THE MECHANISM OF ANTICARCINOGENIC EFFECTS OF TUALANG HONEY (TH) ON INDUCED BREAST CANCER IN RATS

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by

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Introduction: The multifloral Tualang honey (TH) and the monofloral Manuka honey (MH) have been reported to have antimicrobial, anti-inflammatory, antioxidant and anticancer effects. Unlike the MH, TH is not extensively studied.

Objectives: This study was conducted to evaluate the mechanisms of the preventive and therapeutic effects of Tualang honey (TH), Manuka honey (MH) and honey sugars analogue (HSA) on experimental breast cancer induced rats using carcinogen 1-methyl-1-nitrosourea (MNU).

Methodology: A total of 130 female Sprague-Dawley rats were used. Sixty female rats were randomly divided into 6 groups with 10 animals per group in each study. Group '0' (negative control; normal rats); Group 1 (positive control; tumour induction but no treatment). Groups 2, 3 and 4 were fed orally with 0.2, 1.0 and 2.0 g/kg body weight of TH. Group 5 received 1.0 g/kg of MH, and Group 6 received 1.0 g/kg HSA. For the "cancer-preventive" study, honey was given one week prior to MNU-induction and for the "cancer-therapeutic" study; treatment was given after breast cancer development. The treatment continued until the 120^{th} day when the rats were sacrificed for samples collections.

Results: Results showed that TH and MH treated rats of "cancer-preventive" groups had a lower tumor incidence, and a longer latency period compared to the non-treated control group. The tumors developed in all treated groups of preventive and therapeutic measures were lesser in number, size and weight compared to the non-treated control. The majority of the tumors in the treated groups were of better grade (grade I and II) compared to the non-treated control group (grade III). The haematological parameters showed that varying strengths of TH, MH and HSA had a potentiating effect on haemoglobin, red blood cells, packed cell volume, mean corpuscular volume, lymphocytes and eosinophils, and a lowering effect on total white blood cells, red cell distribution width, polymorphs, monocytes and platelets compared to the non-treated control. These treatments showed no hyperglycemic effects and no body weight loss. The systemic

administration of TH, MH and HSA exerts anti-cancer effects through up-regulation of the expression of pro-apoptotic proteins such as caspase 9, Apfa-1 (apoptotic protease activating factor 1), p53, IFN-γ (interferon gamma) and IFNGR1 (interferon gamma receptor 1), and a concomitant down-regulation of the expression of anti-apoptotic proteins such as Bcl-xL (B-cell lymphoma-extra large), TNF-α (tumor necrosis factor alpha), COX-2 (cyclooxygenase-2), E2 (estradiol) and ESR1 (estrogen receptor 1) at serological and or breast cancer tissues levels.

Discussion: Our study shows that the treatment with TH and MH appears to exert cancer-preventive and or cancer-therapeutic effects, is through the modulation tumour grading, body weight, haematological parameters of immune regulatory response, and modulation of pro and anti-apoptotic proteins of mitochondrial apoptotic pathway at serum and breast cancer tissues level. HSA also acts akin to honey.

Conclusion: Tualang honey, Manuka honey and honey sugars analogue can be used as prophylactic cancer-preventive and cancer-therapeutic agents. The mechanism is through the modulation of tumour grading, haematological parameters, and the modulation of pro and anti-apoptotic proteins at serum and cancer tissues level.

Supervisor: Prof. Dr. Nor Hayati Othman

Co-supervisor: Prof. Dr. Siti Amrah Sulaiman

DEDICATIONS

This thesis is dedicated to my beloved father (Malik Faiz Bakhsh), mother (Mehboob Elahi), and my dearest wife (Asma Aftab)

for their encouragement, support and endless patience

They guided the basic principles of life i.e.

“Never shirk away from the responsibilities and live with truth and courage”

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AB	Alveolar buds
AOM	Azoxymethane
Apaf-1	Apoptotic protease activating factor 1
Bcl-xL	B-cell lymphoma-extra large
BDNF	Brain-derived-neurotrophic factor
BW	Body weight
COX-1	Cyclooxygenase 1
COX-2	Cyclooxygenase 2
DCIS	Ductal carcinoma in situ
DEN	Diethylnitrosoamine
DNA	Deoxyribonucleic acid
DSH	Diabetic spontaneously hypertensive
E2	Estradiol
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ESR1	Estrogen receptor 1
FADD	Fas-associated via death domain
FASLG	Fas ligand
g	Gram
Hb	Haemoglobin
HER2	Human epidermal growth factor receptor 2
H_2O_2	Hydrogen peroxide
HSA	Honey sugars analogue
IAP	Inhibitor of apoptosis proteins
IBW	Initial body weight
IGFR	Insulin growth factor receptor
IFN-γ	Interferon gamma
IFNGR1	interferon gamma receptor 1

i.p	intraperitoneal
Kg	Kilogram
L	Litre
MCV	Mean corpuscular volume
MCH	Mean corpuscular hemoglobin
MCHC	Mean corpuscular hemoglobin concentration
MH	Manuka honey
ml	Millilitre
MNU	1-methyl-1-nitrosourea
MMTV	Murine mammary tumor virus
NADP+	Nicotinamide adenine dinucleotide phosphate
NADPH	Reduced form of NADP+
NADH	Nicotinamide adenine dinucleotide
NF-kB	Nuclear factor kappa B
NO	Nitric oxide
NOS	Not otherwise specified
OVX	Ovariectomised
PARP	Poly ADP-ribose polymerase
PAH	Polycyclic aromatic hydrocarbons
PCV	Packed cell volume
pg	Picogram
PR	Progesterone receptor
PS	Percent score
PWG	Percentage weight gain
RBC	Red blood cell
RDW	Mean corpuscular hemoglobin concentration
RNS	Reactive nitrogen species
RS	Reactive species
SD	Sprague-Dawley
SHR	Spontaneous hypertensive rats
STZ	Streptozocin

TD	Terminal ductule
TDLU	Terminal ductal-lobular unit
TEB	nal end buds
TH	Tualang honey
TRAIL	TNF-related apoptosis-inducing ligand
TNF-α	Tumour necrosis factor-α
WBC	White blood cells
WKY	Wister-Kyoto rats
µl	Microlitre
%	Percent

MEKANISME KESAN ANTIKARSINOGENIK MADU TUALANG (MT) PADA TERARUH KANSER PAYUDARA DALAM TIKUS

ABSTRAK

Madu Tualang multiflora / Tualang honey(TH) dan madu Manuka monoflora /Manuka honey (MH) telah dilaporkan mempunyai kesan antimikrob, antiradang, antioksidan dan antikanser. Madu Tualang yang pertama belum dikaji secara meluas. Kami telah menjalankan satu kajian untuk menilai samada TH dan MH boleh dijadikan sebagai pencegahan [kajian 1] dan terapeutik [kajian 2] keatas tikus yang telah dicetuskan dengan kanser payudara menggunakan karsinogen 1-metil-1-nitrosourea (MNU). Kami juga menjalankan kajian terapeutik kanser yang sama menggunakan gula mirip seperti madu/ honey sugars analogue (HSA), cecair yang mengandungi perkadaran gula dalam madu. Untuk kajian 'pencegahan' madu diberi kepada tikus 7 hari sebelum cucukan MNU. Enam puluh ekor tikus betina secara rawak dibahagikan kepada 5 kumpulan dengan 10 ekor bagi setiap kumpulan. Kumpulan '0' tidak menerima MNU dan tidak menerima madu (Kawalan negatif); Kumpulan 1 menerima MNU tetapi tidak madu (Kawalan positif). Kumpulan 2,3 dan 4 telah diberi makan TH melalui mulut/ secara oral dengan 0.2g/kg, 1.0g/kg, and 2.0kg berat badan. Kumpulan 5 menerima 1.0 g / kg berat badan daripada MH Untuk kajian " terapeutik rawatan diberikan apabila tumor pertama dapat dirasakan mencapai saiz 10-12 mm. ", pengrupan tikus adalah serupa seperti kajian "pencegahan" dan ditambah satu kumpulan tikus lagi Group 6 yang diberi makan HSA 1.0 g/kg berat badan tikus.Perkembangan tumor payudara dan berat badan telah dicatatkan sepanjang kajian ini. Tikus dalam semua kumpulan terus menerima rawatan sehingga hari ke-120 apabila mereka dikorbankan. Semasa autopsi, darah telah diambil untuk analisis hematologi dan serologi. Tumor tersebut dikumpulkan untuk pemeriksaan kasar dan ujian mikroskopi dan penentuan ekspresi/ungkapan protein pro dan anti-apoptotic menggunakan kaedah immunohistokimia menggunakan panel antibodi. Keputusan menunjukkan bahawa tikus yang dirawat TH dan MH daripada kumpulan "pencegahan kanser" mempunyai insiden tumor yang lebih rendah (bilangan haiwan yang menghidap tumor), dan tempoh pendaman yang lebih lama (waktu selang apabila tumor yang pertama muncul/terbentuk) berbanding dengan kumpulan kawalan yang tidak dirawat. Bilangan tumor terbentuk dalam kumpulan yang dirawat adalah lebih rendah daripada kumpulan kawalan yang tidak dirawat ($p < 0.05$). Tidak kira sama ada madu atau mirip gula telah diberikan sebelum atau selepas perkembangan kanser payudara, tumor mengalami kenaikan saiz yang lebih perlahan (masing-masing <1.48 cm dan <2 cm untuk pencegahan kanser dan kesan terapeutik) berbanding dengan kumpulan kawalan yang tidak dirawat (masing-masing 2.85 cm dan 3.84 cm). Berat median (g) dan saiz (cm) tumor dalam kumpulan dirawat juga jauh lebih rendah ($p < 0.05$). Kenaikan berat badan sebenar yang signifikan diperhatikan dalam semua kumpulan dirawat berbanding dengan kumpulan kawalan yang tidak dirawat ($p < 0.05$). Ujian histopatologi menunjukkan pelbagai corak tumor; dari benigna, DCIS (ductal carcinoma in situ), kanser invasif micropapillary dan jenis NOS (not-otherwise specified) / (tidak ditetapkan). Kumpulan yang dirawat menunjukkan

