ANTICANCER EFFECTS OF TUALANG HONEY ON 7,12-DIMETHYLBENZ(α)ANTHRACENE (DMBA)-INDUCED MAMMARY CARCINOMA IN RATS

ERAZULIANA BINTI ABD KADIR

Thesis submitted in fulfilment of the requirements for the degree of Master of Science

UNIVERSITI SAINS MALAYSIA

2011

ACKNOWLEDGEMENTS

First and foremost, great to Allah for his gracious blessings bestowed upon me for the completion of this thesis. I am most grateful to Associate Prof Dr. Siti Amrah Sulaiman for being a very generous, supportive and responsible supervisor throughout the years. Thank you for introducing me to the area of cancer research which I later found very interesting. My utmost gratitude to my co-supervisor, Prof. Nor Hayati Othman for all the attentions and guidance at any given time, especially with the pathology work. It is a pleasure to thank Dr. Nurul Khaiza Yahya from Department of Immunology for her honest view on the immunology part of the study.

It is a pleasure to thank those who made my job so much easier, especially to the staff in Pharmacology Lab; Mrs. Siti Alfatihah Fauzi for all the highly appreciated help with the laboratory animal work, and to the staff in Central Research Lab; Mr. Ahmad Kamaludin Muhamad for the help with the histology staining and Mr. Koh Chun Haw for all the other assistance needed in the laboratory. Not to forget Mr. Jamarrudin Mat Asan from Immunology Lab for his valuable help with the blood analysis. I would also like to thank all staffs in Laboratory Animal Research Unit USM; Mr. Nur Mohamad Makhatar for being so helpful throughout the years.

I am indebted to many of my colleagues and friends, who have always giving me support and willing to share the upside and downside of the postgraduate life. To my mother and my other family members, hug and kisses for all the understanding, love and support poured on me throughout my struggle with the project. Last but not least, my

deepest gratitude to USM Fellowship for the sponsorship and to everyone who had been involved in this project right from the beginning of the project until the completion of the thesis. Only Allah can repay all of your kindness.

Thank you and wassalam.

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LIST OF SYMBOLS AND ABBREVIATIONS

%	Percent
°C	Degree of Celcius
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
AB	Alveolar bud
ABW	Actual body weight
ADB	Assay diluent
AI	Apoptotic index
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CD4	Helper T cells
CD8	Cytotoxic T cells
cm	Centimeter
cm^3	Cubic centimeter
DAB	3,3'-diaminobenzidine
DCIS	Ductal carcinoma *in situ*
ddH_20	Double deionized water
DMBA	7,12-dimethylbenz(alpha)anthracene
DNA	Deoxyribonucleic acid
DPX	Synthetic resin
dUTP	2'-deoxyuridine 5'-triphosphate
EC	Endothelial cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FRAP	Ferric reducing ability of plasma
g	Gram

g	Gravitational acceleration
H&E	Hematoxylin and eosin
H_20_2	Hydrogen peroxide
HepG2	Human liver carcinoma cell line
HMF	5-(hydroxymethyl)-furfural
hpf	High power field
HRP	Horseradish peroxidise
IDP	Intraductal proliferation
IqR	Interquartile range
kg	Kilogram
kGy	KiloGray
LMW	Low molecular weight
Lob 1	Lobule type 1
Lob 2	Lobule type 2
Lob 3	Lobule type 3
Lob 4	Lobule type 4
M	Molar
MBT-2	Murine bladder carcinoma cells
MCF-7	Estrogen receptor positive breast cancer cell lines
MDA-MB-231	Estrogen receptor negative breast cancer cell lines
mg/kg	Milligram per kilogram
$MgCl_2$	Magnesium chloride
ml	Milliliter
mM	Millimolar
n	Sample size of animal
NaCl	Sodium chloride
ng/ml	Nanogram per millilitre
NK	Natural killer cells
nm	Nanometer
OD	Optical density
PAH	Polycyclic aromatic hydrocarbon

PBS	Phosphate buffered saline
pg/mg	Picogram per milligram
pg/ml	Picogram per milliliter
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
SD	Sprague-Dawley
SDB	Sample diluent buffer
SEM	Standard error of mean
TD	Terminal ductule
TDLU	Terminal ductal-lobular unit
TdT	Terminal deoxynucleotidyl transferase
TEB	Terminal end bud
TH	Tualang honey
THG	TH-treated groups
TMB	3,3', 5,5'-tetramethylbenzidine
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
TUNEL	Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
UK	United Kingdom
USA	Unites States of America
USM	Universiti Sains Malaysia
VEGF	Vascular endothelial growth factor
WB	Wash buffer
WBC	White blood cell
WR	Working reagent
π	Pi (3.142)

KESAN ANTIKANSER MADU TUALANG KE ATAS KARSINOMA KELENJAR MAMARI ARUHAN 7,12-DIMETHYLBENZ(α)ANTHRACENE (DMBA) DALAM TIKUS

ABSTRAK

Madu Tualang (TH) dilaporkan mempunyai kesan antiproliferatif dan mengaruhkan apoptosis terhadap beberapa jenis titisan sel kanser. Kajian telah dijalankan untuk mengkaji kesan madu Tualang terhadap pembentukan dan perkembangan karsinoma mamari aruhan 7,12-dimethylbenz(α)anthracene (DMBA) dalam tikus.

Satu kajian persediaan telah dijalankan untuk menetapkan dos optimum DMBA untuk aruhan kanser. Pemberian sebanyak satu dos 20 atau 25 mg DMBA kepada tikus betina berumur 45 hingga 48 hari dengan berat badan masing-masing kurang atau lebih daripada 120 g menghasilkan 50% insiden karsinoma. Seterusnya, 40 ekor tikus aruhan DMBA dibahagikan secara rawak mengikut blok kepada empat kumpulan; Kumpulan 1, 2, 3 dan 4 dan menerima 0 (kawalan), 0.2, 1.0 or 2.0 g/kg TH setiap hari bermula pada keesokan harinya sehingga 150 hari. Nodul tumor payudara yang terbentuk diperiksa dua kali seminggu dan berat badan diambil sekali seminggu. Pada hari ke-151, darah telah diambil untuk penentuan jumlah kiraan limfosit dan kemudiannya semua tikus dibunuh. Semasa autopsi, insiden, kriteria, jumlah, berat dan isipadu tumor telah ditentukan. Tumor kemudiannya diperiksa secara histopatologi berserta penentuan indeks apoptosis dan paras faktor pertumbuhan endotelium vascular.

