidak dirawat, tikus yang disuntik dengan DEN/AAF dan dirawat dengan gliserizin, tikus normal, tikus normal yang diberi *Strobilanthes crispus* dan tikus normal yang diberi gliserizin. Bagi menganalisis elemen-elemen antioksidan seperti kalium, kalsium, magnesium dan besi pada peringkat ultra struktur, EFTEM digunakan. Menurut EFTEM, taburan elemen-elemen ini didapati paling banyak dalam kumpulan kanser dengan rawatan *Strobilanthes crispus* jika dibandingkan dengan kumpulan-kumpulan lain. Ini menunjukkan sel-sel tumor mengambil elemen-elemen tersebut dari ekstrak *Strobilanthes crispus* untuk memenuhi keperluannya. Sementara itu, suntikan DEN/AAF ke atas tikus menunjukkan peningkatan aktiviti enzim yang menyahtoksin drug atau karsinogen seperti GST dan ANH pada hepar. Walaubagaimanapun, 5% *Strobilanthes crispus* dapat merencat aktiviti enzim-enzim ini dengan efektif pada tikus yang disuntik dengan DEN/AAF. *Strobilanthes crispus* adalah kaya dengan elemen-

elemen antioksidan seperti kalium, magnesium, kalsium dan besi yang memainkan peranan penting semasa karsinogenesis diaruh. Oleh itu, *Strobilanthes crispus* boleh dianggap berpotensi sebagai agen kemopreventif.

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I certify that an Examination Committee met on 3rd November 2004 to conduct the final examination of Yogespiriya A/P Shreeraman on her Master of Science thesis entitled "Antioxidant Elements Analysis and Enzyme Activities in Hepatocarcinogenesis Induced Rats Treated with *Strobilanthes crispus*" in accordance with University Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at degree at UPM or other institutions.

_____

YOGESPIRIYA D/O SHREERAMAN

Date:

TABLE OF CONTENTS

		Page
DEDICATION		ii
ABSTRACT		iii
ABSTRAK		V
ACKNOWLEDGEMENTS		viii
APPROVAL		x
DECLARATION		xii
LIST OF TABLES		xvii
LIST OF FIGURES		xix
LIST OF PLATES		xx
LIST OF ABBREVIATIONS		xxii
CHAPTER		
1	**INTRODUCTION**	1
2	**LITERATURE REVIEW**	
	2.1 Liver Cancer	4
	2.2 Microscopic features of HCC	5
	2.3 Diethylnitrosamine (DEN)	6
	2.4 2-Acetylaminofluorene (2-AAF)	7
	2.5 Chemical carcinogenesis	8
	2.5.1 Necrosis	9
	2.5.2 Apoptosis	10
	2.6 Diet and cancer	13
	2.7 *Strobilanthes crispus*	14
	2.7.1 The uses of *Strobilanthes crispus*	15
	2.7.2 Chemical composition of *Strobilanthes crispus* leaves	16
	2.8 Antioxidants	17
	2.9 Free Radicals	21
	2.10 Glycyrrhizin	22
	2.11 Minerals / Trace elements	24
	2.12 Transmission electron microscope (TEM)	26
	2.13 Variable pressure scanning electron microscope (VPSEM)	27
	2.14 Phase I and phase II enzymes	29
	2.14.1 Aniline hydroxylase (ANH)	30

2.14.2 Glutathione s-transferase (GSTs) 31

3 GROSS STUDY OF LIVER INDUCED HEPATOCARCINO-GENESIS FROM RATS TREATED WITH *STROBILANTHES CRISPUS*

3.1 Introduction 33
3.2 Materials and Methods 34
3.2.1 Maintaining rats 34
3.2.2 Diethylnitrosamine (DEN) preparation 34
3.2.3 Acetylaminofluorene (AAF) preparation 34
3.2.4 *Strobilanthes crispus* leave extract preparation 35
3.3 *In vivo* bioassay 35
3.3.1 Study group 35
3.3.2 Animal treatment 36
3.3.3 Termination of experiment 37
3.4 Statistical analysis 37
3.5 Results 38
3.5.1 Relative weight of liver to body weight of rat 38
3.5.2 Gross examination of rat liver during induced hepatocarcinogenesis 43
3.6 Discussion 46

4 HISTOLOGY OF RAT LIVER INDUCED HEPATOCARCINOGENESIS FROM RATS TREATED WITH *STROBILANTHES CRISPUS*

4.1 Introduction 48
4.2 Materials and Methods 49
4.2.1 Sample preparation for lesion scoring 49
4.3 Statistical analysis 51
4.4 Result 52
4.4.1 Lesion scoring of portal area 52
4.4.2 Lesion scoring of lobular area of liver during 54
4.4.3 Stages of fibrosis of liver 57