lebih pola tumor benigna berbanding dengan kumpulan kawalan yang tidak dirawat. Majoriti tumor dalam kumpulan yang dirawat adalah dari gred yang lebih baik (gred I dan II) berbanding dengan kumpulan kawalan yang tidak dirawat (gred III). Parameter hematologi menunjukkan bahawa dos yang berbeza bagi TH, MH dan HSA mempunyai kesan lebih besar terhadap Hb (hemoglobin), RBC (red blood cells)/ (sel darah merah), PVC (packed cell volume)/ (jumlah sel dibungkus), MCV (mean corpuscular volume)/ (jumlah min korpuskel), limfosit dan bilangan eosinofil berbanding kawalan positif bukan dirawat. Rawatan dengan TH, MH dan HSA menunjukkan kesan berkurangan pada TWBC (total white blood cells)/ (jumlah sel darah putih), RDW (red cell distribution width)/ (lebar pembahagian sel merah), polimorf, monosit dan bilangan platelet berbanding dengan kawalan yang tidak dirawat positif. Tikus yang dirawat sama ada dengan dos TH, MH dan HSA tidak menunjukkan sebarang kesan hyperglycemic terhadap tahap glukosa darah berpuasa. Serum biokimia menunjukkan bahawa tikus yang dirawat dengan TH, MH dan HSA mempunyai peningkatan tahap protein pro-apoptotic; Apaf-1 (apoptotic protease activating factor 1)/(protease apoptotic mengaktifkan faktor 1) dan IFN-y (interferon gamma), serta penurunan tahap protein anti-apoptotic; TNF-a (tumor nekrosis faktor alpha) dan E2 (estradiol), berbanding dengan kawalan positif yang tidak dirawat ($p < 0.05$). Keputusan immunohistochemistry menunjukkan bahawa rawatan dengan TH, MH dan HSA mempunyai kesan kawal atur positif ke atas spesimen tumor untuk ekspresi/ungkapan protein pro-apoptotic; caspase 9, apaf-1, p53 dan IFNGR1 (interferon gamma reseptor 1), dan kesan kawal atur negatif terhadap ekspresi/ungkapan protein anti-apoptotic; Bcl-xL (B-cell lymphoma-extra large)/(B-sel limfoma-tambahan besar), TNF-a, COX-2 (cyclooxygenase-2) dan ESR1 (estrogen receptor 1), berbanding dengan spesimen tumor daripada kumpulan kawalan yang tidak dirawat ($p < 0.05$). Hasil kajian kami menunjukkan dengan jelas bahawa mekanisme yang TH dan MH memberi kesan pencegahan kanser dan juga sebagai terapi kanser, melalui modulasi parameter hematologi dan serologi oleh tindak balas kawal atur imun. Pemakand oral TH dan MH berupaya memberikan kesan anti-kanser pada sel-sel kanser melalui pengaktifan laluan apoptotic mitokondria melalui kawal atur -atas ekspresi/ ungkapan protein pro-apoptotic seperti caspase 9, Apfa-1, p53 dan IFNGR1, dan mengiringi kawal atur- bawah ekspresi/ ungkapan protein anti-apoptotic seperti Bcl-xL, TNF-a, COX-2 dan ESR1. HSA mempunyai kesan yang serupa tetapi lebih rendah dari madu.

THE MECHANISM OF ANTICARCINOGENIC EFFECTS OF TUALANG HONEY (TH) ON INDUCED BREAST CANCER IN RATS

ABSTRACT

The multifloral Tualang honey (TH) and the monofloral Manuka honey (MH) have been reported to have antimicrobial, anti-inflammatory, antioxidant and anticancer effects. Unlike the Manuka honey, TH is not extensively studied. We conducted a study to evaluate the mechanisms of the preventive and therapeutic effects of TH and MH on experimental breast cancer induced rats using carcinogen 1-methyl-1-nitrosourea (MNU). We also conducted a similar cancer therapeutic study using Honey sugars analogue (HSA), a fluid which contains the proportion of sugars in honey. A total of 130 female Sprague-Dawley rats were used. Sixty female rats were randomly divided into 6 groups with 10 animals per group in each study. Group '0' did not receive MNU and did not receive honey (negative Control); Group 1 received MNU but not honey/honey sugars analogue (positive Control). Groups 2, 3 and 4 were fed orally with 0.2, 1.0, 2.0, 1.0 g/kg body weight of TH, and Group 5 received 1.0 g/kg body weight of MH. Group 6 received 1.0 g/kg body weight of HSA in therapeutic study. For the "cancer-preventive" study, honey was given one week prior to MNU-induction and for the "cancer-therapeutic" study; honey was given when the first palpable tumor reached 10-12 mm in size. The development of mammary tumors and body weights were charted throughout the study. The rats in all treated groups continued to receive the treatment until the 120^{th} day when they were sacrificed. At autopsy, blood was drawn for haematological and serological analysis. The tumors were harvested for gross and histopathological examinations, and determination of pro and anti-apoptotic proteins expression by immunohistochemistry using a panel of antibodies. Results showed that TH and MH treated rats of "cancer-preventive" groups had a lower tumor incidence (the number of animals developing tumors), and a longer latency period (the interval when the first tumor developed after MNU induction) compared to the non-treated control group. The number of tumors developed in the treated groups was lesser than the non-treated control group ($p<0.05$). Regardless of honey treatment given, either before or after the breast cancer development, the tumors had slower size increment (<1.48 cm^3 and <2 cm^3 for cancer preventive and therapeutic effects respectively) compared to the non-treated control group ($=2.85$ cm^3 and $=3.84$ cm^3 respectively). The median weight (g) and size (cm^3) of the tumors in treated groups were also significantly lower ($p<0.05$). A significant actual body weight gain was

observed in all treated groups compared to the non-treated control group ($p<0.05$). Histopathological examination showed various tumor patterns; ranging from benign, DCIS (ductal carcinoma in situ), micropapillary and NOS (not-otherwise specified) type. The treated groups showed more patterns of benign tumors compared to the non-treated control group. The majority of the tumors in the treated groups were of better grade (grade I and II) compared to the non-treated control group (grade III). The haematological parameters showed that different dosages of TH, MH and HSA had an increasing effect on Hb (haemoglobin), RBC (red blood cells), PCV (packed cell volume), MCV (mean corpuscular volume), lymphocytes and eosinophils counts compared to the non-treated positive control. Treatments with TH, MH and HSA presented a decreasing effect on TWBC (total white blood cells), RDW (red cell distribution width), polymorphs, monocytes and platelets counts compared to the non-treated positive control. Various preparations of TH, MH and HSA showed no hyperglycemic effect on fasting blood glucose level. Serum biochemistry showed that the rats treated with TH, MH and HSA had an increased level of pro-apoptotic proteins; Apaf-1 (apoptotic protease activating factor 1) and IFN-γ (interferon gamma), and a decreased level of anti-apoptotic proteins; TNF-α (tumor necrosis factor alpha) and E2 (estradiol), compared to the non-treated positive control ($p<0.05$). Immunohistochemistry results showed that treatments with TH, MH and HSA had a positive regulatory effect on the tumor specimens for the expression of pro-apoptotic proteins; caspase 9, apaf-1, p53 and IFNGR1 (interferon gamma receptor 1), and a negative regulatory effect on the expression of anti-apoptotic proteins; Bcl-xL (B-cell lymphoma-extra large), TNF-α, COX-2 (cyclooxygenase-2) and ESR1 (estrogen receptor 1), compared to the tumor specimens of the non-treated control group ($p<0.05$). The findings of our study clearly show that the mechanisms by TH, MH and HSA appear to exert cancer-preventive and or cancer-therapeutic effects, is through the modulation of haematological and serological parameters of immune regulatory response. The systemic administration of TH, MH and HSA exerts anti-cancer effects on cancer cells via the activation of the mitochondrial apoptotic pathway through up-regulation of expression of pro-apoptotic proteins such as caspase 9, Apfa-1, p53 and IFNGR1, and a concomitant down-regulation of the expression of anti-apoptotic proteins such as Bcl-xL, TNF-α, COX-2 and ESR1.