Pemerhatian klinikal menunjukkan Kumpulan 2 mempunyai peratusan insiden tumor tertinggi (75%) manakala Kumpulan 4 yang terendah (42.9%). Kumpulan 4 juga mempunyai tempoh kependaman tumor paling pendek (Hari ke-101 ± 18) manakala Kumpulan 3 yang terpanjang (Hari ke-140 ± 4). Kumpulan yang menerima TH (THG) menunjukkan peratusan penambahan berat badan sebenar yang lebih rendah berbanding kawalan ($p = 0.012$). Tumor untuk THG mengalami penambahan saiz yang lebih lambat dan min yang lebih kecil (≤ 2 cm^3) berbanding kawalan (≤ 8 cm^3). Jumlah tumor semasa autopsi bagi THG adalah lebih sedikit (12, 5 dan 7 tumor untuk Kumpulan 2, 3 and 4) berbanding kawalan (14 tumor). Jumlah keseluruhan berat dan isipadu tumor per tikus juga lebih rendah bagi THG. Berat tumor untuk Kumpulan 2 dan isipadu tumor untuk Kumpulan 4 adalah dengan signifikannya lebih rendah berbanding kawalan (masing-masing $p = 0.048$ dan $p = 0.018$).

Pemeriksaan histolopatologi menunjukkan majoriti tumor kumpulan TH adalah bergred rendah (1 dan 2) berbanding kawalan (gred 3). Tumor yang dirawat TH menunjukkan kurang bilangan karsinoma duktus *in situ* (DCIS) dengan purata 4, 8 dan 9 DCIS bagi Kumpulan 2, 3 dan 4 manakala 14 DCIS untuk kawalan. Sel-sel kanser THG kelihatan lebih tersusun, bersaiz dan berbentuk yang lebih sekata dan mempunyai nuklei yang lebih padat. Indeks apoptosis (AI) menunjukkan corak peningkatan dengan pemberian dos TH yang lebih tinggi, manakala aras faktor pertumbuhan endotelium vaskular (VEGF) menunjukkan corak penurunan bagi kanser THG berbanding kawalan. Tiada perbezaan yang signifikan bagi jumlah kiraan limfosit di kalangan kumpulan tikus yang mempunyai kanser atau tiada kanser.

Kajian menunjukkan bahawa pemberian madu Tualang diberi bermula sehari selepas pemberian DMBA untuk tempoh selama 150 hari dengan positifnya mengubah perkembangan karsinoma mamari aruhan DMBA pada tikus. Maka, adalah dicadangkan supaya kajian terperinci mengenai penggunaan madu lebah Tualang sebagai agen profilaktik dan terapeutik bagi kanser payudara dikalangan manusia dilaksanakan.

ANTICANCER EFFECTS OF TUALANG HONEY ON 7,12-DIMETHYLBENZ(α)ANTHRACENE (DMBA)-INDUCED MAMMARY CARCINOMA IN RATS

ABSTRACT

Tualang honey (TH) has been reported to have antiproliferative effect and induces apoptosis in various cancer cell lines. The present study was conducted to evaluate the effects of TH on the initiation and progression of 7,12-dimethylbenz(α)anthracene (DMBA)-induced mammary carcinoma in rats.

A preliminary study was conducted to establish the optimum DMBA dose for cancer induction. Single administration of 20 or 25 mg DMBA to 45 to 48 days old female rats with bodyweight of less or more than 120 g respectively was able to induce 50% carcinoma incidence. Later, 40 DMBA induced–rats were block randomized into four groups; Group 1, 2, 3 and 4 and given 0 (control), 0.2, 1.0 or 2.0 g/kg/day of TH respectively starting the next day for 150 days. Breast tumor nodules were assessed twice weekly and body weight was measured once a week. On Day 151, blood was withdrawn for analysis of total lymphocyte count and later the animals were euthanized. During autopsy, the tumor incidence, criteria, amount, weight and volume were assessed. Tumors were then subjected for histopathological examination and determination of apoptosis index and vascular endothelial growth factor level.

Clinical observation revealed that Group 2 presented with the highest percentage of tumor incidence (75%) whereas Group 4 with the lowest (42.9%). Group 4 also had

the shortest tumor latency period (Day 101 ± 18) while Group 3 had the longest (Day 140 ± 4). TH-treated groups (THG) showed lower percentage of actual body weight gain than control ($p = 0.012$). Tumors in THG presented with slower size increment and smaller mean tumor size (≤ 2 cm^3) compared to control (≤ 8 cm^3). Number of tumors during autopsy in THG was lesser (12, 5 and 7 tumors for Group 2, 3 and 4 respectively) than control (14 tumors). Total tumor weight and volume per animal were also lower in THG. Tumor weight in Group 2 and tumor volume in Group 4 were significantly lower than control ($p = 0.048$ and $p = 0.018$ respectively).

Histopathological examinations showed majority of TH-treated tumors were of grade 1 and 2 compared to control (grade 3). TH-treated tumors had less ductal carcinoma *in situ* (DCIS) with average per tumor of 4, 8 and 9 DCIS for Group 2, 3 and 4 respectively whereas 14 DCIS for control. THG cancer cells appeared more orderly arranged, more uniform in size and shape and had denser nuclei. Apoptotic index (AI) was noted to be in elevated pattern towards treatment of higher TH dose, while vascular endothelial growth factor (VEGF) showed pattern of reduced level in THG cancers compared to control. No significant difference was noted in total lymphocyte count among groups of rats with or without cancer.

