UNIVERSITI PUTRA MALAYSIA

THE EFFECT OF ALOE VERA, VITAMIN C AND E ON TUMOUR MARKER ENZYMES IN HEPATOCARCINOGENESIS

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By

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LIST OF ABBREVIATIONS

AAF	2-Acetylaminofluorene
ALP	Alkaline phosphatase
BSA	Bovine Serum Albumin
DEN	Diethylnitrosamine
DMBA	7,12-dimetylben(a)antracene
DMN	Dimetylnitrosamine
GGT	Gamma-glutamyl transferase
GST	Glutathione-S-transferase
GSH	Reduced glutathione
GPx	Glutathione peroxidase
GRx	Glutathione reductase
NADH	Reduced nicotinamide adenine dinucleotide
NDEA	N-nitrosodietylamine
UDPGT	Uridyl diphosphoglucuronyl transferase
UV	Ultra violet
e.u.	enzymes per unit
C	Control
AV	*Aloe vera*
VC	Vitamin C
E	γ-Tocotrienol
T	α-Tocopherol
CPH	Control rats with partial hepactetomy
DA	DEN + AAF
DAV	DEN + AAF + *Aloe vera*
DVC	DEN + AAF + Vitamin C
DE	DEN + AAF + γ-Tocotrienol
DT	DEN + AAF + α-Tocopherol
DEAV	DEN + AAF + γ-Tocotrienol + *Aloe vera*
DEVC	DEN + AAF + γ-Tocotrienol + Vitamin C
DTAV	DEN + AAF + α-Tocopherol + *Aloe vera*
DTVC	DEN + AAF + α-Tocopherol + Vitamin C

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

THE EFFECT OF *ALOE VERA*, VITAMIN C AND E ON TUMOUR MARKER ENZYMES IN HEPATOCARCINOGENESIS.

By

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February 1998

Chairman: Associate Professor Nor Aripin Shamaan, Ph.D.

Faculty: Science and Environmental Studies

The effects of *Aloe vera* (AV), vitamin C (VC), alpha-tocopherol (T) and gamma-tocotrienol (E) on the activities of six tumour marker enzymes; alkaline phosphatase (ALP), glutathione-S-transferase (GST), gamma-glutamyl transferase (GGT), glutathione peroxidase (GPx), glutathione reductase (GRx) and uridyl diphosphoglucuronyl transferase (UDPGT) in rat liver carcinogenesis were studied. Liver cancer was induced by diethylnitrosamine (DEN) and 2-acetylaminofluorene (AAF) followed by partial hepatectomy. Ninety male rats (*Rattus norwegicus*, 120-150g, 6-7 weeks old) were divided into 15 groups. Six groups comprised the normal and supplemented-control groups. Hepatocarcinogenesis was induced in the other nine groups. Four groups were supplemented with AV, VC, E and T respectively and the other 4 groups were given T/VC, T/AV, E/VC and E/AV respectively. The last group remained as the cancer-control group.

Aloe vera and vitamin C were administered *ad libitum* at doses of 0.1 mg/L water and 41.7 mg/L water respectively, in both control and cancer groups. Alpha-tocopherol and gamma-tocotrienol were administered at a dose of 34 mg/kg diet and 30 mg/kg diet, respectively. The rats were sacrificed by cervical dislocation after 16 weeks.

An increase ($p<0.05$) in all six tumour marker enzymes was observed in the cancer-treated group compared to the normal-control group. *Aloe vera* supplementation significantly ($p<0.05$) decreased the tumour marker enzyme activities in cancer-induced liver compared to the cancer-control group. Gamma-tocotrienol exerted a better effect than alpha-tocopherol in reducing the enzyme activities in cancer-induced liver. Vitamin C significantly ($p<0.05$) decreased the enzyme activities in GPx, GRx, GST and UDPGT. However, non of the supplementations decreased the alkaline phosphatase activity in the liver cancer.

In the four cancer-induced groups that received combined supplementation, the tumour enzyme marker activities were greatly reduced ($p<0.05$) compared to the single supplementation. A combined supplementation of alpha-tocopherol and vitamin C gave the best result compared to other combinations.

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

KESAN *ALOE VERA*, VITAMIN C DAN E KE ATAS AKTIVITI ENZIM PENANDA TUMOR DALAM KANSER HEPAR.