CHAPTER 1

INTRODUCTION

Brest cancer is an abnormal and uncontrolled growth of breast epithelial cells, which invade the ducts and lobules of the tissue. It has been recognized as the second most common cancer after lung cancer, the fifth most common cause of cancer death and the leading cause of cancer death in women worldwide, surpassing the cervical cancer (Parkin and Fernandez, 2006; Ferlay *et al.*, 2010; Jemal *et al.*, 2010; Pinder, 2010; Jemal *et al.*, 2011; Vivien V Ng et al., 2014). In Malaysia, a recent report shows breast cancer as the most frequently diagnosed cancer and the International Agency for Research in Cancer (GLOBOCAN) estimated the ASR (age-standardized rate) of breast cancer in Malaysia as 38.7 per 100,000 with 5410 new cases in 2012 (Yip *et al.*, 2014). Considering demographic changes with the lack of access to early diagnosis and treatment in the developing world, it is estimated that the mortality rate from breast cancer will increase by over 100% in developing countries by 2020 (Howell, 2010).

The current treatment modalities against breast cancer include surgery, radiation therapy, hormone therapy and widely used chemotherapy (Turner and Jones, 2008). Chemotherapy drugs had an obstacle of collateral damage to the normal cells and tissues (Yaacob and Ismail, 2014). The relapse after chemotherapy, second primary tumours and resistance to chemotherapeutic drugs have also been reported in breast cancer patients (Tsao *et al.*, 2004; Bansal *et al.*, 2012; Raffoul *et al.*, 2012b). This urges to explore alternative measures for cancer therapy and prevention. The alternative measure to the recent modalities and some of their unavoidable side effects, is the use of natural products such as honey (Othman, 2012a; Othman, 2012c; Ahmed

and Othman, 2013a). This study was aimed to evaluate the cancer-preventive and cancer-therapeutic activity of honey against breast cancer in in vivo. “Cancer-preventive” is termed when honey was given one week prior of tumour induction, while “cancer-therapeutic” termed when honey treatment was given after breast cancer development.

Honey has been used as a traditional medicine to cure several ailments since ancient times in different cultures (Allsop and Miller, 1996). Honey has been shown to have anti-inflammatory (Cooper *et al.*, 2001), anti-microbial (Sherlock *et al.*, 2010), antioxidant (Al-Mamary *et al.*, 2002; Erejuwa *et al.*, 2010b; Erejuwa *et al.*, 2012b) anti-tumour (Swellam *et al.*, 2003; Tomasin and Gomes-Marcondes, 2011; Othman, 2012b), and antidiabetic effects (Erejuwa *et al.*, 2010a; Erejuwa *et al.*, 2011). Honey has also been demonstrated as a natural anticancer vaccine (Othman, 2012a; Othman, 2012c). For that reasons, a type of Malaysia’s own local honey called Tualang honey (TH) has been selected for this study. To capitalize the understanding of its efficacy at broad spectrum level, effects of Manuka honey (MH) and Honey sugars analogue (fructose, glucose, maltose and sucrose) were also investigated for cancer-prevention and cancer-therapy in breast cancer induced animal models.

Tualang honey (TH) is a multi-floral jungle honey. It is produced by “*Apis dorsata*” bee species which build their hives high on Tualang trees (*Kompassia excelsa)*, found mainly in Malaysian tropical rainforests (Erejuwa *et al.*, 2010b; Mohamed *et al.*, 2010a; Ahmed and Othman, 2013b)**.** Published data has shown that TH exhibits antimicrobial (Nasir *et al.*, 2010; Sukur *et al.*, 2011), anti-inflammatory (Bashkaran *et al.*, 2011), antioxidant (Mohamed *et al.*, 2010b; Khalil *et al.*, 2012; Tan *et al.*, 2014) and antidiabetic effects (Erejuwa *et al.*, 2010b; Erejuwa *et al.*, 2012a). TH has been shown to have anticancer effects against oral squamous cell carcinoma (Ghashm *et al.*, 2010b), human osteosarcoma cell lines (Ghashm *et al.*, 2010b),

human breast cancer cell lines MCF-7 cells (Fauzi *et al.*, 2011), cervical cancer cell lines (Fauzi *et al.*, 2011) and leukemic cell lines K562 (Rosline *et al.*, 2010). Recent studies have shown that TH exhibits breast anticancer activity, reduces tamoxifen-induced cytotoxicity in vitro and demonstrates preventive effects against breast cancer in vivo (Kadir *et al.*, 2013; Yaacob *et al.*, 2013; Yaacob and Ismail, 2014).

Manuka honey (MH) unlike Tualang honey is a mono-floral honey. It is produced by honey bees from nectars of Manuka bush (*Leptospermum scoparium*) throughout New Zealand and Australia (Yao *et al.*, 2003). Published literature on Manuka honey indicates its numerous therapeutic properties against several ailments (Russell *et al.*, 1990; Molan, 2001a; Visvadia *et al.*, 2008; Old, 2013; Kamaratos *et al.*, 2014), including breast cancer (Fernandez-Cabezudo *et al.*, 2013). Literature lacks reports on HSA, namely honey sugars analogue (fructose, glucose, maltose and sucrose), and has not been previously reported. Glucose and fructose account for major part of honey (Shin and Ustunol, 2005; Ahmed and Othman, 2013b); exhibit anti-mutagenic and anti-cancer effects in different models (Wang *et al.*, 2002; Speicher *et al.*, 2010). This supports the hypothesis that general honey sugars analogue may exhibit anticancer activity.

Breast cancer induction using carcinogen MNU (1-methyl-1-Nitrosourea) in female rats is one of the most frequently used animal models. It has several advantages such as 1) reliability of tumour induction, 2) organ site specificity, 3) the tumour developed is of ductal origin, 4) resembles human mammary carcinoma, and 5) easy to examine tumour promotion and histopathological characterizations (Welsch, 1985; Thompson and Adlakha, 1991; Thompson *et al.*, 1995; Russo and Russo, 2000; Tsubura *et al.*, 2011). This is the reason that MNU-induced mammary carcinoma in the Sprague–Dawley rat model was chosen in this study.

The potential “preventive” and “therapeutic” effects of TH, MH and HAS are based on these parameters; the tumour growth characteristics; the histological features, the tumour grades and the histological patterns were evaluated in the present study. Assessment of histological grading of breast carcinoma along with the histological type have pivotal importance for the prognosis to determine the appropriate treatment and to anticipate the post-treatment survival in breast cancer (Harvey *et al.*, 1995; Boiesen *et al.*, 2000; Dalton *et al.*, 2000; Ignatiadis and Sotiriou, 2008).

Broad spectrum pre and post treatment studies have revealed a deranged or altered full blood count pattern in breast cancer patients (Sheikh *et al.*, 2011; Akinbami *et al.*, 2013). It is well-established that the functional status of the immune system at haematological and serological level has direct influence on breast cancer (Hamidullah *et al.*, 2012). Research has shown that honey feeding significantly modifies the haematological parameters in normal individuals (Al-Waili *et al.*, 2006; Fiorani *et al.*, 2006). Similarly, Manuka, Pasture, Nigerian Jungle and royal jelly honeys have been found to increase immune factors such as IL- 1β, IL-6 and TNF-α production (Tonks *et al.*, 2001; Timm *et al.*, 2008; Fukuda *et al.*, 2011). This study also addresses the haematological and serological response modification by TH, MH and HSA against breast cancer, which has not been reported yet.

The effects of TH, MH and SM administration on the expression of pro and anti-apoptotic proteins in tumour specimens were also evaluated to elaborate their mechanism of action. Apoptosis is a program that leads to cell death. Apoptotic cell death of cancer cells is the prime purpose of cancer treatment and plays a vital role in immune response resolution, and elimination of dysfunctional cells (Muradian and Schachtschabel, 2001; Pollack *et al.*, 2002).

Honey induces apoptosis via regulation of pro and anti-apoptotic proteins of intrinsic apoptotic pathway (Jaganathan and Mandal, 2010; Fauzi *et al.*, 2011; Ahmed and Othman, 2013a).

1.1 Objectives

1.1.1 General objective

To evaluate the cancer-preventive (study I) and cancer-therapeutic effects (study II) of Tualang (TH) and Manuka (MH) honeys on MNU-induced breast cancer rats, and to understand the underlying mechanisms. Concomitantly, the cancer-therapeutic efficacy of honey sugars analogue (HAS) was also investigated.