This study shows that TH administered starting a day after DMBA administration and continuously for 150 days was positively modulating the progression of DMBA-induced mammary carcinoma in rats. Hence, TH should be investigated further as prophylactic and therapeutic agent against breast cancer in human.

CHAPTER 1

INTRODUCTION

Breast cancer or mammary carcinoma is a type of disease caused by uncontrollable growth of ductular epithelium of the breast. The mass of cancerous cells forms a lump and could metastasize to other organs. The incidence of breast cancer in women is alarmingly high especially in developed countries. In Malaysia, breast cancer is the most frequently diagnosed cancer among women which represents 31.3 % of all female cancers from 2003 to 2005 (Lim *et al.*, 2008).

Early diagnosis and treatment of breast cancer is very important for a successful disease recovery. Nonetheless, the common scenario in Malaysia and several other developing countries is that the cancer patients abscond or present late for hospital treatment. The deterring factors are usually related to social and cultural perception of breast cancer, such as preference for noninvasive traditional or alternative therapy over modern treatment which could be related to fear for surgery, chemotherapy or radiotherapy, living in doubt or denial of the disease, or having financial problem (Sandelin *et al.*, 2002, Hisham and Yip, 2004, Clegg-Lamptey *et al.*, 2009). Often, by the time the patients seek hospital treatment, the cancer is already at advanced stage of III or IV which is difficult to cure.

Therefore, one of the best options to solve the problem is to prevent the disease from occurring. For that matter, it is important to determine the factors that could contribute to the prevention of breast cancer initiation and development. Chemoprevention of cancer involves the use of natural or synthetic chemicals to arrest or reverse the carcinogenic process (Tsao *et al.*, 2004). One of the experimental approaches in search of new chemopreventive agent for breast cancer is by assessing the effect of the test substance on chemically-induced mammary carcinoma in an animal model (*in vivo)*. It is a suitable model to study the potential new therapeutic agent in a wide-scope view involving physiological processes in a living body. DMBA which is the short form for 7, 12-dimethylbenz(α)anthracene has been commonly used to induce mammary carcinoma in rats for testing of various potential substances in preventing or curing breast cancer.

Honey has been known for centuries for its medicinal value and health promoting properties. This notion was supported by various religious beliefs. Back in ancient times, Egyptians, Assyrians, Chinese, Romans and Greeks have been known to use honey for the treatment of wounds and diseases in guts (Zumla and Lulat, 1989). In Islam, as stated in the Holy Qur'an, Surah Al-Nahl (The Bee), verses 68 to 69, honey has been emphasized as medicine for various kind of diseases of mankind. For that reason, a type of Malaysia's own local honey called Tualang honey (TH) has been chosen for the study and is aimed to discover its potential in prevention of breast cancer development in animal model.

Recent *in vitro* studies have shown that TH possesses significant anticancer effect against breast and cervical cancer (Fauzi *et al.*, 2010) and antiproliferative effect

against oral cancer (Ghashm *et al.*, 2010). TH is also known to possess strong antioxidant activity (Mohamed *et al.*, 2010) which could largely contribute to the prevention of breast cancer development. Therefore, effects of TH administration on the tumor growth characteristics were evaluated in the present study. Among the parameters included in the assessment were morphological features and tumor grades. Histological grading was performed as it is an important prognostic factor for the disease (Ignatiadis and Sotiriou, 2008). It has been reported that mammary carcinomas in rats share similar features with the adenocarcinomas in human (Russo and Russo, 2000, Singh *et al.*, 2000)

The effects of TH administration on the apoptotic and angiogenesis activity of the mammary tumor were also evaluated. Apoptosis or "programmed cell death" is a process of removing damaged cells without disturbing normal cells. Apoptotic activity is a good indicator to assess the efficacy of TH which could be used as a target in the cancer treatment. Angiogenesis is responsible for the formation of new blood vessel from pre-existing vascularization in order to support the tumor growth and metastases (Harris, 2003). An agent with anti-angiogenic property could play a role in suppressing the tumor development. For that matter, an angiogenic growth factor known as vascular endothelial growth factor (VEGF) which could be detected in breast cancer (Harris *et al.*, 1996) was measured quantitatively in order to assess the aggressiveness of the tumor.

Neoplastic cells occurrence somehow could be explained by suppressed, inadequate or malformed state of spontaneous immune response, modifying the normal T and B lymphocytes roles to fight against the abnormal cells formation and

development. Honey is however proven to cause an increase in proliferation of both B and T lymphocytes *in vitro* (Abuharfeil *et al.*, 1999). This gives a good opportunity to test if TH has the same positive effect in *in vivo* study in a way to improve the immune defense system by destroying and/or eliminating the tumor cells. Assessment of circulating lymphocytes in the peripheral blood is a good parameter as it could represent capability of mammary cancer-induced subjects to produce immunity cells from the effect of TH administration.

1.1 Objectives

1.1.1 General objective

The general objective of this study was to evaluate the efficacy of Tualang honey in preventing the initiation and progression of DMBA-induced mammary carcinoma in rats.

1.1.2 Specific objectives

a) To study the effect of TH administration on the incidence, latency, multiplicity, weight and volume of DMBA-induced tumors in rats.

b) To determine the effect of TH administration on the grades and morphology of DMBA-induced breast carcinomas in rats.

c) To determine the effect of TH administration on apoptotic cell death in DMBA-induced breast carcinomas in rats.

d) To determine the effect of TH administration on rat's vascular endothelial growth factor (VEGF) in DMBA-induced breast carcinomas in rats.

e) To determine the effect of TH administration on the total lymphocyte count of rats induced with DMBA.

1.2 Significance of research

This study could provide information on the benefits of Tualang honey in preventing breast tumor formation and development. Further study could be done to identify the effective compound in TH that contributes to its beneficial effects.