4.5 Discussion 60

5 ELEMENTAL ANALYSIS BY ENERGY FILTER TRANSMISSION ELECTRON MICROSCOPE (EFTEM)

5.1 Introduction 63

5.2 Materials and Methods 64
5.2.1 Sample preparation for transmission electron microscope 64
5.2.2 Sectioning 64
5.2.3 Elemental mappings by energy filter Transmission electron microscope (EFTEM) 64
5.2.4 Scoring of elemental distribution 65
5.3 Statistical analysis 65
5.4 Results 66
5.4.1 Mean score of calcium distribution 66
5.4.2 Mean score of potassium distribution 68
5.4.3 Mean score of magnesium distribution 70
5.4.4 Mean score of iron distribution 72
5.5 Discussion 79

6 ELEMENTAL ANALYSIS BY VARIABLE PRESSURE SCANNING ELECTRON MICROSCOPE (VPSEM) ATTACHED TO ENERGY DISPERSIVE X-RAY (EDX)
6.1 Introduction 86
6.2 Materials and Methods 87
6.3 Statistical analysis 87
6.4 Results 88
6.4.1 Percentage distribution of magnesium 88
6.4.2 Percentage distribution of potassium 90
6.5 Discussion 92

7 ANILINE HYDROXYLASE AND GLUTATHIONE S-TRANSFERASE ENZYMES ACTIVITIES DURING HEPATO-CARCINOGENESIS TREATED *STROBILANTHES CRISPUS*
7.1 Introduction 94
7.2 Materials and Methods 95
7.2.1 Liver cytosol and microsomal preparation 95
7.2.2 Aniline hydroxylase (ANH) enzyme assay 95
7.2.3 Glutathione s-transferase (GST) assay 96
7.2.4 Protein analysis 97
7.3 Stastistical analysis 97
7.4 Results 98
7.4.1 Aniline hydroxylase (ANH) activity 98
7.4.2 Glutathione s-transferase (GST) activity 100
7.5 Discussion 102

8	**GENERAL DISCUSSION**	105
9	**CONCLUSION**.	111
BIBLIOGRAPHY		113
APPENDICES		128
BIODATA OF THE AUTHOR		132

LIST OF TABLES

Table		Page
1.	Experimental design of rats during induced hepatocarcino genesis	36
2.	Relative weight (liver weight/body weight) of rat during hepatocarcinogenesis treated with *Strobilanthes crispus*	40
3.	Number of rats with nodules in liver during induced hepatocarcinogenesis	44
4.	The ratio of acetone: resin during sample preparation for infiltration technique.	50
5.	The lesion score of portal area of the liver during induced hepatocarcinogenesis in rats treated with *S.crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction	54
6.	The lesion score of lobular area of the liver during induced hepatocarcinogenesis in rats treated with *S.crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction	56
7.	The stages of fibrosis of liver during induced hepatocarcino-genesis in rats treated with *S.crispus* and glycyrrhizin at 2, 4, 10 and 12 weeks post induction	58
8.	Scoring of antioxidant elements distribution using EFTEM	66
9.	Mean score of calcium distribution in liver during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus* by EFTEM	68
10.	Mean score of potassium distribution in liver during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus* by EFTEM	70
11.	Mean score of magnesium distribution in liver during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus* by EFTEM	72

12.	Mean score of iron distribution in liver during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus* by EFTEM	74
13.	Percentage distribution of magnesium in rat liver during induced hepatocarcinogenesis	89
14.	Percentage distribution of potassium in rat liver during induced hepatocarcinogenesis	91
15.	The activity of aniline hydroxylase (ANH) (nmol *p*-aminophenol liberated/mg protein/min) during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus*	99
16.	The activity of glutathione s-transferase (GST) (nmol/min/mg protein) during induced hepatocarcinogenesis in rats treated with *Strobilanthes crispus*	102

LIST OF FIGURES

Figure		Page
1.	The chemical structure of diethylnitrosamine	6
2.	Tumor promoters and progressors	12
3.	The mechanisms of cancer chemoprevention by antioxidants	20
4.	Major sources of free radicals in the body and consequence of Free radical damage	22
5.	The role of Phase I and Phase II enzymes in carcinogen metabolism	29

LIST OF PLATES

Plate		Page
1.	The features of *Strobilanthes crispus* leaves	15
2.	The gross view of liver surface which treated with DEN/AAF	44
3.	The gross view of DEN/AAF induced liver surface after treated with *Strobilanthes cripus*	45
4.	Light micrograph of the portal tract surrounded by severe necrotic cells (score +4)	59
5.	Light micrograph of bridging necrotic cells between portal tracts (score +4)	59
6.	Light micrograph of septa fibrosis (score +3)	60
7.	Transmission electron micrograph of calcium distribution in liver section from rat induced with DEN/AAF	75
8.	Transmission electron micrograph of calcium distribution in liver section from rat induced with DEN/AAF and treated with *Strobilanthes crispus*	75
9.	Transmission electron micrograph of calcium distribution in liver section from normal rat	75
10.	Transmission electron micrograph of potassium distribution in liver section from rat induced with DEN/AAF	76
11.	Transmission electron micrograph of potassium distribution in liver section from rat induced with DEN/AAF and treated with *Strobilanthes crispus*	76
12.	Transmission electron micrograph of potassium distribution in liver section of normal rat	76
13.	Transmission electron micrograph of magnesium distribution in liver section from rat induced with DEN/AAF	77
14.	Transmission electron micrograph of magnesium distribution in liver section from rat induced with DEN/AAF and treated	

with *Strobilanthes crispus* 77

15. Transmission electron micrograph of magnesium distribution in liver section from normal rat 77

16. Transmission electron micrograph of iron distribution in liver section from rat induced with DEN/AAF 78

17. Transmission electron micrograph of potassium distribution in liver section from rat induced with DEN/AAF and treated with *Strobilanthes crispus* 78