1.1.2 Specific objectives

1. To determine the growth inhibitory effects of TH, MH and HSA (tumour incidence, latency, multiplicity, size and weight).
2. To determine the effects of TH, MH and HSA administration on tumour grading, patterns and haematological parameters of rats.
3. To determine the effects of TH, MH and HSA on the expression of pro-apoptotic proteins; Apaf-1 (apoptotic protease activating factor 1) and IFN-γ (interferon gamma), and anti-apoptotic proteins; TNF-α (tumour necrosis factor alpha) and E2 (estradiol) at serum level.
4. To determine the effects of TH, MH and HSA on the expression of proapoptotic proteins; caspase-9, Apaf-1, p53, IFNGR1 (interferon gamma receptor 1), FASLG (Fas ligand) and FADD (Fas-associated via death domain), and anti-apoptotic

proteins; Bcl-xL, (B-cell lymphoma-extra large), TNF-α, COX-2 (cyclooxygenase-2) and ESR1 (estrogen receptor 1) at cancer tissues level.

The above objectives are conducted for both studies, study 1 where honey treatment was given one week prior to cancer induction and for study 2 where honey treatment was given when the first breast cancer mass reached 10-12mm in size.

1.2 Problem statement

Chemotherapy drugs cause collateral damage to the normal cells and tissues. Honey is a probable alternative as an adjunct to conventional treatment

1.3 Hypothesis

Tualang and Manuka honeys have anti breast cancer activity. The mechanism of action is through certain regulatory genes of cellular growth.

1.4 Significance of research

This study could provide information on the benefits of Tualang and Manuka honeys in prevention and therapy of breast cancer. The exploration of the mechanisms of their action will probe the different molecular targets and pathways involved, possibly to facilitate the use as complementary targeted-therapy against this cancer.

CHAPTER 2

LITERATURE REVIEW

2.1 Breast cancer

Generally, cancer is an abnormal growth of cells. It starts as an onset from a single transformed cell. Its genesis is characterized by the swift proliferation, invasion and metastasis (Shishodia *et al.*, 2003). This dynamic process is activated by various carcinogens, tumour promoters and inflammatory agents. The whole modulation is controlled through transcription factors, pro-apoptotic proteins, anti-apoptotic proteins, protein kinases, cell cycle proteins, cell adhesion molecules, cyclooxygenase-2 (COX-2) and other molecular targets (Wang and Lenardo, 2000; Shishodia *et al.*, 2003; Aggarwal and Shishodia, 2006).

Breast cancer is an abnormal and uncontrolled growth of epithelial cells involving the ducts and lobules of the breast tissue. It develops in cells from the lining of ducts and the lobules which supply milk. Mammary carcinoma usually results from the new formation of cells or neoplasm in ducts and lobules, which undergo rapid transformation and growth and eventually form a lump or mass which is known as tumour (Damjanov, 2006; Banin Hirata *et al.*, 2014; NCI, 2014). Breast tumours are classified histologically based on the location of origin. Cancers developing from the ducts are known as ductal carcinomas, whereas those developing from lobules are known as lobular carcinomas. The ductal tumours represent 80% of tumours and lobular tumours account for 10 to 15% of cases. Other subtypes represent less than 10% of cases diagnosed per year (Banin Hirata *et al.*, 2014).

Neoplasia or tumours arising from breast tissue are commonly adenocarcinoma of the cells lining the terminal duct lobular unit. Tumours can be classified into benign or malignant. Benign tumours are identified as localized growth with no distant spread or metastasis to other body parts. The malignant tumours are known as cancer which invade stroma and infiltrate or metastasize to other parts of the body such as lymph nodes, bone, liver, and lung (Weigelt *et al.*, 2010; Beaumont and Leadbeater, 2011; Peto *et al.*, 2012) . Other sites of metastasis may include the skin and brain (Beaumont and Leadbeater, 2011; Peto *et al.*, 2012).

Breast cancer development usually involves progression through a series of intermediate processes that start from ductal hyperproliferation, and followed by subsequent evolution to carcinoma in situ, invasive carcinoma and finally into metastasis (Polyak, 2007). The process of metastasis comprises of a series of sequential steps and failure to complete any of these steps will arrest this process (Fidler, 2003). Metastasis starts with the local invasion of surrounding host tissue cells originating from the primary tumour and continues until the tumour cells invade and intravasate into lymphatic vessels or blood (Hunter *et al.*, 2008; Talmadge and Fidler, 2010). The tumour cells are disseminated *via* the lymphatic vessels or the blood stream to distant organs. Consequently, the tumour cells undergo cell cycle arrest and adhere to capillary beds within the target organs. Before extravagating into the organ parenchyma, these cells proliferate and promote angiogenesis within these organs (Hunter *et al.*, 2008). These tumour cells must simultaneously evade the host's apoptotic signals and immune response in order to survive and proliferate (Fidler *et al.*, 1978; Hunter *et al.*, 2008). When the tumour cells succeed in completing these steps, the process can be repeated to produce secondary metastases or metastasis of metastases (Fidler, 2003; Talmadge and Fidler, 2010).

2.1.1 Incidence and prevalence

Breast cancer is recognized as the second most common cancer after lung cancer, the fifth most common cause of cancer death and the leading cause of cancer death in women worldwide (Parkin and Fernandez, 2006; Ferlay *et al.*, 2010; Jemal *et al.*, 2010; Pinder, 2010; Jemal *et al.*, 2011; Ferlay J *et al.*, 2013). In 2013, breast cancer has been continued to be the most common female cancer and its incidence is still on rise (Vivien V Ng et al., 2014). In Malaysia, a recent report shows breast cancer as the most frequently diagnosed cancer, and the International Agency for Research in Cancer (GLOBOCAN) 2012 estimated the age standardized rate (ASR) of breast cancer in Malaysia as 38.7 per 100,000 with 5410 new cases in 2012 (Yip *et al.*, 2014). Breast cancer is among the most common four types of cancer followed by lung, female breast, bowel and prostate cancer **(**Figure 2.1**)** (Ferlay J *et al.*, 2013). A report generated from the Malaysian National Cancer Registry (NCR) department lists breast cancer at top among the five leading cancers in general population of Malaysia **(**Figure 2.2**)** (Ariffin and Saleha, 2011).

In many developing countries including Malaysia, the incidence of breast cancer is now rising sharply. More than half of incident cases occur in the developing world and combined with still high case-fatality rates (Hisham and Yip, 2004; Shulman *et al.*, 2010; Yip *et al.*, 2014). In Peninsular Malaysia, data from 2003 to 2005 shows that the incidence was highest among women of age between 50 to 60 years old, and the incidence of breast cancer among women in Malaysia is still lower than most of Western countries or developed world (Chye *et al.*, 2008). Considering demographic changes with the lack of access to early diagnosis and treatment in the developing world, there will be a continuous marked increase in the incidence and mortality

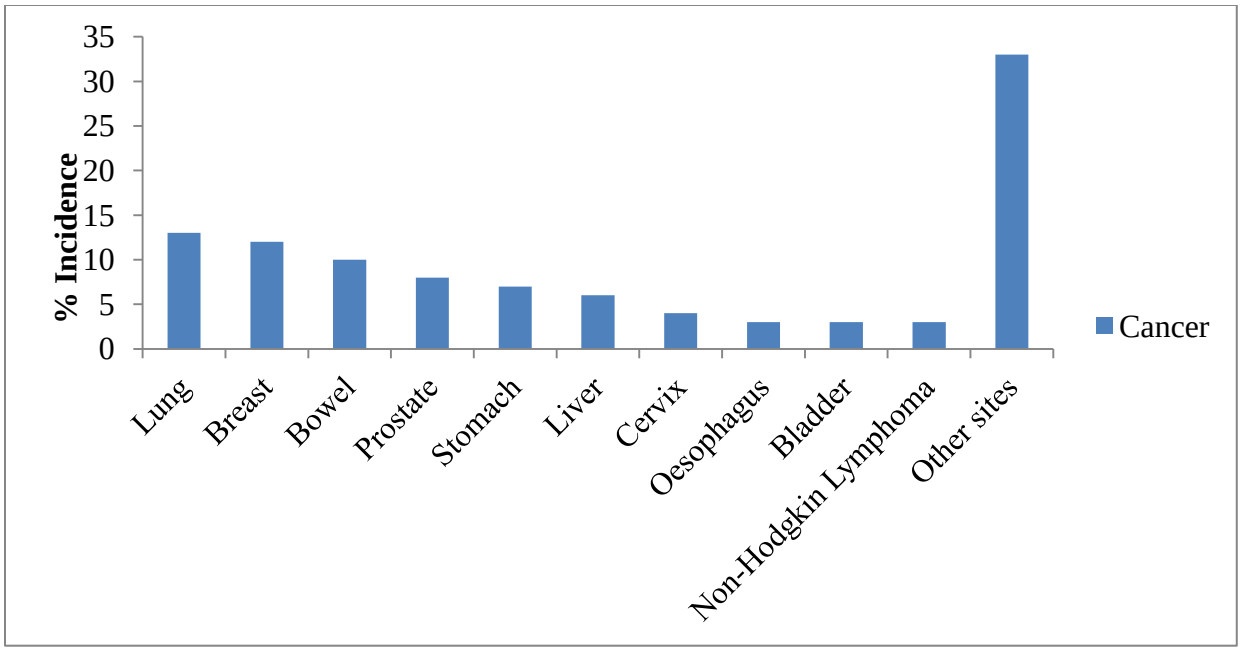

Figure 2.1: The most commonly diagnosed cancers worldwide. Source reference (Ferlay J *et al.*, 2013).