1.3 Hypothesis

Tualang honey is capable of preventing the initiation and progression of DMBA-induced mammary carcinoma in rats.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

2.1.1 Background

In general, cancer is defined as a group of abnormal cells that grow and divide without control. Originated from the breast tissue area, breast cancer or mammary carcinoma which usually occur in women resulted from the new formation of cells (neoplasm) that are most commonly from ducts and lobules area which undergo rapid transformation and growth and thus eventually causing the formation of lump or mass, known as tumor (Damjanov, 2006). Tumors can be benign or malignant. Benign tumors can be identified based on characteristics such as localized growth and no distant spread i.e. metastasis to other organs. On the other hand, malignant tumors, known as cancer involve cells infiltrate or metastasis to other parts of the body such as to lymph nodes, bones, liver, lungs and brain via blood vessels and lymphatic channels (Crowley, 2007). Metastasis can compromise the normal function of the involved tissue or structure.

2.1.2 Prevalence

Breast cancer as the commonest type of cancer among women of all races in Malaysia was represented by age-standardized rate of 47.4 per 100,000 women compared to the incidence of breast cancer in men of only 1.2 per 100,000 men. According to the same source which was mainly based on cases in Peninsular Malaysia from 2003 to 2005, the incidence was highest among women of age between 50 to 60 years old, except for Indians which has the highest incidence among women aged above 60 years old. However, the incidence of breast cancer among women in Malaysia is still lower than most Western countries such as USA, Canada, England, Netherland and Denmark (Lim *et al.*, 2008).

2.1.3 Risk factor

Breast cancer is believed to be caused by a single or multiple endogenous and exogenous factors. The chances of getting breast cancer is higher in older age women especially those reaching the age of menopause which is of 50 years old and above (Marchant, 1982). The risk is higher for women with earlier age of her first menstrual period (menarche) especially before the age of 12, experienced menopause after the age of 55, never had or late in having a full-term pregnancy which is approximately after the age of 35, never breast fed or having larger area of dense mammary tissue appeared on the mammogram assessment (Hsieh *et al.*, 1990, Kelsey *et al.*, 1993, Boyd *et al.*, 2002). The risk is also higher if there is any immediate family member or first degree relatives including mother, sister or daughter had breast cancer, especially if they had the disease

before the age of 50 (Colditz *et al.*, 1996). The same risk also implies if they had history of ovarian cancer (McPherson *et al.*, 2000).

Other than that, personal health history of having benign breast lump or certain types of abnormal breast disease such as atypical hyperplasia also increases the risk of breast cancer (London *et al.*, 1992). Women who had a history of breast cancer in one breast have a risk of developing cancer in the other breast (Helmrich *et al.*, 1983). Detection of gene mutation on breast cancer-related genes such as breast cancer susceptibility gene 1 (BRCA1), breast cancer susceptibility gene 2 (BRCA2) and tumor suppressor gene p53 in the genetic make-up substantially increases the chance of getting breast cancer (Martin and Weber, 2000). Other exogenous factors that increase the risk of breast cancer include long term treatment of oral contraceptive pills and menopausal hormone replacement therapy (Pike *et al.*, 1993), undergone radiation therapy to the chest before the age of 30, being overweight or obese after menopause and last but not least, practicing unhealthy lifestyle with lack of physical activity and excessive consumption of high-fat, low fiber diet and alcohol beverages (Howe *et al.*, 1990).

2.1.4 Structure of the human breast

The anatomy of a normal woman's breast is comprised of two breasts on the anterior chest wall. Each breast is structured by 15 to 20 lobes that each has smaller sections of lobules. Lobules contain milk-producing glands and ducts (lactiferous duct) that connect the lobules to the nipple as a passage for breast milk deliverance. Fatty tissue and fibrous connective tissues (stroma) that contain blood capillaries and nerves,

surround both lobule and ductal units (Figure 2.1). Breasts are also surrounded by a network of lymph vessels that drains into lymph nodes at the chest wall and underarm (axilla) area (Ganshow *et al.*, 2004).

The lobular structure is comprised of ducts that are divided into club-shaped terminal end buds and these buds later cleave into two smaller structures of alveolar buds. A cluster of alveolar buds that surrounds the terminal ducts forms the lobule type 1 (Lob 1) or also known as terminal ductal-lobular unit (TDLU) or virginal lobule. TDLU is the functional unit of human mammary gland and it contains abundance of highly proliferative stem cells population (Russo and Russo, 1978). The other type of lobules, namely lobule type 2 (Lob 2) and type 3 (Lob 3) are the gradual sprouting of the alveolar buds, which are also called as ductules. Progressive branching of Lob 2 and 3 from Lob 1 makes them more developed and differentiated (Figure 2.2). Lobule type 4 (Lob 4) which is present during lactational period of the mammary gland is the most differentiated lobule structure. In nulliparous women, more undifferentiated structure of lobule type 1 and terminal ducts are found; which will remain throughout their nulliparous life with only scarce appearance of Lob 2 and 3 and complete absence of Lob 4. On the other hand, breast of parous women are predominated with fully developed and differentiated Lob 3 since the breast lobules reached its maximum development during pregnancy after a rapid progression from Lob 1 to Lob 2 and finally to Lob 3 (Russo and Russo, 2004).

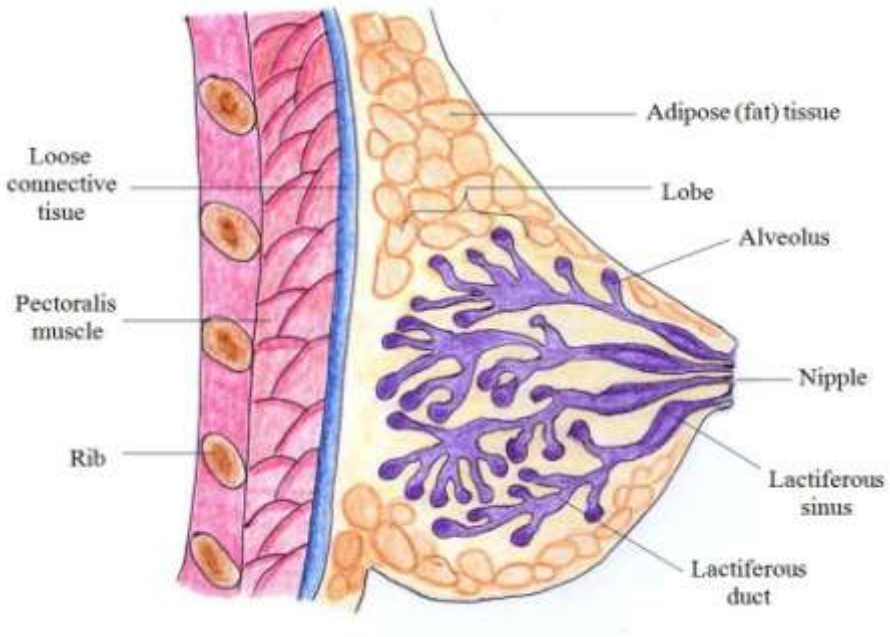

Figure 2.1: **Anatomy of a normal human breast**. The figure is illustrated in cross-sectional view as adapted from Ali and Coombes (2002).