Oleh

ABDAH MD AKIM

FEBRUARI 1998

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Fakulti: Sains dan Pengajian Alam Sekitar

Kesan *Aloe vera* (AV), vitamin C (VC), alfa-tokoferol (T) dan gama-tokotrienol (E) ke atas aktiviti enzim penanda tumor; alkaline fosfatase (ALP), glutation-S-transferase (GST), gama-glutamiltransferase (GGT), glutation perosidase (GPx), glutation reduktase (GRx) dan uridil difosfoglukuronil transferase (UDPGT) di dalam tikus teraruh hepatokarsinogenesis telah dikaji. Aruhan kanser ialah melalui pemberian dietilnitrosamina (DEN) dan 0.02% 2-asetilaminofluorena (AAF) diikuti hepatektomi separa. 90 tikus albino jantan (*Rattus nowegicus*, 120-150g, berusia 6-7 minggu) telah dibahagikan kepada 15 kumpulan. 6 kumpulan adalah untuk kawalan normal. Hepatokarsinogenesis telah diaruh di dalam 9 kumpulan di mana 4 kumpulan diberi AV, VC, E dan T dan 4 kumpulan lagi diberi T/VC, T/AV, E/VC dan E/AV. Kumpulan terakhir adalah kawalan kanser. *Aloe vera* dan vitamin

C diberi dengan dos 0.1 mg/L air dan 41.7 mg/L air secara *ad libitum*. Alfa-tokoferol dan gama-tokotrienol diberi pada dos 34 mg/kg diet dan 30 mg/kg diet. Semua tikus dibunuh secara dislokasi servikal selepas 16 minggu.

Kesemua aktiviti penanda tumor enzim didapati meningkat ($p<0.05$) pada hepar kanser berbanding dengan kumpulan kawalan. Pada hepar kanser, *Aloe vera* didapati mengurangkan dengan signifikan ($p<0.05$) semua aktiviti enzim kecuali aktiviti alkalin fosfatase. Gama-tokotrienol menunjukkan kesan yang lebih ketara daripada alfa-tokoferol dalam mengurangkan aktiviti enzim pada hepar kanser. Vitamin C mengurangkan ($p<0.05$) aktiviti enzim GPx, GRx, GST dan UDPGT.

Kesemua empat kumpulan yang menerima suplementasi berkombinasi mengurangkan ($p<0.05$) semua aktiviti enzim dengan lebih ketara berbanding dengan kumpulan yang menerima hanya suplementasi tunggal. Kombinasi alfa-tokoferol dan vitamin C memberi kesan perlindungan yang paling baik berbanding dengan kombinasi lain.

CHAPTER 1

INTRODUCTION

In some parts of the world especially tropical Africa and parts of Asia, primary cancer of the liver is one of the commonest forms of malignant tumour. In the Western world, alcohol is the most recognised cause whereas in Asia, the possible role of chemical carcinogens such as aflatoxin has been identified (Alfin-Slater and Kritchevsky, 1991). Diagnosis is difficult since there is no specific symptoms; in all cases, there is enlargement of the liver. Chemotherapy and radiotherapy has little effect to help alleviate the situation and removal of the affected part of the liver may be feasible.

As the familiar proverb "prevention is better than cure", the prevention of this disease is obviously the aim of all those who are concerned with health. A number of studies had been done on various putative anticarcinogenic substances, including the supplementation used in this study; *Aloe vera*, vitamin C, α-tocopherol and γ-tocotrienol.

Aloe vera, which is commercially used in skin care product, has only been tested recently for its anticarcinogenic properties. Although vitamin C has been long known to be a potential anticarcinogen, it gains less interest in this

subject because it is more commonly used in preventing scurvy and cold. The effect of two types of vitamin E; α-tocopherol and γ-tocotrienol, which are purified from palm oil, on liver cancer had been extensively studied where γ-tocotrienol possesses a higher anti-cancer property than α-tocopherol (Ong et al, 1994). There had been individual studies on the effect of each supplementation previously (Beattie and Sherlocks, 1976). Therefore, this study aims to examine the simultaneous effect of the supplementations.

Measuring the known tumour marker enzymes; alkaline phosphatase (ALP), uridyl diphosphoglucuronyl transferase (UDPGT), gamma glutamyl transferase (GGT), glutathione-S-transferase (GST), glutathione reductase (GRx) and glutathione peroxidase (GPx) will obviate the effect of these supplementations. Each tumour marker enzyme used was known to increase its activity in cancer-induced liver. However, liver ALP is not being used as extensively as the plasma ALP in detecting cancer whereas GST and GGT are now the most used as markers. GPx and GRx are involved in the glutathione redox cycle, which is the major endogenous protective system against radical species.