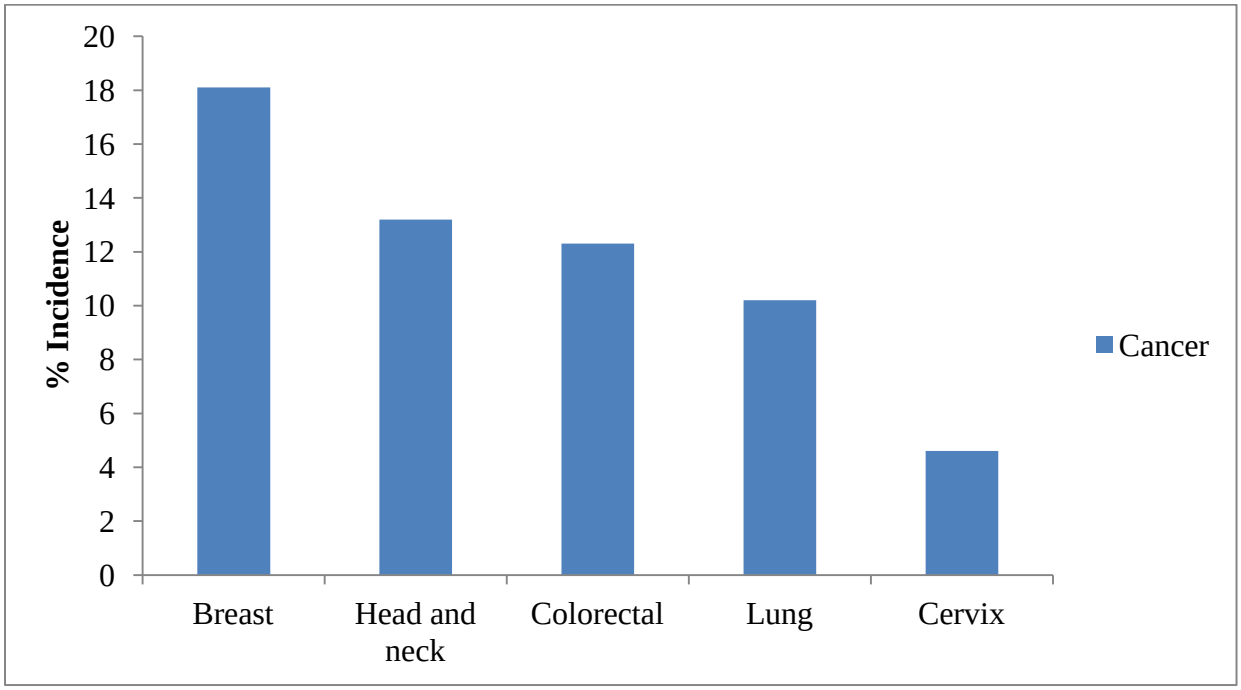

Figure 2.2: The most commonly diagnosed cancers in Malaysia. Source reference (Ariffin and Saleha, 2011).

from breast cancer. It is estimated that this mortality rate from breast cancer will increase by over 100% in developing countries by 2020 (Howell, 2010).

2.1.2 Risk factors

Several research studies have explored a wealth of risk factors among women in different countries which impact on the countries' incidence and prevalence. These risk factors can be classified as demographic-socioeconomic related, genetic-racial-related, hormone-reproductive, behavior-related and lifestyle-related. Many other factors, including prenatal conditions, physical activity, diet, body mass index, estrogen exposure, depression and quality of life have been demonstrated as breast cancer risk factors. A positive family history has also been described as one of the main risk factors (Ruder *et al.*, 2008; Press and Pharoah, 2010; Ostad and Parsa, 2011).

Hormonal conditions are also considered pivotal among the risk factors. Prolonged exposure to higher concentrations of endogenous estrogen; which is thought to be controlled and modulated by menarche, pregnancy and menopause; causes to increase the risk of breast cancer. Higher testosterones level has also shown some higher rate of breast cancer in some studies, although not in all of them. Younger age of menarche and older age of first full-term pregnancy have also been found to be associated with a higher risk of breast cancer. Research has shown an increased risk of breast cancer in oral contraceptive users, whereas some other researchers report no significant difference. It has also been reported that long term use of postmenopausal hormone therapy is associated with higher risk of breast cancer. Exposure to environmental toxic organochlorines agents such polychlorinated biphenyls (PCB's), dioxins, organochlorine pesticides have been demonstrated to increase the risk of breast cancer. Age and gender are

considered among the strongest risk factors for breast cancer. Breast cancer occurs 100 times more frequently in women compared to men. Incidence rates increase with age until about the age of 45 to 50 which are thought to be more susceptible. In Malaysia, more than half of new cases of breast cancer are found to be diagnosed in women under the age of 50 years (Hisham and Yip, 2004).

Ethnic difference has been described as another factor affecting breast cancer prevalence. For instance, in United States, breast cancer prevails more among white people. Much of these differences arise from social conditions and lifestyle factors. There are marked variations in breast cancer incidence and mortality among different countries. Women with higher educational, occupational and economic level are at higher risk because of the reproductive patterns, including age of first birth and age of parity. Ethnic differences in estrogen and progesterone receptor subtypes have also been documented as important factors that may affect the probability of breast cancer in women (Setiawan *et al.*, 2009; Ostad and Parsa, 2011). Familial history of breast cancer carrying gene mutations in breast cancer related genes such as gene 1 (BRCA1) and gene 2 (BRCA2), may also cause women at high risk to develop breast cancer (Martin and Weber, 2000). Research has also shown that risk factors may include the excessive use of alcohol, obesity and physical inactivity which account for 21% of all breast cancer deaths worldwide (Danaei *et al.*, 2005). It has also been reported that reduction in breast size reduces the risk for breast cancer (Jansen *et al.*, 2014).

A number of lifestyle and genetic factors have been shown to cause an increased risk of breast cancer in Malaysian women. These risk factors include, family history, nulliparity, not breastfeeding and use of oral contraceptives are observed to be associated with a greater risk of breast cancer in Malaysian women. The other risk factors are not significantly associated such as

age at menarche and first childbirth. Genetic predisposition may also play a role in the aetiology of breast cancer. It has been estimated that approximately 15% of breast cancer patients in Malaysia report family history of breast and ovarian cancers, with the most significant genetic predisposition of genes identified are BRCA1 and BRCA2 (Yip *et al.*, 2014).

2.2 Morphogenesis and structure of the normal human breast

The breast or mammary gland is one of the defining characteristics among mammals. Rudimentary mammary glands or breasts develop in the embryo and grow after birth with body growth. The gland undergoes a rapid expansion with the ductal tree growing and branching until the limits of the fat pad at puberty in response to steroid hormones. The glandular tissue undergoes a round of proliferation and differentiation during each menstrual cycle and caused ductal tree to produce tertiary branches and alveolar structures (Figure 2.3). The breast develops fully at pregnancy when milk-producing lobuloalveolar structures develop. The lactating breast comprises a branched network of epithelial ducts leading from the nipple and expanded to secretory alveoli embedded within an adipocyte rich stroma (Figure 2.4). (Shackleton *et al.*, 2006; Stingl *et al.*, 2006; Sleeman *et al.*, 2007; Taddei *et al.*, 2008).

The detailed anatomy of breast reveals that it is composed of fifteen to twenty lobes and each lobe further dividends into smaller sections of lobules. Lobules contain lactiferous ducts and milk-producing glands that lead the lobules to the nipple as a passage for milk delivery. Further, fatty tissues and fibrous stroma or connective tissues that contain blood capillaries and nerves, surround both lobules and ductal units (Ganschow, 2004). The lobular structure is found to be comprised of ducts that are divided into club-shaped terminal end buds, and these buds

later form two smaller structures called alveolar buds. These alveolar buds surround the terminal ducts and form the terminal ductal-lobular unit (TDLU).