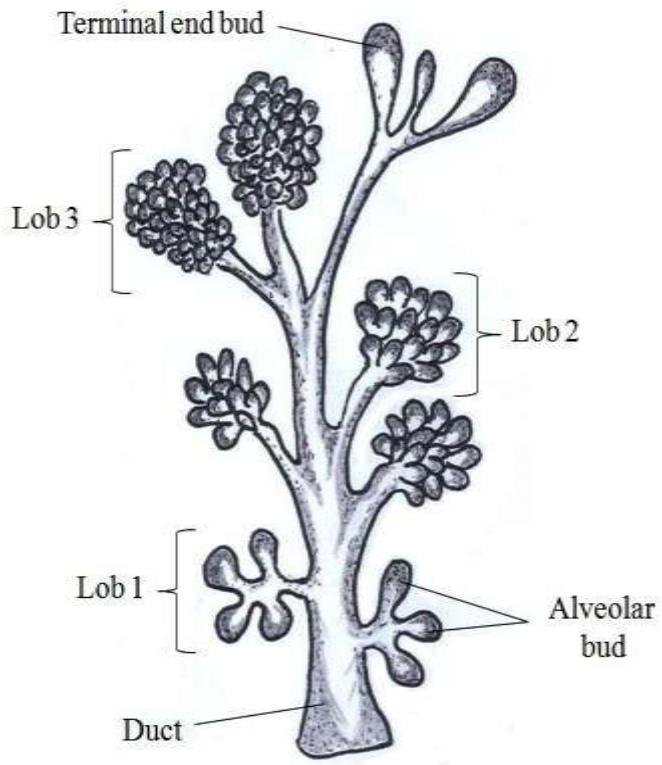

Figure 2.2: **Lobular structure in human breast.** This diagram of lobular structure is showing Lob 1, 2 and 3 in the human breast (adapted from Russo and Russo, 2004).

2.2 Basic carcinogenic process of breast cancer

All pathogenesis of cancer started with multistep process of carcinogenesis including the pathogenesis of breast cancer. It is well known that carcinogenesis is the result of interaction between exogenous (environmental) factors and endogenous process, which triggers the genetic or epigenetic alteration of multiple genes (Kyrtopoulos, 2006). Most common scenario is the exposure of somatic cells or occasionally germ or stem cells to various exogenous factors, either chemically, such as exposure to polycyclic aromatic hydrocarbon (PAH) substance, or physically, such as ionizing radiation causing the stem cells to undergo random mutations.

Genes most commonly affected by the mutation are called proto-oncogenes and tumor suppressor genes. The activation of proto-oncogenes into oncogenes results in the production of proteins which increase the transformation and proliferation of genetically-insulted tumor cells. Altered tumor suppressor genes (e.g. BRCA1, BRCA2, and p53) as occurred in most hereditary or familial breast cancer, lose its sole function to prevent tumor formation of blocking or limiting the cell growth (McPherson *et al.*, 2000, Croce, 2008).

Mutated genes in the stem cell causes inheritable spontaneous changes in DNA structure and affects the DNA replication and subsequent cell division. This will cause imbalance to the rate of cell proliferation and cell death thus alters the normal homeostasis environment. The increased cellular multiplication of the mutated cells or so-called ‘initiated cells’ with reduced programmed cell death mechanism (apoptosis)

for many cell generations causes the formation of excessive amount of new abnormal cells, later known as neoplasia (Bertram, 2000).

Carcinogenesis can be simplified into three main stages; initiation, promotion and progression. Initiation stage is when the cells are induced with various carcinogens into irreversible genetically altered or DNA-damaged cells consequent to the mutation activation. Usually, only multiple genes mutation can result in cancer formation since the huge damage will causes many disruption of bodily restore system including its own DNA repair mechanism. During promotion, the altered cells are stimulated to grow and proliferate faster to form a new population of cells of which is usually benign or preneoplastic lesion. Hormones such as estrogen are known as one of the promoter in human breast cancer. The progression stage involves more additional mutations and other heritable changes to the genetically-fragile tumor cells for transformation into malignant cells (Barrett, 1993). After the individual first exposure to the carcinogens, it usually takes many years or even decades for the promotion and progression to complete before the cancers are formed (Tsao *et al.*, 2004).

2.2.1 Pathogenesis of human breast cancer

The site of origin for breast cancer has been acclaimed to arise from TDLU that comprises the most undifferentiated structure unit of Lob 1 which is also the origin site for the most common type of malignancy in human breast known as invasive ductal carcinoma (Russo and Russo, 1978, Wellings *et al.*, 1980, Russo *et al.*, 1998). The high proliferative activity and fragility upon genetic alteration by exogenous mutagenic

influence of Lob 1 makes it highly susceptible for preneoplastic and neoplastic process (Russo and Russo, 1978).