Details on liver cancer, possible anticarcinogenic properties of *Aloe vera*, vitamin C, α-tocopherol and γ-tocotrienol and the tumour marker enzymes were introduced in the literature review. The hypotheses that vitamin C and E act as anticarcinogens via their antioxidant properties with the elevation of the tumour marker enzymes was tested. Therefore, the objective of this research was to evaluate the activities of the tumour marker enzymes in *Aloe vera*, vitamin C, α-tocopherol and γ-tocotrienol-supplemented as well as their combination in liver cancer.

CHAPTER 2

LITERATURE REVIEW

Cancer in General.

Cancer may be considered as an alteration of the control mechanisms involved in cell division and differentiation. It is a multi-step process which involves chemical modification of cellular or genomic DNA (Arcos et al, 1961). Once modified, the affected DNA is replicated and transcribed differently from the normal DNA. This hereditary transmittable modification (from neoplastic cells to daughter cells) leads to a permanent and anarchic multiplication of transformed cells which may be different from that of the normal cells. The carcinogenic process may be initiated by several agents called initiators. Once carcinogenesis is initiated; the second step, termed "promotion", may continue and finally the third stage of the process, called "progression", occurs. Carcinogens may act as initiators, promoters or a combination of both (Boyland and Sydnor, 1963).

Liver Cancer.

Hepatocellular carcinoma is a common cancer in sub-Saharan Africa and parts of Southeast Asia where liver cancer may account for 5-8% of all

deaths. The risk of getting hepatoma is high in Asia and is more common in men compared to women (Laidlaw and Swendseid, 1991). Liver cancer is rare in western countries where it accounts for only 2% of cancer cases in the United States annually.

The first type of liver cancer is primary liver cancer. Secondary cancer occurs when the liver becomes the seat of secondary deposits of cancers arising primarily in other organs. Metastases in the liver often lead to enlargement of the organ with discomfort or pain below the ribs on the right and in the flank. There may be jaundice arising from interference with the drainage of bile. Other symptoms of liver disease such as irritation of the skin, twitching and drowsiness could be detected (Scott, 1979).

Two forms of liver cancer have been distinguished. The first form is called hepatoma, which originates in the cells of the liver cortex and forms ninety percent of primary liver cancer. The second is cholangiomas, which line the hepatic ducts that drain the bile from the liver and forms 5-10 percent of liver cancer (Manahan, 1989). Rapid enlargement of the liver can also be diagnosed as cancer, though not necessarily so. Others are pains under the ribs on the right side, loss of weight and jaundice. Biopsy of the liver will confirm the precise diagnosis. Treatment such as chemotherapy and radiotherapy is of little help and at present, liver transplantation has been successful and considered as one of the best treatment available.

Causes of Liver Cancer.

The reason for the frequent occurrence of liver cancer is because the liver is often the main target for chemically induced toxicities by carcinogens. Its susceptibility may be due to several factors; firstly, the liver is the primary site for

biotransformation of xenobiotics. Secondly, the liver is the first major site of metabolism for carcinogens absorbed from the gastrointestinal tract, which is the major route of absorption for most xenobiotics (Boyland and Sydnor, 1963).

Furthermore, the liver is strategically located between the intestinal tract and the general circulation. Through the portal vein, the liver receives a large volume of venous blood from the digestive tract while it drains blood through the hepatic veins. Compounds absorbed from the intestines, including products of digestion as well as toxicants pass through the liver to be metabolised. This renders it to be particularly vulnerable to the toxic effects of metabolites (Hodgson and Levi, 1994). Specific hepatocarcinogens that have been identified are alcohol, safrol, pyrrolizidine alkaloids, cycasin, aflatoxin and several steroid hormones.

Cancer is the second leading cause of death after heart disease. From epidemiological studies, about 35% of cancer is related to diet (Table 1, Hodgson and Levi, 1994). The diet probably accounts directly for liver cancer. Carcinogens, both natural and synthetic may affect hormone levels, causing other cancers, e.g., chemicals in food components that alter oestrogen level in the breast and endometrium may cause breast and uterine cancer whereas alteration in testosterone level can cause prostate cancer.