TDLU is the functional unit of human mammary glands and it contains an abundance of highly proliferative population of stem cells (Russo and Russo, 1978). At extended level, ducts divide into ductules, lobules and TDLU, comprising of epithelium. The epithelium seems to be comprised of two cell types, outer basal cells and inner luminal cells (Figure 2.5). The basal cells consist of differentiated myoepithelial cells and may have many features of both smooth muscle and epithelium. This compartment has been shown also to exhibit mammary stem cells (Shackleton *et al.*, 2006; Stingl *et al.*, 2006; Sleeman *et al.*, 2007; Taddei *et al.*, 2008). These stem cells further give rise to the mature epithelium of myoepithelial or the luminal lineage. The luminal cells are mostly comprised of ductal or alveolar milk-secreting cells and a subpopulation of these cells are hormone receptor-expressing cells (Sleeman *et al.*, 2007).

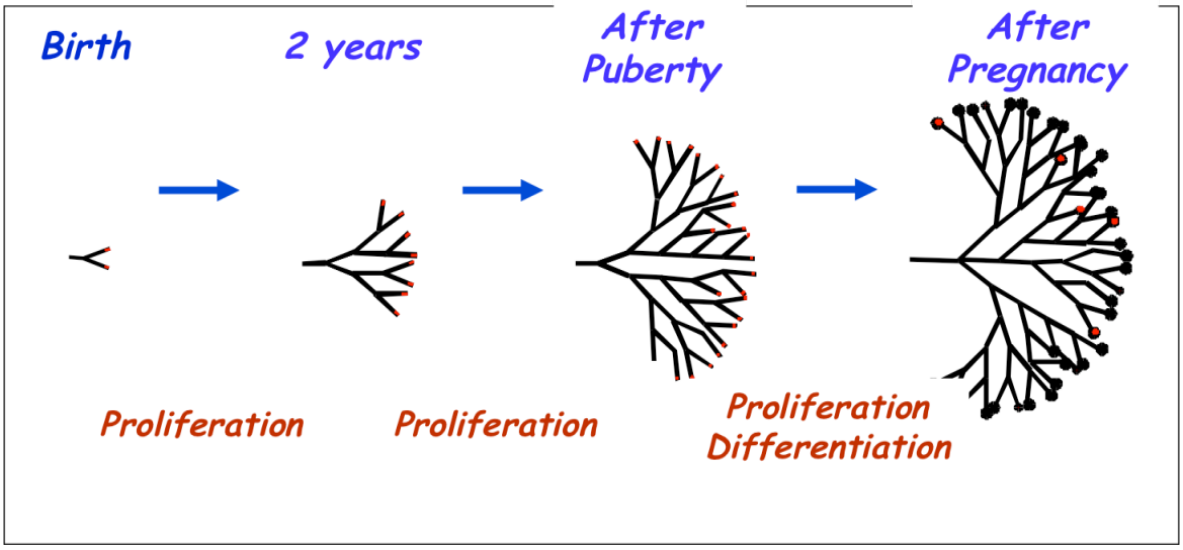

Figure 2.3: Development of the breast ductal tree after birth. Source reference (Sternlicht, 2006).

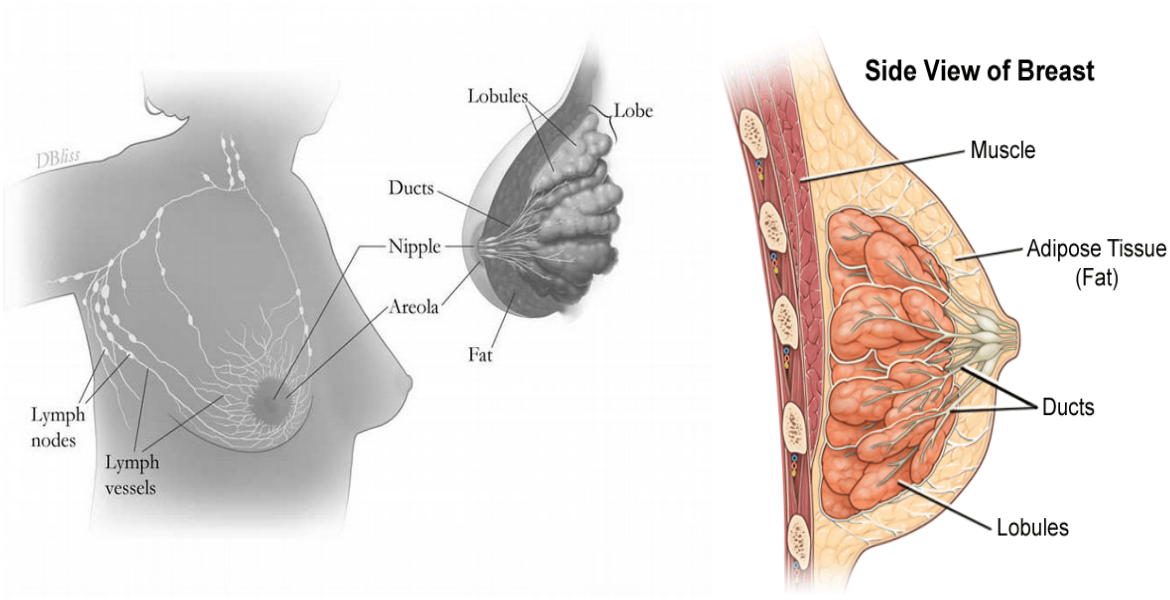

Figure 2.4: Structure of normal human breast. Source reference (Martini *et al.*, 2009).

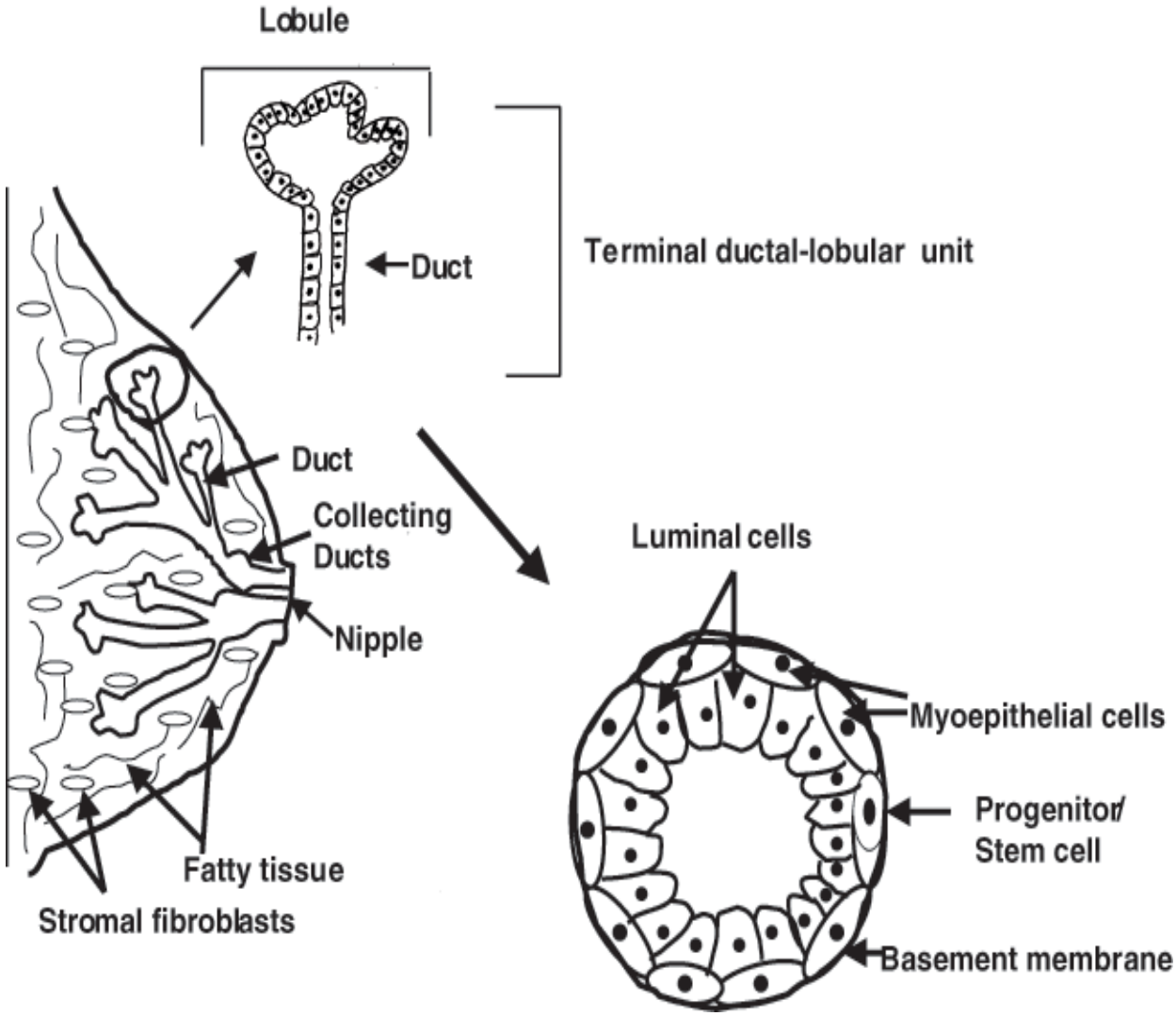

Figure 2.5: Structure of the mammary gland. Terminal ductal–lobular unit (TDLU) comprised of ductal cells. The stroma is composed of adipocytes (fatty tissue) and fibroblasts. Two primary types of cells in normal ducts: outer contractile myoepithelial and inner columnar luminal cells are also shown. A putative progenitor or stem cell is also indicated. Source reference (Dimri *et al.*, 2005).