When the cells are mutagenic, instead of progressing into Lob 2 type of cells, Lob 1 cells congregated in ductules and progressed into atypical type of TDLU to become the precursor for the neoplastic transformation. The preneoplastic lesion is later developed into ductal carcinoma *in situ* (DCIS) and eventually turns into aggressive type of invasive ductal carcinoma of which cells began to metastasize to other parts of the body. More differentiated lobule structures of Lob 2 and 3 are prone to pathogenic changes under carcinogenic influence as well. Lob 2 originates the formation of atypical lobules once it progress into lobular carcinoma *in situ* and later into metastatic lobular invasive carcinoma. Lob 3 tends to progress into more benign type of lesion compared to other type of lobules such as into hyperplastic lobules, fibroadenomas, apocrine cysts, sclerosing adenosis and other type of adenomas. Most differentiated Lob 4 on the other hand seems to show the highest resistance against neoplastic formation as it usually progressed into lactating adenomas only (Russo and Russo, 1978).

2.3 Chemoprevention studies

The general objective of chemoprevention is to interfere with the basic process of carcinogenesis by chemical agents that may block the initial neoplastic induction or prevent the progression of transformed cells into malignant type of neoplasm. Another objective is to prevent development of second primary tumors that may arise from people who has been cured from the initial cancer (Tsao *et al.*, 2004). The multistep of

carcinogenesis process over a long period of time gives the possibility for intervention of natural or synthetic chemopreventive agents at various specific stages of carcinogenesis. The long latent period of cancer formation might be the reason why the high occurrence of breast cancer is among the elderly women of whom might have been exposed to potential carcinogenic agent at their early lifetime.

In brief, the concept for application of this theory is to give the potential chemopreventive agents to the subjects who have been exposed to carcinogen during their early age. Many chemoprevention studies to date have applied this principle. The potential agents can be given either before or shortly after exposure to the carcinogen, depending on the objectives of the study. Results from the experimental model can later be classified as an anti-initiating agent if the intervention is during the initiation stage or anti-promoting agent if the intervention is during the promotion and progression of carcinogenesis. The intervention must have the purpose of enhancing or improving the protective physiological mechanism against the disease (Mehta, 2000). Figure 2.3 shows the overview of appropriate intervention time of chemopreventive agents at specific stage of carcinogenesis as proposed by Garro *et al.* (1992) and Mehta (2000).

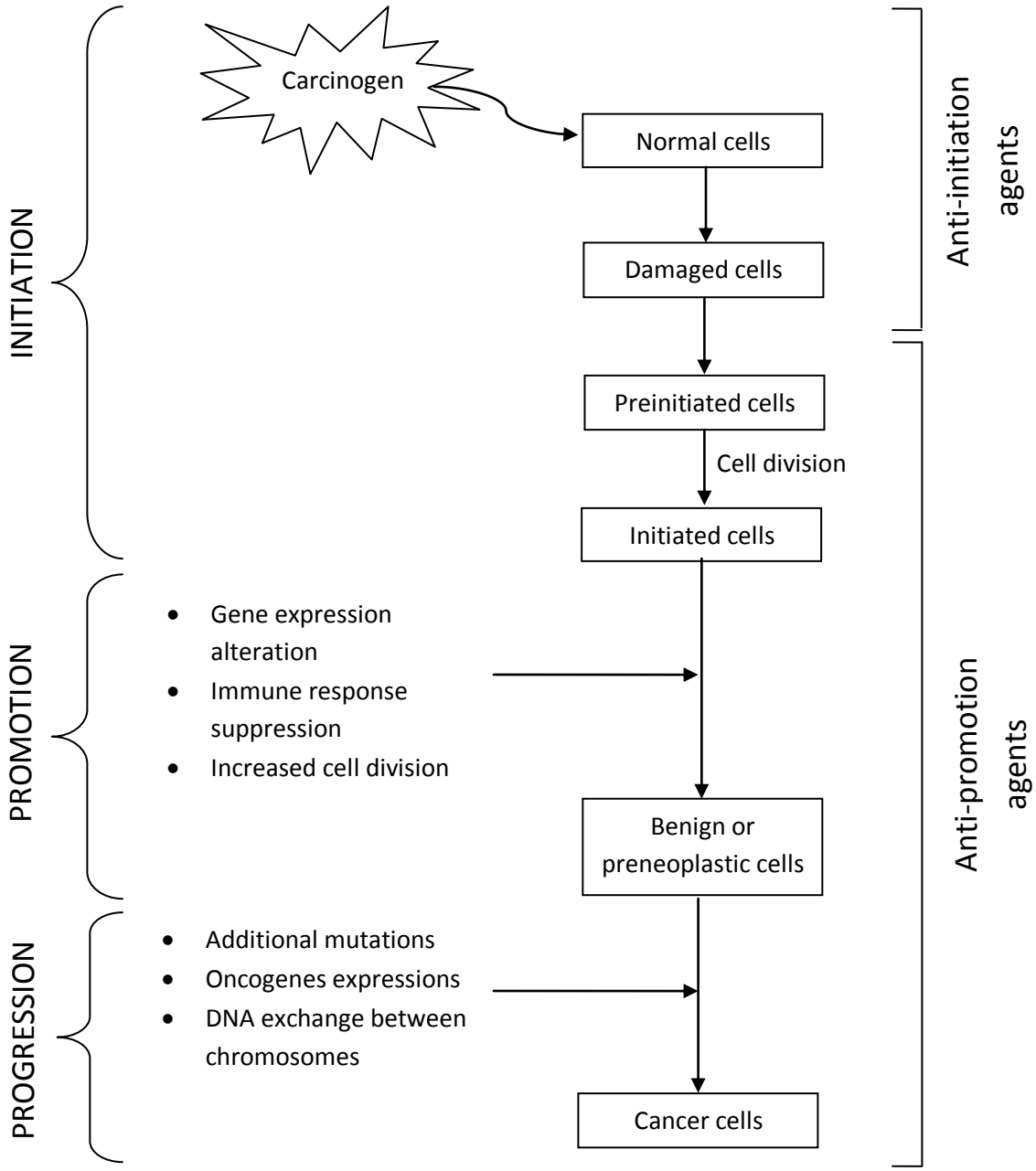

Figure 2.3: **Stages of carcinogenesis and intervention by chemopreventive agent.** The three main stages in carcinogenesis could be intercepted with chemopreventive agent at two probable time points corresponding to the respective stages.