18. Transmission electron micrograph of potassium distribution in liver section of normal rat 78

LIST OF ABBREVIATIONS

HCC	hepatocellular carcinoma
DEN	diethylnitrosamine
AAF	acetylaminofluorene
PCD	programmed cell death
WHO	World Health Organization
DMBA	7,12-di-methylbenz[a]anthracene
EGCG	epigallocatechin gallate
AFB	aflatoxin B1
TLC	thin layer chromatography
UV	ultraviolet
ROS	reactive oxygen species
BHA	butylated hydroxyanisole
BHT	butylated hydroxytoluene
ATP	adenine triphosphate
NF	nuclear factor
SARS	severe acute respiratory syndrome
HIV	human immunodeficiency virus
PTH	parathyroid hormone
EELS	electron energy loss spectroscopy
rpm	revolution per minute
DNA	deoxyribonucleic acid
TEM	transmission electron microscope
EFTEM	energy filter transmission electron microscope
VPSEM	variable pressure scanning electron microscope
SEM	scanning electron microscope
ESEM	environmental scanning electron microscope
EDX	energy dispersive x-ray
NADPH	reduced nicotinamide adenine dinucleotide phosphate
ANH	aniline hydroxylase
GST	glutathione s-transferase
GSH	glutathione
GGT	γ-glutamyl transpeptidase
ALP	alkaline phosphate
GPT	glutamate pyruvate transaminase
RDA	recommended daily allowance
ANOVA	analysis of variance
w/v	weight/volume
e	electron
·OH	hydroxyl
OH	hydroxide

CHAPTER 1

INTRODUCTION

Around 10 million new cancer patients are diagnosed worldwide each year and these rates will increase to 20 million by the year 2020 (Sikora, 1999). In 1995, malignant neoplasm is the major cause of death (45%) in Government Hospitals of Malaysia, which is 2.8 times higher than that of heart disease (16%) (Malaysia's Ministry of Health, 1995). Thus, despite advances in early detection and treatment, overall death rates from cancer have remained largely unchanged since the early 1970s, suggesting the need for a stronger research focus on prevention (Nestle, 1997).

Hepatocellular carcinoma is a cancer arising from the liver. Although the liver is made up of different cell types (e.g. Kupffer cells, hepatocytes, endothelial cells, sinusoidal and fat-storing cells) but the majority of primary liver cancers (over 90 to 95%) arises from hepatocytes and is called hepatocellular carcinoma (Fong, 2002). Hepatocarcinogenesis is a transformation process of normal liver cell into a cancerous cell which involved initiation, promotion and progression stages (Lai and Peter, 1999).

More than 3, 000 plant species have been used as anticancer agents (Lewis and Elvin-Lewis, 1977). This subject has received a lot of attention from both consumers and scientists during recent decades (Poppel and Berg, 1997). A lot of studies reported that various kinds of fruits, green and yellow vegetables, particularly cruciferous vegetables

have their anticancer activity towards human (Kusamran *et al*., 1998). In Malaysia, about 1200 plant species have been reported to have potential pharmaceutical value and some of these have been used as herbal medicine (Soepadmo, 1991). One example of medicinal plant found in Malaysia and Indonesia which has pharmacological potential is *Strobilanthes crispus*. In Indonesia, this plant has been used as antidiabetic, diuretic, antilytic and laxative agent (Sunarto, 1977).

In this study, the anticancer effect of *Strobilanthes crispus* was investigated, *in vivo*. The anticarcinogenic effect of 5% (w/v) *Strobilanthes crispus* during hepatocarcinogenesis has been reported by Elizabeth, (1999) and at this concentration, the extract was found to be very effective in treating hepatocarcinogenesis. Another study by Suherman *et al*. (2004) who tested 1%, 2.5%, 5%, 7.5% and 10% doses of *Strobilanthes crispus* extract on rats during hepatocarcinogenesis reported that among the doses, 5% (w/v) is optimum for supplementation

Thus, in this present study, 5% (w/v) *Strobilanthes crispus* crude extract was chosen to treat DEN/AAF induced hepatocarcinogenesis. Atomic absorption spectroscopy (AAS) was not chosen to analyze the elements in this present study because in a study by Maznah *et al*. (2000), AAS has been used to analyze elements in *Strobilanthes crispus* crude extract. Thus in this study, EFTEM and VPSEM was chosen because this electron microscopes can analyze the antioxidant element at ultracellular level in liver tissue and no previous study has been carried out using these hi-technology equipments so far. While, gas chromatography mass spectroscopy (GCMS) also was not chosen because this