2.3 Carcinogenesis of human breast cancer

Carcinogenesis results from the interaction of exogenous factors leading to initiation of endogenous process. This process triggers the genetic and or epigenetic alteration of multiple genes (Kyrtopoulos, 2006). These genetic alterations are produced by the exposure of somatic cells or normal stem cells to various exogenous factors which either may be chemically- induced such as exposure to polycyclic aromatic hydrocarbons (PAH), or physically-induced such as ionizing radiation causing mutations in the stem cells. This is considered to be the first step in carcinogenesis, where the cellular genome undergoes mutations for neoplastic development.

The somatic genetic changes in cells that contribute to tumour development usually involve sequential mutation of different classes of genes such as proto-oncogenes, tumour suppressor genes, genes involved in cell cycle regulation, and genes that play roles in maintaining normal genomic stability. The human DNA sequences responsible for transformation are called oncogenes. Although the activation of more than one oncogene appears to be essential for neoplastic transformation, but the data imply that initiation may be induced with one hit kinetic. The activation of these oncogene results in the production of proteins which ultimately trigger the increased transformation and proliferation of genetically-insulted neoplastic or tumour cells (McPherson et al., 2000, Croce, 2008).

At extended level, biochemical interactions between tumour gene mutations may destabilize the genome, which compromise control of cell signaling, proliferation and differentiation interfering with the normal interaction of cells in tissues (Karp and Broder, 1995; Osborne *et al.*, 2004). Mutated genes are supposed to cause inheritable spontaneous changes in DNA structure by affecting the DNA replication and cell divisions. These changes result in

imbalance to the rate of cell proliferation and cell death, thus increasing the cellular proliferation of the mutated cells with reduced programmed cell death mechanism. Many cell cycle generations of mutated cells cause the formation of excessive amount of new abnormal cells known as neoplasia or tumours (Bertram, 2000).

Carcinogenesis is a multistep process and can be divided into three main stages; initiation, promotion and progression (Figure 2.6). Initiation phase involves one or more irreversible cellular DNA-damaged changes arising spontaneously or induced by exposure to a carcinogen. This genetic change results in mutations activation. This is the first step in carcinogenesis where the mutations result in neoplastic development. During promotion, the mutated initiated cells are stimulated to undergo further proliferation by upsetting the cellular balance to form a new population of cells, which may be a benign or preneoplastic lesion. The progression is the advanced phase through which successive changes in the neoplasm give rise to increasingly malignant sub-populations or malignant cells. Molecular mechanisms of tumour progression are not fully known, but chromosomal and genes level aberrations or mutations are thought to be involved (Barrett, 1993). After the first exposure to the carcinogens, it may take many years or even decades for the promotion and progression to be completed until the cancer formed (Tsao *et al.*, 2004).

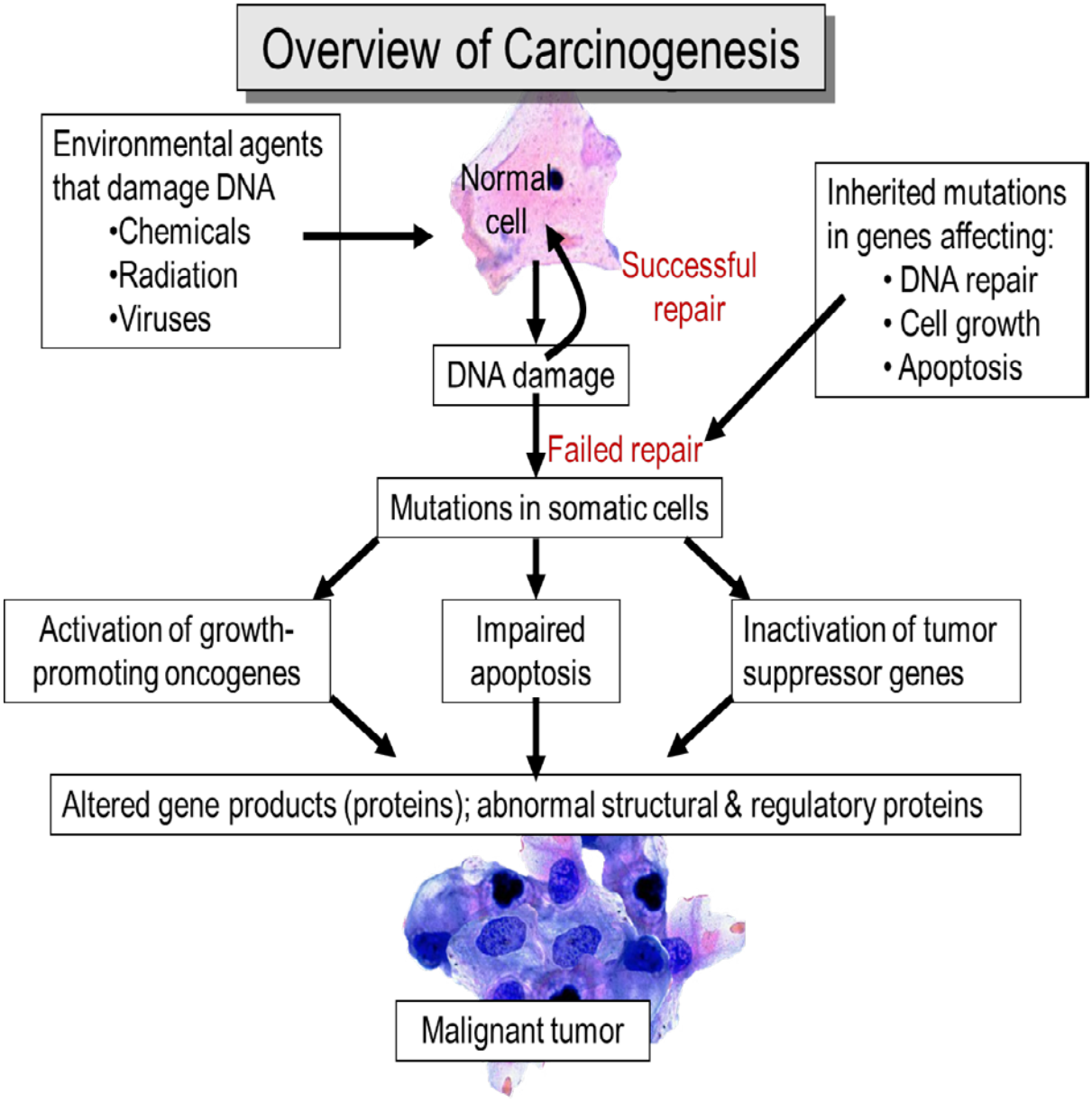

Figure 2.6: Overview of breast cancer carcinogenesis. Source reference (LaMorte, 2005).

2.4 Pathogenesis of human breast carcinoma

Breast cancer arises from a series of mutations that may accumulate over years (Hayat, 2005; Kwei *et al.,* 2010). Several studies have demonstrated that the development of breast cancer has been attributed to numerous factors, including internal such as endocrine or hormonal related factors and external such as genetic factors, early menarche, late menopause, parity and several others (Russo *et al.,* 2000).

The site of origin for breast cancer has been proclaimed to arise from lobular unit that comprises the most undifferentiated structure, and 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, 1980; Russo *et al.*, 1998; Russo *et al.*, 2000). The high proliferative activity and fragility upon genetic alteration by exogenous mutagenic influence of lobules, makes it highly susceptible for preneoplastic and neoplastic processes (Russo *et al.*, 2000).

Generally, cancer cells exhibit six characteristics to ensure their survival namely (1) self-sufficiency in growth signals, (2) insensitivity to apoptosis or cell death (3) uncontrolled replicative potential, (4) resistance to growth-inhibitory factors (5) sustained angiogenesis and (6) tissue invasion and metastasis (Hanahan and Weinberg, 2000; Liu *et al.*, 2005) . One of the key hallmarks of breast carcinoma is the loss of the ability to control growth within organized bilayered ducts or lobules (Mallon *et al.*, 2000; Guinebretière *et al.*, 2005). Normal mammary ducts, lobules and alveoli consist of a single layer of epithelial cells lining the lumen and myoepithelial cells lining the basement membrane (Mallon *et al.*, 2000; Polyak, 2001; Guinebretière *et al.*, 2005). The close cellular contact of luminal and myoepithelial cells enables autocrine and paracrine interaction potentially mediated by chemokines either between luminal

and epithelial cells or between luminal epithelial cells and stromal cells including fibroblasts, adipocytes, macrophages, lymphocytes, eosinophil granulocytes and endothelial cells (Polyak, 2001). An aberrant proliferation of epithelial cells and myoepithelial cells lead to the formation of benign lesions such as fibrocystic diseases, fibroadenomas, epithelial hyperplasia, sclerosing lesions and tubular adenomas, and intraductal papillomas (Courtillot *et al.*, 2005; Bateman, 2010). Generally, these types of lesions are non-detrimental, but have the potential to develop into breast carcinoma in situ.