With the basic knowledge that normal cells can be transformed chemically into cancer phenotype, experimental basis for chemoprevention studies is categorized into *in vitro* or *in vivo. In vitro* or cell culture method is a famous method in breast cancer studies that involves growing specific cell lines, e.g. MCF-7 or MDA-MB-231 for breast cancer, in a strictly-controlled environment. This method is usually chosen for experiment that focused on the mechanism of action of specific cells through their molecular pathway. For studies of finding new drugs or drug development that involves monitoring full scale pharmacological effects of drug to the body system in aspects of immune response, tissues and protein signaling, angiogenesis and apoptosis reaction, *in vivo* is the most suitable model since it is able to display most physiological process of a living animal. Besides, the multiple stages of carcinogenesis are only possible to be observed in animal model (Gupta and Kuperwasser, 2004).

2.4 Breast cancer induction in animal model using DMBA

It is crucial to apply a study model that resembles the physiopathology of the human cancer condition. *In vivo* model that uses rodents, specifically by using rat is a very useful method since its mammary gland is highly susceptible to chemical carcinogen, able to develop spontaneous mammary carcinoma, and most importantly, its pathogenesis and origin site of mammary cancer is quite similar to that of human (Russo and Russo, 1978, Medina, 1996).

A genotoxic agent called 7,12-dimethylbenz(α)anthracene (DMBA) has been recognized as a potent agent to elicit mammary carcinoma in rodents. A successful

induction of breast cancer in rodent depends on the species, strain and age of the host, along with its reproductive history, hormonal status and diet. Most of the mammary malignancy developed in female rats with DMBA induction is hormone-dependent and arise from mammary glandular epithelium later known as adenocarcinoma which is a type of malignancy most commonly diagnosed in human (Russo and Russo, 1996a).

Rat of Sprague-Dawley strain is the top choice for its high susceptibility to DMBA induction compared to other strains (Isaacs, 1986). Single administration of DMBA at appropriate dose through oral or gastric gavage is sufficient for a rat to develop mammary carcinoma (Huggins *et al.*, 1961). The administration should be done within a specific window of age which is between 40 to 60 days of age (Russo and Russo, 1978, Rogers and Lee, 1986). It is crucial to use nulliparous rats for DMBA induction since parous rats with more differentiated structure of lobular unit develop some resistance against the formation of chemically-induced mammary carcinoma.

2.4.1 Structure of mammary system in rats

The mammary glands of a female rat are subcutaneously aligned in ventrolateral position from the cervical to the inguinal regions forming six pairs of glands with one nipple for each gland (Figure 2.4). The first pair of gland is at the cervical region, both the second and third pairs are at the thoracic region and the other three pairs are at the abdomino-inguinal region (Russo and Russo, 1996a). Whole mount preparation of complete mammary organ shows complex branching of stroma and parenchyma forming duct structures which extends from the nipple into the fat pad. The primary or main

branch of duct is called lactiferous duct from which it continuously branch into secondary ducts and further up to six generation of branches. The further branches are smaller ducts, or known as ductules. The end structures of the ductules are later classified into several types; alveolar buds (AB), blunt end buds or terminal end buds (TEB) (Russo and Russo, 1978, Masso-Welch *et al.*, 2000).

The mammary duct is a tubule structure with walls of two types of cells; epithelial cells that line the lumen and the inner layer of myoepithelial cells. The basement membrane separates the parenchyma layers from the stroma (connective tissue). During the differentiation process of rat mammary gland, immature state of club-shaped TEB differentiates into AB and finally into matured lobules structure. TEB is composed of three to six layers of highly proliferating epithelial cells and responsible for branching and elongation of the duct. Cavitations of TEB causes formation of wide-hollow duct called the blunt end buds or terminal ductule (TD), which is the target site of carcinogen in older animals for the development of carcinoma (Masso-Welch *et al.*, 2000).