Although the effect of dietary protein on hepatic tumour development has been shown in several animal models (Emmelot and Muhlbock, 1964), no human epidemiological studies has shown the associations between protein intake and liver cancer. The hypothesis that liver cancer was related directly to kwashiorkor (protein-calorie malnutrition) has now been discarded since the discovery of aflatoxin in contaminated foods in Africa and Asia. Although aflatoxin ingestion has been correlated closely with liver cancer (Alfm-Slater and Kritchevsky, 1991), the

hepatitis B virus also appears to be an important cofactor with carriers having a 200-fold higher risk compared to non-carriers in Taiwan (Becker, 1975). Prevention is possible through a reduction or elimination of aflatoxin in the diet and vaccination against the hepatitis B virus; now being undertaken in Gambia (Brennan, 1977).

Table 1 Causes of Cancer.

Factors involved in cancer	
Major Factors	**Best Estimates**
Diet	35%
Tobacco	30%
Reproductive and sexual behavior	7%
Viruses	5%
Occupation	4%
Geophysical Factors	3%
Alcohol	3%
Pollution	2%
Food Additives	1%
Medicines	1%
Industrial Products	1%
Unknown	?

(Source taken from Hodgson and Levi, 1994).

The major factor in liver cancer in North America and Europe appears to be caused by excessive alcohol ingestion (Greenberg and Harper, 1960). A threshold effect has been suggested where liver cancer occurs only when the level of ingestion is sufficient to cause alcoholic hepatitis and consequent cirrhosis (Harris, 1991). It

could be concluded that excessive alcohol consumption is the major factor in Western countries and North America for cancer of the liver.

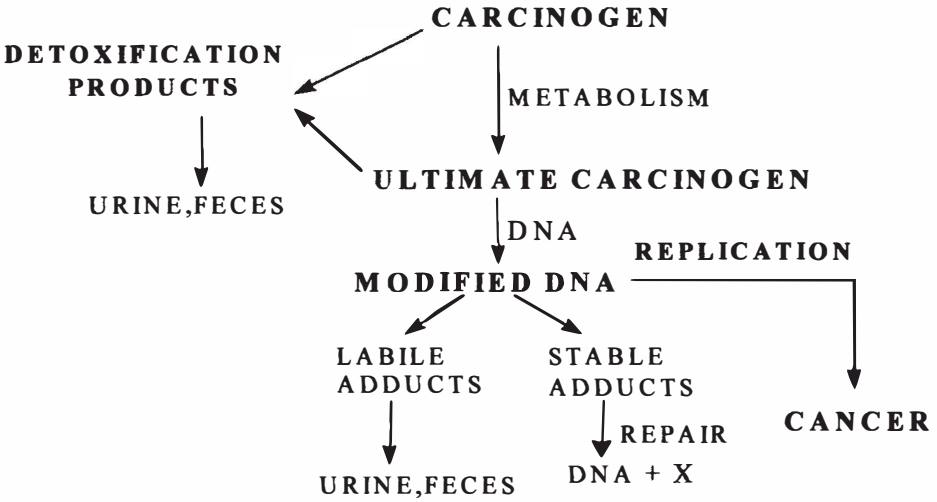

Figure 1 Pathway of carcinogen leading to cancer.
(Source taken from Hodgson and Levi, 1994).

Multistage Carcinogenesis.

From animal and human studies, it is clear that carcinogenesis is a multistage process (Harris, 1991). Carcinogenesis can be divided into at least three stages termed initiation, promotion and progression (Figure 2). Generally, it is thought that initiation is a genotoxic event, one in which the primary sequence of DNA is altered while promotion is considered a non-genotoxic or epigenetic event (Dragan and Pitot, 1992).

Initiation involves the exposure of normal cells to chemical or physical carcinogens, which results in a permanent heritable change in the cell's genome. The affected cell is called the initiated cell. The initiated cell may have a decreased

response to the inter- and intracellular signals that maintain normal tissue architecture and homeostatic growth (Harris, 1991). The initiated cells may remain dormant until exposed to a tumour promoter.

Tumour promotion results in the proliferation of initiated cells and enhancement of genetic damage. The development of malignancy from a benign tumour encompasses the third step of carcinogenesis termed progression. It involves the survival and proliferation of cancer cells and the process of maintenance and evolution of malignancy.