Carcinoma *in situ* is a pre-malignant proliferation of the breast epithelial cells confined within the basement membrane (Russo and Russo, 2000; Thompson and Singh, 2000), and can be classified as ductal carcinoma *in situ* (DCIS) which is originated from the ductal region or lobular carcinoma in situ (LCTS), originating from the lobular region (Meijnen *et al.*, 2006; Pinder, 2010). DCIS exhibited several histological subtypes including cribriform, comedo, solid and micropapillary, and LCIS does not possess any one (Mallon *et al.*, 2000; Guinebretière *et al.*, 2005). In addition, both carcinomas in situ are known to be precursor of invasive carcinoma (Polyak, 2001; Hanby, 2005; Polyak, 2007) (Figure 2.7).

Invasive carcinoma retains similar characteristics as carcinoma in situ, but the tumour cells breach the basement membrane invading the surrounding tissues (Russo and Russo, 2000; Bateman, 2010) (Figure 2.7). Invasive ductal carcinoma (IDC) which is the most common breast carcinoma in humans has been histologically classified as special-type and Not-otherwise specified (NOS) type. It is estimated that 75% of breast carcinoma cases in humans are diagnosed as IDC-NOS (Hanby, 2005), as the tumour exhibits no specific characteristics. The remaining special-type of invasive carcinoma which have distinctive characteristics such as tubular carcinoma, cribriform carcinoma, solid papillary carcinoma and many more, rarely occur

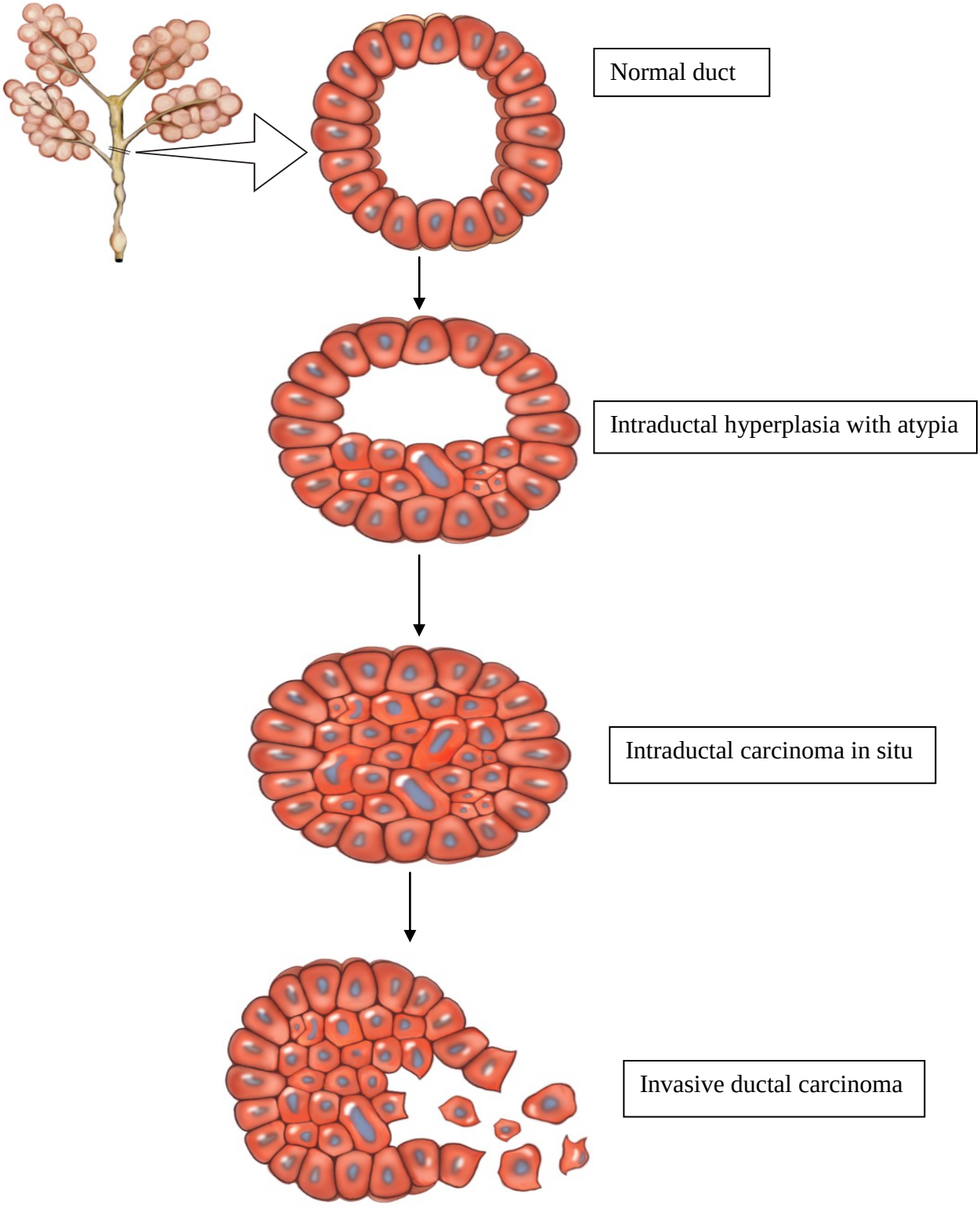

Figure 2.7: Pathogenesis of breast cancer. Source references (Polyak, 2001; Hanby, 2005; Polyak, 2007).

in breast carcinoma cases. In advanced stage, breast carcinoma has been seen to be metastasized to a distant organs such as liver, lungs, brain, adrenal gland and bones through the lymphatic and systemic vascular invasion (Mallon *et al.*, 2000; Hanby, 2005).

2.5 Molecular classification of breast cancer

Breast cancer is a heterogeneous disease and comprised of numerous distinct cell types with different clinical, histological and biological features. Thus, classification of breast cancer cannot be limited to the localization and the proliferative extent of the tumours. The research in recent era has classified the breast cancer on molecular basis by utilizing different molecular techniques and comprehensive genes profiling analysis. The molecular classification of breast cancer has revealed five major subtypes of breast cancer; luminal B, HER2+/ER–, basal-like, luminal A and normal breast–like (Perou *et al.*, 2000; Sorlie *et al.*, 2001; Lakhani, 2002; Hu *et al.*, 2006; Schnitt, 2010). These molecular differences have brought revolutionary changes with distinct clinical outcomes and response to treatments. Amongst the molecular subtypes, luminal A-type tumours have been demonstrated as the low-grade tumours with the best prognosis (Sorlie *et al.*, 2001). The luminal B type tumours have been described as more aggressive with more proliferation, and are of high-grade. Basal-like tumours show relatively poor prognosis expressing some molecules found in the myoepithelial/ basal compartment of normal breast tissues. These tumours are usually negative for HER2 (human epidermal growth factor receptor 2) and ER (Estrogen Receptor) (Lakhani, 2002; Vuong *et al.*, 2014). The recent advances in molecular subtypes of breast cancer have made easy the choice of therapeutic regimen for the patients, and ensure more specificity to the target specific treatment.

2.6 Cancer-preventive studies

The purpose of cancer-prevention or chemoprevention is to interfere with the basic process of carcinogenesis through chemical agents or regimens that may block the initial neoplastic induction, resulting in prevention of the progression of transformed cells into malignant types. The other purpose is to prevent the development of second primary tumours that may arise from patients who had already been cured from the initial cancer (Tsao *et al.*, 2004) . The multistep carcinogenesis process over a long period of time allows the possibility to be intervened through natural or synthetic chemopreventive agents at various stages of carcinogenesis (Raffoul *et al.*, 2012b). The long latent period of cancer formation might be the reason why the high occurrence of breast cancer is among the elderly women, who might have been exposed to potential anti-carcinogenic agent in their early lifetime (Raffoul *et al.*, 2012b).

The concept of cancer-preventive studies is to give the potential chemopreventive agents to the subjects who have been exposed to carcinogens at their early age or any stage. Several cancer-preventive or chemoprevention studies to date have applied this principle (Raffoul *et al.*, 2012b). The potential preventive agents can be given either before or shortly after exposure to the carcinogen, depending on the purposes of trials (Mehta, 2000; Raffoul *et al.*, 2012b). Results obtained from the experimental models 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 carcinogenesis interventions must have the purpose of enhancing or improving the protective physiological mechanism against the disease (Mehta, 2000; Raffoul *et al.*, 2012b).