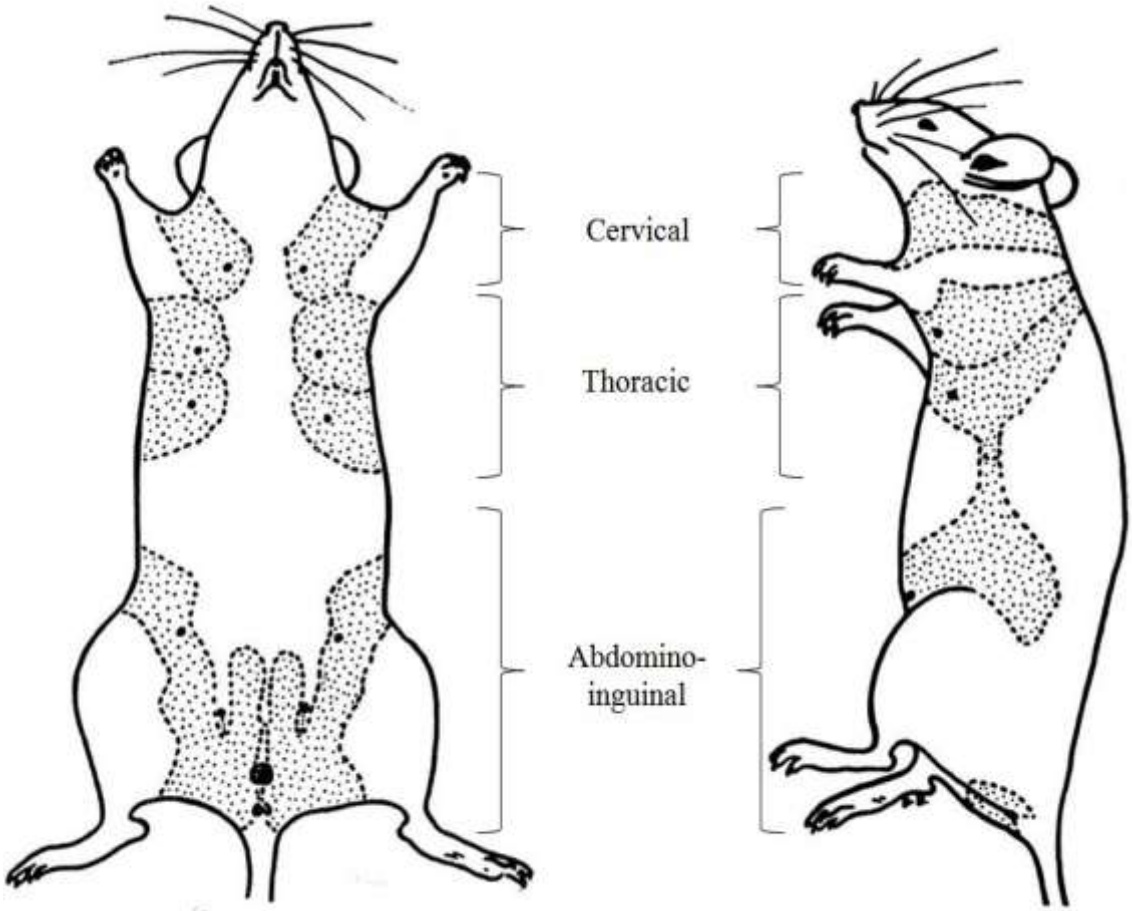

Figure 2.4: **Position of mammary glands in the female rat**. The glands are shown to its maximum extent lateral and ventrally at specific position in the body. The gland is seen oriented from the six-paired nipples. Cervical, thoracic and abdomino-inguinal regions are stated when denoting the position of tumor on the rat mammary pad.

The density of TEB is highest when the rat is approximately 21 days old and decrease sharply until 63 days old, and slowly until 84 days old. This decrease is either due to the change from TEB into AB and later to lobules, or TEB evolution into TD. The amount of AB on the other hand, increases steadily until it reaches a plateau state at the age of between 70 to 84 days old (Russo and Russo, 1978).

2.4.2 Pathogenesis of rat mammary carcinoma

Administration of DMBA to the rats when the amount of proliferating TEB is at the peak and actively differentiating into AB causes the highest incidence of mammary carcinoma and number of tumor formation (Russo *et al.*, 1983b). After the exposure to DMBA, instead of progressing into AB, the lining epithelium of TEB undergoes intraductal proliferation (IDP) thus becoming larger in size. This continuous and rapid DNA synthesis (Russo *et al.*, 1983a) and cell proliferation causes further increase in size and later known as microtumors. With the right microenvironment, these tiny tumors will enlarge and become a more aggressive type of adenocarcinoma. In correlation with the human mammary gland, TEB in young virgin rats is equivalent to TDLU (Lob 1) structure in nulliparous women (Russo and Russo, 1978, Russo and Russo, 1996a), which is believed to be the origin site of breast cancer. Therefore, it is proven that rat mammary carcinoma model is sufficient to mimic the breast carcinoma disease in human.

However, not all carcinogen-exposed TEB will turns into IDP. This minority group of TEB is still able to progress into AB. In certain occasion, some TEBs are

already differentiated into AB at the time of DMBA exposure. These ABs would either remain normal or proliferate at moderate pace forming more benign type of tumor such as adenomas, cysts, or undergoes lumen dilatation, thus giving rise to hyperplastic alveolar nodules (HAN) (Russo *et al.*, 1983b).

2.4.3 Carcinogenicity of 7,12-dimethylbenz(α)anthracene (DMBA)

DMBA is a polycyclic aromatic hydrocarbon (PAH) substance, an environmental pollutant that can be found basically from afterburning products and certain industrial process such as tobacco smoke, industrial and domestic oil furnaces, gasoline and diesel engines. For laboratory purposes, DMBA is usually produced synthetically. In order to exert its biological activity, DMBA needs to undergo metabolic activation which is quite contrary to other types of carcinogens that commonly act as direct-acting carcinogen, such as ionizing radiation and another type of chemical carcinogen used for mammary carcinoma induction called N-methyl-N-nitrosourea (MNU).

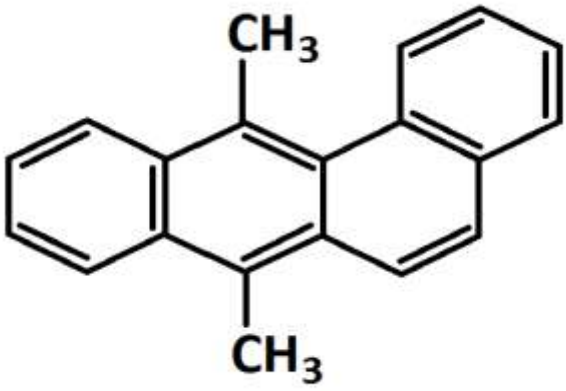

Figure 2.5: **Chemical structure of DMBA** (Melendez-Colon *et al.*, 1999).

2.4.4 Metabolic activation of DMBA

In rats, DMBA is metabolized in hepatic microsomes through oxidation and hydroxylation by P450 monooxygenase enzymes into reactive electrophilic metabolites that includes 7-hydroxymethyl-12-methylbenz(α)anthracene, 12-hydroxymethyl-7-methylbenz-(a)anthracene and many other diol-epoxides (Flaks *et al.*, 1972, Yang and Dower, 1975). These metabolites which are responsible for the mutagenic and carcinogenic initiation, later bind to cellular macromolecules, particularly the DNA within the mammary cells especially the ones in the TEB epithelial cells (Russo *et al.*, 1979) forming covalent DNA adducts such as DMBA-3,4-dihydrodiol 1,2-epoxides (DMBADEs) (Melendez-Colon *et al.*, 1999).

The formation of DMBA-DNA adduct causes impairment to the DNA structure itself, amplified with the impaired DNA repair mechanism, thus proper replication cannot be done and the abnormal DNA will be the starting point of mutagenesis. Oxidative metabolism of carcinogens also leads to the formation of reactive oxygen species (ROS) such as hydrogen peroxide which is an established tumor promoter (Frenkel *et al.*, 1995), hydroxyl radicals and other free radicals that can cause oxidative DNA damage and stimulates the cancer initiation (Xue and Warshawsky, 2005).