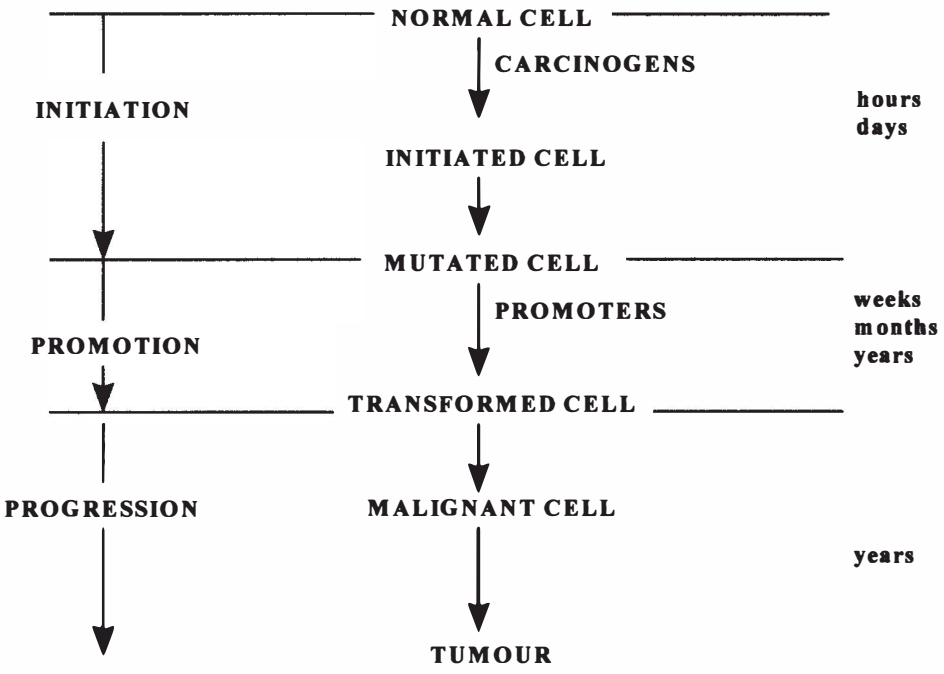

Figure 2 Multistage of cancer. (Source taken from Voiculet et al, 1991).

Carcinogen Metabolism.

Electrophilic Theory

In the metabolism of chemical carcinogens, the electrophilic theory states that carcinogens are metabolically activated to electrophilic metabolites that are capable of interacting with nucleophilic sites in DNA, RNA and protein (Iversen, 1988). Most of the chemical carcinogens are electrophilic reactants. The formation of electrophiles generally involves a two-electron oxidation to a carbonium ion catalysed by a cytochrome P_{450}-dependent mixed function oxidase. Most of the radicals formed probably do not react with DNA. There are also compounds which do not form electrophiles but undergo biotransformation and produce neoplasm, for example saccharin and nitrilotriacetic acid. From this observation, two classes of chemical carcinogens are distinguishable; genotoxic and epigenetic compounds (Iversen, 1988).

Classes of Chemical Carcinogens.

Chemical carcinogens can be divided into two major categories; genotoxic agents which produce alterations in the genetic material of the host (e.g. DEN) and epigenetic (nongenotoxic) agents which do not alter the primary sequence of DNA (for e.g. saccharin). The genotoxic agents possess initiator activity whereas the epigenetic compounds possess promoter activity (Hodgson and Levi, 1994).

Genotoxic agents are further sub-divided into four categories (Iversen, 1988):

Direct-acting carcinogens which do not require metabolic activation to interact with DNA (e.g. beta propiolactone).

Indirect-acting carcinogens which require metabolic activation (e.g. DEN).

Radiation which directly ionise DNA.

Inorganic agents such as arsenic which interfere with the enzymes associated with DNA replication.

Terms in Metabolic Activation.

The terms parent, proximate and ultimate carcinogen were developed to differentiate the action of carcinogens. A parent carcinogen is a compound that must be metabolised in order to have carcinogenic activity. A proximate carcinogen is an intermediate metabolite requiring further metabolism steps resulting in the ultimate carcinogen which is the actual metabolite that covalently binds to the DNA (Nakahara et al, 1972). Diethylnitrosamine (DEN) and 2-Acetylaminofluorene (2-AAF) are examples of chemical carcinogens that require metabolic activation before they exert their effect. After metabolic activation, both DEN and 2-AAF bind to DNA to form DNA adducts. It is further suggested that if the DNA adduct is not repaired before the cell replicates, a mutation may be permanent in the daughter cell (Emmelot and Muhlbock, 1964).